Centrosome-Mediated RNA Segregation: a Novel Patterning Mechanism in *Ilyanassa*

by

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Dedication

This dissertation is dedicated to

Raina, Miller and Reese,

my TASC family,

with all my heart.
Curriculum Vitae

The author was born in Southampton, NY on March 17\textsuperscript{th}, 1982. He attended Cornell University from 2000 to 2004, and graduated with a Bachelor of Science degree in 2004. He came to the University of Rochester in the Fall of 2004 and began graduate studies in Biology. He pursued his research in developmental biology under the direction of Professor J. David Lambert and received a Master of Science degree from the University of Rochester in 2006.
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Abstract

All multicellular organisms require asymmetric cell divisions for normal patterning during embryogenesis. Spiralian embryos are thought to be particularly reliant on autonomous cues for embryonic patterning and thus represent potentially useful models for understanding asymmetric cell division. The series of asymmetric divisions that produce the micromere quartets are particularly important for patterning, because they subdivide the animal-vegetal axis into tiers of cells with different developmental potentials. Recently, a novel segregation mechanism has been found in the embryo of the spiralian mud snail *Ilyanassa obsoleta*: centrosome-mediated RNA segregation. This mechanism results in each quartet inheriting a unique subset of patterning RNAs and has been proposed to be the molecular event responsible for patterning the quartets. In the experiments reported here, I examine how this mode of asymmetric RNA division is established in the *Ilyanassa* embryo and the development impact of these segregated RNAs.

In Chapter 1 I discuss the pertinent background information for this work and discuss the advantages of using *Ilyanassa* as a model to study RNA segregation. In Chapter 2 I report the work on a conserved patterning gene, Nanos, which was used to develop the tools necessary to do molecular studies in the snail. The major focus of my thesis is discussed in Chapter 3. I show that one of the segregated RNAs, IoLR5, is required in the lineage which inherits it and I characterize two cis-acting elements in localized RNAs, IoLR5 and IoLR1, that mediate transport to the centrosomes. Importantly, these results demonstrate that micromere quartet identity, a hallmark of the ancient spiralian developmental program, is controlled in part by specific RNA localization motifs. I report my attempts to identify trans-acting proteins required for centrosome-mediated RNA segregation in Chapter 4 and summarize my conclusions in Chapter 5.
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For Chapter 2 of my dissertation I collaborated with Professor J. David Lambert, with fellow graduate student Xin Yi Chan, and also with Evan P. Kingsley and Yingli Duan. This chapter has been published in Current Biology, March 2008, pages 331-336.

Figures 1.1 and 1.2 are adapted from Lambert and Nagy, 2001
Chapter 1

Introduction: Asymmetric cell division
and the spiralian Ilyanassa

Asymmetric cell division

Development of all multicellular organisms requires the generation and maintenance of polarity within and between cells. Such asymmetries can be established either intrinsically through the asymmetric segregation of proteins, RNAs and/or organelles during mitosis, or extrinsically through spatially dependent cell-cell signaling events after mitosis. The existence of asymmetrically inherited factors was first witnessed by embryologists over a century ago. For example, detailed observations of early embryogenesis in the snail Crepidula and in three ascidians (Conklin, 1902; Conklin, 1905), led Conklin to propose that the differential distribution of cytoplasmic regions (the centrosomal “sphere substance” in Crepidula and “yellow protoplasm” in ascidians) was a possible mechanism to control cell fate specification. Another more obvious example came from the snail Ilyanassa where a large vegetal protrusion, the polar lobe (PL; discussed in detail below), was found to segregate into one of the daughter cells from the first mitotic division of the egg (Crampton, 1896). Although these observations helped shape the hypothesis that cell polarity or specification was generated by the asymmetric distribution of determination factors, it was many decades before scientists began to dissect the process at the molecular level.
Embryonic manipulations in two insect species showed that when an insect embryo is divided into two parts at an early stage, in the middle of the anterior-posterior axis, each half continues to develop into anterior and posterior structures respectively (Sander, 1976). Then, technically challenging transplantations were performed in *Drosophila* where cytoplasm from the anterior region of the egg was injected into the posterior region. This caused the posterior of the embryo to develop into anterior fates. These experiments led to the prediction that factors were localized in the anterior cytoplasm that were required for anterior development. Thus, if these factors were mutated in an embryo, one would expect that no anterior structures would develop. Indeed, forward genetic screens identified mutants with this predicted phenotype. In this case, molecular cloning of the disrupted gene led to the discovery of the anterior morphogen Bicoid (Frohnhofer and Nusslein-Volhard, 1986). Similarly, posterior cytoplasm transported into the anterior caused a posteriorization of the embryo, and this effect was explained at a molecular level with the identification of Nanos as a posterior patterning gene in the fly (Frohnhofer et al., 1986).

Bicoid and nanos mRNAs were found to be localized to opposite poles of the embryo, bicoid at the anterior and nanos in the posterior, and corresponding gradients of protein levels were found to occur along the anterior-posterior axis. These gradients create the basic coordinate system for patterning the anterior posterior axis of the embryo. Detailed analyses of these and other localized RNAs has greatly expanded our knowledge of fly
embryogenesis and has provided considerable knowledge about conserved aspects of how RNAs are localized, cytoskeleton architecture, molecular transport motors and germline specification (Becalska and Gavis, 2009; Kugler and Lasko, 2009; Lehmann and Ephrussi, 1994; Martin and Ephrussi, 2009; St Johnston, 2005). However, early *Drosophila* embryogenesis involves a syncytial blastoderm stage, when RNAs and proteins can diffuse between cells, allowing the formation of protein and RNA gradients. It is not clear how RNA segregation functions in embryos where cell fates are specified earlier, and in the context of normal cell division.

**Subcellular localization and asymmetric segregation of RNA**

At the start of my thesis, numerous localized RNAs had already been found in various model systems. In the yeast *S. cerevisiae*, 22 mRNAs were found to be asymmetrically segregated to the bud tip in dividing cells (Shepard et al., 2003), and in mammals, ~400 transcripts were found to be specifically localized at the dendrites in neurons (Aakalu et al., 2001; Eberwine et al., 2001). With the development of more sophisticated high-throughput methods for *in vivo* visualization of RNAs in recent years, these numbers have greatly increased. For example, in a study of over 3000 mRNAs in *Drosophila*, 71% were found to be localized to discrete subcellular regions (Lecuyer et al., 2007). These findings suggest that many more transcripts are localized within cells than previously thought and that subcellular compartments exist
which have been largely unappreciated in the past (Martin and Ephrussi, 2009).

There are many possible reasons why RNA localization might occur and several possibilities have been covered in depth in recent reviews (Du et al., 2007; Kloc and Etkin, 2005; Martin and Ephrussi, 2009; St Johnston, 2005). One advantage is that mRNAs can be translated many times to rapidly create large quantities of proteins in a specific region. Cells only need to transport a few RNA molecules to a given location to achieve high concentrations of protein as compared to cells translating each peptide and individually transporting the proteins to the appropriate region. Spatially controlled translation is also beneficial for proteins that are cell fate determinants because misexpression of these proteins will cause defects during development. For example, Nanos is restricted to the posterior of the Drosophila egg where it induces posterior development in the fly embryo, but if it is expressed in the anterior, it will induce formation of posterior structures in this region (Gavis and Lehmann, 1992). Another advantage of mRNA localization is for restricting the localization of proteins that are deleterious to cells when expressed in certain subcellular regions, such as myelin basic protein (MBP) (Boggs, 2006). If MBP mRNA remains in the cell body of a neuron and becomes translated, the MBP protein will cause the cell to die. Also, some proteins are incapable of being transported, such as Tau and MAP2 (Aronov et al., 2001). These types of proteins can be properly localized by first transporting their mRNAs to the appropriate subcellular
regions. A fifth advantage is that localized transcripts can be activated by local stimuli to provide cells with a high temporal resolution of protein expression. Rather than environmental cues signaling to the nucleus, initiating transcription of RNA, export of the RNA, translation of the RNA and then transport of the protein, localized mRNAs are already in the appropriate region and only need to be translated (Martin and Ephrussi, 2009).

There are fewer cases known where segregation of RNA is related to cell fate specification events. In *Drosophila*, the well studied cases of subcellular RNA localization lead to embryonic patterning through morphogen gradients, rather than cells being specified by the inheritance of particular RNAs. Similarly, in *Xenopus laevis* embryos, gradients of protein are created by localization of RNAs and proteins to the vegetal pole, followed by subdivision of the embryo by early cleavages (King et al., 2005). In budding yeast, segregation of the ASH1 RNA into the bud tip is required for proper control of mating type switching of the daughter cell (Long et al., 1997; Takizawa et al., 1997). In fly neuroblasts, several RNAs have been characterized that become segregated directly into one of the two daughter cells and participate in generating proper cell fates (e.g. Broadus et al., 1998). Extensive work on asymmetric cell division in *C. elegans* largely involves the asymmetric segregation of proteins, except in the germline, where segregated RNA-protein particles called P-granules are involved in germline specification or maintenance (Updike and Strome, 2010).
RNA elements required for localization and segregation

Localization of mRNAs occurs through two phases; establishment and maintenance. As transcripts leave the nucleus, they must be targeted and transported to their preferred destination (establishment). Then, they must be anchored there to ensure proper localization of the translated protein (maintenance). From the research carried out on establishment of RNA localization, one important commonality has emerged - the presence of cis-acting elements within the transcript sequence that are essential to mediate transport. These elements are typically found in the 3' UTR of transcripts, but they vary greatly in their complexity. The simplest known cis-acting element is found in mammalian oligodendrocytes, where localization of the mRNA for myelin basic protein is based on a single 11-nucleotide region, the A2RE element (Ainger et al., 1997). It has also been demonstrated that localization is sometimes dependent on the presence of small, repeated nucleotide sequences. One example is seen in the D. melanogaster nanos RNA which has four partially redundant nucleotide regions necessary for localization to the posterior (Bergsten et al., 2001; Crucs et al., 2000). Such redundancies are most striking in Xenopus where clusters of a small repeated CAC motifs are found in all RNAs localized to the vegetal cortex in the egg (Betley et al., 2002). Furthermore, large-scale sequence analysis has suggested that similar clusters with CA-containing motifs are found in hundreds of mRNAs in mammalian neurons and this could explain the large numbers of localized transcripts seen in these cells (Andken et al., 2007).
RNA has a high propensity to bind itself and thus create complex secondary structures. Not surprisingly, specific secondary structures have also been implicated in the localization of messages. ASH1 mRNA contains multiple redundant localization elements; however, mutational analyses have shown that transport of this RNA requires the secondary structure formed by these elements and not the primary sequence (Gonzalez et al., 1999). One of the most complex cases is seen with bicoid localization to the anterior of the fruit fly oocyte. The bicoid RNA contains five stem loop secondary structures which are required for transport. One of the proteins necessary for this transport is Staufen, a dsRNA binding protein. Not only does Staufen bind stem loops III, IV and V of the bicoid RNA, but the same Staufen molecule binds two different bicoid RNA molecules through separate regions of stem loop III, causing bicoid dimers. This dimerization is essential for efficient transport and reveals a quaternary structure required for mRNA localization (Wagner et al., 2001).

Cis-acting localization elements interact with trans-acting proteins to accomplish transport (i.e. establishment), and tethering (i.e. maintenance), to specific subcellular domains. In many cases these interactions will lead to directed transport along cytoskeletal filaments. Typically, proteins bind to the transcript’s localization element(s) and form ribonucleoprotein (RNP) aggregates which are then carried by molecular motor proteins, such as dynein or kinesin, to the appropriate destination. The Staufen protein, as described above, is one of the best characterized trans-acting factors
involved in RNA transport. This protein mediates transport of RNAs through both microtubule-dependent (Bicoid and Oskar in the fly egg), and actin-dependent (Prospero in the fly neuroblasts) mechanisms (St Johnston, 2005). Furthermore, Staufen is a highly conserved protein that has also been shown to mediate vegetal cortex localization of RNAs in the frog (such as Vg1) and other localized transcripts in mammalian dendrites (such as CaMKIIα mRNA) (St Johnston, 2005). One RNA, Ash1 in yeast, was actually shown to bypass such mediator proteins and bind directly to the motor protein myosin for transport to the bud tip (Bohl et al., 2000). However, not all messages are localized through molecular motors. For example, Nanos localization in the Drosophila posterior is achieved through the combination of cytoplasmic flow and anchoring to the posterior cortex. These two factors alone only provide a slight enrichment of posterior Nanos mRNA (Bergsten and Gavis, 1999); however, when coupled with translational control and stability, the effective levels of functional Nanos mRNA are more than a hundred times more concentrated in the posterior (Forrest and Gavis, 2003; Zaessinger et al., 2006).

**Ilyanassa as a model to study asymmetric cell division**

The embryo of the mud snail *Ilyanassa obsoleta*, like others that display spiralian development (see below), is heavily reliant on asymmetric cell division. In this section, I will describe the basic events of early development,
focusing on the three main kinds of asymmetric cell divisions that are observed in the embryo. The first are the “polar lobe divisions.” During the first cleavage in this snail, an anucleate vegetal protrusion called the polar lobe (PL) is sequestered into one of the two daughter cells; resulting in one smaller cell (AB) and one larger cell (CD). In the next division, the PL material is again distributed to only one of the two daughter cells. At this stage, the embryo is composed of 4 cells known as the A, B, C and D macromeres, with the D macromere being the largest due to its inheritance of the PL (Figure 1.1). The specification of the D macromere is a key early patterning event, since it determines the secondary axis (“dorsal-ventral”) of the embryo, and it is the source of the embryonic organizer. Ablation experiments demonstrated that the PL material is required for D macromere specification (Clement, 1952; Crampton, 1896), but it is still unknown what factors in the PL mediate this.

The next kind of asymmetric divisions are those that generate the micromere quartets. After the 4-cell stage, the large macromeres go through several rounds of highly asymmetric, synchronous divisions to produce the small micromeres cells. The cells within each quartet have
Figure 1.1 Early cleavage divisions of *Ilyanassa*

(A) The polar lobe (PL) begins as an anucleate vegetal protrusion in the zygote. (B) At the end of the first cleavage division, the PL is inherited by the CD blastomere. (C) During the second cleavage division, the PL is again inherited by only one cell, the D macromere. (D) At the 4-cell stage, the D macromere is the largest of the four macromeres, due to the PL material. (E) The macromeres divide synchronously towards the animal pole, in a clockwise orientation, to produce the first quartet micromeres (shown in blue). (F) Then, the macromeres divide again in a counter-clockwise direction to produce the second quartet (shown in green). (G) Lastly, the macromeres divide in the clockwise direction to produce the third quartet micromeres (shown in red).
Figure 1.2 Larval fates of the micromeres
(A) Each of the micromeres present at the 24-cell stage gives rise to specific tissues within the 7-day old larva. (B-D) Fate maps for some of the individual micromeres are shown in these color-coded animals.
similar developmental properties, and these differ from those of other quartets. Ultimately, the first, second and third quartets will give rise to all of the ectoderm, as well as some other tissues (Figure 1.2). Thus, the production of the quartets is an important early patterning event: it subdivides the animal-vegetal axis of the embryo into tiers of cells with different developmental potentials. The molecular basis of this process is a major focus of this thesis (i.e. Chapters 3 and 4).

The angle of the micromere divisions, relative to the animal-vegetal axis, alternates between clockwise and counter-clockwise during these divisions. First, the macromeres divide in a clockwise direction towards the animal pole, giving rise to the first quartet micromeres. Then, the cleavage plane shifts ~90° and the macromeres divide in a counter-clockwise direction producing the second quartet micromeres. The cleavage plane shifts again and the macromeres divide in a clockwise orientation to form the third quartet micromeres. When viewed from the animal pole at this stage, the alternating cleavage divisions result in the embryo having the characteristic geometric chirality which originally inspired the term spiral cleavage (Figure 1.1).

The last class of asymmetric cell divisions that we consider is the teloblastic divisions of the 4d micromere, the fourth micromere born from the D macromere. 4d divides to produce two bilaterally symmetric cells, 4d^L and 4d^R. These are relatively large cells that undergo a series of asymmetric divisions to produce much smaller cells that each develop with distinct cell fates. 4d progeny will give rise to nearly all of the mesoderm, as well as a
large portion of the endoderm (Lambert, 2008). The 4d blast cell divisions are probably homologous to the teloblast divisions seen in *Helobdella* (Gline et al., 2009) and are very similar to the neuroblast divisions seen in *Drosophila* (Yu et al., 2006). Teloblastic divisions are most obvious in the 4d lineage, but may also occur in other micromere lineages. Additionally, this process may be functionally related to the micromere divisions, since both involve a large cell generating a series of smaller cells with different fates. The 4d lineage is examined in Chapter 2.

The *Ilyanassa* embryo presents several practical and experimental advantages for the study of asymmetric cell division and RNA segregation. The divisions take place when the cells are large and amenable to high-resolution imaging. The cells are injectable at various defined times in the cell cycle, and the synchronous cleavage within and among embryos allows time-series to be created with high temporal resolution. The synchrony of these divisions also allows biochemical analysis of cells at particular parts of the segregation and localization process, since protein can be extracted from a large number of cells at precisely the same stage. These advantages make *Ilyanassa* a useful complement to existing models used for studies of RNA localization and segregation.
Patterning the early *Ilyanassa* embryo

Fate mapping experiments demonstrate that the different quartets each give rise to unique tissues of the *Ilyanassa* larva. For example, the 1a micromere always gives rise to the left eye and the 2d cell always gives rise to the shell gland (Render, 1991; Render, 1997). The reproducibility of the fate map lead many early embryologists to propose that spiral cleaving embryos were completely reliant on cell autonomous specification. Indeed several embryological experiments have provided direct evidence for autonomous specification. First, blastomeres isolated from different spiralian embryos were shown to still develop into their respective tissues (Costello, 1945; McCain and Cather, 1989; Wilson, 1904). Second, micromere ablation studies showed that each micromere is born with a specified fate, and other cells rarely regulate after ablation (Clement, 1967; Clement, 1976; Clement, 1986a; Clement, 1986b). However, the *Ilyanassa* embryo is not entirely patterned autonomously, as cell ablation and more recent molecular techniques have shown that proper development requires signaling from the D quadrant organizer (Clement, 1952; Crampton, 1896; Lambert and Nagy, 2001; Lambert and Nagy, 2003). Therefore, development in spiralian embryos appears to be controlled by a combination of extrinsic and intrinsic cues.

The stereotyped nature of the *Ilyanassa* fate map indicates that the fates of cells in the blastula are established very early. The mechanisms that accomplish this are the major focus of work on this embryo, and of my thesis.
One important component of cell fate specification in the early spiralian embryo is the establishment of developmental differences between micromere quartets. For instance, in *Ilyanassa*, the first quartet micromeres generate the eyes and the second quartet generates the shell. Crucial evidence about how this is established came from difficult cell transplantation experiments (Sweet, 1998). When the first quartet micromeres were transplanted into the position of second quartet cells, they would still develop into eyes, and not shell material. Conversely, when second quartet micromeres were transplanted into the first quartet, rather than generating eyes, these transplanted cells would produce shell material where they eyes were supposed to form (Sweet, 1998). These manipulations showed that the micromeres are specified at, or shortly after, birth; however, there was no molecular data explaining how spiralian embryos regulate asymmetric cell division during early cleavage.

One important clue about how quartet-specific developmental potentials might be established came with the discovery that many RNAs are asymmetrically segregated into micromere quartets via localization to macromere centrosomes (Lambert and Nagy, 2002). This process involves RNA localization to the centrosomes during cytokinesis, where it remains for the duration of interphase. At prophase, the RNA moves to the cortex directly above the centrosome. Then the mitotic spindle orients perpendicular to the RNA and during the subsequent cytokinesis, the cortical material where the RNA is anchored is delivered specifically to one of the two daughter cells.
Drug treatments revealed that transport to the centrosome is microtubule-dependent and transport to the cortex is actin-dependent. Recent work has shown that this phenomenon involves many RNAs, and the regulation of localization and segregation is very intricate, such that each quartet inherits a particular population of RNAs (Kingsley et al., 2007).

Aside from the insight that can be gained in subcellular transport by studying this novel form of division, this process is interesting due to its similarity to a highly conserved structure - the Balbiani body (BB). During oogenesis in animals, patterning RNAs produced maternally are first localized to this structure, before being transported and anchored to the cortex in the egg (Cox and Spradling, 2003; Heinrich and Deshler, 2009; Kloc et al., 2004b; Marlow and Mullins, 2008). The ultrastructure of the developing Balbiani body has been followed in frogs and it was discovered that the BB arises from the oocyte centrosome (Kloc et al., 2004a). This functional homology has lead to the proposal that *Ilyanassa* has co-opted this structure to asymmetrically segregate many RNAs during all of early cleavage (Lambert, 2009). Therefore, this embryo serves as a useful model to study this conserved type of RNA transport.

**Ilyanassa as a representative for the Lophotrochozoa**

The Lophotrochozoa are one of three main clades of organisms found within the Bilateria. The other two major groups are the Ecdysozoa and
Deuterostomia. Nearly all of the major model systems currently studied are members of these other two groups. The ecdysozoans include powerful genetic model systems such as *D. melanogaster* and *C. elegans*. The deuterostomes contain the major vertebrate models like *D. rerio*, *X. laevis* and *G. gallus*, as well as the mammalian models *M. musculus* and *H. sapiens*. While there is a large diversity of adult body plans within the Lophotrochozoa, several different phyla, including the molluscs, nemerteanes, sipunculans, annelids and platyhelminthes flatworms, use the same mode of early patterning, spiral cleavage. This contrasts with development in the Ecdysozoa and Deuterostomia where most phyla can be easily identified strictly based on their pattern of early cleavage during embryogenesis.

The strong conservation of spiralian development was first noted in 1892 by E.B. Wilson:

“Up to a late stage in the spiral period (twenty-eight cells) every individual blastomere and every cell division is represented by a corresponding blastomere and a corresponding cell division in the embryo of the polyclad and in that of the gasteropod. The same practically may be said of the annelid.” (Wilson, 1892; pg 439)

Using microinjection of fluorescent lineage tracers, biologists have validated and expanded upon these observations from early embryologists, demonstrating that across different phyla with spiral cleavage, homologous cells and quartets generate similar fates (Ackermann et al., 2005; Boyer et al., 1996; Damen and Dictus, 1994; Hejnol et al., 2007; Henry and Martindale, 1998b; Henry et al., 2004; Maslakova et al., 2004; Render, 1997; Weisblat and Shankland, 1985). The first quartet typically gives rise to the head
ectoderm. The second quartet usually develops into the trunk ectoderm. The third quartet generates ventral structures and the foregut. Lastly, most of the mesoderm comes from the 4d cell.

Importantly, recent phylogenetic evidence suggests that spiral cleavage was used by the last common ancestor to the Lophotrochozoa (Dunn et al., 2008; Giribet, 2008). The early development of *Ilyanassa*, described above (Figures 1.1 and 1.2), typifies spiralian development, and due to several experimental advantages, it is perhaps the most capable model system currently available for studying this mode of patterning (Gharbiah et al., 2008). Animals are easy to maintain in the laboratory, and high quality embryos can be obtained year round, which is not true of most marine invertebrates. The embryos have a number of experimental advantages, especially for the study of asymmetric cell division (described above). Most importantly, recent work in our lab has established several methods for specific perturbations of particular genes, which had not been possible in organisms with typical spiralian development (see Chapters 2 and 3).

Lophotrochozoan embryos were extensively studied by embryologists more than a century ago, but with the rise of developmental genetics, these embryos were neglected because no genetic model was established from this group. As research in genetic models progressed, it became clear that a limited number of developmental patterning genes and pathways were highly conserved among animals, and these pathways were slightly modified to give
rise to diverse adult body plans. By comparing similar developmental pathways between organisms, it is possible to make predictions about how development occurred in the last common ancestors, thereby broadening our knowledge on how different body plans have evolved. In recent years, developmental biologists have started to fill this large gap in our knowledge of the lophotrochozoans and some intriguing patterns are emerging. For example, work from two snail species has shown the importance of the Nodal pathway in left/right asymmetry in molluscs, suggesting that this pathway was used by the last common ancestor to all of the Bilateria (Grande and Patel, 2009). Thus, we expect that, in addition to expanding our knowledge of asymmetric RNA segregation, studies of *Ilyanassa* embryogenesis will have implications for understanding the evolution of the Lophotrochozoa, and by extension, the evolution of the Bilateria.

For my thesis, I aimed to learn how the centrosome-mediated RNA segregation mechanism described in *Ilyanassa* is established and to determine the development significance of these segregated RNAs. In Chapter 2 I report work on the *Ilyanassa* Nanos homologue that was used to develop techniques necessary to bring *Ilyanassa* up to date as a model molecular system. In Chapter 3 I show that the first quartet requires the segregated RNA IoLR5 for its proper development. Also, I describe my findings on cis-acting elements that mediate RNA transport. In Chapter 4, I discuss my progress on attempting to identify the trans-acting proteins that
drive RNA localization. In Chapter 5 I summarize the major findings of my thesis work.
Chapter 2

Nanos is required in somatic blast cell lineages in the posterior of a mollusc embryo

Introduction

During animal development, blast cell lineages are generated by repeated divisions of a mother cell into a series of daughter cells, often with a specific series of distinct fates. Nanos is a translational regulator that is involved in germline development in diverse animals (Forbes and Lehmann, 1998; Subramaniam and Seydoux, 1999; Tsuda et al., 2003; Wang et al., 2007), and also involved in somatic patterning in insects (Lall et al., 2003; Lehmann and Nusslein-Volhard, 1991). Recently, Nanos was found to be required for maintenance of stem cell divisions in the *Drosophila* germline (Gilboa and Lehmann, 2004; Wang and Lin, 2004). We have found that in the mollusc *Ilyanassa*, Nanos mRNA and protein are specifically localized in the mesendodermal blast cell lineage derived from the strongly conserved 4d cell. Nanos activity is required for differentiation of multiple tissues that are derived from the 4d cell, showing that IoNanos is required for somatic development in this embryo. At the cellular level, we show that IoNanos activity is required for the highly stereotyped cleavage pattern of the 4d lineage, the proliferative capacity of the blast cells, and the marked asymmetry of the blast cell divisions. These results suggest that IoNanos is involved in regulating the blast cell behaviors in the 4d lineage.
Results and Discussion

We recovered an *Ilyanassa obsoleta* ortholog of Nanos (IoNanos) while screening unsequenced embryonic cDNA clones by *in situ* hybridization for patterns of localization in the early embryo (Kingsley et al., 2007). The IoNanos mRNA exhibited a specific pattern of RNA distribution during cleavage stages that suggested a function in the lineage of the 4d cell (this lineage is shown in Figure 2.1). During the first five cleavage cycles the IoNanos RNA is found in all cells of the embryo (Figure 2.2). However, between the 24 and 36-cell stages the pattern of RNA distribution becomes restricted to two cells (Figure 2.3C): the yolk-rich 4D macromere which is nutritive and dispensable for normal development (Clement, 1962), and the 4d micromere. Among the many protostome phyla with spiralian development, the 4d micromere, or mesentoblast, is one of the most striking developmental commonalities (Lambert, 2007). In molluscs and most other spiralian phyla, the 4d cell divides bilaterally, and the daughter cells become teloblasts, executing a series of asymmetric cell divisions which generate a number of smaller cells with mesodermal and endodermal fates. In the veliger larva of gastropods, this lineage develops into the visceral mesoderm (including the heart and several other organs), a portion of the main larval retractor muscle, and the intestine, or hindgut (Hejnol et al., 2007; Render, 1997).
Figure 2.1 The cell lineage and cleavage pattern of the mesentoblast cell 4d in Ilyanassa.

The cells descended from 4d are shown in green, and the other cells in the embryo are outlined for orientation. The sister cells from the immediately preceding divisions are indicated by hatch marks. (A) 28 cell embryo after the birth of 4d. (B) Two hours after the birth of 4d (4d+2h), the cell has divided along the embryo’s axis of bilateral symmetry to generate 4dL and 4dR. (C) At 4d+8h, the 4d lineage has five cells on each side, and each teloblast has divided three times: once toward the vegetal pole (to produce 4dL&R11), once towards the animal pole (to produce 4dL&R111, whose daughters 4dL&R1111 and 4dL&R1112 are shown), then once again towards the vegetal pole (to produce 4dL&R122). (D) At 4d+24h, each teloblast has divided three more times to make a band of cells that leads back towards the future dorsal-posterior side of the embryo. In order of birth, these cells are 4dL&R1212, 4dL&R12112, and 4dL&R121111. The cells 4dL&R112 have divided, and their daughters 4dL&R1112 have also divided, to make four cells on each side that are derived from the 4dL&R11 cells. The cleavage pattern and nomenclature is based on (Goulding, 2001) and unpublished observations (X.Y.C. and J.D.L.).
The IoNanos mRNA is found in a subset of 4d derivatives during the first three rounds of division in this lineage (Figure 2.3C, E, G). We generated polyclonal antibodies against the IoNanos protein, and examined the patterns of protein distribution in early cleavage. The protein largely followed the pattern of RNA localization (Figures 2.3 and 2.4). During the first five cleavage cycles the protein is present in all cells, but between the 24 and 36-cell stages the protein becomes increasingly enriched in 4d. During cleavage of 4d derivatives, IoNanos protein is detected in a subset of these cells. After the fourth division in the 4d lineage, the protein is segregated into the 4d^{L&R112} cells, where it accumulates in the nuclei. After this stage, both the mRNA and the protein are undetectable for the remainder of early cleavage and gastrulation.

To test the function of IoNanos in development, we devised a robust method for pressure injection of the *Ilyanassa* embryo, which has been lacking in this system, and designed a morpholino oligo against the predicted translational start site of the mRNA. Injection of IoNanosMO into zygotes caused reproducible dose-dependent phenotypes in the larvae (Figures 2.5 and 2.6; Table 1). Injection of 0.1 mM solutions (approximately 1 uM after injection) always resulted in larvae that lacked all of the derivatives of 4d that can be reliably scored. These animals never had hearts, normal larval reductor muscles, or intestine. In contrast to these endomesodermal derivatives, ectodermal structures were largely normal. IoNanosMO larvae
Figure 2.2 IoNanos RNA during early cleavage.

(A) Early cleavage stages of *Ilyanassa*. During the first cleavage, a lobe of cytoplasm called the polar lobe (PL) is formed at the vegetal pole, and the contents of the lobe are inherited by one cell at the two cell stage. A similar lobe forms at the next cleavage cycle (not shown), so that the four cell embryo has one larger cell, called the D macromere and three smaller macromeres (A, B and C). In the next cleavage cycles, the four macromeres divide together to produce sets (or *quartets*) of smaller cells called micromeres. The first quartet is shown in blue, the second in green, and the third in orange. After the third quartet is born, the D macromere divides to produce the 4d cell (see Figure 1). (B-E) RNA is detected by *in situ* hybridization followed by chromogenic detection (blue-black) and nuclei are stained with DAPI (bright blue). The animal pole is oriented towards the camera. (B) Mature oocyte, with RNA detected around the germinal vesicle. (C-E) During the early cleavage stages, the RNA is present in all cells of the embryo. (C) Four-cell stage. (D) Eight-cell stage. (E) 15 to 16-cell stage. In (E), some RNA is localized to centrosomes in the macromeres (arrowhead). Scale bar represents 50 µm.
Figure 2.3 (legend follows)
Figure 2.3 IoNanos RNA and protein are localized to the 4d lineage.

(A, C, E, G) RNA is detected by in situ hybridization followed by chromogenic detection (blue-black) and nuclei are stained with DAPI (bright blue). (B, D, F, H, I, J) The anti-IoNanos antibodies were detected with a fluorescent secondary (red), the nuclei were stained with YOYO-1 (green), and projections were constructed from Z-stacks of each embryo. In all figures, the animal pole is towards the viewer, and the D quadrant is down. (A) After the fifth cleavage cycle (24-cell stage) the RNA is most abundant in the cytoplasm of the 3D macromere cell, and is also found on the centrosomes of the other macromeres (3A, 3B and 3C). The message is also detected in the cytoplasm of the third quartet micromeres (3a-3d). (B) The protein at this stage is found throughout the embryo, but is most abundant in 3D. (C) After the division of 3D into 4d and 4D, IoNanos RNA is found in 4d (in telophase in this embryo, with the DNA of the daughter cells indicated), and 4D (not visible in this view). (D) The protein is abundant in the newly born 4d cell, but is also found elsewhere in the embryo, including the third quartet micromeres 3a-3d. (E) After the division of 4d (4d + 2hr), the RNA is abundant in its two daughter cells 4dL and 4dR, and detectable in 4D. Nuclei of multiple ectodermal cells overlie the staining in 4dL and 4dR. (F) The protein at 4d+2hr is largely restricted to the daughter cells 4dL and 4dR. (G) After the next cleavage cycle in the 4d lineage (4d+4hr), the RNA is again found in two cells of the 4d lineage, 4dL1 and 4dR1. Again, nuclei of multiple ectodermal cells overlie the staining in these two cells. (H) Like the RNA at 4d+4hr, the protein is restricted to 4dL1 and 4dR1. (I) After another cleavage cycle in the 4d lineage (~4d+6hr), the protein is found in 4dL1, 4dL12, 4dR11 and 4dR12. (J) After an additional cleavage cycle in the 4d lineage (~4d+8hr), the protein is largely restricted to the nuclei of the cells 4dL112 and 4dR112. The scale bars represent 50 µm. (K) Summary diagram of IoNanos RNA and protein distribution in the 4d lineage. The dotted red lines indicate protein localization in the nucleus.
**Figure 2.4 IoNanos protein during early cleavage stages.**
The anti-IoNanos antibodies were detected with a fluorescent secondary (red), the nuclei were stained with YOYO-1 (green), and projections were constructed from Z-stacks of each embryo. The animal pole is towards the viewer. (A) In the oocyte, the protein is enriched in a region above the germinal vesicle. (B-D) In the two cell (B), eight cell (C) and 16 cell (D), the protein is found in all cells of the embryo. Scale bar represents 50 µm.
had two velar lobes and most had two eyes. They developed relatively normal shells, but these were smaller than controls and rotated counterclockwise relative to the head and foot. Development of the larval foot was variable, with many larvae having foot structures like statocysts or operculum but poor organization of the foot as a whole. Several lines of evidence indicate that this phenotype results from specific knockdown of IoNanos activity in the embryo. First, injection of ten-fold higher concentrations of a five-mismatch control morpholino oligo results in wildtype larvae (Figure 2.5 and Table 1), and morpholinos targeted to other RNAs give different phenotypes (unpublished observations, J.S.R, X.Y.C. and J.D.L.) indicating that these phenotypes are not generic effects of morpholino oligos. Second, levels of IoNanos protein were strongly reduced in embryos injected with 0.1 mM IoNanosMO at timepoints after the birth of 4d, indicating that IoNanos translation was repressed (Figure 2.6). Finally, zygotic injection of RNA anti-sense to the IoNanos transcript generated an identical phenotype to the 0.1 mM IoNanosMO injection (Figures 2.7 and 2.8; Table 1). Injection of this RNA results in the loss of detectable IoNanos protein but not mRNA, suggesting that it inhibits translation. Injection of RNAs that are antisense to other transcripts give different phenotypes (unpublished observations, J.S.R and J.D.L.). Taken together, these results suggest that loss of IoNanos activity in the embryo blocks normal development of 4d derivatives, and has
Figure 2.5 (legend follows)
Figure 2.5 The effects of IoNanos knockdown and 4d deletion on various larval organs.

(A) Anterior and (B) dorsal views of seven day old larvae showing normal development after zygotic injection of 1 mM 5-mismatch control morpholino. The intestine is naturally pigmented. (C,D) Larvae from zygotes injected with 0.1 mM IoNanosMO. These animals are lacking intestine and heart (scored in live animals), and have larval retractor muscles that are reduced compared to controls (Figure S3). They have relatively normal heads except that the velar lobes are smaller than controls and eyes are sometimes missing. They have small shells that are rotated to the left side compared to controls, and variable development of foot structures. (E,F) Larvae that developed after ablation of the 4d micromere also lack intestine, heart, and normal retractor muscles. They also have variable development of head and foot structures, and shells that are rotated to the left side. The scale bar represents 100 µm. (G) All organs that can be reliably scored in the larva were examined in 20-23 larvae from three different clutches for each treatment, and the fraction of larvae with the indicated tissue or condition was plotted. The velum and eyes derive mainly from the first quartet of micromeres. The shell derives mainly from the second quartet and the foot structures like operculum and statocysts derive from the third quartet. The heart and retractor muscle are mainly derived from 4d, and the intestine is derived completely from 4d. Since the heart data was recorded before fixation, and larvae were sometimes lost during fixation or processing, the fraction with hearts for each treatment is normalized to the number of animals scored after fixation.
Figure 2.6 Effects of IoNanos morpholino on IoNanos protein levels. (A) Control and (B) IoNanosMO-injected embryos at around 4d+2h. (C) Control and (D) IoNanosMO-injected embryos at around 4d+4h. All images are single confocal sections at the level of the teloblast cells, with the anti-IoNanos protein staining in red and nuclei in green.
Figure 2.7 Effects of IoNanos morpholino oligo knockdown and 4d deletion on the larval retractor muscle.
Phalloidin-Alexafluor 488 staining of 7 day-old veligers. The main larval retractor muscle is a bundle of actin-staining muscle fibers on the left side of the animal (white arrowheads in all panels). (A) Wild-type veliger viewed from the left side. (B) Larva after ablation of 4d. B, D and F are viewed from the dorsal side, but give an equivalent view of the larval retractor to A, C and E, since the posterior of the larva does not rotate ventrally in the animals shown in the former panels. (C) Wild-type larva developed from zygote injected with 1 mM IoNanosMO mismatch control. (D) Larva that developed from zygote injected with 0.1 mM IoNanosMO (The diffuse background stain in this animal and in F is not muscle-specific). (E) Larva after zygotic injection of sense control RNA corresponding to positions 2013-3490 of the IoNanos 3'UTR in the sense orientation. (F) Larva after zygotic injection of IoNanos anti-sense RNA. The larval retractor is reduced but not absent in all experimental treatments, and the effect is more severe in the IoNanos knockdowns (D and F) than after 4d deletion (B). Scale bar represents 100 um.
### Table 1.1 Differentiation of larval tissues after IoNanos knockdown or 4d deletion.

<table>
<thead>
<tr>
<th>Control injection</th>
<th>2 velar lobes</th>
<th>2 eyes†</th>
<th>Normal foot</th>
<th>0 statnysts</th>
<th>1 statnyst</th>
<th>2 statnysts</th>
<th>Operculum material present</th>
<th>Normal shell</th>
<th>Retractor muscle present</th>
<th>Retractor muscle normal size</th>
<th>Heart*</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM 5-mismatch control MO**</td>
<td>20/20</td>
<td>20/20</td>
<td>20/20</td>
<td>0/20</td>
<td>0/20</td>
<td>20/20</td>
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<td>20/20</td>
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</tr>
<tr>
<td>0.025 mM IoNanosMO</td>
<td>16/16</td>
<td>16/16</td>
<td>16/16</td>
<td>0/16</td>
<td>0/16</td>
<td>18/16</td>
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<td>18/16</td>
<td>18/16</td>
<td>18/16</td>
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</tr>
<tr>
<td>0.05 mM IoNanosMO</td>
<td>21/21</td>
<td>21/21</td>
<td>21/21</td>
<td>0/20</td>
<td>0/20</td>
<td>21/21</td>
<td>21/21</td>
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<td>21/21</td>
<td>21/21</td>
<td>21/21</td>
<td></td>
</tr>
<tr>
<td>0.1 mM IoNanosMO</td>
<td>23/23</td>
<td>23/23</td>
<td>16/23†</td>
<td>0/23</td>
<td>0/23</td>
<td>23/23</td>
<td>23/23</td>
<td>17/23†</td>
<td>23/23</td>
<td>23/23</td>
<td>16/23†</td>
<td></td>
</tr>
<tr>
<td>4d deletion IoNanos anti-sense RNA</td>
<td>20/20</td>
<td>24/24</td>
<td>24/24</td>
<td>3/20</td>
<td>12/20</td>
<td>5/20</td>
<td>12/20</td>
<td>0/20</td>
<td>20/20</td>
<td>0/20</td>
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</tr>
</tbody>
</table>

### Notes for Table 1.
* Larvae were scored while living for hearts, and the same larvae were not necessarily scored for other organs after fixation.
† All of the animals with missing or abnormally developed tissues in the 0.05 mM injections series were from one of the three capsules used.
‡ All larvae had at least one eye.
** One animal was completely abnormal; it lacked all structures scored, and was not counted in this series.
Figure 2.8 Effects of IoNanos anti-sense RNA injection.
(A) Control embryo at 4d+4h, stained with the IoNanos anti-sera. (B) Embryo injected with antisense IoNanos RNA at 1 ug/ul stained in parallel to embryo in (A), showing no specific staining. (C) Anterior and (D) dorsal views of control larvae injected with 525 ng/ul of a sense RNA corresponding to positions 2013-3490 of the IoNanos transcript. (E and F) Larvae derived from zygotes injected with IoNanos anti-sense RNA. See also Table 1.
less severe and less penetrant effects on some ectodermal organs, especially the foot.

Since the IoNanos knockdown seemed to have specific effects on 4d-derived organs, we directly compared this phenotype to the effects of 4d ablation. We killed 4d with a glass needle, removed the cell, and scored the larvae in parallel to the IoNanosMO animals. As expected from lineage tracing and ablation data (Clement, 1986c; Render, 1997), 4d deletion embryos were always lacking heart, intestine and normal retractor muscle (Figures 2.5 and 2.7; Table 1). More surprisingly, these larvae also had some mild ectodermal defects that would not be predicted from the clonal contribution of 4d. Their shells were sometimes smaller, and rotated counterclockwise relative to the head, and the development of the foot mass was often perturbed. Thus, the 4d ablation phenotype is very similar to the effect of 0.1 mM IoNanosMO injection, and shows that 4d is required for the normal development of some ectodermal structures. Some of the effects on ectodermal structures may reflect events in organogenesis. The loss of retractor muscle may cause the abnormal shell orientation by perturbing the process of torsion (Wanninger et al., 2000), and other defects in the shell may reflect requirements for 4d-derived tissues like intestine in normal shell development (Cather, 1967). However, the effects of 4d ablation on the foot structures are not easily explained as a secondary effect of losing 4d-derived structures, and are more likely caused by a failure of 4d to complete organizer signaling in the early embryo. Organizer signaling is a well-established role
for the mother cell of 4d, the 3D macromere (Clement, 1962; Lambert and Nagy, 2001), but has not been generally appreciated for 4d. However, one report of 3D ablations done shortly before the cell divides indicates it does not complete induction, implying that induction may be completed by 4d (Sweet, 1996). Also, ERK1&2 MAPK activation, which is required for organizer function, is observed in 4d (Lambert and Nagy, 2001). In the closely related snail *Crepidula fornicata* all of the organizer signaling seems to be carried out by 4d (Henry et al., 2006).

There are differences between the effects of IoNanos knockdown and 4d deletion. Most notably, knockdown results in smaller velar lobes and loss of eyes in some cases. These effects are not seen after 4d deletion (Figure 2.5 and Table 1). One possible explanation is that in the deletion experiments 4d is intact for 10-15 minutes after its birth (to ensure that we do not kill its sister cell 4D) and 4d could be signaling in this interval. Another possible explanation is that IoNanos expression observed before 4d in other lineages may be required for normal development.

The patterns of RNA and protein localization, in combination with the congruence of the IoNanosMO phenotype with the 4d fate map and deletion data, indicate that IoNanos is required for normal development of the 4d lineage in *Ilyanassa*. This is striking since clear roles for Nanos in the development of somatic tissues have not been found outside of the insects. Our results are similar to those recently reported in the leech *Helobdella robusta*, where Nanos knockdown causes embryonic arrest during
gastrulation, and defects in the behavior of the ectodermal and mesodermal
germinal bands, which are derived from teloblast cells (Agee et al., 2006). Thus, it may be that the role of Nanos in somatic patterning dates to the base
of the spiralian clade, which makes up a large part of the lophotrochozoan
assemblage (Halanych, 2004). The similarities between the role of Nanos in
spiralian and insects (Lall et al., 2003; Lehmann and Nusslein-Volhard,
1991) suggests that it may have been in place at the base of the protostome
bilaterians. Notably, the 4d lineage roughly corresponds to the posterior of
the fate map during gastrulation in spiralian.

Nanos expression is associated with the germline across the Metazoa,
and in some cases Nanos function has been shown to be required for
germline development (Forbes and Lehmann, 1998; Subramaniam and
Seydoux, 1999; Tsuda et al., 2003; Wang et al., 2007). In some spiralian,
the germline appears to derive from 4d (Rebscher et al., 2007; Woods, 1932).
However, it is not clear whether the germline is specified during
embryogenesis in Ilyanassa. Primordial germ cells have not been identified in
the larva and expression of a germline marker, Vasa, does not persist after
the first few divisions of 4d (Swartz et al., 2008). Since we currently cannot
assess the differentiation of germline derivatives, the question of the effect of
Nanos knockdown on the germline remains open.

The effect of IoNanos knockdown on multiple organs derived from 4d
suggests that the protein may be required for fundamental properties of this
lineage, rather than simply acting as a switch for a particular fate or subset of
fates. By 24 hours after the birth of 4d, the highly asymmetric and stereotypical divisions of the two large 4d teloblast cells have generated ten smaller cells on each side of the embryo in a regular arrangement (Figure 2.1). To examine the effects of the IoNanos knockdown on the behavior of this lineage, we labeled 4d with the lineage tracer Dil in embryos that had been injected with 0.1 mM IoNanosMO as zygotes (Figure 2.9). In these embryos, 4d is born on schedule, 3.5 hours before the other fourth-order micromeres (4abc). However, after knockdown the ensuing divisions in the lineage lack the regularity and the cell size asymmetry observed in wild-type embryos. At 24 hours after the birth of 4d, the arrangement of 4d lineage cells in knockdown embryos is highly variable, and does not resemble wildtype lineages. The large size disparity in control lineages between teloblasts and other cells was reduced by IoNanos knockdown, indicating that the asymmetry of the divisions in the lineage is reduced (Figure 2.9E). The IoNanosMO lineages have around 40% fewer cells than controls (Figure 2.9F), indicating that the proliferative capacity of the teloblasts is impaired. Together, these results indicate that the cleavage program and division asymmetry of the teloblast cells of the 4d lineage are disrupted after IoNanos knockdown. This is similar to the effects of Nanos knockdown in the leech *Helobdella*, which disrupts the proliferation of the teloblasts and the behavior of the teloblast progeny cells. These results imply that Nanos has a conserved role in blast cell function in spiralian, and suggest that Nanos orthologs may be involved in teloblast function in other protostomes. In the
Drosophila ovary Nanos is required to maintain self-renewal of germline stem cells (Forbes and Lehmann, 1998; Gilboa and Lehmann, 2004; Wang and Lin, 2004). The requirement for IoNanos for normal blast cell proliferation, division asymmetry and progeny cell differentiation suggests that this protein may be involved in maintaining stem cell-like properties in some somatic lineages as well.
Figure 2.9 (legend follows)
Figure 2.9 The effects of IoNanos knockdown on the teloblastic cleavage pattern.
The 4d lineage was labeled by Dil injection of 4d in control embryos (A and B) or IoNanosMO1-injected embryos (C and D). Embryos were fixed at 4d+24h. Dil is red, and nuclei are stained with DAPI (blue). The cells in the lineages are shown in B and D. The cells are identified in B (with the teloblast highlighted in red), but the cleavage pattern is irregular after IoNanosMO injection so cells could not be identified. (E) Cell volumes were measured by confocal microscopy of IoNanosMO and control embryos that were labeled with Dil in 4d as above. After injection, they were reared to 4d+24h, fixed, and stained with phalloidin to visualize cell boundaries. Each labeled cell was traced in those Z-sections where it appeared, and sections were summed to estimate total volume. Since the teloblasts were not identifiable in knockdown embryos, we ranked cells in each lineage by volume and plotted the mean size across embryos for each size rank. In control embryos, the teloblasts were always the largest two cells. IoNanos knockdown reduces the size asymmetry of cells within the 4d lineage, by reducing the size of the largest cells and increasing the size of the smaller cells. Measurements are from 10 embryos of each type, and error bars show 95% confidence intervals around the mean, which were calculated for all size ranks where n=10; so size ranks 21-23 in the control and size ranks 11-16 in the IoNanosMO curves do not have error bars because not all ten embryos in the respective samples had the requisite number of cells. (F) Mean cell number in the 4d lineage at 4d+24h, in controls and IoNanosMO-injected embryos. Error bars show 95% confidence intervals around the mean. Controls ranged from 17-23 cells in the 4d lineage, and had a mean number of 19.9 cells, with 11/16 embryos having 20 cells. IoNanosMO embryos ranged from 10-20 cells in the 4d lineage, and had a mean number of 12.2 cells. The embryos where cell volumes were calculated for (E) are a subset of those where cell numbers were determined for (F).
Materials and Methods

Animal collection and husbandry

Adult *Ilyanassa obsoleta* were collected at Western Bay, near Mount Desert Island, ME or Peconic Bay, NY, or obtained from the Marine Resources Center at the Marine Biological Labs, Woods Hole MA. Animal care and embryo collection have been described (Collier, 1981).

Nanos cloning

The IoNanos clone recovered in a previously described *in situ* screen (Kingsley et al., 2007) was 2236 bp. Northern blotting showed that the mRNA was ~3.5 kb, so we performed 3' RACE on a poly-T primed cDNA pool and obtained a 3' fragment that extends the original clone 1238 bp, and performed 5' RACE on a pool primed from a ligated linker and recovered 14 additional bases; the final clone is 3490 bp (Genbank EU087572).

In situ hybridization and antibody staining

*In situ* hybridization was performed as described (Kingsley et al., 2007). Polyclonal antibodies were generated to a peptide corresponding to a portion of the IoNanos amino acid sequence and affinity purified against the peptide. Embryos were fixed for 20 minutes in PEM (100 mM PIPES, pH 6.9, 10 mM EGTA and 1 mM MgSO$_4$) with 4% paraformaldehyde and 0.1% Triton-X, then washed and stored in PBTw (Phosphate buffered saline with 0.1% Tween),
Antibody staining and YOYO-1 (Molecular Probes) staining of nuclei were carried out as described (Lambert and Nagy, 2001). Z-stacks of stained embryos were captured on a Leica SP confocal microscope and projected using Leica software. For the fluorescent images presented, all collection and processing parameters were kept constant to allow comparisons between panels.

IoNanos knockdown, 4d ablation and scoring

Zygotes were injected with the indicated concentrations of IoNanosMO (Gene Tools, LLC) in 1X injection buffer and 1% FITC-dextran. Full length antisense IoNanos RNA was synthesized with T3 RNA polymerase, and 5’ caps were added (mScript, Epicenter). Antisense RNA was injected at 1 ug/ul. Control sense RNA was a fragment corresponding to positions 2013-3490 in the IoNanos transcript (a portion of the 3’ UTR), and was injected at 525 ng/ul (roughly equimolar to the antisense RNA). For ablations, 4d cell was pricked freehand with a pulled glass needle, which caused it to detach from the embryo. Manipulated or injected embryos and controls were reared to the larval veliger stage in separate dishes of .22 um filtered artificial sea water (FASW) for 7 days (hatching stage). While still alive, 7-day old veliger larvae were individually scored for beating hearts and then relaxed in 2:1 FASW:saturated chlorobutanol FASW at 4°C, then fixed together using 3.7% formaldehyde in FASW. Veligers were fixed overnight at 4°C and then washed twice with PBTw. The fixed veligers were co-stained with DAPI (500
ng/ml) and phalloidin (33 nM) and mounted in 80% glycerol in PBS. Veligers were then individually scored at 400X for each of the structures noted in Figure 2.5 and Table 1. Sketches of larvae were made by tracing from images taken at several focal planes of the same sample.

**Lineage tracing and cell volume analysis**

The fluorescent dye DiL was resuspended in EtOH at 100 mg/ml, then diluted 1/20 in soybean oil (Henry and Martindale, 1998a). For confocal analysis, we mixed DiL 1:1 with DiD, which emits in the far red range. We pressure-injected 4d with 1-2 small drops of oil, then fixed embryos in 3.7% formaldehyde in filtered artificial sea water with 100 mM EDTA to preserve the label (Henry et al., 2001). To calculate the cell volumes, we stained embryos with phalloidin-Alexafluor-488 (33 nM) to visual cell boundaries, then optically sectioned embryos at 1 um intervals on a Leica SP confocal. Cross-sectional areas of stained cells were measured in sections where they appeared using ImageJ (Rasband, 2007), and cell volumes were estimated as the sum of the areas of sections for a cell.

**Injection of Ilyanassa embryos**

Prior attempts in several laboratories to develop a pressure injection protocol for *Ilyanassa* have been unsuccessful, essentially because the cells invariably lose a substantial amount of cytoplasm from the point of injection and do not heal. We have optimized several parameters to develop a robust injection
protocol with a high survival rate. Injections are carried out at a pressure of 2000 hPa, using a Narshige micromanipulator and Eppendorf Femptotip needles, or needles pulled to similar specifications using thin wall glass and a Sutter horizontal puller. *Ilyanassa* zygotes produce three polar lobes (PL), vegetal extrusions that accompany the meiotic divisions of the fertilized egg (PL1 and PL2), and the first mitosis (PL3). In our hands, consistently good survival rates were only obtained during a 15 minute window following the retraction of PL2. Since zygotes are extremely fragile during the extended phase of each polar lobe, they are removed from their capsule at least 20 minutes before the start of PL2. Then, immediately after the lobe retraction, embryos are pipetted in a groove scratched into a polystyrene dish with the corner of a glass slide. We inject in FASW supplemented with 5% Ficoll 400, which suppresses cytoplasm loss, perhaps because of the increased viscosity. Control embryos left in 5% Ficoll-FASW solution for more than 15 minutes often suffered developmental abnormalities. At the correct stage, the zygotes normally orient with animal pole up in the injection dish, and in our hands, the highest survival rates were obtained when the site of injection was at an angle ranging from 10° - 60° from the animal pole. Injections deliver around 1% of the total zygote volume. Injection solutions are made in injection buffer (150mM KCl, 10mM Hepes) and supplemented with either 1% FITC-dextran or 100 uM sulforhodamine 101 as a tracer. Before loading microinjection needles, the solutions are filtered through a 0.22 um spin column (Costar). Immediately after the injection, zygotes are transferred to a
new dish of FASW. Then, 3-8 hours post-injection, embryos that have normal morphology and are close to the same stage as synchronously fertilized controls (from the same egg capsule) are transferred to a new dish with FASW. In control injected embryos (1x injection buffer, 1% FITC-dextran, dH2O), 100% of embryos that are morphologically normal and close to the stage of the controls when checked 3-8 hours post-injection have developed into wildtype veligers. In contrast, most embryos that appear morphologically normal but are delayed in cell number at this stage have developed abnormally.

**Antibody development and validation**

We raised antibodies in two rabbits against a peptide corresponding to amino acid positions 237-356 in the IoNanos sequence (NNPDPHTHDVILQARWKVYTR). After three boosts, antibodies from one rabbit were affinity purified against the peptide. The affinity purified sera recognized three bands on Western blots of *Ilyanassa* total protein, one of which was ~47 kD, compared to the 41 KD predicted size of IoNanos protein. Using a gradient of increasingly stringent elutions, we could increase the strength of the 47 kD band relative to the others but we could not remove them. In whole mounts, the staining observed was lost after IoNanosMO injection, indicating that it is specific to IoNanos protein. Pre-incubation of the affinity purified protein with the peptide completely blocked staining in embryos. To verify that the staining pattern we report here is accurate, we
expressed the C-terminal 165 amino acids of IoNanos in *E. coli*, purified protein under denaturing conditions, and inoculated and boosted two rabbits. These sera also recognize the 47 kD band, among others, and also stain the 4d staining pattern described here, in addition to some background staining.
Chapter 3
Developmental role of localized RNAs and cis-acting factors that mediate their transport

Introduction

Spiralian embryogenesis is defined by the characteristic asymmetry and chiral geometry of the early cell divisions (e.g. Figure 3.1). It is also distinguished by similarities in the fate maps of the blastulae produced by these divisions, even across long phylogenetic distances (Henry and Martindale, 1999; Lambert, 2010). Spiralian development is found in several phyla in the large clade of bilateral animals called the Lophotrochozoa. These include the annelids, molluscs, nemerteans, sipunculans, and platyhelminth flatworms. Recent phylogenetic evidence suggests that the last common ancestor of the Lophotrochozoa had spiral cleavage (Dunn et al., 2008), raising the possibility that the taxon Lophotrochozoa should revert to the earlier taxonomic term Spiralia (Schleip, 1929). These results indicate that spiralian development was found in the stem group of one of the three great clades of bilateral animals. This highlights the importance of understanding spiralian development for reconstructing the early evolution of development in protostomes, and by extension, in the bilateral metazoans.

The strong conservation of the cleavage pattern and fate map in these embryos allows homologous cells to be identified in distantly related groups. One of the hallmarks of spiralian development is the regionalization of the
Figure 3.1 IoLR5 mRNA localization and segregation during early cleavage stages

(A) IoLR5 mRNA localization and segregation from 2-cell interphase to the 24-cell stage. The RNA is green, DNA is blue and microtubules are gray. The RNA is localized on the centrosomes during 2-cell stage interphase, and again at 4-cell stage interphase. During prophase, the transcript is transported to the cortex above the nuclei where it resembles a large plaque. In metaphase, the spindle pole orients perpendicular to the RNA plaque and a hole appears in the RNA where the spindle pole approaches the cortex. At the 8-cell stage, the IoLR5 RNA has been inherited entirely by the first quartet micromeres, where it is localized to centrosomes. At the 16-cell stage, the RNA is on the centrosomes in 1q\(^1\), and is also diffuse in the cytoplasm of 1d\(^2\). At the 24-cell stage, the RNA is cytoplasmic in 1q\(^1\) and 1d\(^2\), and is weakly expressed in 1a\(^2\), 1b\(^2\) and 1c\(^2\) where it is localized to the centrosomes. In these embryos, animal pole is towards the viewer and the D quadrant is down. (B) The first quartet micromeres give rise to head ectoderm and the contribution from each micromere (1a, 1b, 1c, and 1d) is shown on an anterior view of a 7-day old larva. (C) Total early cleavage RNA probed with IoLR5 identified bands at ~3.0 and ~3.5 kilobases. (D) Full length IoLR5 transcript. (E) At ~16.5 hours AEL, we can still identify cells in the embryo and in situ hybridization shows abundant IoLR5 expression in 1a\(^{121}\), 1b\(^{121}\) and 1c\(^{121}\) (see Supplemental Figure S1 for a larger image and the identities of other cells in this embryo).
Figure 3.2 IoLR5 in situ on ~16.5 hours AEL embryo

In situ hybridization was carried out using digoxigenin-labeled RNA probes, detected with chromogenic dyes (dark blue/purple), and contrasted with green autofluorescence that appeared after this version of the in situ hybridization protocol. Nuclei were stained with DAPI (blue) and are individually labeled. Question marks indicate uncertainty in cell identification for this embryo. IoLR5 is only seen in 1a<sup>121</sup>, 1b<sup>121</sup> and 1c<sup>121</sup> in this embryo; however, expression was also seen in 1d<sup>121</sup> in a few embryos at this stage.
animal-vegetal axis by the production of the three micromere quartets. The first two cleavages divide the embryo into four macromeres. The micromeres are produced when the four macromeres divide synchronously towards the animal pole in three successive divisions, producing the first, second, and third quartet micromeres (Figure 3.1A). Fate mapping experiments have shown that across spiralian phyla, each quartet generates similar sets of fates (Ackermann et al., 2005; Boyer et al., 1996; Damen and Dictus, 1994; Hejnol et al., 2007; Henry and Martindale, 1998b; Henry et al., 2004; Maslakova et al., 2004; Render, 1997; Weisblat and Shankland, 1985). Typically, the first quartet gives rise to the head ectoderm, including neural structures like eyes, as well as the majority of the primary ciliated band. For instance, in molluscs the first quartet cells generate the ciliated lobes of the head (the velum), as well as anterior neural structures including the eyes (Figure 3.1B). The second quartet usually makes a posterior portion of the ciliated band and trunk ectoderm, while the third quartet normally generates ventral structures and foregut. Most of the mesoderm, including the heart and larval retractor muscle are derived from the 4d cell, the fourth micromere formed by the D macromere.

Embryological experiments indicate that each quartet is born with a distinct developmental potential (Sweet, 1998). After transplantation, members of the first quartet, but not the second quartet, can develop into eyes, consistent with the normal fate of the first quartet. Conversely, second quartet cells, but not first quartet cells, can produce shell material. These
landmark experiments showed that the spiralian micromeres acquire quartet-specific developmental potentials at or shortly after birth, but the molecular basis of this is not known. In the spiralian *Platynereis dumerilii* asymmetries in the levels of nuclear beta-catenin are required in different quartets for normal development. However, this appears to be a general property of divisions in the early embryo and does not provide quartet-specific cues (Schneider and Bowerman, 2007). In *Ilyanassa*, a number of quartet-specific RNAs have been discovered (Kingsley et al., 2007; Lambert and Nagy, 2002). In all cases examined thus far, these RNAs are inherited in a quartet-specific manner after first localizing to macromere centrosomes (Figure 3.1A). Through this segregation mechanism, each RNA becomes localized to distinct sets of micromeres, so that each quartet inherits different subsets of RNAs. This suggests that segregated RNAs may be involved in quartet-specific developmental potential but none of these RNAs have been functionally implicated in development, and the mechanisms that restrict them to particular cells are not known.

The present study focuses on the development of the first quartet micromeres in *Ilyanassa*. First, we show that a particular RNA that is segregated to the first quartet, IoLR5, is required for the normal development of structures derived from this group of cells, the velum and the eyes (Clement, 1967; Render, 1991). Then, we define a narrow interval when centrosomal localization of RNA can occur, and show that this event is necessary for segregation to the first quartet. Finally, we identify a discrete
secondary structure in the IoLR5 RNA that mediates localization to the centrosome and segregation to the first quartet. Our characterization of this structure, and a similar one from another first quartet RNA, suggests a model for how first quartet specificity is achieved.

Results

The IoLR5 mRNA

A cDNA clone named IoLR5 (Ilyanassa obsoleta Localized RNA 5) was recovered during a screen for centrosomally localized RNAs during early cleavage stages (Kingsley et al., 2007). Northern blot hybridization identified transcripts at approximately 3.5 and 3.0 kilobases (Figure 3.1C). Using RACE PCR we extended the original 2055 bp IoLR5 cDNA to 3.5 kb (Figure 3.1D). The two bands on the Northern appear to be due to allelic size variation in a microsatellite (see Experimental Procedures for details).

The pattern of IoLR5 transcript distribution through the 24-cell stage has been described (Kingsley et al., 2007) and is summarized in Figure 3.1A. Briefly, the mRNA is maternally deposited, and then becomes localized to the centrosomes at the 2-cell stage and again at the 4-cell stage. During prophase of the 4-cell stage, the mRNA moves to a region the cortex that will be entirely inherited by the first quartet micromeres during the third cleavage cycle. In the next cleavage of the first quartet cells, the mRNA is again centrosomally localized and segregated to the daughters of the first quartet
cells closest to the animal pole, called \(1a^1\), \(1b^1\), \(1c^1\) and \(1d^1\) or collectively, \(1q^1\). During the 24-cell stage the mRNA is no longer localized to the \(1q^1\) centrosomes and is distributed throughout the cytoplasm. We followed the pattern of IoLR5 further, until the development of the larva known as the veliger. Around 16.5 hours after egg lay (AEL) (~70 cells), the transcript is abundant in \(1a^{121}\), \(1b^{121}\) and \(1c^{121}\) (Figures 3.1E and 3.2). At around 24 hours AEL, all four of the \(1q^{121}\) cells contain the mRNA (Figure 3.3A), and they will shortly divide to produce the \(1q^{1211}\) and \(1q^{1212}\) cells (unpublished results). The A, B and C quadrants have strong expression while the D quadrant is weaker. Also at this stage, expression briefly appears in the four trochoblast cells \(1q^2\) that will make a major contribution to the ciliated band of the head. At ~2 days AEL, IoLR5 mRNA expression is found in 10 cells; two anterior cells bilaterally symmetric at the ventral end of the apical plate (B quadrant cells), and two groups of 4 cells at the anterior that are bilaterally symmetric around the middle of the apical plate (A and C quadrant cells) (Figure 3.3C). At 3 days AEL, the two groups of 4 cells have begun to divide and the mRNA expression is weaker and variable among these cells. Between 3 and 4 days AEL, mRNA becomes less abundant in these cells and their descendants, and is sometimes undetectable. By 5 days AEL, IoLR5 mRNA is no longer detectable by \textit{in situ} hybridization.
Figure 3.3 (legend follows)
Figure 3.3 Late stage IoLR5 mRNA and protein expression

In panels A-G, animal pole is towards the viewer and the D quadrant (dorsal side) is up. (A) *In situ* hybridization on ~1 day AEL embryos shows IoLR5 mRNA is expressed in all 4 cells of 1q^{121} and also in 1q^{2}. (B) Anti-IoLR5 protein staining at a stage slightly later than in (A) when 1a^{121}, 1c^{121} and 1d^{121} have divided. ~2 days AEL, (C) mRNA expression and (D) protein expression, are lost in 1q^{2} and the D quadrant descendants, but are still on in the A, B and C quadrants. An *in situ* hybridization time course with 4 hour intervals ranging from 24 to 48 hours AEL revealed that the IoLR5 expressing cells in panel C are the descendants of 1a^{121}, 1b^{121} and 1c^{121} (unpublished data). After 2 days AEL, mRNA expression decreases in all cells and is totally lost within 3-4 days AEL. (E) By 3 days AEL, the IoLR5-expressing cells in the A and C quadrants have divided to form 9 cells each. Two bilaterally paired cells on the dorsal side of the head also have IoLR5 expression. (F) By ~4.5 days AEL, the A and C quadrant clusters of cells have each divided and can be seen as two distinct clusters on each side of the head. (G) At the early veliger stage, some of the cells in the A and C quadrant clusters are internal, including two putative axons extending from the cerebral ganglia to the foot. H-K are dorsal views of an ~4.5 day AEL embryo stained with the IoLR5 antibody (red) and phalloidin (green) (anterior is down). (H) Projection of Z-stacks for phalloidin only showing the apical plate (white arrow) and the regions where the cerebral ganglia will form (blue arrows). (I) A merged projection of a subset of Z stacks showing the two clusters of the C quadrant lineage that are internalized in the region that forms the cerebral ganglia. The more lateral group is outlined in blue and shown in (J) and and the more medial group is outlined in white and shown in K. Scales bars in A, I and K, represent 100um, 50um and 10um, respectively.
**IoLR5 protein distribution**

While the IoLR5 transcript is 3450 bases long, it has only a single predicted open reading frame (ORF) larger than 50 codons. This ORF is 87 amino acids long (Figure 3.1D) and has a strong translational start site consensus sequence (Kozak, 1987), but no significant homology to database sequences using BLAST searches (Altschul et al., 1990). We raised and validated an anti-IoLR5 antibody (Experimental Procedures), and examined expression on Western blots. Although IoLR5 RNA is maternally deposited, protein is not detected until 24 hours AEL. Protein levels reach their maximum expression at 2 days AEL and begin to decline by 4 days AEL until they are no longer detectable on the Western after 6 days AEL (unpublished results).

Antibody staining on whole mounts shows the IoLR5 protein is cytoplasmic in the same cells as the RNA at 1-2 days AEL, and persists after the RNA is no longer detectable, allowing us to follow the fates of IoLR5-expressing cells into organogenesis (Figure 3.3B, B’, D and D’). The bilaterally paired dorsal IoLR5 cells do not divide in the interval we examined (to 7 days AEL). When eye structures can be recognized, these cells are located directly above the eyes, in regions which likely develop into the tentacles. The paired ventral IoLR5-expressing cells also do not divide in this interval, and they become the ventral-most cells of the apical plate, the region of large cells in the middle of the developing head. The ventral and dorsal pairs of cells both maintain protein expression through 5.5 days AEL. Unlike the dorsal and ventral pairs of cells, each medial cluster of 4 cells begins to
divide ~2.5 days AEL and by 3 days AEL, 9 IoLR5-expressing cells are apparent on each side (Figure 3.3E and 2E’). By 4.5 days AEL, each cluster of 9 cells has split such that the ventral portion (4-5 cells) is a few cell diameters from the dorsal group on each side (Figure 3.3F and F’). Around 5-6 days AEL, the ventral group of cells is completely internal and is part of a densely packed mass of cells that form the cerebral ganglion. Only a subset of the dorsal group of cells is internalized. These cells also appear to join the growing cerebral ganglion, while the remaining cells stay on the velar surface directly ventral to the developing eye, until at least 7 days AEL (Figure 3.3H-K). IoLR5 staining is also observed in two long processes that extend from the cerebral ganglia into the distal region of the foot (Figure 3.3G and G’). These processes include at least two cell bodies, and appear to be axons. Indeed they are in the location reported for the pedal connectives (Dickinson and Croll, 2003). Together, these results show that IoLR5 expressing cells generate several regions of head ectoderm and provide a major contribution to the cerebral ganglion, suggesting that IoLR5 is involved in development of the nervous system in the head.

**Phenotypic effects of IoLR5 knockdown**

To determine the functional role of IoLR5, we injected translation blocking antisense morpholino oligonucleotides (IoLR5MO1) into zygotes and reared embryos to the veliger stage. Injection of IoLR5MO1 resulted in reproducible, dose-dependent phenotypes in the larvae. At a concentration of 0.25mM,
there was no visible effect on development and at concentrations of 1.50mM or greater all animals arrested during or immediately after gastrulation (data not shown). At concentrations ranging from 0.50mM to 1.00mM, animals suffered an increasingly high penetrance of defects in the velar lobes and eyes (head structures; Figure 3.4). A low penetrance of defects in such tissues as the larval retractor muscle, intestine and statocysts were seen at similar levels across various morpholino oligonucleotides (MO) concentrations (Figure 3.4G, individual scoring data are shown in Table 3.1).

The eye phenotypes consisted of animals with no eyes, one eye, or synopthalmia (where the eyes are abnormally close; Figure 3.4A-E’). At 1.00mM, 86% (n= 22) lacked one or both eyes, and 95% had some kind of eye defect when synopthalmia was included. Surprisingly, injected animals that had only one eye were ~3 times more likely to be missing the right eye compared to the left, but the cause of this is not clear (Table 3.1). These defects are extremely similar to those observed after ablation of first quartet cells. For instance, ablation of 1b causes synopthalmia, and ablation of 1a or 1c causes a smaller velar lobe and the lack of an eye on the left or right side, respectively (Clement, 1967). The other phenotypes observed after IoLR5MO1 injection are less penetrant and less dose dependent. The stomodeum and operculum always developed normally. The foot, shell, statocysts, and esophagus developed normally in at least 90% of injected animals. Animals injected with a 1.00mM Standard Control MO always had wild-type development.
Figure 3.4 Knockdown of IoLR5 by injection of IoLR5MO1

(A) Wildtype larva from a zygote injected with the Standard Control MO. Animals injected with IoLR5MO1 developed with several head defects including synopthalmia (B), one eye (C), no eyes (D), and small velar lobes with no eyes (E) (anterior views with dorsal up; scale bar is 100um). (F) Two replicates of Western blot analysis show that IoLR5 protein levels are reduced in IoLR5MO1 injected animals. For replicate 1, total protein was extracted from 47 injected embryos from a single capsule and 47 uninjected embryos from the same capsule. For replicate 2, protein was isolated from 25 embryos from each of two capsules in each lane. (G) Graph showing the percentage of animals with the indicated tissue for each concentration of injected morpholino (Scoring data is in Supplemental Table S1). Effects of the MO were rescued by coinjection with IoLR5MOres mRNA (H). After IoLR5MO1 injection 5/80 larvae (6%) developed with two wildtype eyes, but after coinjection with the IoLR5MOres mRNA, 98/157 (62%) developed two wildtype eyes.
Figure 3.5 Other structures in IoLR5MO knockdown animals

In animals injected with the Standard Control MO, the intestine (blue arrowhead) is seen when viewing from the dorsal side (A and A', anterior is up). Typically the intestine is also seen in IoLR5MO1 injected animals (B and B'). Viewing from the left lateral side, in Standard Control MO animals (C and C'), one can score the wildtype esophagus (orange arrowhead), stomach (green arrowhead) and the style sac (red arrowhead). In IoLR5MO1 injected animals, the shell was occasionally filled with yolk which made scoring internal organs difficult (D and D'). When yolk-filled animals were stained with DAPI, we could occasionally detect many internal structures, based on observations of the nuclei (E and E', D and E are the same animal). For this reason, the stomach, style sac and the digestive gland could not be reliably scored.
Table 3.1. Development of Larval Organs after IoLR5 Knockdown

† Eyes that were only partially formed (small patches of pigment) were not counted as wildtype and account for the missing eye percentages for the 0.50mM, 0.75mM and 1.00mM IoLR5MO1 injections
* These percentages include 1 animal with synopthalmia at 0.75mM and 2 animals at 1.00mM
‡ These structures were not always reliably scored due to presence of yolk within the shell (see 3.5)
≠ The n=’s here only include animals that survived microinjection and developed into animals resembling veligers. Embryos that survived microinjection but died during, or shortly after, gastrulation were not included. For the Std. Control MO and 0.25mM IoLR5MO1 injections, all embryos grew to veligers. Two embryos died with 0.50mM MO injections and 12 embryos died at 0.75mM and 1.00mM each – these animals were not scored.
Two lines of evidence indicate that the IoLR5MO1 phenotype is the result of a specific knockdown of IoLR5 translation. First, Western blot analysis showed that IoLR5 protein in IoLR5MO1 injected animals was either completely absent or strongly diminished as compared to control animals (Figure 3.4F). Second, the eye phenotypes were rescued when the IoLR5MO1 was coinjected with an IoLR5 mRNA with 7 mismatches introduced in the morpholino target site (IoLR5MOres). In this experiment, only 6% of morpholino-injected animals had 2 wild type eyes (n= 80). When IoLR5MOres was coinjected with IoLR5MO1, 62% of the animals developed 2 wild type eyes (n= 157) (Figure 3.4H). Injection of IoLR5MOres by itself had no effect on development and none of the rescue animals developed more than 2 eyes (data not shown), indicating that the transcript is not sufficient for ectopic eye development.

**Segregation requires centrosomal localization**

Since IoLR5 RNA is required for development of first quartet derivatives, we turned to the question of how it is specifically segregated to these lineages. We first addressed whether centrosomal localization *per se* is a required step for asymmetric segregation, by examining the fate of RNAs when centrosomal localization was bypassed.

We found that localization did not occur if RNA was injected after the interval when localization normally occurred. Preliminary experiments showed that an *in vitro* synthesized RNA corresponding to bases 1-2055 of
IoLR5 and labeled with Alexa Fluor 488-5-UTP could recapitulate localization to 4-cell stage centrosomes and segregation to the first quartet micromeres when it was injected into the zygote. To ask whether later-injected RNA also localized, the RNA was injected at various time points from late 2-cell stage until 15 minutes into the 4-cell stage. To enable more precise staging, a lineage tracer (sulforhodamine 101) was co-injected, and embryos were sorted based on whether injection occurred at the 2-cell stage (tracer in two of four cells) or 4-cell stage (tracer in one of four cells). RNA localization was examined every 10 minutes until the mid 8-cell stage, to ensure that only embryos with normal division were included in the analysis. Embryos with representative staining patterns were fixed, stained and imaged with confocal microscopy. When RNA was injected during the 2-cell stage, it always localized to the centrosome (n=16; Figure 3.6A-B). Of the embryos injected after the end of cytokinesis, around half (14/30) had a striking pattern of localization, where RNAs localized to a shallow zone on the surface of the centrosome (Figure 3.6F). The remainder of the injections in this class showed no localization (16/30, Figure 3.6J). To determine whether these two patterns were related to timing of injection, we performed additional injections staged based on the distinct morphology of the embryo in the first five minutes of the four cell stage (Figure 3.6E). We found that (21/24) injections in this interval showed localization to the surface of the centrosome (as in Figure 3.6F), with the remainder showing no localization (as in Figure 3.6J), and that injections 10 minutes later showed no localization (n=33).
shallow localization suggests that the RNA-containing pericentriolar matrix accumulates progressively on the outside surface of the centrosome, with minimal mixing. The presence of both shallow localization and no localization in the embryos injected during the first five minutes of the 4-cell stage indicates that transport to the centrosome ends during this interval.

To evaluate the importance of centrosomal localization for subsequent RNA segregation, we followed the behavior of injected RNAs later in the cell cycle. When RNA did localize to the centrosome it moved to the cortex during prophase and was predominantly asymmetrically segregated into the first quartet micromeres, as observed for IoLR5 RNA injected into zygotes (Figure 3.6C-D'). When shallow localization was observed (as in 4 cell +0-5 min injections), the labeled RNA moved to the cortex as above, but as individual speckles of signal, and only a portion of the RNA was segregated (Figure 3.6G-H'). When RNA did not localize during interphase (i.e. 4 cell +10-15 min), it did not move to the cortex at prophase, and was not segregated asymmetrically (Figure 3.6K-L'). The RNA remained cytoplasmic, until the next interval when it localized to the micromere and macromere centrosomes at the 8-cell stage interphase. This demonstrates that the RNAs remain competent to localize even if they miss the window for transport at the third cleavage (Figure 3.7, also see Figure 3.10). The simplest interpretation of these results is that localization to the centrosome is a required step in the RNA segregation process in this system.
Figure 3.6 (legend follows)
Figure 3.6 Centrosomal localization is required for asymmetric segregation

An in vitro synthesized RNA corresponding to bases 1-2055 of IoLR5 and labeled with Alexa Fluor 488-5-UTP can recapitulate the trafficking of the endogenous RNA when it is injected during the two cell stage (A, bright field lateral view). (B) When RNA was injected at the 2-cell stage, as determined by the presence of the lineage tracer sulforhodamine 101 (red) in two macromeres, the RNA (green) was localized to the centrosomes during 4-cell stage interphase. (projection of confocal stacks; inset shows a single section through the centrosome; DNA is blue; B’ shows the RNA alone). (C) The RNA is on the cortex at prophase, (D) and segregated to the micromeres during cytokinesis. (E) The first five minutes of the four cell stage are characterized by the presence of a constriction in the large D cell (arrowhead). (F) When RNA is injected during this interval, localization is on the surface of the interphase centrosome (inset shows a single section through the centrosome). (G) RNA then moves as speckles to the cortex in prophase. (H) A portion of the detectable RNA in the mother cell is segregated to the micromere daughter. (I) 10 minutes after the start of the 4 cell stage, the constriction of D is not present. (J) Injections during 4-cell +10-15 min. result in no centrosomal localization, (K) or cortical localization, (L) or segregation. Sample sizes indicated in the text are for embryos that were scored in vivo and followed through the next cleavage; they do not include the representative embryos shown here, which were fixed at the indicated time points. A, E, and I are lateral views; the remainder are from the animal pole. Scale bar is 100um.
Fig. 3.7 8-cell localization of 2 and 4-cell injected embryos
To determine if RNAs which did not localize at the 4-cell stage (due to the timing of injection) were still capable of localization, we examined the fluorescently-labeled transcripts (green) at the 8-cell stage. Animals injected at the end of the 2-cell stage (as determined by presence of sulforhodamine 101 – red) segregated most of the transcript to the first quartet and strong centrosomal localization was seen in these cells, while weak centrosomal localization was also seen in the macromeres (A; this animal was originally injected in the AB blastomere). Animals injected at 4-cell +10-15 minutes did not have proper IoLR5 localization to the first quartet cell and the transcript became evenly distributed between the micromere and macromere (B). At 8-cell interphase, strong localization could be seen in both of these cells (B). To highlight the localization of the fluorescent transcript, A’ and B’ show the green channels only for A and B respectively.
The IoLR5 localization element

The large number of RNA localization and segregation patterns in the Ilyanassa embryo suggests that the control of the process in this system is very intricate. One way that this could be generated is by quartet-specific RNA localization elements (similar to Martin and Ephrussi, 2009; St Johnston, 2005). To test if RNA segregation in Ilyanassa relies on such a localization element (LE), we adapted the fluorescent assay described above to map RNAs for regions involved in centrosomal localization. Briefly, digoxigenin-labeled RNAs transcribed from various regions of the cDNA were injected into zygotes and detected at 4-cell interphase to test for localization. Using the original IoLR5 clone (bps 1-2055) we were able to recapitulate centrosomal localization in vivo after injection into zygotes (compare Figure 3.8B to 3.8A), while an antisense transcript of IoLR5 bases 1-2055 failed to localize (data not shown). When labeled IoAlpha-Tubulin was injected, the labeled transcript remained ubiquitous in the cytoplasm (Figure 3.8D), like its endogenous transcript (Figure 3.8C). Together, these results show that this approach is effective and specific.

We mapped the localization activity of the original clone by testing various fragments, and found that localization required a region around base 1260 (Figure 3.8E). In silico folding of the entire IoLR5 RNA with RNAstructure 4.6 (Mathews, 2006) predicted a large stem loop consisting of 118 bases at bases 1194-1312 (Figure 3.8P). This structure was assigned the highest probability of any in the sequence, and was the only structure
found in each of the top 20 secondary structure predictions, suggesting that
the 118 base stem loop is likely present in vivo. Using primers flanking the
stem loop (1174-1334), we tested this small RNA and discovered that it was
in fact able to localize to the centrosome (Figure 3.8F). We then deleted this
118 base region from IoLR5(1091-1802) and this caused the RNA to remain
ubiquitous in the cytoplasm (Figure 3.8I). These results demonstrate that this
region is necessary for localization to four cell centrosomes, and sufficient to
localize itself. To test whether the predicted secondary structure was
sufficient to localize a normally unlocalized RNA, we inserted the stem loop
structure into the IoTis11 RNA, which does not normally localize to the 4-cell
stage centrosomes (Kingsley et al., 2007 and Figure 3.8L), and found that the
resulting RNA does localize to 4-cell stage centrosomes, demonstrating that
the stem loop is sufficient to confer localization (Figure 3.8L and M). We refer
to this 118 base region as the IoLR5 localization element (IoLR5LE).

To examine whether the proper secondary structure of the LE is
important for its role as a centrosomal localization signal, we took advantage
of the fact that MOs can be used to disrupt RNA secondary structure (e.g. in
splicing or miRNA biogenesis, Draper et al., 2001; Kloosterman et al., 2007).
We designed a MO targeted to a region near the center of the IoLR5LE stem
loop (IoLR5StemBlockMO; Figure 3.8P). Injection of this molecule into
zygotes caused a significant impairment of localization of the endogenous
IoLR5 transcript (Figure 3.8O), while injection of the translation blocking MO
did not affect localization (Figure 3.8N). This novel approach suggests that
the secondary structure of the IoLR5LE is important for centrosomal localization. Despite the partial inhibition of centrosomal localization at the 4-cell stage, these animals still developed into apparently wildtype veligers.

After centrosomal localization, endogenous RNAs move to the cortex, and are segregated to micromere daughters. We injected zygotes with digoxigenin-labeled IoLR5LE and allowed embryos to develop to 4-cell stage prophase (when the IoLR5 transcript is localized to the cortex) and to 8-cell stage interphase (when the IoLR5 transcript would be asymmetrically segregated to the micromeres). The IoLR5LE localized to the cortex during prophase and was asymmetrically segregated into the 8-cell stage micromeres (Figure 3.8F-H). In contrast, the LE deletion transcript remained cytoplasmic during the entire 4-cell stage and was never asymmetrically segregated (Figure 3.8I-K). These data demonstrate that the IoLR5LE is necessary for all phases of the segregation process. While the IoLR5LE was capable of recapitulating all of the movements seen with the segregation of the endogenous RNA, we note that it does so with decreased specificity. When the endogenous RNA is segregated during 4-cell stage cytokinesis, all of the transcripts are delivered to the first quartet and none of the RNA is detectable in the macromeres. For the RNAs which we inject, we have high specificity for centrosome localization during interphase; however, not all of the injected transcripts become asymmetrically segregated as some are still visible in the macromeres at the 8-cell stage (see Figures 3.8H, 3.7 and 3.10).
Figure 3.8 (legend follows)
Figure 3.8 Mapping the IoLR5LE

(A) *In situ* hybridization shows that IoLR5 mRNA is localized to the centrosomes at 4-cell interphase, (C) while IoAlpha-Tubulin mRNA is cytoplasmic. (B) An RNA transcribed *in vitro*, corresponding to bases 1-2055 of IoLR5 and labeled with digoxigenin-UTP, was injected into zygotes and found to be centrosomally localized at 4-cell interphase, (D) while a labeled IoAlpha-tubulin mRNA remained cytoplasmic. (E) Various regions of IoLR5 were transcribed *in vitro* and tested for centrosomal localization. Regions that localized are shown in green and regions that did not are shown in red. (P) The smallest region capable of transport to the centrosome (1174-1334) is predicted to form a single large stem loop secondary structure from bases 1194-1312 (IoLR5LE). (F) The labeled IoLR5LE RNA is able to localize to the centrosomes at the 4-cell stage, (G) to the cortex during metaphase, (H) and becomes asymmetrically segregated into the first quartet after cytokinesis. In H, not all of the injected RNA was asymmetrically segregated and the transcripts which were left behind in the macromeres localized to the centrosomes at the 8-cell stage (the 8-cell centrosomes are out of the plane of focus). (I) A region of IoLR5 with the LE deleted bases (1091-1802) was unable to localize to the centrosome, (J) or cortex, (K) and did not become asymmetrically segregated after cytokinesis. (M) The LE was sufficient to drive centrosomal localization of an RNA (IoTis11+IoLR5LE) that is not normally localized at the 4-cell stage. (L) No localization occurs when a digoxigenin-UTP labeled IoTis11 transcript is injected. (N and N’) Embryos from the same capsule were injected with either the IoLR5MO1, or (O and O’) a morpholino designed to block the center of the LE (StemBlockMO; target site is indicated by red bracket in P) with a fixable dextran marker (Oregon green). Embryos were fixed at the 4-cell stage, mixed, and processed for *in situ* hybridization together in the same well. The embryos injected with the StemBlockMO were identified by the presence of Oregon green (O’, green channel). Bright field images for N and O were taken with the same settings. Scale bar is 100uM. Nuclei are stained with DAPI (light blue) and RNAs are shown in dark blue/purple.
Another first quartet RNA requires an element with a similar secondary structure for localization

During the third cleavage, theIoLR1 RNA is temporally and spatially coincident with IoLR5 (Kingsley et al., 2007). The original cDNA clone was 3.6 kb, and the complete transcript is ~9.6 kb, based on Northern blotting (unpublished data). We tested several fragments of the original 3.6 kb clone for localization to 4-cell stage centrosomes (Figure 3.9A-C, and E), and found a 1 kb region that could localize. We folded this fragment using RNAsstructure 4.6 and three large stem loops were found to be highly probable. Each of these was individually tested for 4-cell localization, but only one localized (Figure 3.9C, E and F). When this region was deleted from the 1 kb fragment, centrosomal localization was abolished (Figure 3.9D), showing that this 88 nucleotide sequence is necessary for localization of IoLR1. We refer to this region as the IoLR1LE.

We have attempted to directly test whether the two localization elements are using the same machinery through competition experiments. Through overexpression of a localized RNA, we attempted to saturate the transport machinery and see if localization for other RNAs was adversely affected. Several strategies for this approach have been tested and these methods are detailed below.

The first method involves injecting unlabeled IoLR5LE RNA into zygotes, at a 1000x molar excess over the endogenous IoLR5 transcript levels. These embryos were then fixed at 4-cell interphase. In situ
hybridizations for several RNAs which are normally found localized at 4-cell interphase, including IoLR1, IoLR5 and Io-Ubiquitin-ligase, showed that none of these transcripts had any apparent localization defects in the presence of the injected IoLR5LE (data not shown).

In an effort to have more quantitative in situ analyses in the above experiment, a similar approach was used by injecting the unlabeled-IoLR5LE into the CD macromere of 2-cell embryos and fixing at 4-cell interphase. This allowed for a direct comparison of transcript levels between injected and uninjected cells in the same embryo. As above, there was no visible difference in the localization efficiency of the tested RNAs in C and D, as compared to A and B. This suggests that the transport machinery is not limiting, or that the IoLR5 RNA-protein particles are stable and not accessible to the injected RNA. To test the latter possibility, I attempted to inhibit centrosomal transport for an exogenous RNA. Zygotes from the same capsule were injected with either digoxigenin-labeled IoLR1(1059-2045) alone, or with digoxigenin-labeled IoLR1(1059-2045), the unlabeled-IoLR5LE and Oregon-green dextran. These embryos were fixed at 4-cell interphase and processed together to see if coinjection of IoLR5LE was able to inhibit exogenous IoLR1 transport. I was unable to detect any difference in centrosomal localization between the two sets of embryos (data not shown).

Injected IoLR1 RNA does not completely localize to the centrosomes at the 4-cell stage, resulting in detectable levels of the transcript in the cytoplasm. Comparing the cytoplasmic levels for embryos injected with
digoxigenin-labeled IoLR1 alone, or levels when IoLR1 was coinjected with the IoLR5LE was difficult. To obtain a more precise measurement of the exogenous RNA, we repeated the above experiment using a fluorescently-labeled IoLR1(1059-2045) RNA. These animals were injected as zygotes, fixed at 4-cell interphase and examined on a confocal microscope. A region of interest (ROI) taken in the cytoplasm underneath the centrosome in the D macromere, showed no significant difference in the presence of cytoplasmic IoLR1 RNA between the two experiments. RNA was found to be localized to the centrosome in all cases examined and embryos that were coinjected with the competing IoLR5LE RNA, actually showed a higher mean intensity of IoLR1 RNA localized to the centrosomes (data not shown).

In the various competition experiments that were tested, one fact seems to be apparent – RNA transport to centrosome is not limiting. However, this contradicts my results showing that injected IoLR5 RNA is not completely segregated to the first quartet, as some remains behind and localizes to the centrosomes in the 8-cell macromeres (Chapter 4). One possibility that could satisfy these conflicting results could be that while centrosomal transport is not a rate limiting process, subsequent transport to the cortex and eventual segregation, is limiting. In all the competition experiment described above, embryos were only checked for competitive inhibition of centrosomal transport at 4-cell interphase. Repeating these competition experiments, and observing the later stages from prophase
through cytokinesis, will show if later phases of RNA transport are indeed capable of being competed.
Figure 3.9. Mapping the LE for another 4-cell localized RNA, IoLR1
(A) In situ hybridization of the IoLR1 RNA on the centrosomes at the 4-cell stage. (B) A digoxigenin-labeled transcript corresponding to the original cDNA clone for IoLR1 (bases 1-3614) localized at the 4-cell stage. (E) Several regions of the RNA were tested for localization and the smallest region capable of localization was found to be IoLR1(1654-1742) (C). (D) When this region was deleted, the transcript failed to localize. (F) The IoLR1LE is also predicted to form a single large stem loop secondary structure.
Localization of LEs at other stages

We have focused on the control of localization at the 4-cell stage, but examination of the behavior of localization elements at other stages is informative about how the specificity and complexity of localization are achieved. The endogenous IoLR5 and IoLR1 RNAs are both localized to centrosomes at the 4-cell stage, but at the 2-cell stage, only IoLR5 is localized to the centrosomes (Kingsley et al., 2007). We mapped the sequences required for localization of IoLR5 at the 2-cell stage using fluorescently labeled RNAs (see Materials and Methods). We discovered that the same region required for 4-cell localization, IoLR5LE, is also necessary for centrosomal localization at the 2-cell stage (Figure 3.10A and B). We next tested 2-cell stage localization for IoLR1(1059-2045), which contains the LE for 4-cell stage localization. Surprisingly, this injected RNA was occasionally capable of weak localization at the 2-cell stage (Figure 3.10C). Since the IoLR1 fragment only had weak localization and was variable, we did not attempt fine-scale mapping for this region.

The endogenous IoLR5 and IoLR1 transcripts are highly specific for the first quartet and are not found in the second or third quartet micromeres. Since our injected RNAs do not have the same specificity for segregation as the endogenous transcripts, we were able to follow injected RNAs later in development and test for localization in cells where these messages are not normally found. We discovered that the injected RNAs are capable of localization to macromere centrosomes at the 8-cell stage (Figure 3.10D and
E). Furthermore, at the 24-cell stage, when the first, second and third quartets are all present, we found that the injected RNAs were centrosomally localized in all cells of the embryo (Figure 3.10F and G), even though the endogenous RNAs are never observed in many of these cells. Similarly, the IoLR5LE was even centrosomally localized in the 1q<sup>1</sup> cells at the 24-cell stage, even though the endogenous message is always cytoplasmic at this stage. These results indicate that transcript abundance is tightly regulated to ensure proper distribution of segregated RNAs and they hint that other mechanisms are in place that can prevent transcript localization, even when a LE is present.
Figure 3.10 (legend follows)
Figure 3.10 IoLR5 and IoLR1 localization at the 2, 8 and 24-cell stages
(A) Using fluorescently labeled segments of IoLR5 RNA, we mapped the region required for centrosomal localization at the 2-cell stage (green bars represent regions that localized and red bars indicate regions that did not localize; dotted lines indicate inferred required region). (B) Projection of confocal stack showing centrosomal localization of fluorescently labeled IoLR5(1194-1802) fragment (RNA is green, DAPI is blue). (C) A fragment of IoLR1(1059-2045) containing the 4-cell LE could localize to 2 cell centrosomes, but this was only observed occasionally and was very weak. At the 8-cell stage, IoLR5(1194-1802) (D) and IoLR1(1059-2045) (E) were localized in the first quartet micromeres, as well as the macromeres. At the 24-cell stage, IoLR5LE (F) and IoLR1(1059-2045) (G) RNAs were localized in the first quartet micromeres (yellow arrowheads show 1a₁), the second quartet micromeres (blue arrowheads show 2d₂) and the third quartet micromeres (red arrowheads show 3d). While fluorescent RNAs show the same pattern of 24-cell localization, digoxigenin-labeled probes were used here due to increased sensitivity (see Supplemental Material).
Discussion

One of the hallmarks of spiralian development is the patterning of the animal-vegetal axis by the production of tiers of cells, called quartets, with different developmental potentials, but the mechanisms that control this are unknown. Our results show that a segregated RNA is functionally important for quartet-specific developmental potential. The IoLR5 mRNA is specifically segregated into the first quartet of micromeres, which generate the larval head. Inhibiting IoLR5 translation impairs the development of larval head structures, particularly the eyes and velum, demonstrating the functional role of the protein in development. There is no IoLR5 expression in the cells that become the eyes, indicating that the effect is non-autonomous. The eyes develop in the optic ganglia which arise in contact with the cerebral ganglia where IoLR5 protein is found (Gibson, 1984; Lin and Leise, 1996). Thus, our results suggest that proper development of the eyes or optic ganglia may require previously unappreciated signaling cues from proneural or neural tissues.

Our experiments suggest a model of how first quartet-specific localization arises. We characterized an RNA element that mediates localization to the centrosome at the 4-cell stage, and segregation to the first quartet. We found a similar element in another RNA that is segregated to the first quartet, and we propose that these two elements define a class of RNA sequences that mediate localization to the first quartet and thus contribute to first quartet identity. These elements are also capable of mediating
segregation of injected RNAs to other quartets, even though the endogenous messages are only segregated into the first quartet. Thus, our data indicate that specific first quartet localization is achieved by ensuring that no significant population of a first quartet RNA molecule remains in the macromeres to be segregated in the next mitosis. This seems to be achieved by highly efficient transport mechanisms, and in some cases, by RNA decay. The high capacity and efficiency of transport is supported by several lines of evidence. First, for most first quartet RNAs, like IoLR5 and IoLR1, we cannot detect RNA in the macromeres right after the division occurs. Second, we have found that centrosomal localization can occur as late as 5 minutes after the beginning of the 4-cell stage, but that endogenous IoLR5 and IoLR1 RNAs are localized well before this, during cytokinesis (see Kingsley et al., 2007). Finally, we have directly attempted to saturate the transport machinery by injecting high molar excesses of IoLR5LE RNA, but are unable to competitively inhibit endogenous IoLR5 or IoLR1 RNA, or injected IoLR1 RNA, from localizing to the centrosome. RNA decay also appears to play a role in maintaining quartet-specificity. In at least one case, where a fraction of RNAs do remain in the macromeres, specificity can be achieved by decay of the message in the macromeres. This occurs with the IoEve mRNA, which is then restricted to the first quartet (Lambert and Nagy, 2002). First quartet-specific segregation thus appears to be controlled by a combination of specific RNA secondary structural elements and regulation of transcript abundance.
We have showed that RNAs can only be segregated if they are present in the cell at a time when they can go to the centrosome. It may be that there is an event that is critical for segregation that is concurrent with centrosomal localization but not dependent on it, but this seems improbable. Segregation might require centrosome localization because the RNA undergoes processing when bound to the centrosome which allows for the subsequent cortical localization. A simpler model is that all of the pericentriolar matrix material is transported *en masse* to the cortex.

There are a number of aspects of RNA localization in the early *Ilyanassa* embryo that remain unclear. We do not understand why our injected RNAs can perfectly recapitulate the specificity of centrosomal localization, but are less specific later in the cell cycle and during segregation. We also do not understand how the transition to cytoplasmic distribution is controlled: injected IoLR5 is centrosomal in the 1q\(^1\) cells of the 24 cell stage, but endogenous IoLR5 is cytoplasmic in these cells. Finally, despite the fact that we have identified sequence elements that will segregate into the second and third quartets, we do not know how localization to other quartets occurs. We have injected putatively full-length RNAs for several transcripts that are segregated to the second and third quartets and never observed localization (unpublished results), suggesting that the mechanism is not analogous to first quartet localization.

For the localized RNAs that have been characterized in other organisms, such as Bicoid, Oskar and Vg1, translation of the transcript is
typically required soon after localization or fertilization of the oocyte (Kugler and Lasko, 2009; Martin and Ephrussi, 2009; St Johnston, 2005). The IoLR5 mRNA is asymmetrically segregated very early during embryogenesis, during the third cleavage, and it is reiteratively segregated through several more rounds of divisions, resulting in its localization in particular descendants of the first quartet at the 24-cell stage. After this, the mRNA is no longer asymmetrically segregated and protein expression is seen in just a few hours. The protein is maintained in these cell lineages for several days and is eventually expressed in differentiated neurons; it is also required for development of the eyes, which form relatively late in organogenesis. The long interval of progressive segregation is striking. It suggests that for at least some factors, very fine scale patterning is achieved by sequential segregation, rather than a hierarchical network of patterning factors that progressively subdivide the embryo, as observed frequently in somatic patterning of other systems. While it is uncommon for somatic RNAs to be regulated in this manner during embryogenesis, with the exception of several RNAs studied in ascidian embryos (Nishida and Sawada, 2001), this mode of patterning is very common for germ plasm. In many organisms, determinants for the germ plasm are often segregated through several rounds of cell division to the lineage which will form the germ line (Lehmann and Ephrussi, 1994; Seydoux and Schedl, 2001). This relationship to germ plasm is intriguing because the centrosome-mediated RNA localization seen in *Ilyanassa* may be homologous to a structure involved in patterning oocytes
across the animal kingdom - the Balbiani Body (Lambert, 2009). In *Xenopus laevis, D. melanogaster, and D. rerio* RNAs localize to this structure during oogenesis before becoming transported and anchored to the cortex, similar to the asymmetrically localized RNAs in *Ilyanassa* (Cox and Spradling, 2003; Heinrich and Deshler, 2009; Kloc et al., 2004b; Marlow and Mullins, 2008). Balbiani bodies are involved in localization of factors that are important for somatic patterning and germline formation. At least in frogs, where the ultrastructure of the developing Balbiani body has been followed, it arises from the oocyte centrosome (Kloc et al., 2004a). We propose that *Ilyanassa* has extended this conserved patterning mechanism from the oocyte to early cleavage divisions.

This work raises the possibility that all spiralian embryos rely on centrosome-mediated RNA segregation to pattern the micromere quartets. Consistent with this, several putative patterning genes have been shown to be localized to centrosomes and then asymmetrically segregated in the snail *Crepidula fornicata* (Henry et al., in press). Similarly, multiple RNAs are localized to the centrosomes in the surf clam *Spisula*, although this work did not follow the RNAs during cell divisions to see if they are asymmetrically segregated (Alliegro et al., 2006). Given the strong conservation of the cell fates produced by spiralian micromere quartets, it seems likely that there are also regulatory factors whose role in generating these fates is also conserved across this group. As such factors are identified, it will be interesting to see how their quartet-specificity is established in diverse spiralian embryos.
Materials and Methods

Animal Collection and Husbandry

Adult *Ilyanassa obsoleta* were collected from the Bass River on Cape Cod, MA, the Peconic Bay in Riverhead, NY, Ocean Isle Beach, NC, or ordered from the Marine Resources Center at the Marine Biological Labs in Woods Hole, MA. Animal care and collection have been described (Collier, 1981; Gharbiah et al., 2009)

IoLR5 cloning

The IoLR5 clone isolated from a previously described *in situ* screen (Kingsley et al., 2007) was 2055 bp. Northern blot hybridization identified two transcripts approximately 3.5 and 3.0 kilobases. We performed 3’ rapid amplification of cDNA ends (RACE) on a cDNA pool primed from a ligated linker and obtained an additional 1395 bp. 5’ RACE on a pool primed from a ligated linker yielded no additional bases. A large TC microsatellite was uncovered in the new 3’ fragment that was too large to fully sequence. Attempts to sequence from the 5’ and 3’ ends of the microsatellite revealed ~75-100 continuous TC repeats before the signal deteriorated. In addition, we were unable to cut this region smaller than 850 bps using a mixture of four 4-cutter restriction enzymes (Alu1, Msp1, Sau3a1, Mse1, not shown), further suggesting that the TC repeat is continuous through the microsatellite. PCR
performed on genomic DNA, using primers flanking the microsatellite, produced two bands ~850 and ~400 bps. Consequently, the final clone is ~3450 bp (EU087578.1) and the 3.0 bp band seen on the Northern is likely due to allelic variation in the microsatellite. The full IoLR5 construct was made using standard restriction digestion and ligation techniques. We made a morpholino resistant IoLR5 construct (IoLR5MOres) by using fusion-PCR (Szewczyk et al., 2006) to alter 7 nucleotides in the morpholino binding site. IoTis11+IoLR5LE was made by inserting the IoLR5LE at base pair 47 in the original IoTis11 cDNA clone, using standard restriction digestion and ligation techniques.

Antibody development and validation

We raised antibodies in one rabbit against amino acids 71-82 (QMRMRERNYRVG) of the IoLR5 predicted protein, using the QuickDraw protocol at Pocono Rabbit Farm and Laboratory Inc. Antibodies were affinity purified from the rabbit’s serum against the synthesized peptide. In Western blot analysis using Ilyanassa total protein (isolated from zygote through larval stage embryos), the affinity purified antibody (at 1:1000 dilution) recognized two distinct bands at ~12kD and ~14kD, close to the 9.6kD predicted size of the IoLR5 protein. When animals were injected with the IoLR5MO1, both bands on the Western were lost. Antibody staining of 1 day old whole mount embryos stained the same cells where the RNA was identified by in situ analysis. Colocalization of RNA and protein continued through 48 hours AEL,
after which RNA expression was gradually lost and protein expression was maintained for ~3.5 more days in the developing head. All antibody staining was lost in whole mounts injected with IoLR5MO1, further indicating the specificity of this antibody (unpublished data).

In situ hybridization and antibody staining
Embryos were fixed and processed for in situ hybridization as previously described (Kingsley et al., 2007). For antibody staining, embryos were fixed with 3.7% formaldehyde in FASW for 1-2 hours, washed twice with PBTw and stored at 4°C. Some animals were washed in MeOH before the PBTw washes, but this had no visible effect on whole mount staining. Antibody and DAPI staining were carried out as previously described (Lambert and Nagy, 2001), using a 1:800 dilution of the affinity purified IoLR5 antibody.

IoLR5 knockdowns and scoring
Zygotes were injected with the indicated concentration of IoLR5MO1 (sequence: 5’-GCTGGTGATACAACTCTTGATGACA-3’) or the Standard Control MO (sequence: 5’-TATAAATTGTAACTGAGGTAAGAGG-3’) (Gene Tools, LLC), 1x injection buffer and 1% fluorescein isothiocyanate (FITC)-dextran. Microinjections were performed as previously described (Gharbiah et al., 2009; Rabinowitz et al., 2008). Injected embryos were reared for 7-15 days in .22 µM filtered artificial seawater (FASW) (Instant Ocean). Prior to fixing or scoring live, larvae were anesthetized in a solution of two parts
FASW and one part FASW saturated with trichlorobutanol. Formaldehyde was then added directly to this mixture to a final concentration of 3.7%. After 1-2 hours, fixed animals were washed 2x with PBTw and stored at 4°C. Fixed animals were individually scored at 400x with a Zeiss Axioplan 2. For the knockdown rescue experiment, capped and tailed IoLR5MOres mRNA was transcribed using the mScript kit (T7 polymerase) from Epicenter and coinjected with IoLR5MO1 at a concentration of ~450 ng/µL. For each manipulation, a minimum of 5 embryos were scored from each of at least 3 different capsules to control for between-capsule variation.

RNA transcription, labeling and imaging
For LE mapping experiments, regions of the RNA to be tested were amplified by PCR, using a forward primer with an attached T3 polymerase site. PCR products were cleaned with phenol/chloroform extraction, concentrated by ethanol precipitation, and used as templates for in vitro transcription. Digoxigenin-11-UTP (Roche) was incorporated during transcription as per Roche’s supplied protocol. Transcription reactions were incubated at 37°C for 2-4 hours, treated with DNAse I (Roche) for an additional 20 minutes and purified using the MEGAclear kit (Ambion). For RNAs under 500 bases, the transcription reaction was heat inactivated and the RNA was precipitated with NaOAc and ethanol. For fluorescently labeled RNAs, ChromaTide Alexa Fluor 488-5-UTP (Invitrogen) was incorporated during transcription. All brightfield images were taken on a Zeiss Axioplan 2 with a Canon 300D.
DSLR and Optem (Qioptiq, Fairport, NY) coupler. Fluorescently-labeled RNAs were imaged with a Leica SP5 confocal microscope with Z-stacks projected using Leica software.

**MO inhibition of centrosomal transport**

Capsules were split so that half of the embryos were zygotically injected with 1.0mM IoLR5StemBlockMO (sequence: 5’-CACTGTCCCCTCTAGGCAACATAGG-3’) and 1.0% Oregon Green 488 70,000MW lysine-fixable dextran (Invitrogen). The remaining embryos were injected with 1.0mM IoLR5MO1 and 100uM sulforhodamine 101 (Invitrogen). Embryos from each manipulation were combined and fixed together at the 4-cell stage. *In situ* hybridizations were performed on the combined set of embryos and the two sets of manipulations could only be distinguished by the presence of fluorescence in the green channel, due to the presence of Oregon Green dextran. This allowed for qualitative comparison of transcript abundance in the cytoplasm after the *in situ* hybridization.

**Fluorescently labeled RNA transcription**

To transcribe RNAs labeled with ChromaTide Alexa Fluor 488-5-UTP, we used a ratio of 1:12.5 for ChromaTide-UTP to unlabeled-UTP. When we used the manufacturer’s suggested ratio (1:3), we were unable to obtain *in vivo* localization for any injected RNAs. This may be due to the fluorescent label interfering with the proper secondary structure of the LEs, although this has
not been fully tested. In addition, we could not see localization of fluorescent transcripts less than 500 bases in length and we believe this is due to the low incorporation rates we used during transcription. At the 4, 8 and 24-cell stages, the fluorescently-labeled RNAs we tested had the same localization pattern as digoxigenin-labeled transcripts; however, at the 24-cell stage, digoxigenin-labeled probes were easier to visualize due to higher sensitivity. In 24-cell embryos, most of the injected RNAs are initially segregated to the first quartet so there are only low levels of RNA in the second and third quartets. Due to the green autofluorescence of the yolk, the fluorescently-labeled RNAs are more difficult to see in these quartets. Surprisingly, we discovered a difference in ability of the fluorescent and digoxigenin transcripts to localize at the 2-cell stage. We tested all of the IoLR5 RNA with digoxigenin-labeled RNAs but were unable to recapitulate 2-cell centrosomal localization. Using fluorescently-labeled transcripts (corresponding to the same RNA sequences) we were able to obtain 2-cell localization. Digoxigenin-UTPs are incorporated at higher concentrations than the ChromaTide-UTPs, which may indicate that the digoxigenin label disrupts a secondary structure that is required for 2-cell localization, but not in subsequent stages.
Chapter 4

Trans-acting factors that mediate RNA transport to the centrosome

Introduction

Asymmetric cell divisions are used by all multicellular organisms to generate polarity during embryogenesis. This is often accomplished by the unequal subcellular distribution of RNAs (reviewed in Chapter 1). Many RNAs in the mud snail *Ilyanassa* are asymmetrically segregated through centrosome-mediated segregation, resulting in each quartet inheriting distinct subsets of transcripts (Kingsley et al., 2007; Lambert and Nagy, 2002). I have shown the developmental importance of one of these localized transcripts, IoLR5 (Chapter 3), and identified cis-acting elements required for centrosomal transport in IoLR5 and IoLR1 (Chapter 4). However, it is still unknown what trans-acting factors mediate transport to the centrosome.

As discussed in Chapter 1, subcellular localization of RNAs can be established actively through directed transported or passively through diffusion and anchoring. In *Ilyanassa*, several lines of evidence indicate that transport occurs in an active manner. First, localization to the centrosome was shown to be microtubule dependent (Lambert and Nagy, 2002). Second, I have shown that RNAs are capable of being transported during a window of ~15-20 minutes but all endogenous transcripts are localized immediately at the beginning of this window. Lastly, during *in vivo* time lapse imaging of
fluorescently-labeled IoLR5 RNA, labeled transcripts only moved in the direction of the centrosome (data not shown).

Several proteins have been shown to play conserved roles in the directed transport of RNAs in animals, such as Staufen, ZBP1 and hnRNP A/B (Martin and Ephrussi, 2009; St Johnston, 2005), and it is possible some of these may be functioning in *Ilyanassa*. Like many model systems, *Ilyanassa* is not amenable to forward genetic screens; rather, reverse genetic approaches must be used to test gene function. I chose to test two conserved genes for roles in the centrosome-mediated RNA segregation seen in the *Ilyanassa* embryo: Staufen and Bicaudal-C (BicC). Staufen was first discovered in genetic screens searching for patterning formation defects in *Drosophila* (St Johnston et al., 1991). As described in Chapter 1, more recent research has implicated this protein in RNA transport in the frog egg and mammalian neurons (St Johnston, 2005). BicC codes for another RNA binding protein that was also discovered through study of patterning defects in the fly embryo (Mohler and Wieschaus, 1985). This protein was originally implicated in the subcellular localization of RNAs in *Drosophila* (Mahone et al., 1995). It is now believed that BicC’s role in transport is indirect and it functions as a translational regulator (Chicoine et al., 2007). In either case, BicC has a conserved role in binding RNA and is often founded in the RNP complexes which are transported in the *Drosophila* embryo.

While reverse genetic approaches can lead to novel insights for new organisms, these approaches are inherently biased and can never be used to
discover all of the factors involved in particular molecular interactions. Therefore, to identify all of the trans-acting factors required for centrosome-mediated RNA segregation, it seems advisable to try alternate less-biased techniques. Many methods have been established for identifying novel protein-protein interactions, but only two methods have been adapted to screen for novel RNA-protein interactions. These are the yeast-three hybrid system (Bernstein et al., 2002) and tandem RNP affinity purification (TRAP; Hogg and Collins, 2007). These screens are very challenging and are prone to spurious interactions between the affinity matrices, the requisite RNA binding motifs and the tested proteins. Thus, they are prone to high false positive results. Despite these difficulties, these are the best characterized methods available for identifying proteins that bind certain RNA motifs.

In this chapter I report my attempts to identify the trans-acting factors involved in RNA localization in Ilyanassa. I have tried to functionally test the conserved RNA binding proteins Staufen and BicC and I have performed two TRAP experiments searching for proteins that can bind the IoLR5LE.

Results

Knockdown of conserved transport proteins

The genome of Ilyanassa has not yet been fully sequenced; however, the Lambert lab has now undertaken three large scale sequencing projects and gained a considerable amount of genomic information. Sequencing was
carried out on EST libraries made from early cleavage embryos using three separate methods: Sanger sequencing, 454 and Solexa (unpublished data). Importantly, clones for many RNA binding proteins have been identified in the snail, including Staufen, BicC and numerous hnRNPs.

Due to the conserved roles known for Staufen and BicC, I set out to characterize their expression during early cleavage and study their respective loss-of-function phenotypes. We do not currently have the full sequences for these genes, but the cDNA clones identified were sufficient to make in situ probes and examine transcript distribution. The mRNA for Staufen was found to be ubiquitously expressed at high levels throughout early cleavage (data not shown). The mRNA for BicC was found to utilize centrosome-mediated RNA segregation. BicC is segregated to the first quartet, the second quartet, and the 3d cell in the third quartet (data not shown). These data show that the mRNAs for these two genes are expressed at the appropriate time to potentially function in centrosomal transport.

In Chapter 2, I described two independent knockdowns methods that were successful in *Ilyanassa*; injection of morpholinos (MO) and antisense RNA (Rabinowitz et al., 2008). The BicC cDNA clone we isolated included the 5’ translational start site, so a morpholino was designed targeting this region and injected into zygotes. Embryos were raised for 7-10 days, to the veliger stage, but no visible defects were seen in these animals. The Staufen cDNA clone did not contain the translational start site sequence; therefore, we could not try MO knockdown for this gene. Therefore, I injected antisense
RNAs for Staufen into zygotes and reared these to the veliger stage. Unfortunately, these injections also failed to produce any visible phenotypes in the larvae. The antisense RNA injections were also tried against BicC, but again larvae from these injections displayed no visible phenotypes. Since the efficacy of knockdown was not assessed, and it is possible that proteins for these genes are deposited maternally into the zygote, it is still not clear whether these genes play a role in centrosome-mediated RNA segregation in *Ilyanassa*.

**Tandem RNP affinity purification to identify trans-acting factors**

Of the two characterized methods for discovering novel RNA-protein interactions, I chose to proceed with the TRAP method for 3 reasons. First, the yeast-three hybrid system was prohibitive because of the need to construct a special library for the screen. Second, a recent study using TRAP was successful in identifying a novel trans-acting factor essential for the localization of Nanos mRNA (Jain and Gavis, 2008). Third, we felt that given the likelihood of false positives, it might be more efficient to screen these out with the TRAP method.

For the “bait” in this screen, I used the IoLR5LE. This 118 bp sequence was inserted into the vector supplied with the Dual TRAP kit. This vector contains two RNA aptamers: S1 recognized by streptavidin and the tobramycin-aptamer recognized by tobramycin. The RNA containing these three RNA elements is transcribed *in vitro*, bound to isolated protein and
purified in tandem, first over the tobramycin matrix, then followed by the streptavidin matrix. The experiment was attempted twice using protein isolated from two populations of cells. In the first experiment, I isolated total protein from early cleavage stages ranging from the zygote through the 24-cell stage. For the second, I isolated protein from the ovaries. In both experiments, I was unable to identify any proteins, based on SDS-PAGE gels, which were specifically enriched through interactions with the IoLR5LE (data not shown). I discuss ways to improve this experiment in the Discussion.

**Discussion**

The trans-acting factors that mediate RNA segregation in *Ilyanassa* remain mysterious; however, the approaches explained above still hold promise for discovering these proteins.

The centrosome-mediated RNA segregation machinery is active as early as the 2-cell stage (IoLR5 – Chapter 3). If Staufen and/or BicC are essential for transport then they are likely be deposited maternally. This is a likely reason why the translational knockdown strategies I tested for Staufen and BicC failed. I have not attempted antibody staining for the Staufen or BicC proteins, but broadly cross-reactive antibodies are available for each and it is possible that these antibodies could be reactive in *Ilyanassa*. If so, it would be easy to determine when and where these proteins are expressed. Furthermore, having a specific antibody for either of these would potentially
provide a means of inhibiting protein activity directly. The active sites for Staufen protein, the dsRNA binding domains, are the most conserved regions of the protein (Miki et al., 2005). Any antibodies found to be cross-reactive might be targeted to these regions and could inhibit Staufen activity.

Several modifications to the TRAP protocol can greatly enhance the sensitivity of the method. First, there appeared to be very faint bands present in the first set of experiments and simply starting with more protein will increase the yield for these bands. Based on the protein concentrations used in the Nanos TRAP experiment, (Jain and Gavis, 2008), I would advise using a minimum of 3-fold more protein than my initial experiments – at least 1000 capsules per purification. Second, in both of the experiments I attempted, there were large quantities of high molecular weight proteins found in both control purification (no IoLR5LE in the vector) and experimental purification (IoLR5LRE in the vector). By incubating the extracted protein with both streptavidin and tobramycin, before starting the affinity purification, it may be possible to greatly reduce spurious binding ("pre-clearing" the protein extract). The pre-clearing procedure was also used by Jain and Gavis, 2008. Lastly, during the second matrix binding step in the tandem affinity purification, adding low concentrations of a cross-linker (such as formaldehyde) could preserve weak RNA-protein interactions once they have formed.
Materials and Methods

Knockdown methods for Staufen and Bicaudal-C

Microinjections and transcription of antisense RNAs were done as previously described (Rabinowitz et al., 2008). The BicC MO sequence is (5’-TCCGCCATAGCATACTGGAAGCTGC -3’) (GeneTools, LLC).

Protein isolation

Embryos were cut from capsules and pooled together in a single 1.5 ml Eppendorf tube. Embryos were washed once with lysis buffer (20 mM HEPES pH 7.4, 150 mM KCl, 1 mM MgCl2, 1mM DTT, 0.1% NP-40, 0.5% Triton X-100, 10% glycerol and 1x protease inhibitor tablet). The lysis buffer was removed and an equal volume of lysis buffer per volume embryos was added to the tube. Cells were homogenized with a pestle and spun for 10 minutes at ~14,000 RPM in a refrigerated centrifuge. Supernatant was transferred to a new tube and glycerol was added to 10%. Protein was then frozen in liquid nitrogen and stored at -80°C.

Dual TRAP protocol

One copy of the IoLR5LE was cloned into the pTRAP V5 vector (from CytoStore) using standard restriction digestion and ligation techniques. The affinity purification was performed as per manufacturer’s instructions (CytoStore). The supplied protocol states that the tandem purification can be done in either direction: tobramycin first and then streptavidin, or vice versa. I
chose to bind the “bait” RNA to the tobramycin matrix first and the streptavidin matrix second.

*Labeled-RNA transcription*

All digoxigenin- (Roche) and Alexafluor-488-UTP-labeled (Invitrogen) RNAs were transcribed as previously described (see Chapter 4).
Chapter 5

Conclusions

Asymmetric cell division is one of the most fundamental mechanisms in developmental biology and was first witnessed over a century ago when the field of embryology was still in its infancy. Most studies of asymmetric cell division have followed a similar experimental pathway. First, careful observations of embryogenesis led researchers to propose the existence of asymmetrically distributed factors. Then, embryological manipulations provided direct evidence that segregated factors were essential. Lastly, molecular genetics allowed the discovery of the specific genes responsible for generating these polarizing events. In insects, the pathway from observations to genes took a single decade. In molluscs, after 100 years, we can finally say we are making progress.

The field of embryology began with a concerted study of many different animal phyla, many within the Lophotrochozoa. The observations made from diverse organisms allowed researchers to draw comparisons between different modes of development and this led to a variety of predictions about how development is regulated. Importantly, early studies of lophotrochozoans were responsible for derivation of the asymmetric cell division hypothesis. With the discovery of DNA, and later patterning genes, many of these predictions were shown to be correct. However, as the power
of genetic mutational analyses became apparent, many researchers turned to genetic model systems, such as *D. melanogaster* and *C. elegans*, to study developmental processes. Because of this, very little progress has been made beyond observations and classic embryological manipulations in spiralian embryos. Since the major model systems are distantly related to each other, it has been difficult to use comparisons between them to understand many aspects of the evolution of animal development. The spiralian lophotrochozoans are a key group to explore to improve sampling across the animal kingdom for evolutionary inferences. In addition, because they have a significantly different mode of development from other systems, they are expected to provide a different and informative perspective on conserved developmental and cell biological processes.

During my thesis work, I have helped to establish protocols that allow molecular genetics studies in one of the best characterized models within the Lophotrochozoa, the mud snail *Ilyanassa obsoleta*. Using these techniques, I have now demonstrated for the first time a link between patterning molecules and the conserved mode of development known as spiral cleavage. Also, I have provided evidence for how the snail generates polarity during embryogenesis and highlight that this is a potentially conserved mechanism in spiralian embryos.
Conserved role of Nanos in patterning

Since forward genetic screens are not feasible in *Ilyanassa*, development of a microinjection protocol was of vital importance. With a focus on IoNanos, I helped design this protocol and opened the door for functional genetic studies in the snail. The results from the IoNanos knockdown were very intriguing for two main reasons. First, the requirement for IoNanos in the development of somatic tissues derived from the 4d cell, demonstrated one of the first examples of Nanos involved in somatic patterning outside of insects. Due to the evolutionary divergence of these organisms, this suggests that Nanos may have functioned in somatic patterning of the last common ancestor of the Bilateria. Furthermore, phenotypic effects of IoNanos knockdown resulted in the loss of blast-cell potential in the 4d cell. The Nanos gene has been implicated in germline stem-cell maintenance in many organisms (Draper et al., 2007; Sunanaga et al., 2008; Wang and Lin, 2004). Blast cells have many stem cell-like qualities and they are likely to be functionally homologous cell types. The finding that MO knockdown of IoNanos results in a loss of the stem cell characteristics found in the 4d lineage, suggests that Nanos may have a more conserved role in maintaining pluripotency in stem cells.

Specification of the spiralian quartets

As I have already discussed, the spiralian mode of development is a highly conserved mechanism within the Lophotrochozoa. Spiral cleavage is
inherently asymmetric as the large macromere cells give rise to the much smaller micromere cells, through successive rounds of cleavage towards the animal pole. The micromere quartets pattern the animal-vegetal axis of the embryo and were shown to be specified at, or shortly after, their birth. The finding that many RNAs are subcellularly localized and segregated in a quartet-specific manner led to the hypothesis that these RNAs could be the patterning molecules that specify the micromeres. Notably, my studies of IoLR5 function have provided clear evidence that proper development of the first quartet micromeres requires this segregated RNA and also demonstrates the first molecular evidence supporting the above hypothesis. In addition, the finding that transcripts are localized to the centrosomes in other spiral cleaving embryos, such as Crepidula and Spisula, suggests that centrosome-mediated RNA segregation may represent the basis for how the micromeres are specified in all spiralian phyla. Although this hypothesis requires more taxonomic sampling, after a century of research, we can now being to explain how spiralian embryos become polarized during early cleavage.

**Cis-acting localization elements**

I have mapped the LEs for two transcripts that localize to the 4-cell stage centrosomes, IoLR1 and IoLR5. These LEs are predicted to fold into similar secondary structures and I demonstrated that they are sufficient for centrosomal transport in all early cleavage cells of the embryo. Since
endogenous IoLR1 and IoLR5 RNAs are not found localized in all early cleavage cells, other mechanisms, such as transcript abundance and stability, must function to regulate the subcellular patterning of these RNAs.

One of the initial goals of my thesis work was to determine if quartet-specific LEs mediate the localization of certain RNAs into particular quartets. With the results from IoLR1 and IoLR5, I have indeed found evidence that suggests a class of localization elements exist that drive localization in the 4-cell stage, and are also sufficient for transport in other stages. However, I have tried to map the LEs for several messages that are localized specifically to the second quartet and not the first. Surprisingly, I have not been able to achieve second quartet (or first quartet) localization for any of these transcripts. Most of the RNAs I tested during these experiments were transcribed from cDNA clones and it is therefore possible that the LE sequences are not present in the cDNA clones we have isolated. However, we do believe that we have the full transcript sequence for one of these genes, IoTis11, but this too is unable to recapitulate centrosomal localization (data not shown and Xin Yi Chan). Perhaps, RNAs which are specifically localized to the second quartet, require trans-acting factors for their localization that are bound during posttranscriptional processing events in the nucleus. Such a mechanism has been shown to be involved in the posterior localization of Oskar mRNA. This transcript can only be appropriately localized when splicing factors from the exon-exon junction complex are initially bound to it in the nucleus (Hachet and Ephrussi, 2004).
The PAR genes: conserved proteins involved in asymmetric cell division

Groundbreaking work from Kemphues et al. (1988) identified a class of partitioning defective genes (PAR) that had unique cytoplasmic localizations within the C. elegans zygote and were necessary to generate polarity in the embryo. Further studies on the PAR proteins have greatly expanded our understanding of how the anterior-posterior axis and germline are set up during early embryogenesis in C. elegans (Kemphues, 2000), but the more remarkable finding has been the high level of conservation seen with PAR genes across the animal kingdom (Goldstein and Macara, 2007; Wodarz, 2002). These genes appear to have conserved biochemical capabilities and control the polarization of multiple cell types, such as eggs and epithelia, through the use of diverse downstream effectors in the respective tissues (St Johnston and Ahringer, 2010). The PAR proteins typically interact directly with the actin cytoskeleton at the cortex and are often involved in spindle orientation for cells that undergo asymmetric division (Gonczy, 2008; Siller and Doe, 2009).

Through the ongoing sequencing efforts in the Lambert lab, we have identified some of these conserved genes. While none of these have yet been studied in the snail, based on the conserved nature of these genes, it seems likely that the PAR proteins are directly involved in centrosome-
mediated RNA segregation. The second phase of this process involves RNAs being transported to the cortex in an actin dependent manner. I propose that the PAR genes likely function in the anchoring of these RNAs to the cortex. Furthermore, I expect that the PAR genes are directly involved in aligning the mitotic spindles to this same region of this cortex, as has been shown in other systems (Siller and Doe, 2009). The precise alignment of the spindle is what ultimately results in the RNA-containing cortex material being inherited by only one of the two daughter cells and is also directly responsible for generating the stereotypic divisions seen in spiralian embryos.

**Trans-acting factors**

The proteins responsible for mediating transport to the centrosome are still unresolved. However, there is great potential with the reverse genetic approaches and the pull-down screening methods to identify these trans-acting factors (Chapter 4). Staufen is currently the most conserved protein involved in the subcellular localization of RNAs. Importantly, characterization of this protein has shown it is involved in the microtubule-dependent transport of anterior and posterior localized RNAs in the fly embryo, as well as actin-dependent transport in fly neuroblasts. This ability for Staufen to act as a mediator between RNAs and motor proteins on both microtubules and actin filaments is intriguing because the RNA localization seen in the snail uses both of these cytoskeletal elements, within the same cell. Therefore, the use
of Staufen during both phases of RNA localization in the snail could be an efficient method to segregate the localized transcripts.

Studies in *Drosophila* have shown that the BicC protein actually binds its own mRNA (Chicoine et al., 2007). In the snail, BicC mRNA undergoes centrosome-mediated segregation and this brings up the possibility that the BicC protein is also being transported to the centrosome. Importantly, while BicC is no longer thought to be directly involved in transporting RNAs, the protein is commonly found in the RNP complexes that do become transported. If BicC is found to be aggregated with RNAs undergoing transport, then it may be possible to use the BicC protein to pull-down other proteins found in these RNPs. Identifying novel protein-protein interactions is considerably easier than finding RNA-protein partners. Therefore, BicC could be used indirectly to discover the trans-acting factors involved in transport to the centrosome.

I have already proposed several advances in the TRAP protocol to increase the specificity of RNA-protein interactions. The only major worry with this approach is that since *Ilyanassa* currently lacks a fully sequenced genome, it is possible that even if a protein band is isolated, mass spectrometry may not be sufficient to determine what the protein is. Also, due to the small yield of protein that is likely from this experiment, it is not likely that there will be enough purified product to directly sequence the protein using Edmund degradation. Nonetheless, I think this approach is a vital step in unraveling how RNAs are segregated in the *Ilyanassa* embryo.
References


