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SHORT COMMUNICATION

Localization of *Vasa* mRNA during early cleavage of the snail *Ilyanassa*

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Abstract Members of the Vasa family of helicases are specifically localized to germ line lineages in embryos of many animal groups and, in some cases, have been shown to be required for germ line formation. Despite considerable attention to the embryology of gastropod molluscs, the germ line has not been identified in the early cleavage stages of these embryos. We have cloned a Vasa ortholog in the snail *Ilyanassa* and examined the distribution of *IoVasa* mRNA during early cleavage. Initially, the transcript is present in all cells and non-specifically localized to centrosomes in a subset of cells. The *IoVasa* mRNA becomes progressively more enriched in the dorsal quadrant of the embryo, and then becomes restricted to particular cells in the 4d lineage. At the 64-cell stage, *IoVasa* mRNA is detected in $4d^{L11}$, $4d^{L12}$, $4d^{R11}$, and $4d^{R12}$. Following another round of division in the 4d lineage, the mRNA is restricted to two cells: $4d^{L121}$ and $4d^{R121}$. By the 108-cell stage, *IoVasa* mRNA is no longer detectable. Because the germ line is thought to arise from the 4d lineage in spiralians, these data are consistent with the hypothesis that the *Ilyanassa* germ line is marked by inheritance of *IoVasa* and derived from the cells $4d^{L121}$ and $4d^{R121}$. Alternatively, *IoVasa* may be required in somatic lineages where it is expressed, and the germ line may be specified later in development.

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Introduction

During embryonic development, many animals set aside a population of cells called the germ line that ultimately gives rise to the gametogenic tissues of the adult gonad. In some animals, such as *Drosophila melanogaster*, *Xenopus sp*., and *Caenorhabditis elegans*, the germ line is determined early in development by the inheritance of determinants by specific cells. In other organisms, including mammals, the germ line is specified later in development by inductive mechanisms (reviewed in Extavour and Akam 2003).

Relatively little is known about how the germ line is specified in embryos of the large clade of animal phyla called the Lophotrochozoa, which includes spiralians such as molluscs, annelids, and turbellarian flatworms, and lophophorates like phoronids, bryozoans, and brachiopods (Halanych 2004). In planarian flatworms, the germ line cells are specified conditionally late in development or after regeneration (Wang et al. 2007). In the leech *Helobdella robusta*, germ cells arise from general segmental mesodermal precursors relatively late in development, suggesting that the germ line is not specified by determinants in early cleavage (Kang et al. 2002). In the polychaete annelid *Platynereis*, germ cells derive from a subset of posterior mesodermal precursors (Rebscher et al. 2007).

Among molluscs, there is evidence for both early specification of the germ line by determinants and specification later in development. Observations of the clam *Sphaerium* provide the strongest support for the role of determinants in specifying the germ line of a mollusc (Woods 1932). In this embryo, a population of distinctive

mitochondria, which are present in the oocyte, marks a lineage that is likely the germ line. This special cytoplasm is segregated into the 4d cell and is then inherited by particular 4d derivatives. The 4d cell is conserved among lophotrochozoans with spiralian development in terms of its cleavage pattern and the fates of its progeny (reviewed in Lambert 2007). In other molluscs, it appears that the germ line is specified later. In the gastropod *Viviparus*, primordial germ cells have not been identified in early cleavage stages and the germ line cells reportedly arise from the mesodermal pericardial epithelium, indicating a later specification of the germ line, perhaps by inductive mechanisms (Griffond 1977).

Vasa was first identified in *Drosophila* as a maternally supplied factor required for posterior patterning and germ line development (Schupbach and Wieschaus 1986). The Vasa protein is a component of the polar granules, subcellular structures in the oocyte pole plasm—the region that gives rise to the germ line. It encodes an ATP-dependent RNA helicase (Hay et al. 1988; Lasko and Ashburner 1988). In mice, loss of function of the vasa ortholog *mvh* prevents development of the male but not female gonads (Tanaka et al. 2000). In *C. elegans*, the vasa-related genes, GLH-1 and 4, are redundantly required for normal gametogenesis (Kuznicki et al. 2000). In *Drosophila,* Vasa also plays roles in somatic development. Vasa is required for the translation of the *Drosophila* posterior determinant *nanos* (Gavis et al. 1996). It has also been shown to play a role in establishing oocyte polarity by regulating *gurken* (Styhler et al. 1998; Tomancak et al. 1998).

The snail *Ilyanassa obsoleta* is one of the best-studied models of lophotrochozoan development. The early cleavages of the *Ilyanassa* embryo are shown in Fig. 1. The clonal contributions of the cells up to the 24-cell stage have been determined by lineage tracing, and the embryo is amenable to a variety of experimental manipulations. Because the role of Vasa is conserved across the animal kingdom, its ortholog, *IoVasa*, could serve as a marker of the *Ilyanassa* germ line and provide insight into lophotrochozoan germ line formation in general. In this study, we report the recovery of an *Ilyanassa* ortholog of Vasa and describe the mRNA distribution at the level of individual identified cells.

Methods and materials

Vasa cloning

While sequencing expressed sequence tags (ESTs) from a 0- to 48-h (fertilization to gastrulation) cDNA library, we found several EST sequences that were nearly identical in their regions of overlap and had highest similarity to Vasa orthologs. The largest of these clones was sequenced entirely and named *IoVasa*, and the sequence was deposited in Genbank (EU047801). A 361-bp fragment containing 316 bp of non-primer sequence had been cloned previously by degenerate PCR from *Ilyanassa* cDNA (J.D.L. unpublished data). This clone corresponds to base pairs 31–392 in the *IoVasa* sequence and was used as a template for generation of digoxigenin-labeled probe for the in situ hybridization experiments shown here. We also synthesized probe from the full-length clone and verified that it gave the same staining pattern as the shorter fragment for the stages shown here.

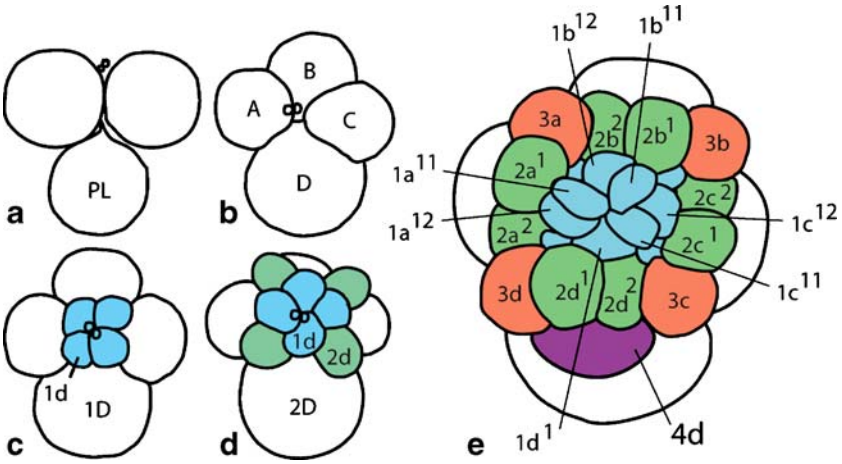

Fig. 1 Early cleavage in *Ilyanassa*. During each of the first two cleavages, a polar lobe is produced (as in **a**). These lobes sequentially segregate the vegetal pole of the embryo into the *D* macromere at the four-cell stage (**b**). After the four-cell stage, the *A*, *B*, *C*, and *D* macromeres divide synchronously toward the animal pole to produce sets of four smaller cells called micromeres. The first quartet is shown in *blue* (**c**), second quartet in *green* (**d**), and the third in *orange* (**e**). After the birth of the third quartet, the *D* macromere divides earlier than the other three macromeres to produce the *4d* micromere (**e**), which gives rise to the major mesodermal organs of the larva and the intestine

Vasa phylogeny

We assembled a set of broadly sampled metazoan Vasa and PL10 protein sequences and aligned them by eye. After excluding regions of ambiguous alignment, we conducted a phylogenetic analysis on a region corresponding to amino acid positions 214–612 in the *Drosophila melanogaster* sequence (accession numbers are below). The analysis was carried out in Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the WAG model, running four chains for 10^6 generations and sampling of trees every 100 generations. We used the following sequences in our alignment: *Mus musculus* EIF4a, accession number NP_001407; *Ephydatia fluviatilis* PL10, BAB13309; *D. melanogaster* belle (PL10), NP_536783; *Crepidula fornicata* PL10, ABD59346; *Danio rerio* PL10, NP_571016; *Platynereis dumerilii* PL10a, CAJ15140; *Nematostella* vectensis PL10, AAW29072; Mus *musculus* PL10, NP_149068; *Xenopus laevis* PL10, NP_001080283; *Drosophila melanogaster* Vasa, NP_723899; *Crassostrea gigas* vasa-like protein, AAR37337; *Ciona intestinalis* Vasa, BAA36710P; *Ephydatia fluviatilis* Vasa, BAB13310; *Strongylocentrotus purpuratus* Vasa, XP_781494; *Danio rerio* vasa-like protein (Vasa), AAI29276; *Platynereis dumerilii* Vasa, CAJ38803; *Nematostella vectensis* Vasa1, AAW29073; *Nematostella vectensis* Vasa2, AAW29074; *Artemia franciscana* Vasa, BAD99523; *Mus musculus* Ddx4 (Vasa), NP_034159; and *Ilyanassa obsoleta* IoVasa, EU047801.

RNA in situ hybridization and cell identification

Animal maintenance, embryo collection, in situ hybridization, and microscopy are described in Kingsley et al. (2007). Sense probe gave no staining (e.g., Fig. 3d,f insets). The cleavage pattern is extremely regular and so the identities of cells can be determined after examination of DAPI-stained embryos. All cell identifications here are verified on a range of stages that were examined along with those presented. All cells were identified in all embryos presented, except Fig. 4g and h, which show a stage later than detectable IoVasa staining.

We use the nomenclature for the 4d lineage proposed earlier (Goulding 2001), where the left and right daughter cells of 4d are called $4d^L$ and $4d^R$, respectively, rather than M^1 and M^2 or some variant. The main advantages of this are (1) that it does not conflict with the convention in spiralian nomenclature of using m^1 and m^2 for animal and vegetal micromere daughters, respectively, and (2) it does not presuppose a fate of particular 4d derivatives, which have not been determined using modern methods in any spiralian embryo. This nomenclature should also facilitate phylogenetic comparisons of the 4d lineage because the behavior and cleavage pattern of mesoteloblast (or the large M) cells varies across taxa, and this is obscured by always referring to these cells as M^1 and M^2.

Visualizing the 4d lineage

Dil was resuspended in EtOH at 100 mg/ml, then diluted 1/20 in soybean oil (Henry and Martindale 1998). We pressure-injected 4d with one to two small drops of Dil oil then fixed embryos at various time points using 3.7% formaldehyde in filtered artificial sea water with 100 mM ethylenediaminetetraacetic acid to preserve the label (Henry et al. 2001). Embryos were treated with a mixture of RNAses A and T at 10 ug/ml for 1.5 h then stained with YOYO-1 (Molecular Probes) at 2.5 nM.

Results and discussion

A Vasa ortholog in Ilyanassa

We recovered an *Ilyanassa* cDNA with highest sequence similarity to Vasa orthologs. The PL10-belle family of helicases is closely related to Vasa proteins. To test whether our sequence was more closely related to Vasa sequences than PL10 sequences, we aligned a set of Vasa and PL10 amino acid sequences from various metazoans and inferred a phylogeny using a Bayesian approach (Ronquist and Huelsenbeck 2003). The consensus tree has a strongly supported clade containing all of the previously identified PL10 orthologs in the tree and not *IoVasa* (Fig. 2). This result indicates that *IoVasa* is an ortholog of other Vasa sequences, rather than PL10 sequences.

Localization of IoVasa RNA during early development

We determined the spatial distribution of *IoVasa* RNA during embryonic development, using in situ hybridization of RNA probes. During the first seven cleavage cycles, the RNA is present throughout the embryo (Fig. 3). At these stages, the RNA is diffusely distributed in the cytoplasm, but it is also always non-specifically localized to particular centrosomes (e.g., Fig. 3c). In *Ilyanassa*, specific centrosomal localization (i.e., with little or no cytoplasmic RNA) has been observed for several mRNAs and is usually associated with segregation in the ensuing division (Lambert and Nagy 2002; Kingsley et al. 2007). Non-specific centrosomal localization, as observed for *IoVasa* during early cleavage (Fig. 3), is found with about 45% of embryonic transcripts and is not always associated with asymmetric segregation (Kingsley et al. 2007). Initially, the *IoVasa* RNA distribution is radially symmetrical but, as the early cleavages progress, the RNA becomes somewhat more abundant in cells of the D quadrant. At the two-cell

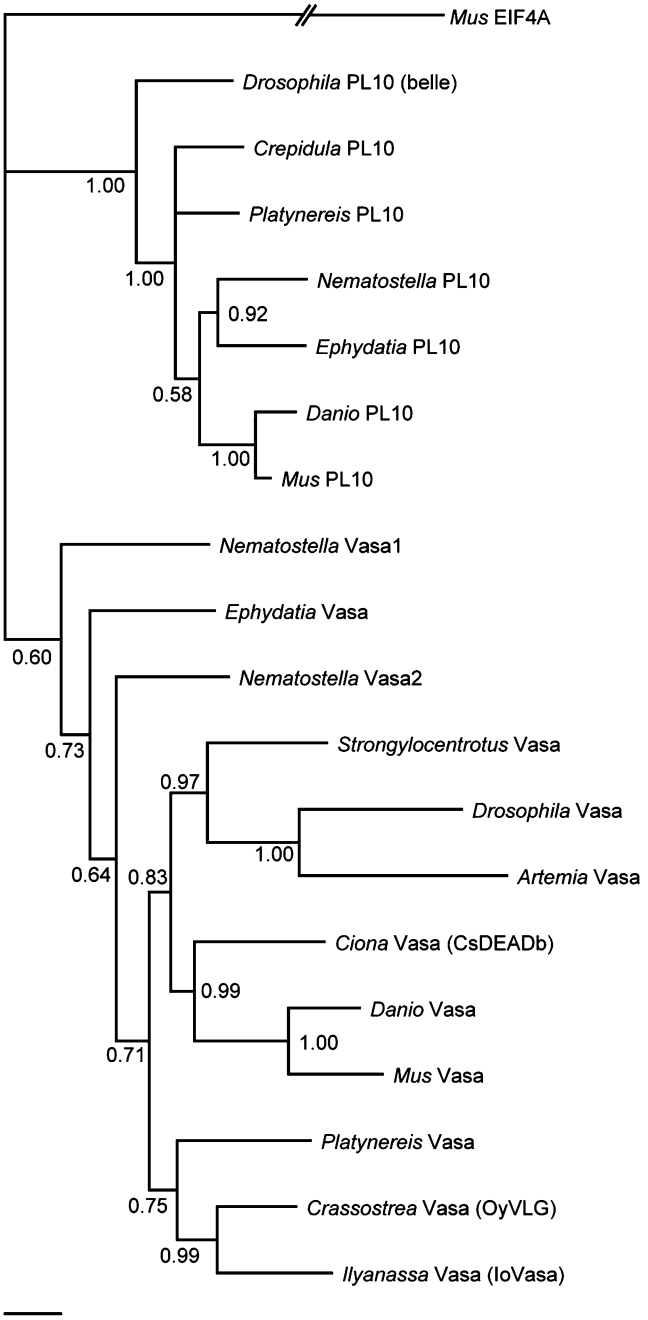

Fig. 2 Phylogenetic analysis of *IoVasa* sequence. The phylogram depicts the most probable relationship between a diverse set of metazoan Vasa and PL10 amino acid sequences, based on Bayesian inference (see "Methods and materials" for details). The posterior credibility values for each node are indicated. The *PL10* sequences form a monophyletic group with a 100% credibility value that does not include *IoVasa*. The actual length of the out-group branch is 1.7 expected changes per site

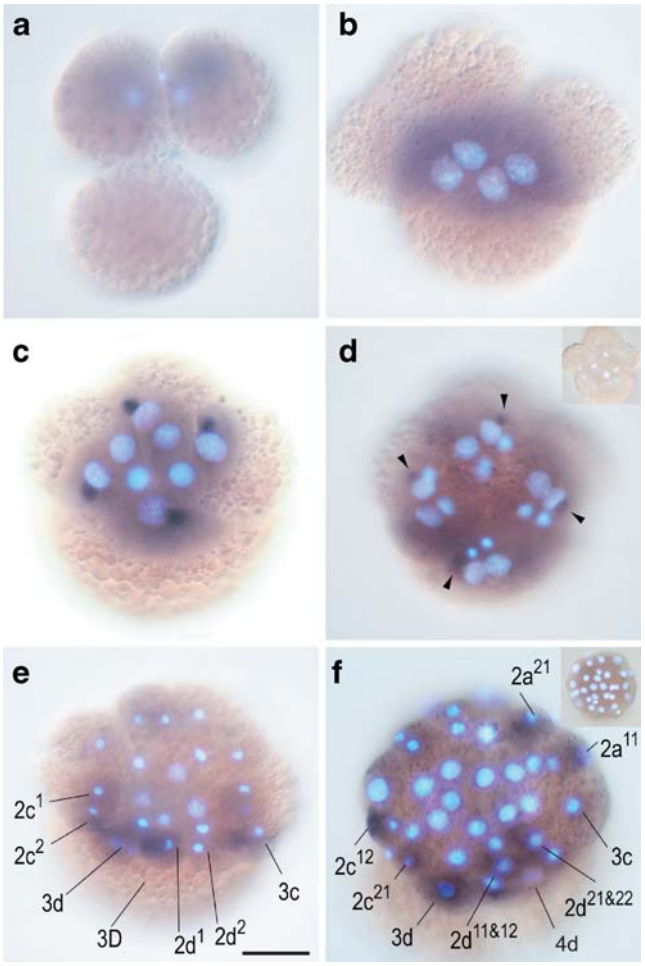

Fig. 3 *IoVasa* RNA in early cleavage embryos. *Blue black* chromogenic stain indicates RNA hybridization, and nuclei are counterstained with DAPI (*bright blue*). **a** In a lateral view of a two-cell embryo, *IoVasa* RNA is detected in AB and CD macromeres but not in the polar lobe. In **b** through **f**, the embryos are viewed from the animal pole, with the **D** quadrant towards the bottom of the page. **b** In a four-cell embryo, cytoplasmic staining is detected in all four macromeres. **c** In an eight-cell embryo, *IoVasa* RNA is present throughout the cytoplasm and localized to macromere centrosomes (the four darkly staining spheres adjacent to the four outermost nuclei). **d** In a 16-cell embryo, *IoVasa* transcripts are detectable in the cytoplasm throughout the embryo, and are also enriched on the centrosomes in the macromeres (*arrowheads*). *Inset*: RNA in situ hybridization to an embryo at a similar stage (12- to 15-cell stage) using the sense strand control probe. **e** During the fifth cleavage cycle (16- to 24-cell stage), IoVasa RNA is cytoplasmic throughout the embryo and enriched in the **D** quadrant. **f** In a 28- to 36-cell embryo, the mRNA is enriched on the dorsal side of the embryo and is present in *4d*. *Inset*: RNA in situ hybridization to an embryo at a similar stage using the sense strand control probe. Scale bar corresponds to 50 μm

stage (Fig. 3a), hybridization was detected in the AB and CD macromeres but not in the polar lobe. This pattern differs from the oyster *C. gigas*, where the *Oyvlg* RNA is localized to the perinuclear area of the CD macromere at the first division (Fabioux et al. 2004).

In the cleavage cycles after the birth of 4d, *IoVasa* RNA becomes increasingly enriched in particular 4d derivatives and becomes undetectable in the rest of the embryo (Fig. 4). After two rounds of cell division in the 4d lineage, *IoVasa* mRNA is strongest in $4d^{L1}$, $4d^{R1}$ (Fig. 4a). The nomencla-

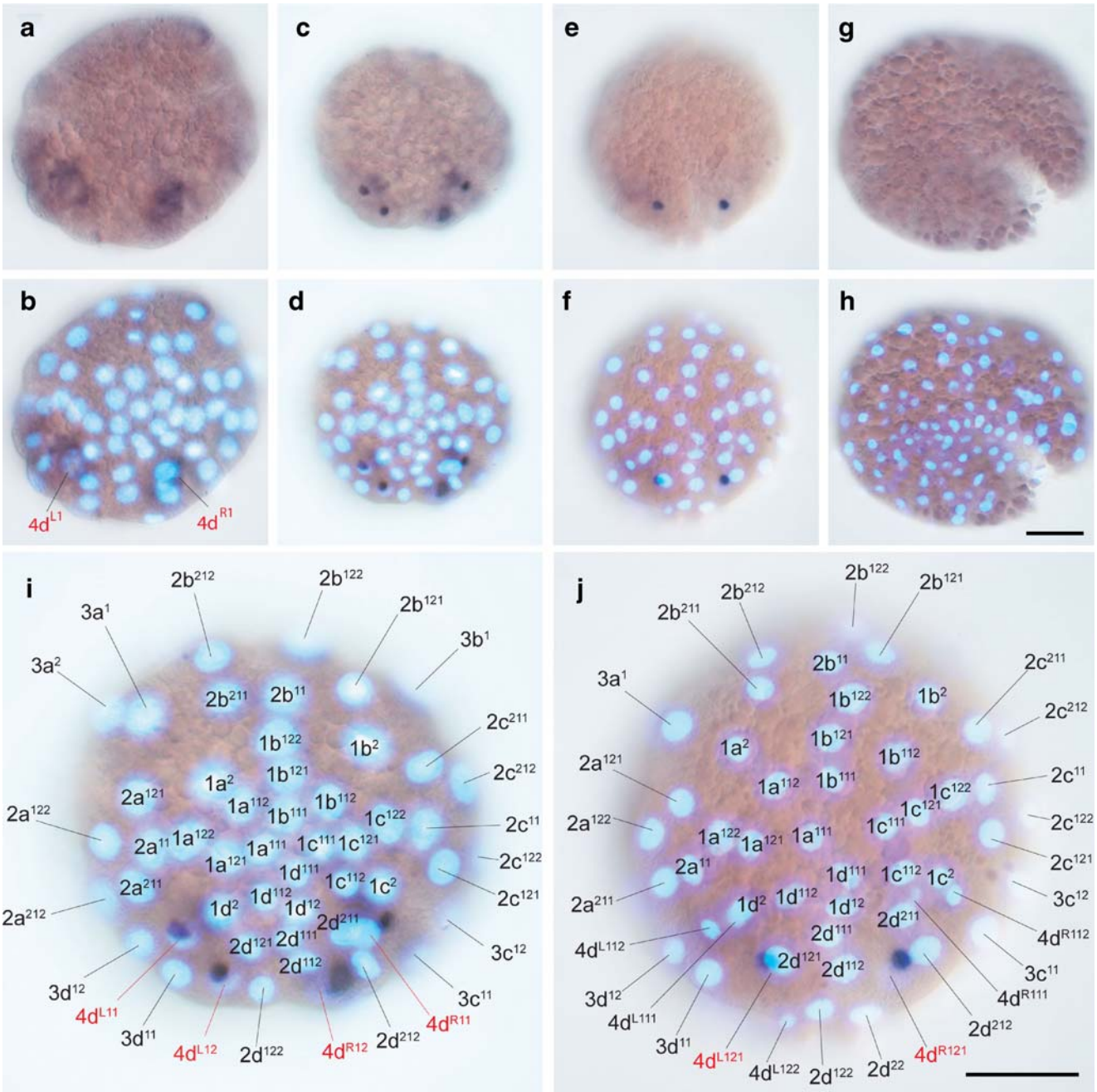

Fig. 4 *IoVasa* localization during gastrulation, counterstained with DAPI (in *blue*). Cells in the *4d* lineage with detectable *IoVasa* are labeled in *red*. **a, b** In a 60-cell embryo, staining is strongest in $4d^{L1}$ and $4d^{R1}$, but it is also detected in $2b^{122}$, $3d^{12}$, $2a^{212}$, and $2a^{211}$. **c, d, i** In a 64-cell embryo, staining is detected in $4d^{L11}$, $4d^{L12}$, $4d^{R11}$, and $4d^{R12}$. **e, f, j** In a 70-cell embryo, *IoVasa* is detected in $4d^{L121}$ and $4d^{R121}$. **g, h** In an embryo with approximately 108 cells, *IoVasa* mRNA is no longer detectable. The cleft in the yolk of this embryo (lower right side) is due to compression of the embryo for microscopy. Scale bar corresponds to 50 μm

ture of the 4d lineage is explained in the "Methods and materials", and the lineage is shown in Fig. 5. At the 64-cell stage, four discrete patches of *IoVasa* are detectable, which correspond to the cells $4d^{L11}$, $4d^{L12}$, $4d^{R11}$, and $4d^{R12}$. At this stage, there is little staining elsewhere in the embryo (Fig. 4c,d,i). When $4d^{L11}$ and $4d^{R11}$ divide, the RNA appears to be segregated into the $4d^{L112}$ and $4d^{R112}$ daughter cells but then degrades (not shown). When $4d^{L12}$ and $4d^{R12}$ divide, the RNA appears to be segregated into the $4d^{L121}$ and $4d^{R121}$ daughter cells (not shown) so that at the 70-cell stage, *IoVasa* staining is essentially restricted to these two cells (Fig. 4e,f,j) where it is specifically localized to spherical structures that are likely to be the centrosomes. *IoVasa* is no longer detected in slightly later embryos at 108-cell stage (Fig. 4g,h). We have not been able to detect *IoVasa* mRNA in later stages, including various time points

during gastrulation and organogenesis, and in the hatching stage veliger larva.

IoVasa in the mesoblastic teloblasts

Notably, $4d^{L\&R121}$—the last cells where *IoVasa* mRNA is observed—may be homologous to the primordial germ cell lineage founder cells identified in the bivalve mollusc *Sphaerium*. In most spiralians examined, 4d divides equally, and the daughter cells become founders for bilateral teloblastic lineages; Conklin referred to the larger mother cells that carry out these asymmetric cell divisions as the *mesoblastic teloblasts* (Conklin 1897). In *Ilyanassa*, the latest detectable *IoVasa* mRNA is in the cells $4d^{L\&R121}$, when the 4d lineage includes ten cells. To find out whether IoVasa is localized to the mesoblastic teloblasts, we injected 4d with the fluorescent lineage tracer DiI and examined the cleavage pattern in this lineage in living and fixed embryos. We determined which cells were the mesoblastic teloblast at each stage (Fig. 5), namely $4d^{L\&R1}$, $4d^{L\&R12}$, and $4d^{L\&R121}$. This cleavage pattern is the same as described in *Crepidula* (Conklin 1897). These results show that IoVasa mRNA is specifically localized to the mesoblastic teloblast cells of the endomesodermal lineage in *Ilyanassa*.

In the clam *Sphaerium*, the mitochondrial cloud was inherited by 4d daughter cells $4d^{L}$ and $4d^{R}$ (in our notation) then stayed in the teloblastic cells during three rounds of asymmetric division (Woods 1931, 1932). After those cleavages, these cells stopped dividing asymmetrically and assumed a primordial germ cell morphology, at which point they remained inactive until reaching their position in the definitive gonad. Thus, the last cells where *IoVasa* mRNA is observed occupy a similar position in the cell lineage as the primordial germ cells in *Sphaerium*. Both are the mesoblastic teloblasts after three rounds of asymmetric division. We note that it is unclear whether the mesoblast cells at this stage in *Sphaerium* would be named $4dL\&R^{121}$ using our notation because the orientation of the mesoblast divisions was not specified.

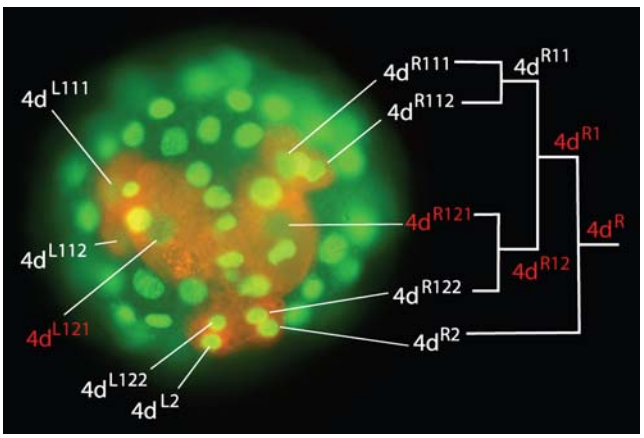

Fig. 5 The cells $4d^{L+R121}$ are the mesoblastic teloblasts in *Ilyanassa*. The *4d* cell was injected with the lineage tracer DiI and allowed to develop to approximately 70 cells. DiI is *red*, and nuclei are stained with YOYO-1 (*yellow green*). The labels of the mesoblastic teloblast cells are *red*

In the oyster *C. gigas,* Vasa RNA is restricted to one cell at each of the early cleavage stages examined, until a stage during gastrulation when it is observed in two cells (Fabioux et al. 2004). While the identities of the cells were not determined, this pattern is consistent with the RNA being segregated in the lineage leading to 4d, as observed for the putative germ line determinants in *Sphaerium,* and similar to what we observe in *Ilyanassa*. Together, these observations suggest that localization of Vasa mRNA to 4d may be conserved among molluscs. As in *Ilyanassa*, the Vasa RNA in the polychaete annelid *Platynereis* is ubiquitous during early cleavage then becomes increasingly specific to a subset of cells in the mesoderm (Rebscher et al. 2007). However, the restriction to particular mesodermal cells happens much later in *Platynereis* than in *Ilyanassa*. Furthermore, the polychaete Vasa RNA is continuously detectable between early cleavage stages and later stages when it is expressed in identified germ line cells. This differs from *Ilyanassa,* where no primordial germ cells have been discovered in the larva and Vasa RNA is not detectable after the first few cleavage cycles of the 4d lineage.

IoVasa and the germ line in Ilyanassa

The gastropod mollusc where the origin of the germ line has been best described is Viviparus, a basal member of the Caenogastropod group to which *Ilyanassa* belongs. In *Viviparus*, the germ line cells arise from pericardial mesoderm and do not appear to be specified early in embryogenesis. Thus, it is somewhat surprising to find IoVasa RNA localized to particular cells during early cleavage of *Ilyanassa*. Our data is consistent with the hypothesis that the IoVasa RNA localization we observe is directly involved in germ line specification, provided that as-yet unidentified primordial germ cells derive from IoVasa-expressing cells later in development. Previously unrecognized germ line cells have been discovered in adults of the annelid *Enchytraeus* and have been shown to contribute to the gonads during regeneration (Tadokoro et al. 2006). This highlights the possibility that similar cryptic germ line cells might exist in the *Ilyanassa* larva.

Of course, the expression of *IoVasa* in early development may not be involved with germ line specification. Vasa has been shown to be required for normal suppression of transposon levels in *Drosophila* (Vagin et al. 2004). It is possible that the Vasa expression we see in *Ilyanassa* is related to this function. In the 4d lineage and the early macromere divisions, large cells produce a series of smaller

daughter cells with different fates. In this context, it is also possible that *IoVasa* is involved in maintaining pluripotency or self-renewal in these somatic lineages.

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