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vasa and *nanos* expression patterns in a sea anemone and the evolution of bilaterian germ cell specification mechanisms

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SUMMARY Most bilaterians specify primordial germ cells (PGCs) during early embryogenesis using either inherited cytoplasmic germ line determinants (preformation) or induction of germ cell fate through signaling pathways (epigenesis). However, data from nonbilaterian animals suggest that ancestral metazoans may have specified germ cells very differently from most extant bilaterians. Cnidarians and sponges have been reported to generate germ cells continuously throughout reproductive life, but previous studies on members of these basal phyla have not examined embryonic germ cell origin. To try to define the embryonic origin of PGCs in the sea anemone *Nematostella vectensis*, we examined the expression of members of the *vasa* and *nanos* gene families, which are critical genes in bilaterian germ cell specification and development. We found that *vasa* and *nanos* family genes are expressed not only in presumptive PGCs late in embryonic development, but also in multiple somatic cell types during early embryogenesis. These results suggest one way in which preformation in germ cell development might have evolved from the ancestral epigenetic mechanism that was probably used by a metazoan ancestor.

INTRODUCTION

The establishment of a viable germ line is a crucial step in the development of all sexually reproducing animals. Studies carried out in genetic model organisms have provided models of some aspects of the molecular mechanisms of germ cell specification (Extavour and Akam 2003). Germ cells are sometimes specified at the beginning of embryonic development by inheritance of cytoplasmic determinants (preformation), as is the case in the fruit fly *Drosophila melanogaster* (Williamson and Lehmann 1996), the nematode worm *Caenorhabditis elegans* (Kimble and White 1981), and the zebrafish *Danio rerio* (Yoon et al. 1997). Differentiation of germ cells can also be induced at later stages of embryonic development by inductive signals from neighboring tissues (epigenesis), as has been demonstrated in mice (Lawson and Hage 1994; Tam and Zhou 1996) and axolotls (Nieuwkoop 1947).

Data available for most bilaterian species studied suggest that regardless of which of these two mechanisms is used, germ cells usually have a single embryonic origin in development, meaning that the founder population of germ cells specified during embryogenesis (primordial germ cells or PGCs) is not significantly amplified, renewed or replaced during adult reproductive life (Extavour and Akam 2003). The use of molecular markers to identify germ cells upon their first appearance during embryonic development is generally accepted as one of the most reliable ways to establish the embryonic origin of germ cells (Yoon et al. 1997; Nakao 1999; Shinomiya et al. 2000; Tsunekawa et al. 2000; Carré et al. 2002; Chang et al. 2002; Takamura et al. 2002). However, for metazoans other than the bilaterians, there is little data on how germ cells are specified during development, and no studies to date have used molecular markers to identify germ cells upon their first appearance during basal metazoan embryogenesis.

Members of basal metazoan phyla such as platyctene ctenophores, acoelomorph flatworms, and cnidarians are capable of asexual reproduction by budding, but also have distinct germ cell populations with male and female individuals that mate (Brusca and Brusca 2003). The study of germ cell segregation in such species is therefore crucial to understanding how the germ line of a metazoan ancestor might have evolved as a distinct cell population, how somatic tissues lost the potential to make gametes, and how modification of these developmental programs may have given rise to both the epigenetic and preformistic modes of germ cell specification observed in bilaterians.

The phylum Cnidaria includes hydroids (Hydrozoa), true jellyfish (Scyphozoa) and sea anemones, corals, and sea pens

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(Anthozoa). The bulk of the cnidarian biological literature deals with members of the Hydrozoa, the most well-known members of which are species of the genus *Hydra*. Defining a unique germ line in *Hydra* is somewhat problematic, as gametes derive from a subpopulation of pluripotent stem cells called interstitial cells (I cells), which have traditionally been thought to be capable of giving rise not only to male and female gametes but also to multiple somatic cell types (nematocytes, neurons, and gland cells) (Weismann 1883; Hargitt 1919; Berrill and Liu 1948; Halvorson and Monroy 1985; Thomas and Edwards 1991; Bode 1996). Although studies applying the cytological and ultrastructural criteria (high nuclear:cytoplasmic ratio; nuage) that are typically used to identify germ cells have failed to distinguish a putative uniquely gametogenic population of I cells (Noda and Kanai 1977), more recent studies based on clonal isolation of I cells have established that there is an I cell subpopulation that is uniquely responsible for producing gametes (Littlefield 1985, 1991; Littlefield and Bode 1986; Nishimiya-Fujisawa and Sugiyama 1993, 1995). However, the heterogeneous population of unipotent stem cells found in adult individuals may share a common origin in embryogenesis or earlier in development (Littlefield 1991).

A few molecular markers have been identified that are expressed in presumed unipotent germ line stem cells during or just before gametogenesis in adults, but these have helped to identify neither the developmental origin of these cells, nor their relationship with other I cells (Littlefield et al. 1985; Miller and Steele 2000; Miller et al. 2000; Mochizuki et al. 2000, 2001). For example, using the conserved germ line genes *vasa* and *nanos* (see below) as molecular markers to identify germ cells has proven useful in sexually active adult *Hydra* (Mochizuki et al. 2000, 2001), but the expression patterns of these genes during earlier stages of development are currently unknown. Similarly, Seipel and colleagues have analyzed the expression of the conserved stem cell gene *Piwi* during embryogenesis and medusa formation in the hydrozoan *Podocoryne carnea* (Seipel et al. 2004), but because this gene is expressed in somatic stem cells as well as the germ line, this study likewise did not identify the embryonic origin of germ cells.

The cytological and molecular similarity of all I cells to germ cells makes searching for the embryonic origin of germ cells in hydrozoans a difficult prospect. Moreover, although I cells are thought to arise from somewhere in the endodermal core during embryogenesis, the exact timing and location of their embryonic origin remains unclear (Kumé and Dan 1968; Martin et al. 1997; Pilato 2000). Furthermore, with respect to several biological and life history characteristics, the hydrozoans are a derived group of cnidarians, and thus may be poor representatives for generalized cnidarian development. The anthozoans (sea anemones, corals, and sea pens) have been suggested by several phylogenetic analyses to be the oldest extant representatives of the Cnidaria (Bridge et al. 1992, 1995; Medina et al. 2001; Collins 2002). We have therefore chosen the sea anemone *Nematostella vectensis* as a model system for studying embryonic development, and specifically germ cell development, in a basal cnidarian.

N. vectensis is easily cultured in the laboratory, can be spawned year round by simple regulation of the light–dark cycle, and produces thousands of gametes in a single spawning, which can be fertilized to give large synchronous populations of developing embryos (Frank and Bleakney 1976; Hand and Uhlinger 1992; Fritzenwanker and Technau 2002). Although this species may have at least one population of self-renewing ectodermal cells, *N. vectensis* is not thought to have I cells of the type seen in *Hydra*, which can give rise to both somatic cell types and gametes. Reproduction can occur asexually by fission, or sexually by external fertilization of gametes produced by individual male and female adults (Hand and Uhlinger 1992). Studies using only cytological characteristics to identify the germ line have suggested that germ cells are generated continuously throughout adult reproductive life, instead of being uniquely segregated during embryogenesis, as appears to be the case in most bilaterians studied (Extavour and Akam 2003).

In this study, we used the products of genes of the *vasa* and *nanos* families as molecular markers to identify germ cells throughout the embryonic development of *N. vectensis*. The *vasa* family of DEAD box helicases are conserved genes whose expression is generally restricted to germ cells for all metazoans for which data are available (Mochizuki et al. 2001; Extavour and Akam 2003). They are thought to have originated from a group of helicases constituting the *PL10* family, whose expression is found in both germ cells and pluripotent somatic stem cell types (Mochizuki et al. 2001). The *vasa* genes may have acquired a germ cell-specific role after their divergence from the *PL10* founder family (Mochizuki et al. 2001). *Nanos*-like genes are also widely conserved across the Metazoa, and have been shown to play important roles in both germ cell development and the development of some somatic tissues (Wang and Lehmann 1991; Pilon and Weisblat 1997; Mochizuki et al. 2000; Extavour and Akam 2003).

We show that *N. vectensis vasa* and *nanos* family genes are expressed in broad somatic domains during early embryonic development, and later are restricted to putative PGCs. Combining this gene expression data with characteristic germ cell morphology suggests that germ cells first appear late in development, in the same time and place as the development of the endodermal mesenteries. Finally, we note that some, but not all, *vasa* and *nanos* genes are expressed maternally, suggesting a possible mechanism for the evolutionary change in the mode of germ cell specification from epigenesis in late embryogenesis to preformation earlier in development.

MATERIALS AND METHODS

Cloning of *N. vectensis vasa* and *nanos* genes

N. vectensis vasa- and *nanos*-related genes were isolated via degenerate PCR using embryonic cDNA. Nested degenerate primers were designed to isolate an 867 base pair fragment within the highly conserved RNA helicase domains of Vasa- and PL10-related proteins. These primers are as follows: upstream primers MACAQTG (5′-ATGGCNTGYGCNCARACNGG-3′) and QTGSGKTA (5′-CARACNGGNWSNGGNAARACNGC-3′); and downstream primers HRIGRTG (5′-CCNGTNCKNCCDATNCKRTG-3′) and EYVHRIG (5′-CCDATNCKRTGNACRTAYTC-3′). Similarly, degenerate primers were designed to isolate a 160 base pair fragment within the zinc finger domain of Nanos-related proteins. The sequences for these primers are as follows: upstream primer CVFCRNN (5′-TGYGTNTTYTGYMGNAAYAA-3′) and downstream primer HTIKYCP (5′-GGRCARTAYTT-DATNGTRTG-3′).

PCR fragments were cloned in the pGEM-T easy plasmid vector (Promega, Madison, WI, USA) and sequenced at Gene Gateway (Hayward, CA, USA). Sequences from authentic *nanos* clones were used to design nested sets of non-degenerate primers for RACE (rapid amplification of cDNA ends). Both 3′-RACE and 5′-RACE were performed using the Smart Race cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA, USA). Overlapping 3′ and 5′ RACE fragments for each gene were conceptually spliced and submitted to GenBank as composite transcripts.

Sequence alignments and phylogenetic analysis

The *N. vectensis nanos* and *vasa* nucleotide sequences were analyzed via BLASTX searches of the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Amino acid alignments of the *vasa/PL10* helicase domain and nanos CCHC zinc finger domain (available upon request) were made using MacVector (CLUSTALW) and corrected by hand for obvious alignment errors. A Bayesian phylogenetic analysis was conducted using MrBayes 3.01 (Huelsenbeck and Ronquist 2001) using the "jones" amino acid model option with 1,000,000 generations sampled every 100 generations and 4 chains. A majority rule "consensus tree" was produced with PAUP*4.0b10 (Swofford 2002) from the last 9001 trees representing 900,000 stationary generations. Posterior probabilities were calculated from this "consensus." Additionally, Neighbor Joining (using mean AA distances) and parsimony analyses were conducted with PAUP* v4.0b10 using 1000 bootstrap replicates. Accession numbers for species and genes used in the analyses are as follows: *Nvnos1* AY730693; *Nvnos2* AY730694; *NvPL10* AY730695; *Nvvas1* AY730696; *Nvvas2* AY730697.

In situ hybridization

To characterize gene expression, in situ hybridization on whole mounts of *N. vectensis* was carried out as described previously (Finnerty et al. 2003). Digoxigenin-labeled RNA probes were constructed using the MegaScript Kit (Ambion, Austin, TX, USA). For *Nvvas1*, *Nvvas2*, and *NvPL10*, the 867 base pair fragments originally isolated by degenerate PCR were used to construct probes. For *nanos* genes *Nvnos1* and *Nvnos2*, probes were constructed from 3′ RACE fragments and were 1.2 and 1 kb in size, respectively.

Immunohistochemistry

Adult animals or primary polyps at various stages of gametogenesis were fixed by gradual addition of 37% formaldehyde to the 1/3 × seawater solution in which the animals were suspended. Fixation was at room temperature, with 30 min incubations each of 1%, 2%, and 4% formaldehyde. Animals were washed 3 × 30 min with 1 × PBS, and stored in 1 × PBS at 4°C, or in 70% EtOH or MeOH at −20°C for at least 2 months before staining. For antibody staining, before incubation with primary or secondary antibodies, animals were washed in 1 × PBS +0.1% Triton X-100 (PBT) for for at least 1 h, followed by washes in PBT+0.1% BSA (PBTB) for at least 30 min, and blocked in PBTB+4% normal goat serum (NGS; Sigma, St. Louis, MO, USA) for at least 30 min at room temperature or at 4°C overnight. Incubation with both primary and secondary antibodies was overnight at 4°C. Counterstains TO-PRO-3 iodide, YO-PRO-1 iodide, phalloidin-Alexa 647 (Molecular Probes, Eugene, OR, USA), and phalloidin-FITC (Sigma, St. Louis, MD, USA) were added to the secondary antibody incubation. After incubation with secondary antibody and counterstains, animals were washed at least 1 h in 1 × PBS +0.01% Triton X-100, and cleared in the dark in VectaShield (Vector Laboratories, Burlingame, CA, USA) or in 70% glycerol in 1 × PBS with 1 μg/ml DAPI at 4°C or at −20°C until mounting, which was done in the same medium as for clearing. Primary antibodies used were rabbit For2 (anti-Vasa) (Chang et al. 2002) 1:30 and rabbit anti-Vasa (gift of Paul Lasko) 1:100. Secondary antibodies used were goat anti-rabbit Alexa 488 1:500 (Molecular Probes) and goat anti-rabbit horseradish peroxidase (HRP) 1:300 (Jackson ImmunoLabs, Westgrove, PA, USA).

Image capture and processing

Embryos were examined using a Zeiss AxioPhot (Zeiss, Jena, Germany) and images captured with a Leica DCF 300F camera driven by either OpenLab or Leica FireCam software, or using a Leica TCS confocal scanning microscope (Leica, Wetzlar, Germany). Images were assembled using Adobe Photoshop 7.0 and Macromedia Freehand.

RESULTS

Cloning and characterization of *N. vectensis vasa* and *nanos* genes

Fragments of three DEAD-box helicase genes were isolated by degenerate PCR from embryonic *N. vectensis* cDNA. BLASTX searches of the NCBI database were utilized for both orthology assignments and in the creation of an amino acid alignment of the helicase domain from a variety of metazoan, plant, and fungal taxa. Based upon predicted amino acid sequence of the three *N. vectensis* gene fragments, all three possess six of the eight characteristic amino acid motifs within the helicase domain (Mochizuki et al. 2001) found in both Vasa- and PL10-related proteins. Within the helicase domain, one gene, which we name *NvPL10*, shares the greatest amino acid identity with PL10-related genes, with the

highest homology to coral (*Acropora CnPL10*) and sponge (*Ephydatia PoPL10*) genes (Fig. 1C). The other two *N. vectensis* DEAD box helicases show greatest amino acid identity to other cnidarian and sponge *vasa* genes (Fig. 1C); we therefore name them *Nvvas1* and *Nvvas2*.

Phylogenetic analyses of the helicase domain from metazoan, plant, and yeast *vasa* and *PL10* genes support the orthology established by amino acid similarity within the helicase domain (Fig. 1A). The *NvPL10* gene clearly clusters with other metazoan *PL10* related proteins, to the exclusion of both plant *PL10* genes and other helicase genes (*P68* and *vasa* genes) in agreement with previous analyses (Mochizuki et al. 2001). Within the *vasa* gene family, the vertebrate members clearly cluster together with 100% posterior probability, >50% bootstrap support, utilizing multiple methods of phylogenetic analysis (Bayesian, distance, and parsimony), with branching order reflecting known evolutionary relationships. The cnidarian genes group into two main clades, one containing only hydrozoan *vasa* genes, and another containing both anthozoan and hydrozoan *vasa* genes, with the *Nvvas1* gene clustering with *Hydra* Cnvas1, and *Nvvas2* branching with the coral (*Acropora*) *Cnvas* gene. From both amino acid identity and phylogenetic analysis (Fig. 1, A and C), it appears likely that the *vasa* genes duplicated early in cnidarian evolution, as multiple members have been identified in both anthozoans and hydrozoans. The absence of a second gene in other cnidarians (e.g., *Acropora, Tima, Hydractinia*) is likely due to incomplete PCR sampling, although gene loss is a possibility.

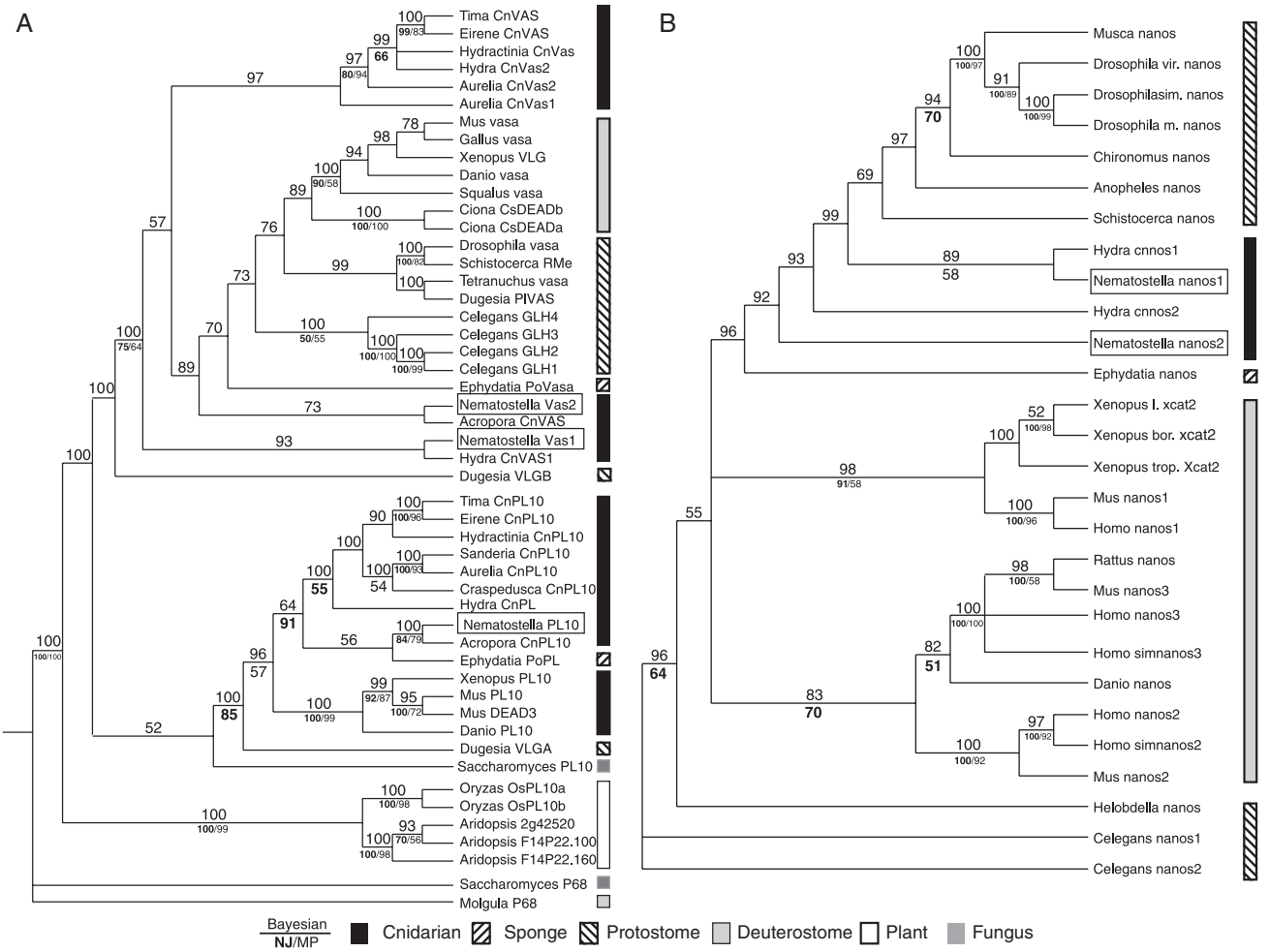
Two *nanos* genes were isolated by degenerate PCR and additional sequence information was obtained using RACE (*R*apid *A*mplification of *c*DNA *E*nds) PCR strategies. The *Nvnos1* and *Nvnos2* genes encode putative proteins of 216 and 248 amino acids. Both *Nvnos* genes possess two putative CCHC zinc finger domains, displaying a high degree of homology with other metazoan nanos-related proteins (Fig. 1D). The CCHC domains of *Nvnos1* and *Nvnos2* share the greatest amino acid identity with the *nanos* genes from the hydrozoan *Hydra magnipapillata Cnnos1* and *Cnnos2*, respectively (Fig. 1D).

Phylogenetic analyses based upon an alignment of the two CCHC zinc finger domains in metazoan *nanos* genes confirmed the orthology of the two *N. vectensis* genes (Fig. 1B). The *Nvnos1* gene groups with the *Cnnos1* gene from the hydrozoan, *H. magnipapillata*, whereas the *Nvnos2* gene falls immediately basal to the *Cnnos2* gene. This suggests that there was an early duplication of *nanos* genes in cnidarian evolution. Additionally, phylogenetic analysis shows three main clades of metazoan *nanos*-related genes. Two of these clades are unique to deuterostomes, particularly chordate vertebrates, containing *nanos-1* genes from vertebrates (including the frog *Xcat2* gene) and vertebrate *nanos-2* and *-3* genes. It appears that the *nanos-2* and *-3* genes resulted from a vertebrate-specific gene duplication event, with additional duplication events having occurred within the mammals. The third clade contains both arthropod and the basal metazoan *nanos*-related genes from cnidarians and sponge.

Embryonic and polyp development in *N. vectensis*

Fertilized *N. vectensis* eggs divide to form a hollow blastula stage and gastrulate by unipolar invagination at the future oral pole 12–15 h after fertilization (AF). A ciliated, swimming, bilayered planula stage embryo is formed by the end of the second day AF. The first tentacle buds of the juvenile appear at the future oral pole (posterior end of swimming planula) approximately 4 days AF. The first two (so-called "directive") mesenteries begin to form during the planula stage, followed by development of the remaining six mesenteries. Adult *N. vectensis* males and females possess eight mesenteries, which are involved in digestion, circulation, and reproduction (Frank and Bleakney 1976; Fautin and Mariscal 1991). Each of the eight mesenteries is a fold of the endodermal gastrodermis that runs along the oral–aboral axis and attaches to the pharynx. Gametogenesis takes place within the mesogleal compartment of the mesenteries. All eight mesenteries empty into the coelenteron aboral to the pharynx, and gametes are extruded through the oral opening. A full complement of tentacles are formed by 2–3 weeks AF.

Fig. 1. Phylogenetic analysis of *Nematostella vectensis vasa* and *nanos* genes. (A) Bayesian consensus tree of metazoan, fungal, and plant DEAD box helicase genes. The three *N. vectensis* genes isolated in this study are shown boxed in red. Multiple methods of phylogenetic analyses confirm the presence of the single *PL10* class gene and two *vasa* genes in *N. vectensis*. (B) Bayesian consensus tree of the CCHC zinc finger domain of metazoan *nanos* genes. Phylogenetic analyses suggest a relationship between the *nanos* genes of the hydrozoan *Hydera magnipapillata*, and the two *nanos* genes from the anthozoan *N. vectensis* isolated in this study. Colored bars indicate shared taxonomic relatedness for both gene trees. Numbers above branches indicate posterior probabilities of a Bayesian analysis (consensus of 9001 trees from 900,000 stable generations), whereas numbers below branches indicate bootstrap support (1000 iterations) from both neighbor joining and parsimony analyses. See Supplementary Data (Figs S1 and S2) for details of phylogenetic analyses, sequences studied, and GenBank accession numbers. (C) Helicase domain alignment showing percent identity in the helicase domain (boxed in yellow) of metazoan *vasa* and *PL10* genes. Amino acid identity is shown relative to the three helicase genes isolated from *N. vectensis*, *Nvvas1*, *Nvvas2*, and *NvPL10*, respectively. *N. vectensis vasa* and *PL10* genes share the greatest amino acid identity (in bold) with other cnidarian and sponge *vasa* and *PL10* genes. (D) Metazoan *nanos* gene alignment showing percent identity in the CCHC zinc finger domains. Amino acid identity is shown relative to the two *nanos* genes isolated from *N. vectensis*. The two *N. vectensis nanos* genes share the greatest amino acid identity (in bold) within their zinc finger domains with the *nanos* genes from the hydrozoan *H. magnipapillata*.

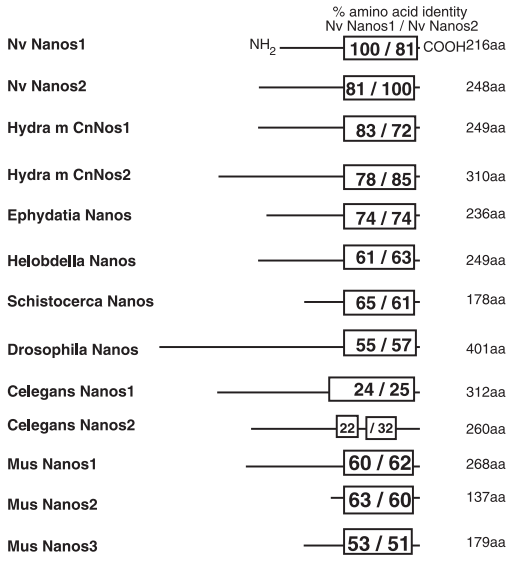


C

% amino acid identity
Nv Vasa1/ Nv vasa2/ Nv PL10

Nv Vasa-1	100/62/48
Nv Vasa-2	**62**/100/50
Nv PL10	48/50/100
Acropora CnPL10	41/43/79
Acropora CnVas	55/**66**/45
Hydra m CnPL10	53/56/77
Hydra m CnVas1	58/59/50
Hydra m CnVas2	56/62/47
Ephydatia PoPL10	52/54/**80**
Ephydatia PoVasa	**62**/**66**/53
Drosophila vasa	51/53/46
Dugesia VLGA	54/56/68
Dugesia VLGB	53/56/54
Dugesia PlVas	41/44/38
Celegans GLH1	42/43/42
Celegans GLH2	42/43/43
Celegans GLH3	40/40/42
Celegans GLH4	33/36/34
Mus Vasa	54/60/44

D



Expression of *N. vectensis vasa* genes

We studied the expression of two *N. vectensis vasa* genes and a *PL10* gene throughout all stages of embryogenesis using in situ hybridization. *Nvvas2* is not expressed in fertilized eggs or during early cleavages (Fig. 2, A–C). Its transcript is first detected just before gastrulation in a group of cells spanning approximately half of the blastula, including those cells destined to invaginate and form the endodermal core of the gastrula (Fig. 2D). As gastrulation begins, all of the ingressing endodermal cells as well as some of the surface cells close to the blastopore express *Nvvas2* (Fig. 2E). At later stages of gastrulation, *Nvvas2* expression is detected only in endodermal cells but no longer in ectodermal cells (Fig. 2, F and G). As development proceeds, *Nvvas2* expression becomes concentrated in the developing endoderm of the first two directive mesenteries (Fig. 2H). In the planula stage, *Nvvas2* expression becomes further refined to two clusters of cells in the presumptive directive mesenterial rudiments, as levels in the surrounding endoderm decrease (Fig. 2I). As tentacles begin to form, *Nvvas2* expression remains restricted to two clumps of presumptive mesenterial rudiment cells (Fig. 2J). At the early polyp stage, all endodermal expression of *Nvvas2* has disappeared, and expression remains only in two cell clusters (Fig. 2, K and L). The *Nvvas2*-positive cells at this stage have large round nuclei, characteristic of germ cells. As mesentery development proceeds at the polyp stage, *Nvvas2* expression is

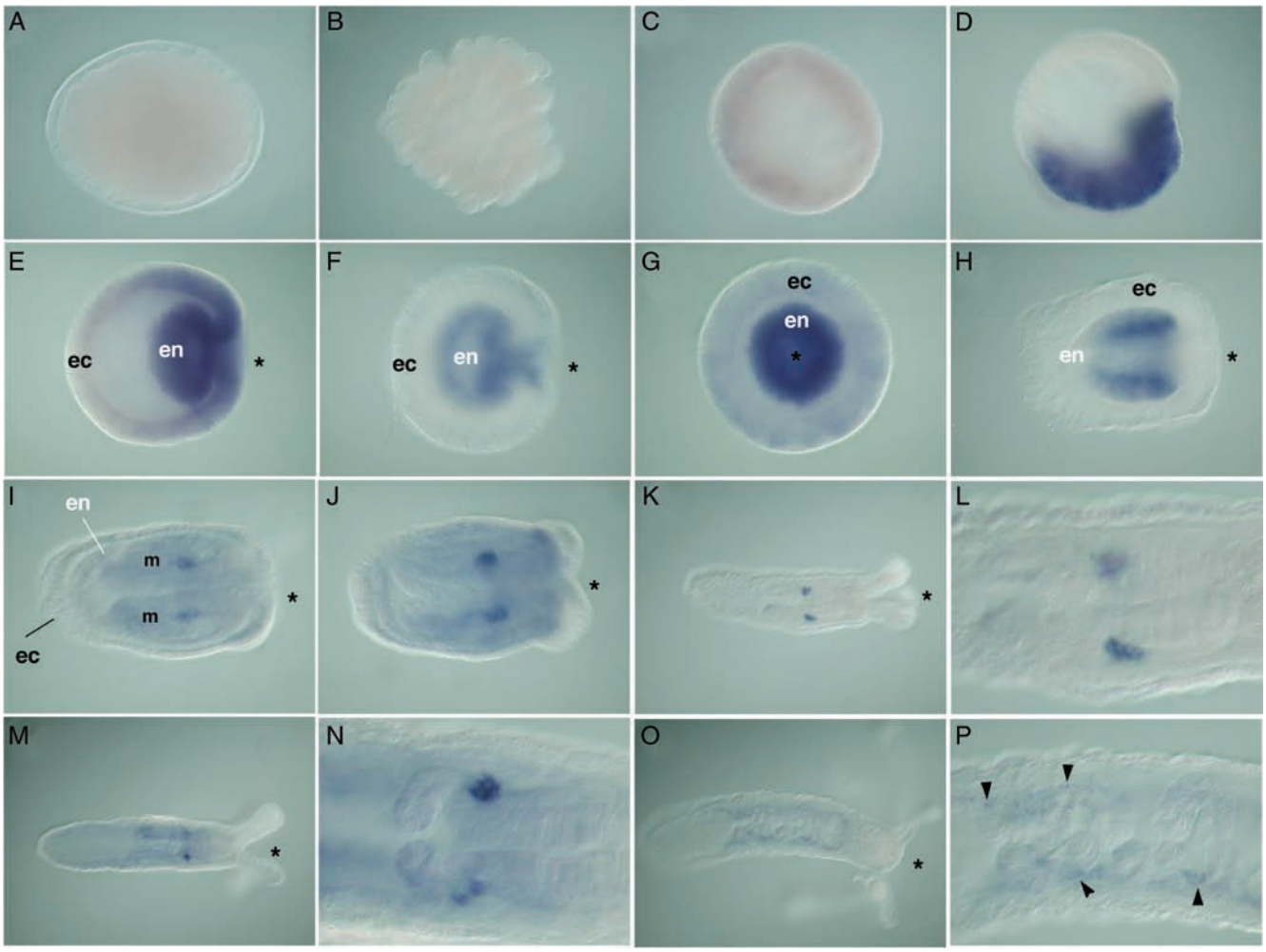


Fig. 2. Expression of *Nvvas2* during *Nematostella vectensis* embryogenesis. In all panels, the blastopore is to the right and/or marked with an asterisk, and views are lateral unless otherwise indicated. ec, ectoderm; en, endoderm; m, directive mesenteries. (A) Fertilized egg. (B) Early cleavage stage. (C) Blastula stage. (D) Blastula just prior to gastrulation. (E) Early gastrula. (F) Slightly older gastrula. (G) Blastopore view of a gastrulating embryo of the stage shown in (F). (H) Early planula. (I) Planula. (J) Planula showing initiation of tentacle development. (K) Early polyp. (L) Higher magnification of the *Nvvas2* expression clusters of the polyp shown in (K). (M) Advanced polyp. (N) Higher magnification of the anterior mesenteries of the polyp shown in (M). (O) Late polyp stage with many developed tentacles (not all tentacles are in the plane of focus). (P) Higher magnification of the polyp shown in (O) with scattered mesenterial cells expressing *Nvvas2* (arrowheads).

detected in individual cells throughout the length of the mesenteries (Fig. 2M), and nests of several highly expressing *Nvvas2*-positive cells remain in the oral portions of the developing mesenteries (Fig. 2N). In advanced polyps, *Nvvas2* is expressed uniformly in putative germ cells along the length of the mesenteries (Fig. 2O). Individual *Nvvas2* expressing cells in the mesenteries have cytoplasmic *Nvvas2* expression and large nuclei, a conserved characteristic of metazoan PGCs (Fig. 2P). The expression of *NvPL10* is almost identical at all stages of embryogenesis to that of *Nvvas2* (Fig. 3).

The expression of *Nvvas1* differs strikingly from that of *Nvvas2* at early embryonic stages. Maternal expression of *Nvvas1* is detected in the fertilized egg (Fig. 4A), and during early cleavage stages, *Nvvas1* is detected at low levels in all blastomeres (Fig. 4B). At the onset of gastrulation, *Nvvas1* is expressed intensely in the ingressing endoderm, and in some ectodermal cells around the oral pole that will eventually give rise to the endoderm at later stages (Fig. 4C), but becomes restricted exclusively to the endoderm at later gastrula stages (Fig. 4, D and E). As development proceeds, *Nvvas1* expression becomes concentrated in two discrete, symmetrical areas of the developing endoderm, and is still excluded from the ectoderm (Fig. 4, F and G). In the planula stage, *Nvvas1* expression becomes further refined to two elongated regions in the developing mesenteries (Fig. 4, H and I). As the tentacles begin to form, *Nvvas1* expression is reduced to two spots in the presumptive mesenterial rudiments (Fig. 4J). In the young polyp, *Nvvas1* is expressed in a patchy pattern corresponding to discrete cells, which may be germ cells, scattered throughout the mesenteries (Fig. 4, K and L).

Expression of *N. vectensis nanos* genes

We used in situ hybridization to study the expression of two *N. vectensis nanos* genes throughout embryonic development. *Nvnos2* is expressed uniformly in the cytoplasm of fertilized eggs (Fig. 5A). During early cleavage, *Nvnos2* expression is detected in all blastomeres (Fig. 5B), but is subsequently downregulated. At the onset of gastrulation, *Nvnos2* expression is detected in endodermal cells at the site of ingression (Fig. 5, C and D). As gastrulation continues, *Nvnos2* expression appears to increase in presumptive endodermal cells, and very low levels of *Nvnos2* are detected in the apical tuft at the aboral end of the embryo throughout swimming stages (Fig. 5, E–L). As gastrulation continues, endodermal expression becomes restricted to the endodermal components of the pharynx. The strength of expression subsequently increases in scattered endodermal cells, in two bilaterally symmetrical regions that will give rise to the first two (directive) mesenteries, and in ectodermal cells of the apical tuft located at the aboral pole (Fig. 5, H and I). As tentacle formation begins, expression of *Nvnos2* fades in the apical tuft and body wall endoderm but remains strongly expressed in the developing mesenteries (Fig. 5, J and K). At later stages of tentacle formation, *Nvnos2* expression in the presumptive mesenteries is largely concentrated in a central ring of endoderm around the pharynx and in the endoderm of the two directive mesenteries (Fig. 5, K and L). In early polyps, no *Nvnos2* expression is detected in the ectoderm, while mesenterial expression persists (Fig. 5M). As the polyp matures and elongates, the expression of *Nvnos2* remains essentially unchanged (Fig. 5N).

Nvnos1 is not expressed maternally or in early cleavage stages. It becomes upregulated for the first time in a scattered group of ectodermal cells at gastrula stages, but is absent from all endodermal cells (Fig. 6). As discussed above, these ectodermal cells may be a population of nematocyst precursors with stem cell characteristics, but are unlikely candidates for PGCs.

Identification of germ cells in late stage reproductive mesenteries

In order to determine whether the patchy mesenterial signal seen at the late polyp stage with probes against Nvvas2 (Fig. 2, O and P), NvPL10 (Fig. 3, I–L), Nvvas1 (Fig. 4, K and L), and Nvnos2 (Fig. 5, L–N) coincided with cells of characteristic germ cell morphology, we used a combination of Nomarski and fluorescent optics, together with antibodies against Vasa protein (Lasko and Ashburner 1990; Chang et al. 2002). As a test of the specificity of these cross-reactive antibodies in *N. vectensis*, we stained whole gravid adult females with anti-Vasa antibodies observed to be specific to Vasa family proteins (Strand and Grbic' 1997; Batalova and Parfenov 2003; Extavour 2004). Figure 7E shows an entire adult female *N. vectensis* stained with the anti-Vasa antibody, in which several darkly staining circles are seen in the region of the body close to the mesenteries. Dissection of the mesenteries shows that Vasa immunoreactivity is expressed in developing oocytes in all eight mesenteries (Fig. 7, F and F′). In late stage oocytes removed from the mesenteries, Vasa immunoreactivity is concentrated in a perinuclear ring in the ooplasm (Fig. 7I). In primary polyps, Vasa immunoreactivity is detected in clumps of cells in the forming mesenterial walls (Fig. 7, A and B). The patchy distribution of Vasa immunoreactivity is similar to the signal seen in the in situ hybridizations of the *Nvvas* genes, suggesting that the cells of the mesentery that express *Nvvas* genes are primordial germ cells. Higher magnification of Vasa-positive cells shows that these cells bear striking similarities to the cells observed to cluster in the mesenterial walls of adult females (described below). Vasa immunoreactivity is located in the cytoplasm of the clumps of cells in the primary polyp mesenteries, which have large round nuclei with diffuse chromatin and a high nuclear:cytoplasmic ratio, characteristic of PGCs (Fig. 7, C and D).

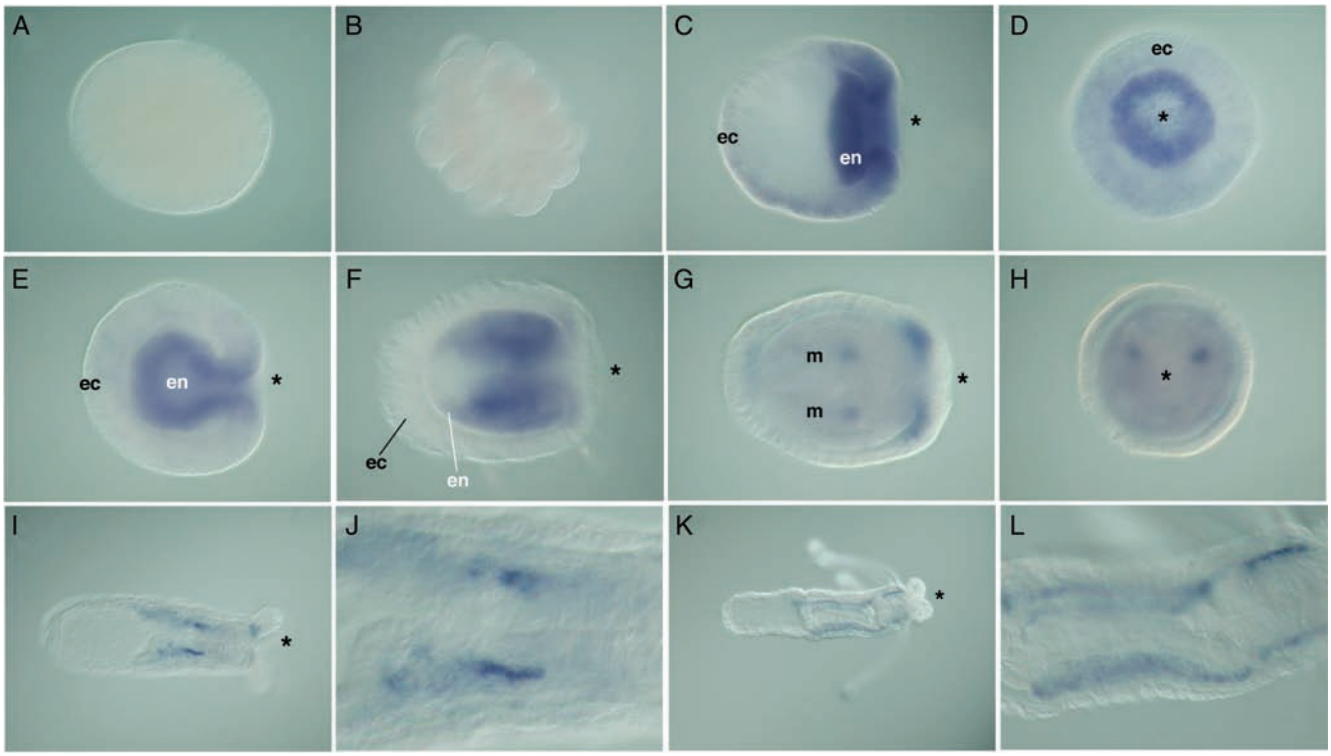
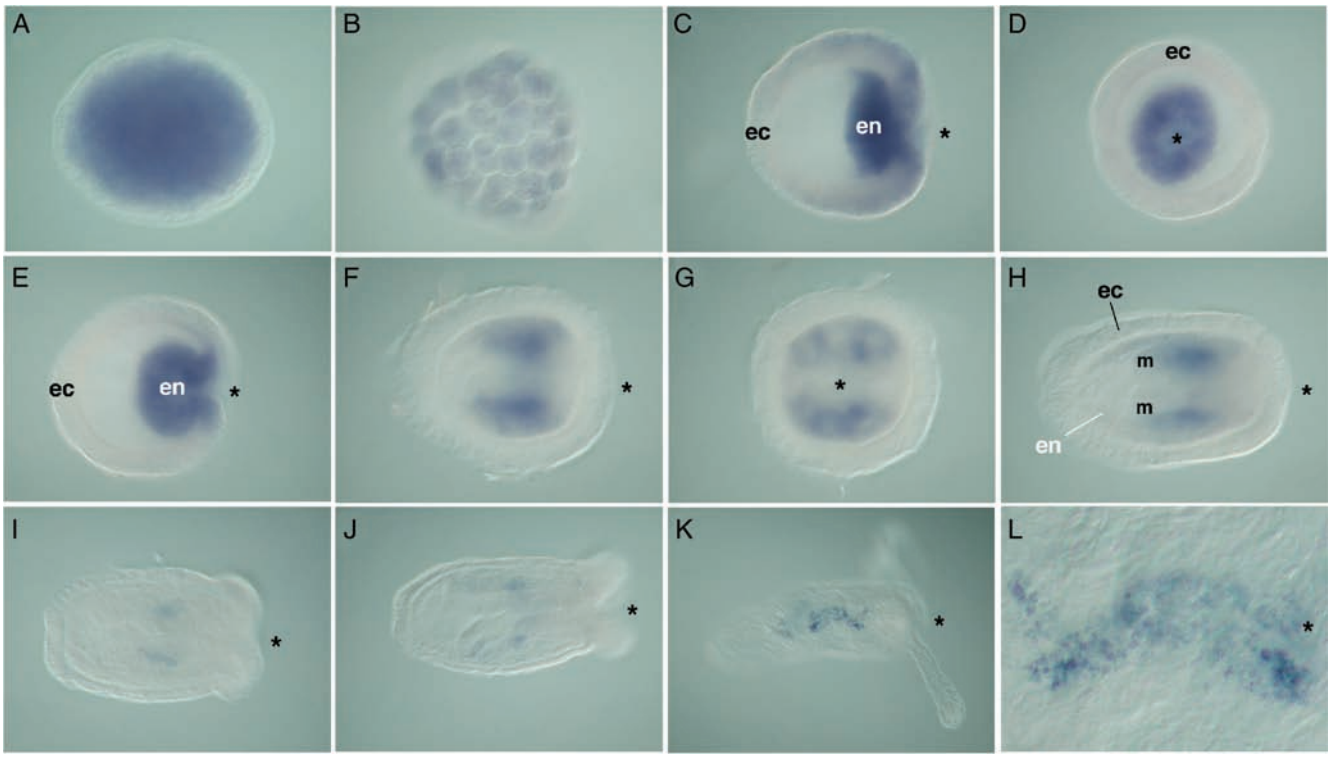


Fig. 3. Expression of *NvPL10* during *Nematostella vectensis* embryogenesis. In all panels, the blastopore is to the right and/or marked with an asterisk, and views are lateral unless otherwise indicated. ec, ectoderm; en, endoderm; m, directive mesenteries. (A) Fertilized egg. (B) Early cleavage stage. (C) Initiation of gastrulation. (D) Blastopore view of an embryo of the age shown in (C). (E) Mid-gastrula stage. (F) Early planula stage. (G) Planula stage. (H) Blastopore view of the embryo seen in (G). (I) Mid-polyp stage. (J) Higher magnification of the polyp shown in (I). (K) Late polyp stage with well developed tentacles. (L) Higher magnification of the polyp shown in (K).



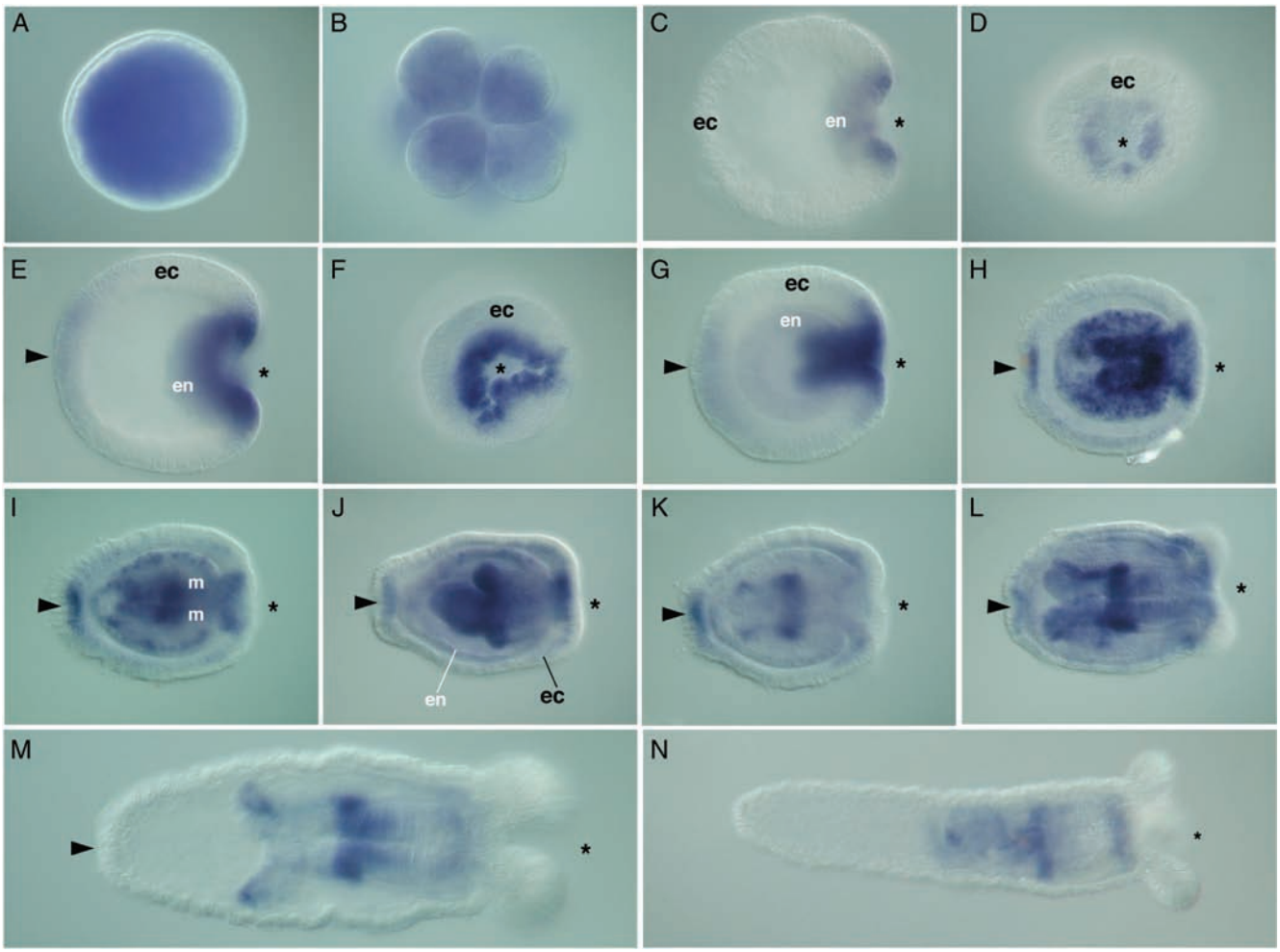


Fig. 5. Expression of *Nvnos2* during *Nematostella vectensis* embryogenesis. In all panels, the blastopore is to the right and/or marked with an asterisk, and views are lateral unless otherwise indicated. Arrowheads in E–L indicate ectodermal expression in the apical tuft region. ec, ectoderm; en, endoderm; m, directive mesenteries. (A) Fertilized egg. (B) Eight cell stage. (C) Initiation of gastrulation. (D) Blastopore view of an early gastrulating embryo of the stage shown in (C). (E) Progression of gastrulation. (F) Blastopore view of a gastrulating embryo of the stage shown in (E). (G) Mid-gastrula stage. (H) Late gastrula stage. (I) Elongation of early planula. (J) Early planula at beginning of tentacle formation. (K) Early tentacle formation. (L) Progression of tentacle formation. (M) Early polyp with few developing tentacles. Ectodermal expression is no longer detectable (arrowhead). (N) Elongated polyp.

Examination of the mesenterial epithelia of adults shows that the walls of the mesenteries contain early stage oocytes (Fig. 7G) with cytoplasmic Vasa immunoreactivity (Fig. 7H). Early oocytes are recognizable by their size, large nucleus, and diffuse chromatin. Obvious perinuclear localization of Vasa protein is not observed at this stage. The borders of the mesenteries contain immunopositive areas (Fig. 7, J–L), which closer inspection reveals to be clusters of Vasa-positive cells whose nuclei contain chromatin more diffuse than that of the surrounding somatic nuclei of the mesentery (Fig. 7, M–O). High magnification of one such Vasa-positive cluster (Fig. 7P) shows cells with large round nuclei, diffuse chromatin, cytoplasmic Vasa immunoreactivity distribution, and high nuclear/cytoplasmic ratio, consistent with the interpretation that these are primordial germ cells.

Fig. 4. Expression of *Nvvas1* during *Nematostella vectensis* embryogenesis. In all panels, the blastopore is to the right and/or marked with an asterisk, and views are lateral unless otherwise indicated. ec, ectoderm; en, endoderm; m, directive mesenteries. (A) Fertilized egg. (B) Early cleavage stage. (C) Early gastrula. (D) Blastopore view of a gastrulating embryo. (E) Mid-stage gastrula. (F) Early planula. (G) Blastopore view of an embryo of the stage seen in (F). (H) Planula. (I) Planula at early stages of tentacle development. (J) Late planula stage. (K) Young polyp. (L) Higher magnification of the polyp in (K).

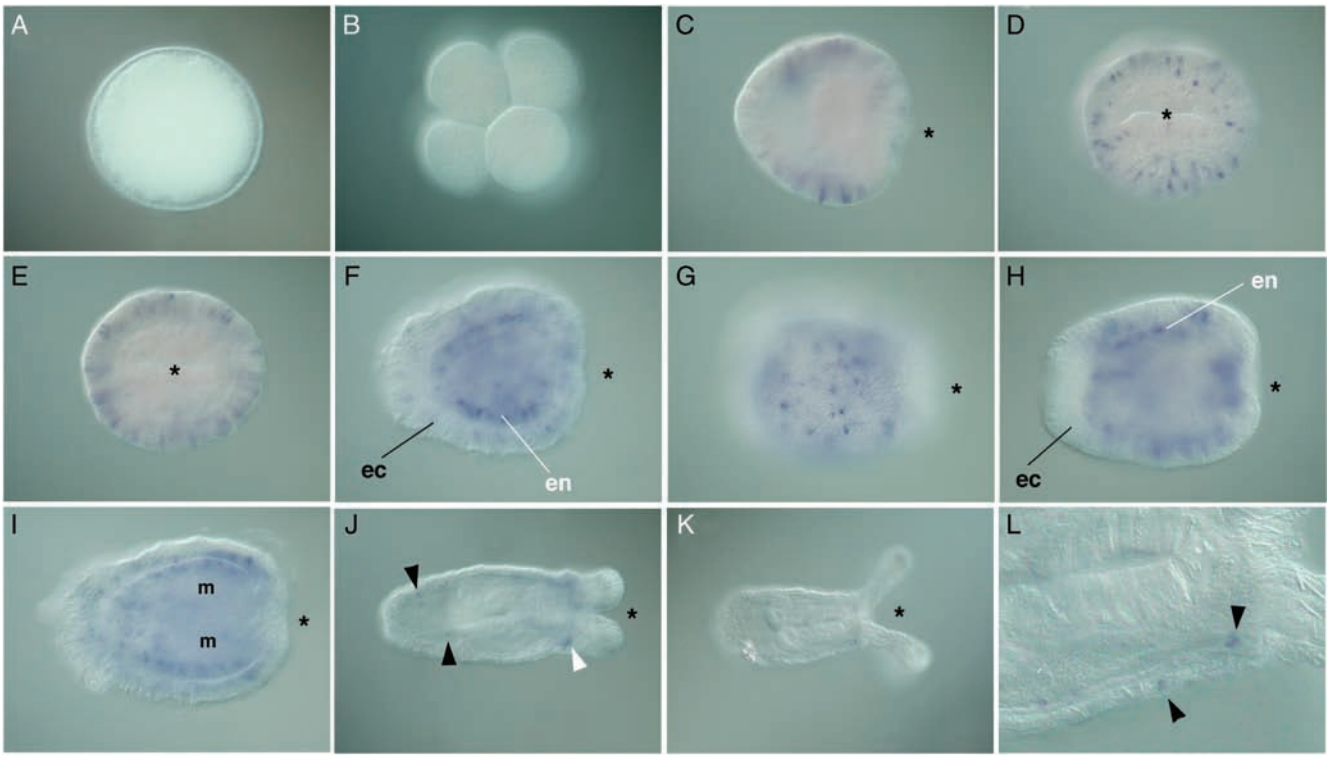


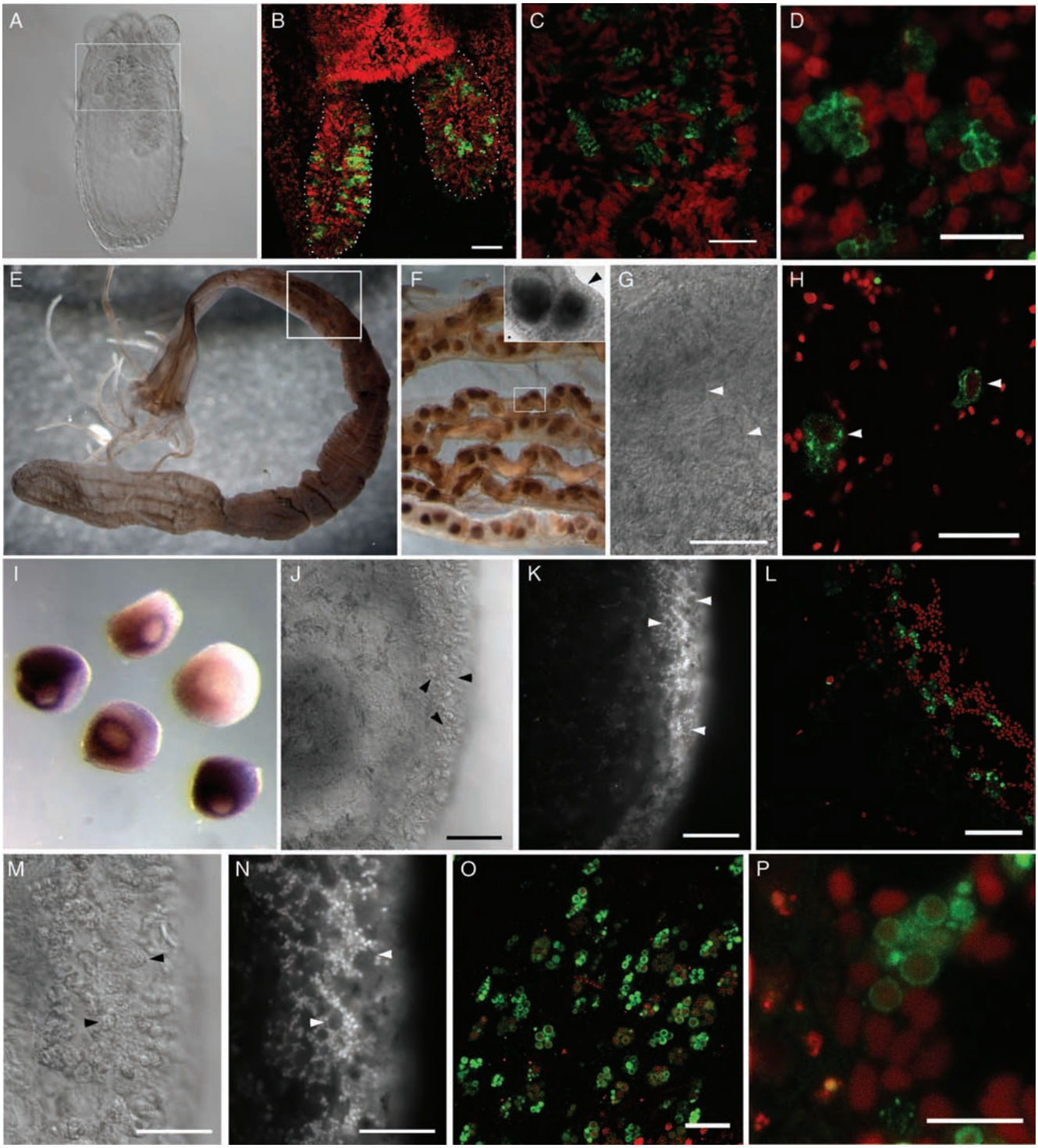
Fig. 6. Expression of *Nvnos1* during *Nematostella vectensis* embryogenesis. In all panels, the blastopore is to the right and/or marked with an asterisk, and views are lateral unless otherwise indicated. ec, ectoderm; en, endoderm; m, directive mesenteries. (A) Fertilized egg. (B) Four cell stage. (C) Early gastrula. (D) Blastopore view of a gastrulating embryo of the stage shown in (C). (E) Blastopore view of the same embryo shown in (D), a deeper plane of focus. (F) Early planula stage. (G) The same embryo shown in (F) focused on the surface of the embryo. (H) Planula stage at the beginning of tentacle formation. (I) Later planula. (J) Early polyp stage: *Nvnos1* expression is concentrated in endodermal cells near the anterior of the polyp (white arrowhead), but is also found in single cells scattered throughout the ectoderm (black arrowheads). (K) Later polyp. (L) Higher magnification of the polyp shown in (K). Some ectodermal cells still express *Nvnos1* at this stage (arrowheads).

Fig. 7. Vasa protein expression in *Nematostella vectensis* primary polyps and reproductive females. Red: nuclei. Green: Vasa immunoreactivity. (A–D) Oral is up in all panels. (A) DIC image of early polyp stage showing directive mesentery development (boxed area). (B) Higher magnification confocal image of region boxed in (A), showing the directive mesenteries (outlined with white dots) containing clumps of Vasa-positive cells. (C) A magnified view of the developing mesenteries showing the scattered clusters of Vasa-positive cells. (D) High magnification of clumps of Vasa-positive putative primordial germ cells (PGCs) of the developing mesenteries. (E, F, I) Whole mount images showing Vasa expression detected by immunohistochemistry with anti-Vasa antibody (Lasko and Ashburner 1990). (E) Whole mount view of mature female *N. vectensis*. (F) Dissection and higher magnification of area boxed in (E). (F′) Higher magnification of area boxed in (E), showing differences in morphology between the site of attachment to the body wall (asterisk) and side of mesentery facing the inner lumen (arrowhead). (G) High magnification of unstained mesenterial epithelium containing early stage oocytes (arrowheads). (B–D, H, L, O, P) Confocal images showing Vasa staining detected by immunofluorescence. (H) Early stage oocytes (arrowheads) in the mesenterial epithelium expressing Vasa protein in the cytoplasm. (I) Whole oocytes removed from lumen of mesenteries shown in (E). (J) DIC view of the inner edge of the mesentery (indicated by the arrowhead in (F′)), showing distinct clump-like structures (arrowheads). The edge of the mesentery is to the right and the mesenterial cavity is to the left. (K) DAPI stain of the tissue shown in (J) reveals that these clumps (arrowheads) have more diffuse nuclei than surrounding cells. (L) Confocal image of the same type of tissue shown in (J, K). (M) Higher magnification DIC view of the area shown in (J), the clumps are groups of rounded cells (arrowheads). (N) DAPI stain of the tissue shown in (M), arrowheads indicate the same clumps of rounded cells as in (M). (O) Higher magnification of the mesenterial edge shown in (M, N). Vasa expression in these rounded cells suggests that they are PGCs. (P) High magnification of a clump of Vasa-positive putative PGCs of the mesenterial epithelium. Scale bars: A = 40 μm; C, G, H, M, N = 16 μm; B, J, K, L = 20 μm; O = 8 μm; D, P = 4 μm. C and D are single 1.0 μm confocal sections; B is a maximum projection of 25 confocal sections of 2.2 μm each; H, L, O, and P are single confocal sections through 1.7, 2.5, 3.0, and 1.9 μm, respectively.

DISCUSSION

Multiple roles of *vasa* and *nanos* genes in *N. vectensis*

Both *Nvvas* genes and *NvPL10* appear to play early roles in endoderm development during gastrulation, evidenced by their broad expression in the ingressing cells at the future oral pole and later in the developing mesenteries. At late planula stages, expression of both *Nvvas* genes and *NvPL10* is restricted to cells with characteristic PGC morphology, which appear at this stage in the developing reproductive mesenteries. Sequence analysis of both *Nvvas1* and *Nvvas2* indicates



that these are true *vasa* family members, and not members of a different DEAD box helicase family (Fig. 1A). In most metazoans, the *vasa* family genes are localized specifically to the germ line. However, in some metazoans, *vasa* genes have been implicated in aspects of pluripotent somatic cell development as well, specifically in the development of I cells in *Hydra* (Mochizuki et al. 2001) and the neoblasts (cells involved in regeneration) of the flatworm *Dugesia japonica* (Shibata et al. 1999). The possible dual somatic and germ line role of *vasa* family members observed in *N. vectensis*, whereas rare in bilaterians, is thus not unusual among basal metazoans.

NvPL10 belongs to the PL10 family of helicases, a conserved group of yeast (Chuang et al. 1997; Kawamukai 1999), animal (Leroy et al. 1989; Gururajan et al. 1991), and plant (Lin et al. 1999) proteins that are very similar to the *vasa* helicases, but have been shown to be involved not just in the germ line, but also in the development of a variety of somatic tissues. The apparent role of *NvPL10* in the development of both germ cells and endoderm is thus not surprising. Our preliminary phylogenetic analysis of *N. vectensis vasa*-like genes (Fig. 1A) suggests that the duplication and divergence of a putative ancestral *PL10* family gene (Mochizuki et al. 2001) occurred before the cnidarian lineages arose, and that *vasa* family genes may then have undergone further duplications within individual cnidarian lineages. The early endomesodermal expression of the *Nvvas* genes may therefore be a reflection of their ancestral somatic role. In the evolution of higher metazoans, as genetic subroutines defining individual somatic cell fate decisions arose, the function of such genes in somatic development may have been lost, resulting in *vasa* family member genes with only specialized germ line role. We might therefore predict that the roles of *Nvvas1* and *Nvvas2* in endoderm development would be at least partially redundant with *NvPL10* function, but that *NvPL10* function alone would not be sufficient for proper germ line development. Further experiments testing the possible function of individual *Nvvas* genes using RNAi or morpholino knockdown approaches should allow us to test these predictions, and to determine whether or not *Nvvas1* and *Nvvas2* has entirely dispensed with their somatic role.

Nvnos2 expression is detected early in endodermal cells during gastrulation, and later in two restricted domains: somatically in a group of aboral ectodermal cells, and in putative germ cells as they appear in the developing mesenteries. *Nvnos1* is not expressed in germ cells at all, but rather in a subset of ectodermal cells that most likely correspond to a population of nematocyst precursors. Because available data on *nanos* genes indicate a role in germ cell development in all animals studied, but a somatic role only in some animals, it has been suggested that basal metazoan *nanos* genes may have functioned exclusively in the germ line, and acquired somatic roles more recently in the evolution of bilaterians. Although tentative, our phylogenetic analysis of *N. vectensis nanos* genes supports this view, as *nanos1* genes with somatic function appear to be the result of gene duplication within the cnidarian lineage of an ancestral *nanos2* gene with germ line function (Fig. 1B).

Nanos genes have been implicated in germ line development in both invertebrates and vertebrates from Hydra to humans (Kobayashi et al. 1996; Pilon and Weisblat 1997; Forbes and Lehmann 1998; Asaoka-Taguchi et al. 1999; Deshpande et al. 1999; MacArthur et al. 1999; Subramaniam and Seydoux 1999; Koprunner et al. 2001; Sano et al. 2001; Kang et al. 2002; Jaruzelska et al. 2003; Lall et al. 2003; Tsuda et al. 2003). However, an additional somatic role for *nanos* in axial patterning has been experimentally demonstrated in *D. melanogaster*, and the expression patterns of *nanos* genes in metazoans from leeches to grasshoppers suggest that these genes can play roles in the development of a range of somatic tissues (Lehmann and Nusslein-Volhard 1991; Mosquera et al. 1993; Curtis et al. 1995; Kang et al. 2002; Haraguchi et al. 2003; Lall et al. 2003). Interestingly, in those animals in which *nanos* genes have been implicated in somatic development, the somatic function is carried out by maternally inherited gene product, and zygotic transcription is usually restricted to the germ line (Pilon and Weisblat 1997; Subramaniam and Seydoux 1999; Kang et al. 2002; Haraguchi et al. 2003; Lall et al. 2003; Torras et al. 2003; Tsuda et al. 2003). Although we do not know with certainty when zygotic transcription begins in *N. vectensis* embryogenesis, the difference between maternal and zygotic *nos* function also appears to be the case for *Nvnos2*, whose maternal expression is implicated in endoderm development, whereas presumptive zygotic expression may be principally in germ cells. The presumed duplication of *Nvnos2* that gave rise to *Nvnos1* seems to have involved a change in regulatory region function, such that *Nvnos1* is transcribed exclusively zygotically, and in a completely different somatic cell population. Whether or not this is a general characteristic of the duplication and functional divergence of *nanos* genes cannot be determined with the data currently available in the literature, as where phylogenetic data are available, information on maternal expression is absent (*Hydra*) (Mochizuki et al. 2000), and where full expression profiles are available, phylogenetic resolution is poor (mouse, *C. elegans*) (Subramaniam and Seydoux 1999; Haraguchi et al. 2003; Tsuda et al. 2003).

Development of germ cells in *N. vectensis* and comparison with other basal metazoans

Our data do not allow us to determine unambiguously whether *N. vectensis* germ cells are specified late in embryonic development, or by inheritance of a special cytoplasm containing determinants during early embryonic cleavage. However, our observations are not inconsistent with those of previous studies on sexually reproducing adult sea anemones,

using only cytological and/or ultrastructural analyses to identify cell types. Such studies have all suggested that germ cells originate in the endodermally derived gastrodermis of the mesenteries, and then move into the mesoglea to undergo gametogenesis (Campbell 1974; Jennison 1979; Fautin and Mariscal 1991; Hinsch and Moore 1992). Germ cell origin in the gastrodermis followed by gametogenesis in the mesoglea has also been reported for various species of corals (Halvorson and Monroy 1985; Ryland 1997; Goffredo et al. 2000) and a sea pen (Eckelbarger et al. 1998).

The data available for other basal metazoans indicate that epigenesis is the most common germ cell specification mechanism for non-bilaterian animals. The germ cells of acoelomorph flatworms appear to share a mesenchymal origin with the neoblasts, pluripotent somatic stem cells similar in potential to I cells of hydrozoans (Gschwentner et al. 2001). Ctenophore germ cells are first identified in early larval stages, in the endodermal canals where the gonad rudiments form (Dunlap Pianka 1974; Hernandez-Nicaise 1991), although their exact embryological origins are not known. Sponges are known to possess genes of the *vasa*, *PL10*, and *nanos* families (Mochizuki et al. 2001), and although expression data are not available for these genes, sponge gametes are known to derive from various subpopulations of pluripotent mesenchymal cells (Tuzet et al. 1970; Gaino et al. 1984). In summary, basal metazoans all derive their germ line from populations of endodermal or mesenchymal cells that are not specified at the beginning of embryogenesis by inheritance of cytoplasmic determinants. This epigenetic mechanism is clearly different from that observed in, for example, flies and nematode worms (Kimble and White 1981; Williamson and Lehmann 1996), but may be regulated in a similar way to germ cell specification in mice (Tam and Zhou 1996). In the mouse, BMP-2, -4, and -8 family members have been shown to provide the signal for cells of the proximal epiblast to become germ cells (Lawson et al. 1999; Ying et al. 2000; Ying and Zhao 2001). If this signaling pathway reflects the ancestral epigenetic germ cell specification mechanism, then we might expect expression of *N. vectensis BMP* genes to be localized at the planula stage to ectodermal and/or endodermal cells in the region of the directive mesenteries, immediately preceding the differentiation of germ cells, including stabilization and/or zygotic transcription of *vasa* and *nanos* genes, in that region. Future studies on the protein distribution of Nanos and Vasa proteins, as well as expression and function of *N. vectensis BMP* genes, could address the question of whether this epigenetic mechanism is ancestral or not in metazoans.

Evolution of bilaterian germ cell specification mechanisms

Whether germ cell specification is accomplished by inductive signaling between embryonic cells, or maternal localization of cytoplasmic factors, the molecules that signal germ cell differentiation are highly conserved across diverse phyla. What appear to differ are the upstream signals regulating the expression of genes such as *vasa* and *nanos*, and not the expression of those genes themselves. Comparison of germ cell specific gene expression from different species has therefore failed hitherto to suggest how a mechanism ensuring early asymmetric localization of such genes could have evolved from a developmental program that triggers expression in a small group of cells late in embryogenesis.

The expression profiles of *vasa* and *nanos* genes in *N. vectensis*, however, suggest one possibility for the evolution of preformation. Four of the five genes are expressed in endodermal precursors at the time of gastrulation. Two of the four genes implicated in germ cell development, *Nvvas2* and *NvPL10*, become localized to putative germ cells at the time of their formation in the late planula stage. The other two genes, *Nvvas1* and *Nvnos2*, are additionally expressed in fertilized eggs, probably maternally, possibly reflecting a role in oogenesis. Experiments in flies and mice have shown that the products of both *vasa* and *nanos* genes are necessary not only for germ cell embryonic specification but also for gametogenesis (Styhler et al. 1998; Tsuda et al. 2003). If expression of some germ cell-specific genes was not turned off at the end of oogenesis (as may be the case for *Nvvas2* and *NvPL10*), but instead remained in mature oocytes until early cleavage stages (as observed for *Nvvas1* and *Nvnos2*), then their expression would not have to be induced de novo in developing germ cells later in embryogenesis. Instead, germ cell fate could be inherited in the form of cytoplasm containing the products of these germ cell-specific genes. Our data from *N. vectensis* lead us to speculate that changes during gametogenesis in the transcriptional and translational regulation of key genes could provide an explanation for the evolution of preformation of germ cell specification from an ancestral epigenetic mechanism.

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SUPPLEMENTARY MATERIAL

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/EDE/EDE05023/EDE05023sm.htm.

Fig. S1. Alignments of vasa genes. (A) Amino acid alignment of the DEAD box helicase domain from representative plant, fungal, and metazoan genes used for phylogenetic analysis of *vasa* genes. The three DEAD box helicase genes isolated from *N. vectensis* share six of the eight characteristic amino acid motifs within the helicase domain (shown in *bold*) (Mochizuki et al. 2001).

Fig. S2. Alignments of nanos genes. (A) Amino acid alignment of the two CCHC zinc finger domains found in metazoan *nanos* genes used for phylogenetic analyses. Conserved cysteines and histidines are shown in *bold*.

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Supporting Information for

***vasa* and *nanos* expression patterns in a sea anemone and the evolution of bilaterian germ cell specification mechanisms**

Extavour, C.G., Pang, K., Matus, D. Q. & Martindale, M. Q., *Evolution and Development* **7(3)**: 201-215 (2005)

Supplementary Information Figure S1A:

Vasa/PL10 Alignments of DEAD box helicase domain used in phylogenetic analyses:

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Molgula_P68         SGKTLAFILPAIVHIN-AQPYLDP----------GD------GPIVLVLCPTRELAQQVQ [43]
Tima_CnPL10         SGKTAAFLVPILSRIFEEGPFENA-GTI----RSGXS-RRKQFPIAIVLAPTRELASQIY [54]
Sanderia_CnPL10     SGKTAAFLLPIMNRIYQEGPYDTGYAGG----GGGRTGRRKQFPFCLILAPTRELASQIY [56]
Aurelia_CnPL10      SGKTAAFLLPIMSRIYEEGPYDQQYGGA----GG-RPGRRKQFPFCLILAPTRELASQIF [55]
Craspedusca_CnPL10  SGKTAAFLVPIMSQIFTEGPFDNTYSDS----RSGG--RRKQFPIALVLAPTRELASQIY [54]
Eirene_CnPL10       SGKTAAFLVPILSRIFEEGPFENA-GTI----RGGTS-RRKQFPIAVVLAPTRELASQIY [54]
Hydractinia_CnPL10  SGKTAAFLVPILSRIFEEGPFEGA-SNN----RSGG--RRKQFPIALVLAPTRELASQIY [53]
Tima_CnVAS          SGKTAAFLLPVLTGMMEFKDEF-TSQ----------LSEVQAPLALVIAPTRELATQIF [48]
Hydractinia_CnVas   SGKTAAFLLPVLASIMQHKDQL-TSQ----------LSEVQAPLGLIIAPTRELANQIY [48]
Eirene_CnVAS        SGKTAAFLXPVLTGMMEFREEF-SSQ----------LSEVQAPLALIIAPTRELATQIF [48]
Aurelia2_CnVas      SGKTAAFLLPVLTKMMEDGLSG--SK----------FSEVQAPAALIISPTRELTVQIH [47]
Aurelia1_CnVas      SGKTAAYLLPVISTLLKNGVTEA--D----------SCECASPNALIIAPTRESAIQIF [47]
Oryzas_OsPL10a      SGKTAAFCFPIISGIMSSRPPQRPR----------GSRTAYPLALILSPTRELSVQIH [48]
Oryzas_OsPL10b      SGKTAAFCFPIISGIMRSRPPPRSR----------GSRTAYPLALILSPTRELSVQIH [48]
Mus_vasa            SGKTAAFLLPILAHMMRDGITASRFK----------ELQEPECIIVAPTRELINQIY [47]
Danio_r_vasa        SGKTAAFLLPILQRFMTDGVAASKFS----------EIQEPEAIIVAPTRELINQIY [47]
Squalus_vasa        SGKTAAFLLPIIEMLLKGNAASSR----------FKELQEPEVVIVAPTRELINQIY [47]
Gallus_vasa         SGKTAAFLLPIVDRMMKDGVTAS----------FPKQQDPQCIIVAPTRELINQIF [46]
Xenopus_l_VLG       SGKTAAFLLPILSYMMNEGITASQYL----------QLQEPEAIIIAPTRELINQIY [47]
Ciona_CsDEADb       SGKTAAFLLPVLTKLITNGLQSSQFS----------EKQTPRAIVVGPTRELIYQIF [47]
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Celegans_GLH4       SGKTLAFLIPFVIKLMEEFEKDRDVTD----------EKPSPRLLIVAPTRELVNQTF [48]
Celegans_GLH3       SGKTAAFLLPIMSRLILEKDLNYGAE----------GGCYPRCIILTPTRELADQIY [47]
Celegans_GLH2       SGKTAAFLLPIMARLIDENDLNTAGE----------GGCYPRCIILTPTRELTDQIY [47]
Celegans_GLH1       SGKTAAFLLPIMTRLIDDNNLNTAGE----------GGCYPRCIILTPTRELADQIY [47]
Dmelanogaster_      SGKTAAFLLPILSKLLEDPHELELG----------RPQVVIVSPTRELAIQIF [43]
Schistocerca_RMe    SGKTAAFLLPIINTILNDPRELVMTG----------QGCEPHAVILSPTRELALQIF [47]
Tetranuchus_vasa    SGKTLAFILPILQDLFNDKSLQTNYG----------QTPQTPSAVIVTPTRELAVQIY [48]
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Nematostella_Vas2   -----AYMLPVLTSLIKQGLNAPPR----------SPLALCVAPTRELAKQIY [38]
Nematostella_Vas1   -----AFLLPVMTSMMNAGLTSSSF----------SETQTPQAMCIAPTRELANQIY [42]
Hydram_CnVas2       SGKTAAFLIPVLNTLMQFRSELTSS-L----------SEVQAPLALVIAPTRELAVQIQ [48]
Hydram_CnVAS1       SGKTASFLLPIITNLMNEGLDNIDSNI----------DGVALPLAAILAPTRELVVQLF [49]
Dugesia_PlVAS       SGKTAAFLIPIIKGLHGTVLETDSSN----------TSSTAFPRALIMTPTRELCRQIF [49]
Dugesia_VLGB        SGKTAAFLIPIINHLVCQDLNQ----------QRYSKTAYPKCLILAPTRELAIQIL [47]
Dugesia_VLGA        SGKTAAFLIPLLSMMYQDGPGN---SLS----HS---GYKKEYPVALILAPTRELAVQIY [50]
Ephydatia_PoVasa    SGKTAAFLLPAITKLIKEQVPGGSQ----------AETQSPQVLIISPTRELTLQIY [47]
Acropora_CnPL10     SGKTAAFLIPILSRIFEEGPPP-LPEAR----QL---SRRKQFPICLVLAPTRELACQIF [52]
Acropora_CnVAS      SGKTAAFLLPVMTGMLQKGLTSSSIMG----------GPQCPQALIISPTRELACQIY [48]
Hydram_CnPL         SGKTAAFLVPILSRIFEEGPFENPSNVR----QG---GKKKQYPIALVLAPTRELASQIY [53]
Ephydatia_PoPL      SGKTAAFLIPILDLVFQQGCPRPPSDSR---YS----GRRKQYPTALVLGPTRELAVQIF [53]
Aridopsis_2         SGKTAAFCFPIISGIMKDQHVERPRGSR----------AVYPFAVILSPTRELACQIH [48]
Aridopsis_3         SGKTAAFCFPIISGIMKDQHVQRPRGSR----------TVYPLAVILSPTRELASQIH [48]
Aridopsis_1         SGKTAAFCFPIISGIMKDQHIERPRGVR----------GVYPLAVILSPTRELACQIH [48]
Yeast_PL10          SGKTGGFLFPLFTELFRSGPSPVPEKAQ----S---FYSRKGYPSALVLAPTRELATQIF [53]
Xenopus_PL10        SGKTAAFLLPILSQIYADGPGDAMKHLK----DNGRYGRRKQFPLSLVLAPTRELAVQIY [56]
Mus_PL10            SGKTAAFLLPILSQIYTDGPGEALRAMK----ENGKYGRRKQYPISLVLAPTRELAVQIY [56]
Danio_PL10          SGKTAAFLLPVLSQIYTDGPGEALQAAKNSAQENGKYGRRKQYPISLVLAPTRELALQIY [60]
Mus_DEAD3           SGKTAAFLLPILSQIYADGPGEALRAMK----ENGRYGRRKQYPISLVLAPTRELAVQIY [56]

Yeast_P68           QECTKFGKSSRIRNTCVYGGVPRGPQIR-DLIRG--VEICIATPGRLLDMLDSNKTNLRR [100]
Molgula_P68         QVAAEFGSSSHIKNTCVYGGASKGPQLR-DLERG--CEIVIATPGRLIDFLEQKKTNLRR [100]
Tima_CnPL10         DEARKFVYRSRMRPCVVYGGA-DVGTQMRDIDRG--CHILVATPGRLVDMIQRGKIGLEA [111]
Sanderia_CnPL10     DEAKKFSYRSALRPCVVYGGA-DAGAQMRDLDRG--CHLLVATPGRLVDMIQRGKIGLES [113]
Aurelia_CnPL10      DEARKFAYRSMVRPCVVYGGA-DVGAQMRELDRG--CHILVATPGRLVDMIQRGKIGLES [112]
Craspedusca_CnPL10  DESRKFAYRSCIRPCVVYGGA-DVSTQMRDLERG--CHILVATPGRLVDMIQRGKIGLDS [111]
Eirene_CnPL10       DEARKFVYRSRMRPCVVYGGA-DVGTQMRDIDRG--CHILVATPGRLVDMIQRGKIGLEA [111]
Hydractinia_CnPL10  DEARKFVYRSCIRPCVVYGGA-DVGTQMKDIDRG--CHIIVATPGRLVDMVQRGKIGLEC [110]
Tima_CnVAS          AEARKFSTGSNIRPVVVYGGV-SVGHQLRQVESG--CHLLVGTPGRLKDFLGRRRISLEN [105]
Hydractinia_CnVas   QEARKFSFQTSVRPVVVYGGV-SVAYQLRQVQNG--CHLLVGTPGRLKDFIGKRKISLEN [105]
Eirene_CnVAS        SEARKFSYNTNIRPVVVYGGV-SVAHQLRQVESG--CSLLVGTPGRLKDFLGRRKISLEN [105]
Aurelia2_CnVas      MEARKFSHQTSLRAVVCYGGV-SVAHQLRQIENG--CHMIVATPGRLKDFAEKRKLSLAS [104]
Aurelia1_CnVas      LEARKFAYGSSLRTVVVYGGV-SVSHQVGELCDG--CNLLVATPGRLNDFIGRGRVSLSK [104]
Oryzas_OsPL10a      EEARKFAYQTGVRVVVAYGGA-PIHQQLRELERG--VEILVATPGRLMDLLERARVSLQM [105]
Oryzas_OsPL10b      EEARKFAYQTGVKVVVAYGGA-PITQQLRELERG--VEILVATPGRLMDLLERARVSLQM [105]
Mus_vasa            LEARKFSFGTCVRAVVIYGGTQFGHSV-RQIVQG--CNILCATPGRLMDIIGKEKIGLKQ [104]
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Supplementary Information Figure S2A:
Amino Acid Identity between the zinc finger CCHC domains of metazoan nanos genes:

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Musca_nanos           QKRYNGPKNEKYSSAKHCVFCENNN--------------EPDAVVKSHAVRDSMGRVLCP [46]
Chironomus_nanos      KKMDKKNSIKKKKMDDHCVFCKNNG--------------ADEILYKSHTVKDLKGRVLCP [46]
Drosophila_vir_nanos  NKRYNSGK-FKELVPRHCVFCENNN--------------EPEAVVNSHTVRDAYGRVLCP [45]
Hydram_cnnos2         TLYNSHDSLTLRASSNVCVFCRNNG--------------ESENVYASHVLKDTDGRTSCP [46]
Drosophila_sim_nanos  YKRYNS---KAKEISRHCVFCENNN--------------EPEAVINSHSVRDNFNRVLCP [43]
Hydram_cnnos1         QQQIQSKALKNLSKTSVCVFCRNNG--------------ESREFYSSHTLKDNEGNTMCP [46]
Xenola_xcat2          ----ES------VGHKGCGFCRSNR--------------EALSLYTSHRLRALDGRVLCP [36]
Xenobor_xcat2         ----EG------RGYKGCGFCRSNK--------------EAMSLYSSHRLRSLDGRVLCP [36]
Xenotrop_Xcat2        ----EA------HGHKGCGFCRSNR--------------EAQSLYSSHRLRAPDGRVLCP [36]
Anopheles_nanos       KCRNKS-----TCELDHCVFCFNNK--------------ADREVYESHRCKDEAGNVTCP [41]
Helobdella_nanos      --GKSG------EPALVCVFCRNNK--------------EPECVANSHLVKDEKGQVTCP [38]
Drosophila_nanos      YKRYNS---KAKEISRHCVFCENNN--------------EPEAVINSHSVRDNFNRVLCP [43]
Ephydatia_nanos       -TFSKLPTQQPIKKQQVCVFCRNNG--------------ESESFYTSHYLKDAEGKVTCP [45]
Schistocerca_nanos    -PSIYA------KKGTWCAFCRNNG--------------ESDKIFRSHQLKDNYGKTVCP [39]
Rattus_nanos1         -ESSA-------APERLCSFCKHNG--------------ESRAIYQSHVLKDEAGRVLCP [38]
Danio_nanos           -PKSSP------AERKFCSFCKHNG--------------ETEAVYTSHYLKNRDGDVMCP [39]
Homo_nanos2           -PGANG------GLGTLCNFCKHNG--------------ESRHVYSSHQLKTPDGVVVCP [39]
Mus_nanos2            -EGYPG------CLPTICNFCKHNG--------------ESRHVYTSHQLKTPEGVVVCP [39]
Homo_nanos3           -ESSP-------APERLCSFCKHNG--------------ESRAIYQSHVLKDEAGRVLCP [38]
Mus_nanos3            -ESSA-------APERLCSFCKHNG--------------ESRAIYQSHVLKDEAGRVLCP [38]
Mus_nanos1            -ARLLK------PELQVCVFCRNNK--------------EAVALYTTHILKGPDGRVLCP [39]
Homo_nanos1           -ARLLK------PELQVCVFCRNNK--------------EAMALYTTHILKGPDGRVLCP [39]
Homo_simnanos2        -PGANG------GLGTLCNFCKHNG--------------ESRHVYSSHQLKTPDGVVVCP [39]
Homo_simnanos3        -ESSP-------APERLCSFCKHNG--------------ESRAIYQSHVLKDEAGRVLCP [38]
Nematostella_nanos2   LGKPTARSSAPGANRQVCVFCRNNG--------------ESEEVYASHVLKSADGKTTCP [46]
Nematostella_nanos1   -NRENK----KNRNANVCVFCRNNG--------------ESKKVYSSHVLKDAEGNTTCP [41]

Celegans_nanos1       KLRSMV-CGICGATGDNAHTTKHHLEAFGDD----------------------------- [82]
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Musca_nanos           KLRTYI-CPICKASGDKAHTVKYCPQKPIITMEDAVN-----AESFRLSKGTYYKQQMKV
[100]
Chironomus_nanos      KLRAYQ-CPICGADGDQSHTVKYCPKKPIVTMEDLKK-----LDASKMINGYASTRF--- [97]
Drosophila_vir_nanos  KLRTYV-CPICGASGDSAHTIKYCPKKPIVTMEDAIK-----AESFRLAKSNYYKQQMKV [99]
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[104]
Drosophila_sim_nanos  KLRTYV-CPICGASGDSAHTIKYCPKKPIITMEDAIK-----AESFRLAKSSYYKQQMKV [97]
Hydram_cnnos1         ILRAYT-CPLCKSHGNQSHTIKYCPKYTPKPK-----------TDKLLGISMPLL----- [89]
Xenola_xcat2          VLRGYT-CPLCGANGDWAHTMRYCPLRRLLRD----------PQSNSNNPKLRH------ [79]
Xenobor_xcat2         VLRGYT-CPLCGANGDWAHTMRYCPLRQLLRN----------PQSPRNGQ---------- [75]
Xenotrop_Xcat2        VLRGYT-CPLCGANGDWAHTMRYCPLRHFLRH----------PHSPRDGQ---------- [75]
Anopheles_nanos       VLQTFV-CMRCKATGTKAHTAKYCPLKPVITPEDCLA------MELRRHKIHRKGVCT-- [92]
Helobdella_nanos      ILYIYT-CPICGATGKAAHTIKYCPYNTGERFYVPPLTRKTGNRSQDNVGPVRSSFGVSI [97]
Drosophila_nanos      KLRTYV-CPICGASGDSAHTIKYCPKKPIITMEDAIK-----AESFRLAKSSYYKQQMKV [97]
Ephydatia_nanos       VLRAYT-CPLCGANGDGAHTIKYCPENSQSVRNGGIG-----KRQAVAAAAAAAAAIITRK [99]
Schistocerca_nanos    ILQKYV-CPPCKATGPEAHTVKYCPKNPNPLPVALMN----VLKAQRSETSKARVKRNRY [94]
Rattus_nanos1         ILRDYV-CPQCGATQEHAHTRRFCPLTGQGYTSVYC-------YTTRNSAGKKLTRPDKA [90]
Danio_nanos           YLRQYK-CPLCGATGAKAHTKRFCPMVDKNYCS----------VYAKSTW------- [78]
Homo_nanos2           ILRHYV-CPVCGATGDQAHTLKYCPLN-GGQQSLYR-------RSGRNSAGRRVKR---- [86]
Mus_nanos2            ILRHYV-CPLCGATGDQAHTLKYCPLN-SSQQSLYR-------RSGRNSAGRRVKR---- [86]
Homo_nanos3           ILRDYV-CPQCGATRERAHTRRFCPLTGQGYTSVYS-------HTTRNSAGKKLVRPDKA [90]
Mus_nanos3            ILRDYV-CPQCGATQEHAHTRRFCPLTSQGYTSVYC-------YTTRNSAGKKLTRPDKA [90]
Mus_nanos1            VLRRYT-CPLCGASGDNAHTIKYCPLSKVPPPTVRPP-----PRSNRDSLPSKKLR---- [89]
Homo_nanos1           VLRRYT-CPLCGASGDNAHTIKYCPLSKVPPPPARPP-----PRSARDGPPGKKLR---- [89]
Homo_simnanos2        ILRHYV-CPVCGATGDQAHTLKYCPLN-GGQQSLYR-------RSGRNSAGRRVKR---- [86]
Homo_simnanos3        ILRDYV-CPQCGATRERAHTRRFCPLTGQGYTSVYS-------HTTRNSAGKKLVRPDKA [90]
Nematostella_nanos2   ILRAYT-CPICKASGDDSHTIKYCPQNQQTQGNGQLPP--P-PVKPPTSTAQPIARSTRG
[102]
Nematostella_nanos1   ILRAYT-CPLCKASGSQSHTIKYCPKNKNGSK--------------LQAKV--------- [77]

Celegans_nanos1       ---------------------------------------- [82]
Celegans_nanos2       FYKHSSLIQKIYASSEDSF---------------------- [104]
Musca_nanos           ---------------------------------------- [100]
Chironomus_nanos      ---------------------------------------- [97]
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Supplementary Data Tables (available on request)

Supplementary Table 1. Gene sequences used for *vasa* alignments

Species Name	Common Name	Gene Name	Accession Number
Acropora digitifera	Staghorn coral	CnVas	**BAB13683**
Aurelia aurita	Moon jellyfish	CnVas1	**BAB13682**
Aurelia aurita	Moon jellyfish	CnVas2	**BAB13688**
Caenorhabditis elegans	Nematode worm	Glh-1	**P34689**
Caenorhabditis elegans	Nematode worm	Glh-2	**AAB03337**
Caenorhabditis elegans	Nematode worm	Glh-3	**AAC28388**
Caenorhabditis elegans	Nematode worm	Glh-4	**AAC28387**
Ciona savignyi	Sea squirt	CsDEAD1A	**BAB12216**
Ciona savignyi	Sea squirt	CsDEAD1B	**BAB12217**
Craspedacusta sowerbyi	Freshwater jellyfish	Cnvas	**BAB13684**
Danio rerio	Zebrafish	Vlg	**CAA72735**
Drosophila melanogaster	Fruit fly	Vasa	**P09052**
Dugesia dorotocephala	Flatworm	PlVas1	**BAB13313**
Eirene sp.	Marine hydroid	CnVas1	**AB048855**
Ephydatia fluviatilis	sponge	PoVas1	**BAB13310**
Gallus gallus	Chicken	Cvh	**BAB12337**
Homo sapiens	Human	Vasa	**XP_003654**
Hydra magnapapillata	Hydra	CnVas1	**BAB13307**
Hydra magnipapillata	Hydra	CnVas2	**BAB13308**
Hydractinia echinata	Colonial hydroid	CnVas1	**AB048856**
Mus musculus	Mouse	Vasa	**NP_039159**
Schistocerca gregaria	Locust	RMe	**AAO15914**
Squalus acanthias	Spiny dogfish	Vasa	**AF432868**
Tetranuchus urticae	Spider mite	Vasa	**AY167036**
Tima Formosa	Elegant jellyfish	CnVas	**AB048857**
Xenopus laevis	Frog	XVLG1	**AAC03114**

Supplementary Table 2. Gene sequences used for *PL10* alignments

Species Name	Common Name	Gene Name	Accession Number
Acropora digitifera	Staghorn coral	CnPL10	**BAB13676**
Aribidopsis thaliana	Thale cress	At2g42520	**CAB68195**
Aribidopsis thaliana	Thale cress	F14P22.100	**CAB68189**
Aribidopsis thaliana	Thale cress	F14P22.160	**AAD23001**
Aurelia aurita	Moon jellyfish	CnPL10	**BAB13675**
Craspedusca sowerbyi	Freshwater jellyfish	CnPL10	**BAB13677**
Dugesia dorotocephala	Flatworm	PlvlgA	**AB047386**
Dugesia dorotocephala	Flatworm	PlvlgB	**AB047387**
Eirene sp.	Marine hydroid	CnPL10	**BAB13678**
Ephydatia fluviatilis	Sponge	PoPL10	**BAB13309**
Hydra magnipapillata	Hydra	CnPL10	**BAB13306**
Hydractinia echinata	Colonial hydroid	Cn PL10	**BAB13679**
Molgula oculata	Ascidian	P68	**AAD38874**
Mus musculus	Mouse	PL10	**NP_149068**
Mus musculus	Mouse	Dead3	**Q62167**
Oryzas sativa	Rice	OsPL10a	**AB042643**
Oryzas sativa	Rice	OsPL10b	**AB042644**
Saccharomyces cerevisiae	Yeast	Dbp1p	**NP_015206**
Schizosaccharomyces pombe	Yeast	P68	**NP_596523**
Sanderia malayensis	Malaysian jellyfish	CnPL10	**BAB13680**
Tima formosa	Elegant jellyfish	CnPL10	**BAB13681**
Xenopus laevis	Frog	AN3	**P24346**

Supplemementary Table 3. Gene sequences used for *nanos* alignments

Species Name	Common Name	Gene Name	Accession Number
Anopheles gambiae	Mosquito	ENSANGP00000020428	**XP_316157**
Caenorhabditis elegans	Nematode worm	Nos-2	**NM_063051**
Caenorhabditis elegans	Nematode worm	Nos-1	**NM_063957**
Chironomous samoensis	Midge	Csnos	**AAA87459**
Danio rerio	Zebrafish	Nanos	**NM_131878**
Drosophila melanogaster	Fruit fly	Nanos	**M72421**
Drosophila simulans	Fruit fly	Nanos	**AAF68506**
Drosophila virilis	Fruit fly	Dvnos	**AAA87460**
Ephydatia fluviatilis	Sponge	PoNos	**AB052596**
Helobdella robusta	Leech	Hro-nos	
Homo sapiens	Human	Nanos1	**NM_199461**
Homo sapiens	Human	Nanos2	**XM_371181**
Homo sapiens	Human	Nanos3	**XM_29819**
Homo sapiens	Human	SimNanos2	**XP_371181**
Homo sapiens	Human	SimNanos3	**XP_292819**
Hydra magnipapillata	Hydra	Cnnos1	**AB037080**
Hydra magnipapillata	Hydra	Cnnos2	**AB037081**
Mus musculus	Mouse	Nanos1	**NM_178421**
Musca domestica	House fly	Mdnos	**AAA87961**
Rattus norvegicus	Rat	Nanos1	**XM_222459**
Schistocerca Americana	Grasshopper	Nanos	**AY179887**
Xenopus borealis	Kenyan clawed frog	Xcat2	**AAK49296**
Xenopus laevis	African clawed frog	Xcat-2	**CAA51067**
Xenopus tropicalis	Western clawed frog	Xcat2	**AAK49295**