

## Vasa protein expression is restricted to the small micromeres of the sea urchin, but is inducible in other lineages early in development

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### Abstract

Vasa is a DEAD-box RNA helicase that functions in translational regulation of specific mRNAs. In many animals it is essential for germ line development and may have a more general stem cell role. Here we identify vasa in two sea urchin species and analyze the regulation of its expression. We find that vasa protein accumulates in only a subset of cells containing vasa mRNA. In contrast to vasa mRNA, which is present uniformly throughout all cells of the early embryo, vasa protein accumulates selectively in the 16-cell stage micromeres, and then is restricted to the small micromeres through gastrulation to larval development. Manipulating early embryonic fate specification by blastomere separations, exposure to lithium, and dominant-negative cadherin each suggest that, although vasa protein accumulation in the small micromeres is fixed, accumulation in other cells of the embryo is inducible. Indeed, we find that embryos in which micromeres are removed respond by significant up-regulation of vasa protein translation, followed by spatial restriction of the protein late in gastrulation. Overall, these results support the contention that sea urchins do not have obligate primordial germ cells determined in early development, that vasa may function in an early stem cell population of the embryo, and that vasa expression in this embryo is restricted early by translational regulation to the small micromere lineage. © 2007 Elsevier Inc. All rights reserved.

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### Introduction

Vasa is an ATP-dependent DEAD box helicase, similar to the eukaryotic initiation factor 4A (eIF4A). Vasa unwinds double-stranded RNA, though *in vivo*, DEAD-box proteins appear to unwind only local RNA–RNA interactions of a few base pairs or to dissociate proteins from the RNA, which in turn allows other interactions to occur (Linder and Lasko, 2006). The exact mechanism of function is not known although the recent crystal structure of *Drosophila* vasa suggests that the winding activity is actually the result of contortion of the dsRNA by bending (Sengoku et al., 2006). Extensive work in *Drosophila* indicates

that vasa acts as a translational regulator of two mRNAs that are localized in the oocyte: *gurken* (*grk*), which directs both anterior/posterior and dorsal/ventral polarity, and *oskar* (*osk*), which directs germ plasm assembly at the posterior of the oocyte (Mahowald, 2001; Riechmann and Ephrussi, 2001). Both *grk* and *osk* mRNAs are translationally repressed by the well-conserved RNA-binding protein bruno until they reach their destination in the oocyte resulting in localized protein accumulation (Castagnetti et al., 2000; Kim-Ha et al., 1995; Webster et al., 1997; Yan and Macdonald, 2004). Bruno binds the BRE (bruno response element) in the 3'UTR of the *osk* message and recruits the protein Cup which then binds the cap-binding protein eIF4E (eukaryotic initiation factor 4E; Nakamura et al., 2004). With Cup bound to eIF4E, the translational machinery cannot be recruited and translation is blocked. Vasa appears to

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function in this process by relieving the *bruno*/*cup* repression of *gurken* and *oskar* mRNA (Johnstone and Lasko, 2004). Mutations in the helicase domain of *vasa* cause a lack of *gurken* and *oskar* translation, and result in germ line defects and female sterility in *Drosophila* (Lasko and Ashburner, 1988; Styhler et al., 1998). *Vasa* mutations in mice result in male sterility, largely due to defective primordial germ cell proliferation and differentiation (Tanaka et al., 2000). Although the regulation of expression and the molecular function of *vasa* is not understood in most animals, given the wide conservation of *vasa*, *bruno*, and the translational machinery, it is likely that *vasa*'s role as a translational activator is conserved.

In most of the deuterostomes studied, including fish, frogs, and ascidians, *vasa* mRNA is localized to discreet regions of oocytes and/or early embryos. For example, in the ascidian *Ciona intestinalis*, for which *vasa* is a stringent marker of the germ line, *vasa* mRNA is present in eggs, and by the second cleavage division, it is concentrated at the posterior region of the embryo (Fujimura and Takamura, 2000). *Vasa* mRNA further concentrates to the posterior region of the B3 blastomeres at the 4-cell stage, and is subsequently inherited by the posterior-most blastomeres, B4.1, at the 8-cell stage, B5.2 at the 16-cell stage, B6.3 at the 64-cell stage, and B7.6 at the early gastrula stage. In *Xenopus*, the *vasa*-like genes *DEADsouth* (RNA) and *Xvlg1* (protein) are localized to the granules of the germ plasm at the vegetal pole of oocytes, are segregated to the four vegetal cells during the initial cell divisions and accumulate specifically in primordial germ cells (PGCs) of the tadpole (Komiya et al., 1994; MacArthur et al., 2000). Zebrafish *vasa* RNA is also localized to germ plasm granules, but these granules are associated with the cortex of the animal pole of late-stage oocytes. During the first two cleavage divisions in zebrafish embryos, *vasa* RNA granules are concentrated to the distal parts of the cleavage furrows, resulting in four *vasa* RNA containing aggregates. These aggregates are asymmetrically segregated during every cell division such that only four PGCs are present in the late zebrafish blastula. Shortly before gastrulation, these four cells begin to divide symmetrically and start to migrate to the future site of the gonads (Knaute et al., 2002; Yoon et al., 1997). In each case, *vasa* serves as a specific marker for the birth, development, and migration of primordial germ cells.

Mice and sea urchins, however, have no detectable localized germ line determinants in oocytes or early embryos, either functionally (e.g. Ransick et al., 1996; Tam and Zhou, 1996) or by molecular markers (e.g. Hayashi et al., 2007; Juliano et al., 2006). In mice, removal of the proximal epiblast region, the source of precursors for the primordial germ cells, results in a re-specification of the germ cells and normally gravid adults (Hayashi et al., 2007; Tam and Zhou, 1996). The mRNA of the mouse *vasa* homolog is present in oocytes and early embryos, but is then not detectable until the primordial germ cells begin to migrate into the genital ridge at 10.5 days post-coitum (dpc) (Toyooka et al., 2000). Thereafter, it remains specific for the primordial germ cells in this animal. Similarly, removal of the cells thought to contribute to the germ line in sea urchins did not prevent fertility of the resulting animals (Ransick et al., 1996). The small micromeres were hypothesized to contribute to the

primordial germ cells in sea urchins because they had characteristically enlarged nuclei, slow cell divisions (Pehrson and Cohen, 1986; Tanaka and Dan, 1990), and specifically accumulated mRNA for *nanos*, *vasa* and *piwi* (Fujii et al., 2006; Juliano et al., 2006). That embryos lacking small micromeres still developed germ cells argues that the small micromeres could not be the obligatory primordial germ cell line in this animal, and/or that the primordial germ cells are not actually determined until later in development (Ransick et al., 1996).

*Vasa* may also function in a broader context of stem cells in development. For example, in polychaetes (annelid worms), *vasa* and its close family member PL10, are each present in a large population of mesodermal cells, only a fraction of which become primordial germ cells (Rebscher et al., 2007). The multipotent i-cells of hydrozoans and the neoblasts of flatworms similarly express germ plasm components along with stem cell markers (Mochizuki et al., 2000, 2001; Shibata et al., 1999). Setting aside a population of undifferentiated multipotent cells, from which the PGCs are segregated later, may constitute an ancestral mechanism that has been lost in species exhibiting premature specification of the adult body plan by localized maternal determinants (Johnson et al., 2003). Further, Hayashi et al. (2007) have speculated a stem cell model for germ cell determination in mice in which only a few of the precursor cells actually become primordial germ cells, through progressive restriction, whereas other progeny diversify into somatic stem cell fates.

Here we examine the expression and regulation of *vasa* protein in the sea urchin, a basal deuterostome. We find that although all cells of the embryo contain *vasa* mRNA, protein expression is under specific cellular control, and that its translation is inducible throughout the embryo. The cells that normally accumulate *vasa* are the small micromeres, and results herein suggest that these cells may fit the two step (Rebscher et al., 2007), or progressive restriction mechanism (Hayashi et al., 2007) of stem cells that contribute to several adult cell types, including the germ line.

## Materials and methods

### Reagents

Reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted.

### Animals

Adult husbandry was managed as described (Voronina et al., 2003). Treatment of embryos with LiCl (30 mM) was performed as described previously (Logan et al., 1999), as was rearing of advanced larvae and juveniles (George et al., 2004). Microinjections of *in vitro* transcribed mRNA were performed as described (Oliveri et al., 2003) and were done three times, in different batches of eggs. Micromere removal experiments were performed three times as described (Ransick et al., 1996), with different batches of embryos. Controls included untreated embryos, and embryos treated with dissociation medium but not surgically altered.

### cDNA cloning

A partial *Strongylocentrotus purpuratus* *vasa* sequence was found in the genome (<http://sugp.caltech.edu/>). Gene-specific primers (5'-GGTCGAGACA-

GGCCCAAAAATATAC-3' and 5'-CACAGGTCGTATGGTGGTGC-3') were used to screen an *S. purpuratus* ovary cDNA library (described in Wessel et al., 1998) by PCR and the amplification product was cloned into pGEMT-Easy (Promega, Madison, WI). DNA sequencing was performed by the macromolecular sequencing facility at Brown University, using ABI 377 prism automated sequencers (Perkin-Elmer, Foster City, CA). This sequence was identified as vasa based on homology of the encoded protein to vasa in the region conserved specifically in vasa, as opposed to other DEAD-box helicases. The initial isolated fragment was used to design gene-specific primers for 5' and 3' RACE procedures. The consensus sequence of the resulting cDNAs is entered in the GenBank (accession numbers to be obtained) and the actual *S. purpuratus* vasa gene (<http://annotation.hgsc.bcm.tmc.edu/Urchin/>) is a modified version of SPU\_08908. To isolate vasa-like sequences from *Lytechinus variegatus*, we utilized degenerate PCR with primers specific for DEAD-box motifs using a gastrula cDNA library. The screen resulted in a single sequence containing vasa-specific DEAD box motifs, which was then extended to full open reading frame by 5' and 3' RACE protocol, from both gastrula and ovary cDNA.

### Cell and embryo labeling

We designed and obtained several vasa-specific antibodies. To design peptide-specific vasa antigens, we utilized the *S. purpuratus* vasa sequence information. The vasa-specific motif within the DEAD-box region, KPTPV QKYGMPIISC, was synthesized, coupled to KLH, and used to generate polyclonal antibodies in two rabbits (Sigma Genosys; The Woodlands, TX). Additionally, a fragment representing the DEAD-box of *S. purpuratus* vasa (amino acids 300 to 583, submitted to GenBank) was used to produce a 6-histidine-tagged protein fusion and the resulting antiserum was affinity-purified

on a column containing the protein immunogen. Additional antibodies used in this study included an anti pan-insect vasa, called For2 (Chang et al., 2002), and an anti-zebrafish vasa antibody obtained from Dr. Zivkovic at the Hubrecht Laboratory, Netherlands Institute for Developmental Biology (Braat et al., 2000). Immunofluorescence localization was performed as described (Laidlaw and Wessel, 1994; Voronina et al., 2003).

Protein samples of *S. purpuratus* eggs and several embryonic stages were prepared, subjected to SDS-PAGE and immunoblot analysis as described (Voronina et al., 2003). For antibody competition experiments, the diluted anti-fragment antiserum was incubated for 1 h with 100 µg/ml purified antigenic protein and then pelleted at 10,000×g for 15 min. The signal intensities on the resulting blots were quantified using Metamorph software (Universal Imaging Corporation, Downingtown, PA), and the values were normalized per intensity of duplicate Coomassie-stained gel or per intensity of a loading control, yolk protein YP30 band.

Whole-mount *in situ* RNA hybridizations were performed as previously described (Minokawa et al., 2004).

## Results

### Sea urchin vasa homologs

Full length vasa cDNA homologs were isolated from two sea urchin species, *S. purpuratus* and *L. variegatus*. These proteins are 762 and 796 amino acid residues, respectively, and were concluded to be vasa based on sequence similarity to known vasa proteins and the characteristic domain architecture (Fig. 1).

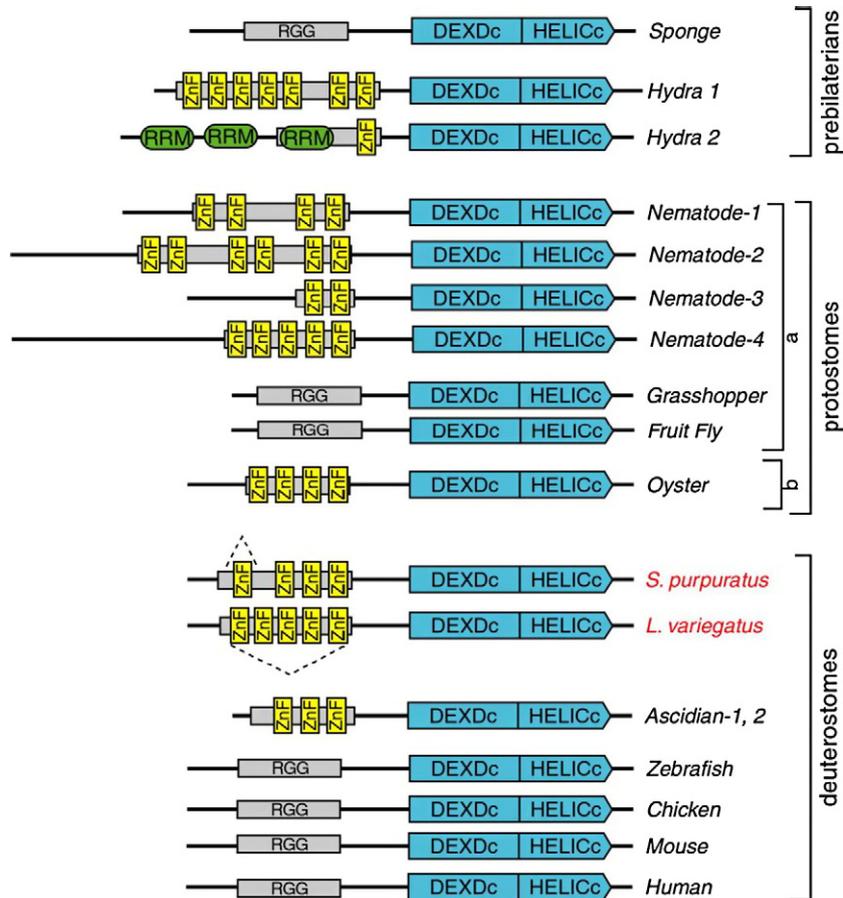


Fig. 1. Functional conservation of protein domains across vasa subfamily of DEAD box helicases. Pictured domain structures of the proteins were predicted by SMART (Letunic et al., 2006). Cloned sea urchin vasa homologs are identified with species name in red. Major splice variants are pictured; exons missing in minor splice variants are indicated with dotted lines. a – ecdysozoa, b – lophotrochozoa, RGG – glycine-rich region containing RGG repeats, ZnF – zinc finger motif, RRM – RNA-binding domain, DEXDc/HELICc – DEAD box RNA helicase domain.

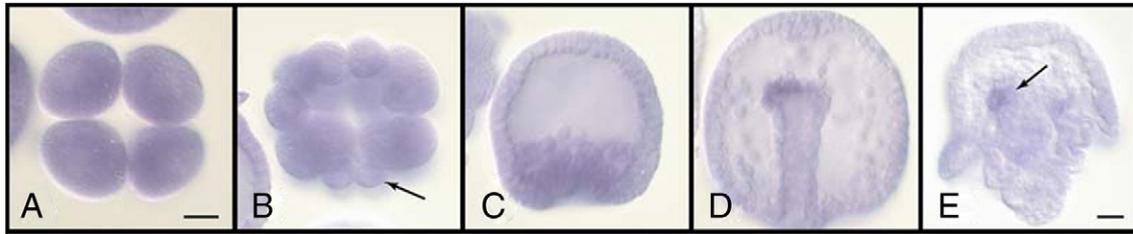


Fig. 2. *Vasa* mRNA accumulates uniformly in early embryos. *In situ* RNA hybridization detects uniform *vasa* mRNA accumulation in early embryonic stages (A, four cells; B, 16 cells, with arrow indicating the micromeres) becoming restricted to the vegetal region in mesenchyme blastula (C) and during gastrulation (D) the mRNA is restricted to small micromere descendants that in larvae (E) accumulate selectively in the left coelomic pouch (E, arrow indicates the small micromere descendants in the left coelomic pouch). Scale bar=20  $\mu$ m.

These included: Zn-fingers, glycine-rich regions, and a C-terminal DEAD-box motif. Despite these conserved structures, the N-terminal halves of *vasa* orthologs have considerable amino acid divergence (Fig. 1). Computational searches failed to identify additional *vasa* isoforms in the *S. purpuratus* genome, however we did detect two *vasa* splice forms in each species. These isoforms differ in the N-terminus of the transcript, and produce protein isoforms with varying number of Zn finger domains (Fig. 1 and data not shown).

#### *Vasa* mRNA analysis

As previously shown (Juliano et al., 2006), *in situ* RNA hybridization of *vasa* in *S. purpuratus* demonstrates that embryos developing through blastula stage have uniform *vasa* mRNA accumulation (Figs. 2A, B), which becomes restricted to the vegetal plate of mesenchyme blastulae (Fig. 2C), and then to a small population of about 6–8 cells at the tip of the archenteron in gastrulae (Fig. 2D). In the larvae, *vasa* mRNA is detected in a subset of cells initially in both coelomic pouches, and subsequently only in the left pouch (Fig. 2E). This is significant since the embryonic rudiment is largely derived from this same region (see e.g. Pearse and Cameron, 1991). Similar *vasa* mRNA

accumulation was detected in *L. variegatus*, and were supported by qPCR results (data not shown, and Juliano et al., 2006).

#### *Vasa* protein analysis

To analyze the distribution of *vasa* protein throughout embryogenesis, we made and obtained several different anti-*vasa* antibodies. We generated antibodies to an internal 20-amino acid peptide (aa 330–344) and to the C-terminal RNA helicase fragment, both from the *S. purpuratus* sequence. We also obtained antibodies against zebrafish *vasa* (Braat et al., 2000) and anti-pan-*vasa* antibody developed originally against grasshopper *vasa* (Chang et al., 2002). Each of these antibodies yielded similar results in immunolocalizations (see below), and western blots show that the approximately 80 kDa *vasa* band does not change in abundance substantially during embryogenesis (Fig. 3).

#### Accumulation of *vasa* protein during embryogenesis and larval development

*Vasa* protein is present in unfertilized eggs, consistent with mRNA accumulation in oocytes (Juliano et al., 2006), and is enriched along the periphery of the cell (Fig. 4A). This pattern of

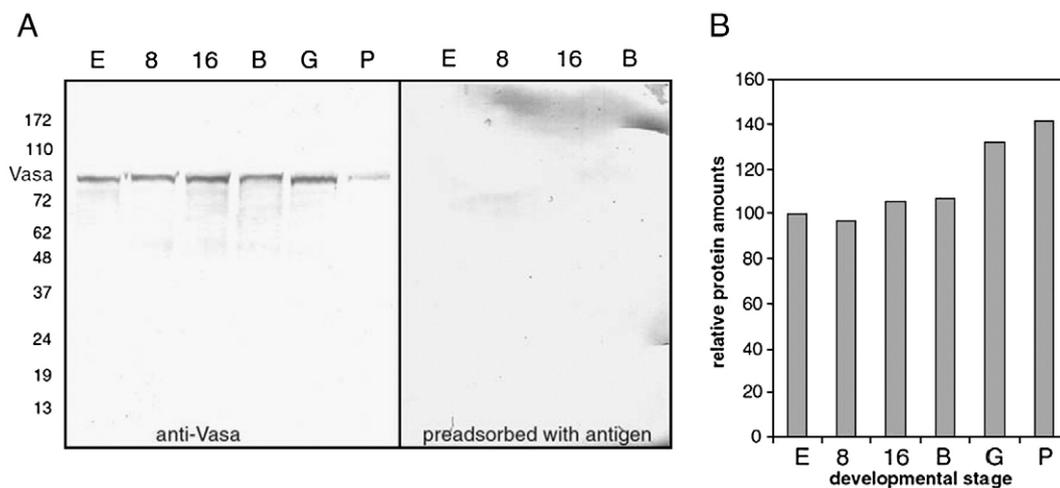


Fig. 3. *Vasa* protein is expressed throughout embryonic development. (A) 120 micrograms of protein lysates were loaded per lane and probed with anti-DEAD box domain affinity-purified antiserum. *Vasa*-specific bands are competed away by preincubation of the antibody with the antigen. (B) Quantification of the western blot signal in panel A normalized per Coomassie staining intensity of the duplicate gel (levels of *vasa* in the egg are set to 100%). Developmental stages used: E, egg; 8, 8-cell embryos; 16, 16-cell embryos; B, blastula; G, gastrula; P, pluteus.

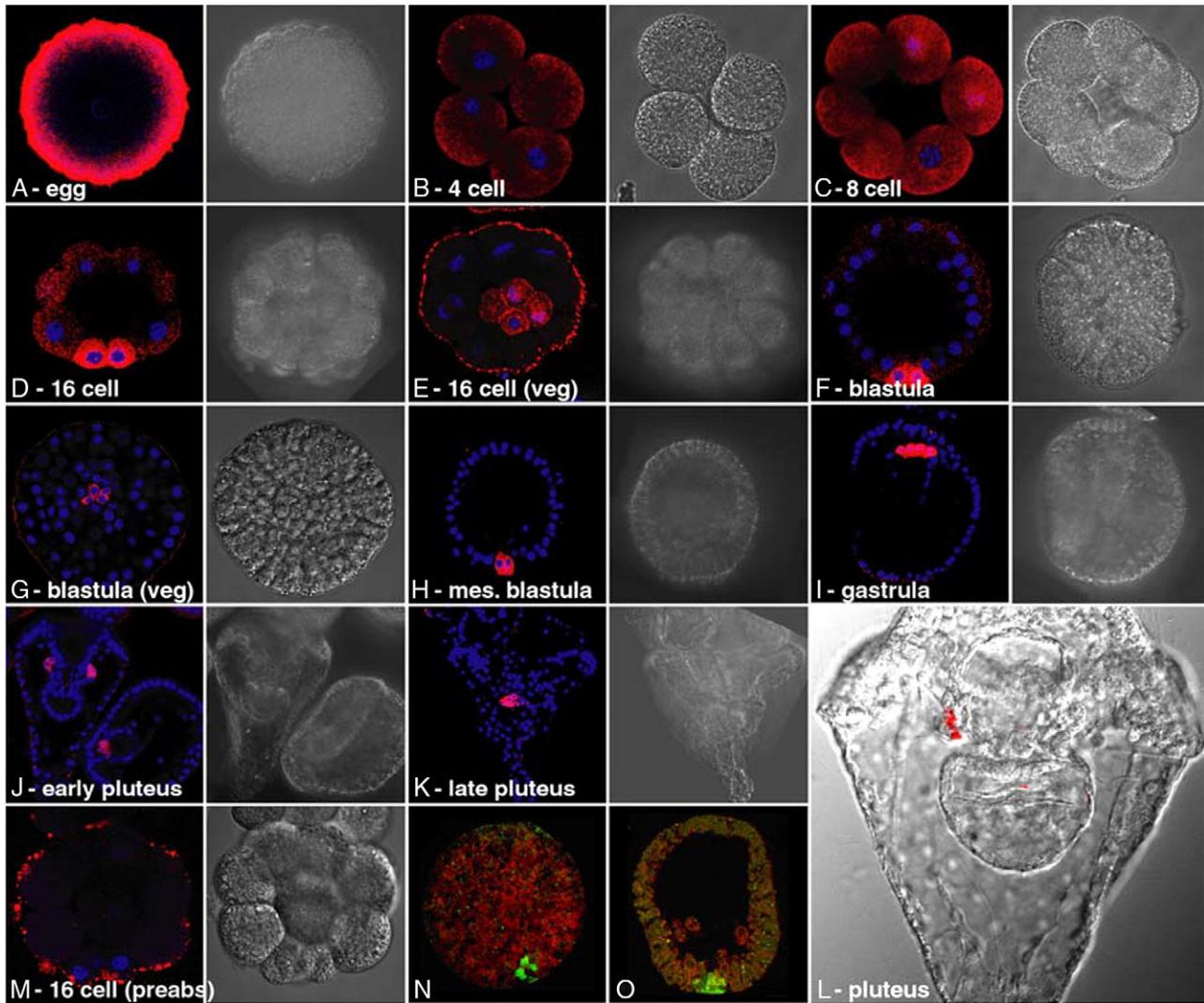


Fig. 4. Uniform distribution of vasa protein in the early embryonic stages is followed by restriction of vasa to micromeres at the 16-cell stage, the small micromeres at the 32-cell stage, and remains associated with the small micromeres throughout embryonic development. Indicated embryonic stages were fixed and immunolabeled with anti-vasa DEAD-box antibody (red in panels A–M), or anti-pan insect vasa For2 (green in panels N, O). DNA was counterstained with Hoechst (blue). Corresponding DIC images of the embryos are shown at right. (A–L) Vasa localizations in *S. purpuratus*. (M) 16-Cell control, primary antibody following preadsorption with the antigenic protein does not exhibit specific staining pattern in the micromeres. (N, O) For2 antibody (Chang et al., 2002) labels small micromeres in *L. variegatus* blastula.

labeling is not due to limited antibody permeability, as it is similar on immunolabeled sections of eggs (data not shown). The immunolabeling during early cleavage is homogenous through the 8-cell stage (Figs. 4B and C), similar to the mRNA distribution. After the first asymmetric division in the embryo at 16 cells, in contrast to the distribution of the vasa mRNA (Fig. 2B), vasa protein is selectively enriched in the micromeres (Fig. 4D). The fifth cell division, generating small micromeres and large micromeres (32-cell stage) further restricts vasa to just the small micromeres; the large micromeres lose vasa signal and the small micromeres remain vasa-positive through gastrulation (Figs. 4F–I). Since the overall amount of protein appears to stay relatively constant through early development (Fig. 3), we conclude that translation of new vasa protein likely continues in the small micromere lineage, accompanied by protein turnover in the non-small micromere lineages. In gastrulae, the eight vasa-positive cells (four small micromeres after a single round of cell division)

are located at the tip of the invaginating archenteron and in early larval stages they are associated with the developing coelomic pouches (Figs. 4I–K). In more advanced larvae (approximately 5 days in *S. purpuratus*, 3 days in *L. variegatus*), the vasa-positive cells are restricted to the left coelomic sac (Fig. 4L). We do not know if this change in the location of vasa-positive cells reflects a turnover of vasa in cells of the right coelomic pouch, migration of vasa-positive cells from the right to the left pouch, selective death of the vasa-positive cells in the right coelomic pouch, or a combination thereof. The number of vasa-positive cells subsequently increases significantly in the left coelomic pouch of larvae and following metamorphosis, vasa is expressed in the germ cells of the developing juvenile gonads (data not shown). This vasa immunolabeling pattern was similar in other sea urchins, e.g. *L. variegatus* (and data not shown), and with other anti-vasa antibodies (Figs. 4N, O, and data not shown), suggesting a stereotypic and specific vasa distribution common to sea urchins.

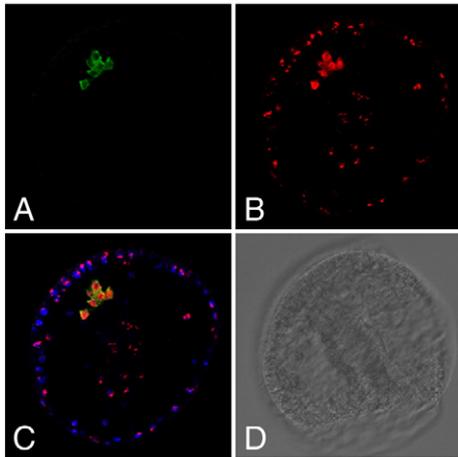


Fig. 5. BrdU incorporation marks small micromeres that are vasa-positive during gastrulation. (A) Anti-Vasa antibody staining (green). (B) Anti-BrdU antibody staining, red. (C) Red and green channels overlay; blue is nuclear staining of the embryo (Hoechst). (D) Brightfield view of the embryo.

Following gastrulation, we find a strict coincidence of vasa RNA and protein expression in each species. In contrast, early in development the vasa mRNA is distributed uniformly throughout the embryo, as the vasa protein becomes restricted to the small micromeres. Progressive restriction of vasa protein expressing cells begins at the 16-cell stage, and we focused our attention in the regulation of this mechanism.

*Vasa-positive cells of the embryo are exclusively the small micromere lineage*

Based on morphological criteria, the vasa-positive cells in the sea urchin embryo appear to be small micromeres. This is a reliable determination up to 128-cell stage, but recognition is lost later in development. To test this premise using molecular markers, we employed BrdU pulse-chase labeling of the sea urchin embryos, as described previously (Tanaka and Dan, 1990). BrdU is used to label replicating DNA during the first embryonic cell cycle which becomes diluted out with subsequent cell divisions. By virtue of the slow cell cycle in small micromeres, these cells preferentially retain BrdU relative to the other, more rapidly dividing cells. Embryos were pulsed with BrdU following fertilization and then cultured until late gastrula stage when they were processed for both BrdU and vasa immunolabeling. The results show a perfect correlation of BrdU retention and vasa protein (Fig. 5). Thus, by using both morphological and molecular criteria, we conclude that the vasa-positive cells in the embryo and larvae are the small micromeres and their immediate descendants.

*Inherent vasa expression patterns*

To test the lineage restrictions of vasa we cultured blastomeres isolated from 2-, 4- or 8-cell stages as pairs or as individuals, until untreated siblings reached the early blastula stage (Fig. 6). This end point was chosen as an easily recorded

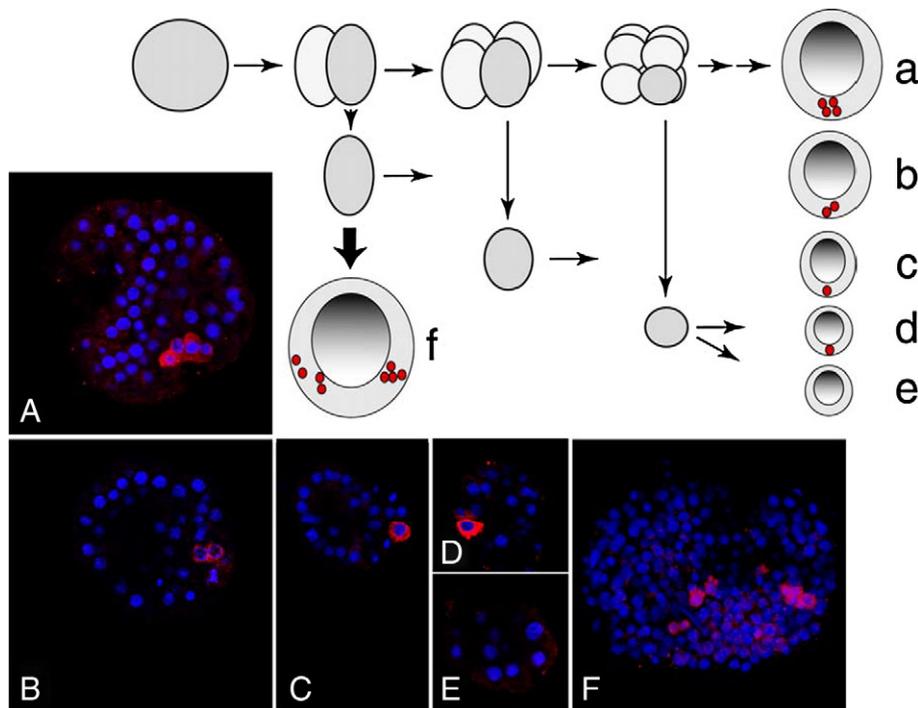


Fig. 6. Vasa expression following blastomere dissociation. Schematic of the experiments in panels A–E is shown on top. Fertilized eggs were dissociated at the 2-, 4- or 8-cell stages, or left untreated. At 20 h of development, embryos arising from blastomeres or controls were fixed and labeled with anti-Vasa antibodies (red); DNA is stained with Hoechst (blue). Representative embryos are shown for each treatment group. (A) Control embryo, 4 vasa-positive cells. (B) Progeny of blastomere dissociated at the 2-cell stage; 2 vasa-positive cells (100%). (C) Progeny of blastomere dissociated at the 4-cell stage; 1 vasa-positive cell (100%). (D and E) Progeny of blastomeres dissociated at the 8-cell stage; 1 vasa-positive cell (50%) or no vasa-positive cells (50%). (F) After dissociation at 2-cell stage, 4 blastomeres (2 embryos worth) were induced to aggregate and were cultured for 20 h, resulting in 2 clusters of 4 vasa-positive cells.

and identifiable 4-*vasa*-cell stage (the 4 small micromeres). The results show that the number of *vasa*-positive cells is imper-turbable: a blastomere isolated from a 2-cell stage had precisely half the number of small micromeres, two, as normal embryos (Fig. 6B); and a blastomere from a four cell embryo developed into a yet even smaller embryo and had one *vasa*-positive cell (Fig. 6C). Tiny blastulae resulting from an 8-cell stage blas-tomere had either none or one *vasa*-positive cell, reflecting the equatorial cleavage that occurred at this time resulting in an animal and vegetal tier of blastomeres (Figs. 6D, E). No changes were detected in *vasa* accumulation in other cells of the emb-ryoids. Furthermore, dissociation of 2-cell embryos followed by reaggregation into 4-cell embryos results in a blastula that contains two clusters of 4 *vasa*-positive cells (Fig. 6F).

#### Regulation of *vasa* protein expression

To understand the mechanism of *vasa* protein localization selectively in small micromeres, we tested disruptions of emb-ryonic patterning on *vasa* protein expression. A widely used experimental perturbation to test mechanisms of embryonic pattern formation is LiCl exposure. This treatment uniformly activates the *wnt/*  $\beta$ -catenin signaling pathways by blocking GSK3 activity, resulting in a vegetalized, embryonic pheno-type as a result of nuclearizing  $\beta$ -catenin (Logan et al., 1999). Nuclear  $\beta$ -catenin is first detected in the nuclei of both large and small micromeres after 5th cleavage, and persists until gastru-lation, driving expression of the micromere gene program, starting with a paired homeodomain family transcription factor *Pmar1* (Logan et al., 1999; Oliveri et al., 2002). This pattern could implicate nuclear  $\beta$ -catenin in the upkeep of high levels of *vasa* protein expression.

To test the mechanism of selective *vasa* protein expression, embryos first were treated with 30 mM LiCl from the 2-cell stage to the late gastrula stage of the control group (48 h), and several time points were taken along this interval and the resulting *vasa* protein patterns were compared with those in untreated embryos (Figs. 7A–D). That the embryos were indeed vegetalized was determined by morphological and molecular criteria including a preponderance of exogastrulae, and aberrant *Endo 1* expression (data not shown). The results show that LiCl treatment did not disrupt the pattern of *vasa* expression: protein was found enriched in the four small micromeres in both control and the treated group. However, LiCl treatment did induce more *vasa* protein in the embryos overall (Figs. 7A–D). This was also apparent by immunoblot analysis of treated and control samples indicating a 50% increase in overall *vasa* protein levels in the treated sample (data not shown). We do not, however, know if the detected increase in *vasa* expression represents an overall increase in synthesis from the ubiquitous transcript, or a lack of protein turnover normally seen in non-small micromeres.

To further examine this *vasa* inducibility, we then micro-injected into embryos mRNA encoding a dominant-negative cadherin that inhibits nuclear beta-catenin localization (Logan et al., 1999). Down-regulation of  $\beta$ -catenin by DN-cadherin decreased expression of *vasa* protein at the 16-cell and 32-cell stage (5 h) to the blastula stage (9 h) by about 30%, both in the

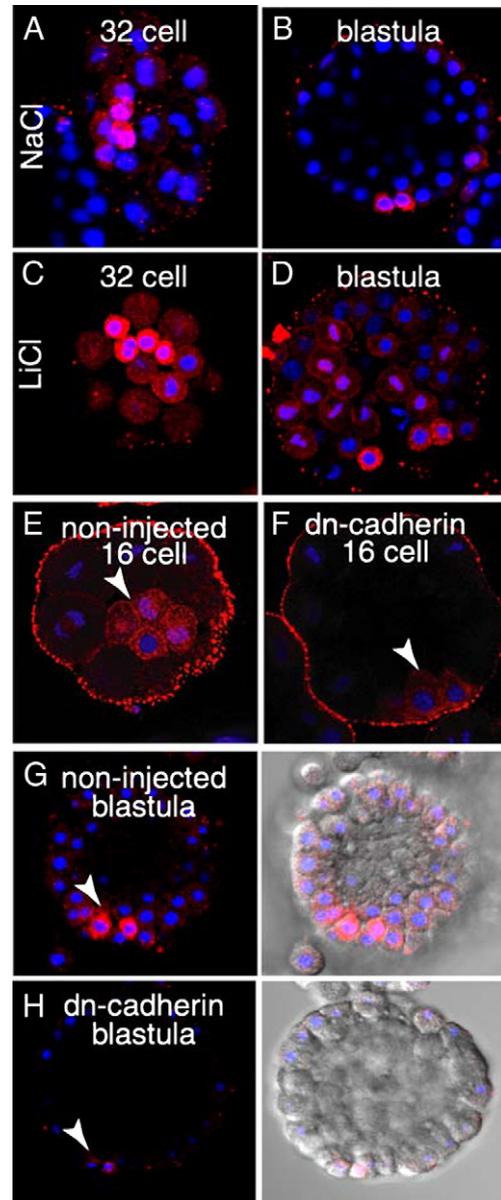


Fig. 7. Regulation of *vasa* protein expression. LiCl induces *vasa* protein accumulation. Embryonic cultures were treated with 30 mM LiCl or NaCl (as a control) in seawater from the 2-cell stage (1.5 h) until blastula (24 h). Samples of the treated embryos were removed, fixed at several stages, and the *vasa* protein expression pattern was detected by immunolocalization (red). DNA is counterstained with Hoechst (blue). In contrast to control (NaCl), LiCl-treated embryos show higher general expression of *vasa* protein (A, C). By blastula, increased *vasa* protein level outside of the small micromere population is still apparent (B, D). Conversely, repression of the  $\beta$ -catenin signaling pathway by over-expression of a dominant-negative cadherin fragment, resulted in significantly less *vasa* protein, particularly in the micromeres in 16-cell embryos (E and F), and in the small micromeres of the blastula (G and H). Micromeres and small micromeres are indicated with an arrowhead in each panel.

micromeres, and overall throughout the embryo (Figs. 7E–H). These changes were apparent both by *in situ* immunolabeling as well as by immunoblots (data not shown). Further, the homeo-domain containing protein *pmar*, a downstream target of  $\beta$ -catenin and a skeletogenic determinant by virtue of its repression of *HesC* (Revilla-i-Domingo et al., 2007), appears to

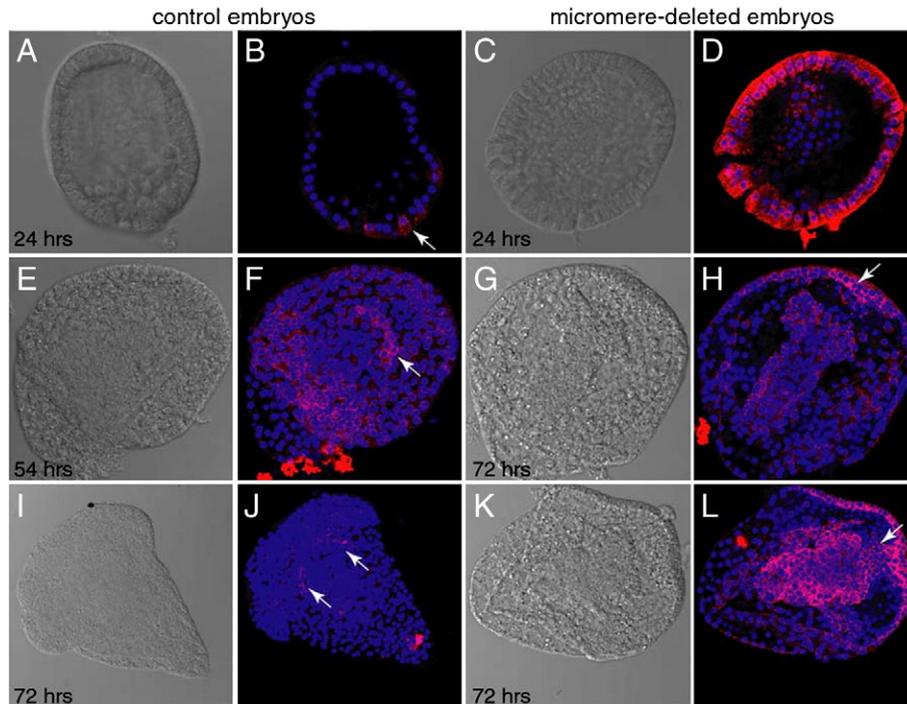


Fig. 8. Compensatory vasa expression upon micromere deletions. Upon surgical removal of micromeres, vasa protein is expressed broadly in the embryo, and subsequently is restricted to a smaller domain of expression. Here, manipulated embryos were fixed and immunostained with anti-Vasa (red); DNA is stained with Hoechst (blue). Arrows mark vasa expression domains. (A, B) Following 24 h of culture, control embryos reach mesenchyme blastula stage (vasa only in small micromeres). (C, D) Deleted embryos form blastula at 24 h (broad vasa expression). (E, F) Control embryos at 54 h, gastrula (vasa at the tip of archenteron). (G, H) Deleted embryos gastrulate following 72 h (vasa in oral ectoderm). (I, J) Control embryos form plutei by 72 h (vasa in coelomic pouches). (K, L) Late gastrula/prism stage deleted embryos at 72 h (vasa at junction of gut/oral ectoderm).

generate a transient stimulus for vasa protein expression (data not shown). Overall, it appears that interference with the function of  $\beta$ -catenin signaling changes the total levels of vasa protein expression throughout the embryo without disturbing the selective vasa expression in the micromeres. In particular, nuclear  $\beta$ -catenin appears essential for significant vasa translation in the micromeres and small micromeres. This result argues for a  $\beta$ -catenin-sensitive gene transcribed in the micromeres and the small micromeres that directs vasa translation. Several such candidates have been identified (Ransick et al., 2002). Again, we do not know if the altered vasa protein accumulation represents strictly an overall increase in synthesis from the

ubiquitous transcript, and/or a lack of protein turnover normally seen in non-small micromeres. It is clear that although these manipulations impinge on the accumulation of vasa protein throughout the embryo, the small micromeres remain distinguishable from all other cells by their higher level of vasa protein accumulation.

*Vasa regulation – micromere removal causes ectopic vasa translation*

Ransick et al. (1996) surgically removed the micromeres (the vasa-positive cell lineage) from 16-cell embryos, yet the adults

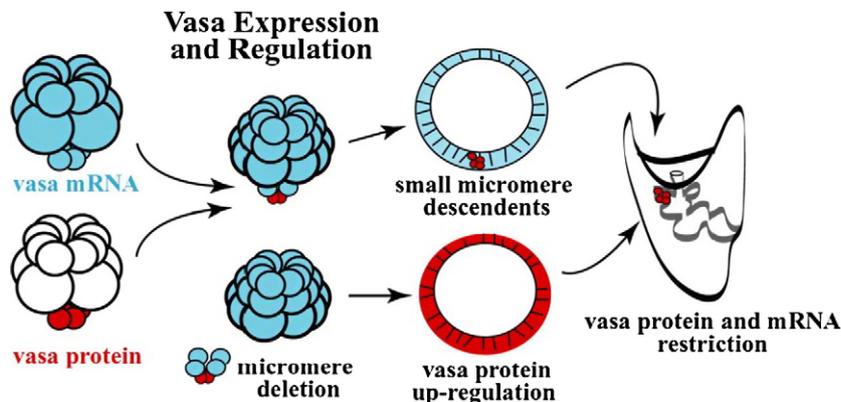


Fig. 9. Summary diagram of vasa regulation during early embryonic development. Blue shading represents mRNA accumulation, whereas red shading indicates vasa protein.

resulting from these embryos made gametes normally, a finding inconsistent with the vasa-positive, small micromeres being the obligate primordial germ cells. We assessed the fate of vasa protein in micromere depleted embryos cultured to early blastula, gastrula, or to larval stages. When blastulae resulting from micromere removal were examined for vasa expression we found that vasa protein expression dramatically increased throughout the embryo (Fig. 8D) without a detectable change of vasa mRNA levels compared to controls (data not shown). When gastrulae resulting from micromere depletion were examined, we found that vasa signal overall was still increased, but was now enriched within the gut and oral ectoderm (Fig. 8H). Finally, when larvae resulting from micromere depletion were analyzed, vasa expression was again restricted to cells of the coelomic region, the endoderm, and to the oral ectoderm. Thus, the micromeres must in some way repress the translation and accumulation of vasa throughout the embryo and their removal enables a compensatory induction leading to vasa expression (Fig. 9).

## Discussion

Germ line determinants are conserved between animals, but how cells of the embryo acquire such determinants, and their function in cell fate are very different. Some animals localize determinants, proteins and/or mRNAs, in distinct regions of the egg or early embryo, which then dictates a germ cell lineage to the cells that acquire this cytoplasm (Seydoux and Braun, 2006). Other animals employ inductive interactions and synthesize these same determinants in a particular cell lineage that then forms the primordial germ cells. It is becoming apparent that in animals using inductive interactions for germ cell formation that a progression occurs from a general embryonic stem cell population that produces somatic tissues, as well as separate populations of somatic stem cells and germ-line progenitors. This progression was demonstrated both in polychaetes and in mice (Hayashi et al., 2007; Rebscher et al., 2007). The results presented here, and by others, support the contention that the small micromeres of the sea urchin are an early stem cell of the larval rudiment. These cells contain germ line determinants, do not divide frequently early in development, but later in larval growth, they begin to proliferate and diversify, likely into both somatic cells of the adult rudiment, and into primordial germ cells. Surprisingly, the micromeres (shown here), and presumably the small micromeres somehow repress vasa translational up-regulation in other cells of the embryo (Kurihara and Amemiya, 2005). Vasa up-regulation in the absence of micromeres appears to be largely a result of translational regulation since we do not see any increased vasa mRNA accumulation to otherwise explain the massive increase in vasa. As far as we know, this is the only known translational response from micromere removal. By virtue of vasa function in translational regulation, we hypothesize that the micromere-null embryos increase translation of ubiquitous mRNAs, the products of which may regulate additional reprogramming of developmental fate decisions. It is hard to imagine that the entire embryo becomes stem-cell-like but perhaps the increase in vasa

protein allows for retention of plasticity in the embryonic cells for lack of the original signaling center. Vasa restriction to a small percentage of cells then occurs during gastrulation and may indicate committed fate decisions, except for the vasa-positive cells. Remaining vasa-positive cells presumably have taken over the stem cell function and will contribute to the adult rudiment.

Vasa protein accumulation in the small micromeres appears to be the result of two selective activities. First, an increase in vasa translation likely occurs specifically in the small micromeres from the maternal mRNA; the mRNA levels in the embryo are uniform whereas vasa protein accumulates only in the small micromeres. Future studies will address the mechanism of this selective translational activation with the hypothesis that 3'UTR control elements are responsible for this selection. Second, vasa protein appears to selectively turnover in non-small micromere cells. Vasa is present uniformly in early embryos, and beginning after the 16-cell stage, vasa protein is lost in all cells except the small micromeres. This turnover mechanism is not known, though it should be pointed out that in *Drosophila*, a well-conserved SOCs-box protein, gustavus, associates with vasa and may be involved in vasa protein longevity (Styhler et al., 2002). The sea urchin embryo also contains this putative ubiquitin ligase, and intriguingly, its mRNA accumulates throughout the early embryo, and then encircles the vasa-positive, small micromeres (data not shown). Perhaps then vasa protein is selectively degraded in non-small micromeres by a gustavus-dependent ubiquitination pathway.

It is not yet known whether the small micromeres have a repressive function on vasa expression on their own, or whether the micromeres (including the large micromeres) have this common function. One possibility is that the large micromeres repress trans-fating of the skeletal lineage later in development (mesenchyme blastula), and that the small micromeres repress only the alternative vasa expression pathway. A series of transplants using only large or small micromeres followed by vasa immunolabeling may help resolve this functional difference.

It is noteworthy that only one other animal, the larva of ascidians, has been shown to be able to rescue vasa-positive cells upon their removal (Takamura et al., 2002). In all other animals reported, removal of the vasa-positive cells results in adult sterility. For example, removal of pole cells in *Drosophila*, or a small piece at the posterior end of the primitive streak of a 7-day-old mouse embryo, the remainder of the embryo loses the ability to make germ cells (McLaren, 1981). The “rescue” revealed in this sea urchin by micromere removal may reflect an ancestral mechanism of vasa expression and stem cell formation retained by the typically developing sea urchin.

Primordial germ cells become highly proliferative when they reach the gonad of the juvenile, giving rise to a large number of germ cells that differentiate into eggs and/or sperm. Thus, they are true stem cells, but are restricted to a single, germ line fate. Recent evidence points to vasa association with stem cells that give rise to the primordial germ cells and somatic stem cells (e.g. Agata et al., 2006; Rebscher et al., 2007). In the polychaete, *Platynereis dumerilii*, vasa is present in a population of

mesodermal stem cells that proliferate in the posterior growth zone of annelids, the so-called MPGZ cells. These cells are highly proliferative, give rise to several different cell fates – including primordial germ cells – and express vasa. Vasa expression is limited to the unspecified MPGZ cells, and the primordial germ cells that emanate from them. Of additional interest is that these cells also express other germ cell markers, including nanos, piwi, and the stem cell marker, PL10. Therefore, primordial germ cells may have a common origin with certain somatic stem cells such as those found in *Planaria* and *Cnidaria* (e.g. Extavour and Akam, 2003; Mochizuki et al., 2001) and thus may reflect an ancestral mode of germ cell specification. The sea urchin is a basal deuterostome and perhaps shares this ancestral mechanism in germ cell and somatic stem cell function.

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