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The DEAD-box RNA helicase Vasa functions in embryonic mitotic progression in the sea urchin

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SUMMARY

Vasa is a broadly conserved ATP-dependent RNA helicase that functions in the germ line of organisms from cnidarians to mammals. Curiously, Vasa is also present in the somatic cells of many animals and functions as a regulator of multipotent cells. Here, we report a mitotic function of Vasa revealed in the sea urchin embryo. We found that Vasa protein is present in all blastomeres of the early embryo and that its abundance oscillates with the cell cycle. Vasa associates with the spindle and the separating sister chromatids at metaphase, and then quickly disappears after telophase. Inhibition of Vasa protein synthesis interferes with proper chromosome segregation, arrests cells at M-phase, and delays overall cell cycle progression. Cdk activity is necessary for the proper localization of Vasa, implying that Vasa is involved in the cyclin-dependent cell cycle network, and Vasa is required for the efficient translation of *cyclinB* mRNA. Our results suggest an evolutionarily conserved role of Vasa that is independent of its function in germ line determination.

KEY WORDS: Sea urchin, Translational regulation, Cell cycle, Vasa, Embryonic cells

INTRODUCTION

Vasa was among the first germline genes to be identified (Lasko and Ashburner, 1988). Its DEAD-box motif, its physical interaction with eIF5B and the observation that several proteins do not accumulate in Vasa-mutant flies suggests that it functions in translational regulation (Linder, 2003; Johnstone and Lasko, 2001; Carrera et al., 2000). Curiously, Vasa appears to be inactivated by phosphorylation in response to activation of a meiotic checkpoint during *Drosophila* oogenesis (Ghabrial and Schüpbach, 1999), implying an involvement of Vasa in meiotic cell cycle progression. Furthermore, recent work suggests that Vasa functions in mitotic activity in *Drosophila* germline stem cells (Pek and Kai, 2011). Although target mRNAs reported for Vasa function so far are limited to the germ line of a few animals, Vasa orthologs are present throughout the animal kingdom. In addition to its role in the germ line, it has been reported to function in multipotent and somatic stem cell lineages in early embryonic cells, and even to function in tumor progression (e.g. Raz, 2000; Linder and Lasko, 2006; Rosner et al., 2009; Pfister et al., 2008; Shibata et al., 1999; Oyama and Shimizu, 2007; Rebscher et al., 2007; Juliano and Wessel, 2010; Janic et al., 2010). Its function outside of the germ line, though, has not been resolved.

Echinoderms are a sister group to the chordates. Echinoderm embryos demonstrate highly regulative developmental programs, and they exhibit several rapid cell divisions following fertilization, going from M-to-S phases without intervening G-phases (Horstadius, 1950). Vasa protein and mRNA are present uniformly throughout the egg and early embryo (Voronina et al., 2008) (see Fig. S1 in the supplementary material), becoming lineage restricted only at the 5th cleavage to the small micromeres. These cells are formed by two asymmetric divisions and eventually contribute to the adult rudiment, including the germ line (Yajima and Wessel, 2011). We report here that Vasa specifically accumulated around the mitotic apparatus during M phase in all blastomeres throughout early embryogenesis and inhibition of new Vasa translation selectively inhibited normal cell division. We believe this non-germline function of Vasa may reflect a broader and conserved role than previously anticipated.

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MATERIALS AND METHODS

Animals

Strongylocentrotus purpuratus and *Patiria miniata* were collected in Long Beach, CA, USA, and *L. variegatus* were from Florida, USA.

Immunolabeling

Immunolabeling was conducted as previous described (Yajima, 2007). Briefly, embryos were fixed with 90% methanol, washed with PBS and treated with antibodies. The primary antibodies, Anti-SpVasa (Voronina et al., 2008), anti-phospho-Histone H3 (Millipore) and anti-Tubulin (Sigma) were used at 1:250, 1:100 and 1:50, respectively, and Hoechst was used at a final concentration of 0.1 μg/ml. The secondary antibodies Cy3 and Alexa488 (Invitrogen) were used at 1:300 and 1:500, respectively. For triple immunolabeling, *L. variegatus* embryos were fixed as described above and LvCyclinB antibody (Voronia et al., 2003), previously labeled with blue flourophore by APEX antibody labeling kit (Invitrogen), was used at 1:50. Anti-tubulin (Sigma) antibody was used as described above; SpVasa antibody was used at 1:200 and Cy3-Fab secondary antibody was used at 1:10. Use of a monovalent Fab for this secondary antibody is to avoid cross-reactivity (Wessel and McClay, 1986).

Drug treatments

For chemical treatments of the cytoskeleton, 16-cell stage embryos were treated for one cell cycle (40 minutes) with 5 μM Cytochalasin D (Sigma), with 16 ng/ml Nocodazole (Sigma) or with 0.1 μM Roscovitine (LC Labs, cat. R-12340).

mRNA

Morpholinos were made by Gene Tools (Oregon, USA) and were designed in the 5′UTR of *Spvasa* (GCTTTACTGAGCAGAAATCTAGTTC), *Sppl10* (CTCATAACTACTGATTTTCTCTGAC) and *Spnanos1* (Juliano

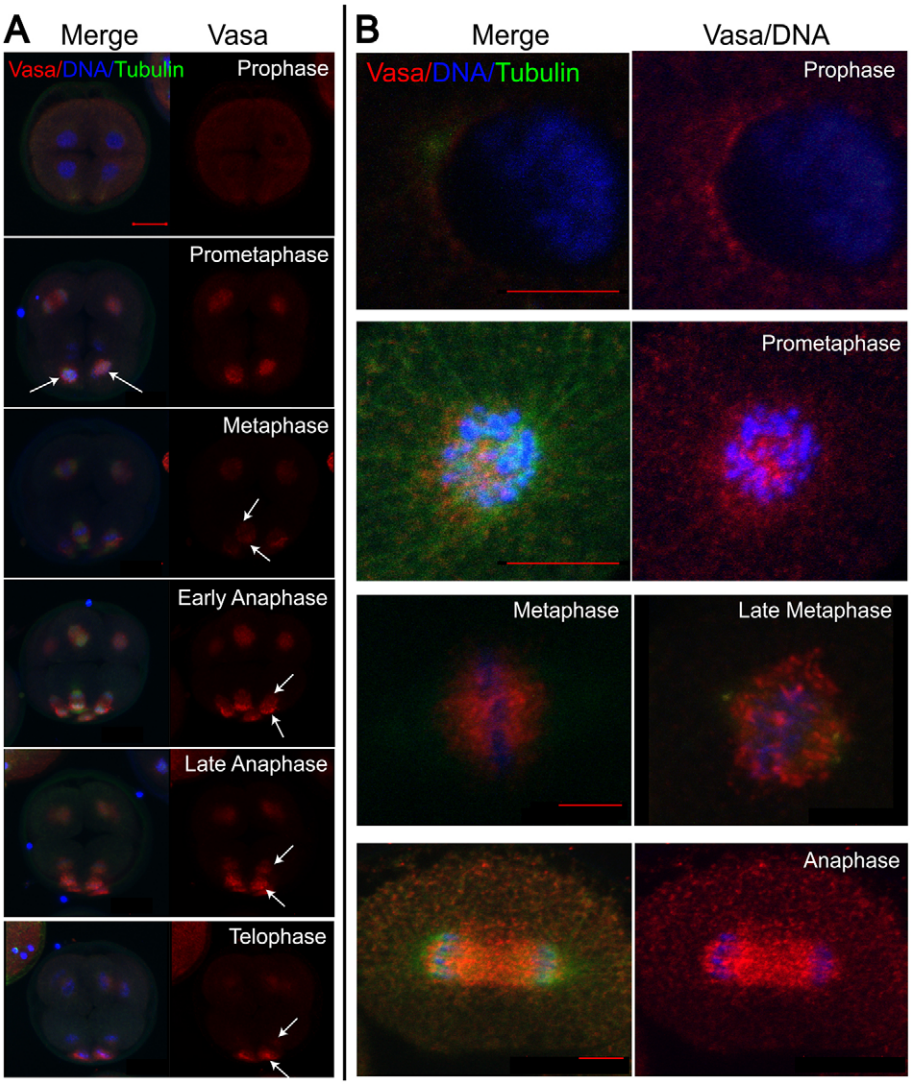

Fig. 1. Vasa is present in each cell of the early embryo and its localization changes dynamically during the cell cycle. (**A**) Embryos at the 4th cell division were immunostained with anti-Vasa antibody (red), and co-stained with anti-β-tubulin antibody for spindles (green) and Hoechst for DNA (blue). Vasa is perinuclear at early prophase, enters the nucleus at prometaphase (arrows), evenly surrounds the aligned chromosomes by metaphase (arrows) and becomes asymmetrically enriched in the vegetal cells at early and late anaphase (arrows). In telophase, Vasa remains in the micromeres, but is not enriched in other cells (arrows). Scale bar: 20 μm. (**B**) Detailed observations of Vasa localization during M phase in a symmetric cell division. Scale bars: 5 μm.

et al., 2009), respectively. For mRNA rescue experiments and for time-lapse imaging, SpVasa-mRNA and a series of deletion constructs were made and used as previously reported (Gustafson et al., 2011).

Microscopy

Fluorescent images and time lapse video were taken by confocal laser microscopy (Zeiss LMS510) or wide-field fluorescence microscopy (Zeiss Axioplan). To measure the DNA content, *z*-stack confocal projections were reconstructed and the integrated fluorescence intensity of each cell was calculated using Image J.

Protein analysis

Western blotting was performed as described previously (Voronia et al., 2008), and the experiment was performed three independent times; 25 embryos were collected for each lane and immunoblotted (Gustafson et al., 2011; Wessel et al., 2000).

Luciferase assay

A dual-luciferase assay was performed as described (Oulhen et al., 2007) with slight modifications. Briefly, to produce Vasa antibody beads (VasaAB-beads), 20 μl of SpVasa antibody was incubated with 200 μl of ProteinA-beads (Invitrogen) at room temperature for 1 hour, and washed with PBS for four times. The four- to eight-cell stage lysate was treated with VasaAB-beads to deplete Vasa or with pre-immune IgG antibody-beads as a control for 1 hour at 16°C. Renilla luciferase mRNAs containing either the UTRs of *Sp-cyclinB* or of *beta-globin*, and the reference firefly luciferase mRNA containing *beta-globin* UTRs were added to these lysates and the relative luciferase activity was measured as described (Promega, Dual Luciferase Assay system).

RESULTS AND DISCUSSION

Vasa accumulates on the spindle complex during M-phase

We found that Vasa was dynamically expressed in all cells of the early embryo in a cell-cycle dependent manner (Fig. 1A). Vasa accumulated peri-nuclearly at prophase and became enriched around the developing aster (Fig. 1B). It entered the nuclear region promptly following nuclear envelope breakdown at prometaphase and associated with the condensing chromosomes. At metaphase, Vasa accumulated adjacent to both aligned sister chromatids, as well as to the spindle, and filled the inter-chromatid region at late metaphase and anaphase. This pattern of Vasa accumulation on the spindle complex in M phase (Fig. 1B) was similar for all blastomeres in both live and fixed cells, including the unequal cleavages at the fourth and fifth cell divisions to form the micromeres and small micromeres, respectively (see Fig. S2A and Movies 1 and 2 in the supplementary material). This cell-cycle dependent localization of Vasa was observed in later blastomeres, but disappeared after the blastula stage when the cell cycle slowed by introduction of G phase.

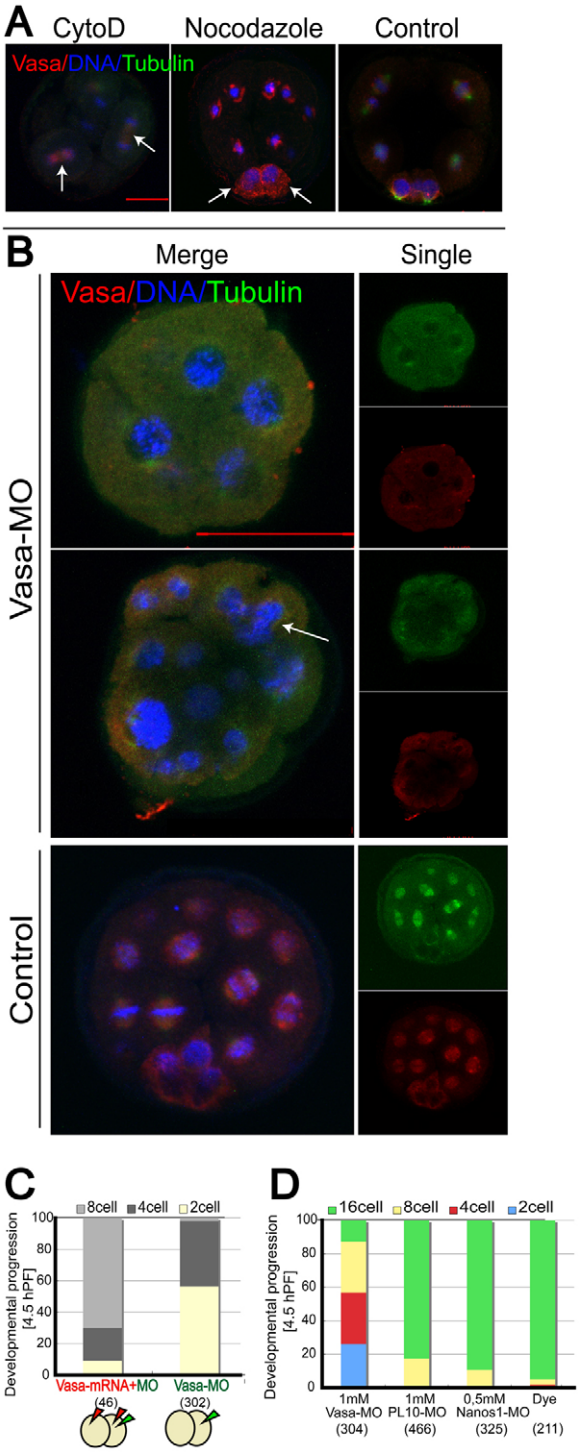

Fig. 2. Vasa functions in cell-cycle progression. (**A**) Actin filament disruption by cytochlasin D treatment had no effect on Vasa localization during M phase (arrows). Nocodazole treatment impaired proper subcellular localization of Vasa but did not disrupt its accumulation in the micromeres (arrows). Scale bar: 20 μm. (**B**) Vasa-depleted embryos exhibited a slow cell cycle with large nuclei (first panel), and lacked normal spindle formation, even following chromosome condensation (middle panel, arrow). Embryos (including control) were each fixed at 5 hours postfertilization. Scale bar: 50 μm. (**C**) Synthetic Vasa mRNA lacking the MO site rescues the cell-cycle delay in Vasa MO-injected embryos. Vasa mRNA was injected into fertilized eggs and Vasa MO was injected into one of two blastomeres at the two-cell stage. The percentage of embryos that reach the given stage (two cell, four cell, eight cell) by 4.5 hours postfertilization is quantified, and the non-MO-injected lineage was used as an indicator of healthy development to control for injection damage. (**D**) mRNA-specific MOs were injected into fertilized eggs and the number of embryos successfully completing their cell cycles and reaching each stage was determined at 4.5 hours postfertilization. Only 10% of Vasa MO-injected embryos successfully reached the 16-cell stage, whereas PL10 MO- and Nanos1 MO-injected embryos showed a success rate similar to the control embryos.

Vasa interacts with the mitotic apparatus during M-phase

Embryos treated with cytochalasin D (actin destabilizing) at the 16-cell stage failed during cytokinesis, although they proceeded with nuclear divisions (Schroeder, 1978) and Vasa accumulated normally between the separating sister chromatids (Fig. 2A). Cells treated with nocodazole (microtubule destabilizing) arrested at M phase and their chromosomes were condensed as reported (Larkin and Danilchik, 1999). In these embryos, Vasa accumulated abnormally in large clumps around the nucleus (Fig. 2A), suggesting that microtubules, but not actin filaments, are important for Vasa localization.

When Vasa accumulation was attenuated by injection of Vasa morpholino antisense oligonucleotide (MO; 1 mM stock) in the early embryo, the cells showed a severe delay in their cell cycle progression in a dose-dependent manner (Fig. 2B; see Fig. S2B in the supplementary material), and lacked normal spindles even following chromosome condensation (Fig. 2B). Deficiencies resulting from Vasa depletion were, however, rescued with Vasa mRNA that was insensitive to the Vasa MO (Fig. 2C), and overexpression of Vasa in a wild-type background had no effect on the cell cycle (data not shown) (Gustafson et al., 2011). The requirement for Vasa in cell cycle progression was tested further by comparing its role with PL-10, a closely related DEAD-box protein with significant sequence similarity to Vasa. In PL-10-deficient embryos, however, a cell cycle delay/arrest was never observed. Furthermore, a MO against another germline marker, Nanos1 (Juliano et al., 2009), also failed to alter cell cycle dynamics in early embryos (Fig. 2D). We conclude from these results that Vasa functions in cell cycle progression.

To understand which regions of Vasa are responsible for these cell-cycle functions, a series of deletion constructs were tested for their ability to rescue the MO-induced cell cycle abnormality (Fig. 3). We found that the C-terminal conserved domain of the protein was necessary both for spindle localization of Vasa and for rescue of the MO-induced cell cycle phenotype (Fig. 3G,H, arrows). N terminus deletions of Vasa showed subcellular accumulation similar to the full-length Vasa, with a granular-like perinuclear accumulation at interphase and spindle-enriched localization during M phase (Fig. 3B-D, arrows). The MO-induced cell-cycle deficiency was also rescued by these proteins (Fig. 3I), suggesting that spindle-associated accumulation of Vasa is independent of the N terminus region, at least to amino acid residue 203. Importantly, this region is the most divergent of Vasa sequence among animals, and is essential for dynamics in cell specific accumulation following the fourth cell division (Gustafson and Wessel, 2010; Gustafson et al., 2011). The C

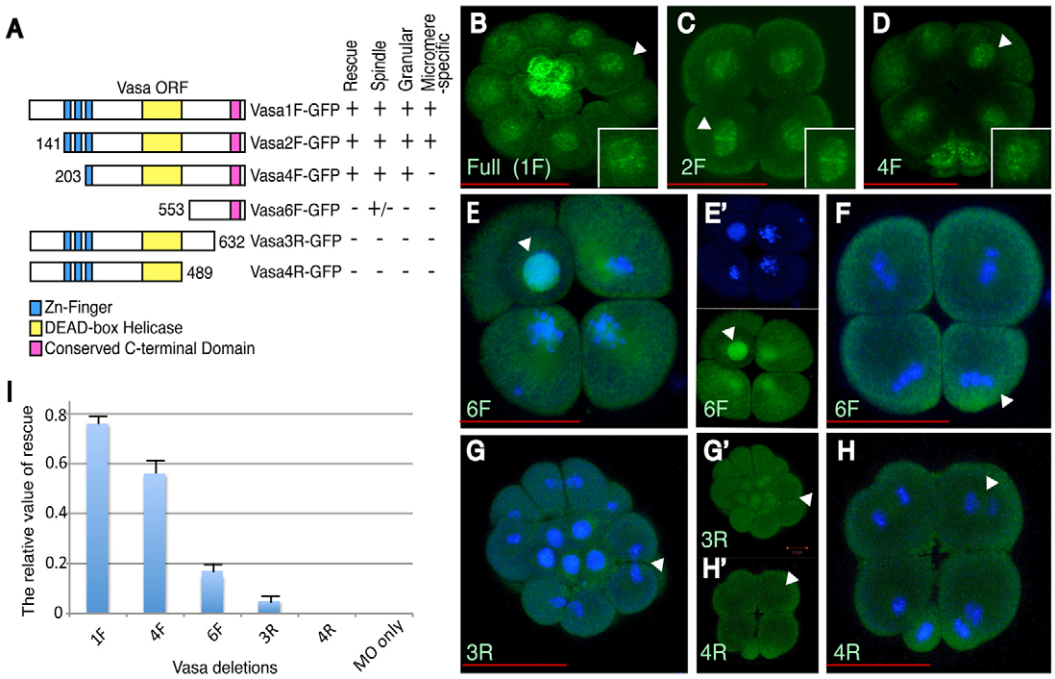

Fig. 3. The Vasa C terminus is responsible for its localization to the spindle. (**A**) A series of deletion constructs injected into eggs. Each construct can be characterized as follows: a 'Rescue' function in the presence of Vasa MO; localization to the 'Spindle'; a characteristic 'Granular' appearance perinuclearly; 'Micromere-specific' accumulation of Vasa during embryogenesis. Each character was evaluated as + or –; +/– indicates an incomplete penetrance. (**B-H**) Each mutation construct was injected into eggs and Vasa-GFP expression was observed at the eight- to 16-cell stage. Scale bars: 50 μm. Arrowheads in each image indicate cell cycle-dependent localization of Vasa (B-D), unusual nuclear localization of Vasa during interphase (E,F) or non-specific localization of Vasa during M phase (G,H), respectively. Insets (E′,G′,H′) are the single channel recordings for either DNA or Vasa-GFP of E, G, H, respectively. (**I**) Vasa mutation constructs were injected into eggs, the Vasa MO was injected into one of two blastomeres, and the level of the rescue for the cell-cycle deficiency was measured at 4.5 hours postfertilization. Each value was normalized to MO-only embryos. Data are mean±s.e.m.

terminus instead is highly conserved and is essential for anchoring Vasa around the nucleus, for its spindle association and for its cell-cycle function.

To delineate the cell cycle defect in embryos lacking Vasa, one of two blastomeres at the two-cell stage was depleted of Vasa (see Fig. S3A in the supplementary material), the embryos were cultured and several molecular metrics were evaluated in these sibling cells. Phospho-H3 labeling, an M-phase indicator (Adams et al., 2001), demonstrated that Vasa-depleted cells have a prolonged M phase (see Fig. S3B in the supplementary material). DAPI quantitation of nuclei indicated that the amount of DNA in Vasa-deficient cells was up to four times greater than in control cells, demonstrating that Vasa is not required for DNA replication (see Fig. S3C in the supplementary material). Time-lapse observation of Histone-2B-GFP also indicated that the chromosomes of Vasa-null cells replicated but did not segregate (see Movie 3 in the supplementary material). Furthermore, Vasa protein levels oscillated with the cell cycle: Vasa was degraded following fertilization but then increased significantly at first metaphase (Fig. 4A). This cycling behavior was apparent for the first few divisions and was similar to the original cycling seen of the cyclins in this same animal (Evans et al., 1983; Voronina et al., 2003). This oscillation continued during subsequent divisions, suggesting that Vasa may function within, or be regulated by, the network of cell-cycle factors. To test this general theme, we used the well-known Cdk1 and 2 inhibitor roscovitine (Meijer et al., 1997). In addition to halting cell cycle progression, reduction of Cdk1 and 2 activities also diminished the peri-nuclear accumulation of Vasa in S-phase (Fig. 4B). Taken together, these data suggest that Vasa is involved in cell cycle progression in early embryogenesis, and it may be associated with cell-cycle components important for M phase.

Vasa regulates translation of cyclin B

As Vasa and CyclinB colocalize on the spindle complex during M phase (Fig. 4C), we tested whether Vasa is necessary for the translation of *cyclinB* mRNA by measuring *cyclinB* translation. We used a dual-luciferase assay in four- to eight-cell stage lysates in the presence or absence of Vasa (Fig. 4D). Vasa was depleted from the lysate by Vasa antibody-linked beads and in the absence of Vasa, the translational level of the introduced *cyclinB-luc* mRNA was significantly lower than the control (treated only with IgG-linked beads). The level of translation in control mRNA (*beta-globin-luc*) was unaffected by Vasa. These results suggest that, although Vasa may interact with many mRNAs and proteins, it functionally regulates cyclinB translation, and possibly other mRNAs located on the spindles during M phase in the rapid cell divisions of these large cells during early embryogenesis.

Vasa function in cell cycle progression is evolutionary conserved

We surveyed the cell-cycle-dependent Vasa localization in other echinoderms and found that Vasa protein was localized in the nuclear region of cells in M phase with a strong morphological

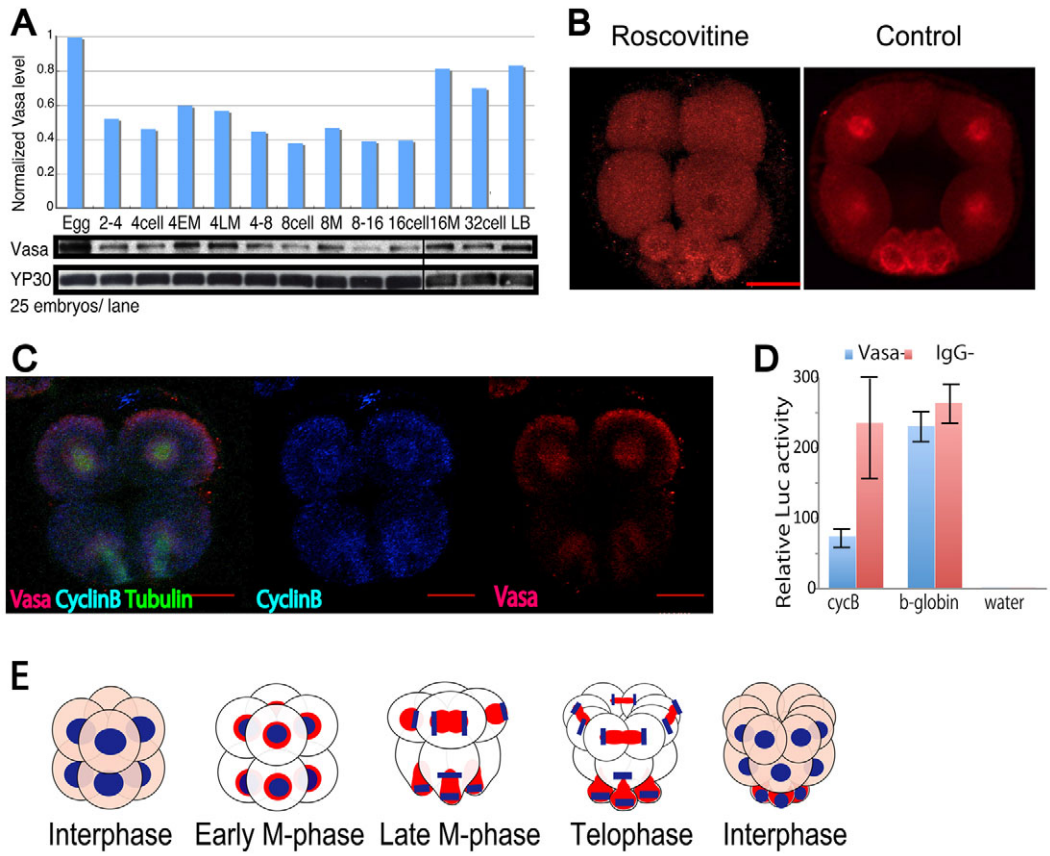

Fig. 4. Vasa oscillates with the cell cycle and regulates cyclinB translation. (**A**) Twenty-five embryos per lane were subjected to an immunoblot to relatively quantify the amount of Vasa at each step in the cell cycle. Maternal Vasa protein significantly decreases after fertilization and embryonic Vasa levels oscillate during the cell cycle in a similar pattern to Cyclin B. Vasa protein levels in the graph were quantified by normalizing to Yolk Protein 30 (YP30). Schematic drawing of embryos represent the Vasa distribution (red) during the cell cycle. (**B**) Roscovitine, a Cdk inhibitor, disrupts proper localization of Vasa. Scale bar: 20 μm. (**C**) Vasa (red) and CyclinB (blue) colocalize on spindles (green) during M phase. (**D**) Dual luciferase assay. CyclinB translation is dependent on Vasa (Vasa depleted), compared with the control (IgG-minus depletion). Data are mean±s.e.m. (**E**) A summary diagram of the dynamic localization of Vasa (red) during cell cycle progression.

consensus, and that MO treatment of sea star Vasa resulted in similar cell-cycle defects (see Fig. S4 in the supplementary material; data not shown). Vasa association with spindles also has been reported in other animal phyla, such as annelids and chaetognaths (Oyama and Shimizu, 2007; Carré et al., 2002), and a recent report in *Drosophila* demonstrates the involvement of Vasa in chromosome condensation (Pek and Kai, 2011).

Other studies revealed that many of the mitotic cyclins, including *cyclinB* mRNAs, are locally translated on microtubules in many organisms (Blower et al., 2007). Therefore, we hypothesize that Vasa functions to regulate the translation of cell cycle-associated cyclins during M phase on spindles that enhances the rapid cell cycle of early embryos. Our results, however, do not exclude the model that Vasa also functions during interphase in the cytoplasm. Furthermore, Vasa accumulation in cells is not sufficient for a rapid cell cycle. Indeed, Vasa in the sea urchin embryo accumulates to its highest levels in the small micromeres, the very cells with the slowest of cell cycles in the embryo. The small micromeres, however, also accumulate Nanos, which is known to bind to the 3′UTR of *cyclinB* in a pumillio-dependent fashion and to repress *cyclin B* translation (Barker et al., 1992). Thus, Nanos activity trumps the Vasa function in the small micromeres and Nanos null-small micromeres display more rapid cell cycling and eventually apoptosis, a result very similar to the phenotype seen in the pole cells of *Drosophila* (Juliano et al., 2009; Juliano et al., 2006; Fujii et al., 2009; Sato et al., 2007; Tsuda et al., 2003; Kobayashi et al., 1996; Hayashi et al., 2004).

We hypothesize that an ancient function of a Vasa-like DEAD-box protein may have been in the regulation of a rapid cell cycle. This hypothesis is supported by the cell-cycle activity of Yeast DED1 (Grallert et al., 2000). Yeast does not have Vasa/PL10, and DED1 is the one similar to Vasa/PL10 in sequence within the same DDX4 family (Hay et al., 1988). Diversification of this gene family may then have segregated activities so that the DEAD-box member we call Vasa selectively functions in multipotent cells, or selectively in the germ line cells – the site of its original identification. In animals with a rapid cell cycle lacking G1 and G2 phases, Vasa appears to have been retained for roles in facilitating translation of cell cycle regulators, particularly when the cell is multipotent and large, such as an early embryo. Taken together, we propose that the function of Vasa in cell cycle progression (summarized in Fig. 4E) is a fundamental and ancient function of this gene, which has been retained by diverse cells throughout the animal kingdom.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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