Published in final edited form as:
Bioessays. 2010 July ; 32(7): 626–637. doi:10.1002/bies.201000001.

Vasa genes: Emerging roles in the germ line and in multipotent cells

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Abstract

Sexually reproducing metazoans establish a cell lineage during development that is ultimately dedicated to gamete production. Work in a variety of animals suggests that a group of conserved molecular determinants function in this germ line maintenance and function. The most universal of these genes are vasa and vasa-like DEAD box RNA helicase genes. However, recent evidence indicates that vasa genes also function in other cell types, distinct from the germ line. Here we evaluate our current understanding of vasa function and its regulation during development, addressing vasa's emerging role in multipotent cells. We also explore the evolutionary diversification of the amino-terminal domain of this gene and how this impacts the association of vasa with nuage-like perinuclear structures.

Keywords

vasa; germ line; multipotent cells; zinc-knuckle; primordial germ cell

Introduction

Segregation and maintenance of the germ line is required for all sexually reproducing metazoans. In many animals, a population of primordial germ cells (PGCs) is set aside early in embryogenesis that is dedicated for the germ cell lineage while other cells in the embryo differentiate into soma. PGCs migrate to the somatically-derived gonads and proliferate into germ line stem cells that can self renew and differentiate into gametes. At least 3 general mechanisms are used to specify PGCs within the animal kingdom. The germ line can form (1) early in embryogenesis from an inheritance of maternal factors (maternally derived, also referred to as preformation) used in flies and nematodes, (2) by cell-cell interactions early in embryogenesis (inductive, also referred to as epigenetic) as seen in mice, and (3) any time in the animal's life, even in adulthood, from a multipotent stem cell precursor (persistent multipotent cell derived germ cells), such as in planaria and hydra.[1–4] Despite these developmental differences, animals employ a group of conserved molecular determinants for PGC specification. The most common of these is the gene vasa.[5–7] While the exact function of Vasa is unclear, its extensive conservation underscores its universal importance in germ line development (Table 1).

Vasa is a member of the DEAD box protein family which functions in a broad range of molecular events involving duplex RNA. Nine conserved sequence motifs typify all DEAD-box genes (Figure 1A.[8] Biochemical analyses show how these motifs, in Vasa and other

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DEAD-box proteins, confer its ATP-dependant RNA helicase catalytic activity. Structural data also suggest that Vasa unwinds duplex RNA in a non-processive manner.[8–11] DEAD-box proteins may operate as chaperones that unwind local secondary structures to facilitate proper RNA folding and interactions with accessory proteins. [12,13] These features are evident in examples encompassing pre-mRNA splicing, ribosome biogenesis, nuclear export, translational regulation and degradation.[8]

Functional analyses of Vasa

What is Vasa's role in germ line development? Vasa-null animals have been generated in *Drosophila*, *Caenorhabditis elegans* and mice by gene knockout, by reduction of Vasa mRNA by RNA-interference (RNAi) and by Vasa protein reduction by antisense morpholino treatment (Knockdown).[14–20]

Drosophila leads the way in understanding vasa function

Genetic screens for maternal-effect genes in *Drosophila* first revealed vasa function in oocyte development.[21] Subsequent mutational and gene inactivation studies showed vasa function in posterior patterning and in germ cell specification in the embryo.[15,22,23] Identifying the molecular targets of this DEAD-box helicase though has proven difficult. Vasa produced from bacterial recombinant can bind RNA and has ATP-dependent RNA helicase activity *in vitro*. This activity is absent with mutations in the conserved DEAD-box sequence motifs.[10,24] Structural analysis of the DEAD-box region in *Drosophila* Vasa suggests that it unwinds duplex RNA in a nonprocessive manner by binding and bending short stretches of the duplex.[10] Consistent with its RNA-binding ability, biochemical and genetic data suggest *Drosophila* Vasa acts as a translational regulator. Indeed, its direct binding to the translational initiation factor eIF5B is required for proper translation of maternal gurken transcripts.[15,25–28]

Does Vasa bind RNA in a sequence specific manner?

Recently, Liu *et al*., (2009) screened for mRNAs that co-purified with Vasa from Drosophila embryos. They identified 221 candidate mRNAs that bound to Vasa, 24 of which were mRNAs in the pole cells – where Vasa is *in vivo*. *Mei-P26* was one of the candidates – its protein product represses microRNA activity and promotes differentiation of the germ line stem cells. Liu *et al*. found that in *vasa* mutants, mei-P26 translation is substantially reduced.[29] This is intriguing in that mei-P26 was previously shown to interact with one of the Argonaut proteins of the miRNA pathway (Ago1) to repress the miRNA interference of target mRNAs in the germ line. Thus, the absence of vasa resulted in low mei-P26 synthesis, and therefore miRNA interference was functional in the germ line. Perhaps more important was that this Vasa/mei-P26 mRNA interaction was shown to be sequence specific; Vasa bound specifically to a (U)-rich motif in the mei-P26 39 untranslated region *in vitro*, and expression of a GFP-mei-P26 transgenes *in vivo* was dependent on the same (U)-rich 39 UTR domain. Moreover, mei-P26 translation was significantly reduced by a mutation in Vasa that reduced its interaction with the translational initiation factor eIF5B.[29] These results are important for several reasons: 1) it provides an important gene regulatory link to understand miRNA regulation in the germ line, 2) it suggests that Vasa interacts with mRNAs selectively, and 3) Vasa interacts with mRNAs in a sequence selective fashion, perhaps linking the sequences in the 3′UTR target mRNA to the initiation factor important for translation of the sequence. Further identification of other sequences in this Vasa-interactome will be important to understand the consensus mRNA sequences for its interaction and the resulting sequences important for germ line development.

Overlapping functions of multiple vasa genes in *C. elegans*

Germ line development in the nematode *Caenorhabditis elegans* is dictated by the inheritance of localized cytoplasmic determinants in the egg and early embryo. Despite some notable differences in germ plasm composition between *Drosophila* and other organisms, such as the lack of an Oskar homolog outside of diptera, and the presence of the unique PLG-1 gene in *C. elegans*, the localization of Vasa in the germ plasm remains a conserved feature.[4] *C. elegans* has four germ line helicases (GLH 1-4), each are Vasa homologs, each present in the germ granules (P-granules) and each are present exclusively in germ line blastomeres during development.[16–18] Loss of function analyses suggests that GLH-1 is most important for germ line development; *glh-1* mutants have a dramatic decrease in germ cells and mature gametes.[18,19] Although GLH 2–4 transcripts are present in the germ line and their proteins localize to P-granules, deletion of glh-2, glh-3 or glh-4 genes alone are not sufficient to cause adult sterility.[17,19] Unfortunately, the mechanism of GLH-function is not known, and may require a biochemical approach as recently accomplished in *Drosophila*.[29]

The picture of vasa function in mammals is dim

Vasa-null mice develop normally and the females are completely fertile. In contrast, male vasa-null mice are infertile due to deficiencies in male germ cell proliferation and differentiation.[14] The Vasa-like gene, DBY in humans, also appears to be required for male fertility.[30] These examples of sex-specific phenotypes in mammals are just the opposite to vasa mutants in *Drosophila*, where females are sterile and males are fertile. Mutants of the vasa-related gene (PL10, also called Belle) in *Drosophila*, however, are male infertile.[31] Thus, although vasa is present in both male and female germ lines, it must either have different targets of function or is regulated differently in the two germ line types. The major impediment in our progress here is that we have so few clues as to the functional mRNA targets of vasa. Overcoming this deficiency will likely require the biochemical approaches of Vasa-mRNA co-purification. Such an approach is difficult though in many animals in which only a small amount of relevant tissue is accessible.

Vasa function is required in diverse organisms – studies with loss of function approaches

While work in *Drosophila*, *C. elegans* and mice constitutes the most extensive analysis of any vasa gene, a growing body of data from several different animals have contributed to our understanding of Vasa function. Abrogating Vasa expression by utilizing RNAi in embryos and adults has been useful for collecting functional data in animals lacking stable transgenics. For example, the flatworm *M. lignano* displays Vasa expression in the multipotent neoblast stem cells in addition to germ cells. However, RNAi knockdown of Vasa had no effect on stem cell maintenance, neoblast proliferation, gonad formation or gonad development.[32] This suggests either a nonessential role of Vasa in adults or a functional overlap with other Vasa-like genes in flatworm gonads and neoblasts, similar to that seen in the germ line of the roundworm. So too in colonial ascidians,[33–35] oysters,[36] in teleosts,[20,37] *Xenopus*,[38] the parasitic wasp,[39] and the crustacean *Parhyale hawaiensis*,[40] vasa function and or association in germ cell development is widespread.

Vasa function is required in diverse organisms – gain of function in the chicken and the human

Recent Vasa gain-of-function analyses in human and chicken cell lines have provided more insight into Vasa's role in development. Ectopic Vasa expression has been reported in epithelial ovarian cancer cells where it abrogates the DNA damage-induced G2 checkpoint by down regulating 14-3-3σ expression.[41] However, it is unclear whether the abnormal presence of Vasa reflects a causative role in ovarian tumorigenesis. This could be tested in

mice by restoring 14-3-3σ expression in a Vasa-expressing ovarian cancer cell line, reintroducing these cells into a host animal and analyzing whether it abolishes tumorigenicity. Nonetheless, this observation illustrates the importance of correct Vasa expression in development. Ectopic Vasa expression in chicken embryonic stem cells (ESCs) induces expression of specific germ line and meiotic genes.[42] As a consequence, following their injection into chick embryos, these ESCs exhibit improved germ line colonization and adopt a germ cell fate. This supports a fundamental role of Vasa in germ line identity and function.

Together these data suggest Vasa has an essential and evolutionarily conserved role in many aspects of germ line development including germ cell specification, proliferation and maintenance. Identification and analyses of Vasa targets will help resolve the mechanistic details behind this conserved germ line function as well as additional roles outside the germ line. The results also show that no one approach (gene inactivation versus gene over-expression), no one cell-type, no one animal, and no one gene will likely solve this problem. Instead, using diverse experimental approaches in many different animals will hopefully enable the most efficient approach to definitely identify vasa function.

Beyond the germ line: Vasa in multipotent stem cells

Although Vasa has proven a reliable marker for the germ lineage in several animals, these examples mostly consist of insects and vertebrates (Table 1).[5] Emerging data have provided a broader phylogenetic perspective on vasa and demonstrate its function also in multipotent cell types. In Cnidarians, for example, the multipotent interstitial cells (I-cells) contribute not only to the germ line, but also to somatic cell types such as nematocytes (nerve cells) and gland cells.[7,43,44] Flatworms possess remarkable regenerative capabilities due to their pool of multipotent stem cells (neoblasts).[45] Vasa is expressed in the ovaries, the testes and the neoblast stem cells.[32] The function(s) of Vasa in these persistent multipotent cells of cnidarian and flatworm is yet unknown, but with recent technological advances in the experimentation of both of these animals, vasa's function can be tested by RNAi or morpholino knock-down of Vasa followed by testing the ability of I-cells or neoblasts not only to proliferate and differentiate into somatic cell types, but also into a germ line.

While no examples of Vasa expression are reported outside of germ line cells in vertebrates and insects, data from other deuterostomes and from various arthropods prove otherwise. For example, the crustacean *Polyascus polygenea* (a colonial barnacle) contains Vasa in a cluster of multipotent stem cells in its buds and stolon during the parasitic stage of its life cycle.[46] Vasa is present in the auxiliary cells of the oyster ovary, in the somatic cells of the reconstructing gonadal tissues during its development for the next spawning cycle,[47] and in the snail, is present in non-germ-line lineages.[48] In the polychaete annelid, Vasa is enriched in the progenitor mesodermal posterior growth zone (MPGZ). The MPGZ cells are multipotent somatic stem cells, highly proliferative and contribute to both mesodermal tissue and PGCs.[49] In the oligochaete as well, Vasa is present in non-genital segments during its development.[50]

Tunicates are close relatives to vertebrates and analyses in the colonial ascidians (*Botryllus primigenus and Polyandrocarpa misakiensis*) show vasa mRNA expression in their germ lines along with cell aggregates containing somatic-derived multipotent hemoblasts.[51,52] Regenerating buds induce vasa expression *de novo* at every budding cycle suggesting that vasa may have functions in regeneration activities independent of the germ line.[2,35,53] Indeed, many of the animals with Vasa in their multipotent cells are capable of tissue regeneration in the adult to varying degrees.[54,55] This is also true in the more limited

regenerative context for oyster gonad regeneration as well as asexual reproductive budding in colonial ascidians and the parasitic barnacle. However, Vasa expression in multipotent cells outside of a regenerative context in the polychaete annelid may be indicative of a more general germ cell developmental phenomenon where localized cytoplasmic factors may help set aside a multipotent stem cell population of which a subset are later designated for the germ line. Overall, the presence of Vasa in non-germ line cells in various taxa is becoming standard, but still lacking is an experimental test of vasa function during the regeneration, the development, or in the maintenance of potency of non-germ line cells.

Regulation of Vasa expression

Transcriptional and epigenetic control of vasa expression

Localized Vasa expression is a common feature throughout phylogeny and in order to control this, animals employ a variety of mechanisms to regulate both vasa mRNA and protein accumulation. Several studies suggest vasa transcriptional regulation contributes to cell and tissue-specific Vasa expression in developing embryos and adults. [56] In *Drosophila* embryonic development, zygotic transcription of vasa occurs specifically in the pole cells immediately after gastrulation and remains germ line-specific into adulthood.[57] DNA methylation is one form of epigenetic regulation directing differential gene expression, where hypermethylation is associated with gene silencing.[58] A recent study in humans suggests that the methylation state of the vasa gene promoter controls its specific transcription in the testes. The vasa promoter is hypomethylated in the testes and methylated in all other tissues, which do not express vasa.[59] A clinical study showed that spermatogenesis defects such as idiopathic azoospermia or severe oligospermia were also associated with a hypermethylated vasa promoter in some individuals.[60] Transcriptional regulation during gonad development and germ cell maturation likely also involves hormonal signaling.[61,62]

Post-transcriptional regulation of vasa

The presence of different vasa splice forms in several animals such as *M. lignano, S. purpuratus, L. variegatus* and *P. dumerilii*, suggests RNA processing contributes to vasa regulation (Table 1).[32,49,63] Differential vasa expression can also result from regulation of vasa mRNA stability. For example, the vasa transcript in the amphipod crustacean *Parhyale hawaiensis* is maternally provided and uniformly distributed during early cleavage stages before localizing in 32-cell stage embryos. These Vasa-positive cells were determined to be PCGs by lineage tracing analysis and vasa transcript localization is dependent on its 3′UTR to preferentially stabilize it in the germ line.[40,64] Differential vasa transcript stability has been seen in several other animals, such as zebrafish and other teleosts, and thus appears to be an important general process for post-transcriptional regulation.[37,65–67]

Translational repression is another regulatory mechanism that allows localized protein production from a ubiquitous transcript during embryonic development.[68] Sequences within a transcript 5′ and 3′UTRs may contain *cis*-regulatory elements which form secondary RNA structures or which bind *trans*-acting factors that inhibit its translation.[69] Relief of this translational repression can direct localized vasa expression. For instance, in mice, Dazl protein binds the 3′UTR of mouse vasa mRNA *in vivo*, stimulates its translation in *Xenopus* oocyte extracts and Dazl knockout mice have reduced levels of Vasa protein, suggesting this regulation is crucial for Vasa translation and spermatogenesis.[70]

Post-translational regulation of Vasa

Work in *Drosophila* has detailed multiple mechanisms that regulate Vasa protein localization to the pole plasm. For example, Vasa directly interacts with Oskar protein *in*

vitro and this interaction may facilitate Vasa anchoring to polar granules in the posterior pole of the oocyte.[71] Indeed, all mutant oskar alleles are defective in Vasa protein localization.[72,73]

Evidence also suggests that proteolysis may play a regulatory role in Vasa localization. In *Drosophila*, Vasa is ubiquitinated in the oocyte and its pole plasm accumulation is dependent on the deubiquitinating enzyme Fat facets.[74] Since ubiquitylation can target a protein for degradation, Fat facets may stabilize Vasa in the pole plasm. Normal Vasa pole plasm localization also depends on Gustavus and Fsn, two paralogous B30.2/SPRY domain proteins.[75,76] Both Gustavus and Fsn directly interact with Vasa through their B30.2/SPRY domain and share several features indicative of an E3 ubiquitin ligase function.[76–78] Gustavus contains a SOCS-box domain that complexes with Elongin B/C *in vitro* and Cullin-5 *in vivo*.[75–77]Fsn has an F-box domain that interacts with Cullin-1 *in vivo*.[76] While Gustavus and Fsn both appear to target Vasa for ubiqutination, mutational and overexpression analyses suggest their functions are not identical and may contribute to a delicate regulatory balance of Vasa ubiquitination required for its proper localization.[76] These results raise several important questions concerning this regulation. What is the nature of Vasa ubiquitination by Gustavus and Fsn? Are there mono-ubiquitination, K48-ubiquitin chains or K63-ubiquitin chains species of Vasa and do these impart different stability and localization properties?

Regulation of proteolysis may also direct Vasa localization during embryonic development in the sea urchin *Strongylocentrotus purpuratus*. Vasa transcript is present uniformly during early embryogenesis through blastula stage, but Vasa protein is strongly enriched in the 16-cell stage micromeres and subsequent small micromeres.[63,79] The Vasa coding region is sufficient for its small micromere accumulation and proteasome inhibition increases Vasa protein levels throughout the embryo (Gustafson *et al*., in preparation). Vasa expression is also regulated by proteolysis in *C. elegans*.[19] The Jun N-terminal kinase member KGB-1 and COP9 signalosome subunit 5 (CSN-5) both bind GLH-1.[80] Phosphorylation of GLH-1 by KGB-1 targets it for proteasomal degradation, whereas CSN-5 association in GLH-1 enhances its stability in the germ line.[80] In addition, recent data from Vasa orthologs in mice, *Xenopus* and *Drosophila* suggest arginine methylation is a conserved aspect of Vasa regulation. Furthermore, in *Drosophila*, the arginine methyltransferase Capsuleen (dPRMT5/csul/dart5) is required for symmetric dimethyl-ariginine modifications of Vasa *in vivo*.[81] However, additional work is required to elucidate the functional consequences of Vasa arginine methylation. Does arginine methylation influence Vasa's binding specificity to target mRNAs and other proteins or are they important for Vasa's localization and protein stability?

Sub-cellular Vasa localization suggests a dynamic role in mRNA association - the Nuage-like structures

In addition to its cell type and tissue-specific expression, another universal feature of Vasa expression is its subcellular localization. Germ cells in all metazoan animals contain perinuclear electron-dense ribonucleoprotein (RNP) structures.[82] These RNP rich structures are often called nuage, the mitochondrial cloud, polar granules, P-granules, chromatoid bodies and in some somatic cells, P bodies. [82,83] While the various names of these structures correspond to differences in morphology, composition and animals in which they were first identified, it is believed they are related entities.[83,84] Several of the proteins identified as nuage components are known to function in mRNA regulation.[4] Although it is still unclear whether these structures are centers for translation, polysomes have been reported adjacent to nuage in *Drosophila* and rats.[83,85] Ultrastructural analyses in *C. elegans* show nuage structures are frequently associated with nuclear pores.[86] One of the most common nuage components is Vasa (Figure 2).[17,86–90] Examples in several animals

suggest that at some point in development, either Vasa mRNA or protein displays a nuage association or nuage-like localization (Table 2).

It is unclear however whether nuage is the location of Vasa function. Instead, this subcellular localization may be used to direct a lineage-specific segregation of Vasa during early embryonic development. In many animals, Vasa-containing nuage structures are thought to be exclusively germ line-specific. However, the presence of Vasa-positive nuage-like structures in multipotent neoblasts of the flatworm *Magnostatum lignano* suggests they may be more widespread (Table 2).[32] Now that at least one vasa target, mei-P26, has been identified, its relative association with the nuage will be important to determine in order to resolve the consistent role of vasa in this specialized cellular structure.

Does vasa interact with the RNAi pathway?

Several studies indicate a functional relationship between Vasa and both the small interfering RNA and micro-RNA processing pathways. One essential component in both of these pathways is the RNase III endonuclease Dicer,[91] which, in mice, colocalizes with Vasa in nuage.[89] Furthermore, ectopically expressed Vasa and Dicer protein interact in COS cell lysates and this interaction requires the C-terminal portion of Vasa.[89] The C-terminal RNaseIII region of Dicer is sufficient to interact with Vasa and the remaining N-terminal ATPase/helicase-PAZ domain region appears independent of Vasa.[89]

PIWI proteins represent a subgroup of Argonautes required for germ line stem cell maintenance and fertility in several animals.[92] *Drosophila* PIWI is a polar granule component that interacts with Vasa and PIWI-mutant flies have normal Vasa protein expression and abdominal patterning of the embryo, but exhibit a severe deficiency in pole cell formation.[93] Over-expressing PIWI results in a dose-dependent increase in Vasa protein levels and pole cell formation. These data suggest that a PIWI-mediated piRNA pathway regulates the levels of Vasa and Oskar proteins and possibly other genes involved in the germ line determination pathway in *Drosophila*.[93] A similar interaction is found in mouse, where Vasa protein binds to both recombinant and endogenous MIWI and MILI, which are mouse PIWI homologs.[81,94] Indeed, MILI and Vasa knockout mice have similar phenotypes and defects in spermatogenesis indicative of cooperative molecular functions. [14,94] In MIWI knockout mice, Vasa protein does not localize to the nuage structures.[94] However, it is still unknown whether MIWI is required for nuage and ultrastructural studies in MIWI knockout mice are needed. Exactly how these specific interactions influence Vasa, MIWI or MILI function is unclear.

Recent work has identified Maelstrom as a nuage component that interacts with both mouse Vasa and MIWI, is required for spermatogenesis and also is involved in silencing transposable elements.[95,96] In *Drosophila*, Maelstrom protein localizes to nuage in a Vasa-dependent manner. In *maelstrom* mutant oocytes, a higher molecular weight Vasa protein species is evident indicating that Maelstrom is required for proper Vasa modification or processing.[87] Although still not definitive, the consistent association in multiple animals of vasa and members of the RNAi pathway argues strongly that they have a functional relationship. This may not be surprising since both are postulated to be involved in translational regulation, and the engagement of each mRNA with the ribosome is likely a continuously evaluated process that responds with incremental increases, decreases, and rates of translation. Researchers often have difficulty in dissecting overlapping pathways by classic genetic means, so an alternative approach is to make use of blossoming *in vitro* cell free lysate assays both for mRNA translational activity, as well as for mRNA stability as a result of miRNAs.[97,98] In the context of vasa function in the RNAi pathway, it is hard to

ignore the intersection here that the small RNAs are 20–30 bases in length, and that vasa is capable of unwinding 20–25 bases of dsRNA.

Vasa evolution resulted in divergent N-termini while retaining the conserved helicase domain

Comparative phylogenetic data suggests that the Vasa gene family originated from a duplication in a PL10-related DEAD-box gene early in metazoan evolution.[7] While animals have both vasa and PL10 genes, plants and fungi have only PL10 genes and lack vasa genes (Figure 1B). At some point after this gene duplication, vasa genes acquired CCHC Zn-knuckle domains in the region N-terminal of the conserved DEAD box. The number of Zn-knuckle domains found Vasa sequences vary from 1 to 8. However, vertebrates and insects may have both independently lost these Zn-knuckle domains (Figure 1C, Table 1).

CCHC Zn-knuckles can be categorized as a “classical zinc-finger” based its zinc chelation topology of a short β-hairpin followed by an α-helix.[99] They can bind single and double-stranded DNA or RNA and may be involved in transcriptional regulation.[100,101] Most examples of CCHC Zn-knuckles come from human retroviruses which all require Zn^{2+} binding for proper nucleocapsid protein folding.[102–104] The CCHC Zn-knuckle domains of retroviral nucleocapsid proteins interact with specific structures in the viral RNA genome during packaging.[105–109] A non-viral CCHC Zn-knuckle example includes the *C. elegans* Lin28 protein, which has 2 CCHC Zn knuckles that are crucial for its localization to P-granules and stress granules.[110] While the exact functional significance of these Zn-knuckle domains is unknown, their RNA-binding properties point to a role in Vasa’s RNA target specificity. Zn-fingers are versatile and can also target binding to other proteins.[99]

Do the CCHC Zn-knuckles impart additional functional dimensions to Vasa proteins? While the presence of the 9 conserved DEAD-box sequence motifs in Vasa allow inference into their RNA helicase catalytic activities, the highly divergent N-terminal regions are more cryptic. The presence of Vasa Zn-knuckles correlates, in many animals, with an expanded expression pattern (and possible functional role) outside of the germ line. The presence or absence of Zn-knuckles may reflect differences in RNA or protein target binding. These different target interaction properties may be important to potential expanded functions outside of the germ line. One exception to this notion is the presence of Zn-knuckles in the 4 Vasa homolog Germ-line helicase (GLH 1-4) genes in *C. elegans*, whose expression is restricted to the germ line.[16–18] Alternatively, it is possible that the loss of Zn-knuckles in insect and vertebrate vasa genes coincided with the emergence of Zn-knuckle containing cofactor proteins which now confer the target specificity. However, no such Vasa cofactors have been identified yet in either insects or vertebrates.

Summary and Future directions

Although vasa has been known to be an important gene in development for almost 30 years, we know remarkably little about how it works. With recent progress and technological breakthroughs recently, however, we anticipate that the next few years will result in a plethora of answers and new understanding. We feel the following are the most important questions to address:

What are the mRNA targets of vasa in translational regulation?

Does vasa function similarly in germ cells as it does in multipotent stem cells?

What role does the highly divergent N-terminus of vasa play in its function?

Is the nuage a site of vasa function or storage?

Is vasa an integrator of RNAi and translational regulation?

Despite the tremendous diversity in embryonic development displayed in metazoan animals, the broad conservation of Vasa underscores its importance for reproduction. Despite numerous functional analyses though, our understanding of Vasa's specific molecular function in translational regulation is meager. Further Vasa functional analyses will greatly expand our understanding of Vasa's purpose during development and how it is localized so effectively. Analysis of Vasa expression in less-studied animals outside of vertebrates and insects imply that the role of Vasa is not strictly confined to the germ line, which coincides with the divergence of its N-terminus. Perhaps the animals containing Zn-knuckles on their N-termini have different functional capabilities than those without a defined N-terminal structure. Alternatively, vasa may simply interact with a Zn-knuckle containing protein and thereby have similar functions. Ectopic expression studies and gene swapping experiments will certainly help in this regard. Most importantly, if Vasa utilizes its helicase activity in translation, does it function as a general translation factor or does it interact with specific target transcripts? Although Vasa is capable of binding RNA *in vitro*, it is currently unknown whether Vasa interacts with mRNAs *in vivo*.[10,24] Gurken is one of the only examples for which Vasa has been shown to be required for translation.[23] However, we still do not know if Vasa directly binds Gurken transcript. A screen for mRNAs that interact with Vasa proteins will help identify its targets. With the break in this wall by Liu *et al*., 2009, we may now be able to crack the function of vasa in both the germ line, and multipotent cells.

Abbreviations

PGC	primordial germ cell
DEAD-box	single-letter amino acid code (aspartic acid, glutamic acid, alanine, aspartic acid) within the helicase domain of a family of conserved proteins
MPGZ	mesodermal posterior growth zone

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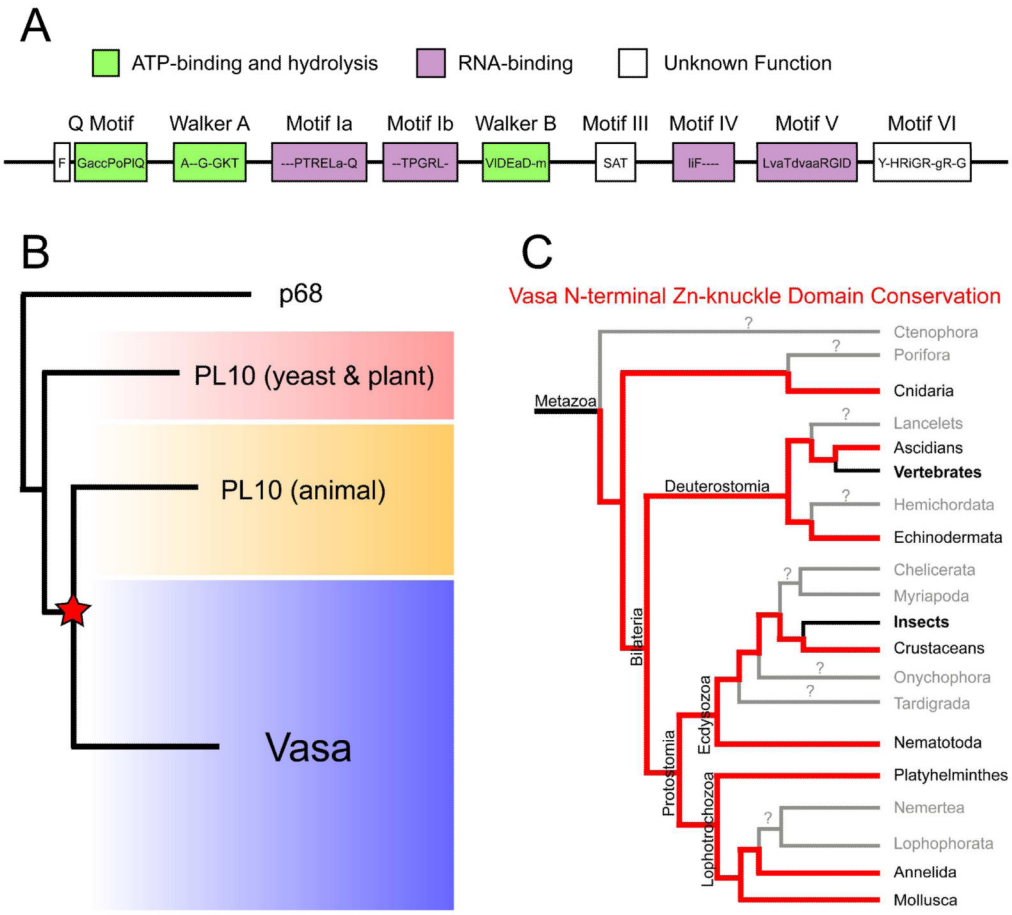

Figure 1. Vasa gene family conservation

(A) Nine conserved sequence motifs specific to all DEAD-box genes. Residues important for ATP-binding and hydrolysis are indicated in green and residues involved in RNA binding are indicated in purple. The function of the remaining conserved motifs in white is unclear. (B) A schematic representation of the phylogenetic relationship between Vasa genes, PL10 genes and the closest related DEAD box gene, p68 (Adapted from Mochizuki *et al*., 2001). The star indicates the evolutionary split between Vasa and PL10 genes. (C) CCHC Zn-knuckle domain-containing Vasa genes from Table 1 were used to illustrate the phylogenetic conservation of this motif in the N-terminal portion of Vasa proteins. Animals with Vasa CCHC Zn-knuckles are indicated in red, animals lacking Vasa Zn-knuckles are shown in black and animals with insufficient Vasa sequence information are indicated in grey (Phylogenetic tree adapted from Dunn *et al*., 2008).

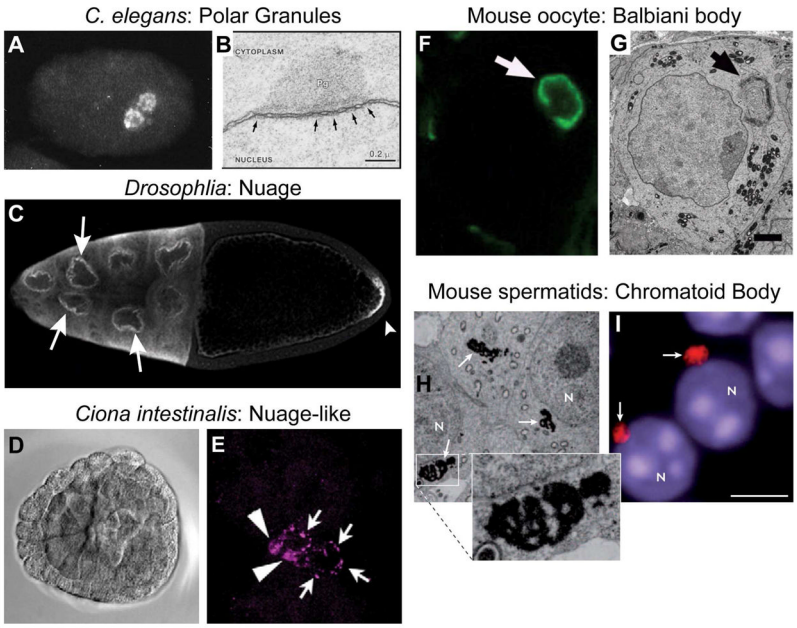

Figure 2. Vasa protein association with nuage-like structures

Vasa protein localizes to perinuclear structures in a wide variety of metazoans. *C. elegans* immunofluorescent imaging of Vasa protein (A) and TEM image of polar granules (B) (black arrows indicate nuclear pores). (C) In *Drosophila melanogaster* developing egg chamber, Vasa protein localizes to the pole plasm in the oocyte (white arrowhead) and the pernuclear nuage in nurse cells (red arrows). DIC (D) and Vasa immunoflourescence (E) imaging of a *Ciona intestinalis* gastrula embryo with both punctate (arrows) and diffuse (arrowheads). Vasa protein (F, immunofluorescence) localizes to the Balbiani body (G, TEM) in mouse oocytes. In mouse spermatids, chromatoid bodies (black arrows) (H, TEM) contain Vasa protein (white arrows) (I, immunoflourescence).

Table 1

Full-length vasa ortholog cDNAs

Group	Common name	Gene name	Polypeptide length	CCHC Zn-fingers	Genebank accession
Porifera	**Sponges**	N/A	N/A	N/A	N/A
Cnidaria	**Hydra**				
Hydra magnipapillata		CnVasl	797	7	BAB13307
		CnVas2	890	1	BAB13308
Hydractinia echinata		HeVas	680	3	ABO93350
Platyhelminthes	**Flatworms**				
Macrostomum lignano		Macvasa splice-form 1	929	3	CAL91031
		Macvasa splice-form 2	860	2	CAL91030
Neobenedenia girellae		Ngvlg3	634	7	BAF44661
Paragonimus westermani		vasa2n	606	0	ABM30180
		vasa3n	606	0	ABM30181
Echinoderms					
Strongylocentrotus purpuratus	Purple urchin	SpVasa splice-form 1	766	4	ACM80369
		SpVasa splice-form 2	732	3	
Lytechinus variegates	Green urchin	LvVasa splice-form 1		5	
		LvVasa splice-form 2	679	0	ACM80368
Eucidaris tribuloides	Pencil urchin	EtVasa	498	1	ACM80367
Asterias forbesi	Forbes's Starfish	AfVasa	715	3	ACM80365
Asterina miniata	Batstar	Am Vasa	730	4	ACM80366
Tunicates	**Sea Squirts**				
Ciona intestinalis		DEAD1	669	3	BAA36710
Ciona savignyi		CsVHa	688	3	BAB12216
		CsVHb	770	6	BAB12217
Halocynthia roretzi		Vasa	691	3	ACJ64200
Botryllus primigenus		BpVas	687	5	BAE44472
Botryllus schlosseri		BS-Vasa	547	1	ACJ69403

Group	Common name	Gene name	Polypeptide length	CCHC Zn-fingers	Genebank accession
Polyandrocarpa misakiensis		PmVas	705	5	BAE94497
Botrylloides violaceus		Vasa protein	630	4	ABM74410
Lancelets	N/A	N/A	N/A	N/A	N/A
Vertebrates					
Homo sapiens	Human	Ddx4	690	0	AAH47455
Mus musculus	Mouse	Ddx4	728	0	AAI37602
Gallus gallus	Chicken	Cvh	662	0	BAB 12337
Danio rerio	Zebrafish	Vasa protein	688	0	AAI29276
		Vasa-like protein	715	0	AAL89410
Sus scrofa	Wild boar	Vasa-like protein	722	0	AAT46129
Oncorhynchus mykiss	Rainbow trout	Vasa	647	0	BAA88059
Oryzias latipes	Medaka	Vasa	617	0	BAB61047
Rattus norvegicus	Rat	Ddx4	713	0	Q64060
Xenopus laevis	Clawed frog	Vlgl	700	0	AAC03114
Rana lessonae	Pond frog	DEAD box protein	724	0	CAH56439
Carassius auratus	Silver crucian carp	CagVasa	701	0	AAV70960
Oreochromis niloticus	Nile tilapia	Vasa	645	0	BAB 19807
Monopterus albus	Swamp eel	Vasa-like protein	618	0	ABA54551
Silums meridionalis	Southern catfish	Vasa	662	0	ACD62525
Pan troglodytes	Chimpanzee	Vasa	703	0	XP 517757
Macaca fascicularis	Crab-eating macaque	Ddx4	725	0	Q4R5S7
Thunnus orientalis	Pacific bluefin tuna	Vasa	644	0	ABY77970
Trachurus japonicus	Japanese jack mackerel	Vasa	657	0	BAG72093
Salvelinus leucomaenis	Whitespotted char	Vasa	662	0	ACA33927
Leucopsarion petersii	Ice goby	Vasa homolog	645	0	BAD04052
Cyprinus carpio	Carp	Vasa	691	0	AAL87139
Am by stoma mexicanum	Axolotl	AxVH	724	0	AAT09162
Molluscs					
Crassostrea gigas	Pacific oyster	Oyvlg	758	4	AAR37337

Group	Common name	Gene name	Polypeptide length	CCHC Zn-fingers	Genebank accession
Chlamys farreri	Japanese scallop	Vasa	801	5	ABE27759
Haliotis asinina	Vetigastropod	Vasa	763	2	ACT35657
Pogonophora	N/A	N/A	N/A	N/A	N/A
Vestimentifera	N/A	N/A	N/A	N/A	N/A
Annelids					
Platynereis dumerilii	Dumeril's clam worm	Vasa homolog	712	3	CAJ15139
		Vasa protein isoform	732	4	CAJ38803
Enchytraeus japonensis		Vasa-related	990	8	BAF767096
Rotifers	N/A	N/A	N/A	N/A	N/A
Nematodes					
Caenorhabditis elegans		Germ-line helicase 1	763	4	AAB52901
		Germ-line helicase 2	974	6	AAB03510
		Germ-line helicase 3	720	2	AAC28388
		Germ-line helicase 4	1156	5	AAC28387
Caenorhabditis briggsae		GLH-1 related	795	4	CAP31774
Onychophora	N/A	N/A	N/A	N/A	N/A
Tardigrades	N/A	N/A	N/A	N/A	N/A
Myriapods	N/A	N/A	N/A	N/A	N/A
Insects					
Drosophila melanogaster	Fruit fly	Vasa	661	0	CAA31405
Anopheles gambiae	African malaria mosquito	Vasa-like protein	596	0	AAY41942
Apis mellifera	Honey Bee	Vasa protein	630	0	NP 001035345
Acyrthosiphon pisum	Pea aphid	Vasa-like protein	669	0	XP 001948608
Culex quinquefasciatus	Southern house mosquito	Vasa	641	0	XP 001850751
Tribolium castaneum	Red flour beatle	Vasa	627	0	NP 001034520
Bombyx mori	Silkworm	BmVLG	601	0	BAA19572

Group	Common name	Gene name	Polypeptide length	CCHC Zn-fingers	Genebank accession
Drosophila virilis	Fruit fly	Vasa	625	0	AAM49782
Aedes aegypti	Yellow fever mosquito	Vasa-like protein	638	0	AAY41941
Melipona scutellaris	Stingless bee	Vasa	624	0	ABQ96192
Melipona quadrifasciata	Stingless bee	Vasa	624	0	AC102436
Scaptotrigona postica	Stingless bee	Vasa	624	0	ABQ96191
Frieseomelitta varia	Stingless bee	Vasa	624	0	ACI02437
Copidosoma floridanum	Parasitic wasp	Vasa	708	0	AAT12450
Gryllus bimaculatus	Two-spotted cricket	Vasa	650	0	BAG65665
Crustaceans					
Parhyale hawaiensis	Amphipod crustacean	Vasa	676	5	ABX76969
Daphnia magna	Water flea	DmaVas	779	6	BAE00180
Litopenaeus vannamei	Pacific white shrimp	Vasa-like protein	703	3	AAY89069
Fenneropenaeus chinensis	Chinese white shrimp	Vasa	712	3	ABQ00071
Macrobrachium rosenbergii	Giant freshwater prawn	MrVLG	710	3	ABC87271
Moina macrocop	Water flea	MmVas	843	6	BAD99524
Artemia franciscana	Brine shrimp	AfVas	726	3	BAD99523
Horseshoe Crabs	N/A	N/A	N/A	N/A	N/A
Arachnids	N/A	N/A	N/A	N/A	N/A

Table 2

Subcellular localization of Vasa during development

Species	Nuage-localization
Cnidaria	
Hydractinia echinata (Hydrazoa)	**Protein:** Perinuclear nuage material in oocytes and early embryos[44]
Platyhelminthes	
Macrostomum lignano (Flatworm)	**Protein:** Perinuclear localization in developing germ cells in ovaries and testes as well as multipotent neoblasts[32]
Echinoderms	
Strongylocentrotus purpuratus (Purple sea urchin)	VasaGFP Localizes to perinuclear granular structures resembling nuage in small micromere cells (Gustafson *et al*., unpublished)
Annelids	
Platynereis dumerilii (Dumerili's clam worm)	**Protein:** Localizes to a perinuclear ring in oocytes corresponding to previously described nuage material[49,111]
Tunicates	
Ciona intestinalis (Ascidian)	**Protein:** Perinuclear nuage-like particles during the late tailbud stage of embryonic development[90]
Vertebrates	
Homo sapiens (Human)	**Protein in Gonocytes and oogonia:** Not expressed in first trimester fetuses, but progressively increases in developing female germ cells between weeks 12–20 and coincides with a change from a uniform cytoplasmic distribution to a punctuated perinuclear ring localization[112–114]
	Protein in Oocytes: Localizes to a single compact perinuclear body in developing embryos[113]
Mus musculus (Mouse)	**Protein in Spermatocytes:** Localizes to the cytoplasm in a perinuclear fashion resembling the chromatoid body[115]
Gallus gallus (Chicken)	**Protein:** Localizes to spherical mitochondrial cloud structures in developing oocytes[116]
Sparus aurata (Gilt head sea bream)	**mRNA:** Localizes to the perinuclear cytoplasm in early midvitellogenic and vitellogenic oocytes[62]
Oryzias latipes (Medaka)	**mRNA:** Localizes to patches with the early oocyte cytoplasm[117]
Danio rerio (Zebrafish)	**Protein:** Granular localization in PGCs and Vasa[20]
	mRNA: localizes to nuage-like structures in 1000-cell embryos[118]
Xenopus Laevis (African clawed frog)	**Protein:** Localizes to germ granules and germ plasm during early embryonic development[38,119]
Chaetognaths	
Sagitta inflata (Arrow worms)	**Protein:** Localizes to the large germ granule formed in fertilized eggs [120]
Insects	
Drosophila melanogaster (Fruit Fly)	**Protein:** Found in perinuclear nuage of nurse cells in developing egg chambers[72,73,121]
	mRNA: Localizes to a perinuclear granules in the egg[122]
Bombyx mori (Silkworm)	
Nematodes	
Caenorhabditis elegans	**Protein:** Localizes to P-granules during embryonic development[16–18]
Molluscs	
Crassostrea gigas (Oyster)	**mRNA:** Localizes distinctly to cytoplasmic granules at the vegetal pole in unfertilized oocytes[47]