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ORIGINAL ARTICLE

C-terminal residues specific to Vasa among DEAD-box helicases are required for its functions in piRNA biogenesis and embryonic patterning

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Abstract The DEAD-box RNA helicase Vasa (Vas, also known as DDX4) is required for germ cell development. In *Drosophila*, analysis of hypomorphic mutations has implicated maternally expressed Vas in germ cell formation and posterior embryonic patterning. *vas*-null females, which rarely complete oogenesis, exhibit defects in mitotic progression of germline stem cells, Piwi-interacting RNA (piRNA)-mediated transposon silencing, and translation of Gurken (Grk), an EGFR ligand. The carboxy-terminal region of Vas orthologs throughout the animal kingdom consists of several acidic residues as well as an invariant tryptophan in the penultimate or ultimate position (Trp660 in *Drosophila melanogaster*). Using CRISPR/Cas9 gene editing, we made a substitution mutant in this residue. Replacing Trp660 by Glu (W660E) abolishes the ability of Vas to support germ cell formation and embryonic patterning and greatly reduces Vas activity in piRNA biogenesis, as measured by transposon silencing, and in activating Grk translation. A conservative substitution (W660F) has much milder phenotypic consequences. In addition, females expressing only a form of Vas in which the seven C-terminal amino acids were replaced with the corresponding residues from Belle (Bel, also known as DDX3) show defects in perinuclear nuage assembly and transposon silencing. Oogenesis in females expressing only the chimeric Vas arrests early; however, in a vas^{1} background, in which early expression of endogenous Vas supports oogenesis, the chimeric protein supports posterior patterning and germ cell specification. These results indicate that the unique C-terminus of Vas is essential for its function in piRNA biogenesis and that the conserved Trp660 residue has an important functional role.

Keywords Vasa · piRNA biogenesis · Germ plasm · Embryonic patterning · Germ cell specification

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Introduction

In many organisms, the DEAD-box helicase Vasa (Vas) is essential for specification of primordial germ cells (Castrillon et al. 2000; Ikenishi and Tanaka 2000; Knaut et al. 2000; Kuznicki et al. 2000; Tanaka et al. 2000; Voronina et al. 2008). In some metazoans, Vas orthologs are also involved in the function of somatic multipotent stem cells (Alié et al. 2011; Shibata et al. 1999; Yajima and Wessel 2015). In *Drosophila*, Vas accumulates in the pole plasm, a specialized cytoplasmic region at the posterior pole of the oocyte and embryo that contains determinants for germ cell specification and posterior patterning (Lasko and Ashburner 1990). In the absence of functional posterior-localized Vas, determinants of germ cell specification and posterior pattern such as *oskar* (*osk*) and *nanos* (*nos*) messenger RNAs (mRNAs) are not stably localized or effectively translated in the pole plasm, and the resulting embryos lack posterior pattern elements and germ cells (Hay et al. 1988; Lasko and Ashburner 1990; Wang et al. 1994). In addition, earlier in oogenesis, Vas regulates translation of Grk, an EGFR ligand also involved in patterning of the oocyte (Styhler et al. 1998; Tomancak et al. 1998). As a result, embryos produced by females homozygous for vas^{PH165}, a null allele, have severe

defects in dorsal appendage formation (Tomancak et al. 1998). vas^{PH165} females produce very few eggs, because of additional roles for Vas in the progression of germline mitotic divisions and in differentiation of germline stem cells (Liu et al. 2009; Pek and Kai 2011a). Vas interacts with eIF5B, a translation initiation factor required for ribosome subunit joining (Carrera et al. 2000), and this interaction is required for Vas-mediated translational activation of *grk* and *mei-P26* mRNAs (Johnstone and Lasko 2004; Liu et al. 2009).

Germ cells in many animals contain a unique structure termed the nuage, which is a compartmentalized site for Piwi-interacting RNA (piRNA) processing. piRNAs are small RNAs that inhibit expression of transposon-encoded genes, thereby safeguarding genome integrity against deleterious effects of excessive mobility of transposable elements (Pek et al. 2012). piRNAs can also target mRNAs for translational repression and degradation (Barckmann et al. 2015; Rouget et al. 2010). In *Drosophila*, *vas* is essential both for nuage assembly and for piRNA biogenesis (Liang et al. 1994; Xiol et al. 2014; Zhang et al. 2012). *vas* is involved in the transfer of piRNA precursors across the nuclear envelope and is a component of an amplifier complex participating in the ping-pong cycle that produces piRNAs (Xiol et al. 2014; Zhang et al. 2012).

RNA helicases of the DEAD-box family, including Vas, have crucial roles in a wide range of cellular processes including ribosome biogenesis, translation initiation, pre-mRNA splicing, and mRNA decay (Jankowsky 2011). DEAD-box proteins bind and hydrolyze ATP and catalyze unwinding of secondary structures in RNA, promoting strand separation (Cordin et al. 2006; Jarmoskaite and Russell 2011; Luking et al. 1998). In the helicase core, these proteins are composed of two tandemly repeated RecA-like domains, which share a set of highly conserved motifs. Belle (Bel), which belongs to a subfamily including yeast Ded1p, *Xenopus* An3, mouse PL10, and human DDX3, is the closest paralog of Vas (Johnstone et al. 2005). These two proteins have analogous roles in germ line and soma. Vas interacts with Aubergine (Aub) and Spindle-E (SpnE) to promote mitotic chromosome segregation in the female germ line (Pek and Kai 2011a), while Bel executes a similar role in the somatic cells through interaction with components of the endogenous siRNA (endo-siRNA) pathway, such as Ago2 (Pek and Kai 2011b).

Aside from their conserved helicase domain, most DEAD-box proteins have flanking N- and C-terminal sequences, which vary significantly in their length and content. Most structural analyses have excluded these, often disordered, terminal sequences (Carmel and Matthews 2004; Caruthers et al. 2000; Kim et al. 1998; Korolev et al. 1997; Sengoku et al. 2006; Subramanya et al. 1996; Zhao et al. 2004); therefore, little is known about their contribution to the functions and structures of DEAD-box proteins. The C-terminal region of Vas contains several motifs conserved among Vas/DDX4 orthologs. Amino acids 636–646 in *Drosophila melanogaster* Vas are shared among some closely related *Drosophila* species and are required for precisely concentrating Vas in the pole plasm (Dehghani and Lasko 2015). More notably, the most C-terminal seven amino acids from even distant Vas orthologs are highly acidic. In *Drosophila*, deletion of the last seven amino acids (655–661), which include five acidic residues, results in severe defects in transposable element (TE) silencing, Grk translation, posterior patterning of embryos, and germ cell formation (Dehghani and Lasko 2015).

There is a highly conserved Trp (Trp660 in *D. melanogaster*), which in Vas orthologs is usually the penultimate amino acid but occasionally is found at the absolute C-terminus. This conserved Trp is specific to Vas/DDX4 and is absent from the sequences of most other DEAD-box proteins in *Drosophila* and beyond. Thus, it seems unlikely that this residue contributes to the general helicase activity of Vas orthologs; rather, it might be involved in specific interactions between Vas and the proteins or RNAs that interact with it. To gain insight into Vas functions that are dependent on Trp660, we used the CRISPR-Cas9 system to mutate this residue in endogenous *vas* and expressed enhanced green fluorescent protein (eGFP)-tagged Trp660-mutated Vas from transgenes, to study the consequences of these mutations in vivo. In addition, we investigated which functions of Vas could be supported by a chimeric Vas-Bel protein containing the seven C-terminal residues of Bel. Finally, we explored whether any known protein-protein interactions involving Vas were dependent on the C-terminal domain or on Trp660 specifically.

Materials and methods

Endogenous *vas* alleles

vas^{PH165} is a null allele in which the entire coding sequence of *vas* has been deleted (Styhler et al. 1998). vas^{1} is a hypomorphic allele, generated by ethyl methanesulfonate (EMS) mutagenesis (Schüpbach and Wieschaus 1986). This allele does not carry any mutation in the coding sequence of *vas*; however, expression of Vas in the ovaries is undetectable after the germarium stage (Lasko and Ashburner 1990; Liang et al. 1994).

vas^{W660E} was generated using CRISPR-Cas9-mediated homologous recombination according to the method described by Port et al. (2014). A stable transgenic line expressing the guide RNA (gRNA), targeting a sequence including the Trp660 codon (AGCAATGGGATTGAAATGTA), was generated. The transgenic males were then crossed to *nos-cas9* females, and their embryos were used for injection of a donor DNA encoding the W660E mutation. The donor DNA was composed of a synthetic ssDNA oligonucleotide (Integrated DNA Technologies, Inc.) corresponding to 80 base pairs on either side of Trp660 codon and substituting that with GAA

(Glu). The PAM sequence was mutated from TGG to TGA in the donor DNA to prevent its degradation by Cas9 in the cells.

Transgenic *vas* alleles

egfp-vas^{+} and *egfp-vas*$^{\Delta 15–75}$ have been described previously (Dehghani and Lasko 2015). *egfp-vas*^{W660E}, *egfp-vas*^{W660F}, and *egfp-vas*$^{c.bel}$ were generated by a PCR-based mutagenesis approach from a full-length *vas* cDNA.

In vivo functional assays

Functional assays including egg-laying and hatching tests as well as the statistical analysis of the data were conducted as described in Dehghani and Lasko (2015). Immunostaining, in situ hybridization, and reverse transcription quantitative PCR (RT-qPCR) tests were also described in Dehghani and Lasko (2015).

Yeast two-hybrid assays

The Matchmaker Gold Yeast Two-Hybrid System (Clontech Inc.) was used to test direct interactions between *vas* and other candidate genes. The coding sequences of *vas*$^{+}$, *vas*$^{\Delta 655–661}$, and *vas*^{W660E} were cloned between *NdeI* and *PstI* restriction sites in the pGBKT7 vector. The entire coding sequences of *gus*, *aret*, *Dynein light intermediate chain* (*Dlic*), *fsn*, *piwi*, *barr*, *cap-D2*, *spn-E*, *aub*, *ago3*, *csul*, and *vls* were amplified and inserted between *NdeI* and *BamHI* restriction sites in the pGADT7 AD vector. For *osk* short isoform, *eIF5B* and *qin*, fragments encoding amino acids 5–468, 491–1144, and 275–1857, respectively, were cloned. Media preparation, yeast handling, and interaction assays were performed according to the manufacturer's instructions.

Results

Vas orthologs from different species contain an invariant tryptophan in their C-terminal region

Comparison among amino acid sequences from different Vas orthologs, as far apart as sea urchins and humans, reveals that a Trp residue (W660 in *Drosophila*) is highly conserved within the last two amino acids of these sequences (Fig. 1a). This Trp is always flanked by several acidic residues; for example, in *D. melanogaster*, there are four Glu and one Asp residues preceding and following the conserved Trp, respectively. Such a conserved Trp, however, is rarely found among DEAD-box proteins other than Vas orthologs (Fig. 1b). In *D. melanogaster*, for example, Bel, which has the highest sequence similarity to Vas, is the only other DEAD-box protein that contains Trp in its C-terminus. Rm62 contains a

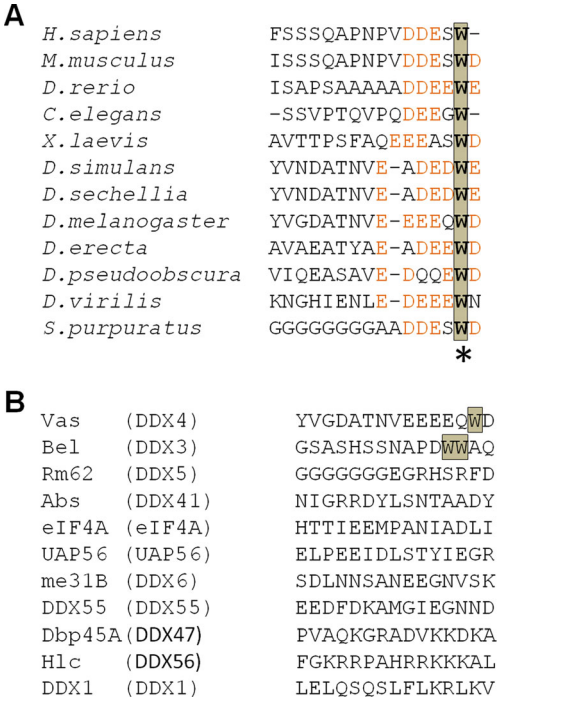


Fig. 1 Vas orthologs share a conserved tryptophan (W) in their C-terminal end, which is not common between the other DEAD-box proteins. **a** Sequence alignment of the last 15 amino acids in Vas orthologs from different species indicates an invariant Trp in the penultimate or ultimate position. Acidic residues, shown in *red*, are also abundant in the C-terminus of Vas orthologs. **b** The last 15 amino acids in the C-terminal end of different DEAD-box proteins in *Drosophila melanogaster* are compared. This list includes at least one representative from each main group of DEAD-box proteins. The names of the human orthologs are shown in parentheses. Bel is the only other DEAD-box protein containing two consecutive Trp residues in its C-terminus, which also exhibits the highest sequence homology to Vas

phenylalanine (Phe; F), another aromatic amino acid, in a similar position. However, this Phe residue is not conserved across species; for example, DDX5 (P68), the human or mouse orthologs of Rm62, do not carry a Phe within the last five amino acids.

Substitution of Trp660 with Glu abolishes Vas function in germ cell formation and embryonic patterning

Using CRISPR/Cas9 technology, we generated an endogenous allele of *vas*, *vas*^{W660E}, in which Trp660 is replaced by Glu. We also generated a transgenic allele of *vas*^{W660E}, which encodes a protein N-terminally tagged with eGFP, to examine how the mutant protein localizes in ovaries. Similarly, we constructed an *egfp-vas*^{W660F} allele which encodes Phe as a conservative substitution for Trp660. All the mutations were confirmed through amplifying and sequencing the genomic DNA. Expression of these proteins was confirmed by western blots (Fig. 2b).

We investigated the significance of Trp660 for Vas function in germ cell development by counting the proportion of

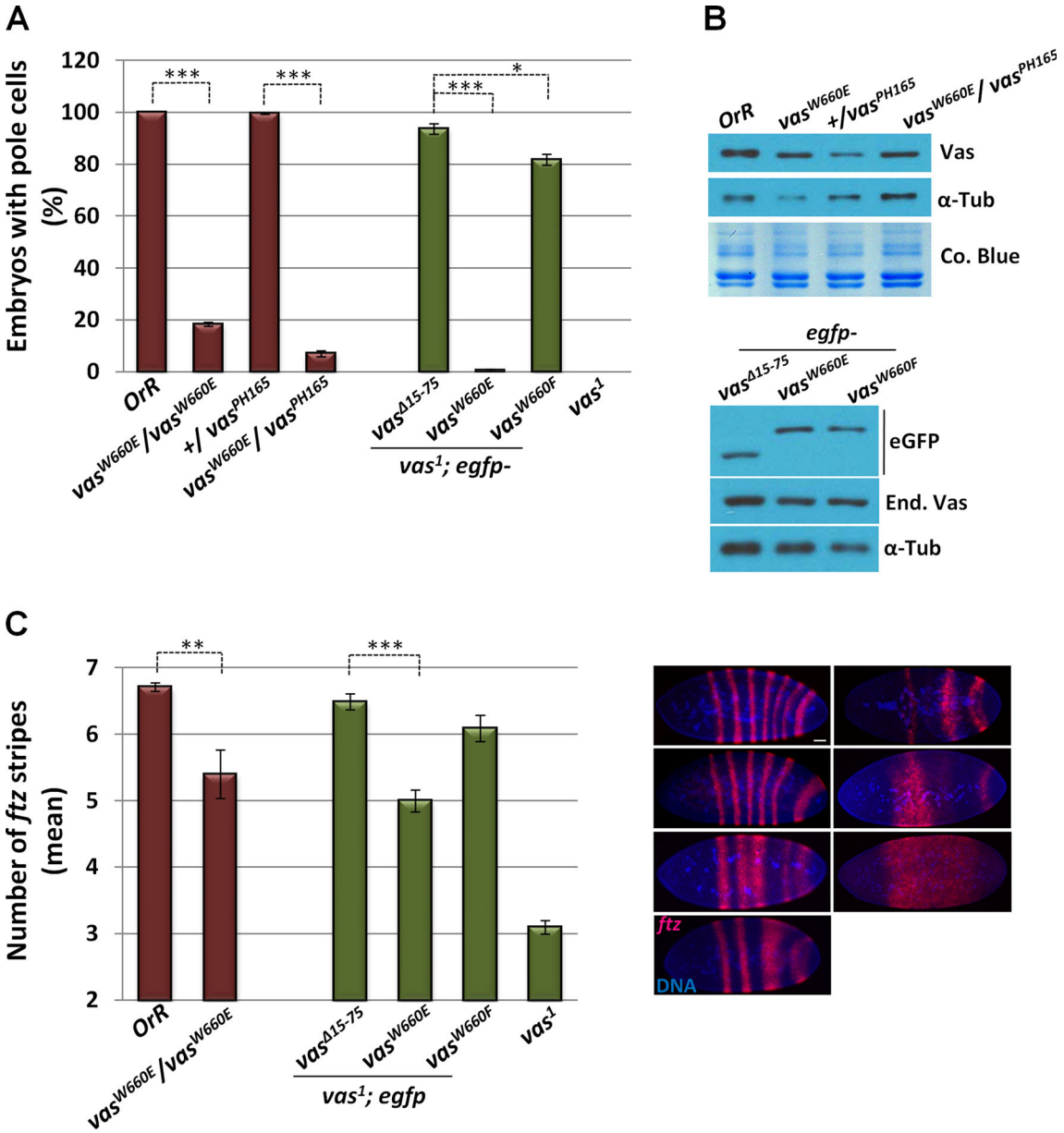


Fig. 2 Substitution of tryptophan (W) with glutamic acid (E), which is an abundant residue in the C-terminus of Vas, is associated with severely reduced germ cell formation and impaired posterior patterning. **a** The percentage of stage 5–6 embryos with pole cells is compared between different genotypes carrying endogenous wild-type, vas^{W660E}, and vas^{PH165} alleles (*red bars*) or between transgenic alleles of *egfp-*$vas^{\Delta 15-75}$ (positive control), *egfp-*vas^{W660E}, and *egfp-*vas^{W660F} expressed in vas^{1} background (*green bars*). One, two, or three asterisks represent $P < 0.05$, <0.005, or <0.0005, respectively. **b** The top western blot indicates expression of Vas in ovaries carrying different endogenous alleles, corresponding to the red bars in **a**. The bottom western blot compares expression levels of the egfp− alleles. α-Tubulin (α-Tub), Coomassie Blue staining, and endogenous (end.) Vas are used as the loading controls. **c** W660E, but not W660F, significantly reduces the average number of *ftz* stripes (illustrated on the right) in the embryos produced by the mutant females, reflecting compromised posterior patterning. Scale bar indicates 50 μm

stage 5–6 embryos from vas^{W660E} homozygous females with pole cells. All embryos from females with two copies of wild-type *vas* (*OrR*) produce pole cells, which in number vary around an average of 34.5 (Fig. 2a). In contrast, we found that only 18 % of embryos produced by vas^{W660E} homozygous females (subsequently referred to by the maternal genotype) form pole cells with an average of only 1.7 pole cells per embryo ($n = 83$). To confirm that this is specifically associated with the vas^{W660E} mutation and to rule out second-site genetic effects, we examined vas^{W660E}/vas^{PH165} embryos and observed a similarly significant decrease in the number of embryos producing pole cells compared to $+/vas^{PH165}$ (7 % with pole cells, average of 0.24 pole cells per embryo, $P = 5.7E{-}05$).

We next investigated if Phe, a different hydrophobic amino acid with an aromatic ring, could replace Trp in position 660 of Vas. To address this question, we used an *egfp-vas*^{W660F} transgene to rescue germ cell formation in a *vas*^{1} background. We compared this construct with *egfp-vas*$^{\Delta 15-75}$, as a positive control, since both lines expressed eGFP-Vas at similar levels (Fig. 2b) and it has been shown previously that *egfp-vas*$^{\Delta 15-75}$ mimics full-length *vas* in these in vivo functional assays (Dehghani and Lasko 2015). Our analyses indicate that the average number of *vas*^{1}; *egfp-vas*^{W660F} embryos with pole cells is 82 %, which is significantly different from only 0.5 % of *vas*^{1}; *egfp-vas*^{W660E} embryos that formed pole cells ($P = 4.0E{-}04$; Fig. 2a). In contrast, *egfp-vas*^{W660F} was much closer to, if still significantly lower than, *egfp-vas*$^{\Delta 15-75}$ control (94 %) for its ability to induce germ cells in *vas*^{1} embryos ($p = 0.01$). The average number of pole cells per embryo was 8.2 ($n = 127$), 0.005 ($n = 393$), and 9.3 ($n = 90$) for *egfp-vas*^{W660F}, *egfp-vas*^{W660E}, and *egfp-vas*$^{\Delta 15-75}$, respectively.

To investigate the effects of Vas660 mutations on embryonic patterning, we examined the expression pattern of *fushi tarazu* (*ftz*), a marker for segmentation, in *vas*^{W660E} embryos (Fig. 2c) (Hafen et al. 1984) . The average number of *ftz* stripes in stage 5–6 *vas*^{W660E} embryos was 5.4, which is significantly lower than the average of 6.7 stripes in *wt* ($P = 0.001$). Our analysis using *egfp* transgenic alleles also indicates that unlike the W660E mutation, which decreased the number of *ftz* stripes from 6.5 to 5 ($P = 5.5E{-}12$), W660F did not have a significant effect on posterior patterning. We also tested the viability of *vas*^{W660E} homozygous and *vas*^{W660E}/*vas*^{PH165} heterozygous embryos. Our analyses show that no more than 1 % of the embryos from either of these two groups are viable, whereas +/+ or +/*vas*^{PH165} embryos have a hatching rate of 90 and 60 %, respectively ($n > 10{,}000$). We also found that the majority of *vas*^{W660E} or *vas*^{W660E}/*vas*^{PH165} eggs do not undergo any development, suggesting that *vas*^{W660E} females have additional defects in oogenesis and many of their eggs are not fertilized.

W660E substitution perturbs stable localization of Vas in the nuage and pole plasm

In early stages of oogenesis, Vas normally localizes to the perinuclear region of the nurse cells, also known as the nuage. At stage 10, Vas starts to be transported to the oocyte and accumulates at the pole plasm. To examine the effect of the W660E mutation on Vas localization, we used eGFP-tagged Vas fusions, as antibodies do not efficiently penetrate late-stage oocytes for immunostaining. Confocal images show that in the *vas*^{1} background, eGFP-Vas^{W660E} not only partially localizes to the perinuclear nuage but also forms abundant cytoplasmic foci, which may indicate instability of the nuage structure (Fig. 3a). Accumulation of eGFP-Vas^{W660E} in the pole plasm is initially normal in the *vas*^{1} background, but it is less stably maintained there, as the GFP signal was diminished in stage 14 oocytes compared to stage 13 (Fig. 3a). Consistent with this, immunostaining of *vas*^{W660E} embryos shows that the majority (82 %) of stage 1–2 embryos contained no detectable Vas in their posterior region, while the others had considerably decreased levels compared to *wt* ($n \geq 250$; Fig. 3b). In contrast, we found that, in a wild-type genetic background, eGFP-Vas^{W660E} localizes to the nuage of wild-type egg chambers comparably to eGFP-Vas^{+} (Fig. 3c) and persisted there stably throughout oogenesis.

We also investigated whether W660E affects protein stability by quantifying levels of *egfp-vas*$^{+}$ and *egfp-vas*^{W660E} transcripts, using RT-qPCR, and comparing that with the protein levels on the western blot (Fig. 3d, e). These experiments indicate that the level of *egfp-vas*^{W660E} transcript is about two times higher than *egfp-vas*$^{+}$ in the corresponding transgenic lines which were tested. As western blotting also shows a relatively high level of eGFP-Vas^{W660E} protein in the ovaries we conclude that the mutation does not have major effects on protein stability.

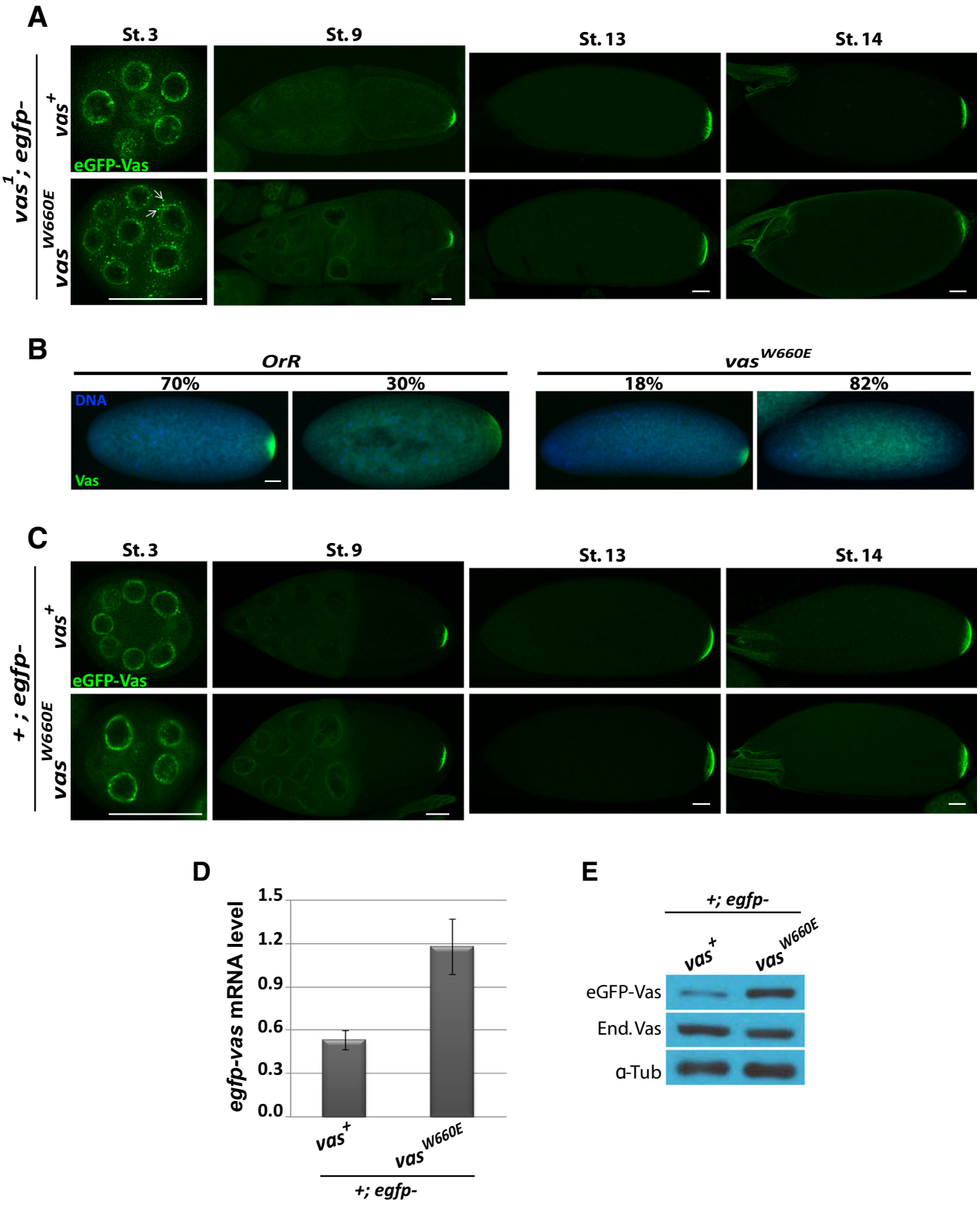
Mutation of Trp660 to Glu reduces Vas function in piRNA biogenesis as measured by HeT-A silencing

Vas is required for nuage assembly, and its critical role in piRNA biogenesis has been confirmed both in *Drosophila* and in mammals. Females homozygous for a *vas*-null allele, *vas*^{PH165}, overexpress transcripts from several TEs including HeT-A (Zhang et al. 2012). Elevated levels of HeT-A transcripts also have been reported for most *vas* alleles with mutations in their conserved helicase and C-terminal domains (Dehghani and Lasko 2015). To investigate whether substitution of Trp660 with Glu affects Vas function in the piRNA pathway, and therefore transposon silencing, we compared levels of HeT-A transcript in *vas*^{W660E} ovaries with wild type and found a more than tenfold increase in the mutant ($P = 0.006$; Fig. 4). HeT-A levels in *vas*^{W660E} fall within the same range as those observed for females carrying mutations in the conserved helicase motifs of Vas or deletion of the seven C-terminal amino acids (Dehghani and Lasko 2015).

We confirmed this result by measuring the levels of HeT-A in ovaries of *vas*^{PH165} females expressing either eGFP-Vas^{+} or eGFP-Vas^{W660E}. In contrast to the *wt* construct which is able to fully suppress HeT-A overexpression, *vas*^{PH165} ovaries expressing *egfp-vas*^{W660E} exhibit increased levels of HeT-A at about ten times higher than *wt* ($P = 0.005$). The W660F mutation, on the other hand, did not significantly affect the ability of Vas to suppress HeT-A overexpression in the *vas*^{PH165} background.

vas^{W660E} females produce a similar number of eggs to wild type, but these eggs have dorsal appendage defects

Vas has a critical role in oogenesis by regulating multiple processes including germline mitotic divisions, stem cell



differentiation, and dorsal ventral patterning of the oocytes (Liu et al. 2009; Pek and Kai 2011a; Tomancak et al. 1998). In vas^{PH165}, females all of these pathways are perturbed; these mutants produce very few eggs and their eggs do not form

◀ **Fig. 3** eGFP-Vas^{W660E} accumulates in distinct cytoplasmic foci in the nurse cells of vas^{1} ovaries, and its localization to the pole plasm significantly reduces by the end of oogenesis. **a** eGFP-Vas^{W660E} is compared to eGFP-Vas^{+} in a vas^{1} background for its localization to the nuage (stage 3 egg chambers) and pole plasm of stage 9, 13, and 14 oocytes. Arrows indicate the cytoplasmic foci containing eGFP-Vas^{W660E}. Scale bars indicate 50 μm. **b** Vas immunostaining indicates posterior localization of Vas in all stage 1–2 wt (OrR) embryos, which in 70 % of the cases is strongly concentrated (n = 250). In contrast, only 18 % of vas^{W660E} embryos maintain detectable levels of Vas at the posterior region (n = 545). DAPI staining indicates embryonic stages. **c** In the wt background, localization of eGFP-Vas^{W660E} mimics localization pattern of eGFP-Vas^{+}. **d**, **e** Comparisons between transcript and protein levels of *egfp-vas*$^{+}$ and *egfp-vas*^{W660E} (RT-qPCR and western blot) also indicate that Vas^{W660E} protein level is relatively stable in a *vas*^{1}/+ background. The endogenous Vas and α-tubulin were used as the loading controls for western blot; Act5C and rp49 were used as the reference genes in RT-qPCR

normal dorsal appendages. In addition, our previous analyses indicate that several mutations in the conserved motifs of Vas that are critical for its enzymatic activity do not abolish female fecundity, even though these residues are indispensable for other functions of Vas such as piRNA biogenesis or germ cell formation (Dehghani and Lasko 2015).

To examine if the W660E mutation, in addition to its effects on germ cell formation, embryonic patterning, and transposon silencing, affects female fecundity, we counted the number of eggs laid by individual females within the first 3 days from eclosion (Fig. 5a). *vas*^{W660E} homozygous or hemizygous (over *vas*^{PH165}) produces eggs in numbers comparable to wild-type females. Expression of either *egfp-vas*^{W660E} or *egfp-vas*^{W660F} in the *vas*^{PH165} background also restored fecundity, although rescue by *egfp-vas*^{W660E} was not as robust as *egfp-vas*^{W660F} and the control. We conclude that the W660E mutation has little effect on female fecundity.

We, however, observed that 23 % of eggs from *vas*^{W660E} homozygous females had only a single fused dorsal appendage, a phenotype that we did not observe in wild-type eggs ($P = 1.7E{-}08$; Fig. 5b). This phenotype was aggravated in hemizygous *vas*^{W660E}/*vas*^{PH165} females in that 74 % of the eggs had only one dorsal appendage versus 0.2 % in +/*vas*^{PH165} ($P = 1.3E{-}20$). This indicates that Vas^{W660E} has a significantly decreased activity in establishing the dorsal-ventral axis. Again, our analysis using *egfp* transgenic alleles confirmed these results and as well showed that the conservative substitution, W660F, does not reduce Vas activity in dorsal-ventral patterning. To determine whether these effects were correlated with decreased Grk expression, we immunostained ovaries with an antiserum recognizing Grk. This experiment showed that the defects in dorsal appendages of *vas*^{W660E} or *vas*^{PH165}; *egfp-vas*^{W660E} embryos are indeed correlated with decreased amounts of Grk in stage 7–8 oocytes (Fig. 5c). The dorsal appendage defects observed in *vas*^{W660E} embryos could also account for the large number of these embryos which remain unfertilized.

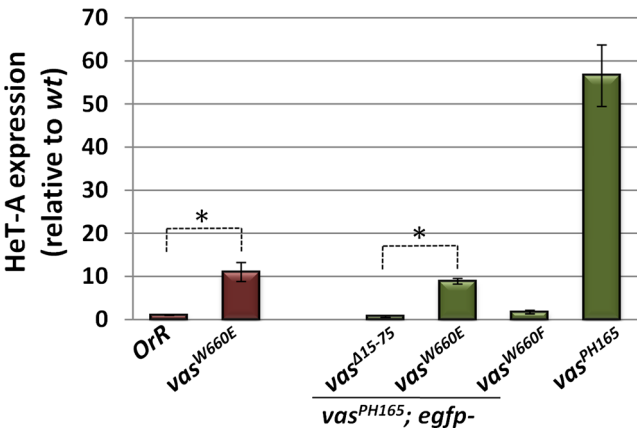
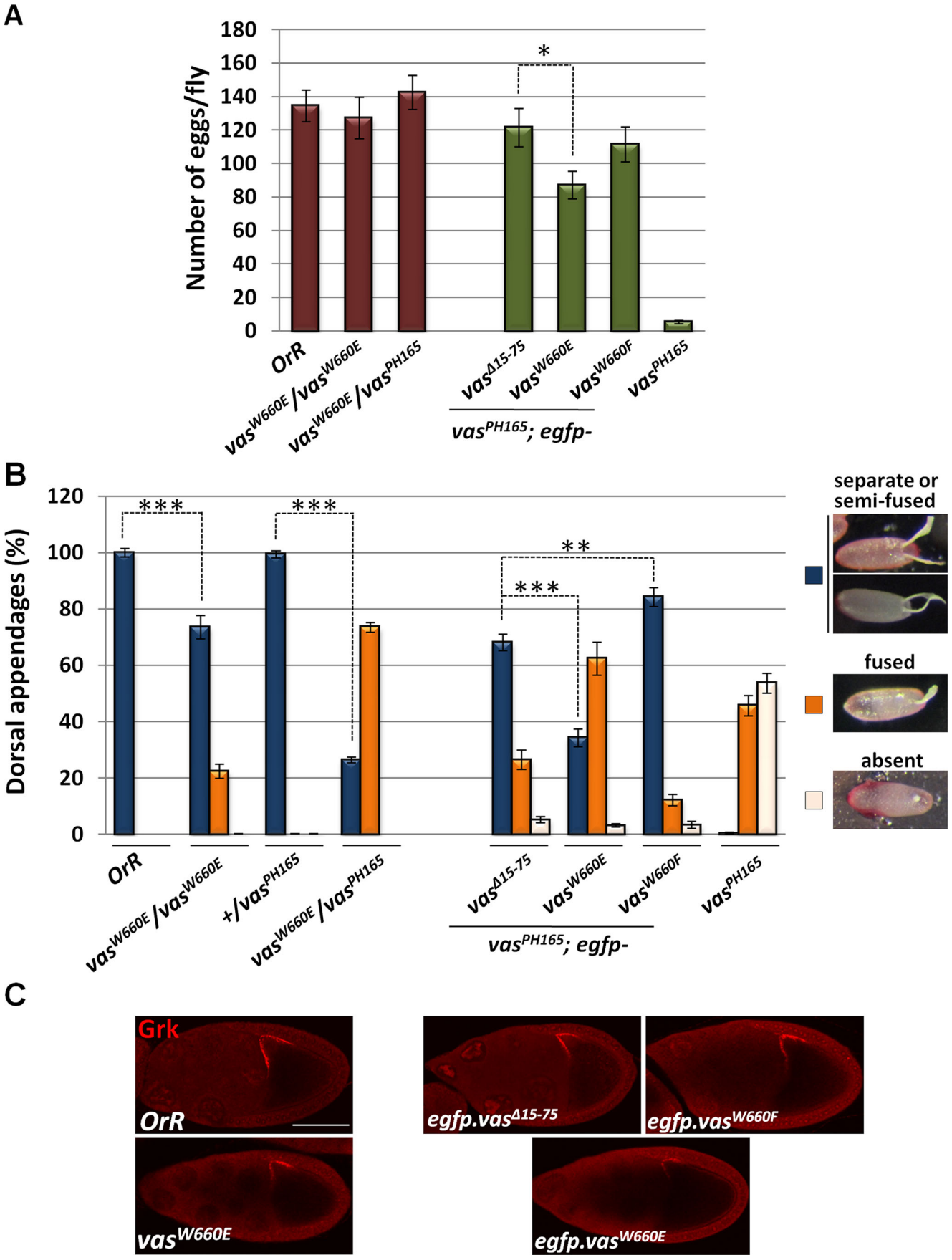


Fig. 4 Substitution of Trp660 with Glu impairs Vas function in transposable element silencing. The retrotransposon, HeT-A, is dramatically overexpressed in *vas*^{PH165} ovaries compared to wild-type (OrR). A consistent overexpression, albeit to a lesser extent than in *vas*^{PH165}, is also observed in *vas*^{W660E} ovaries. *egfp-vas*$^{Δ15–75}$ and *egfp-vas*^{W660F} are able to fully suppress HeT-A overexpression in *vas*^{PH165} ovaries. On the contrary, ovaries expressing *egfp-vas*^{W660E} still exhibit a significant overexpression of HeT-A. HeT-A levels in different genotypes are normalized to the wild-type level. *pre-rp49* and *18S-rRNA* are used as the reference genes. Each bar represents the average from at least three biological replicates. Error bars indicate SEM

Direct binding of Vas to its known interacting partners is independent of Trp660

Previous studies, using different in vitro and in vivo assays, have identified a number of proteins that are associated with Vas in ribonucleoprotein complexes (Table S1). To investigate possible correlations between these protein interactions and the functional defects observed for *vas*^{W660E} and *vas*$^{Δ655–661}$ (Dehghani and Lasko 2015), we tried to co-immunoprecipitate Vas-protein complexes from ovaries or embryos. With this method, however, known interacting partners of Vas, such as Aubergine (Aub), Oskar (Osk), and Valois (Vls), were not detected in the IP eluates from ovaries or embryos expressing either eGFP-Vas or endogenous Vas. We reasoned that this might be due to weak or transient interactions or to low abundance or instability of these proteins in the whole ovary/embryo lysates. As an alternative, we used a yeast two-hybrid assay to compare Vas^{W660E} and Vas$^{Δ655–661}$ with wild-type Vas for their interactions with 16 other proteins that had been identified as Vas interactors by various means in other studies. Our experiments confirmed interactions between Vas and Osk, Gus, and eIF5B in this assay, which have been previously reported (Breitwieser et al. 1996; Carrera et al. 2000; Styhler et al. 2002). These interactions remained unchanged for Vas^{W660E} and Vas$^{Δ655–661}$ (Fig. S1). For the other 13, which were mostly tested as full-length proteins, we did not find an interaction with Vas as measured by this assay.


A
Number of eggs/fly
180
160
140
120
100
80
60
40
20
0
*
OrR
vasW660E/vasW660E
vasW660E/vasPH165
vasΔ15-75
vasW660E
vasW660F
vasPH165
vasPH165; egfp-
B
Dorsal appendages (%)
120
100
80
60
40
20
0
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separate or semi-fused
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OrR
vasW660E/vasW660E
+/vasPH165
vasW660E/vasPH165
vasΔ15-75
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egfp.vasW660F
egfp.vasW660E

◀ **Fig. 5** *vas*^{W660E} females produce a wild-type number of embryos. This mutation, however, has a significant effect on Grk expression and dorsal appendage formation. **a** Females carrying two copies of wild-type *vas* (*OrR*), two copies of *vas*^{W660E}, or only one copy of *vas*^{W660E} (over *vas*^{PH165}) produce a comparable number of embryos (red bars). Similarly, expression of either *egfp-vas*^{W660E} or *egfp-vas*^{W660F} could largely restore female fecundity to *vas*^{PH165}, although *egfp-vas*^{W660E} still had significantly lower fecundity compared to *egfp-vas*$^{\Delta 15-75}$ control. **b** The percentage of embryos with two separate or semi-fused dorsal appendages is significantly reduced in *vas*^{W660E} compared to wild-type; this effect is even more pronounced when females are hemizygous for *vas*^{W660E} (over *vas*^{PH165}). Expression of *egfp-vas*$^{\Delta 15-75}$ or *egfp-vas*^{W660F} increases the number of embryos with two dorsal appendages from 0 % in *vas*^{PH165} to 68 or 84 %, respectively. On the contrary, such embryos produced from *vas*^{PH165}; *egfp-vas*^{W660E} females comprise only 34 % of the total. n (the number of females tested) > 20; error bars represent SEM. **c** Representative images of Grk immunostaining in stage 7–8 egg chambers of homozygous *vas*^{W660E} compared to *OrR* (left) and *vas*^{PH165} egg chambers expressing *egfp-vas*$^{\Delta 15-75}$, *egfp-vas*^{W660F}, or *egfp-vas*^{W660E} (right). Scale bar indicates 50 μm

The Bel C-terminus cannot substitute for the corresponding region of Vas

Among other DEAD-box proteins, Bel has the highest sequence similarity to Vas, and its C-terminal end contains two conserved Trp residues in its third and fourth from last positions. Given the significance of the C-terminal domain and its conserved Trp660 for several functions of Vas, we explored whether the last seven amino acids of Bel could replace those from Vas by testing an eGFP-Vas$^{c.bel}$ chimeric protein (Fig. 6a, b).

Our experiments using this construct show that, similar to eGFP-Vas^{W660E}, in the *vas*^{1} background, eGFP-Vas$^{c.bel}$ exhibits a granular localization in the nuage as opposed to a smooth perinuclear ring in the *wt* background (Fig. 6c). However, unlike eGFP-Vas^{W660E}, localization of eGFP-Vas$^{c.bel}$ in the pole plasm of *vas*^{1} oocytes is maintained until stage 14. Surprisingly, eGFP-Vas$^{c.bel}$ failed to restore any fecundity in *vas*^{PH165} females, which is usually at least partially rescued by different Vas mutants (Fig. 6d; Dehghani and Lasko 2015). As well, the few embryos produced by *vas*^{PH165}; *egfp-vas*$^{c.bel}$ females, like vas null, exhibited severe defects in dorsal-ventral patterning (data not shown). We also observed that *vas*^{PH165} ovaries expressing eGFP-Vas$^{c.bel}$ transcribe high levels of HeT-A similar to *vas* null (Fig. 6e), which is consistent with the defects in localization of this mutant protein to the nuage, and indicates defects in piRNA biogenesis.

Despite its failure to support oogenesis in *vas*^{PH165}, when expressed in *vas*^{1} background, eGFP-Vas$^{c.bel}$ could increase the average number of *ftz* stripes from 3.4 to 5.5 ($P = 1.8E{-}10$; Fig. 6f). These numbers still remained significantly lower than the average number of *ftz* stripes supported by *egfp-vas*$^{\Delta 15-75}$ control; nevertheless, about 10 % of these embryos hatched ($n = 5000$). Interestingly, 15 % of *vas*^{1}; *egfp-vas*$^{c.bel}$ embryos also produced germ cells ranging in number between 2 and 15 ($n = 480$ embryos). These results indicate that replacement of the seven C-terminal residues of Vas with those of Bel has its most profound effects during oogenesis and specifically on piRNA biogenesis, while Vas functions in events that occur later in development, namely posterior patterning and germ cell specification, are less severely affected.

Discussion

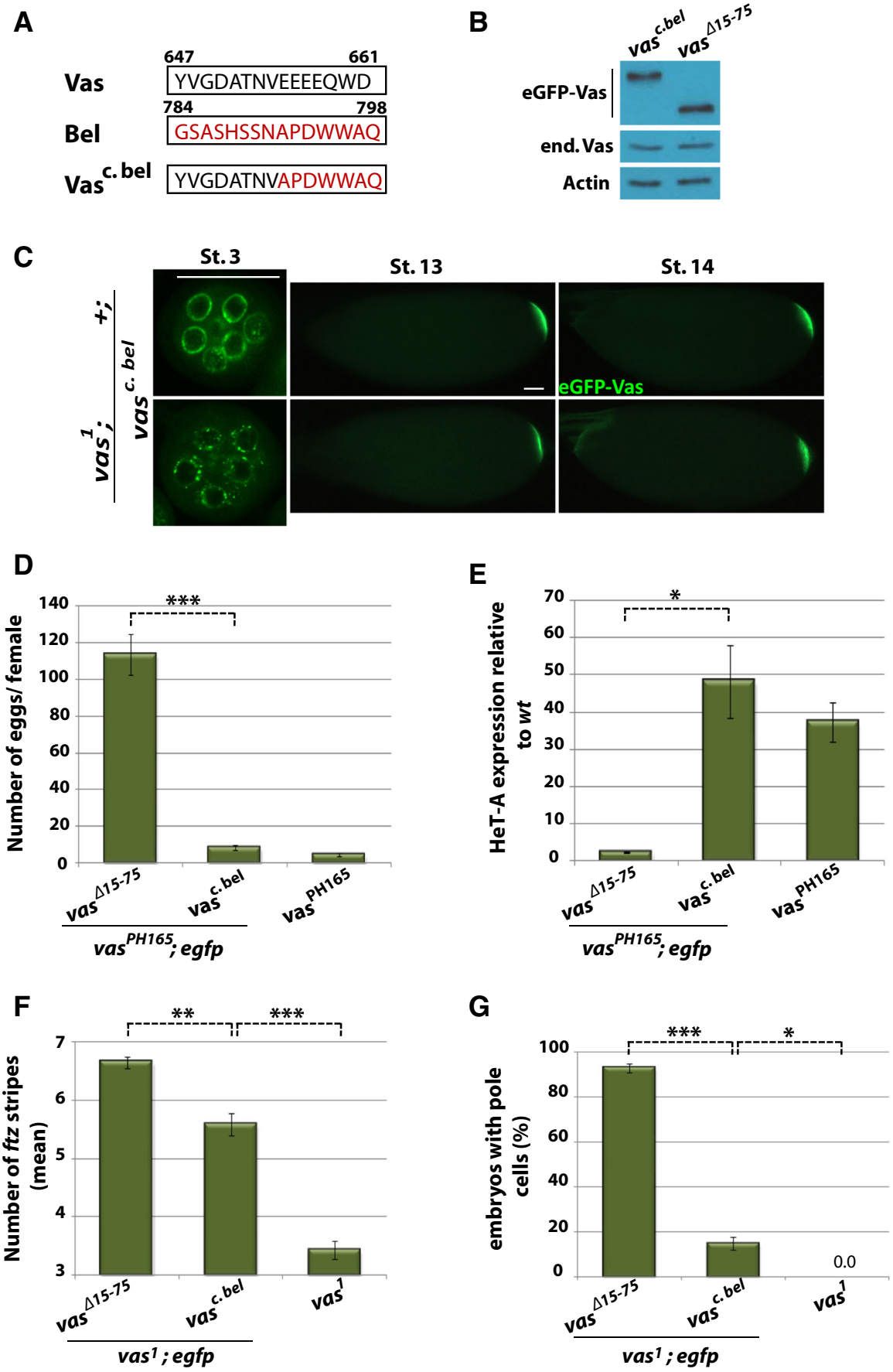
Our previous analysis revealed that the most C-terminal seven amino acids (655–661) of Vas are critical for many of its developmental functions (Dehghani and Lasko 2015). In the current study, we found that a single missense mutation, W660E, behaves similarly to the deletion of motif 655–661, in that it severely compromises most functions of Vas including germ cell specification, oocyte axis determination, and transposon silencing. This mutation alone, however, did not decrease the number of eggs produced by females, implying that the acidic residues that are adjacent to the conserved Trp are essential for oogenesis. In addition, we found that the C-terminal domain cannot be replaced with terminal seven amino acids from Bel, which contains Trp residues but is otherwise different from Vas.

The *vas*^{W660E} phenotype is intermediate between *vas*^{1} and *vas*-null

In this study, for the first time, we edited the endogenous allele of *vas*, and this allowed us to examine the effect of a point mutation by direct comparisons with wild type. Our analyses indicate that *vas*^{W660E} resembles *vas*^{1} in that females produce a normal number of embryos, but these embryos do not form germ cells and are not viable because of posterior patterning defects. However, *vas*^{W660E} ovaries resemble *vas*-null ovaries in that they produce high levels of HeT-A transcripts, indicating that piRNA biogenesis is compromised. Also, like *vas*-null mutations, *vas*^{W660E} oocytes display defects in their dorsal appendages, suggesting that this mutant form of Vas does not optimally activate Grk translation. In addition, *vas*^{W660E} females produce more eggs than do females expressing only a catalytically dead form of Vas (Dehghani and Lasko 2015), which suggests that Vas^{W660E} still retains some enzymatic activity.

A *vas* transgene encoding Phe instead of Trp660 supports Vas functions

Our analysis of *egfp-vas*^{W660F} indicates that Vas functions in oogenesis and germ cell formation generally could tolerate this conservative substitution. This was surprising since Trp660 has remained invariant through evolution. It remains possible that W660F results in small changes in Vas functions that reduce fitness in natural environments. An endogenous


A
647
661
Vas
YVGDATNVEEEEQWD
784
798
Bel
GSASHSSNAPDWWAQ
Vas c. bel
YVGDATNVAPDWWAQ
B
vas c.bel
vas Δ15-75
eGFP-Vas
end. Vas
Actin
C
St. 3
St. 13
St. 14
+;
vas c. bel
vas 1;
eGFP-Vas
D
Number of eggs/ female
140
120
100
80
60
40
20
0
***
vas Δ15-75
vas c.bel
vas PH165
vas PH165; egfp
E
HeT-A expression relative to wt
70
60
50
40
30
20
10
0
*
vas Δ15-75
vas c.bel
vas PH165
vas PH165; egfp
F
Number of ftz stripes (mean)
7
6
5
4
3
**
***
vas Δ15-75
vas c.bel
vas 1
vas 1 ; egfp
G
embryos with pole cells (%)
100
80
60
40
20
0
***
*
0.0
vas Δ15-75
vas c.bel
vas 1
vas 1 ; egfp

◀ **Fig. 6** Replacing the last seven amino acids of Vas with the corresponding residues from Bel results in complete oogenesis failure; however, expression of this protein in the embryos from *vas*^{1} females significantly rescues abdominal patterning. **a** The last 15 amino acids of Vas$^{c.bel}$ composed of fragments from Vas and Bel. **b** Expression level of eGFP-Vas$^{c.bel}$ is compared to eGFP-Vas$^{\Delta 15–75}$, as a positive control used in the subsequent experiments. End. Vas and Actin serve as the loading controls. **c** In contrast to its normal localization to the nuage in a wild-type background, eGFP-Vas$^{c.bel}$ expressed in *vas*^{1} ovaries accumulates in perinuclear and cytoplasmic foci. Localization of this protein to pole plasm, however, is maintained in fully developed oocytes (stage 14) in both wild-type and *vas*^{1} backgrounds. Scale bar indicates 50 μm. **d** eGFP-Vas$^{c.bel}$ fails to support oogenesis progression. **e** Unlike *vas*$^{\Delta 15–75}$ control, which fully represses HeT-A overexpression in *vas*^{PH165} ovaries, expression of *egfp.vas*$^{c\ bel}$ does not restore transposon silencing. **f** Surprisingly, abdominal patterning is significantly restored upon expression of *egfp.vas*$^{c.bel}$ in *vas*^{1} embryos. About 15 % of these embryos also produced germ cells (**g**). One, two, or three asterisks indicate $P < 0.05$, <0.005, or <0.0005, respectively

allele of *vas*^{W660F} would facilitate more accurate quantitative analyses to investigate this.

The nature of interactions between Trp660 and other parts of Vas and its possible role in protein conformation remain to be investigated through crystal structure analyses that would include the C-terminal region. We observed, however, that the non-conservative substitution to Glu, despite its severe effects on most Vas functions, had mild or no effect on female fecundity, suggesting that the mutant protein still retains a conformation that supports some function. Yet, it is possible that a subtle change in Vas folding affects certain functions more than the others.

The acidic C-terminus of Vas is distinct among DEAD-box proteins and essential for its function

The closest paralog of Vas, Bel, in its core region shows 68 % sequence similarity to Vas, and its long glycine-rich N-terminus, like Vas, contains multiple RGG motifs. The conserved helicase core in Bel is followed by a C-terminal domain, about three times longer than Vas C-terminus, which exhibits some sequence similarity to Vas toward the end, including the presence of two Trp residues. Phylogenetic analyses suggest that *vas*-like genes emerged from *PL10*-like genes, including *bel*, through a gene duplication occurring after diversion of plants but before appearance of sponges (Mochizuki et al. 2001). In *Drosophila*, strong *bel* alleles are recessive lethal, consistent with the essential role of Bel in mitotic chromosome segregation in somatic cells (Johnstone et al. 2005; Pek and Kai 2011b). A similar function in the germline is carried out by Vas (Pek and Kai 2011a). Bel is also expressed in *Drosophila* ovaries and is required for female fertility (Johnstone et al. 2005), but most likely in different pathways than Vas, as we found that overexpression of Bel in *vas*^{PH165} ovaries does not rescue any oogenesis defects (data not shown).

vas$^{c.bel}$ is among the very few non-null alleles of *vas* that completely abolish female fecundity (Dehghani and Lasko 2015; Liang et al. 1994; Tomancak et al. 1998). In contrast to severe defects in oogenesis progression and transposon silencing, Vas$^{c.bel}$ supports many of the later functions of Vas. It localizes in the pole plasm of late-stage oocytes and early embryos, and it even partially supports posterior patterning and germ cell formation. This raises the possibility that the acidic motif is involved in specific interactions with the components of piRNA pathway. The divergent C-terminal regions of Bel and Vas could explain why Bel interacts with components of the siRNA pathway while Vas interacts with components of the piRNA pathway. Further analysis of protein-protein interactions involving Vas and Bel, and of how these interactions are affected by C-terminal mutations, will be required to test this hypothesis.

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References

Alié A, Leclere L, Jager M, Dayraud C, Chang P, Le Guyader H, Queinnec E, Manuel M (2011) Somatic stem cells express Piwi and Vasa genes in an adult ctenophore: ancient association of "germline genes" with stemness. Dev Biol 350:183–197

Anand A, Kai T (2012) The tudor domain protein kumo is required to assemble the nuage and to generate germline piRNAs in *Drosophila*. EMBO J 31:870–882

Anne J (2010) Targeting and anchoring Tudor in the pole plasm of the *Drosophila* oocyte. PLoS One 5:e14362

Barckmann B, Pierson S, Dufourt J, Papin C, Armenise C, Port F, Grentzinger T, Chambeyron S, Baronian G, Desvignes JP, Curk T, Simonelig M (2015) Aubergine iCLIP reveals piRNA-dependent decay of mRNAs involved in germ cell development in the early embryo. Cell Rep 12:1205–1216

Breitwieser W, Markussen FH, Horstmann H, Ephrussi A (1996) Oskar protein interaction with Vasa represents an essential step in polar granule assembly. Genes Dev 10:2179–2188

Carmel AB, Matthews BW (2004) Crystal structure of the BstDEAD N-terminal domain: a novel DEAD protein from *Bacillus stearothermophilus*. RNA 10:66–74

Carrera P, Johnstone O, Nakamura A, Casanova J, Jackle H, Lasko P (2000) Vasa mediates translation through interaction with a *Drosophila* yIF2 homolog. Mol Cell 5:181–187

Caruthers JM, Johnson ER, McKay DB (2000) Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. Proc Natl Acad Sci U S A 97:13080–13085

Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP (2000) The human *vasa* gene is specifically expressed in the germ cell lineage. Proc Natl Acad Sci USA 97:9585–9590

Cordin O, Banroques J, Tanner NK, Linder P (2006) The DEAD-box protein family of RNA helicases. Gene 367:17–37

Dehghani M, Lasko P (2015) In vivo mapping of the functional regions of the DEAD-box helicase Vasa. Biol Open 4:450–U457

Hafen E, Kuroiwa A, Gehring WJ (1984) Spatial distribution of transcripts from the segmentation gene fushi tarazu during Drosophila embryonic development. Cell 37(3):833–841

Hay B, Ackerman L, Barbel S, Jan LY, Jan YN (1988) Identification of a component of *Drosophila* polar granules. Development 103:625–640

Ikenishi K, Tanaka TS (2000) Spatio-temporal expression of *Xenopus* vasa homolog, XVLG1, in oocytes and embryos: the presence of XVLG1 RNA in somatic cells as well as germline cells. Dev Growth Differ 42:95–103

Jankowsky E (2011) RNA helicases at work: binding and rearranging. Trends Biochem Sci 36:19–29

Jarmoskaite I, Russell R (2011) DEAD-box proteins as RNA helicases and chaperones. Wiley Interdiscip Rev RNA 2:135–152

Johnstone O, Lasko P (2004) Interaction with eIF5B is essential for Vasa function during development. Development 131:4167–4178

Johnstone O, Deuring R, Bock R, Linder P, Fuller MT, Lasko P (2005) Belle is a *Drosophila* DEAD-box protein required for viability and in the germ line. Dev Biol 277:92–101

Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C, Caron PR (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. Structure 6:89–100

Knaut H, Pelegri F, Bohmann K, Schwarz H, Nusslein-Volhard C (2000) Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. J Cell Biol 149:875–888

Korolev S, Hsieh J, Gauss GH, Lohman TM, Waksman G (1997) Major domain swiveling revealed by the crystal structures of complexes of *E-coli* Rep helicase bound to single-stranded DNA and ADP. Cell 90:635–647

Kugler JM, Woo JS, Oh BH, Lasko P (2010) Regulation of *Drosophila* Vasa in vivo through paralogous cullin-RING E3 ligase specificity receptors. Mol Cell Biol 30:1769–1782

Kuznicki KA, Smith PA, Leung-Chiu WMA, Estevez AO, Scott HC, Bennett KL (2000) Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C-elegans*. Development 127: 2907–2916

Lasko P, Ashburner M (1990) Posterior localization of Vasa protein correlates with, but is not sufficient for pole cell development. Genes Dev 4:905–921

Lerit DA, Gavis ER (2011) Transport of germ plasm on astral microtubules directs germ cell development in *Drosophila*. Curr Biol 21: 439–448

Liang L, Diehljones W, Lasko P (1994) Localization of Vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. Development 120:1201–1211

Liu NK, Han H, Lasko P (2009) Vasa promotes *Drosophila* germline stem cell differentiation by activating *mei-P26* translation by directly interacting with a (U)-rich motif in its 3′ UTR. Genes Dev 23: 2742–2752

Luking A, Stahl U, Schmidt U (1998) The protein family of RNA helicases. Crit Rev Biochem Mol Biol 33:259–296

Megosh HB, Cox DN, Campbell C, Lin H (2006) The role of Piwi and the miRNA machinery in *Drosophila* germline determination. Curr Biol 16:1884–1894

Mochizuki K, Nishimiya-Fujisawa C, Fujisawa T (2001) Universal occurrence of the *vasa*-related genes among metazoans and their germline expression in *Hydra*. Dev Genes Evol 211:299–308

Patil VS, Kai T (2010) Repression of retroelements in *Drosophila* germline via piRNA pathway by the Tudor domain protein Tejas. Curr Biol 20:724–730

Pek JW, Kai T (2011a) A role for Vasa in regulating mitotic chromosome condensation in *Drosophila*. Curr Biol 21:39–44

Pek JW, Kai T (2011b) DEAD-box RNA helicase Belle/DDX3 and the RNA interference pathway promote mitotic chromosome segregation. Proc Natl Acad Sci U S A 108:12007–12012

Pek JW, Patil VS, Kai T (2012) piRNA pathway and the potential processing site, the nuage, in the *Drosophila* germline. Dev Growth Differ 54:66–77

Port F, Chen HM, Lee T, Bullock SL (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. Proc Natl Acad Sci U S A 111:E2967–E2976

Rouget C, Papin C, Boureux A, Meunier AC, Franco B, Robine N, Lai EC, Pelisson A, Simonelig M (2010) Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo. Nature 467:1128–1132

Schüpbach T, Wieschaus E (1986) Maternal-effect mutations altering the anterior-posterior of the *Drosophila* embryo. Rouxs Arch Dev Biol 195:302–317

Sengoku T, Nureki O, Nakamura A, Satoru KI, Yokoyama S (2006) Structural basis for RNA unwinding by the DEAD-box protein *Drosophila vasa*. Cell 125:287–300

Shibata N, Umesono Y, Orii H, Sakurai T, Watanabe K, Agata K (1999) Expression of *vasa*(*vas*)-related genes in germline cells and totipotent somatic stem cells of planarians. Dev Biol 206:73–87

Styhler S, Nakamura A, Swan A, Suter B, Lasko P (1998) *vasa* is required for Gurken accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. Development 125:1569–1578

Styhler S, Nakamura A, Lasko P (2002) Vasa localization requires the SPRY-domain and SOCS-box containing protein, Gustavus. Dev Cell 3:865–876

Subramanya HS, Bird LE, Brannigan JA, Wigley DB (1996) Crystal structure of a DExx box DNA helicase. Nature 384:379–383

Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T (2000) The mouse homolog of *Drosophila* Vasa is required for the development of male germ cells. Genes Dev 14:841–853

Tomancak P, Guichet A, Zavorszky P, Ephrussi A (1998) Oocyte polarity depends on regulation of *gurken* by Vasa. Development 125:1723–1732

Voronina E, Lopez M, Juliano CE, Gustafson E, Song JL, Extavour C, George S, Oliveri P, McClay D, Wessel G (2008) Vasa protein expression is restricted to the small micromeres of the sea urchin, but is inducible in other lineages early in development. Dev Biol 314:276–286

Wang C, Dickinson LK, Lehmann R (1994) Genetics of Nanos localization in Drosophila. Dev Dynam 199:103–115

Webster PJ, Liang L, Berg CA, Lasko P, Macdonald PM (1997) Translational repressor *bruno* plays multiple roles in development and is widely conserved. Genes Dev 11:2510–2521

Xiol J, Spinelli P, Laussmann MA, Homolka D, Yang Z, Cora E, Coute Y, Conn S, Kadlec J, Sachidanandam R et al (2014) RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts. Cell 157:1698–1711

Yajima M, Wessel GM (2015) Essential elements for translation: the germline factor Vasa functions broadly in somatic cells. Development 142:1960–1970

Zhang F, Wang J, Xu J, Zhang Z, Koppetsch BS, Schultz N, Vreven T, Meignin C, Davis I, Zamore PD et al (2012) UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery. Cell 151: 871–884

Zhao R, Shen JP, Green MR, MacMorris M, Blumenthal T (2004) Crystal structure of UAP56, a DExD/H-Box protein involved in pre-mRNA splicing and mRNA export. Structure 12:1373–1381