vasa is expressed in somatic cells of the embryonic gonad in a sex-specific manner in *Drosophila melanogaster*

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Summary

Vasa is a DEAD box helicase expressed in the *Drosophila* germline at all stages of development. *vasa* homologs are found widely in animals and *vasa* has become the gene of choice in identifying germ cells. I now show that *Drosophila vasa* expression is not restricted to the germline but is also expressed in a somatic lineage, the embryonic somatic gonadal precursor cells. This expression is sexually dimorphic, being maintained specifically in males, and is regulated post-transcriptionally. Although somatic Vasa expression is not required for gonad coalescence, these data support the notion that Vasa is not solely a germline factor.

Key words: *vasa*, *Drosophila*, Germline, Somatic gonadal precursor, Germ cell

Introduction

Vasa is the founder member of the class of DEAD box proteins (Hay et al., 1988a; Lasko and Ashburner, 1988) and was originally discovered as a maternal effect gene required for the formation of germ cells and abdominal segments and in *Drosophila* (Schüpbach and Wieschaus, 1986).

Vasa protein is expressed throughout the life cycle of *Drosophila* germ cells and is frequently cited as being germline specific. Germ cells form early in development at the posterior pole of the embryo where Vasa is maternally provided. At stage 10 the germ cells migrate from the posterior midgut pocket to mesodermally derived clusters of somatic gonadal precursor cells (SGPs) which are specified bilaterally (reviewed by Richardson and Lehmann, 2010). During this migration the germ cells become transcriptionally active and switch on zygotic *vasa* transcription (Van Doren et al., 1998). The germ cells associate with the SGPs and coalesce to form two compact rounded embryonic gonads, one on each side of the embryo. In males, the embryonic gonad is already oriented along its anterio-posterior axis, the anterior cells somatic become hub cells (the germline stem cell niche) and a group of male-specific SGPs (msSGPs) occupy the posterior (DeFalco et al., 2003). During larval, pupal and adult stages germ cells continue to express Vasa.

Flies mutant for *vasa* illustrate how Vasa is required at several stages of development. Firstly, Vasa is required during oogenesis. Females homozygous for *vasa* null alleles are viable but sterile due to a number of defects during oogenesis including defects in proper encapsulation of the oocyte by the follicular epithelium, positioning of the oocyte within the egg chamber, and in integrity of the oocyte nucleus (Styhler et al., 1998). In contrast, *vasa* null males are viable and fertile (Lasko and Ashburner, 1990).

Secondly, Vasa is required for germ cell formation and embryonic patterning. Germ cell formation is dependent on pole plasm, a specialised yolk-free cytoplasm containing electron rich structures called polar granules. Vasa accumulates at the posterior pole of developing oocytes and is a component of pole plasm (Hay et al., 1988a; Hay et al., 1988b), and is required for polar granule assembly (Schüpbach and Wieschaus, 1986). Females homozygous for weak alleles of *vasa*, which are sufficient to allow progression through oogenesis, lay embryos which fail to form germ cells (Hay et al., 1988a) and lack posterior segments.

Vasa contains a DEAD motif and DEAD-box family members are generally considered RNA helicases and Vasa shows RNA-binding and helicase activity *in vitro* (Liang et al., 1994). During oogenesis Vasa regulates translational initiation of germline mRNAs including *gurken* (Styhler et al., 1998; Tomancak et al., 1998) and *mei-P26* (Liu et al., 2009) via an interaction with eukaryotic initiation factor 5B (eIF5B) (Carrera et al., 2000; Johnstone and Lasko, 2004). In addition, Vasa has a translation-independent function in regulating mitotic chromosome condensation in the female germline stem cells (Pek and Kai, 2011) and is required for Piwi-interacting RNA (piRNA) mediated transposable element silencing (Vagin et al., 2004; Lim and Kai, 2007; Malone et al., 2009).

vasa homologs are present throughout the animal kingdom and are considered excellent makers to study germ cell formation and germline development (reviewed by Raz, 2000). However, a number of recent studies have observed *vasa* message or protein in somatic cell lineages of other organisms, including the sea urchin *Strongylocentrotus purpuratus* (Yajima and Wessel, 2011), the polychaete *Platynereis dumerilii* (Rebscher et al., 2007), the

annelid *Tubifex tubifex* (Oyama and Shimizu, 2007), the planarian *Dugesia japonica* (Shibata et al., 1999), the flatworm *Macrostomum lignano* (Pfister et al., 2008) the ctenophore *Pleurobrachia pileus* (Alié et al., 2011), the cephalochordate amphioxus *Branchiostoma floridae* (Wu et al., 2011), and the cnidarian *Hydractininia echinata* (Rebscher et al., 2008) leading to the broader concept of Vasa being required for both germline and somatic multipotent or stem cell function (Gustafson and Wessel, 2010).

During our lab's work on germ cell migration in *Drosophila* I noticed that some non-germline embryonic cells were positive for Vasa expression so I investigated whether *vasa* is truly germline specific in *Drosophila*.

Results

vasa is expressed outside of the *Drosophila* germline

Firstly I examined the expression of *vasa* RNA in wild-type *Drosophila* embryos. As previously reported (Hay et al., 1988a; Lasko and Ashburner, 1988), there is strong uniform maternal expression in the syncytial embryo (Fig. 1A) which is degraded upon cellularization in both the somatic cells and germ cells (Fig. 1B). Zygotic expression is detectable in the germ cells by stage 11 (Fig. 1C) and RNA is strongly expressed in the region of the embryonic gonads from stage 13 onwards (Fig. 1D,F).

The embryonic gonad is composed of two cell types, the germ cells and the SGPs, which are closely intermingled making it difficult to assess in which cells *vasa* is expressed. Therefore I made use of an *osk* mutant allelic combination ($osk^{301/CE4}$) in which females lay embryos with normal embryonic patterning but no germ cells are formed (Lehmann and Nüsslein-Volhard, 1986). Surprisingly, in such embryos strong *vasa* RNA expression was still observed in the region of the embryonic gonads at stage 14 (Fig. 1H) which became restricted to a smaller number of cells by stage 16 (Fig. 1J). Thus *vasa* is expressed by somatic cells. At the protein level, Vasa positive cells are present in the region of the embryonic gonads in embryos from *osk* mutant mothers (Fig. 1I), although the staining intensity is lower than in wild-type embryos which appear darker due to the presence of germ cells (Fig. 1E).

To confirm that the somatic *vasa* expression that occurs in embryos that lack germ cells is not specific to *osk* mutants, I examined *vasa* expression in embryos that have very few germ cells due to germ cell death. *wunen2* (*wun2*) encodes a lipid phosphate phosphatase that when over-expressed in somatic cells results in germ cell death, with only a few germ cells remaining by stage 13 (Starz-Gaiano et al., 2001). In such embryos, *vasa* expression was clearly evident in the region of the embryonic gonads (Fig. 1L), the extent of which was too large to be accounted for by a few remaining germ cells. These embryos frequently showed gonads with a large germ cell with strong Vasa staining surrounded by smaller somatic cells with weaker Vasa staining (Fig. 1K, insert). I conclude that somatic cells normally express *vasa* RNA and protein and that this occurs independently of the presence of germ cells.

To verify whether germ cells normally express *vasa* at late embryonic stages I examined *wun wun2* M−Z− mutant embryos in which the germ cells fail to migrate and remain inside of the gut (Renault et al., 2010) (Fig. 1M). At stage 16 in such embryos *vasa* RNA positive clusters of cells were observed inside the midgut (Fig. 1N) confirming that germ cells switch on *vasa* expression and this occurs independently of initiating their migration.

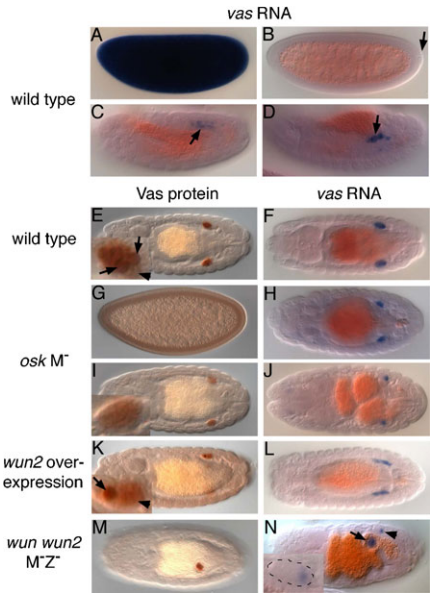

Fig. 1. *vasa* RNA and protein expression in wild-type and germ cell deficient embryos. In wild-type embryos (**A–D**) *vasa* RNA is uniform in the syncitial blastoderm (A), is degraded upon cellularization (B), including in the germ cells (arrow), is zygotically expressed in the germ cells (arrow) at stage 11 (C) and can be seen in the embryonic gonad (arrow) at stage 13 (D) and stage 14 (**F**). Vasa protein can be detected in germ cells at all stages of development including the embryonic gonads at stage 14 (**E**). In embryos laid by $osk^{301/CE4}$ mutant mothers (**G–J**), no germ cells are formed (G, stage 5 embryo in which germ cells are absent from the posterior pole, right); however, *vasa* protein (I) and RNA can be detected in the region of the embryonic gonads at stage 14 (H) reducing to a narrower expression domain by stage 16 (J). Embryos with *wun2* over-expression (using *twiGal4*, *24BGal4* and *UASwun2myc*), causing the majority of germ cells to die during migration, still contain embryonic gonads with *vasa* protein (**K**) and RNA (**L**). The embryo in L is stage 13 in which the SGPs are aligned but not yet coalesced, to show that the extent of *vasa* RNA expression cannot result from a few remaining germ cells. (**M**,**N**) In *wun wun2* maternal and zygotic mutant embryos, the germ cells survive but fail to migrate remaining as a cluster inside the midgut (M). In such embryos the germ cells (arrow in N) and somatic cells in the region of the embryonic gonad (arrowhead in N) express *vasa* RNA. Inset in N shows magnified view of the gonad (outlined) showing the *vasa* positive cells to be at the posterior of the gonad and therefore likely somatic cells rather than germ cells. Embryos are shown in dorsal view except for N which is a lateral view and only one gonad is in the plane of focus. Insets in E,I,K show close-ups of one of the embryonic gonads showing the Vasa expressing cells consisting of larger germ cells (arrows) and smaller somatic cells (arrowheads).

Overall I conclude that *vasa* RNA is highly expressed in both the germ and somatic cells of the embryonic gonads and that Vasa protein is highly expressed in the germ cells and is weakly expressed in surrounding somatic cells. The close association of the germ and somatic cells and the difference in protein levels explains why Vasa expression was not previously reported in somatic cells.

vasa is expressed in the somatic gonadal precursor cells

To verify that the somatic Vasa expression observed in Fig. 1 is not a consequence of the detection method or antibody used, I examined embryos stained fluorescently with an independent anti-Vasa antibody and co-stained with an antibody against Abdominal B (AbdB), a homeotic gene that is expressed in msSGPs (DeFalco et al., 2008). In male embryos, Vasa is present

in both the germ cells and also in smaller AbdB positive cells (Fig. 2B) at the posterior of the gonad (Fig. 2A) although with weaker intensity.

To verify this somatic expression results from the *vasa* locus I examined if this expression was present in male embryos zygotically null for *vasa* ($vas^{PH}/Df(2L)osp29$). Although the germ cells remain positive for Vasa due to pedurance of the maternal provision the somatic expression was never observed in male *vasa* zygotic null embryos (Fig. 2D).

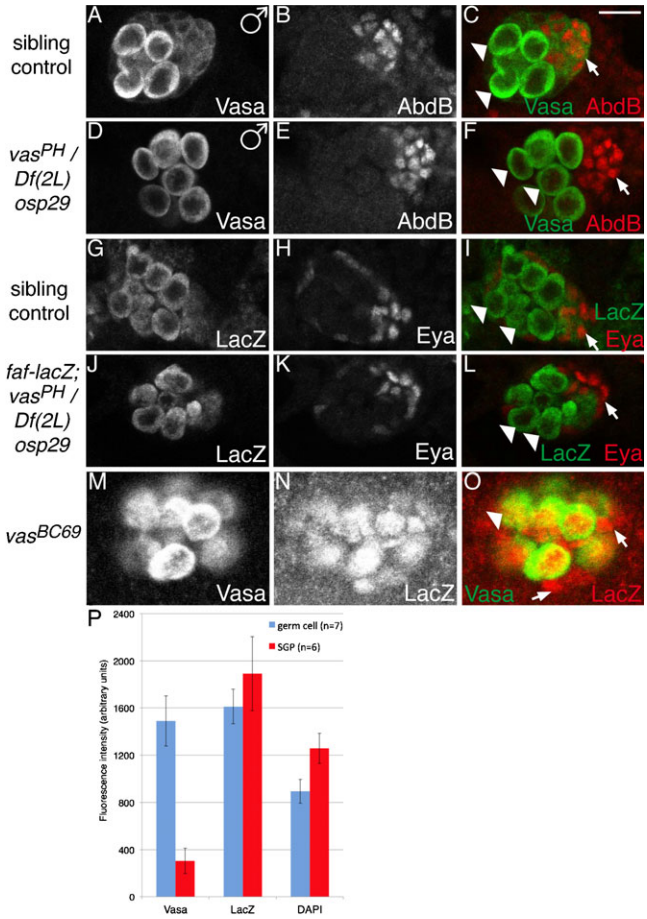

Fig. 2. Vasa protein is expressed in somatic gonadal precursor cells. Embryo anterior is to the left. Scale bar = 10 µm. Arrows indicate somatic cells, arrowheads indicate germ cells. (**A–F**) The embryonic gonad of a male stage 15 sibling control embryo (A–C) stained using a rat anti-Vasa antibody showing Vasa positive somatic cells co-expressing AbdB (B) and that in a *vasa* zygotic null embryo (D–F) no somatic Vasa is detected (D) although the AbdB positive cells are present (E). (**G–L**) The embryonic gonad of a stage 15 sibling control (G–I) or *vasa* zygotic null embryo (J–L) showing wild-type coalescence of Eya marked SGPs (H,K) with LacZ labeled germ cells from a *faf-lacZ* transgene (G,J). Note that the balancer chromosome in the sibling control is marked with a *ftz>lacZ* transgene resulting in LacZ expression in some somatic cells (G). (**M–O**) Embryonic gonad of a stage 15 embryo laid by a wild-type female mated to vas^{BC69} (a nuclear LacZ enhancer trap) male, stained for Vasa (M) and LacZ (N). LacZ expression is observed both in germ and somatic cells of the gonad. (**P**) Graph showing mean fluorescence intensity of Vasa, LacZ and DAPI (which binds DNA) staining of germ cells and SGPs in a stage 15 male embryo laid by a wild-type female mated to vas^{BC69}. The intensity of Vasa staining is much higher in the germ cells compared to the SGPs, although the LacZ staining intensity is similar suggesting that the former is not caused by differences in transcription. The DAPI intensity is slightly higher in the SGPs due to their smaller size. Standard error bars are shown.

Based on their location and co-expression of AbdB the somatic *vasa* positive cells are SGPs and in male embryos are the msSGPs. To ascertain if Vasa is required in SGPs, their behaviour in *vasa* zygotic null embryos was examined using antibodies against AbdB and against the general SGP marker Eyes absent (Eya) also known as Clift (Boyle et al., 1997). SGPs are present and coalesce with germ cells in *vasa* zygotic null embryos (Fig. 2B,E,H,K) indicating that SGP coalescence behaviour is independent of Vasa. In addition the expression of these makers was not altered in the mutant embryos indicating that *vasa* null SGPs maintain appropriate marker gene expression.

To test whether the differences in Vasa expression levels between germ cells and somatic cells reflects differences in transcription I made use of an enhancer trap in the *vasa* locus, vas^{BC69}. In this line, a nuclear *lacZ* containing P-element is inserted into the first exon of *vasa* and anti-LacZ staining recapitulates endogenous *vasa* expression (Sano et al., 2001). To avoid perdurance of LacZ from maternal expression from the *vasa* locus I stained embryos from wild-type females mated to vas^{BC69} males. As expected LacZ positive germ cells were observed, but LacZ positive cells were also seen intermingled around the germ cells (Fig. 2M–O). Thus zygotic transcription from the *vasa* promoter and subsequent translation occurs both in germ cells and SGPs during embryogenesis. The LacZ staining intensity in germ cells and SGPs was comparable whilst the staining intensity of Vasa was much lower in the SGPs (Fig. 2A,N, quantified in Fig. 2P). The intensity of *vasa* RNA staining between germ cells and somatic cells was also similar (Fig. 1N). These data indicate that the difference in Vasa protein levels between these cell types is not due to differences in *vasa* transcription but likely reflect differences in translation or protein stability.

vasa expression shows sex-specific differences

Not all embryos from *osk* mutant mothers expressed *vasa* RNA at late embryonic stages (data not shown). To test if this heterogeneity in detection resulted from sex-specific differences in *vasa* expression I mated *osk* mutant females to males carrying a LacZ expressing transgene on the X chromosome to differentiate between female (XX) and male (XY) embryos, which would be positive and negative for LacZ expression respectively.

At stage 13 in both female and male embryos *vasa* expression was equivalent (Fig. 3A,B). However, whilst this expression decreased and eventually became undetectable in female embryos (Fig. 3C,E), in male embryos this expression was maintained and became restricted to the posterior of the gonad (Fig. 3D,F) the location of the msSGPs.

vasa is required only in the female germline

Given the expression of Vasa in embryonic msSGPs I tested whether the male gonad was affected by the loss of Vasa in larvae and adults. Male 3rd instar larval gonads appeared normal in size in *vasa* null animals (Fig. 4). The msSGPs give rise to the terminal epithelium, which in larval stages appears as a cluster of Eya positive cells at one end of the gonad (Nanda et al., 2009). In wild-type, Vasa is no longer detectable in the terminal epithelium at this stage using either an anti-Vasa antibody (Fig. 4A–D) or the vas^{BC69} enhancer trap (data not shown). In *vasa* null gonads the terminal epithelium is still present (Fig. 4E–H) indicating that Vasa is not essential for survival of these cells.

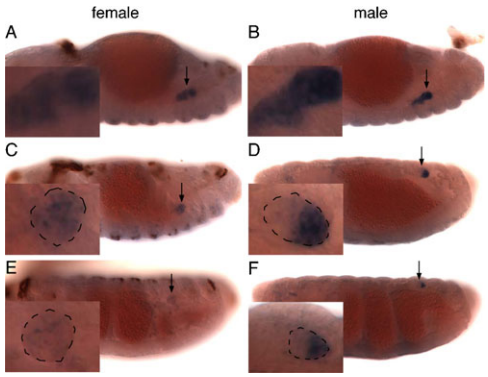

Fig. 3. Sex specific somatic expression of *vasa* RNA. Lateral views of embryos laid by osk^{CE4}/osk^{301} females (which therefore lack germ cells) mated to males carrying a *dfd>lacZ* transgene on the X chromosome stained for *vasa* RNA (blue) and LacZ protein (brown). The XX female embryos will therefore be positive for LacZ (**A**,**C**,**E**) whereas the XY male embryos will be LacZ negative (**B**,**D**,**F**). At stage 13 *vasa* RNA is present in somatic cells in both males and female embryos (A,B), and becomes enriched at the posterior of the gonad in stage 15 (D) and stage 16 (F) males but decreases in stage 15 (C) and is not detected in stage 16 (E) females.

I examined the testis of *vasa* null adults. I found that such testis were phenotypically normal (data not shown) in agreement with these flies being fertile. I conclude, that as previously reported, *vasa* is not essential in the adult testis (Lasko and Ashburner, 1990).

Vasa null females lay only very few eggs (Fig. 5A) which all display a ventralised phenotype as measured by the absence of (Fig. 5C, middle egg) or defects in the number or length of the dorsal appendages (Fig. 5C, upper and lower eggs, respectively). To examine whether *vasa* expression in the soma is required during oogenesis I tested whether germline specific expression of *vasa* using the *nanos-Gal4VP16* driver was sufficient to rescue the number and patterning defects of eggs from *vasa* null females. I found that germline *vasa* expression was able to increase the egg laying rates of *vasa* null females but not to wild-type levels (Fig. 5A). The eggs laid by the rescued females were no longer ventralised but appeared wild-type (Fig. 5D) and indeed many hatched (data not shown). The lack of eggs laid by *vasa* null females is due to a large amount of degenerating ovarioles compared to wild-type (Fig. 5E,F). The ovaries of the rescued females on the other hand appeared mostly wild-type with only the occasional degenerating ovariole (Fig. 5G). I conclude that *vasa* is only essential in the female germline.

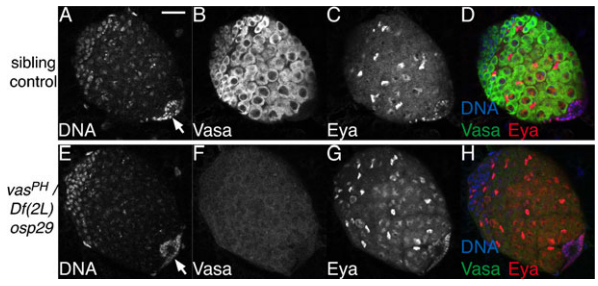

Fig. 4. Larval gonads of *vasa* null males appear wild-type. 3rd instar larval gonads from a sibling control (**A–D**) or *vasa* null males (**E–H**) showing DAPI stained nuclei (A,E), Vasa (B,F) and Eya (C,G) and merged image (D,H). Arrows indicate terminal epithelium precursors. Scale bar = 25 μm.

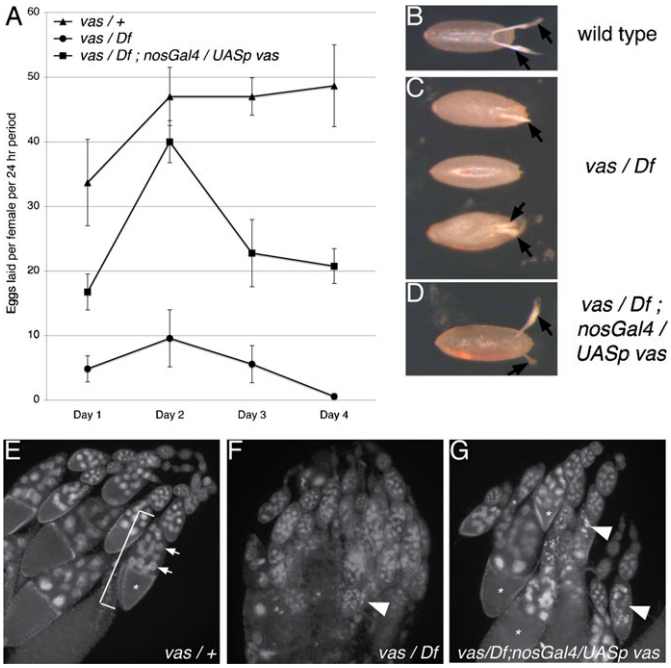

Fig. 5. Germline specific expression of Vasa is sufficient to rescue fertility of *vasa* null females. (**A**) Average number of eggs laid over a 24 hr period by single females either wild-type (triangles), *vasa* null (circles, $vas^{PH}/Df(2L)osp29$) or *vasa* null with *vasa* driven in the germline (squares, $vas^{PH}/Df(2L)osp29;nosGal4VP16/UASp\ vasa$). (**B–D**) Eggs laid by wild-type females have two dorsal appendages (B), whereas *vasa* null females lay eggs with various degrees of ventralization with either no dorsal appendages (C, middle egg), one dorsal appendage (C, upper egg) or short dorsal appendages (C, lower egg). *vasa* null females with *vas* driven ectopically in germ cells lay wild-type looking eggs with two dorsal appendages (D). Arrows indicate dorsal appendages. (**E–G**) 7-day-old ovaries with DNA stained using DAPI. Sibling control ovaries (E) contain wild-type egg chambers (square bracket) with nurse cells (arrows) and developing oocyte (asterisk). *vasa* null ovaries (F) contain mostly egg chambers without developing oocytes and egg chambers often degenerate (arrowhead). In *vas* null ovaries rescued by *vasa* driven in the germline (G) some abnormal egg chambers remain (arrowheads) but wild-type egg chambers with developing oocytes are also observed (asterisks).

Discussion

The *vasa* gene is often cited as the most conserved germline marker in animals. The germline restricted *vasa* expression pattern, first demonstrated in *Drosophila,* has been instrumental for identifying germ cells in many animal species. In zebrafish (Braat et al., 2000; Knaut et al., 2000), the crustacean *Parhyale hawaiensis* (Özhan-Kizil et al., 2009), and mouse (Fujiwara et al., 1994) *vasa* expression is indeed germline specific but increasingly it is being recognized that, for other species, *vasa* is also expressed in somatic cell types.

In this paper I report that *vasa* expression in *Drosophila melanogaster*, both at the RNA and protein level, is not restricted to the germline but is also expressed in a somatic lineage, the embryonic SGPs. This embryonic expression at the RNA level occurs transiently in female embryos but is maintained in the posterior somatic gonadal precursor cells in male embryos. These posterior cells are the msSGPs which give rise to the terminal epithelium.

The situation in late *Drosophila* embryogenesis in which *vasa* positive germ cells are surrounded by *vasa* positive somatic cells is highly reminiscent of the situation in a number of other species. In amphioxus, presumptive germ cells are specified by

inheritance of Vasa containing germ plasm and these cells migrate as a cluster towards the posterior end of the embryo and enter a domain which expresses *vasa* zygotically (Wu et al., 2011). In *Platynereis* germ cells are also formed early in development by inheritance of Vasa containing germ plasm. At the end of embryogenesis the germ cells reside in the mesodermal posterior growth zone which also expresses Vasa and from this position they will begin their migration during larval stages (Rebscher et al., 2012). In both cases the *vasa* positive somatic domains are proliferative zones and the *vasa* positive cells are likely somatic stem cells.

What is the function of somatic *vasa* expression in *Drosophila*? In the embryo, gonad coalescence is wild-type in *vasa* null animals (Fig. 2A–L). Although it cannot be ruled out that there are subtle changes in *vasa* null SGPs, in expression profile for example, these do not affect embryonic SGP behaviour. The terminal epithelium is present in the gonads of *vasa* null male larvae (Fig. 4) indicating that *vasa* expression during embryogenesis is not essential for establishment or maintenance of this tissue. Given that Vasa is expressed by proliferating lineages in a number of different species, it is tempting to speculate that Vasa may also support proliferation of *Drosophila* SGPs. If this is the case, the lack of a discernible phenotype would mean that Vasa acts redundantly with other DEAD-box RNA helicases of which there are several in *Drosophila*.

Germline establishment and maintenance has traditionally been thought to require a conserved set of germline specific genes including *vasa*, *nanos* and *pumilio*. Recent studies indicating a broader expression of these genes in many organisms, in cells with stem cell properties, have argued their role maybe more multipotent than purely germline (Gustafson and Wessel, 2010). The finding that another member of this conserved set of genes is not germline specific, even in the animal it was first discovered in, strengthens this argument and urges caution when using *vasa* expression alone to identify germ cells in non-model organisms.

Materials and Methods

Fly stocks

The following *Drosophila* alleles were used: vas^{PH165} is a *vasa* null allele with a 7.3 kb deletion removing the entire *vasa* coding region (Styhler et al., 1998); *Df(2L)osp29* is a deficiency uncovering *vasa* (courtesy of the Bloomington Stock Center); vas^{BC69} contains a P-element enhancer-trap containing a nuclear *lacZ* transgene inserted the first exon of *vasa* (Sano et al., 2001) and was courtesy of Stephane Ronsseray, Institut Jacques Monod, Paris, France; UASp *vasa* was courtesy of Akira Nakamura, RIKEN Center for Developmental Biology, Kobe, Japan (Sengoku et al., 2006); osk^{CE4} was courtesy of Anne Ephrussi, EMBL, Heidelberg, Germany; osk^{301} is a point mutation, courtesy of the Tübingen stock collection, Tübingen, Germany; *nanos-Gal4VP16* and *UASwun2myc* were courtesy of Ruth Lehmann, New York, USA; *wun wun2* M−Z− embryos were made according to Renault et al. (Renault et al., 2010). A *dfd>lacZ* insertion on the X chromosome (used to sex embryos) and *faf-lacZ* insertion which provides LacZ maternally and localized to the posterior pole where germ cells are formed (used to label germ cells) (Fischer-Vize et al., 1992) were courtesy of the Bloomington Stock Center.

Antibody stainings and *in situ* hybridisation

Embryos were laid at room temperature (except from osk^{CE4}/osk^{301} females which were kept at 18℃) and fixed in 4% formaldehyde, devitellinized in heptane/methanol. Larvae were dissected in Ringers solution and fixed in 4% formaldehyde. For stainings the following antibodies were used: rabbit anti-Vasa (used for all anti-Vasa stainings except Fig. 2A–F, 1:10,000, a gift from Anne Williamson and Helene Zinszner, Lehmann Lab, New York, USA), rat anti-Vasa (1:40, Developmental Studies Hybridoma Bank, Iowa City, USA), mouse anti-lacZ (1:1000, Promega, Mannheim, Germany), rabbit anti-lacZ (1:10,000, Cappel, MP Biomedicals, Solon, USA), mouse anti-Eya (1:12, 10H6, Developmental Studies Hybridoma Bank), mouse anti-AbdB (1:10, 1A2E9, Developmental Studies Hybridoma Bank), anti-rabbit biotin, Cy3 and Cy5 (1:500, Jackson ImmunoResearch, Newmarket, Suffolk, UK) and Alexa-488 (1:500, Invitrogen, Life Technologies GmbH, Darmstadt, Germany) coupled secondaries. A VECTASTAIN® ABC kit (Vector Labs, Burlingame, USA) followed by diaminobenzidine was used to detect biotinylated secondary antibody. Embryos were either mounted in Epon resin and viewed on a Zeiss Axioimager or mounted in Aqua-polymount (Polysciences) and viewed on an Olympus FV-1000.

Relative levels of Vasa and LacZ in the gonads of vas^{BC69} embryos were determined using ImageJ software by measuring the mean fluorescence intensity of Vasa in the cytoplasm (using the rat anti-Vasa antibody) and LacZ (using the rabbit anti-LacZ antibody) and DAPI in the nucleus of several germ cells and SGPs in a single gonad. The result for one representative gonad out of a total of 4 quantified is shown in Fig. 2P.

A DIG-labeled *vasa* RNA antisense probe was synthesized with T7 RNA polymerase using the DIG labeling system (Roche) from a 2.3 kb full length sequence verified *vasa* cDNA clone (corresponding to transcript *vas-RA* in Flybase) in pNB40 (courtesy of Ruth Lehmann, New York, USA) linearized with AvaI. The probe was chopped by carbonate hydrolysis, the embryos were fixed as above and *in situ* hybridization carried out according to Lehmann and Tautz (Lehmann and Tautz, 1994). For a double antibody and *in situ* hybridisation staining, the antibody staining was performed first followed by the *in situ* hybridisation in a protocol adapted from Manoukian and Krause (Manoukian and Krause, 1992).

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Competing Interests

The author has no competing interests to declare.

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