Evidence against a germ plasm in the milkweed bug *Oncopeltus fasciatus*, a hemimetabolous insect

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Summary

Primordial germ cell (PGC) formation in holometabolous insects like *Drosophila melanogaster* relies on maternally synthesised germ cell determinants that are asymmetrically localised to the oocyte posterior cortex. Embryonic nuclei that inherit this "germ plasm" acquire PGC fate. In contrast, historical studies of basally branching insects (Hemimetabola) suggest that a maternal requirement for germ line genes in PGC specification may be a derived character confined principally to Holometabola. However, there have been remarkably few investigations of germ line gene expression and function in hemimetabolous insects. Here we characterise PGC formation in the milkweed bug *Oncopeltus fasciatus*, a member of the sister group to Holometabola, thus providing an important evolutionary comparison to members of this clade. We examine the transcript distribution of orthologues of 19 *Drosophila* germ cell and/or germ plasm marker genes, and show that none of them localise asymmetrically within *Oncopeltus* oocytes or early embryos. Using multiple molecular and cytological criteria, we provide evidence that PGCs form after cellularisation at the site of gastrulation. Functional studies of *vasa* and *tudor* reveal that these genes are not required for germ cell formation, but that *vasa* is required in adult males for spermatogenesis. Taken together, our results provide evidence that *Oncopeltus* germ cells may form in the absence of germ plasm, consistent with the hypothesis that germ plasm is a derived strategy of germ cell specification in insects.

Introduction

In sexually reproducing animals, only germ cells contribute genetic information to future generations. The germ line/soma separation is a cell fate decision shared across Metazoa (Buss, 1987). Despite the fundamental commonality of germ cell function in animals, the molecular mechanisms underlying germ cell specification are remarkably diverse across different taxa (Extavour and Akam, 2003; Extavour, 2007; Ewen-Campen et al., 2010; Juliano et al., 2010).

Primordial germ cells (PGCs) can be specified via different developmental mechanisms; here we call these "cytoplasmic inheritance" and "zygotic induction." (We and others have previously referred to these mechanisms as "preformation" and "epigenesis" respectively (Nieuwkoop and Sutasurya, 1981; Extavour and Akam, 2003; Extavour, 2007). However, these terms can hold different meanings in other contexts of the history and philosophy of biology (e.g. Callebaut, 2008). We therefore avoid them here in favour of more mechanistically descriptive terms.) Cytoplasmic inheritance is characterised by the asymmetric formation of a specialised cytoplasmic region within the oocyte or early embryo, termed "germ plasm." Germ plasm contains maternally provided mRNAs and proteins that are individually necessary and collectively sufficient for PGC formation. Cells that inherit germ plasm during embryogenesis acquire germ line fate. The best understood example of cytoplasmic inheritance occurs in *Drosophila melanogaster*, where germ plasm is maternally synthesised, localised to the posterior of the oocyte during oogenesis, and subsequently incorporated into PGCs (pole cells) during cellularisation. Removing pole cells after their formation, or compromising the molecular components of germ plasm, leads to loss of PGCs and sterility in adulthood (reviewed by Mahowald, 2001). In contrast, zygotic induction of PGCs takes place later in development and requires signalling from neighbouring somatic cells to induce germ line fate. This mode of PGC development is exemplified by *Mus musculus*, wherein PGCs develop from a subset of presumptive mesodermal cells after the segregation of embryonic and extraembryonic tissues in response to local signalling (reviewed by Magnúsdóttir et al., 2012).

Across Insecta, germ plasm has been almost exclusively reported in taxa nested within Holometabola ("higher" insects, which undergo complete metamorphosis) including *D. melanogaster* (reviewed by Kumé and Dan, 1968; Anderson, 1973; Nieuwkoop and Sutasurya, 1981), and in only three species belonging to the sister assemblage to the Holometabola (see below). Thus, although the vast majority of our knowledge of insect germ cell development comes from studies of germ plasm in *D. melanogaster*, this mode of germ cell specification is likely a derived feature of Holometabolous insects and their close sister taxa.

Our present knowledge of PGC specification in basally branching insects (Hemimetabola) is based almost entirely on classical histological studies of insect development conducted over the past 150 years. Nearly all of these report that PGCs arise late in embryogenesis, raising the possibility that they may be specified through inductive mechanisms (Wheeler, 1893; Heymons, 1895; Hegner, 1914; Nelsen, 1934; Roonwal, 1937). Experimental approaches to discovering germ plasm in Hemimetabola are limited, but a study involving destruction of the germ rudiment via irradiation in the cricket *Gryllus domesticus* (Schwalm, 1965) showed that no specific region of early embryos in this species contains a germ line determinant. Functional tests of genes that may specify germ cells in Hemimetabola have been performed in only one insect, the cricket *Gryllus bimaculatus*. In this cricket, the conserved germ line markers *vasa* and *piwi* are dispensable maternally and zygotically for PGC formation (Ewen-Campen et al., 2013). Most evidence available for the Hemimetabola therefore suggests the absence of germ plasm and the operation of zygotic PGC specification mechanisms.

Exceptions have been reported, however, in some members of the Paraneoptera, an assemblage of insect orders (including Hemiptera [true bugs], Psocoptera [book lice], and Thysanoptera [thrips]) that collectively form the sister group to Holometabola (Yeates et al., 2012). Cytological studies of three paraneopteran species, a book louse (Psocoptera (Goss, 1952)), a thrip (Thysanoptera (Heming, 1979)) and an aphid (Hemiptera (Chang et al., 2009)) suggested the presence of germ plasm in oocytes or early embryos, as did expression studies of *vasa*, *piwi* and *nanos* expression during asexual development of the pea aphid *Acyrthosiphon pisum* (Chang et al., 2006; Chang et al., 2007; Chang et al., 2009; Lu et al., 2011). However, *A. pisum* embryogenesis is highly modified relative to that of other hemimetabolous insects and even relative to other members of the same order (Miura et al., 2003). Studies of embryogenesis in most other hemipterans describe absence of germ plasm and PGC origin after cellularisation from the blastopore region at gastrulation stages (Metschnikoff, 1866; Witlaczil, 1884; Will, 1888; Seidel, 1924; Mellanby, 1935; Butt, 1949; Sander, 1956; Kelly and Huebner, 1989; Heming and Huebner, 1994). We therefore wished to examine the expression and function of germ line genes in a hemipteran displaying embryological characteristics more representative of the order.

Here we characterise germ cell formation and migration in the milkweed bug *Oncopeltus fasciatus* (Hemiptera). We examine the expression of 19 molecular markers including *vasa*, *nanos*, and *piwi*, and test the germ cell function of three of these using RNA interference. We show that in striking contrast to *Drosophila*, transcripts of none of these genes localise asymmetrically within *Oncopeltus* oocytes or early embryos. We identify PGCs using multiple criteria, and show that neither *vasa* nor *tudor* are required for PGC specification or oogenesis in this species, but that *vasa* is required for spermatogenesis in adult males. These data show that the PGC specification role of *vasa* has diverged between *Oncopeltus* and the Holometabola, and suggest that *Oncopeltus* PGCs may form in the absence of maternally supplied germ plasm.

Results

Putative germ cells are first detectable in the late blastoderm stage

In contrast to *D. melanogaster*, classical studies of *Oncopeltus fasciatus* embryogenesis have not revealed a germ plasm in oocytes or early embryos, and instead first identify cells with cytological characteristics of PGCs at the posterior of the embryo at the end of the cellular blastoderm stage (Butt, 1949). We used semi-thin plastic sectioning and fluorescence microscopy to confirm these observations, and traced the development of these putative PGCs throughout gastrulation and germ band elongation (supplementary material Fig. S1). Our observations of putative PGC formation in *Oncopeltus* were consistent with historical studies (Butt, 1949), showing that these cells first arise at the blastoderm posterior immediately prior to gastrulation (supplementary material Fig. S1). Unlike pole cells in *D. melanogaster*, presumptive PGCs in *Oncopeltus* arise on the basal side of the blastoderm surface, adjacent to the yolk (supplementary material Fig. S1G–H). In order to obtain further evidence that these cells were PGCs and test for the presence of a maternally supplied germ plasm, we examined the expression of conserved germ line markers.

Cloning *Oncopeltus* germ line markers

We cloned fragments of *vasa*, *nanos*, and *piwi* (Ewen-Campen et al., 2010) and confirmed that each was the best reciprocal BLAST hit to its respective orthologue in *D. melanogaster*. *vasa* was cloned using degenerate primers (supplementary material Table S1). *nanos* and a single *piwi* gene were recovered from the *Oncopeltus* transcriptome, in addition to a single AGO-3 orthologue (an additional PIWI family protein belonging to a separate sub-family; not shown). We believe it is unlikely that *Oncopeltus* possesses additional orthologues of these genes because (1) the *Oncopeltus* ovarian and embryonic transcriptome, which is nearly saturated for gene discovery and has an average coverage of $23\times$ (Ewen-Campen et al., 2011), contained only one orthologue of each gene; and (2) degenerate PCR for *vasa* using primers flanking the conserved DEAD box helicase domain (Rocak and Linder, 2004) recovered only a single *vasa* orthologue.

Phylogenetic reconstruction confirmed that *Oncopeltus vasa* is nested within other insect *vasa* genes (supplementary material Fig. S2A), and that *Oncopeltus piwi* belongs to the PIWI sub-family containing the *Drosophila* genes *piwi* and *aubergine* (which are *Drosophila*-specific duplications) (supplementary material Fig. S2B). The portion of animal Nanos proteins with conservation sufficient for confident alignment (48 amino acids) is too short to yield significant phylogenetic signal (supplementary material Fig. S2C, note low support values), but *Oncopeltus* Nanos does contain the diagnostic $2\times$(CCHC) zinc finger domain found in all Nanos orthologs (supplementary material Fig. S2D).

Our analysis of the *Oncopeltus nanos* sequence produced an unexpected result: we found that a stop codon is present 771 bp upstream of the first CCHC zinc finger domain, although no methionine is found anywhere in this region. This is unlikely to be a sequencing error, as it was identified with high coverage (22 reads/bp at this position) in the transcriptome (Ewen-Campen et al., 2011) and confirmed using Sanger sequencing of independent clones generated from a different cDNA pool than that used to generate the transcriptome. Furthermore, repeated attempts at 5′ RACE using a third independent DNA pool failed to amplify a start codon. Several lines of evidence confirm that this *Oncopeltus nanos* sequence represents a highly expressed mRNA and is therefore unlikely to be a pseudogene: it was recovered from a transcriptome made solely from poly(A)-RNA, and is detected via both RT-PCR (Ewen-Campen et al., 2011) and in situ hybridisation (see below). We hypothesise that a large, unspliced intron downstream of the start codon may have been

present in our mRNA preparations. Alternatively, given that the length of the predicted translated region upstream of the first CCHC zinc finger domain (266 amino acids) is within the range of known arthropod Nanos orthologues (95 to 332 amino acids) (Wang and Lehmann, 1991; Curtis et al., 1995; Calvo et al., 2005; Lynch and Desplan, 2010), it may be that the *Oncopeltus* Nanos N terminus has a non-methionine start codon. Although rare, eukaryotic non-AUG translation initiation can occur in nuclear-encoded genes, including developmentally relevant genes (Hellen and Sarnow, 2001), and can be recognized by insect ribosomes (Sasaki and Nakashima, 2000; Jan et al., 2001). In the absence of a complete genome sequence we cannot distinguish between these hypotheses. Despite this uncertainty, we report *nanos* transcript expression here for the sake of completeness.

vasa, *nanos*, and *piwi* transcripts do not localise asymmetrically in ovaries

The distinct cytoplasm inherited by early-specified PGCs in multiple organisms, including *D. melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis* and *Danio rerio*, contains transcripts of the highly conserved *piwi*, *vasa* and *nanos* gene families. Of these, only *nanos* mRNA is asymmetrically localised to *D. melanogaster* germ plasm, while *piwi* and *vasa* transcripts are ubiquitous throughout the fly oocyte and embryo. However, in several other organisms *vasa* orthologue transcripts are asymmetrically localised germ plasm components (reviewed by Ewen-Campen et al., 2010).

To test whether any of these transcripts were asymmetrically localised to putative germ plasm in *Oncopeltus* oocytes, we conducted in situ hybridisation on adult ovaries. The structure of *Oncopeltus* ovaries is typical of Hemiptera and several other insect orders but differs remarkably from that of *Drosophila* (Fig. 1A) (Büning, 1994). Rather than each oocyte developing together with its own complement of 15 nurse cells as in *Drosophila*, all oocytes in *Oncopeltus* ovarioles share a common pool of syncytial nurse cells located at the anterior of each ovariole in a region termed the "tropharium" (Fig. 1A1). The nurse cell syncytium connects to all oocytes via elongated, microtubule-rich tubes called "nutritive tubes" (Hyams and Stebbings, 1979; Harrison et al., 1991) through which maternal factors, including mRNA, proteins and mitochondria, are transported to developing oocytes (Fig. 1A2,A3) (Stebbings et al., 1985; Stebbings and Hunt, 1987; Anastas et al., 1991; Hurst et al., 1999; Stephen et al., 1999).

vasa, nanos, and *piwi* were expressed at high levels in *Oncopeltus* nurse cells and oocytes of all stages, but at no stage of oogenesis did any of these three transcripts localise asymmetrically within oocytes (Fig. 1B–D). Expression was detected in nurse cells, resting oocytes, nutritive tubes, and developing oocytes, suggesting that these transcripts are synthesised in the nurse cells and subsequently transported to oocytes via nutritive tubes (Fig. 1B–D). *nanos* and *piwi* were expressed throughout the tropharium (Fig. 1C,D), in contrast to *vasa*, whose expression was primarily in nurse cells of the posterior tropharium, resting and developing oocytes (Fig. 1B). In late stage oocytes, expression remained ubiquitous (not shown), similar to the expression in just-laid eggs (see below).

In situ screen of conserved *Drosophila* germ plasm markers fails to reveal a germ plasm in *Oncopeltus*

The expression of *piwi*, *vasa* and *nanos* suggests that a maternally localised germ plasm containing transcripts of these genes is not
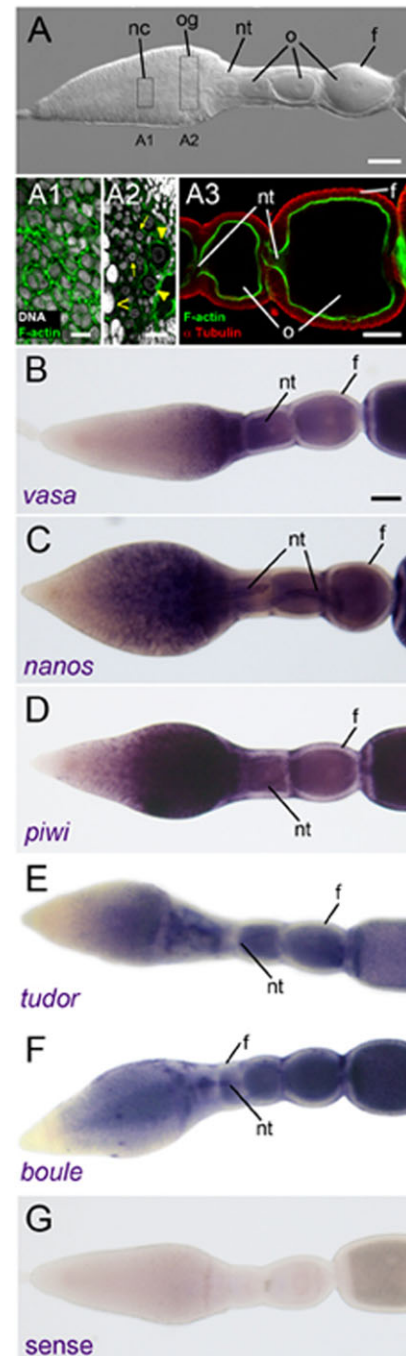

Fig. 1. Germ cell markers do not localise asymmetrically during oogenesis. (A) Overview of a single *Oncopeltus* ovariole. *nc*: nurse cells; *o*: oocytes; *nt*: nutritive tubes; *f*: follicle cells. Boxed regions are enlarged in (A1–A3). (A1) Nurse cell syncytium containing polyploid nurse cell nuclei (white) connected by cytoplasmic bridges (green). (A2) Posterior tropharium containing oogonia (arrows) and resting oocytes (arrowheads). Caret indicates polyploid nurse cells in the anterior of this region. (A3) Nutritive tubes (*nt*) are actin-rich at the end that enters the anterior of each oocyte. Transcripts of *vasa* (B), *nanos* (C), *piwi* (D), *tudor* (E) and *boule* (F) are detected in nurse cells, nutritive tubes, and uniformly in oocytes. (G) A representative sense control (for *vasa*) is shown; sense controls for other genes were similar. Scale bars: 100 μm in A,A3,B (applies to C–G); 25 μm in A1,A2. Anterior to the left in all panels.

present in *Oncopeltus* oocytes. However, a functional germ plasm that contains gene products other than those encoded by these three genes could be present in oocytes or early embryos. To

explore this possibility, we examined the expression of 14 additional genes whose transcripts are enriched in the germ plasm and germ cells of *Drosophila* (supplementary material Table S2) (Tomancak et al., 2002; Lécuyer et al., 2007; Tomancak et al., 2007) that were also recovered from the *Oncopeltus* ovarian and embryonic transcriptome (Ewen-Campen et al., 2011) based on best reciprocal BLAST hit analysis with the *Drosophila* proteome (Zeng and Extavour, 2012). Although several of these genes do not have documented mutant phenotypes for germ cell formation in *Drosophila* (supplementary material Table S2), all are expressed at high levels in germ plasm and/or pole cells and are therefore molecular markers for germ plasm in *Drosophila*. We reasoned that if *Oncopeltus* possessed germ plasm it would likely be revealed by at least one of these genes.

In addition, we examined the expression of *boule* and *tudor*, which have widely conserved functions in germ cells across Metazoa (Eberhart et al., 1996; Ewen-Campen et al., 2010; Shah et al., 2010). *tudor* is one of 23 Tudor domain-containing proteins

in *Drosophila* (Ying and Chen, 2012), but there is no evidence that loss of function of other Tudor domain-containing genes have grandchildless phenotypes in *Drosophila* (Handler et al., 2011; Pek et al., 2012). We therefore focus only on the expression and function of the orthologue of *Drosophila tudor* (CG9450). We examined *boule* and *tudor* transcript expression throughout oogenesis and embryogenesis through mid-germ band stages.

None of these 16 transcripts localised asymmetrically in ovaries (Fig. 1E,F; supplementary material Fig. S3). Instead, like *vasa*, *piwi* and *nanos* (Fig. 1B–D), all of these genes were expressed ubiquitously throughout oogenesis. Half of the genes examined (*sra*, *CycB*, *Bsg25D*, *Uev1A*, *CG16817*, *Unr*, *mael* and *tud*) were expressed, like *vasa* (Fig. 1B), in nurse cells adjacent to resting oocytes, as well as in the resting and early oocytes themselves (Fig. 2E; supplementary material Fig. S3B–H). Five genes (*Gap1*, *eIF5*, *bel*, *orb* and *bol*) were, like *piwi* and *nanos* (Fig. 1C,D), strongly expressed in all nurse cells of the
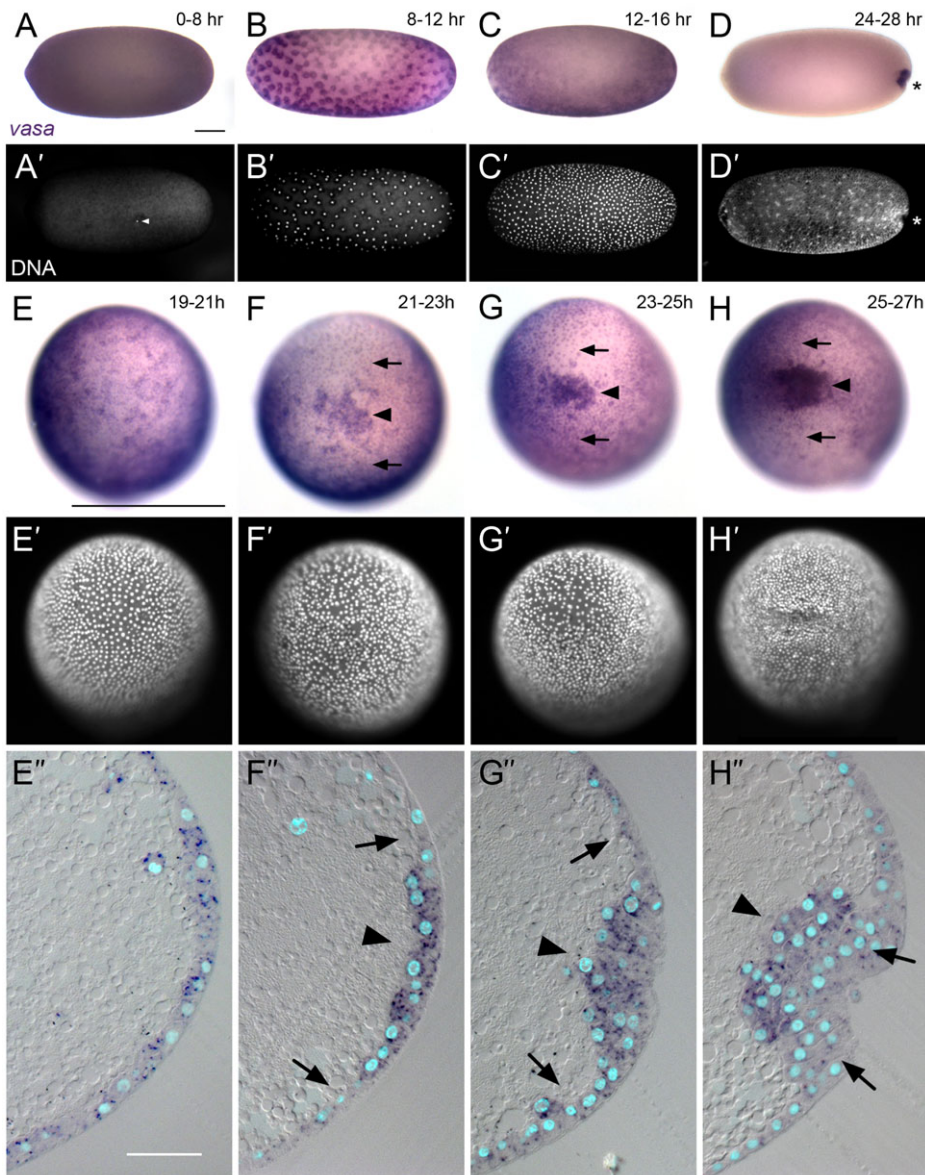

Fig. 2. *vasa* transcript expression first labels PGCs at late blastoderm stages. (A–D) *vasa* transcript expression. (A′–D′) Corresponding images of nuclear stains. (A,A′) Immediately following fertilisation *vasa* is detected ubiquitously. Arrowhead: polar body. (B,B′) In early cleavage stages *vasa* transcripts are associated with all energid nuclei. (C,C′) During early syncytial blastoderm stages, *vasa* expression remains ubiquitous. (D,D′) At cellular blastoderm stages (24–28 h AEL), *vasa* marks putative PGCs at the posterior pit (asterisk). (E–H) End-on perspective of the posterior of *Oncopeltus* embryos showing *vasa* expression during PGC formation. (E′–H′) Corresponding images of nuclear stains. (E″–H″) Medial sections of *vasa*- (purple) and nuclear- (cyan) stained embryos at corresponding time points. (E–E″) In late syncytial blastoderm stages, *vasa* is expressed ubiquitously. (F–F″) In early cellular blastoderm embryos, *vasa* expression increases in some posterior cells (arrowheads in F,F″) while levels in the remainder of the blastoderm decrease (arrows in F,F″). (G–G″) At posterior germ band invagination *vasa*-positive cells (arrowheads) are the first cells to enter the yolk; *vasa* transcripts continue to be cleared from somatic tissue (arrows). (H–H″) As invagination proceeds *vasa* expression is largely restricted to PGCs (arrowhead) and nearly cleared from the soma (arrows). Scale bars: 100 µm in A (applies to B–D,A′–D′); 500 µm in E (applies also to F–H′); 50 µm in E″ (applies also to F″–H″). Anterior is to the left in A–D′ and E″–H″.

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tropharium (Fig. 2F; supplementary material Fig. S3I–L). Two genes (*cta* and *Tao*) were expressed in resting and early oocytes but barely at all in the tropharium (supplementary material Fig. S3M,N), suggesting that these genes may be transcribed by resting oocyte nuclei rather than by nurse cells. Finally, *aret* (aka *bruno*), which is a translational regulator of Oskar in *Drosophila* (Kim-Ha et al., 1995; Webster et al., 1997), was expressed in nurse cells of the posterior tropharium and in early stages of oogenesis but excluded from resting oocytes (supplementary material Fig. S3O), suggesting that it is transcribed by oocyte nuclei after the onset of oogenesis. In summary, although transcripts of most of these genes are likely to be supplied maternally to oocytes, they are not asymmetrically localised within oocytes of any stage.

vasa, *boule* and *tudor* transcripts mark PGCs throughout embryogenesis but are not asymmetrically localised in early embryos

Although none of the genes examined showed asymmetric localisation during oogenesis or early embryogenesis, at late blastoderm stages many of the genes appeared enriched at the posterior pit, where PGCs had previously been identified based on cytological criteria (supplementary material Fig. S1) (Butt, 1949). However, because at this stage of development gastrulation begins at the posterior, this region of the blastoderm is multilayered. Upon close examination, we found that the apparent transcript enrichment was an artifact of tissue thickness for all genes except *vasa*, *tudor* and *boule*, whose transcripts appeared truly enriched in putative PGCs at late blastoderm/early gastrulation stages (Fig. 2D–H″, Fig. 4N,S).

Strikingly, we found that *vasa*, *tudor* and *boule* marked PGCs from the time of their formation at cellular blastoderm stages, but that none of these genes' transcripts were asymmetrically localised prior to PGC formation. Immediately after egg laying, *vasa* transcripts were not localised asymmetrically but rather were ubiquitously distributed throughout the embryo (Fig. 2A). As energid nuclei reached the embryonic surface (Fig. 2B), cytoplasmic islands enriched with these transcripts were distributed evenly across the embryonic surface, remaining there as these energids divided to form the uniform blastoderm (Fig. 2C). Prior to posterior pit formation, *vasa* expression became restricted to putative PGCs at the embryonic posterior (Fig. 2D).

To visualise *vasa* expression in the developing PGCs in greater detail, we collected staged embryos in two-hour intervals over the period during which PGCs arise (19 to 27 hours after egg laying (AEL)), performed in situ hybridisation for *vasa* (Fig. 2E–H), and sectioned the embryos in plastic resin (Fig. 2E″–H″). During this eight-hour period, the blastoderm nuclei undergo two concurrent, dynamic processes: continuing cell divisions increase the nuclear density throughout the blastoderm, and the blastoderm nuclei move towards the posterior pole and ultimately into the yolk (Butt, 1949; Liu and Kaufman, 2004) (Fig. 2E′–H′). From 19–21 hours AEL, the ubiquitous *vasa* expression seen in early embryos remained unchanged (Fig. 2E–E″). However, from 21–23 hours AEL *vasa* expression became enriched in a subset of cells at the blastoderm posterior (Fig. 2F–F″). From 23–25 hours AEL, *vasa*-positive cells increased in density at the blastoderm posterior and began to move into the yolk (Fig. 2G–G″). This movement appeared passive, due to the formation of the posterior pit by invagination of the germ rudiment. However, in the absence of time-lapse data we cannot rule out the possibility of active PGC movement out of the blastoderm epithelium and
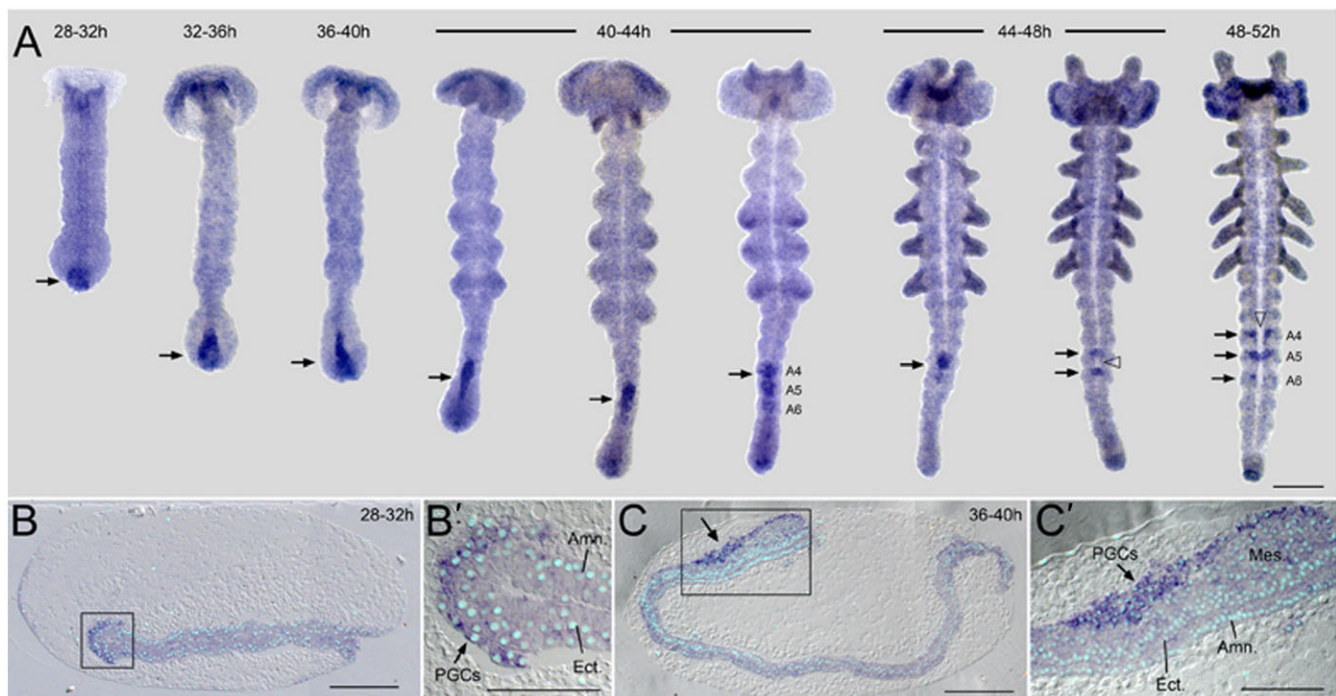

Fig. 3. ***vasa* marks PGCs throughout migration.** (**A**) *vasa* transcript expression during progressive stages of germ band development. Arrows indicate *vasa*-positive PGCs. (**B**) Medial section of an embryo at 28–32 h AEL, showing *vasa* in situ hybridisation (purple) and nuclear stain (cyan). Boxed region enlarged in (**B′**) shows PGCs in contact with ectoderm (*Ect*.) and the amnion (*Amn*.). (**C**) Medial section of an embryo at 36–40 h AEL, when PGCs (arrow) initiate migration along the mesoderm (*Mes*.). Boxed region enlarged in (**C′**). Scale bars: 200 μm in A–C; 100 μm in B′,C′. Anterior is up in A, left in B–C′.

towards the yolk. From 25–27 hours AEL, as the germ rudiment began its invagination into the yolk, *vasa*-positive cells formed a distinct mesenchymal clump within the yolk at the posterior of the embryo (Fig. 2H–H″). During this and all following stages, in addition to the marked enrichment in PGCs, *vasa* transcripts were additionally observed ubiquitously at low levels throughout somatic tissue (Figs 2, 3).

Throughout all subsequent stages of germ band elongation and patterning, *vasa* continued to mark PGCs (Fig. 3). During early stages of germ band elongation prior to limb bud formation (~28–32 hours AEL) *vasa*-positive PGCs remained at the embryonic posterior on the dorsal surface of the newly forming mesoderm (Fig. 3A,B,B′). The PGC cluster then became pear-shaped from 32–42 hours AEL, as the anteriormost PGCs began to move towards the anterior of the embryo (Fig. 3A,C,C′). As the head lobes enlarged (36–40 h AEL), PGCs began to migrate anteriorly on the dorsal surface of the embryo and continued their migration during limb bud stages (40–44 h AEL) (Fig. 3A). During appendage elongation stages (44–48 h AEL) PGCs split into distinct clusters spanning the midline in abdominal segments A4–A6, one cluster per segment. As appendage segmentation became morphologically distinct (48–52 h AEL), the segmental clusters split along the ventral midline into bilateral clusters in A4–A6.

tudor and *boule* were also expressed in PGCs at all stages in a pattern indistinguishable from that of *vasa* (Fig. 4), providing further evidence that the *vasa*-positive cells are *Oncopeltus* PGCs. None of the other genes we examined (supplementary material Fig. S3), including *nanos* and *piwi* (Fig. 4), were enriched in PGCs at any stage of embryogenesis.
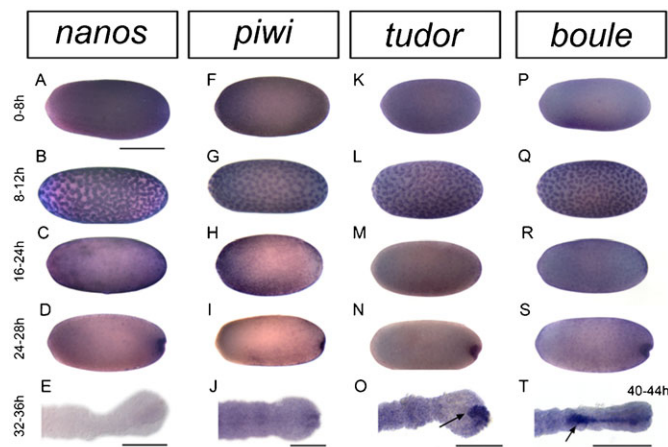

Fig. 4. *Oncopeltus* PGCs express *tudor* and *boule*, but not *nanos* or *piwi*. In early embryos, expression of all four genes remains ubiquitous during energid proliferation (**A,F,K,P**) and blastoderm formation (**B,C,G,H,L,M,Q,R**). During posterior pit formation *nanos* is expressed throughout the length of the embryo (**D**), whereas *piwi* expression is reduced in the presumptive extraembryonic serosal tissue in the anterior of the embryo (**I**). Apparent posterior staining in (D) and (I) is the result of tissue thickness in that location, and is not specific to PGCs. *tudor* (**N**) and *boule* (**S**) transcripts become restricted to presumptive PGCs at the time of their specification. In germ band stage embryos, while *tudor* (**O**) and *boule* (**T**) mark presumptive PGC clusters, *nanos* is not detected (**E**), while *piwi* expression is ubiquitous (**J**). Scale bars: 500 μm in A (applies also to B–D,F–I,K–N,P–S); 100 μm in E,J; 200 μm in O,T. Anterior to the left.

Neither *vasa* nor *tudor* are required for PGC formation

Our gene expression analysis demonstrates that *vasa*, *tudor*, and *boule* are specifically expressed in PGCs beginning at the putative time of their specification at the embryonic posterior just prior to gastrulation. To determine whether these genes were required for PGC formation or development in *Oncopeltus*, we performed maternal RNAi (mRNAi) for each gene. We confirmed that mRNAi effectively reduced zygotic transcript levels in our experiments using RT-PCR (Fig. 5E). PGC presence or absence was determined with in situ hybridisation against PGC markers at ~40–54 hours AEL, when germ cells are visible on the dorsal mesoderm.

RNAi knockdown of *vasa* or *tudor* did not disrupt embryonic patterning or germ band development (supplementary material Table S3), despite the widespread expression of these genes at early blastoderm stages (Figs 2, 4), and their persistent low levels of expression in somatic cells even after PGC formation (Figs 3, 4). Strikingly, germ cells were clearly present in both *vasa* (93.8%, $n=16$) and *tudor* (100%, $n=20$) knockdowns, suggesting that neither of these genes is required for PGC specification (Fig. 5A–C′). It is formally possible that residual *vasa* or *tudor* transcripts that may have escaped destruction by mRNAi could be sufficient to play an instructive role in PGC formation. However, we note that transcript levels of both genes in the progeny of injected mothers were barely detectable in the case of *vasa*, and undetectable in the case of *tudor*, when assessed with RT-PCR even as late as 4 days AEL (Fig. 5E). Moreover, even hypomorphic alleles of *tudor* (Schüpbach and Wieschaus, 1986) and *vasa* (Lasko and Ashburner, 1990; Schüpbach and Wieschaus, 1991; Liang et al., 1994) lead to loss of PGCs in *Drosophila*. We therefore hypothesise that in *Oncopeltus*, *vasa* and *tudor* are required neither maternally nor zygotically for germ cell specification, although they are expressed in the cells specified as PGCs.

To address the possibility of redundancy between these two genes, we performed double knockdown of *vasa* and *tudor*, which reduced transcripts of both genes to undetectable levels (Fig. 5E). Eggs laid by *vasa* + *tudor* double RNAi females had an increased rate of embryonic lethality relative to controls (supplementary material Table S3; 47.4%, $n=19$ vs 10.3%, $n=39$), which may mean that these genes work together to play roles in somatic development. However, embryos that escaped this lethality still had PGCs (100%, $n=10$) (Fig. 5D,D′).

None of the knockdowns caused any qualitative or quantitative change in egg laying by injected females compared to controls, and ovaries of injected females showed neither morphological abnormalities nor signs of disrupted oogenesis (not shown). This indicates that, in contrast to *Drosophila* (Schüpbach and Wieschaus, 1991; Styhler et al., 1998; Tomancak et al., 1998; Johnstone and Lasko, 2004), *vasa* is not required individually or redundantly with *tudor* for *Oncopeltus* oogenesis or egg laying.

boule is necessary for *Oncopeltus* oogenesis and embryonic survival

boule mRNAi caused a complete cessation of egg laying by injected females after four to five clutches (one clutch is laid every one to two days). In contrast, *vasa*, *tudor* and control mRNAi females continued to lay up to 12 clutches. Ovaries of *boule* dsRNA-injected females possessed only a few oocytes at early stages of oogenesis, and few or no mature oocytes (not shown), indicating a requirement for *boule* in the progression of
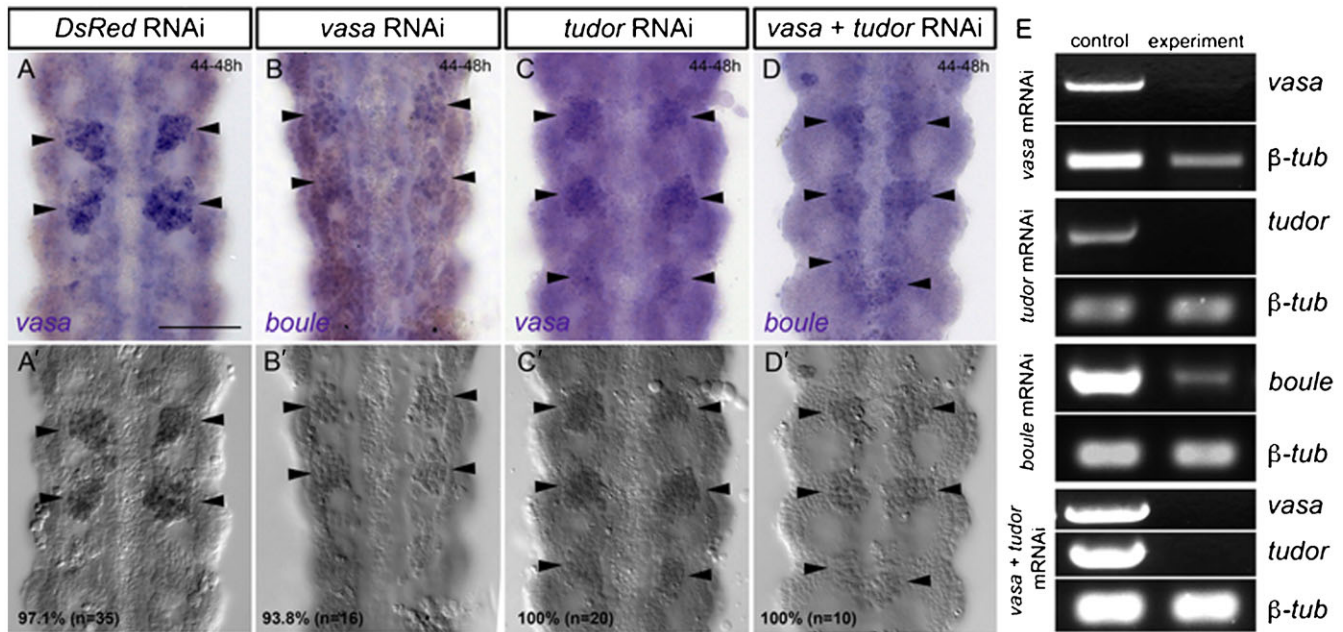
Fig. 5. *vasa* and *tudor* are not required for PGC specification in *Oncopeltus*. (**A–D**) Bright field images of in situ hybridisations for PGC markers in different RNAi conditions; numbers indicate sample sizes and % of embryos with PGCs. Arrowheads indicate PGC clusters in abdominal segments A4–A6. (**A′–D′**) DIC images of the same embryos shown in (A–D) showing distinct PGC cluster morphology. (A,A′) In control embryos *vasa*-positive PGCs are visible on the dorsal surface of abdominal segments 4–5. PGCs are present in *vasa* RNAi (B,B′), *tudor* RNAi (C,C′), and double *vasa + tudor* RNAi (D,D′) embryos. (**E**) RT-PCR validation of RNAi knockdown. Controls are animals injected with *DsRed* dsRNA. Expression of β-tubulin was analysed to confirm cDNA integrity and allow comparison of amounts of template per lane. Scale bar: 100 μm. Anterior is up in A–D.

oogenesis. Eggs laid by *boule* RNAi females displayed nearly complete embryonic lethality (81.8%, $n=22$) in all but the first clutch laid. (The first clutch of *Oncopeltus* eggs laid following mRNAi typically displays no abnormalities, as these eggs have developed their chorion by the time of injection and are therefore impervious to dsRNA (Liu and Kaufman, 2004).) This was a striking increase in embryonic lethality compared to *DsRed* controls (26.8%, $n=190$), *vasa* knockdowns (23.2%, $n=198$) and *tudor* knockdowns (5.6%, $n=54$). The oogenesis requirement for *boule* and resulting embryonic lethality thus prevented us from determining whether *boule* is required for germ cell specification in *Oncopeltus*, and we do not further report on the role of *boule* on oogenesis in the present study.

vasa is required for *Oncopeltus* spermatogenesis

Given that in contrast to *Drosophila*, *vasa* is not required for germ cell specification or oogenesis in *Oncopeltus*, we wished to test for other possible functions of this gene. In mice, despite its expression in the embryonic PGCs of both sexes once they reach the genital ridge (Fujiwara et al., 1994; Diez-Roux et al., 2011). *vasa* is required not for PGC specification, but rather for gametogenesis in males (Tanaka et al., 2000). Similarly, we recently showed that *vasa* plays a role in spermatogenesis in the cricket *G. bimaculatus* (Ewen-Campen et al., 2013). We therefore asked whether *vasa* also functions during spermatogenesis in *Oncopeltus*.

The testes of *Oncopeltus* show an organisation typical of insect testes (Dumser, 1980), with stages of spermatogenesis located in an anterior–posterior progression (supplementary material Fig. S4). Unlike *Drosophila*, which has a single sperm tubule (testiole) per testis (Hardy et al., 1979), each *Oncopeltus* testis

comprises seven testioles (Bonhag and Wick, 1953). In situ hybridisation for *vasa* showed that it is strongly expressed in secondary spermatogonia of each testiole, and at lower levels in early primary spermatocytes and post-spermatocyte stages, but not in primary spermatogonia or somatic cells (Fig. 6A). Adult males injected with dsRNA against *vasa* displayed multiple abnormalities in spermatogenesis. Testioles of *vasa* RNAi males lacked clearly defined cysts and contained large numbers of small, dense nuclei in the anterior region (Fig. 6H,I,I′), which in controls contained only spermatocytes with large, pale nuclei (Fig. 6C,D,D′; supplementary material Fig. S4E). The primary spermatogonial region of *vasa* RNAi testioles contained cysts of irregular size (Fig. 6I, arrowheads) with poorly defined cytoplasmic bridges (Fig. 6I′, arrows). In the spermatocyte region *vasa* RNAi testioles contained large, poorly defined clusters of several hundred cells (Fig. 6J, arrowheads) at varying stages of spermatogenesis (Fig. 6J′). The nuclear morphology of cells in these cysts corresponded to spermatocyte (Fig. 6E; supplementary material Fig. S4E) or early spermatid (Fig. 6F) stages, as well as shell stage-like nuclei (Fig. 6K,K′) typical of the mid-stage spermatids of controls (Fig. 6F′). Cysts of wild type shell stage spermatids are no longer syncytial as the actin-rich cytoplasmic bridges disappear during spermatocyte stages (supplementary material Fig. S4G). In contrast, the anterior shell stage-like nuclei in *vasa* RNAi testioles remained connected by cytoplasmic bridges (Fig. 6K′, red arrows), consistent with precocious spermatid differentiation. Moreover, although they displayed clear shell stage nuclear morphology (Fig. 6K,K′, red arrowheads), they were larger than wild type shell stage nuclei (Fig. 6F′, red arrowheads), suggesting that they had begun spermatid differentiation as syncytial diploid cells without first
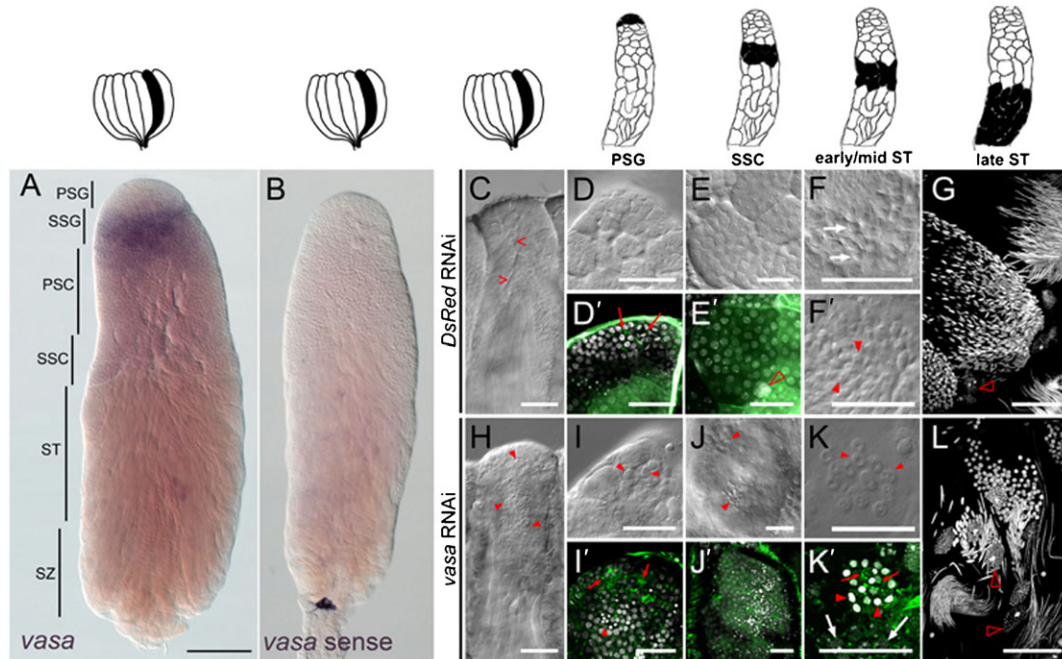
Fig. 6. *vasa* **is expressed in adult testes and required for spermatogenesis in** *Oncopeltus.* Schematics indicate the region of the testis (**A–C**) or testiole (**D–L**) shown in each column. (A) *vasa* in situ of an adult wild-type testiole showing expression in the secondary spermatogonia. PSG: primary spermatogonia; SSG: secondary spermatogonia; PSC: primary spermatocytes; SSC: secondary spermatocytes; ST: spermatids undergoing spermiogenesis; SZ: spermatozoa. (B) *vasa* sense control probe. DIC optics (C–F,F′) and F-actin (green) and nuclear staining (white) (**D′,E′**) of control testioles reveals distinct, synchronized spermatogenic cysts separated by clear cyst boundaries (carets) (C), small cysts of spermatogonia (PSG) at the apex (D,D′), larger cysts of secondary spermatocytes (SSC) posterior to the apex (E,E′; arrowhead in (E′) indicates somatic sheath cells associated with cysts of germ cells), early spermatids with round prominent nuclei (early ST) (F) and mid-stage spermatids with smaller, compact round nuclei (mid ST) (F′). (G) Late spermatid cysts in controls are synchronized in spermiogenesis; hollow arrowheads indicate somatic sheath cells. *vasa* RNAi testioles contain large masses of cells with heterogeneous nuclear morphologies (H; arrowheads). (I) PSG cysts are abnormal in shape and size, contain nuclei of multiple sizes (arrowheads), and (**I′**) have filamentous actin masses interspersed between nuclei (arrows) rather than clearly defined cytoplasmic bridges (compare with D′, arrows). (J,J′) Abnormal cysts contain clusters of small dense nuclei (arrowheads). (K,K′). Aberrant cysts retain cytoplasmic bridges at spermatid stages (red arrows), and contain nuclei with morphologies corresponding to different spermatogenic stages, including both early (white arrows; compare with F) and mid ST (red arrowheads; compare with F′) stages. (L) *vasa* RNAi late spermatid cysts are asynchronous, comprising multiple late spermatid and spermatozoon differentiation stages within a single cyst; cysts remain associated with sheath cells (hollow arrowheads). Scale bars: 200 μm in A (applies to B); 100 μm in C,H; 50 μm in D–E′, G, I–J′, L; 25 μm in F,F′,K,K′. Anterior is up in all panels.

proceeding through meiosis as in wild type. Finally, the posteriormost region of *vasa* RNAi testioles contained irregular groups of cells at mixed stages of late spermatid and spermatozoon differentiation (Fig. 6L), rather than the perfectly synchronised cysts of late spermiogenic stages seen in controls (Fig. 6G). These defects were observed in testes examined 28–29 days following injection of adult males, but are not artefacts of age, as testes of 10 week old wild type adult males showed normal progression through all stages of spermatogenesis (supplementary material Fig. S4B).

Taken together, these data suggest that *vasa* is required for the maintenance of synchrony within cysts at multiple stages of spermatogenesis. In addition, *vasa* may be required for secondary spermatogonia to enter correct meiotic progression as spermatocytes, in the absence of which germ cells are nonetheless able to continue with subsequent stages of spermatogenesis.

Discussion

Oncopeltus germ cell formation
In several cases, analyses of molecular markers such as *vasa* mRNA have revealed the presence of a cryptic germ plasm that had eluded prior histological studies (Yoon et al., 1997; Tsunekawa et al., 2000; Wu et al., 2011). In *Oncopeltus*, we

have shown that none of the transcripts of an extensive suite of conserved germ cell markers localise asymmetrically within oocytes or in early embryos (Figs 1, 2, 4; supplementary material Fig. S3), including transcripts of genes that localise to and are required for the function of germ plasm in *Drosophila*. Gene products of at least one of these conserved germ line markers have been found in the germ plasm of every species where a germ plasm is known to exist (Ewen-Campen et al., 2010), although we note the important caveat that in *Drosophila*, several of these genes (*vasa*, *piwi*, and *tudor*) are localized as proteins rather than mRNAs. Thus, the lack of localisation of transcripts of any of these 19 genes during oogenesis or early embryogenesis suggests that *Oncopeltus* lacks germ plasm. Instead, our data support the hypothesis that *Oncopeltus* germ cells form in the absence of germ plasm, and are not present prior to the onset of posterior invagination at the end of the cellular blastoderm stage. We cannot, however, rule out the possibility that untested molecular markers, including protein products of the genes examined here, could be asymmetrically localised to a putative germ plasm in *Oncopeltus*.

While we provide multiple markers of PGCs, further experiments could be useful to confirm the identity of these cells. However, demonstration that these cells are functional PGCs via ablation experiments is complicated by the fact

that they arise at the inner face of the blastoderm at the gastrulation center, so that their physical disruption would likely also compromise mesoderm formation and subsequent embryogenesis. Moreover, we note that while pole cell removal experiments in *Drosophila* result in sterility, pole cell removal in another insect with germ plasm, the wasp *Pimpla turionellae*, yields fertile adults despite the fact that these pole cells are bona fide PGGs in wild type embryos (Bronskill, 1959; Achtelig and Krause, 1971; Fleischmann, 1975). Further, we are currently unable to genetically ablate these cells and determine their effect on fertility, as our *vasa*, *tudor* and *vasa + tudor* RNAi double RNAi experiments do not disrupt their formation (Fig. 5). Lineage tracing techniques that would permit tracking of the putative PGCs over the six-week period between PGC formation and sexual maturity are not currently available for *Oncopeltus*. These caveats notwithstanding, the molecular and morphological evidence that the cells we identify in this report are bona fide *Oncopeltus* PGCs is comparable to that available for PGC identification in most studied animal species: (1) three conserved germ line genes, *vasa*, *tudor*, and *boule*, are specific germ cell markers in *Oncopeltus* (Figs 1–4); (2) transcripts of these genes first become enriched in germ cells specifically at the time that these cells were previously reported to arise based on morphological and cytological criteria (Figs 2–4) (Butt, 1949); and (3) cells with these molecular markers undergo migration and primordial gonad occupation (supplementary material Fig. S1; Figs 3, 4) consistent with the well-documented behavior of PGCs in many other hemipterans (Seidel, 1924; Mellanby, 1935; Butt, 1949; Sander, 1956; Kelly and Huebner, 1989; Heming and Huebner, 1994).

Although the posterior location of germ cells at the time of their specification is superficially similar to that of pole cells in *Drosophila* and other Diptera, PGC specification and development in *Oncopeltus* differs in several important ways. First, while *Drosophila* pole cells form on the exterior of the posterior syncytial blastoderm before somatic cellularisation, *Oncopeltus* germ cells appear on the yolk side of the cellular blastoderm. Second, while *Drosophila* pole cells are the first cells in the embryo to cellularise (Huettner, 1923), *Oncopeltus* germ cells arise after blastoderm cellularisation is complete (Butt, 1949). Third, because *Oncopeltus* is an intermediate-germ insect, only the gnathal and thoracic segments have been specified at the time that germ cells arise (Liu and Kaufman, 2004), whereas in the long-germ insect *Drosophila*, pole cells form posterior to the abdominal embryonic segments. Lastly, *Oncopeltus* germ cells form on the dorsal surface of the embryo, and remain on the yolk-facing surface of the mesoderm during their migration to the gonad primordium in anterior abdominal segments (Fig. 3). As a result, they do not undergo a transepithelial migration through the hindgut epithelium as in *Drosophila* (reviewed by Richardson and Lehmann, 2010).

The function of "germ line genes" in *Oncopeltus*
Our functional analysis led to the surprising discovery that neither *vasa* nor *tudor* play instructive roles in germ cell specification in *Oncopeltus*. Both of these genes are required for germ cell specification in *Drosophila* (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986) and other species (Sunanaga et al., 2007; Spike et al., 2008). However, *vasa* has widely divergent roles across Metazoa (reviewed by Yajima and Wessel, 2011), and in many cases is dispensable for PGC

specification (Tanaka et al., 2000; Braat et al., 2001; Li et al., 2009; Özhan-Kizil et al., 2009). In several organisms it plays a role in adult gametogenesis (Tanaka et al., 2000; Ohashi et al., 2007; Salinas et al., 2007; Fabioux et al., 2009; Salinas et al., 2012; Ewen-Campen et al., 2013).

Intriguingly, we find that similar to the mouse and the cricket, *vasa* is required for spermatogenesis in adult *Oncopeltus* (Fig. 6), but not for oogenesis. This sex-specific function may relate to a putative role in stem cell function. As in other hemimetabolous insects (Büning, 1994), in *Oncopeltus* germ line stem cells are likely active in the apex of the testes (Schmidt and Dorn, 2004) but are not thought to be present in adult ovaries. One caveat to this hypothesis is that *vasa* transcript was not detected by in situ hybridisation in the primary spermatogonia (Fig. 6A), although it may be present at very low levels in those stem cells. Alternatively, given its strong expression in secondary spermatocytes and the defects in cyst integrity and synchrony caused by *vasa* RNAi (Fig. 6), *Oncopeltus vasa* may play a male-specific role in the onset or synchrony of meiosis. Consistent with a conserved role for *vasa* in bilaterian meiosis, male germ cells in *vasa* knockout mice arrest just prior to meiosis onset (Tanaka et al., 2000), and in human stem cell-derived germ cells *vasa* overexpression enhances meiotic progression (Medrano et al., 2012). *Oncopeltus vasa* RNAi leads to premature spermatid differentiation by some diploid secondary spermatocytes within a cyst, resulting in cyst asynchrony. In *Drosophila*, mutations are known that disrupt meiosis but do not prevent sperm formation (Davis, 1971), consistent with the hypothesis that spermiogenesis can be decoupled from meiotic status.

The evolutionary origins of germ plasm in insects
Together with recent molecular and classical histological data on germ cell specification in other insects, our results are consistent with the hypothesis that germ plasm is a derived mode of germ cell specification that arose in the ancestor to holometabolous insects (Fig. 7) (Lynch et al., 2011; Ewen-Campen et al., 2012). The only other functional genetic analysis of germ line specification in a hemimetabolous insect to date (Ewen-Campen et al., 2013) has also provided evidence that maternally supplied posterior germ plasm is absent, and that *vasa* is dispensable maternally and zygotically for germ cell formation. Our data thus support the notion that germ plasm-driven germ cell specification mechanisms operative in *Drosophila melanogaster* and *Nasonia vitripennis* are derived relative to the Hemimetabola (Fig. 7).

The ubiquitous distribution of germ cell markers in early *Oncopeltus* embryos and their subsequent enrichment in presumptive germ cells at the blastoderm posterior is reminiscent of *vasa* expression in the beetle *Tribolium* (Fig. 7) (Schröder, 2006; C. von Levetzow, Konservierte und divergente Aspekte der *twist*-, *snail*- und *concertina*-Funktion im Käfer *Tribolium castaneum*, PhD thesis, Universität zu Köln, 2008). Further taxonomic sampling, and functional studies in *Tribolium*, will be needed to determine whether the PGC specification mechanisms in these two species may be the result of common ancestry (Fig. 7).

A large number of transcripts that localise to germ plasm in *Drosophila* are expressed ubiquitously in *Oncopeltus* oocytes and early embryos. This suggests that the evolution of germ plasm in Holometabolous insects involved a large-scale change in the localisation of many transcripts in the oocyte. We propose that this likely resulted from a change in the localisation of an upstream component capable of recruiting many downstream
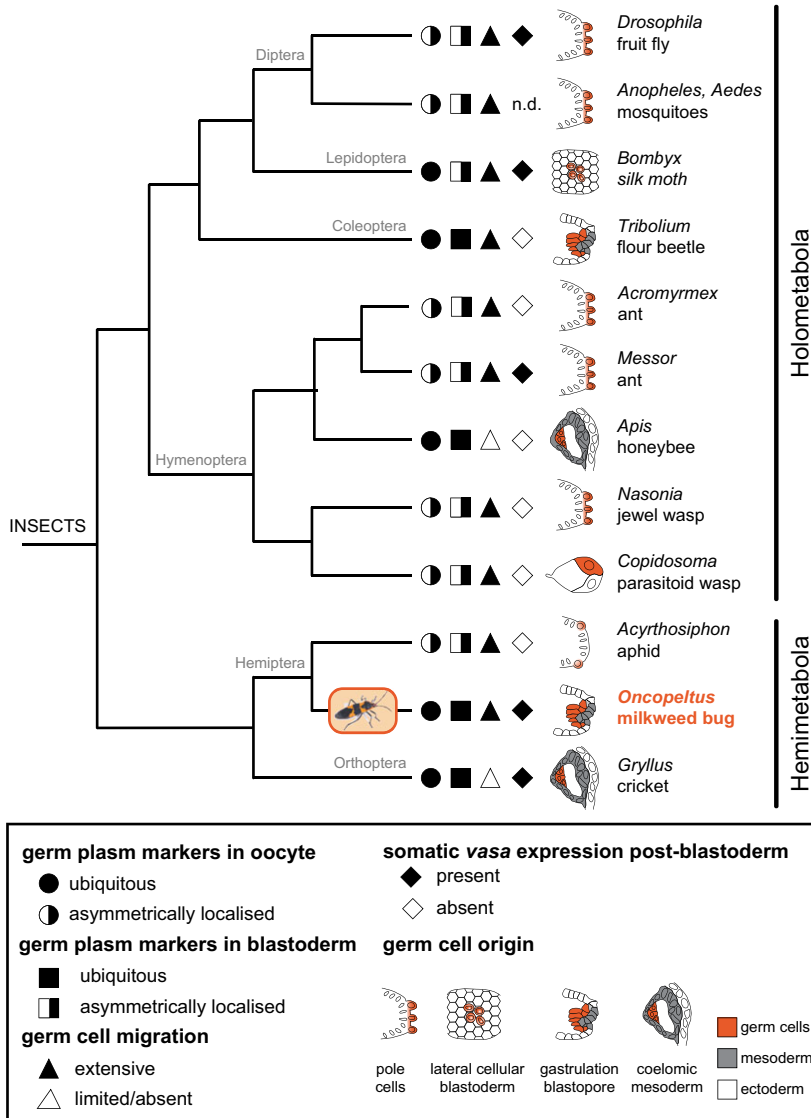
Diptera

Drosophila
fruit fly

Anopheles, Aedes
mosquitoes

Lepidoptera

Bombyx
silk moth

Coleoptera

Tribolium
flour beetle

Acromyrmex
ant

Messor
ant

Hymenoptera

Apis
honeybee

Nasonia
jewel wasp

Copidosoma
parasitoid wasp

Holometabola

INSECTS

Hemiptera

Acyrthosiphon
aphid

Oncopeltus
milkweed bug

Orthoptera

Gryllus
cricket

Hemimetabola

germ plasm markers in oocyte
● ubiquitous
◐ asymmetrically localised

germ plasm markers in blastoderm
■ ubiquitous
◧ asymmetrically localised

germ cell migration
▲ extensive
△ limited/absent

somatic vasa expression post-blastoderm
◆ present
◇ absent

germ cell origin

pole cells | lateral cellular blastoderm | gastrulation blastopore | coelomic mesoderm

■ germ cells
■ mesoderm
□ ectoderm

Fig. 7. Phylogenetic distribution of germ cell specification mechanisms and migration patterns across insects. Species shown are those for which data on the expression and/or function of molecular markers for germ cells during oogenesis and embryogenesis are available. Molecular data suggest absence of germ plasm in oocytes (circles) and early embryos (squares) of some Holometabola (*Tribolium*, *Apis*) and Hemimetabola (*Oncopeltus* and *Gryllus*), and somatic expression of *vasa* at post-blastoderm stages of development (diamonds) is not uncommon. In most species, PGCs undergo extensive migration from the site of specification to the gonad primordia (triangles). Data from this study (also Nakao, 1999; Mahowald, 2001; Donnell et al., 2004; Zhurov et al., 2004; Chang et al., 2006; Dearden, 2006; Juhn and James, 2006; Nakao et al., 2006; Schröder, 2006; Chang et al., 2007; Juhn et al., 2008; Zhao et al., 2008; Chang et al., 2009; Khila and Abouheif, 2010; Lynch et al., 2011; Ewen-Campen et al., 2013; C. von Levetzow, Konservierte und divergente Aspekte der *twist*-, *snail*- und *concertina*-Funktion im Käfer *Tribolium castaneum*, PhD thesis, Universität zu Köln, 2008). Phylogenetic relationships from Yeates et al. (Yeates et al., 2012).

transcripts, rather than via the sequential evolution of distinct localisation mechanisms for individual transcripts. Studies on the genetic mechanism of evolutionary redeployments of multiple downstream genes have largely focused on transcription factors, as individual transcription factors are capable of regulating large numbers of target genes (Hoekstra and Coyne, 2007; Moczek, 2008; Stern and Orgogozo, 2008; Craig, 2009). Interestingly, in the case of germ plasm, transcription factors are unlikely to have been key players in the mechanisms of evolutionary change for a number of reasons. First, regulation of germ line determinants is largely post-transcriptional (Arkov and Ramos, 2010; Richter and Lasko, 2011; Sengupta and Boag, 2012; Nousch and Eckmann, 2013). Second, germ plasm transcript function relies on their subcellular localisation (often mediated via signals in their 3′UTRs) rather than their presence or absence (Rangan et al., 2009). Finally, unlike key transcription factors identified as largely sufficient to induce specific somatic cell fates (e.g. Akam, 1998; Kozmik, 2005; Baena-Lopez and García-Bellido, 2006), there is no single conserved gene that is sufficient to confer germ cell fate across metazoans. The evolution of germ plasm may therefore serve as an example of how a novelty (asymmetrically

localized germ plasm in the oocyte) arose via changes in RNA localisation rather than transcriptional regulation.

Materials and Methods

Animal culture

Oncopeltus fasciatus were cultured at 28℃ as previously described (Ewen-Campen et al., 2011). Timing of embryonic events reported here may differ from that reported in other studies using lower rearing temperatures (e.g. Liu and Kaufman, 2004).

Cloning and phylogenetic analysis

Total RNA was extracted from mixed-stage embryos and ovaries using TRIzol (Invitrogen) and used for first strand cDNA synthesis with qScript cDNA SuperMix (Quanta BioSciences). An *Oncopeltus vasa* fragment was cloned using degenerate primers (supplementary material Table S1). *nanos* and *piwi* fragments were obtained from the *Oncopeltus* transcriptome (Ewen-Campen et al., 2011). Fragments were extended using RACE PCR (SMART RACE cDNA kit, Clontech), and used as templates for DIG-labeled in situ probes and dsRNA fragments following sequence verification (supplementary material Table S1). Genes for the in situ hybridisation screen (supplementary material Tables S1, S2) were obtained from the *Oncopeltus* transcriptome (Ewen-Campen et al., 2011; Zeng and Extavour, 2012) and amplified using primers containing linker sequence (5′-CCCGGGGC-3′) enabling direct addition of a T7 site to the 3′ end in a subsequent PCR reaction. Extended sequences are available from ASGARD (http://asgard.rc.fas.harvard.edu) (Zeng and Extavour, 2012). All coding sequences

reported in this study have been submitted to GenBank [accession numbers KC261571–KC261587] except for *orb* and *Uev1A*, for which we obtained only 3′ UTR sequence.

Maximum-likelihood phylogenetic analysis was performed for *vasa*, *piwi*, and *nanos* as previously described (Ewen-Campen et al., 2012).

Tissue fixation and gene expression analysis

Embryos were fixed and stained as previously described (Liu and Kaufman, 2004; Erezyilmaz et al., 2009; Kainz et al., 2011). Adult gonads were dissected in 1× PBS and fixed in 4% formaldehyde in 1× PBS for at least one hour. Antibodies used were mouse anti-alpha tubulin DM1A (Sigma) 1:50 and goat anti-mouse Alexa Fluor 568 (Invitrogen) 1:500–1:1000, and counterstains were FITC-phalloidin (Invitrogen) 0.5–1 µl and Hoechst 33342 (Sigma) 0.1–0.5 µg/ml.

Plastic sectioning

In situ hybridisation and/or Sytox Green (Invitrogen) staining were performed prior to embedding embryos in Durcupan ACM Fluka (Sigma), mixed at a ratio of 32:27:1:0.6 = components A:B:C:D. Embryos were dehydrated through 10-minute washes in each of 50%, 70%, 90%, 2× 100% ethanol and 100% acetone, transferred to a 1:1 mixture of acetone: catalysed Durcupan, and left uncovered in a fume hood overnight. Embryos were individually transferred to fresh Durcupan in silicone molds (Electron Microscope Sciences NO. 70903) and oriented following a 30-minute initial hardening at 65℃. Resin blocks were baked for 24 hours at 65℃.

Block fronts were trimmed with a razor blade and sectioned at 5–6 µm on a Leica RM2255 microtome with a high-profile knife holder using High-Profile disposable "diamond-edge" steel knives (C.L. Sturkey NO. D554D50). Sections were collected on water droplets on charged slides, dehydrated on a heat block, and mounted in Permount (Fisher Scientific).

Parental RNAi

dsRNA for all genes (supplementary material Table S1) was prepared as previously described (Kainz et al., 2011) and resuspended in injection buffer (5 mM KCl, 0.1 mM NaH$_2$PO$_4$) to a concentration of 2 µg/uL. Male and female adults were injected three days after final molt with 5 µL of 2 µg/uL dsRNA using a Hamilton syringe and size 26 needles. Testes were collected from injected males 27–29 days after injection.

Reverse-transcription PCR

Half of each clutch laid by injected females was fixed for in situ hybridisation, and the other half was homogenised in TRIzol (Invitrogen) and stored at −80℃ before isolation of total RNA. RNA was isolated separately from late blastoderm (24–29 hours AEL), early germ band (24–48 hours AEL) and late germ band (72–96 hours AEL) embryos laid by injected mothers. Genomic DNA was treated with Turbo DNase (Ambion) at 37℃ for 30 minutes, followed by DNase heat-inactivation and phenol/chloroform extraction. cDNA was synthesised from 120 ng of each RNA sample using Superscript III Supermix (Invitrogen). PCR was performed using Advantage 2 DNA Polymerase from 1 µL of cDNA template and primers indicated in supplementary material Table S1 at 60℃ annealing temperature with 35 PCR cycles. RT-PCR results for samples of all three embryonic ages tested yielded indistinguishable results, indicating that maternal RNAi was effective at reducing zygotic transcripts in embryos at least up to four days AEL.

Author Contributions

B.E.-C. and C.G.E. designed the research; B.E.-C., T.E.M.J. and C.G.E. performed experiments, collected and analysed data and wrote the manuscript; C.G.E. obtained funding for the research.

Competing Interests

The authors have no competing interests to declare.

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