

Germ Cell Specification Requires Zygotic Mechanisms Rather Than Germ Plasm in a Basally Branching Insect

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Summary

Background: Primordial germ cell (PGC) specification is a universal process across animals, but the molecular mechanisms specifying PGCs are remarkably diverse. In *Drosophila*, PGCs are specified by maternally provided, asymmetrically localized cytoplasmic factors (germ plasm). In contrast, historical literature on most other arthropods reports that PGCs arise from mesoderm during midembryogenesis, suggesting that an arthropod last common ancestor may have specified PGCs via zygotic mechanisms. However, there has been no direct experimental evidence to date for germ plasm-independent arthropod PGC specification.

Results: Here we show that in a basally branching insect, the cricket *Gryllus bimaculatus*, conserved germ plasm molecules are ubiquitously, rather than asymmetrically, localized during oogenesis and early embryogenesis. Molecular and cytological analyses suggest that *Gryllus* PGCs arise from abdominal mesoderm during segmentation, and *twist* RNAi embryos that lack mesoderm fail to form PGCs. Using RNA interference we show that *vasa* and *piwi* are not required maternally or zygotically for PGC formation but rather are required for primary spermatogonial divisions in adult males.

Conclusions: These observations suggest that *Gryllus* lacks a maternally inherited germ plasm, in contrast with many holometabolous insects, including *Drosophila*. The mesodermal origin of *Gryllus* PGCs and absence of instructive roles for *vasa* and *piwi* in PGC formation are reminiscent of mouse PGC specification and suggest that zygotic cell signaling may direct PGC specification in *Gryllus* and other Hemimetabola.

Introduction

Of the many specialized cell types that comprise an animal's body, only one is capable of contributing genetic information to the next generation: the germ cells. The restriction of reproductive potential to a small subset of cells is a universal process across sexually reproducing animals and represents a profound evolutionary novelty likely required for the evolution of multicellularity [1]. The molecular mechanisms that specify these cells, however, are remarkably diverse between taxa [2–5] and only well understood in a handful of model organisms.

Primordial germ cell (PGC) specification mechanisms have been categorized into two modes: cytoplasmic inheritance and zygotic induction [3, 4, 6]. Cytoplasmic inheritance (e.g.,

in *Drosophila melanogaster*) involves the localization of maternal mRNAs and proteins (germ plasm) to a subcellular region of the oocyte. Germ plasm is necessary and sufficient to induce PGC fate. In zygotic induction (e.g., in *Mus musculus*), by contrast, there is no germ plasm, and PGCs instead form in response to inductive signals from neighboring somatic cells [7].

Within insects, cytoplasmic inheritance appears to be a derived character confined primarily to the holometabolous insects [8] (Figure 1A; see also Table S1 available online), where germ plasm has been demonstrated experimentally in many species (Table S1). Histological studies of insects branching basally to Holometabola (the Hemimetabola), in contrast, have reported the absence of both germ plasm and pole cells in nearly all of these taxa [3, 6] (Figure 1A; Table S1). Studies of molecular markers for PGCs in hemimetabolous insects have been limited to the highly atypical parthenogenetic embryos of the pea aphid *Acyrtosiphon pisum*, a milkweed bug, and several orthopteran species (Table S1), yet there is no conserved pattern of PGC origin across these taxa.

In this study, we use multiple conserved molecular markers and RNAi to characterize PGC formation in the cricket *Gryllus bimaculatus* (Orthoptera), a hemimetabolous model species for studying the development of basally branching insects [9]. We provide several lines of evidence that *Gryllus* PGCs form from the abdominal mesoderm via inductive signaling and discuss the implications of these results for the evolution of germ plasm and the possibility of an ancient relationship between bilaterian PGCs and mesoderm.

Results

Gryllus Germ Cells Express a Suite of Conserved Genes

Within the Orthoptera, neither germ plasm nor pole cells have been reported (Figure 1A; Table S1). Histological examinations of orthopteran embryos conducted by William Wheeler over a century ago [10] suggested that PGCs arise from or among abdominal mesoderm cells during abdominal segmentation (Figure 1C), consistent with reports of germline origin both in other Hemimetabola and in most arthropods [3, 6]. However, conserved molecular markers can reveal a cryptic germ plasm that eludes histological examinations [11–13]. We therefore examined the expression of several conserved molecular PGC markers (*vasa* [14], *piwi* [14], *tudor*, *boule*, and *germ cell-less*) and three additional PIWI family genes (Figures S1A and S1B) in *Gryllus* ovaries and embryos. Because some germ plasm components localize as proteins rather than transcripts (see for example [15, 16, 17]), we also examined the expression of Vasa and Piwi proteins [14].

In fully segmented (stage 9) *Gryllus* embryos (Figures 1B and 1C), we identified cells matching Wheeler's description [10] that express both mRNA and protein of *piwi* and *vasa*, as well as *bol* and *gcl* transcripts (Figures 1D, 1E, and S1C). These cell clusters were found in abdominal segments A2–A3 in all embryos, and in A4–A5 in 45% of embryos (Figures 1D and 1E, arrowheads). Clusters were located on the dorsal medial face of mesodermal structures termed “coelomic pouches,” which are present in every gnathal, thoracic, and abdominal

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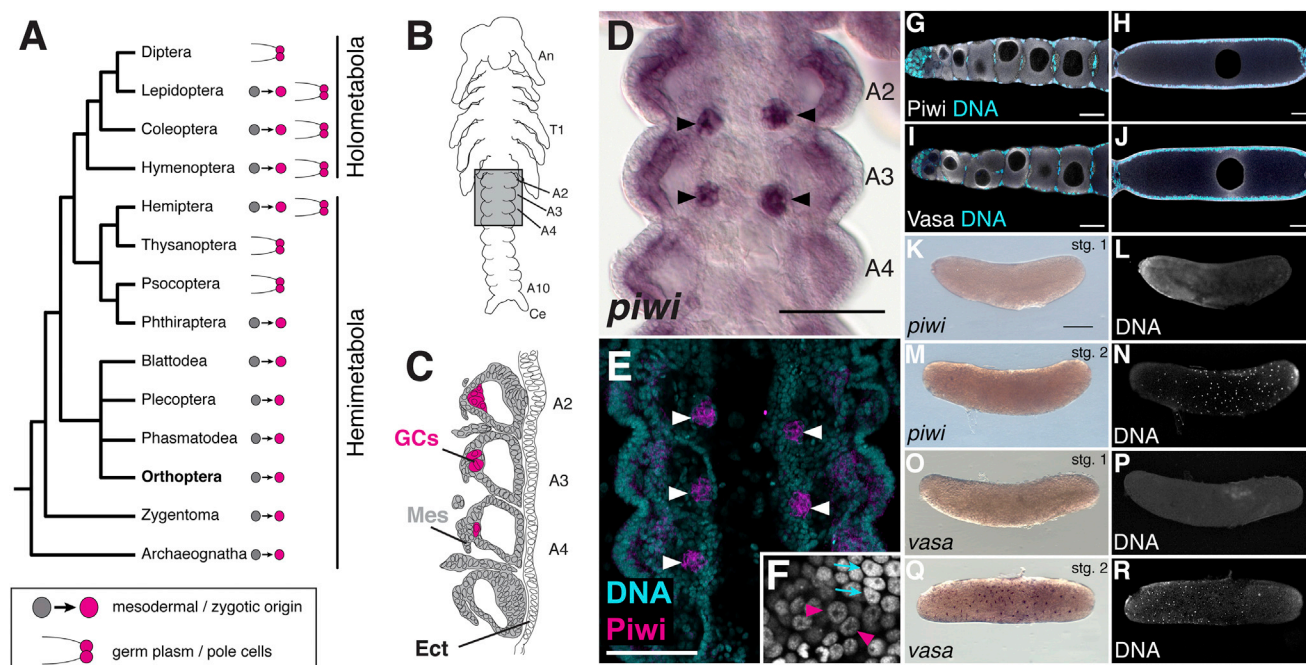


Figure 1. Molecular Markers Suggest Absence of Germ Plasm in *Gryllus*

(A) Phylogenetic distribution of reported PGC specification mechanisms across insects (see Table S1).
(B) Schematic of a stage 9 *Gryllus* embryo, highlighting the region enlarged in (D)–(E) (gray box). A2–A4, abdominal segments 2–4.
(C) Tracing of Wheeler's description of orthopteran PGCs at the earliest time point they could be identified [10]. GCs, germ cells (magenta); Mes, mesoderm (gray); Ect, ectoderm (white).
(D and E) *Gryllus* PGCs (arrowheads) express *piwi* transcripts (D) and protein (E).
(F) PGCs (arrowheads) display nuclear morphology distinct from somatic cells (arrows).
(G–J) Piwi (G and H) and Vasa (I and J) proteins do not localize asymmetrically in the ooplasm.
(K and M) *piwi* transcripts are undetectable during stages 1–2.
(O and Q) *vasa* transcripts are undetectable at stage 1 (O) and associated with all energid nuclei at stage 2 (Q).
(L, N, P, and R) Corresponding nuclear stains.
Scale bar represents 100 μ m in (D) and (E); 50 μ m in (G)–(J); 200 μ m in (K)–(R). Anterior is up in (B)–(F), left in (G)–(R). See also Figures S1, S2, and Table S1.

segment. These cells possessed universal PGC characteristics [3] of large nuclei with diffuse chromatin and a single large nucleolus (Figure 1F). Based on these gene expression, nuclear morphology, and embryonic location data, we conclude that these cells are *Gryllus* PGCs. We also examined the expression of four additional putative candidate PGC marker genes (*tudor*, *piwi-2*, *AGO3-A*, and *AGO3-B*) but found that they were not specific PGC markers in *Gryllus* embryos (Figures S1B and S1F).

Gryllus Germline Markers Do Not Localize within Oocytes or Reveal PGCs in Early Embryos

We next examined the expression of *Gryllus* PGC markers during earlier stages of embryogenesis and oogenesis to test whether they revealed the presence of germ plasm in oocytes or PGCs in early embryos. All genes tested were consistently ubiquitous throughout oogenesis and never localized asymmetrically within the ooplasm (Figures 1G–1J, S1D, and S1F), although Vasa and Piwi proteins were enriched around the oocyte nucleus (Figures 1G–1J). In blastoderm-stage embryos (stages 1–3) and early germband-stage embryos (stage 4), *piwi* (Figures 1K–1N, S1E, and S2G–S2P'), *vasa* (Figures 1O–1R, S1E, and S2Q–S2Z'), *bol*, and *gcl* (Figures S2A–S2F) were expressed ubiquitously at low levels and showed no asymmetric localization within the embryo. These results are in stark contrast to the posterior accumulation of PGC determinants in *Drosophila* oocytes and early embryos [16, 18–20] and

suggest an absence of germ-plasm-driven PGC specification in *Gryllus*.

Gryllus PGCs Arise De Novo during Midembryogenesis

To determine the embryonic origin of *Gryllus* PGCs, we examined the expression of *piwi* and *vasa* transcripts and proteins throughout abdominal elongation and segmentation. During early germband stages (stage 4), we detected low-level ubiquitous expression of both genes in all ectodermal and mesodermal cells (Figures 2A–2B' and S3A–S3B'). It was not until thoracic limb bud enlargement began (stage 5) that *piwi* transcripts were detected at higher levels in two subsets of cells in abdominal segments A2–A4 among the lateral abdominal mesoderm (Figures 2C and 2C'). As appendage elongation began (stage 6), *piwi*-positive cells split into distinct groups along the anterior-posterior axis (Figures 2E and 2E'), and Piwi protein levels rose in these cells (Figures 2E'' and 2E'''). During morphological segmentation of the abdomen (stages 7–9) these cell groups coalesced into four to six distinct clusters adjacent and dorsal to the coelomic pouches in segments A2–A4 and continued to express high levels of *piwi* transcripts and protein (Figures 2F–2H''' and S3D–S3F'''). *vasa* transcript and protein expression was similar to that of *piwi*, but *vasa* became enriched in PGCs slightly later than *piwi* and showed higher expression levels in the soma (Figure S3).

Interestingly, hallmarks of active transcription were observed in PGCs throughout all stages examined (Figures

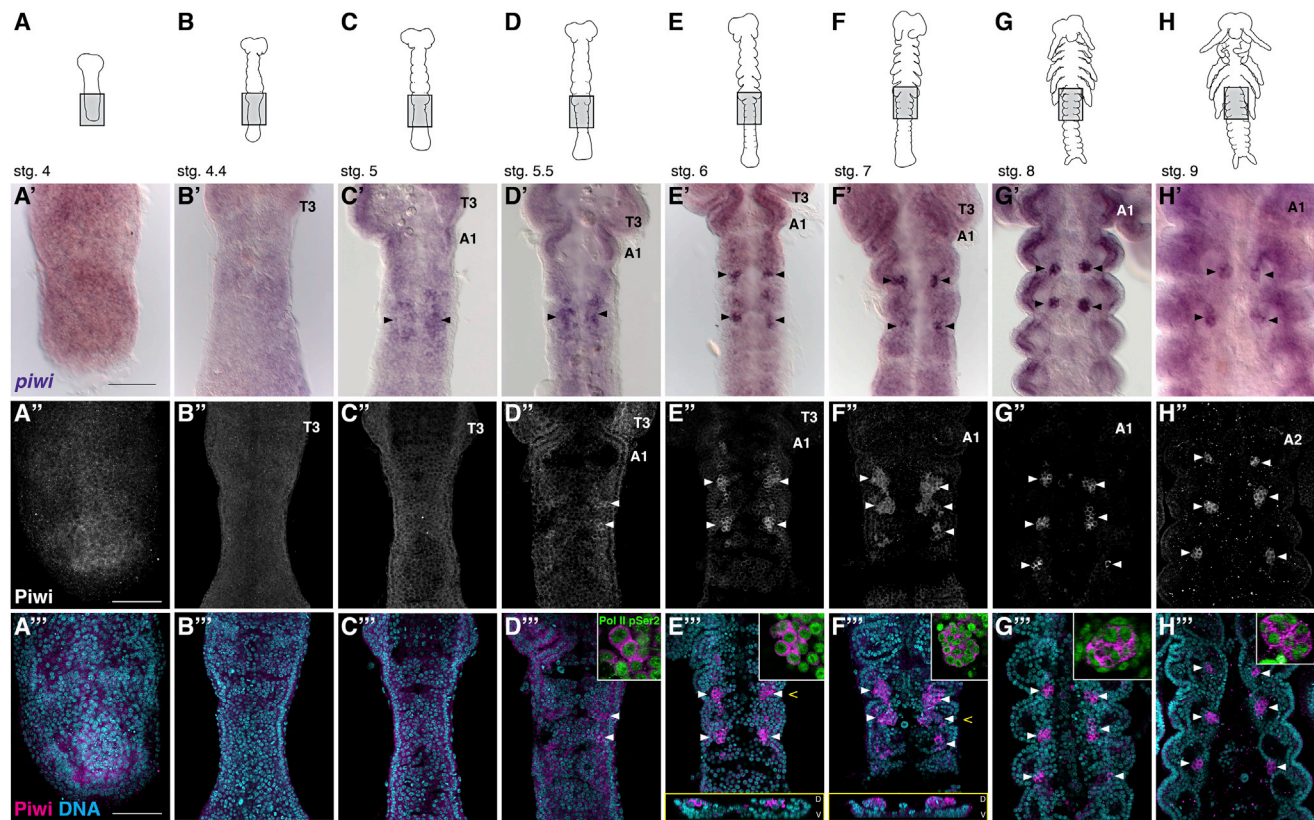


Figure 2. *Gryllus* PGCs Arise during Early Segmentation Stages

Drawings of *Gryllus* embryogenesis (A–H), highlighting the region depicted below (gray boxes). *piwi* transcripts are ubiquitous in stages 4 and 4.4 embryos (A' and B'), but beginning at stage 5 (C'), two bilateral groups of *piwi*-positive cells arise on the dorsal surface of the embryo, then resolve into clusters during later stages in abdominal segments A2–A4 (D'–H'). Piwi protein shows a similar expression pattern to that of *piwi* transcript but is enriched slightly later in development (A''–H''). Piwi (magenta) overlaid on nuclear stain (cyan) reveals that PGCs arise prior to coelomic pouch formation and ultimately reside medial to these mesodermal structures (A'''–H'''). Yellow-framed insets in (E''') and (F''') show orthogonal projections at the position of the caret, illustrating the dorsal location of PGCs. Insets in (D''')–(H''') show expression of RNA polymerase II pSer2 (green) in PGCs. T3, thoracic segment 3; A1 and A2, abdominal segments 1 and 2.

Scale bar represents 100 μ m. Anterior is up in all panels. See also [Figures S2](#) and [S3](#).

2D'''–2H''', insets). This is consistent with *Gryllus* PGC formation via active transcriptional response to inductive signaling between cells rather than PGCs being a transcriptionally quiescent subpopulation of early-segregated cells as seen in *Drosophila* and other species with germ plasm [21].

Consistent with a conversion of presumptive mesoderm cells to PGCs beginning at stage 5, the nuclear morphology of mesodermal cells correlated with the relative levels of Piwi expression throughout development. At stage 4, all mesoderm cells had uniform Piwi expression and nuclear morphology, relatively compact chromatin, and multiple nucleoli ([Figures 3A](#) and [3A'](#)). As Piwi expression increased in presumptive PGCs, their nuclei became larger with increasingly diffuse chromatin, whereas nuclei of neighboring Piwi-poor cells decreased in size, and their chromatin became compact as they progressed through mesoderm differentiation ([Figures 3B–3D](#)). By stages 8–9, PGCs were clearly distinguished by their high nuclear-cytoplasmic ratio, diffuse chromatin, and single large nucleolus ([Figures 3E–3F'](#)), criteria used to identify PGCs in historical studies of Orthoptera and other animals [3]. Following stage 10, PGC clusters merged via short-range cell migration ([Figure 3G](#)) and coalesced into two bilateral gonad primordia ([Figure 3H](#)) located in segments A3–A4. Thus, *Gryllus* PGCs do not undergo long-range

migration, as they do in many other species including *Drosophila* [22], but rather arise near the location of the embryonic gonad.

Knockdown of *Gryllus piwi* or *vasa* Does Not Disrupt PGC Formation or Maintenance

We knocked down *vasa* and *piwi* function using both maternal and zygotic RNAi (mRNAi and eRNAi, respectively) and confirmed knockdown using qPCR and immunostaining ([Figures 4A, 4B, 4E, and 4H](#)). In contrast to *Drosophila*, in which *vasa* and *piwi* are required maternally for embryonic PGC formation [15, 18], mRNAi against *vasa* and *piwi* did not disrupt PGC formation in *Gryllus* embryos ([Figures 4C–4H](#)), and there was no significant difference in the number of PGCs in either *vasa* or *piwi* mRNAi or eRNAi embryos relative to controls ([Figures 4I and 4J](#)). Furthermore, female embryos laid by mothers injected with *vasa* or *piwi* double-stranded (ds)RNA ultimately grew into fertile adults with fully functioning ovaries ([Figure 4K–4M](#)). In contrast to the *Drosophila* requirement for *vasa* and *piwi* in oogenesis and axial patterning [15, 23], *Gryllus* females injected with *vasa* or *piwi* dsRNA displayed no defects in egg laying, oogenesis, or axial patterning ([Figures S4A–S4C](#)). Moreover, double knockdown of *vasa* + *piwi* maternally or zygotically did not

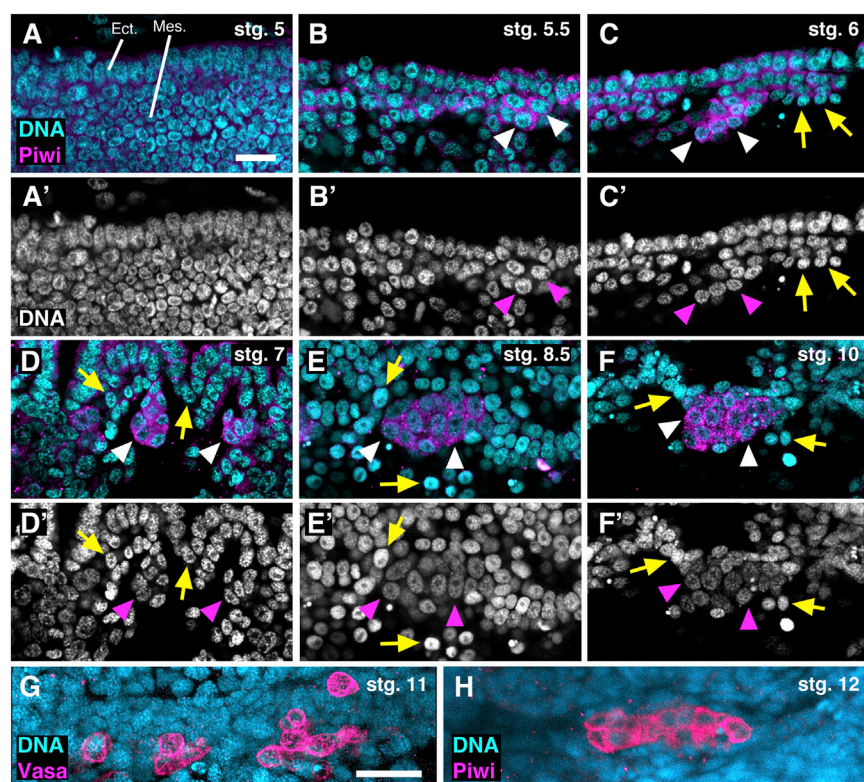


Figure 3. Piwi Expression Correlates with Acquisition of PGC Nuclear Morphology in Abdominal Mesoderm Cells

(A–F') Between stages 5 and 10, Piwi expression increases in PGCs (A–F, arrowheads), and nuclear morphology of Piwi-enriched cells changes accordingly (A'–F', arrowheads). Late stage 5 PGCs have chromatin compaction and multiple nucleoli similar to neighboring mesodermal cells (B and B'). As Piwi enrichment in PGCs increases (arrowheads), their chromatin becomes more diffuse and nuclear size increases (yellow arrows) (C and C'). In subsequent stages chromatin morphology differences become more pronounced (D–F').

(G and H) At stage 11 (G), PGCs commence short-range migration along the anterior-posterior axis toward the intersegmental region of A3–A4 to form a single gonad primordium on each side of the embryo by stage 12 (H).

Scale bars represent 50 μm in (A) (applies to A'–F') and (G) (applies to H). Anterior is to the left.

Mesoderm Is Required for *Gryllus* PGCs

Our observations thus far suggested that PGCs arise from among mesodermal cells during abdominal segmentation. To test this hypothesis, we took advantage of the conserved role of the

disrupt PGC formation or axial patterning (Figures S4C–S4E), indicating that these genes do not act redundantly to direct PGC specification.

vasa and *piwi* Play Roles in *Gryllus* Spermatogenesis

In mice, which lack germ plasm and specify PGCs from presumptive mesoderm via signaling, *vasa* and *piwi* are not required for PGC specification but do mark established PGCs of both sexes and play roles in adult spermatogenesis [24, 25]. We tested whether these genes were required for adult spermatogenesis in *Gryllus* by injecting adult males with dsRNA for *vasa* or *piwi* to achieve paternal RNAi (pRNAi). *Gryllus* testes comprise 200–300 testioles (sperm tubules) [26], within which spermatogenesis proceeds from anterior to posterior (Figures 5A and S5A–S5G). The anterior region of each testiole expresses Vasa and Piwi proteins (Figures S5T and S5U) and contains primary and secondary spermatogonia (Figure 5A). Knockdown of *vasa* or *piwi* via pRNAi severely reduced spermatogonial region length (Figure 5H). In both *vasa* and *piwi* pRNAi testes, meiotic spermatocytes were found in the anterior region of testioles, in some cases almost abutting primary spermatogonia (Figures 5C, 5D, 5F, and 5G, yellow arrowheads), and secondary spermatonial cysts were reduced (Figures 5C, 5D, and 5G, red arrows) or absent (Figure 5F), suggesting that the mitotic divisions of primary spermatogonia were affected. The misregulation of primary spermatocyte divisions was not due to absence of the germline stem cell niche (apical cell), which was present in *piwi* and *vasa* pRNAi testes (Figures 5E–5G, asterisks, and S5B, S5H, and S5N). Postpermatogonial stages of spermatogenesis appeared unaffected (Figures S5I–S5M and S5O–S5S). These data indicate that, as in mice and other animals (see Discussion), *piwi* and *vasa* play a role in *Gryllus* gametogenesis in adult males.

twist gene in mesoderm development [27] to ask whether PGCs could form if mesoderm development was compromised. *Gryllus twist* is expressed in the abdominal mesoderm beginning during axial elongation, including in cells of the region where PGCs arise (Figures S6A–S6D2). In *Drosophila*, *twist* mutants display gastrulation defects [28], yet PGCs form normally because PGC specification occurs via germ plasm well before gastrulation (Figures 6A and 6E). In *Gryllus*, *twist* eRNAi similarly causes disorganization or loss of major mesodermal structures within all body segments (Figures 6F and 6G, compare to 6B and 6C). In contrast to *Drosophila*, however, 49% of *Gryllus twist* eRNAi embryos lack PGCs, compared to 0% of controls ($p < 0.01$, Figures 6D and 6G'), and those *twist* eRNAi embryos that specify PGCs have fewer than controls ($p = 0.05$, Figure 6H). These results are consistent with the hypothesis that PGCs form from a subset of abdominal mesoderm. Alternatively, PGCs may be formed normally at stage 5 but fail to be maintained due to absent or compromised mesodermal surroundings.

Discussion

We have shown that neither *vasa* nor *piwi* are required maternally or zygotically for the formation of functional PGCs (Figures 4 and S4) but instead play a role in spermatogonial divisions in adult males. Our results differ from those of analogous experiments in *D. melanogaster* [15, 18], indicating that the functions of these genes have diverged between *Gryllus* and *Drosophila*. Although these genes are not required for *Gryllus* PGC formation, we propose that, together with *gcl* and *boule* expression (Figure S2) and the transition from mesodermal to PGC-like morphology in situ (Figure 3), *vasa* and *piwi* are nevertheless informative *Gryllus* PGC markers, despite their pleiotropic roles in other developmental

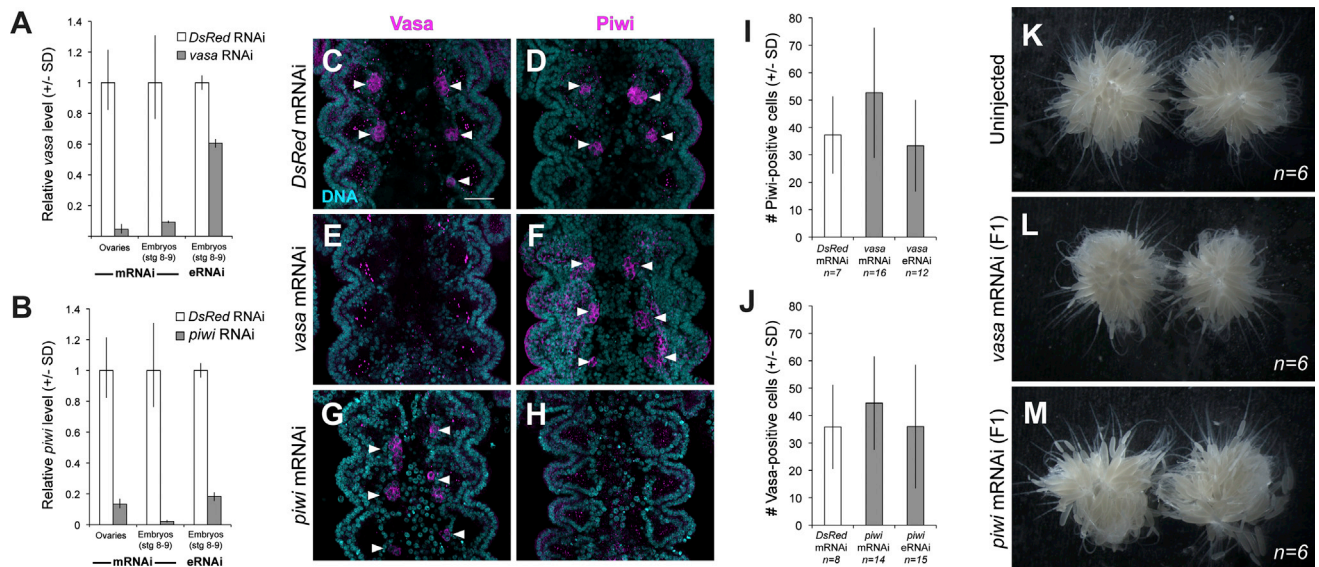


Figure 4. *vasa* and *piwi* Are Not Required for *Gryllus* PGC Specification

(A and B) qPCR validation of *vasa* and *piwi* knockdown following mRNAi and eRNAi.

(C–H) Piwi-positive PGCs (arrowheads) form in *vasa* RNAi embryos, and Vasa-positive PGCs form in *piwi* RNAi embryos. Consistent with qPCR results, *vasa* mRNAi (E; 100%, $n = 9$) and *piwi* mRNAi (H; 60%, $n = 10$) abolished respective protein expression. eRNAi produced similar results (not shown).

(I and J) PGC quantification confirms that PGC formation is not reduced (Student's t test: *vasa* mRNAi $p = 0.07$; *vasa* eRNAi $p = 0.57$; *piwi* mRNAi $p = 0.24$; *piwi* eRNAi $p = 0.77$).

(K–M) Ovaries from adult offspring of *vasa* and *piwi* pRNAi mothers (L–M) are indistinguishable from uninjected controls (K). Scale bar represents 50 μ m in (C)–(H).

See also Figure S4.

processes. We cannot eliminate the possibility that untested marker genes might show an earlier PGC specification event than the one we identify in stage 5 (Figure 2C). However, given the conserved coexpression of the tested genes in PGCs of

multiple metazoans, we believe it unlikely that all four would be absent from *Gryllus* PGCs at the time of their specification.

Evidence from multiple systems suggests that functional divergence of *vasa* and *piwi* is widespread. In

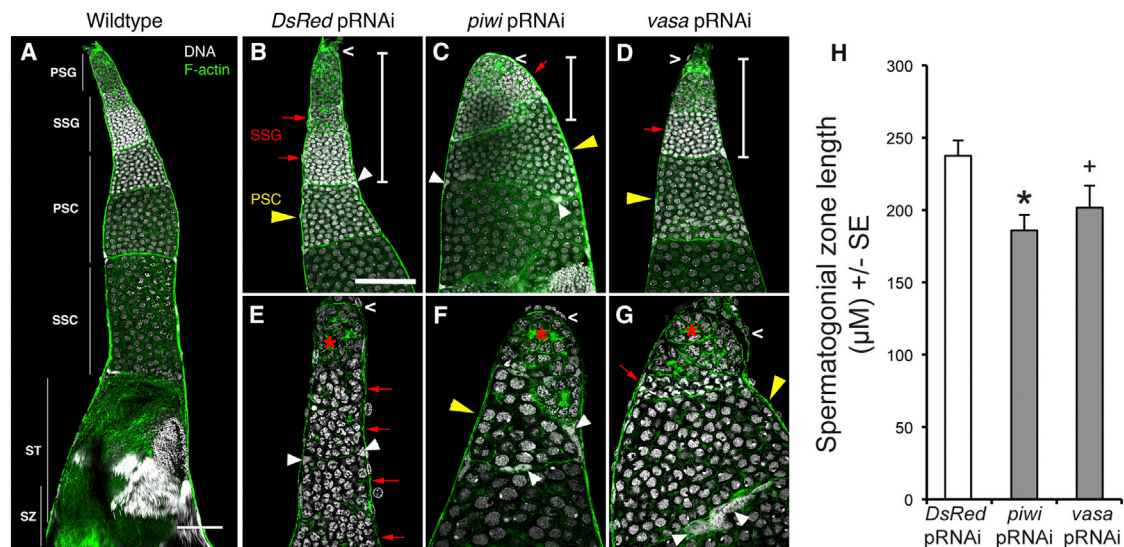


Figure 5. *piwi* and *vasa* pRNAi Causes Defects in Spermatogonial Proliferation

(A) Wild-type *Gryllus* testis showing the stages of spermatogenesis.

(B–G) White bars in (B)–(D) indicate the spermatogonial zone containing secondary spermatogonia (SSG, red arrows). The zone of primary spermatocytes (PSC, yellow arrowheads) nearly abuts the primary spermatogonial zone in *piwi* (C and F) and *vasa* (D and G) pRNAi testes because of the shortened SSG zone but is absent from the anterior region of control testis that have extensive SSG populations (B and E). Higher magnification (E–G) is shown of anterior testis regions in control (E), *piwi* RNAi (F), and *vasa* RNAi (G) testes.

(H) *vasa* or *piwi* paternal RNAi results in a shortened spermatogonial zone compared to controls (Student's t test: $*p < 0.01$, $+p = 0.06$).

Scale bar represents 100 μ m in (A) and 50 μ m in (B) (applies also to C–G). Anterior is up in (A)–(G). See also Figure S5.

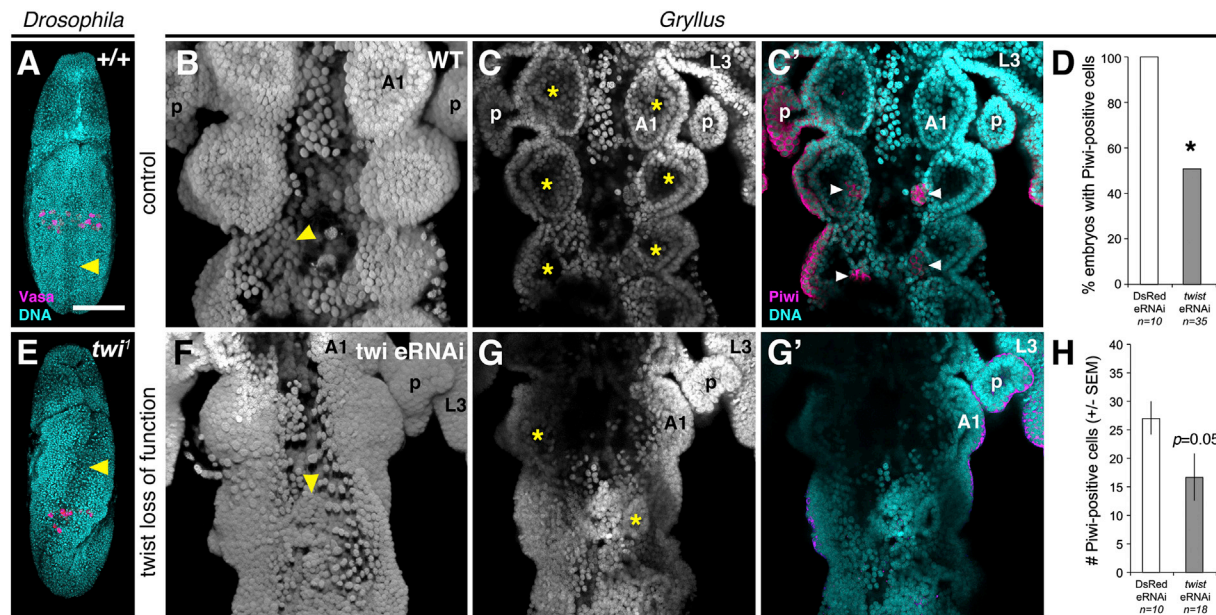


Figure 6. *twist* eRNAi Disrupts *Gryllus* PGC Formation

*Drosophila twist*¹ embryos display gastrulation defects (A and E; arrowhead indicates ventral furrow) but specify PGCs (magenta) properly. In comparison to wild-type (B–C'), *Gryllus twist* eRNAi embryos (F–G') have disorganized mesoderm (F, arrowhead), show absent or rudimentary coelomic pouches (G, asterisks), and fail to specify PGCs (G') significantly more often than controls (D; Fisher's exact test, **p* < 0.01). When they do specify PGCs, *Gryllus twist* eRNAi embryos have fewer PGCs than controls (H; Student's *t* test, *p* = 0.05).

Scale bar represents 50 μ m and applies to all panels. L3, third thoracic leg; A1, first abdominal segment; p, pleuropodia. Anterior is up. See also Figure S6.

D. melanogaster, where both genes were first discovered, mutations in *vasa* or either of the two *piwi* orthologs (*piwi* and *aubergine*) cause defects in germ plasm formation, oogenesis, PGC specification, and posterior patterning [15, 18, 19, 29]. Similarly, *vasa* and *piwi* orthologs are required for PGC specification, development, and oogenesis in *C. elegans*, *D. rerio* [see 2], and medaka [30]. In mice, however, *vasa* is expressed in embryonic PGCs of both sexes, but *vasa*^{−/−} homozygotes display no discernable defects in PGC specification or oogenesis and instead show a male-specific defect in spermatogenesis [25]. Similarly, knockout mice for any of the three PIWI family homologs display spermatogenic defects only, with no defects in females [24, 31, 32]. Our data therefore suggest that the roles of *Gryllus vasa* and *piwi* are similar to those of their mouse homologs. Functional genetic and gene expression data from insects (Table S1) suggest that, in this clade, an instructive role for these genes in PGC formation may be restricted to the Holometabola, perhaps concomitant with the co-option of *oskar* to the top of the PGC specification pathway [14]. Consistent with this hypothesis, *vasa* is dispensable for PGC formation in another hemimetabolous insect, the milkweed bug, *Oncopeltus fasciatus* [33].

Our data indicate that a zygotic mode of PGC specification is likely present in *Gryllus*, whereby PGCs appear to arise from presumptive mesoderm. Because *twist* is expressed broadly in mesodermal cells (Figure S6), our *twist* RNAi results could indicate either that mesoderm gives rise to PGCs directly or that mesoderm is required to maintain PGCs (we note that these interpretations are not mutually exclusive). However, our morphological (Figure 3) and gene expression (Figures 2, S3, and S6) analyses strongly suggest that cells convert from mesodermal to PGC fate in situ. Alternatively, an undifferentiated population of PGC precursors could exist that do not express any of the tested PGC marker genes but are induced

to adopt PGC fate by adjacent mesodermal cells. If this is the case, however, we note that such pluripotent precursors cannot require maternal provision of *vasa* or *piwi* and would most likely be specified by zygotic mechanisms.

Several lines of evidence suggest that a cell lineage relationship between mesoderm and the germline may be a cell type association predating the emergence of Bilateria. Bilaterian germ cells are strikingly similar in gene expression and cytological characteristics to endomesodermally derived stem cells in bilaterian outgroups. Whereas nonbilaterians do not have a dedicated germline per se, their pluripotent stem cell populations serve the function of the germline (reviewed in [34]), and cnidarian pluripotent stem cells are derived from endomesoderm during embryogenesis [35–39]. Within bilaterians, gametogenic cells are consistently described as arising from gonadal epithelia of mesodermal origin in most arthropods and many marine invertebrates (reviewed in [3]). In many spiralian, cytological, cell lineage, and molecular data indicate that PGCs originate from a multipotent mesodermal precursor or precursors (see also [3, 40–47]). Recent studies suggest that mouse PGCs may default to a mesodermal specification program if germline induction signals are absent [48, 49]. The work presented here illuminates broad similarities between PGC specification and *vasa* function in *Gryllus* and in the mouse. Future work will be required to explore this apparent similarity in greater depth and to determine the extent of conservation in the developmental and molecular processes involved in specifying the germline across Bilateria.

Experimental Procedures

Gryllus husbandry, gene expression analysis, mRNAi, eRNAi, and phenotypic analysis were carried out as previously described [50]. For pRNAi, 5 μ l of 3 μ g/ml dsRNA was injected into the coelomic cavity of adult males

1–3 days after the final molt to sexual maturity, and testes of injected males were dissected for analysis 7 days after injection (details in [Supplemental Experimental Procedures](#)).

Accession Numbers

Sequences have been deposited in GenBank (accession numbers KC242803–KC242808).

Supplemental Information

Supplemental Information includes six figures, Supplemental Experimental Procedures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.03.063>.

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Supplemental Information

**Germ Cell Specification Requires
Zygotic Mechanisms Rather Than
Germ Plasm in a Basally Branching Insect**

Ben Ewen-Campen, Seth Donoughe, Donald Nat Clarke, and Cassandra G. Extavour

Supplemental Inventory

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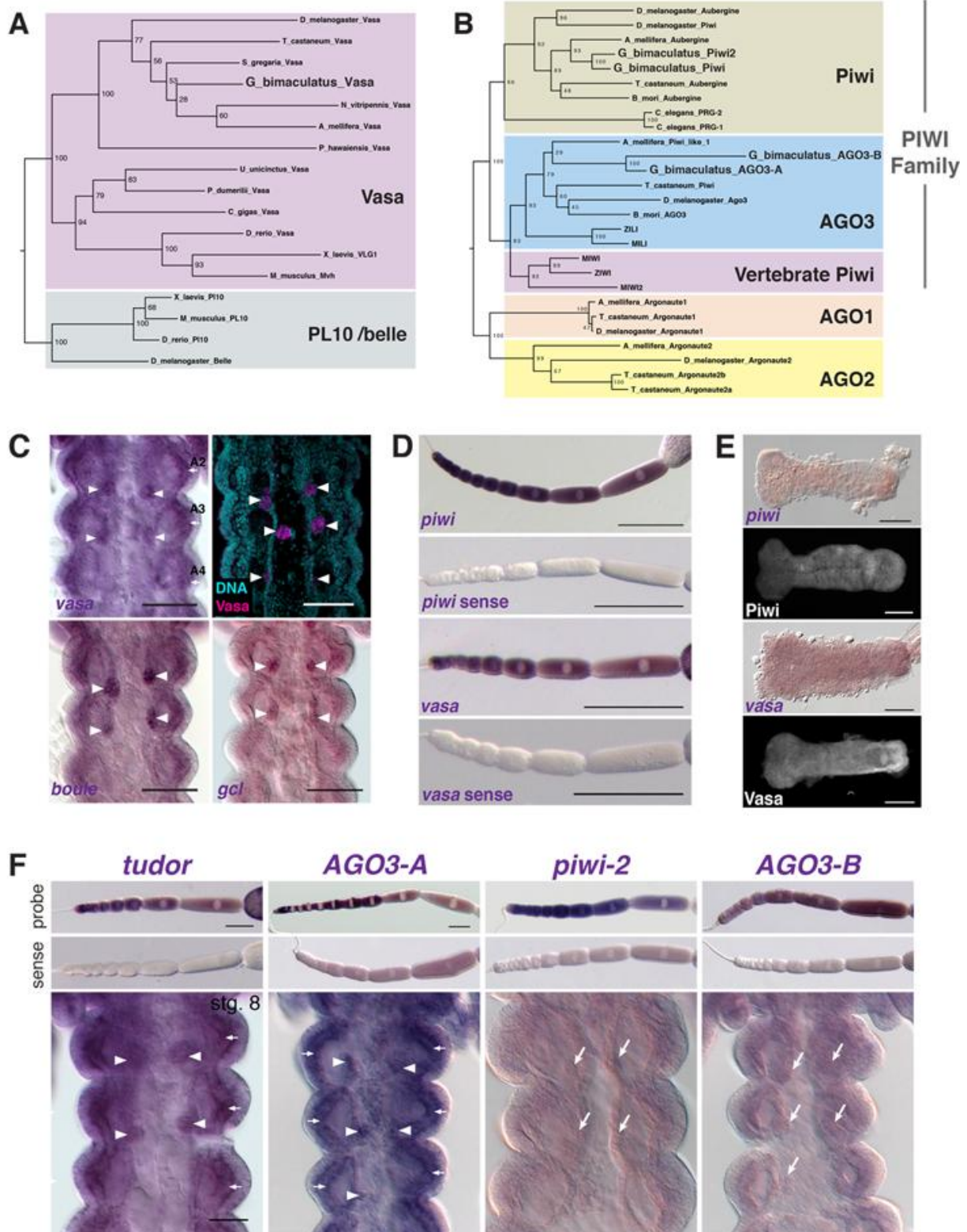


Figure S1, Related to Figure 1. Phylogenetic Analysis of *Gryllus* Germ Line Genes, and Expression Patterns of Additional Germ Line Marker Candidates

(A and B) Maximum-likelihood phylogenetic reconstruction of Vasa and Piwi amino acid sequences. (A) As previously reported [1], *Gryllus* Vasa falls clearly within other insect *vasa* genes, not the PL10/Belle class of RNA helicases. (B) *Gryllus* possesses two *piwi*-like genes and two AGO3-like genes, both of which represent species-specific duplications. As only the first identified *piwi*-like gene [2] was enriched in *Gryllus* PGCs, we focus the present analyses on this orthologue, which we refer to here simply as *piwi* as it is clearly orthologous to other animal *piwi* genes. Note that *aubergine* is a *Drosophila*-specific duplication of *piwi*.

(C) *Gryllus* PGCs (arrowheads) express high levels of Vasa protein and transcripts of *vasa*, *boule* and *germ cell less*. All genes are also expressed at lower levels throughout the somatic tissues of the embryo.

(D) *piwi* and *vasa* transcripts are expressed ubiquitously during all stages of oogenesis and do not localise to the posterior ooplasm.

(E) *piwi* and *vasa* transcripts and protein products are expressed ubiquitously in stage 4 embryos. The apparent increased expression levels at the germ band posterior are an artifact of tissue thickness.

(F) top row: *tudor*, *AGO3-A*, *piwi-2*, and *AGO3-B* transcripts are not localized asymmetrically in oocytes. Bottom row: these genes do not specifically label *Gryllus* PGCs. In the PGC-containing region (Figure 1B-E) at stage 9, *tudor* and *AGO3-A* are detectable in PGCs (arrowheads), but are also expressed throughout the somatic tissues of the embryo (arrows). *piwi-2* and *AGO3-B* are not detected above background levels in stage 8-9 embryos. Arrows mark PGCs recognisable based on morphology and anatomical position independent of gene expression. Anterior is to the left in D and top two rows row of E, and up in C and bottom row of E. Scale bars = 100 μ M in C, 500 μ M in D; 200 μ M in E and top two rows of F; 50 μ M bottom row of F.

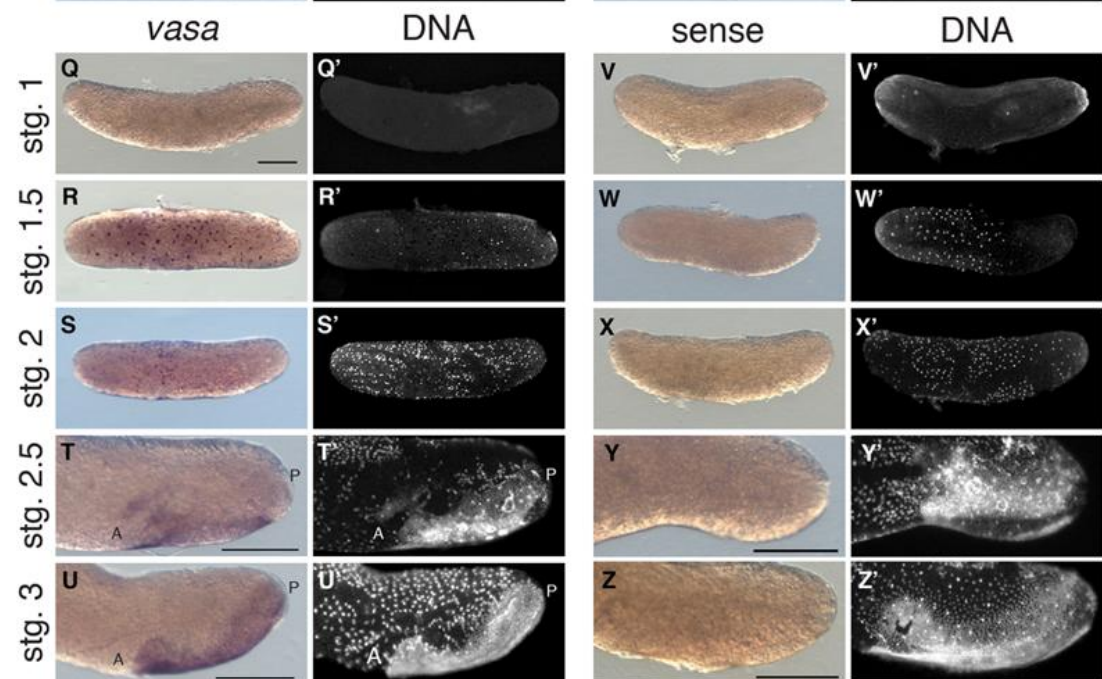
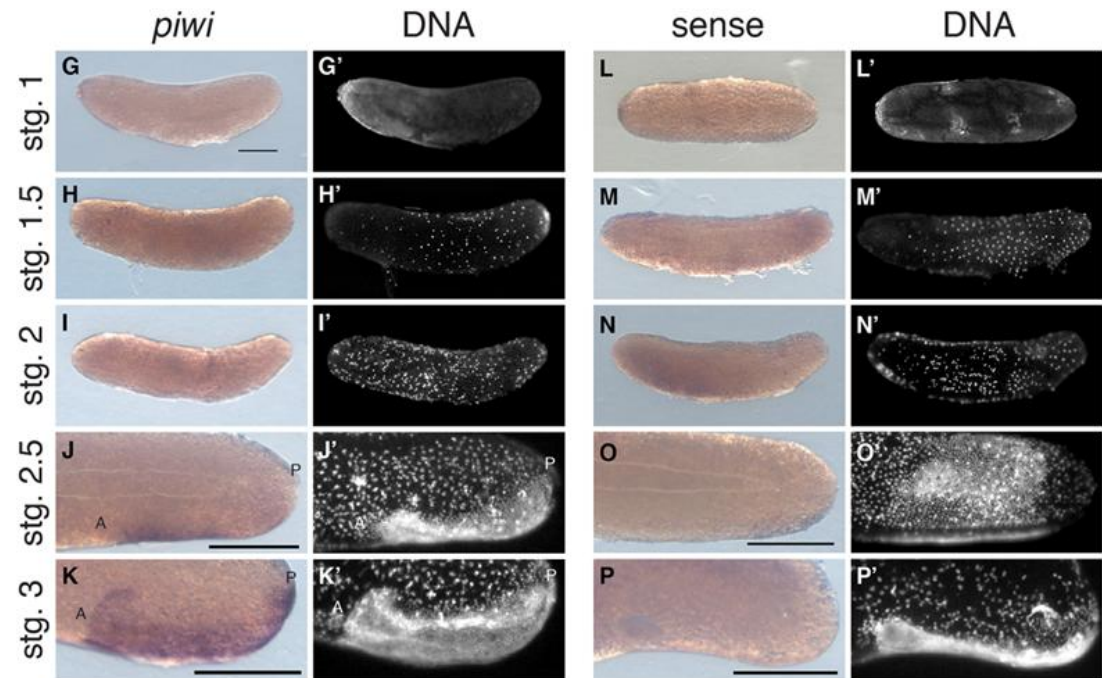
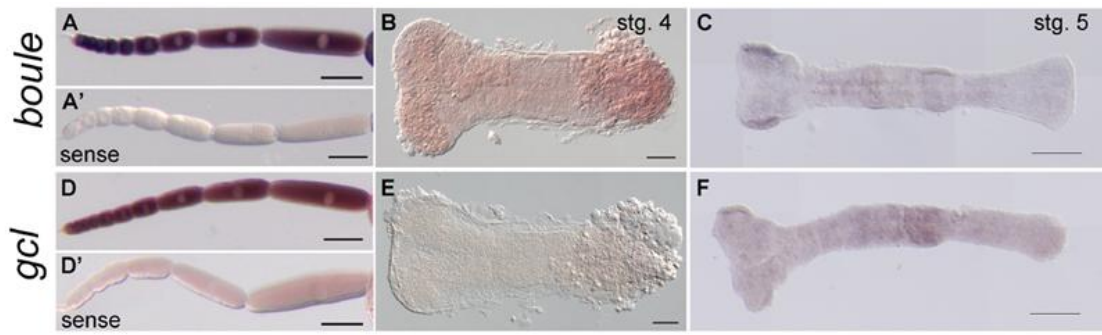


Figure S2, Related to Figures 1 and 2. Additional Gene Expression Data in Support of the Absence of Germ Plasm in *Gryllus*

(A–F) Expression of *boule* and *gcl*, which mark PGCs in stage 9 embryos (Figure 1) during oogenesis and early embryogenesis. Neither gene is asymmetrically localized in oocytes (A and D; A' and D' show sense controls). Both genes are expressed ubiquitously during stages 4 (B and E) and 5 (C and F), and do not reveal any segregated PGCs during these stages.

(G–Z) *piwi* (G–K) and *vasa* (Q–U) expression during blastoderm stages. (L–P and V–Z)

Corresponding sense controls. (G'–Z') Nuclear stains of adjacent panels. *piwi* transcripts are undetectable during blastoderm stages (G–I), and are found ubiquitously at low levels as the germ band condenses (J and K). *vasa* transcripts are undetectable in just-laid eggs (Q), and energids are associated with all nuclei along the A–P axis as they populate the blastoderm surface (R). During subsequent blastoderm divisions (S), *vasa* expression is not localized to any specific subset of nuclei. As the germ band condenses at the posterior of the egg (T, U), *vasa* expression is detected at similar, low levels throughout the germ band but not enriched at the posterior or in any other specific region. A = germ band anterior, P = germ band posterior. Scale bars = 200 μ M in (A)–(A') and (D)–(D'); 100 μ M in (B) (applies also to E); 200 μ M in (C) (applies also to F), (G), and (Q) (applies also to H–I and R–S, respectively), and (J)–(K), (O)–(P), (T)–(U), and (Y)–(Z). Anterior is to the left in all panels.

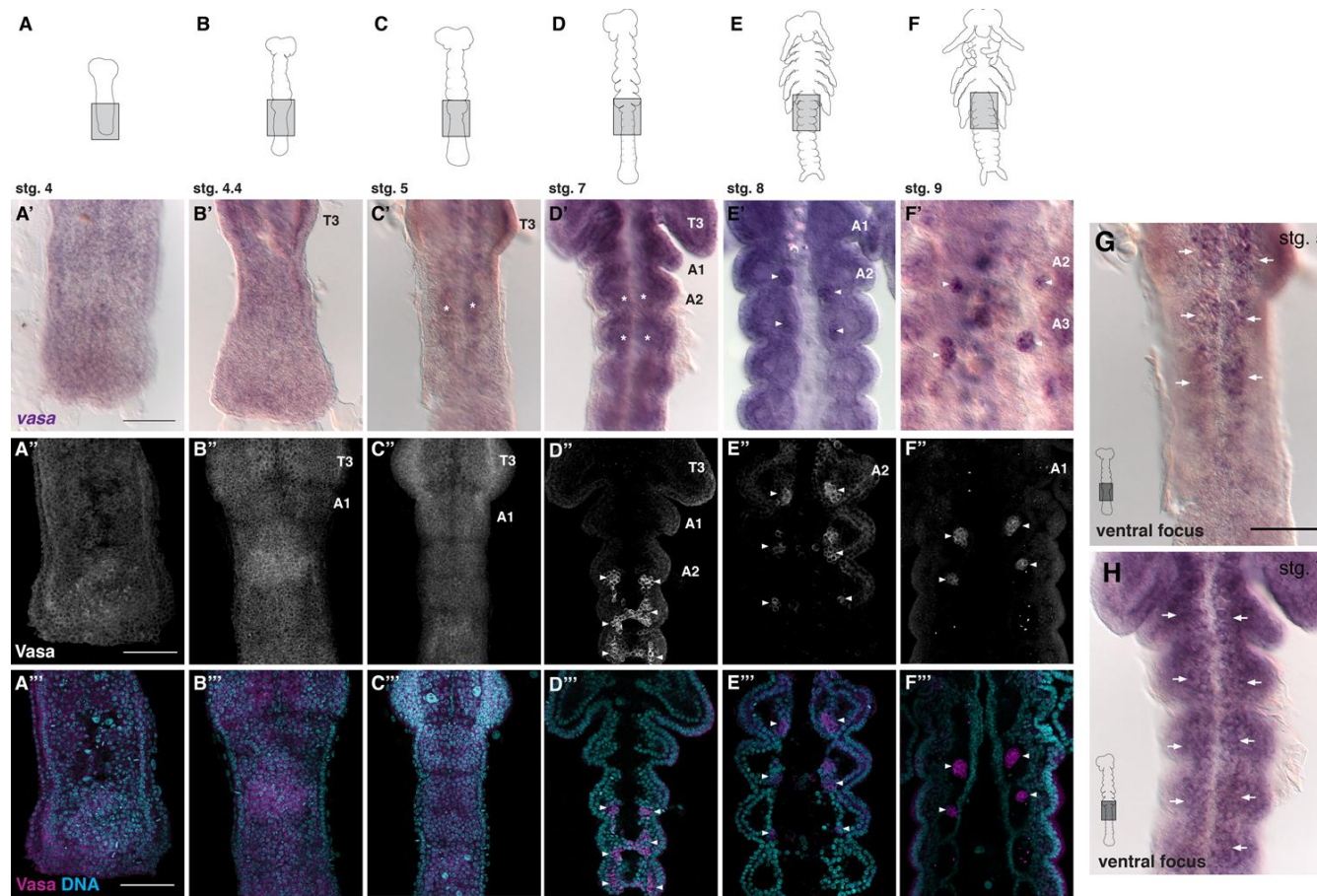


Figure S3, Related to Figure 2. Expression of *vasa* Transcript and Protein throughout Abdominal Segmentation

(A–F) Schematic drawings of *Gryllus* mid-staged embryos of the stages shown here; boxed grey areas indicate regions shown in panels below. *vasa* transcripts are expressed ubiquitously during stages 4–4.4 (A and B), and do not reveal the presence of PGCs at this stage, consistent with *piwi* expression. (C and D) During stages 5 and 7 *vasa* transcripts do not reveal the presence of PGCs. Astrices in (C' and D') denote out-of-focus staining in the ventrally located nervous system, which is shown in focus in (G–H). *vasa* transcripts are detected in PGCs during stage 8 and 9 (E and F). *Vasa* protein is ubiquitously expressed during stages 4–5 (A'–C', A''–C''). In stage 7 embryos (D' and D''), *Vasa* protein is strongly enriched in PGCs, and this expression continues in stage 8 and 9 (E'–F' and E''–F''). Arrowheads indicate PGC clusters. T3 = thoracic segment 3; A1, A2 = abdominal segments 1 and 2. Scale bar = 100 μM. Anterior is up in all panels.

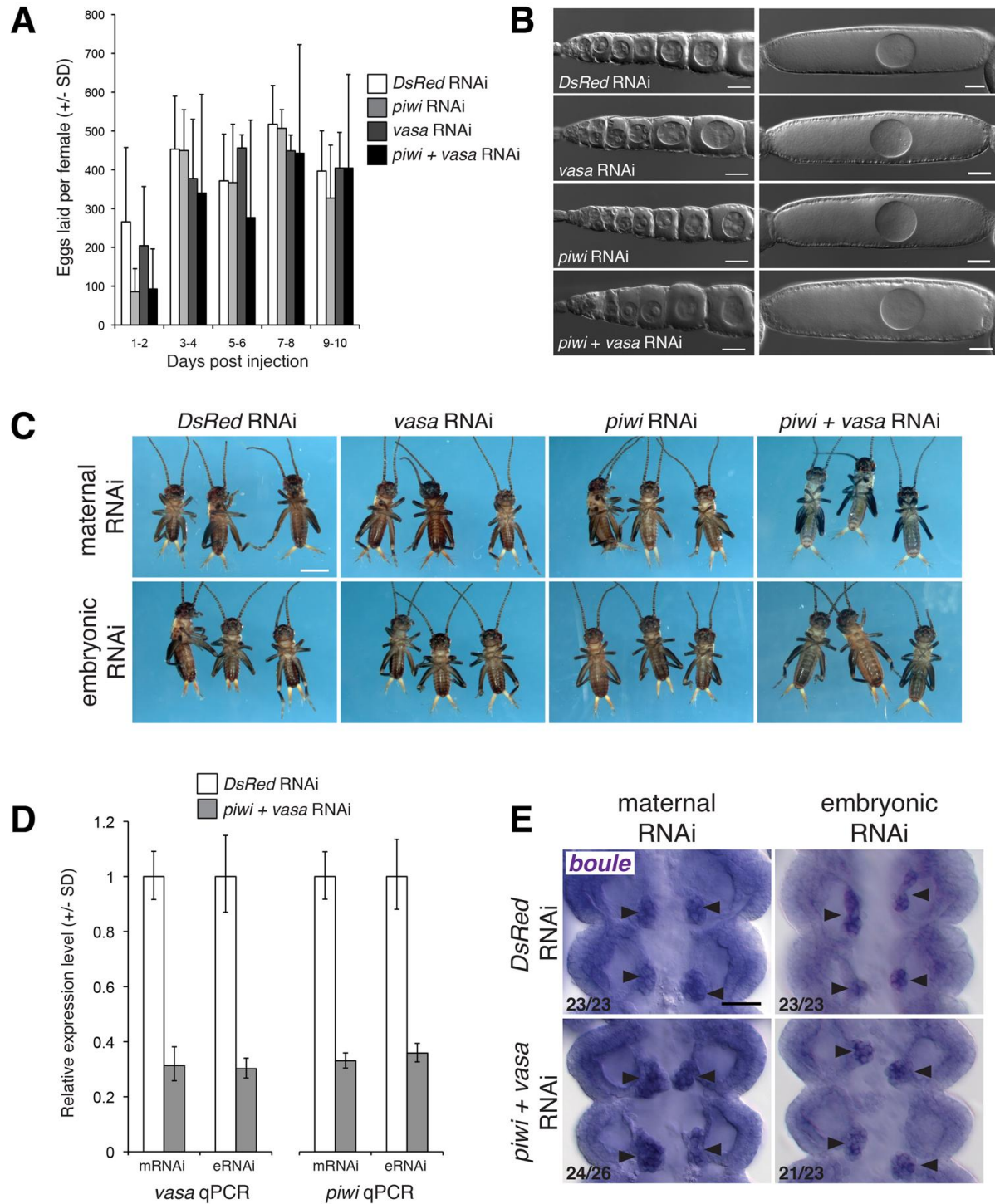


Figure S4, Related to Figure 4. Phenotypic Analysis of *vasa* and *piwi* mRNAi, and *vasa* + *piwi* Double eRNAi and mRNAi, in Ovaries and Embryos

(A) Females injected with dsRNA against *vasa* or *piwi* lay numbers of eggs that do not differ significantly from controls (student's t-test, $p > 0.05$ in every pairwise comparison of *vasa* or *piwi* RNAi with *DsRed* RNAi on the indicated days post-injection).

(B) Ovaries dissected from *vasa* or *piwi* mRNAi females 10 days after injection are morphologically wild type and contain normal oocytes at all stages of development, despite having transcript levels reduced to less than 5% or 10% of wild type levels, respectively (Figure 6A–6B).

(C) Hatchlings of embryos laid by mRNAi or eRNAi females do not display axial patterning defects, and appear morphologically wild type.

(D) qPCR validation of *vasa* + *piwi* double knockdown following mRNAi and eRNAi. Transcript abundance was normalized to expression of *beta-tubulin*.

(E) As in *DsRed* RNAi controls (top), *boule*-positive PGCs form in *vasa* + *piwi* double RNAi embryos (bottom). Numbers at bottom left indicate sample sizes. Scale bars = 50 μ M in (B) and (E); 2 mM in (C). Anterior is to the left in (B); up in (C) and (E).

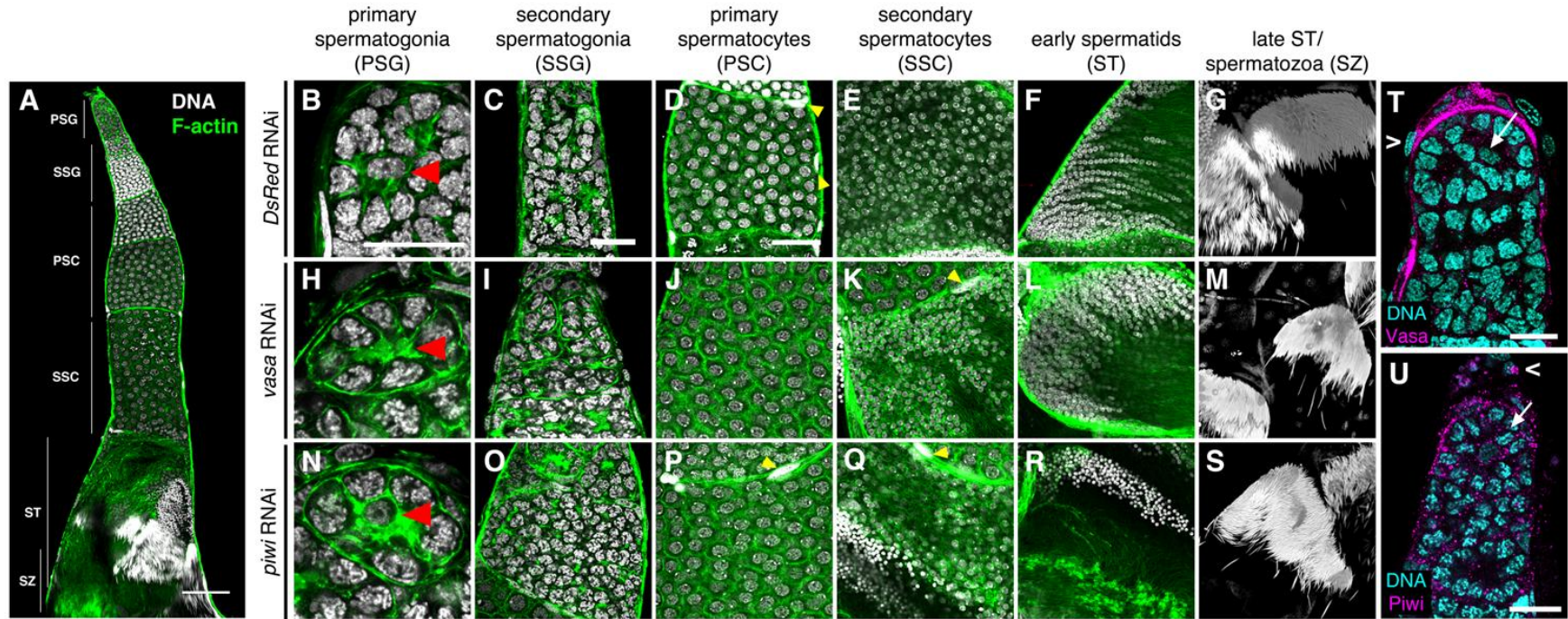


Figure S5, Related to Figure 5. *vasa* or *piwi* pRNAi Does Not Disrupt Postspematogonial Stages of Spermatogenesis in *Gryllus*

Wild type *Gryllus* testis showing the stages of spermatogenesis (A). Primary spermatogonia (PSC) undergo self-renewing divisions, which are thought to occur under the influence of a single apical cell (red arrowheads in B, H, N) that provides a “stem cell niche” analogous to the hub of *Drosophila* testes [3, 4]. Following seven mitotic divisions by cysts of secondary spermatogonia (SSG) enclosed by somatic cell sheaths (yellow arrowheads in D, K, P-Q), the resulting 128 primary spermatocytes (PSC) undergo meiosis (secondary spermatocytes: SSC) to produce 512 clonally related spermatids (ST), which undergo synchronous spermeogenesis to produce bundles of mature spermatozoa (SZ) [5]. Although *vasa* and *piwi* pRNAi testes display a reduction in the number of secondary spermatogonial cysts (Figure 7), they possess normal apical cells (B, H, N, arrowheads), and cysts proceeding normally through all stages of spermatogenesis (D-G, I-M, O-S), which are surrounded by somatic sheath cells (arrowheads) as in controls. Vasa (T) and Piwi (U) proteins are expressed in the anterior region of testioles, which contains primary spermatogonia (arrows) and somatic sheath cells (arrowheads), and is enclosed by a cellular peritoneal sheath (carets). Scale bars = 100 μ m in A; 50 μ m in (D) (applies also to E–G, J–M, P–S); 25 μ m in (B) (applies also to H and N) and (C) (applies also to I and O); 20 μ m in (T)–(U).

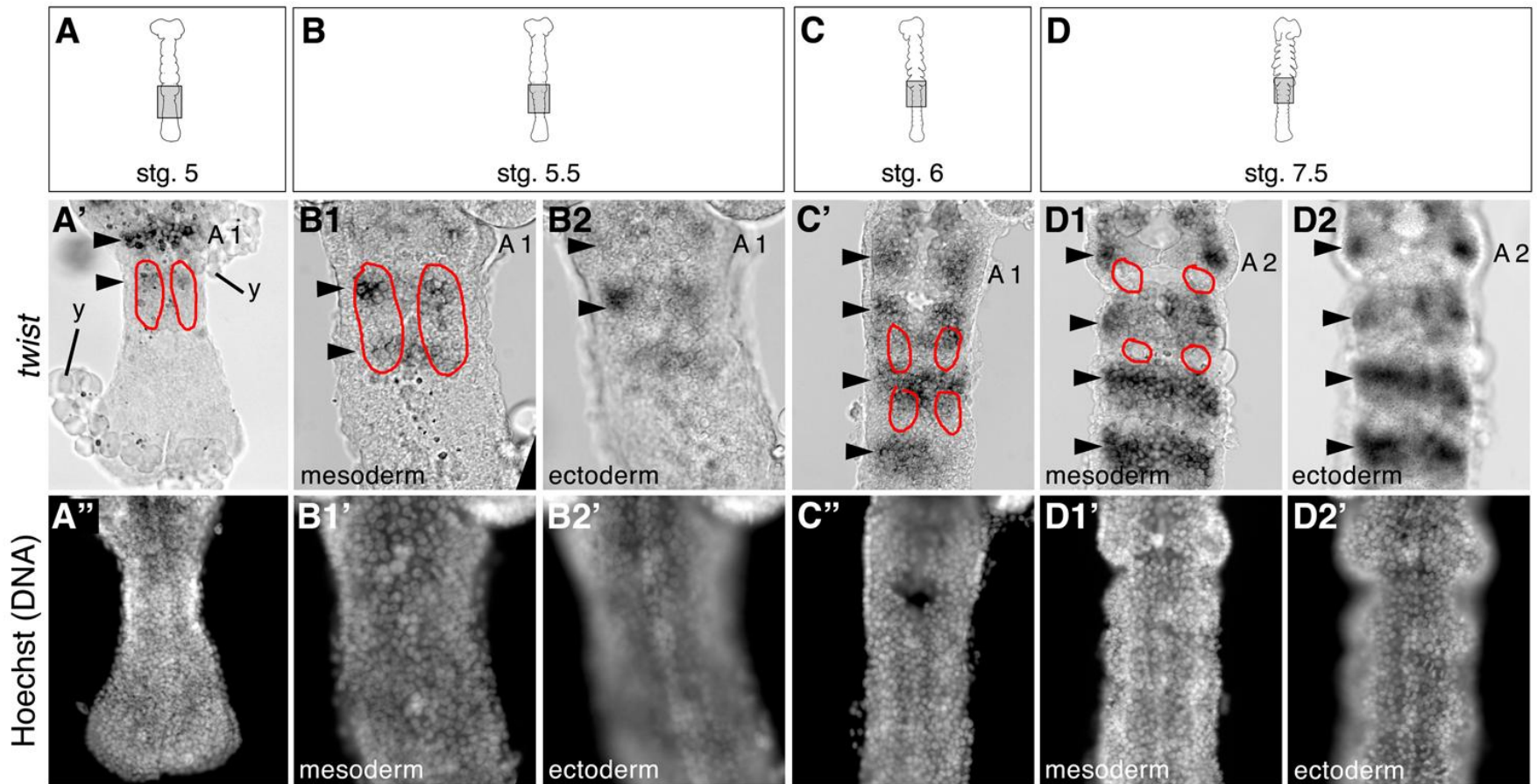


Figure S6, Related to Figure 6. *twist* Expression in the Abdominal Mesoderm where *Gryllus* Germ Cells Arise

(A–D) Schematic drawings of progressive stages of *Gryllus* embryogenesis; grey box indicates region shown in panels below. *twist* transcripts accumulate in an anterior to posterior progression in abdominal segments, indicated by black arrowheads in (A'), (B1)–(B2), (C'), and (D1)–(D2). Red outlines in (A'), (B1), (C'), and (D1) indicate the regions that become enriched for *piwi* expression at these stages, suggesting that these are the sites of PGC origin and showing that these regions express *twist* at the proposed onset of PGC specification (stage 5). Bottom row shows nuclear staining of corresponding bright field images in the row above. Anteriormost abdominal segment is labeled in each panel. y = yolk. *twist* expression is confined to the mesoderm and absent from the ectoderm, as shown in micrographs of mesodermal focal planes in (B1 and D1), and ectodermal focal planes in (B2 and D2).

Table S1, Related to Figure 1. Data on Insect PGC Origin during Embryogenesis

Please see accompanying Excel file. Only studies directly addressing the mechanism and/or description of the first embryonic appearance of PGCs are referenced. Numbered references in the Excel file are listed at the end of this Supplemental Information document.

Supplemental Experimental Procedures

Insect Cultures and Embryonic Staging

Gryllus bimaculatus cultures were maintained as previously described [139] and embryos were staged according to [140]. *Drosophila Oregon R* and *twist*¹ stocks were obtained from the Bloomington Drosophila Stock Center (#5, #2381).

Cloning and Phylogenetic Analysis

Orthologues of *boule*, *tudor*, *germ cell-less*, an additional *piwi*-like gene, and two *AGO3*-related genes were identified in a *Gryllus* developmental transcriptome via reciprocal best BLAST hit analysis against the *Drosophila melanogaster* proteome. *Gryllus twist* was a gift of S. Roth (University of Cologne, Germany).

To resolve the orthology of the four *Gryllus* PIWI family proteins, we used maximum-likelihood based phylogenetic reconstruction as implemented by RAxML v 7.2.8 [141, 142] on the Odyssey Cluster, maintained by the FAS Sciences Division Research Computing Group (Harvard University). The alignment was produced using Muscle [143] and trimmed using Gblocks [144] under the least stringent settings. The best tree and rapid bootstrap analysis were conducted from 2000 independent runs under the WAG model of protein evolution with a gamma distribution of rate heterogeneity.

In Situ Hybridisation

DIG-labeled probes were hybridised at 68° C following standard protocols [139], with 50% polyvinyl alcohol included in the NBT/BCIP development step. Probe lengths were as follows: *vasa*: 1,953 bp; *piwi-1*: 781 bp; *piwi-2*: 821 bp; *AGO3-A*: 760 bp; *AGO3-B*: 832 bp; *germ cell-less*: 1,691 bp; *boule*: 995 bp; *tudor*: 1,707 bp. Our results for *vasa* expression (both mRNA and protein) in *Gryllus* differ from those reported by Mito *et al.* [1], who failed to identify the germ cell clusters that we observed beginning at stages 6/7 (Figures 1, 4, S1, S4). This discrepancy may be due to the strong nervous system expression of *vasa* that can obscure the relatively weaker PGC expression (their Figures 3I, 3J, 4A-D), and to our use of a species-specific Vasa antibody [2] as opposed to the cross-reactive antibody [145] used by Mito *et al.*. Mito *et al.* also reported detection of transient *vasa* mRNA staining at the posterior of stage 4 embryos (their Figure 3C-D), and interpreted it as consistent with Heymons' 1895 claim that germ cell precursors arose among the posterior germ band mesoderm shortly after gastrulation [125]. However, we found that this apparently stronger expression is due to the thickness of the posterior germ band tissue at these stages. In three *in situ* hybridisation replicates and ≥30 early stage embryos, *vasa* did not show consistent enrichment in any specific embryonic region before stage 5. We therefore conclude that germ cells are not specified until this stage, in agreement with the majority of previous authors on orthopteran germ cell origin [131-133].

Immunohistochemistry

Primary antibodies used were rabbit anti-Gb-Vasa and anti-Gb-Piwi [2] at 1:300, mouse anti-RNA polymerase II pSer 6 Mab H5 (Covance MMS-129R) 1:100, FITC-conjugated anti-alpha Tubulin (Sigma F2168) 1:100 and rabbit anti-Drosophila Vasa 1:500 (gift of P. Lasko) following standard procedures. Goat anti-rabbit secondary antibodies conjugated to Alexa 488, Alexa 555 or Alexa 568 (Invitrogen) were used at 1:500 or 1:1000. Counterstains were Hoechst 33342 (Sigma B2261) 0.1 to 0.05 µg/ml and FITC-conjugated phalloidin (Sigma P5282) 1 µg/□l.

RNA Interference

dsRNA injection into adult females (maternal RNAi = mRNAi) and newly laid embryos (embryonic RNAi = eRNAi) was conducted as previously described [2]. dsRNA fragments for *vasa* and *piwi* were 541 bp and 646 bp, respectively. For eRNAi double knockdown experiments, equal volumes of *vasa* and *piwi* dsRNA were mixed prior to injection. For mRNAi double knockdown experiments, twice the volume of dsRNA as that used for single RNAi experiments (15 µg each of *vasa* and *piwi* dsRNA, or 30 µg of the DsRed control dsRNA) was injected into adult females. dsRNA was used at a concentration of 3 µM (mRNAi) and 5 µM (eRNAi and pRNAi).

qPCR Analysis of Knockdown

qPCR was used to verify RNAi efficacy as follows: total RNA was extracted from RNAi-treated ovaries or stage 8-9 (day 4) embryos using TRIzol (Invitrogen) and including a 30-minute DNase digestion at 37° C to remove genomic contamination. Equal volumes of RNA were used as template for first strand cDNA synthesis using SuperScript III (Invitrogen) including a no reverse transcriptase control. cDNA was diluted 1:5 prior to qPCR. qPCR was conducted using PerfeCta SYBR Green SuperMix (Low ROX, Quanta Biosciences) in a Stratagene MxP3005 machine. Primers amplifying single amplicons of *piwi* (129 bp; F: TTCGGCCAACTACTTCAAGC; R: AGAGTTTCCCGATGAACACG), *vasa* (150 bp; F: GAACATTGTGAGCCTCATGC ; R: TTGCTGAGCCTGGTGGTAT) and beta-tubulin (166 bp; F: TGGACTCCGTCCGGTCAGGC; R: TCGCAGCTCTCGGCCTCCTT) were used. Each reaction was conducted in triplicate, and fluorescence measurements were normalised and background-subtracted using the ROX dye present in the PCR reactions.

C_t values were used to calculate fold change compared to *DsRed*-injected controls using the $2^{-\Delta\Delta C_t}$ method [146]. Triplicate C_t values were averaged and the standard deviation was propagated using standard methods.

Imaging and Image Analysis

Micrographs were captured with AxioVision v.4.8 driving a Zeiss Stereo Lumar equipped with an AxioCam MRc camera, Zen Blue 2011 driving a Zeiss Stereo Zoom equipped with an AxioCam HRc camera, a Zeiss Axio Imager equipped with an AxioCam MRm camera using epifluorescence either with or without an Apotome, or an Olympus IX71 equipped with a Hamamatsu C10600-108 camera. Confocal microscopy was performed with a Zeiss LSM 710 or 780 confocal, using comparable gain, offset, and averaging parameters for all samples. Image analyses were performed with AxioVision v.4.8, Zen 2009 or Zen 2011 (Zeiss), and figures were assembled in Photoshop CS4, InDesign CS4, or Illustrator CS4 (Adobe). For confocal images shown in Figures 1E; 2A''-H''; 4C-H; 6C' and G'; S3A'''-F''', a maximum-intensity projection of multiple optical sections of the antibody staining was superimposed over a single optical section of the nuclear counterstain for visual clarity. All other confocal micrographs are maximum intensity projections (Figures 2A''-H''; 3G and H; S3A''-F''), three-dimensional projections (Figure 6B, F) or single optical sections (all other confocal micrographs).

Author Contributions

BE-C and CGE designed research, analysed data and wrote the paper; experiments were carried out by SD (*twist* eRNAi experiment), DNC (pRNAi egg-laying and embryonic survival scoring,

PGC migration analysis), CGE (nuclear morphology analysis, *twist* in situ hybridisation analysis, spermatogenesis analysis of pRNAi experiments) and BE-C (all other experiments); CGE obtained funding for the research.

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Insect Order	Species[1]	Presumptive PGC Origin	Mode of PGC Specification[2]	PGC Identification Criteria[3]	Functional or Experimental Evidence[4]	References
	Stage[5]	Reported Location/Derivation[6]				
Diptera						
	<i>Acricotopus lucidus</i>	Blastoderm	Pole cell[7]	GP	LM, LSM	N [6]
	<i>Aedes aegypti</i>	Blastoderm	Pole cells	GP	LM, MM	N [7, 8]
	<i>Anopheles gambiae</i>	Blastoderm	Pole cells	GP	MM	N [8]
	<i>Anopheles maculipennis</i>	Blastoderm	Pole cells	GP	LM	N [7]
	<i>Calliphora erythrocephala</i>	Blastoderm	Pole cells	GP	LM	N [9]
	<i>Calliphora erythrocephala</i>	Blastoderm	Pole cells	GP	LM	N [10]
	<i>Ceratitis capitata</i>	Blastoderm	Pole cells	GP	LM, TEM, LSM	N [11]
	<i>Chironomus</i> sp.	Blastoderm	Pole cells	GP	LM, MM	N [12, 13]
	<i>Clogmia albipunctata</i>	nd[8]	nd	nd	LM	N [14]
	<i>Comptosia concinnata</i>	Blastoderm	Pole cells	GP	LM	N [15]
	<i>Culex fatigans</i>	Blastoderm	Pole cells	GP	LM	N [16]
	<i>Culex pipiens</i>	Blastoderm	Pole cells	GP	LM	Y [7, 17]
	<i>Culex quinquefasciatus</i>	Blastoderm	Pole cells	GP	MM	N [18]
	<i>Dacus tyroni</i>	Blastoderm	Pole cells	GP	LM	N [19]
	<i>Drosophila melanogaster</i>	Blastoderm	Pole cells	GP	LM	Y [20-29]
	<i>Heteropeza pygmaea</i>	Blastoderm	Pole cells	GP	LM	N [30]
	<i>Lucilia cuprina</i>	Blastoderm	Pole cells	GP	LM	Y [24]
	<i>Lucilia sericata</i>	Blastoderm	Pole cells	GP	LM	N [16]
	<i>Melophagus ovinus</i>	Blastoderm	Pole cells	GP	LM	N [31]
	<i>Miasor americana</i>	Blastoderm	Pole cell	GP	LM	N [32]
	<i>Miasor metraloas</i>	Blastoderm	Pole cell	GP	LM	N [33, 34]
	<i>Musca domestica</i>	Blastoderm	Pole cells	GP	LM, MM	N [13, 35]
	<i>Musca vomitoria</i>	Blastoderm	Pole cells	GP	LM	N [36, 37]
	<i>Mycophila speyeri</i>	Blastoderm	Pole cells	GP	LM	N [38]
	<i>Phoenicia sericata</i>	Blastoderm	Pole cells	GP	LM	N [39]
	<i>Phormia regina</i>	Blastoderm	Pole cells	GP	LM	N [40]
	<i>Rhynchosciara americana</i>	Blastoderm	Pole cells	GP	MM	N [41]
	<i>Sciara coprophila</i>	Blastoderm	Pole cells	GP	LM	N [42]
	<i>Simulium pictipes</i>	Blastoderm	Pole cells	GP	LM	N [43]
	<i>Smittia</i> sp.	Blastoderm	Pole cells	GP	LM	N [44]
Siphonaptera						
	<i>Ctenocephalides felis</i>	Blastoderm	Pole cells	GP	LM	N [45]
	<i>Hystriochystylla diploie</i>	Blastoderm	Pole cells	GP	LM	N [45]
	<i>Neosopsyllus fasciatus</i>	Blastoderm	Pole cells	GP	LM	N [45]
Lepidoptera						
	<i>Antheraea pernyi</i>	Germ band	CP Mesoderm	MZ	LM	N [46]
	<i>Bombyx mori</i>	Blastoderm	Lateral ventral blastoderm	GP	LM, MM	Y [47-54]
	<i>Byasa (Atrophaneura) alcinous alcinous</i>	Germ rudiment	Ventral germ rudiment	MZ	LM	N [55]
	<i>Diacrisia virginica</i>	Germ band	CP Mesoderm	MZ	L	N [56]
	<i>Endoclyta exoreoscens</i>	Germ band	PGB	MZ	LM	N [57]
	<i>Endoclyta signifer</i>	Germ band	PGB	MZ	LM	N [57]
	<i>Endromis versicolora</i>	Germ band	PGB primary ectoderm	MZ	LM	N [58]
	<i>Ephestia kuehniella</i>	Germ band	PGB primary ectoderm	MZ	LM, MM	N [59, 60]
	<i>Epiphyas postvittana</i>	Germ band	Germ band midline	MZ	LM	N [61]
	<i>Eu Vanessa antiope</i>	Blastoderm	Ventral cellular blastoderm	MZ	LM	N [62]
	<i>Heliothis zea</i>	Germ band	Germ band midline	MZ	LM	N [63]
	<i>Luehdorfia japonica</i>	Germ rudiment	Ventral germ rudiment	MZ	LM	N [55]
	<i>Neomicroprocteryx nipponensis</i>	Germ band	CP Mesoderm	MZ	LM	N [64]
	<i>Parnassius glacialis</i>	Germ rudiment	Ventral germ rudiment	MZ	LM	N [55]
	<i>Pectinophora gossypiella</i>	Blastoderm	Pole cells	GP	LM, TEM	N [65]
	<i>Pieris rapae</i>	Germ band	Mesoderm	MZ	LM	N [66]
	<i>Solenobia inquetrella</i>	Germ band	PGB primary ectoderm	MZ	LM	N [67]
Coleoptera						
	<i>Calandra granaria</i>	Blastoderm	Pole cells	GP	LM	N [68]
	<i>Calandra oryzae</i>	Blastoderm	Pole cells	GP	LM	N [69]
	<i>Calandra callosa</i>	Blastoderm	Pole cells	GP	LM	N [70]
	<i>Calligrapha multipunctata</i>	Blastoderm	Pole cells	GP	LM	N [71]
	<i>Corynodes pusis</i>	Blastoderm	Posterior blastoderm	GP	LM	N [72]
	<i>Doryphora decemlineata</i>	Germ band	PGB	MZ	LM	N [73]
	<i>Hydrophilus piceus</i>	Germ band	CP mesoderm	MZ	LM	N [74]
	<i>Leptinotarsa decemlineata</i>	Blastoderm	Pole cells	GP	LM	N [71]
	<i>Rhagophthalmus ohlbei</i>	Germ disc	Posterior germ disc	MZ	LM, SEM	N [75]
	<i>Tenebrio molitor</i>	Germ band	PGB mesoderm	MZ	LM	N [76, 77]

Insect Order	Species[1]	Presumptive PGC Origin	Mode of PGC Specification[2]	PGC Identification Criteria[3]	Functional or Experimental Evidence[4]	References	
	Stage[5]	Reported Location/Derivation[6]					
	<i>Tribolium castaneum</i>	Germ band	PGB	MZ	TEM, LM, SEM, MM	N	[78-81]
	<i>Xyleborus ferrugineus</i>	Blastoderm	Pole cells	GP	LM	N	[82]
Hymenoptera							
	<i>Apanteles glomeratus</i>	Blastoderm	Pole cells	GP	LM	N	[83, 84]
	<i>Apis mellifera</i>	Germ band	Mesoderm	MZ	SEM, MM	N	[78, 85-90]
	<i>Copidosoma</i> sp.	2 nd cleavage	B4 at four cell stage[9]	GP	LM, MM	Y	[91-98]
	<i>Habrobracon juglandis</i>	Blastoderm	Pole cells	GP	LM	N	[99]
	<i>Mormoniella (Nasonia) vitripennis</i>	Blastoderm	Pole cells	GP	LM	N	[100-102]
	<i>Pimpla turionellae</i>	Blastoderm	Pole cells	GP	LM	"+"	[103, 104]
	<i>Pteronidea ribesii</i>	Germ band	PGB	MZ	LM	N	[105]
	<i>Trichogramma evanescens</i>	Blastoderm	Pole cells	GP	LM	N	[106, 107]
	<i>Tritneptis dipronis</i>	Blastoderm	Pole cells	GP	LM	N	[108]
Hemiptera							
	<i>Acyrthosiphon pisum</i>	Blastoderm	Pole cells	GP	LM, MM	N	[109-113]
	<i>Aphis pellargonii</i>	Blastoderm	PGB	MZ	LM	N	[114]
	<i>Aphis plantoides</i>	Blastoderm	PGB	MZ	LM	N	[115]
	<i>Aphis rosea</i>	Blastoderm	PGB	MZ	LM	N	[33]
	<i>Oncopeltus fasciatus</i>	Blastoderm	PGB	MZ	LM, TEM, MM	Y	[116-118]
	<i>Pyrrhocoris apterus</i>	Blastoderm	PGB	MZ	LM	N	[119]
	<i>Rhodnius prolixus</i>	Blastoderm	PGB	GP	LM	N	[120, 121]
Thysanoptera							
	<i>Haplothrips verbasci</i>	Blastoderm	Pole cells	GP	LM	N	[122]
Psocoptera							
	<i>Liposcelis divergens</i>	Blastoderm	PV blastoderm	MZ	LM	N	[123]
Phthiraptera							
		Blastoderm	Pole cells	GP	LM	N	[cited in 122, 124]
Blattodea							
	<i>Periplaneta orientalis</i>	Germ band	Mesoderm	MZ	LM	N	[125]
	<i>Phyllodromia (Blatta) germanica</i>	Germ band	Mesoderm	MZ	LM	N	[125]
Plecoptera							
	<i>Pteronarcys proteus</i>	Germ band	Mesoderm	MZ	LM	N	[126]
Phasmatodea							
	<i>Carausius morosus</i>	Germ band	Mesoderm	MZ	LM, TEM	Y	[127-130]
Orthoptera							
	<i>Gryllus bimaculatus</i>	Germ band	Mesoderm	MZ	LM, MM	Y	This study
	<i>Gryllus campestris</i>	Germ band	PGB	MZ	LM	N	[125]
	<i>Gryllus domesticus</i>	Germ band	PGB	MZ	LM	N	[125]
	<i>Locusta migratoria</i>	Germ band	CP Mesoderm	MZ	LM	N	[131]
	<i>Melanoplus differentialis</i>	Germ band	Lateral abdominal ectoderm	MZ	LM	N	[132]
	<i>Xiphidium enisterum</i>	Germ band	Mesoderm	MZ	LM	N	[133]
Zygentoma							
	<i>Ctenolepisma lineata</i>	Germ band	CP Mesoderm	MZ	LM	N	[134]
	<i>Lepisma saccharina</i>	Germ band	PGB	MZ	LM	N	[135]
	<i>Thermobia domestica</i>	Germ band	CP Mesoderm	MZ	LM, TEM	N	[134, 136]
Archaeognatha							
	<i>Pedetonotus unimaculatus</i>	Blastoderm	PGB	MZ	LM	N	[137]
	<i>Petrobius brevistylis</i>	Germ band	CP Mesoderm	MZ	TEM	N	[138]

[1] Species name is shown as reported in the primary data reference, listed alphabetically within an insect order.

[2] MZ = mesodermal/zygotic origin; GP = germ plasm/pole cells

[3] LM = light microscopic histological analysis, of either whole mounts or sections; TEM = transmission electron microscopy; SEM = scanning electron microscopy; EM = enzymatic markers; MM = molecular markers; LI = cell lineage studies.

[4] Y = yes; N = no. Includes data derived from functional genetic evidence and/or physical perturbation/ablation.

[5] Since direct comparison of the duration of stages of development in different species is not appropriate due to differences in culture conditions, we describe here developmental stages rather than absolute time.

[6] Pole cell(s) = Drosophila-like PGC formation at the blastoderm posterior; CP = coelomic pouch; PGB = posterior germ band (germ layer indicated if specified by author of primary reference); PV = posterior ventral

[7] In embryos of *Acrictotopus lucidus*, *Miastor americana* and *Miastor metraloas*, a single pole cell (PGC) gives rise to all germ line cells.

[8] nd = no data; for *Clogmia albipunctata* pole cells have been specifically noted as absent, but no hypotheses on PGC origin have been proposed in the literature.

[9] *Copidosoma floridanum* is unusual among insects in that it displays holoblastic cleavage rather than syncytial cleavage in early development. B4 is the name given to the small blastomere formed at second cleavage, which inherits the oosome (germ plasm) and is the primordial germ cell.