Hox genes limit germ cell formation in the short germ insect Gryllus bimaculatus

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Hox genes are conserved transcription factor-encoding genes that specify the identity of body regions in bilaterally symmetrical animals. In the cricket Gryllus bimaculatus, a member of the hemimetabolous insect group Orthoptera, the induction of a subset of mesodermal cells to form the primordial germ cells (PGCs) is restricted to the second through the fourth abdominal segments (A2 to A4). In numerous insect species, the Hox genes Sex-combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), and abdominal-A (abd-A) jointly regulate the identities of middle and posterior body segments, suggesting that these genes may restrict PGC formation to specific abdominal segments in G. bimaculatus. Here we show that reducing transcript levels of some or all of these Hox genes results in supernumerary and/or ectopic PGCs, either individually or in segment-specific combinations, suggesting that the role of these Hox genes is to limit PGC development with respect to their number, segmental location, or both. These data provide evidence of a role for this ancient group of genes in PGC development.

Hox | Gryllus bimaculatus | insect | evo-devo | germ cells

The Hox genes are an ancient family of transcription factor-encoding genes that play a conserved role in specifying the body regions of bilaterally symmetrical animals during development (reviewed in ref. 1). In arthropods, Hox genes act to specify the distinct identities of different body segments (reviewed in ref. 2), with mutations in Hox genes usually resulting in switches of segmental type called “homeotic transformations” (reviewed in ref. 3). We previously showed that in the cricket Gryllus bimaculatus, which belongs to the hemimetabolous insect order Orthoptera, the primordial germ cells (PGCs) form from the mesoderm of the second to the fourth abdominal segments (A2 to A4) (4) via a bone morphogenetic protein (BMP)-dependent mechanism (5). Given that BMP activity is not limited to the segments where PGCs form and develop, but rather is present in the dorsal regions of all body segments (5), these data suggested that additional unidentified factor or factors must ensure that PGCs form in the appropriate segments. As Hox genes play a conserved role in establishing segmental identity, here we test the hypothesis that Hox genes contribute to regulating PGC development in A2 to A4.

Data implicating Hox genes in embryonic germ line development in other animal taxa are scarce. In the mouse Mus musculus, as in G. bimaculatus, PGCs are established via BMP signals that activate the transcription factor Blimp-1 (5–7). Mouse embryonic cells that take on PGC fate repress the Hox genes Hoxl and Hoxb1 via activity of the BMP-activated transcription factor Blimp-1 (7–9). This has been interpreted as reflecting the loss of somatic differentiation programs that is associated with adopting PGC fate. Repression of Hox genes during differentiation of human induced pluripotent stem cells into in vitro-derived PGCs (10) is consistent with the hypothesis that Hox gene expression and stable PGC fate within the same cells are mutually exclusive. In a system where germ cells are specified by inductive signals from neighboring cells, the degree or robustness of the PGC differentiation response may be influenced by the degree of concomitant Hox gene knockdown. Indeed, it was recently reported that, among mouse embryonic cells expressing PGC markers, there are some cells that also express hematopoietic markers, including at least one Hox gene (11). One interpretation of these observations is that those putative PGCs that respond to inductive signals by expressing lower levels of germ cell markers may still be prone to express some somatic markers. Thus, it may be that decreased levels, rather than total absence of expression, of somatic markers like Hox genes can facilitate the acquisition of PGC fate.

The relevance of Hox gene function to PGC fate is further exemplified by documented roles for Hox genes in the development of somatic gonad tissue. A role for HoxD genes in patterning and elongation of the external genitalia in mice has long been recognized (12), and HoxA genes are required for the correct development and function of elements of the female reproductive system that are derived from the Müllerian ducts, including the uterus and endometrium (16–19). However, the functions of Hox genes known to be expressed in the genital ridges, the precursors to the gonads (see, for example, ref. 20), has received less attention.

In contrast to G. bimaculatus, in the model insect Drosophila melanogaster PGCs form early in development (21, 22), long before the activation of the Hox genes that establish the identities of the body segments (23–26). Following gastrulation, the PGCs migrate toward the somatic gonad precursors (reviewed in ref. 27), which develop from the mesoderm of embryonic abdominal parasegments 10–12 (28). The Hox genes abdominal-A (abd-A) and Abdominal-B (Abd-B) are necessary for the formation of the gonad precursors, which is independent of PGC specification.

Significance

Hox genes are necessary for the proper placement of organs along animal body axes. In insects, Hox genes are used in a "code" of overlapping expression domains to specify body segments. We previously showed that, in the cricket Gryllus bimaculatus, germ cells are specified exclusively in the second through the fourth abdominal segments. Given the role of Hox genes in establishing segmental identity in insects, we tested the hypothesis that Hox genes control the segment-specific development of germ cells in crickets. We found that a subset of Hox genes limit germ cell development in the PGC-bearing segments. These data suggest a role for Hox genes in regulating germ cell placement.

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Specifically, Abd-A establishes anterior gonad fates, and Abd-A and Abd-B act in concert to establish the posterior gonad fates (28–31). In addition, in adult male *D. melanogaster*, Abd-B is required for correct function of the accessory gland (32), which is a component of the reproductive system that regulates the female response to mating (33). Moreover, this Hox gene is also required to maintain the identity of both germ line and somatic stem cells in the adult testis (34–37). However, these somatic and postembryonic functions of Abd-B do not affect embryonic PGC establishment in *D. melanogaster*, which takes place much earlier in development.

Across insects, the Hox genes Sex-combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), and abdominal-A (abd-A) have a conserved role in establishing the thoracic and abdominal segments during embryogenesis (reviewed in ref. 2). To explore the role of these Hox genes in the development of *G. bimaculatus* PGCs, we used embryonic RNAi (eRNAi) to repress the function of each of these genes individually and also in combination. We found that these Hox genes act to limit PGC formation in specific segments of the abdomen. Reminiscent of their combinatorial action in specifying other aspects of segment identity, including ectodermal patterning and appendage differentiation, these data suggest that specific combinations of Hox genes may also be needed for appropriate numbers of PGCs to develop and/or for them also express in correct segments. These data demonstrate a role for these highly conserved and ancient genes in limiting germ line development in an animal, and provide evidence for an additional embryonic role of Hox genes outside of establishing anterior–posterior segmental identity in an insect.

**Results and Discussion**

*G. bimaculatus* PGCs emerge from the lateral mesoderm of abdominal segments A2 to A4 during embryonic stage 5 (38) and come to form clusters within the mesoderm of either one or both hemisegments (i.e., the left or right halves of the segments) in embryonic stages 8 and 9 (4). Four Hox genes—Antp (Scr), Ubx (Ubx), and Abd-A (abd-A) (SI Appendix, Figs. S1 and S2)—were previously reported (39, 40) to be expressed in the abdomen, including segments A2 to A4, at embryonic stage 5 (38) of *G. bimaculatus* embryogenesis, which is when germ cells are first detectable by molecular enrichment of a number of gene products including Gb-Piwi protein (41) and morphological high nuclear-to-cytoplasmic ratio, loose chromatin compaction (4, 41, 42) criteria. These expression data suggested that these four Hox genes could potentially be involved in early staging of gonad function or development.

To investigate whether these posterior Hox genes were expressed at a time and place that could enable them to regulate the activity of one or more of these Hox genes during development. To test this hypothesis, we used eRNAi to abrogate the activity of one or more of these Hox genes during embryogenesis. We thus examined embryos of embryonic stages 7–9. We compared the PGC number per hemisegment and per embryo, as well as the presence or absence of PGC clusters in each hemisegment, to controls (SI Appendix, Tables S2–S7). Our previous observations of hundreds of wild-type embryos have shown that, by embryonic stages 8 and 9, usually three or four of the A2 to A4 hemisegments, and, less commonly, fewer than three or more than four of these hemisegments, are populated by clusters of PGCs (4, 5). We thus examined embryos of embryonic stages 8 and 9 for this analysis, as these are the earliest stages at which PGCs can be reliably quantified (5, 6). eRNAI against Gb-Scr efficiently depleted Gb-Scr transcripts as assessed by qPCR (SI Appendix, Fig. S4 A and B), although this was not accompanied by homeotic transformation of gnathal appendages to a thoracic identity (n = 36; Fig. 2L), as is often observed upon Scr knockdown in other insects (e.g., refs. 43 and 44). Gb-Scr eRNAi did, however, result in a statistically significant increase in PGC number as well as a significantly higher proportion of segments bearing PGC clusters in A2 to A4 relative.
GB RNAi did not significantly affect PGC number (n = 14; Fig. 2L), and Gb-abd-A transcripts following the GB RNAi and S7 Gabd-A, suggesting a minimal role in PGC development in the segments bearing PGC clusters in A1 to A4 and also increased PGC number in A2 (Fig. 2C and H) and offspring (Fig. 2K). Thus, loss of Gb-Abd-A resulted in both additional PGCs in the correct segments, as did loss of Gb-Scr, and also in ectopic PGCs in A1. Gb-Ubx RNAi resulted in a number of clear somatic homeotic phenotypes that were consistent with Ubx knockdowns in other insects (45–48). Specifically, we observed the transformation of A1 appendages (pleuropodia) toward ectopic walking legs (n = 28/41; Fig. 2L and SI Appendix, Figs. S5B, S6B, F, and F7A), supported by expression of the appendage marker Distal-less (Dll) (49) (SI Appendix, Fig. S6D) and reduced expression of the G. bimaculatus ortholog of nanobrev (ttk), a gene that shows enriched expression in pleuropodia (SI Appendix, Fig. S6F). Accordingly, qPCR analysis indicated that Gb-Ubx transcripts were effectively reduced by the RNAi treatment (SI Appendix, Fig. S4A and B). However, Gb-Ubx RNAi did not significantly affect PGC number, or the numbers of PGC clusters, in any segment, or overall, relative to controls (Fig. 2D, I, and K). Thus, we suggest that Gb-Ubx alone plays no or a minimal role in PGC development in this cricket.

eRNAi targeting Gb-abd-A transcripts resulted in ectopic appendages throughout all abdominal segments (n = 19/25; Fig. 2L and SI Appendix, Figs. S5C, S6C, and G, and S7B). These ectopic outgrowths expressed Dll (SI Appendix, Fig. S6C), but not ttk (SI Appendix, Fig. S6G), and were consistent with outgrowths observed in Abd-A knockdowns in other insects (45, 50–53). qPCR confirmed a decrease in Gb-abd-A transcripts following the RNAi treatment (SI Appendix, Fig. S4A and B). Gb-abd-A RNAi increased both PGC number in A2 and the proportion of hemisegments bearing PGC clusters in A3 (Fig. 2E, J, and K). Together, the results of these single Hox gene knockdowns suggest that Gb-Scr and Gb-abd-A repress mesodermal transformation to PGCs in A2 to A3, and that Gb-Abd-A represses PGC formation in A1 to A4. In arthropods, Hox genes often work in concert to either activate or repress transcriptional targets (reviewed in ref. 2). Moreover, Hox genes often cross-regulate each other’s expression in multiple developmental contexts (e.g., ref. 54). Therefore, we explored the possibility that the aforementioned Hox genes could be acting together in the context of PGC specification. We predicted that, if a combination of Hox genes worked together to modulate PGC formation, a double knockdown of these genes might result in unique PGC defects relative to the defects observed in the single knockdowns discussed above. To test this prediction, we systematically injected embryos with equal amounts of dsRNA targeting each pairwise combination of these posterior G. bimaculatus Hox genes (SI Appendix, Table S1). Consistent with our prediction, for each double knockdown combination tested, we indeed found that the resulting PGC phenotypes could not be simply deduced from the component single-knockdown phenotypes.

Unexpectedly, all double eRNAi treatments that involved Gb-Scr dsRNA as a partner resulted in embryonic lethality 1 d after injection (SI Appendix, Table S1). We therefore could not study its interaction with the other Hox genes, and instead focused on the remaining three possible pairwise double-knockdown combinations. The first of these, eRNAi simultaneously targeting Gb-Antp and Gb-Ubx, resulted in the same embryonic homeotic transformation as Gb-Ubx single knockdowns (i.e., an ectopic fourth leg on A1, n = 27/31; SI Appendix, Figs. S5D and S7C). This double knockdown also resulted in an increase in the presence of PGCs in A2 to A5, as well as an increase in the number of PGCs in segments A2 to A4, and overall, compared with controls (Fig. 3A, D, and E). Comparing these results to the Gb-Ubx and Gb-Antp single knockdowns, the overall PGC increase induced by the double knockdown (Fig. 3D) was not significantly different from that induced by the Gb-Antp knockdown alone (Fig. 2K), suggesting that Gb-Antp acts without Gb-Ubx to repress PGC formation in A2 to A4. However, the reduction in A1 PGC number in the double knockdown (Fig. 3A) relative to Gb-Antp alone (Fig. 2H) suggests that Gb-Antp may act to repress a PGC formation-promoting function of Gb-Ubx in A1. Furthermore, the presence of significantly more PGC clusters in A5 in the double knockdown (Fig. 3A and E) relative to both single knockdowns (Fig. 2H and I) also suggests that these genes act together to repress PGC formation in A5.

RNAi simultaneously targeting Gb-Ubx and Gb-abd-A resulted in embryos with ectopic legs throughout the abdomen (n = 8/14;
Double eRNAi reveals synergistic effects of Hox repression on germ cells. (A–C) PGC cluster quantifications of each eRNAi treatment (double RNAi treatment indicated by label above adjacent panels E–G, respectively). Blue: absent (0 μm²); 0 PGCs); pink: small (<5,000 μm²), 1–5 PGCs); magenta: medium (5,000–10,000 μm²); 6–20 PGCs); purple: large (>10,000 μm²), >20 PGCs). Sample sizes are n = 31, 14, and 20 embryos, and n = 62, 28, and 40 hemisegments scored for A–C, respectively. Asterisks denote significant size differences from DsRed controls (Fig. 2F) and single eRNAi treatments (Fig. 2G–J) in that segment; asterisk colors indicate comparisons to DsRed (black), Gb-Antp eRNAi (dark blue), Gb-abd-A eRNAi (light purple), or Gb-Ubx eRNAi (dark purple). See SI Appendix, Tables S3 and S6, for statistical values. (D) Box plot showing the distribution of total PGC volumes per embryo in each knockdown condition and the control condition (gray) except for Gb-Ubx+Gb-abd-A eRNAi, which displayed no significant total PGC differences compared with controls was not statistically significant (SI Appendix, Table S3). Comparing these results to the Gb-Ubx and Gb-abd-A single knockdowns suggests that Gb-abd-A alone is negatively regulating PGC formation in A4, and that both Gb-Ubx and Gb-abd-A act together to limit PGC formation in A4 (compare Fig. 2F and J with Fig. 5B).

The effect of eRNAi of Gb-Antp and Gb-abd-A together on PGCs was striking: A1 to A6 contained significantly more PGC clusters than in controls (Fig. 3 C, D, and G), and there were significantly more PGCs in A3 to A4 and overall. Comparing this double knockdown to the single Gb-Antp and Gb-abd-A knockdowns revealed that the PGC increase caused by Gb-Antp eRNAi alone (Fig. 2H) was slightly suppressed in A2 (Fig. 3C), suggesting a potential role of Gb-Antp in repressing Gb-abd-A’s ability to promote PGC formation in A2. Furthermore, this double knockdown provided evidence that Gb-Antp and Gb-abd-A act together to suppress PGC development in A3, A6, and overall (Fig. 3 D and G).

Together, our results are consistent with a role of Hox genes in suppressing the formation of PGCs in G. bimaculatus abdominal segments (Fig. 4). We propose that this role is at least partially independent of the role of Hox genes in encoding segmental identity, as the embryonic somatic homeotic phenotypes obtained upon Hox gene RNAi do not always correlate with PGC positioning defects. For example, when we repress Gb-abd-A via eRNAi, A2 to A3 bear pleuropodia-like appendages (SI Appendix, Figs. S5C and S6C). In wild-type embryos, the pleuropodia are on A1, and thus we might expect that, in this eRNAi condition, A2 to A3 are transformed to an A1 identity. As the A1 segment generally lacks PGCs (4, 5), we should not observe PGCs in these segments in Gb-abd-A eRNAi-injected embryos. However, instead we see an increase in PGCs in these segments in this condition (Fig. 3F). In another example, Gb-Ubx+Gb-abd-A eRNAi embryos bear ectopic leg-like structures on all abdominal segments (SI Appendix, Fig. S5E). Given that G. bimaculatus
and eyes absent (eya), gonad primordia markers used in *Drosophila* (57, 58), could identify putative somatic gonad precursors in *G. bimaculatus*. However, in situ hybridization did not show enhanced expression of either gene in cells adjacent to PGCs in A2 to A4 (*SI Appendix, Fig. S1*). These data suggest that either the gonad primordia are specified at later stages, or that these genes are not used to specify gonad primordia in *G. bimaculatus*. As a somatic gonad precursor marker is currently unavailable for *G. bimaculatus*, at this time we cannot determine whether Hox genes regulate both PGC and somatic gonad cell fate independently, or in a coordinated manner. Further work will thus be needed to determine whether the increase in PGCs induced by many of the Hox eRNAi conditions is at the expense of mesoderm, which would have been expected to be somatic gonadal cells in wild-type embryos in this cricket.

Finally, we note that, although we detect expression of these Hox genes in the regions containing PGCs (Fig. 1 and *SI Appendix, Fig. S3*) (39), given the systemic nature of the RNAi technique that we have employed here, we cannot formally rule out the hypothesis that Hox genes affect PGCs indirectly through action in some other body part or extraembryonic territory where Hox genes are also expressed. *Gb-Scr, Gb-Antp, and Gb-Utx* are expressed in thoracic and abdominal segments both anterior and posterior to A2 to A4 during the time of PGC specification and cluster formation, as well as in gnathal segments in the case of *Gb-Scr* (refs. 39 and 40 and *SI Appendix, Fig. S3*). *Gb-abd-A* expression extends through all abdominal segments during this developmental window (refs. 39 and 40 and *SI Appendix, Fig. S3*). To our knowledge, extraembryonic expression of these Hox genes has not been reported to date. Although we consider it more likely that the Hox eRNAi-induced PGC effects that we have reported here are due to Hox gene activity in the specific segments where we observed these effects, we cannot eliminate the possibility of a long-distance, tissue-nonautonomous role for posterior Hox genes in PGC development.

As in other animals with discrete gonads, arthropod PGCs must meet with somatic gonad cells and end up in a specific location in the body to form a functional gonad. In arthropods, the location of the gonad, and often the location of PGCs when they first arise, is tied to specific body segments. In insects like *Drosophila*, where PGCs form much earlier than Hox gene activation and must migrate to the primordial gonad, the somatic gonad precursors rely on Hox genes to form in specific segments (28). In the beetle *Tenebrio molitor*, distinct PGC clusters form in many abdominal segments, and then coalesce into only those segments that will ultimately contain the gonads (59). In the firebrat *Thermobia domestica* and the stick insect *Carausius morosus*, PGCs are thought to originate as a long cluster spanning multiple segments before ultimately becoming confined to the gonads within specific segments (reviewed in ref. 60). Outside of insects, in the spider *Parasteatoda tepidariorum*, the germ cells also arise as clusters, which are situated in the opisthosomal segments 2–6 (61). Considering the functional genetic *G. bimaculatus* data presented here in the context of these data from various arthropods, we speculate that, in addition to their roles in segment identity specification, one or both of (a) assigning the PGC-bearing segments or (b) regulating the development of PGCs within the appropriate stages may be additional ancestral roles for Hox genes in arthropods.

**Materials and Methods**

All Hox genes were cloned using a previously published *G. bimaculatus* transcriptome (62). The predicted translations of the resulting sequences were subjected to phylogenetic analysis to corroborate orthology (*SI Appendix, Fig. S2*). Animal husbandry, eRNAi (*SI Appendix, Table S1*), embryonic staging, cloning, and qPCR (*SI Appendix, Table S8*), in situ hybridizations and immunostainings, statistical tests, and PGC quantifications were performed as previously described (4–6, 38). See *SI Appendix* for detailed methods.

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