



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 some 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 *Hoxa1* 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 (12–15), 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 to occupy the 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 segment) in embryonic stages 8 and 9 (4). Four Hox genes—*Scr* (*Gb-Scr*), *Antp* (*Gb-Antp*), *Ubx* (*Gb-Ubx*), and *abd-A* (*Gb-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 (4)] 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 stages of PGC specification or development.

To investigate whether these posterior Hox genes were expressed at a time and place that could enable them to regulate development of PGCs following their initial specification, we performed in situ hybridization during embryonic stages 7 and 8 (38), the stages during which PGCs are proliferating and forming distinct clusters in each hemisegment (4). As previously described (39, 40), we found that the expression of these Hox genes exhibited spatial collinearity in the ectoderm from gnathal to abdominal segments in wild-type embryos at stage 8, a stage when all segments have been defined and are morphologically distinct (Fig. 1 and *SI Appendix*, Fig. S3). Because PGCs arise from the abdominal mesoderm, we asked whether these Hox genes were also expressed in the mesoderm. *Gb-Scr*, *Gb-Antp*, and *Gb-abd-A* transcripts were expressed in the mesodermal cells of the abdominal segments (Fig. 1 *B*, *C*, *E*, *G*, *H*, and *J*), whereas *Gb-Ubx* transcripts were not detected above background levels in the dorsal mesodermal cells of A2 to A10, but rather appeared to be restricted to the ectoderm in these segments (Fig. 1 *D* and *I*).

To determine whether the mesodermally expressed Hox genes were also expressed in PGCs, we conducted codetection of Piwi protein, a PGC marker in *G. bimaculatus* (4), and Hox gene transcripts. We detected *Gb-Scr*, *Gb-Antp*, and *Gb-abd-A* transcripts in mesodermal cells adjacent to PGCs at stages 7–8.5, but transcripts were undetectable or at very low levels in the PGCs themselves (Fig. 1 *B'*, *C'*, *E'*, *G'*, *H'*, and *J'*). *Gb-Ubx* expression

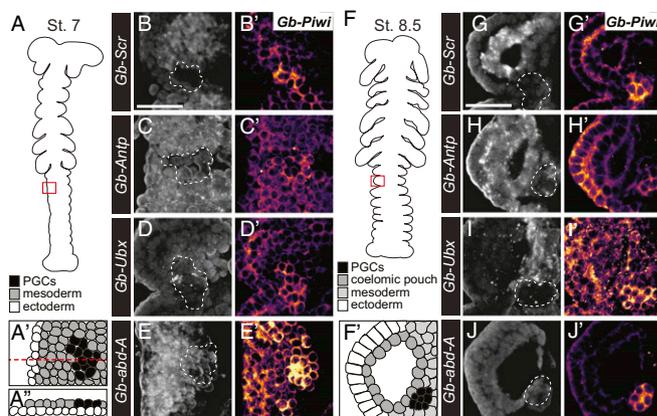


Fig. 1. Posterior Hox gene expression adjacent to but absent from *G. bimaculatus* PGCs. (A and F) Schematic illustration of whole embryos at the embryonic stages (St.) (38) used for expression analysis. Red rectangles in A and F show enlarged areas in A' and B–E' and in F' and G–J', respectively. (A' and F') Schematics of dorsal views of areas contained within red rectangles in A and F showing spatial relationships between mesoderm (gray), coelomic pouch mesoderm (dark gray), ectoderm (white), and PGCs (black). (A'') Schematic of orthogonal section through the plane indicated with the red dotted line in A', dorsal side up, showing spatial relationship between ectoderm (white: ventral) and mesoderm (gray: dorsal). (B–J) In situ hybridization for *Gb-Scr*, *Gb-Antp*, *Gb-Ubx*, and *Gb-abd-A* expression in the third abdominal segment (A3) at St. 7 (B–E) and St. 8.5 (G–J) of wild-type embryos. (B'–J') Costaining with Gb-Piwi protein (yellow: high levels; purple: low levels) in abdominal segment A3. (B and G) Expression of *Gb-Scr* is enriched in the abdominal mesoderm and undetectable above background levels in the ectoderm. (B' and G') Costaining with Gb-Piwi protein reveals *Gb-Scr* expression is undetectable in PGCs. (C and H) *Gb-Antp* expression is detected in abdominal mesoderm and ectoderm. (C' and H') *Gb-Antp* transcript is undetectable in *G. bimaculatus* PGCs at St. 7 and at very low levels at St. 8.5. (D and I) Weak *Gb-Ubx* expression is observed in the ventral mesoderm of A3. (D' and I') PGCs do not express detectable *Gb-Ubx*. (E and J) High *Gb-abd-A* expression is detected in the A3 ectoderm and mesoderm. (E' and J') PGCs display lower expression levels of *Gb-abd-A* than the adjacent mesoderm. [Scale bars, 100 μ m in B (applies to B–E) and G (applies to G–J).]

was also undetectable in the PGCs at these stages (Fig. 1 *D* and *I*). Taken together, these results suggest that, as in mice, *G. bimaculatus* Hox gene transcription is active in somatic cells adjacent to PGCs at this stage, but is repressed or inactive within PGCs (7–9).

Given their expression in cells in close proximity to PGCs, we hypothesized that these Hox genes might play a role in PGC development. To test this hypothesis, we used eRNAi to abrogate the activity of one or more of these Hox genes during *G. bimaculatus* embryogenesis (*SI Appendix*, Table S1) and assessed the effect on PGC number and segmental position. Specifically, 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. 2*L*), 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

to controls (Fig. 2*A, B, F, and G*). Furthermore, the total number of PGCs per embryo increased significantly relative to controls (Fig. 2*K*).

eRNAi against *Gb-Antp*, similarly to the *Gb-Scr* eRNAi treatment, did not result in a homeotic phenotype ($n = 14$; Fig. 2*L*). However, our qPCR results showed that this treatment was also sufficient to reduce *Gb-Antp* transcripts by 4 d after egg laying (AEL), the time at which we scored PGCs (SI Appendix, Fig. S4*B*). *Gb-Antp* eRNAi significantly increased the proportion of segments bearing PGC clusters in A1 to A4 and also increased PGC number in A2 (Fig. 2*C and H*) and overall (Fig. 2*K*). Thus, loss of *Gb-Antp* resulted both in additional PGCs in the correct segments, as did loss of *Gb-Scr*, and also in ectopic PGCs in A1.

Gb-Ubx eRNAi 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. 2*L* and SI Appendix, Figs. S5*B, S6 B and F, and S7A*), supported by expression of the appendage marker *Distal-less* (*Dll*) (49) (SI Appendix, Fig. S6*B*) and reduced expression of the *G. bimaculatus* ortholog of *tramtrack* (*ttk*), a gene that shows enriched expression in pleuropodia (SI Appendix, Fig. S6*F*). Accordingly, qPCR analysis indicated that *Gb-Ubx* transcripts were effectively reduced by the RNAi treatment (SI Appendix, Fig. S4*A and B*). However, *Gb-Ubx* eRNAi did not significantly affect PGC number, or the numbers of PGC clusters, in any segment, or overall, relative to controls (Fig. 2*D, 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. 2*L* and SI Appendix, Figs. S5*C, S6 C and G, and S7B*). These ectopic outgrowths expressed *Dll* (SI Appendix, Fig. S6*C*), but not *ttk* (SI Appendix, Fig. S6*G*), 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 eRNAi treatment (SI Appendix, Fig. S4*A and B*). *Gb-abd-A* eRNAi increased both PGC number in A2 and the proportion of hemisegments bearing PGC clusters in A3 (Fig. 2*E, 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-Antp* 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. S5*D 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. 3*A, 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. 3*D*) was not significantly different from that induced by the *Gb-Antp* knockdown alone (Fig. 2*K*), 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. 3*A*) relative to *Gb-Antp* alone (Fig. 2*H*) 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. 3*A and E*) relative to both single knockdowns (Fig. 2*H 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 ($n = 8/14$;

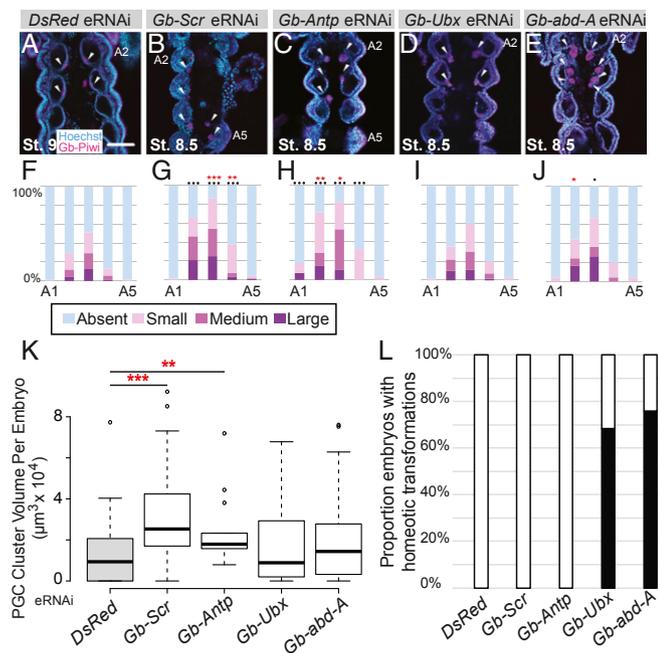


Fig. 2. eRNAi of *Gb-Scr*, *Gb-Antp*, and *Gb-abd-A* increases PGC number. (A–E) Confocal images of the A2–A5 of a representative embryo from each knockdown condition as well as the control condition (*DsRed* eRNAi). *Gb-Piwi* (magenta) marks PGC clusters (arrowheads) (4). The second abdominal segment (A2) is labeled in each panel. Anterior is up. (Scale bar, 100 μm .) (F–J) PGC cluster quantifications of each eRNAi treatment (G–J) compared with (F) *DsRed* controls. Blue: absent ($0 \mu\text{m}^3$, 0 PGCs); pink: small ($<5,000 \mu\text{m}^3$, 1–5 PGCs); magenta: medium ($5,000\text{--}10,000 \mu\text{m}^3$, 6–20 PGCs); purple: large ($>10,000 \mu\text{m}^3$, >20 PGCs). Sample sizes are $n = 82$, 36, 14, 41, and 25 embryos, and $n = 164$, 72, 28, 82, and 50 hemisegments scored for F–J, respectively. Red asterisks denote significant size differences of PGC clusters in that segment compared with controls. Black dots denote significant differences in presence/absence of PGC clusters compared with controls. Hemisegments were scored separately for the presence/absence and number of PGCs since in wild-type embryos a given segment may have PGCs in only one of the two hemisegments that comprise a segment (4, 5). (K) Box plot showing the distribution of total PGC volumes per embryo in each knockdown condition and the control condition (gray). Red asterisks denote significance levels resulting from a Mann–Whitney test. The whiskers extend to data points that are 1.5 times above the interquartile range away from the first or third quartile. The black lines represent the medians. Sample sizes are $n = 82$, 36, 14, 41, and 25 embryos for each condition from left to right. (L) A 100% stacked bar chart showing the proportion of eRNAi embryos displaying homeotic phenotypes. St.: embryonic stage (38). Sample sizes are $n = 82$, 36, 14, 41, and 25 embryos for each condition from left to right. All *P* values for each test are listed in SI Appendix, Tables S2 and S4. ** $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

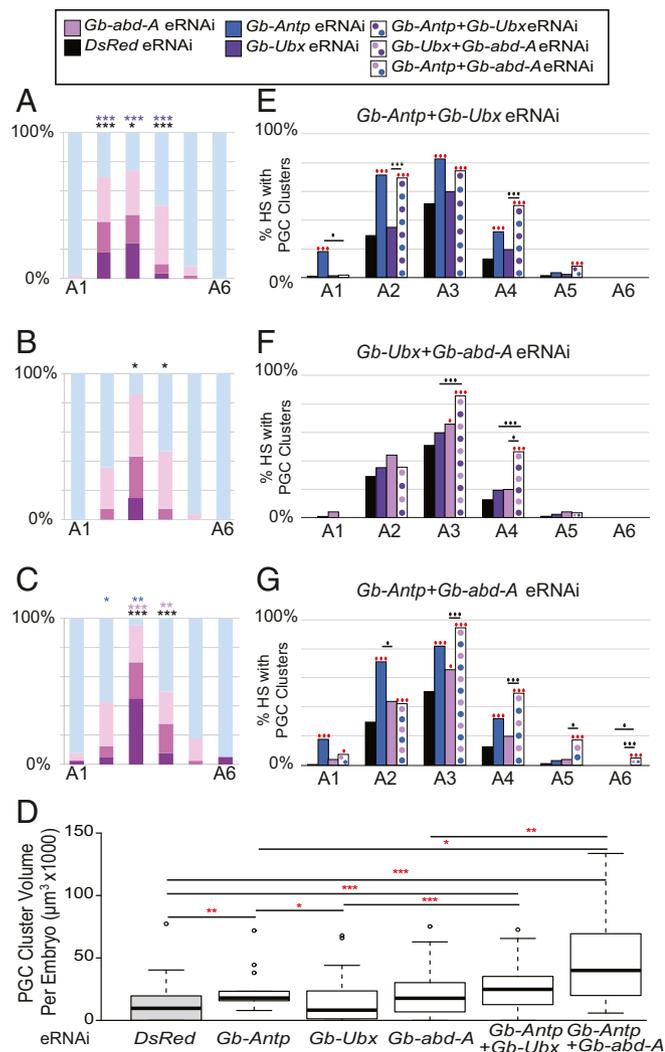


Fig. 3. Double eRNAi reveals synergistic effects of Hox repression on germ cells. (A–C) PGC cluster quantifications of each eRNAi treatment (double RNAi treatment indicated by label above adjacent panels E–G, respectively). Blue: absent ($0 \mu\text{m}^3$, 0 PGCs); pink: small ($<5,000 \mu\text{m}^3$, 1–5 PGCs); magenta: medium ($5,000\text{--}10,000 \mu\text{m}^3$, 6–20 PGCs); purple: (large $>10,000 \mu\text{m}^3$, >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 in total. Red asterisks denote significance levels in PGC number resulting from a Mann–Whitney test for differences in PGC number. The whiskers extend to data points that are 1.5 times above the interquartile range away from the first or third quartile. The black lines represent the medians. Sample sizes are $n = 82, 14, 41, 25, 31,$ and 20 embryos for each condition from left to right. See *SI Appendix, Tables S2, S3, and S6*, for statistical values. (E–G) Bar plots showing the proportion of hemisegments (HS) containing PGC clusters in single vs. double eRNAi treatments. Sample sizes are $n = 31, 14,$ and 20 embryos, and $n = 62, 28,$ and 40 hemisegments scored for E–G, respectively. The dots represent significance levels testing for the presence/absence of PGC clusters using a Fisher’s Exact or χ^2 test; red: compared with *DsRed* control; black: comparisons between single and double eRNAi conditions. See *SI Appendix, Tables S4, S5, and S7*, for statistical values. $^*/P < 0.05$, $^{**}P < 0.01$, $^{***}/P < 0.001$.

SI Appendix, Figs. S5E and S7D), exhibiting a similar phenotype to other studied insects (45, 52). These embryos also have significantly more PGC clusters in A3 and A4 compared with controls, as well as an increase in total PGC number in A4 (Fig. 3B and F). However, the overall increase in PGCs per embryo 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 A3, and that both *Gb-Ubx* and *Gb-abd-A* act together to limit PGC formation in A4 (compare Fig. 2I and J with Fig. 3B).

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. 3C, 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. 3D 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. 2J). 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*

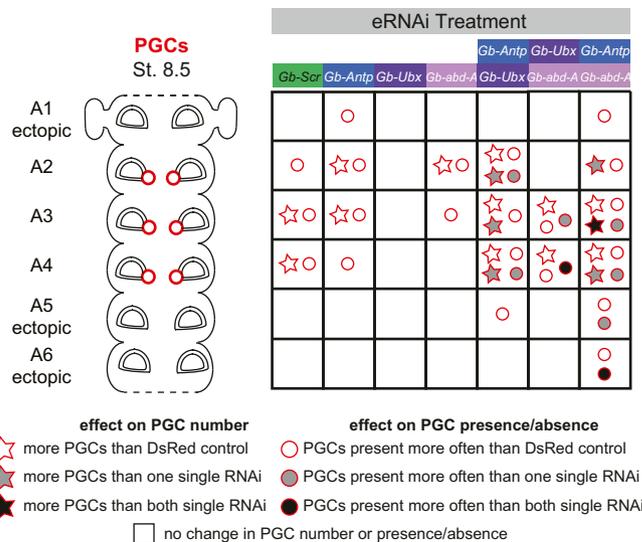


Fig. 4. Hypothesis: Combinatorial action of Hox genes regulates segmental positions of PGCs. Summary of the eRNAi PGC number and presence/absence phenotypes. (Left) In wild-type embryos, PGC clusters (red circles) form in segments A2 to A4 (4). (Right) Statistically significant changes in PGC number (star symbols) and/or statistically significant changes in the presence of PGC clusters (circle symbols) are labeled for each of the segments A1 to A6 in each of the single and double eRNAi conditions tested here.

wild-type embryos lack PGCs on their leg-bearing (thoracic) segments (4), we might expect an absence of Hox genes in A2 to A4 in this eRNAi condition. However, instead we see an increase in the presence of PGCs in these segments (Fig. 3B). Furthermore, we note that the *Gb-Scr* and *Gb-Antp* single eRNAi embryos, although they lacked the somatic homeotic appendage transformations reported in loss-of-function conditions of these genes in other insects (Fig. 2L) (43, 44), nevertheless had significantly more PGCs than controls (Fig. 2K). We hypothesize that PGC number may be more sensitive than appendage differentiation to the levels of expression of these genes. However, we cannot rule out the possibility that off-target RNAi effects, or interactions of our targeted genes with other, unknown genes, account for our observation of supernumerary PGCs despite the apparent absence of somatic homeotic transformation.

In addition, we note that even in conditions where Hox gene RNAi results in a significant increase in total PGC number, including the appearance of ectopic PGCs, PGCs are still largely restricted to segments A2 to A4, and most abdominal segments remain free of PGCs, as in wild type. This suggests that additional, still-unknown mechanisms must act to prevent PGC formation outside of A2 to A4. It is therefore possible that the role of these Hox genes in PGC development is a generally repressive role that acts in concert with or in addition to whatever factors provide the spatial information that restricts germ cells to the A2 to A4 segments.

The single or combinatorial use of Hox genes to pattern organs outside of the “Hox code” that confers regional identity along primary anterior–posterior or proximal–distal axes of animals has been reported in other taxa. In insects, such nonaxial Hox roles have also been previously described, such as those needed for the positioning of the bacteriomes in the seed bug *Nysius plebeius* (51), the formation of the lantern organ in the firefly *Photuris* (*sp.*) (55), and the differentiation of a specific accessory gland cell type in male fruit flies (32). Our results provide an example of Hox genes being used to coordinate cell-type specification, namely the specification of PGCs. In this context, Hox genes may contribute to correct gonad positioning in the context of the body plan, or simply to appropriate gonad function, by ensuring that an appropriate number of PGCs are available to populate the primordial gonad.

Our results suggest that Hox genes may play an indirect role in PGC specification. As has been suggested in the case of mouse PGC specification (7, 8, 56), down-regulation of Hox genes may suppress somatic fate, permitting or facilitating adoption of PGC fate by BMP-responding cells. Alternatively, Hox genes may act in the somatic cells adjacent to PGCs to negatively regulate PGC fate, thus limiting one or both of the number of cells that can adopt PGC fate, or the position where PGCs emerge in the abdominal segments. The stage in development at which PGCs are reliably quantifiable (4 d AEL) is nearly 2 d after their initial specification (2.5 d AEL). Thus, changes in PGC number or placement that are observed at 4 d AEL could be the result of changes in any or all of their initial specification at 2.5 d AEL, subsequent proliferation, putative apoptosis, maintenance, or short-range migration to the correct segmental locations (4). Our eRNAi experiments likely disrupt transcript levels before and during this entire phase of PGC development. As a result we cannot definitively address which of the processes described above may require Hox gene input.

These caveats notwithstanding, one possible role for these Hox genes in A2 to A4 may be to regulate an appropriate number of PGCs in the area where the gonad must form. An alternative, but not mutually exclusive, possibility is that Hox gene expression suppresses PGC fate in somatic gonad precursors such that loss of Hox gene expression results in an expansion of the PGC population at the expense of somatic gonad precursor cells. *G. bimaculatus* PGCs do not undergo long-range migration (5), and the cells of the somatic gonad primordia are thought to lie within A2 to A4 (4). As a first step toward testing these hypotheses, we asked if *G. bimaculatus* orthologs of *Six4*

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. S8). 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 that would have been fated 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-Ubx* 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 segments 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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