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Juvenile hormones direct primordial germ cell migration to the embryonic gonad

Lacy J Barton^{1,2}, Justina Sanny¹, Emily Packard Dawson¹, Marcela Nouzova^{3,4}, Fernando Gabriel Noriega^{3,5}, Matthias Stadtfeld^{1,6}, Ruth Lehmann^{1,7}

¹Department of Cell Biology, Skirball Institute of Biomolecular Medicine, and Howard Hughes Medical Institute, NYU Grossman School of Medicine, NY, USA, 540 First Avenue, New York, NY 10016, USA

²Department of Neuroscience, Developmental and Regenerative Biology, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX, 78249, USA

³Department of Biological Sciences and Biomolecular Sciences Institute, Florida International University, 11200 SW 8th St, Miami, FL 33199, USA

⁴Institute of Parasitology, Biology Centre CAS, Ceske Budejovice, 37005, Czech Republic

⁵Department of Parasitology, University of South Bohemia, České Budějovice, 37005, Czech Republic

⁶Present: Department of Medicine, Weill Cornell Medicine, 413 E 69th St, New York, NY 10021, USA

⁷Present: Whitehead Institute and Department of Biology, MIT, 455 Main Street, Cambridge, MA 02142, USA

Summary

Germ cells are essential to sexual reproduction. Across the animal kingdom, extracellular signaling isoprenoids, such as retinoic acids (RAs) in vertebrates and juvenile hormones (JHs) in invertebrates, facilitate multiple processes in the germline lifecycle. Here we investigated the role of these potent signaling molecules in embryonic germ cell migration, using JHs in *Drosophila melanogaster* as a model system. In contrast to their established endocrine roles during larval and adult germline development, we found that JH signaling acts locally during embryonic development. Using an *in vivo* biosensor, we observed active JH signaling first within

Corresponding authors: Lacy.Barton@utsa.edu and Lehmann@wi.mit.edu. Corresponding author's lab websites: www.germline.dev and www.lehmannlab.com.

Lead contact: Ruth Lehmann

Author Contributions

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Declaration of Interest

The authors declare no competing interest.

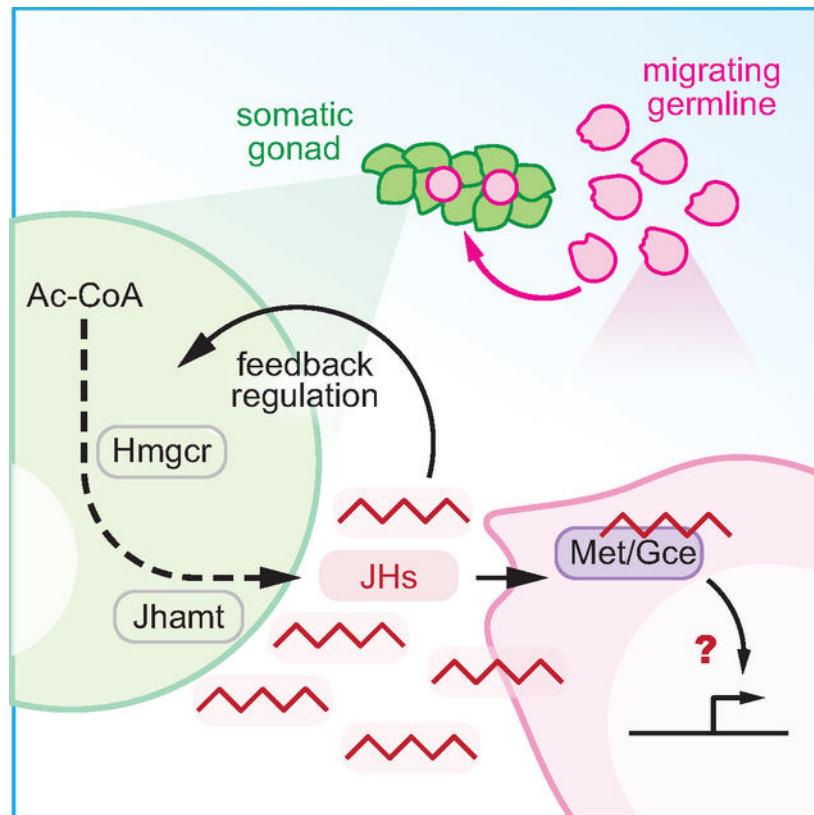
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and near primordial germ cells (PGCs) as they migrate to the developing gonad. Through *in vivo* and *in vitro* assays, we determined that JHs are both necessary and sufficient for PGC migration. Analysis into the mechanisms of this newly uncovered paracrine JH function revealed that PGC migration was compromised when JHs were reduced or increased, suggesting that specific titers or spatiotemporal JH dynamics are required for robust PGC colonization to the gonad. Compromised PGC migration can impair fertility and cause germ cell tumors in many species, including humans. In mammals, retinoids, a JH-related family of signaling isoprenoids, has many roles in development and reproduction. We discovered that retinoic acid, like JH, was sufficient to impact PGC migration *in vitro*. Together, our study reveals a previously unanticipated role of isoprenoids as local effectors of pre-gonadal PGC development and suggests a broadly shared mechanism in PGC migration.

eTOC Blurp

Here, Barton *et al.*, demonstrate that retinoid-like Juvenile hormone signaling is locally active in and around migrating primordial germ cells (PGCs) in the *Drosophila* embryo. Using a combination of *in vivo* and *in vitro* studies, they find that Juvenile hormones are both necessary and sufficient for PGC migration.

Graphical Abstract



Keywords

primordial germ cells; cell migration; gonad; germline; mevalonate pathway; juvenile hormone; retinoids

Introduction

In sexually reproducing species, the germline links one generation to the next. Given the importance to individuals, societies, and species, understanding how germ cells are set aside, develop, and protected until undergoing gametogenesis to yield sperm and egg has been the focus of intense efforts. Through recent advances in single-cell ‘omics and *in vitro* derivation of primordial germ cell (PGC)-like cells from induced pluripotent stem cells, we have gained substantial insights into the molecular programs intrinsically required for germ cell specification and development. However, the yield and gamete quality of *in vitro* derivation approaches falls far short of what is observed *in vivo*¹. This quality gap suggests that we are failing to recapitulate key aspects of germline development. One critical component not yet replicated *in vitro*, nor fully understood *in vivo*, is how germline development is exquisitely coordinated in space and time during embryogenesis by surrounding somatic cells.

Somatic cells coordinate germ cell development and gametogenesis through small, secreted molecules such as steroid hormones and signaling lipids. These potent molecules have multifaceted impacts on reproduction across the animal kingdom. In vertebrate animals, key reproductive small molecules include steroid hormones (i.e., estrogen and testosterone) that are synthesized from cholesterol, and paracrine retinoic acids (RAs), that are derived from carotenoids, a subfamily of isoprenoids. Most animals, though not all², lack the ability to synthesize carotenoids and thus obtain these critical molecules from their diet. Despite differences in biosynthetic origins and structures, invertebrate animals also use steroids and isoprenoids to facilitate many aspects of germline development and reproduction. In *Drosophila*, the steroid hormone, ecdysone, regulates germline stem cell homeostasis, niche development, yolk uptake, and the migratory behavior of certain ovarian somatic cell types^{3–6}. The RA-like isoprenoids, Juvenile hormones, have profound impacts on reproduction. For example, methyl farnesoate is a key sex differentiation molecule in crustaceans⁷. In many insects, JHs act as gonadotropins, with essential roles in vitellogenesis^{8,9}. Recent work in *Drosophila* has revealed that JHs also regulate germline stem cell number and reproductive diapause^{10,11}.

While much is known about how steroid hormones mechanistically control the germline lifecycle, how signaling isoprenoids regulate the germline lifecycle is much less understood. Given the complexity surrounding RAs in germline biology, insights can be gleaned from JHs. These potent sesquiterpenoids, derived from the mevalonate pathway¹², are best known for their influence on juvenile development¹³. They include JH III, JH III bisepoxide (JHB₃), and methyl farnesoate (Figure 1A). As signaling molecules, JHs bind to a basic helix-loop helix/Per-ARNT-SIM (bHLH-PAS) receptor, which in *Drosophila* is encoded by two paralogous genes, *Methoprene-tolerant* (*Met*) and *germ cell-expressed bHLH-PAS*

(*gce*)^{14–16}. Upon ligand binding, these receptors regulate gene expression together with the co-activator Taiman¹⁷. In contrast to paracrine acting RAs, JHs act hormonally such that JH is synthesized in the corpus allatum (CA), a neuroendocrine gland, and released into the circulatory system (Figure 1A). JH production is driven by Juvenile hormone acid methyltransferase (Jhamt), which is expressed in the CA as soon as this gland forms at the end of embryogenesis¹⁸. Curiously, a burst of *jhamt* expression has also been observed in the trunk mesoderm during mid-embryogenesis, before the specification of the CA precursors¹⁸, leaving open the possibility that JHs have additional, non-endocrine roles.

Here we report a paracrine role for JHs during PGC migration. Using an *in vivo* sensor, we detect JH signaling in the proximity of PGCs and found that *jhamt* mutants lacking the methyltransferase activity compromised JH synthesis and compromised PGC migration to the gonad. Extragonadal PGCs are also found in animals lacking HMG Coenzyme A reductase (HMGCR)¹⁹, the rate-limiting enzyme of the mevalonate pathway upstream of Jhamt in JH synthesis¹². Consistent with HMGCR and Jhamt being part of the same synthetic cascade, we found that *jhamt* and *Hmgcr* genetically interact to facilitate PGC migration and that JH, like *Hmgcr*, is both necessary and sufficient for PGC migration. Our further investigations into the function of JH receptors in the embryo revealed a feedback mechanism necessary to maintain JH homeostasis critical for PGC migration. Together, our studies uncovered a new paracrine function for JH signaling in embryonic reproductive development. Studies in mice demonstrated that, like JHs, genes critical to RA synthesis are expressed in the bipotential gonad during PGC development^{20–22}. However, despite shared expression of genes involved in reproductive isoprenoids in vertebrate and invertebrate species, a role for isoprenoid signaling during PGC migration has not been described. Here we demonstrate that RA is sufficient to induce migration of both *Drosophila* and mouse embryonic germ cells, suggesting that this newly identified role for secreted isoprenoids on early germline development may be broadly shared across invertebrate and vertebrate species.

Results

Juvenile hormone signaling is active in the embryonic mesoderm during PGC development.

Juvenile hormone production is driven by Juvenile hormone acid methyltransferase (Jhamt). Jhamt is specifically expressed in the CA, as soon as this JH-producing endocrine gland develops in late embryogenesis¹⁸. Curiously, in *Drosophila* *jhamt* mRNA is also detected during mid-embryogenesis in the trunk mesoderm, before specification and migration of CA precursors¹⁸. Using dual-label fluorescent *in situ* hybridization for mRNAs encoding Jhamt and the germ cell factor, Nanos, we investigated where *jhamt* is expressed relative to PGCs. Consistent with previous work¹⁸, we observed *jhamt* expression in the mesoderm from embryonic Stage 10 (~4 hours after fertilization), continuing through Stage 13 (~11 hours after fertilization) (Figure 1B). At this stage in embryogenesis, PGCs exit the endoderm and sort into two lateral populations as they migrate within the mesoderm toward the somatic gonadal precursors (Figure 1C). This *jhamt* expression pattern suggests that JH synthesis may occur prior to CA formation and be involved in embryonic germline development.

To determine whether mesodermal *jhamt* transcription leads to functional JH production during *Drosophila* embryogenesis, we generated an *in vivo* JH sensor. We placed *eGFP* downstream of eight tandemly repeated copies of a JH response element (JHRE) from the *early trypsin* gene of *Aedes aegypti*²³, which was previously shown to robustly and specifically responds to JH *in vitro* (Figures 1D and S1A)¹⁶. To test the responsiveness of this biosensor to JH *in vivo*, we fed second instar larvae the JH mimic, methoprene, and measured mRNA levels of *GFP* and the endogenous JH signaling transcriptional target, *Krüppel homolog 1* (*Kr-h1*), by real-time-qPCR 24 hours later. We found that *Kr-h1* and *GFP* mRNA levels increased ~two-fold and seven-fold respectively in *JHRE-GFP* larvae that were fed methoprene (Figure 1D). To determine whether this induction by methoprene required JH signaling, we generated animals carrying the JHRE sensor but lacking JH receptors, *Met* and *Gce*. We found that methoprene did not increase expression of either *Kr-h1* or *GFP* mRNA in *Met, gce; JHRE-eGFP* mutant animals (Figure 1D). Together, these data indicate that this new JH sensor faithfully responds to JH signaling *in vivo*.

With this *in vivo* JH sensor, we next investigated if and where JH signaling is active during embryonic development. We stained *JHRE-GFP* embryos with antibodies against GFP and the germ cell-specific RNA helicase, Vasa. GFP signal was first detected in Stage 10 embryos in two locations: in clusters in the anterior portion of the embryo and in bilateral strips of mesoderm within the posterior germband (Figure 1E,F). The anterior signal was still detected in *Met, gce* mutant embryos carrying the JH biosensor, but the mesodermal signal was absent (Figure 1E). To explore whether this difference is due to non-specific, receptor-independent transgene activation, we analyzed animals carrying a JH sensor transgene in which the *Met/Gce* binding motif was mutated (Figure S1A). Anterior GFP was still present in animals with the mutated biosensor; further investigation revealed that this non-JH-specific GFP signal localized to hemocytes (Figure 1B,C). Importantly, the mesodermal signal was not detected in embryos carrying the JH biosensor with mutated receptor binding motifs¹⁶, nor when the functional biosensor *JHRE-eGFP* was tested in *Met, gce* receptor mutants. Thus, the mesodermal signal is due to JH-mediated receptor activation and reflects JH signaling. We found that this specific JH sensitive response began at Stage 10 in the mesoderm (Figure S1D), when PGCs enter this tissue from the endoderm. The JH biosensor was also active in migrating PGCs, though the signal was less intense than in the mesoderm (Figure 1F). JH signaling continued to localize within and near PGCs as they colonized the gonad (Figures 1E, S1C,E). Together, these data demonstrate that JH signaling is active near the mesodermal *jhamt* expression zone and surrounds migratory PGCs during embryogenesis.

Juvenile hormones are required for migrating PGCs to reach the somatic gonad.

To determine whether embryonic JH signaling supports early PGC development, we generated new mutant *jhamt* alleles. Using CRISPR editing, 15 *jhamt* alleles were generated, two of which carried a premature stop codon at amino acid 46 and 13 carried a premature stop codon at amino acid 54 (Figure S2). All new alleles caused a deletion of the methyl transferase domain central to Jhamt-mediated JH biosynthesis (Figure 2A). To avoid confounding effects from second site mutations, phenotypic analyses carried out in this study use the newly generated *jhamt* lines in heteroallelic combinations or *in trans* to an

independently generated small deficiency lacking the entire *jhamt* locus. To verify that Jhamt function was compromised, JH III and JHB₃ titers were directly quantified by liquid chromatography-tandem mass spectrometry²⁴. We found that JH III titers were reduced 50% and JHB₃ titers were reduced >90% in larvae possessing our newly generated alleles (Figure 2B). These results are consistent with previous observations showing that JHs were still detected in animals lacking the entire *jhamt* locus²⁵, suggesting the *Drosophila* genome may contain a second, yet unknown, methyltransferase capable of JH synthesis^{26,27}. To attest the degree by which JH function was compromised, we measured female fecundity. Consistent with previous reports²⁵, the number of eggs laid per day was significantly reduced in females carrying our new *jhamt* mutant alleles (Figure 2C). Together, these data suggest that these *jhamt* mutants significantly affect JH production and display JH loss of function phenotypes.

With new genetic tools, we next compared PGC development in wildtype and *jhamt* mutant embryos. The first detectable germline defect in *jhamt* mutant embryos was an increased number of PGCs that failed to reach the gonad at Stage 14 (Figure 2D). Animals lacking zygotic Jhamt had significantly more extragonadal PGCs than wildtype animals (Figure 2E). This phenotype was rescued when *jhamt* was zygotically expressed with a broad *Tub-GAL4* driver (Figure 2E). These data indicate that *jhamt* expressed in somatic cells facilitates PGC migration to the developing gonad.

PGCs in *jhamt* mutants exited the gut yet some failed to properly colonize the gonad. Closer examination revealed that PGCs remained posterior to the gonad and within the mesoderm, similar to the PGC migratory defect observed in embryos lacking HMG-CoA reductase (HMGCR), the rate-limiting enzyme in the mevalonate pathway (Figure 2D,E)¹⁹. The observation is consistent with previous reports that HMGCR is required for JH synthesis and genetically interacts with *jhamt*²⁵. In *Drosophila*, HMGCR was shown to have a striking, instructive effect on PGC migration: in *Hmgcr* mutant embryos PGCs failed to reach the gonad and when mis-expressed, elevated *Hmgcr* expression alone guided PGCs to the location of ectopic expression (Figures 2F, S2A,B)^{19,28,29}. To determine whether Jhamt functions downstream of HMGCR in PGC migration, we tested for genetic interaction in two ways. First, we measured the effect of genetic *Hmgcr* reduction in sensitized *jhamt* heterozygous animals and found that significantly more PGCs failed to reach the gonad in *jhamt*^{+/-}; *Hmgcr*^{+/-} heterozygous embryos than embryos heterozygous for *jhamt* alone (Figure 2E). Second, we tested whether genetic reduction of Jhamt suppresses the ability of ectopic *Hmgcr* expression to misdirect PGCs. Indeed, loss of one wildtype *jhamt* copy significantly reduced the number of PGCs misdirected by GAL4/UAS-mediated expression of *Hmgcr* (Figure 2F). Together, these data suggest that Jhamt functions downstream of HMGCR to facilitate PGC migration.

Juvenile hormone is sufficient for PGC migration.

HMGCR is sufficient to misdirect migrating PGC to many tissues, even tissues far from the normal migratory path such as the central nervous system (Figures 3A, S3A)^{19,28,29}. To determine whether, like *Hmgcr*, *jhamt* expression is sufficient for PGC migration *in vivo*, we ectopically expressed *jhamt* mRNA using the GAL4/UAS system. We found that

neuronal *Elav-GAL4* driven *jhamt* expression led to a modest, yet significant, increase in PGCs near neuronal Elav-positive cells or even within the central nervous system (Figure 3A,B). Yet overall, significantly fewer PGCs were misdirected by ectopic *jhamt* compared to ectopic *Hmgcr* driven by the same *Elav-GAL4* transgene (Figure 3B). Moreover, unlike *Hmgcr*, ectopic *jhamt* expression was insufficient to attract PGCs to inappropriate locations when *UAS-jhamt* was induced by the endodermal driver, *48Y-GAL4*, or the pan-mesodermal drivers, *twist-GAL4* and *24B-GAL4* (Figure S3B). These data show that Jhamt is sufficient to impact PGC migration *in vivo* in at least one, but not all, tissues, suggesting that other pathway components may be limiting.

HMGCR and Jhamt are both required for JH biosynthesis. There are at least two possible explanations for the difference by which ectopic expression of *jhamt* and *Hmgcr* was sufficient to attract PGCs *in vivo* (Figure 3B). HMGCR may direct PGCs through production of JH and non-JH signals. Indeed, HMGCR is required for the synthesis of many molecules and post-translational protein modifications²⁹. Alternatively, *jhamt* expression may not be sufficient in every tissue due to a lack of precursors derived from the upstream mevalonate pathway. Indeed, HMCR is a rate-limiting enzyme and its expression is spatially restricted during mid-embryogenesis¹⁹. To assess the role of JH directly, we turned to an *in vitro* transwell migration assay, in which *Drosophila* PGCs were obtained from whole embryos, enriched by fluorescence-activated cell sorting (FACS), and placed on a porous filter above media (Figure 3C). Previous work showed that PGCs will translocate in response to serum-free culture media conditioned with Kc167 or S2 embryonic *Drosophila* cells³⁰ (Figure 3D). To explore the impact of JH on PGC migration in this *in vitro* migration assay, we knocked down *jhamt* mRNA in Kc167 cells using two independent dsRNAs (STable 1), collected conditioned media, and measured PGC translocation. Like *Hmgcr* depletion³⁰, significantly fewer PGCs translocated in response to media that was conditioned by *jhamt*-depleted Kc167 cells compared to control conditioned media (Figure 3D). To verify that reduced PGC translocation was not due to fewer Kc167 cells conditioning the media, we quantified Kc167 cell number and found that *jhamt* depletion had no effect (Figure S3B). These data indicate that Jhamt, produced in somatic cells, facilitates PGC migration *in vitro*.

We next asked whether JHs alone are sufficient for PGC migration. To this end, FACS-enriched PGCs were placed on the transwell filter above serum-free, unconditioned culture media supplemented with commercially available JH III with 1% BSA as a non-specific carrier protein. Previous work has shown robust responses to exogenous JHs at concentrations ranging from 1 μ M to 600 μ M in tissue culture, adult feeding, and larval topical application-based assays^{11,16,31,32}. Using the same eight tandem JHREs upstream of luciferase, 1 μ M JH III was required for full response¹⁶. Testing a range of concentrations, we found that 250nM, 2.5 μ M, and 25 μ M of JH III increased expression of the JH signaling target, *Kr-h1* (Figure S3C). Using these concentrations, we found that PGCs translocated in response to JH III, showing a positive correlation with JH concentration except at the highest JH concentration tested (Figure 3E). PGCs did not translocate in response to *in vitro* active concentrations of 20E (Figures 3E, S3C). To further test the specificity of this response, we subjected PGCs to the JH III analogue and insecticide, Methoprene, and found that PGCs

translocated in response to Methoprene at levels comparable to JH III (Figure 3F). Together, these data demonstrate that JHs are sufficient to induce or direct PGC migration.

Compensatory feedback maintains JH homeostasis in *Drosophila melanogaster*.

Our findings suggested that JHs originating from the somatic mesoderm direct PGC migration to the gonad. We next investigated the mechanisms by which PGCs respond to JHs. The canonical JH signaling response involves JHs binding to the Met/Gce receptor, which then associates with the co-activator, Taiman, to promote transcription of target genes (Figure 1A). To determine whether the JH receptors are required for PGC migration, we analyzed embryos mutant for the two, partially redundant, JH receptors^{15,16}. To test the maternal and zygotic contributions of Met/Gce, we generated new *Met^l*, *gce^{Mi02742}* double mutants, since existing *Met*, *gce* double mutant strains die during metamorphosis¹⁵. In *gce^{Mi02742}* mutants, *gce* mRNA is reduced by more than 90% (Figure 4A,B) and *Met^l* carries a point mutation that renders *Drosophila* 33-fold more resistant to lethality inflicted by methoprene³³. This strain was crossed with *Met²⁷*, *gce^{2.5k}* heterozygous females. Even under non-crowding conditions, only ~5% of the expected class of heteroallelic *Met*, *gce* double mutant animals eclosed. As expected^{15,34}, the fecundity of these escaper *Met*, *gce* females was significantly reduced (Figure 4C) but sufficient to permit analysis of Met and Gce requirements in PGC development.

To investigate Met/Gce requirements in PGC migration, we stained maternally and zygotically *Met*, *gce* mutant embryos for Vasa and DAPI. Embryos lacking either maternal or zygotic Met and Gce showed a moderate loss of PGCs relative to wildtype controls at the end of migration (Figure 4D). However, we observed a significant increase in the number of extragonadal PGCs in embryos lacking both maternal and zygotic Met and Gce (Figure 4D). To further explore the relationship between JH receptors and HMGCR in PGC migration, we asked whether loss of Met/Gce suppressed the ability of PGCs to respond to HMGCR-dependent cues. For this experiment, we chose to express HMGCR ectopically in the endoderm, which blocks PGC migration at the beginning of their migratory path at a time when PGCs have only begun to activate the zygotic genome and still mostly rely on maternally deposited mRNAs. Using the endodermal *48Y-GAL4* line to drive *Hmgcr* expression, 50–80% of PGCs were trapped within the gut (Figure 4E). By crossing wildtype or *Met*, *gce* double mutant females to males containing *48Y-GAL4* and *UAS-Hmgcr* transgenes, we could analyze the maternal contribution of Met, Gce receptors to PGC migration. Quantification of the percentage of PGCs remaining in the gut after gonad colonization revealed a small, but statistically significant, suppression of PGCs trapped by ectopic HMGCR expression when the maternally provided JH receptors were depleted (Figure 4E). Together, these data are consistent with a requirement for Met and Gce in PGC migration to the gonad downstream of HMGCR/JH signaling.

Canonical JH signaling leads to transcriptional changes of target genes. To determine whether transcription is required for PGCs to respond to JHs, we returned to the *in vitro* migration assay, where we inhibited transcriptional elongation specifically in PGCs using the P-TEFb-associated CDK9 inhibitor, flavopiridol. To test whether flavopiridol inhibits transcription rapidly and throughout the entire duration of the *in vitro* migration assay,

which includes both a thirty-minute pre-incubation and a 1.5–2-hour incubation in the transwell plates, we stained PGCs for phosphorylation of RNA Polymerase II at Ser2 and Ser5 fifteen minutes and three hours after flavopiridol treatment. As expected³⁵, flavopiridol rapidly and robustly inhibited transcriptional elongation in isolated PGCs (Figure S4). We then subjected control and transcriptionally deficient PGCs to conditioned media, serum-free media, or serum-free media containing JH III and found that inhibition of transcription did not alter PGC translocation in response to any condition tested (Figure 4F). These data suggest that the migratory response of PGCs to JH does not depend on classical transcription-dependent JH receptor signaling.

We were surprised that PGC migration to the gonad was compromised in *Met*, *gce* mutant animals *in vivo*, yet transcription was not required for JH-mediated PGC migration JHs *in vitro*. However, we noticed that extragonadal PGCs in *Met*, *gce* mutant embryos were often located centrally and anterior to the gonad, rather than posterior to the gonad as observed in *jhamt* and *Hmgcr* mutant embryos (Figures 2D,4D). The central, anterior location of extragonadal PGCs in *Met*, *gce* mutant embryos suggested that migration to the gonad may be compromised at an earlier stage than in *jhamt* mutant embryos. One possible explanation is that altered JH homeostasis within the embryos, rather than loss of JH signaling response in migrating PGCs, were the cause of extragonadal PGCs in *Met*, *gce* mutants. To test this possibility, we measured JH titers by quantitative mass spectrometry. Interestingly, we found that both JH III and JHB3 titers were dramatically increased in *Met*, *gce* mutant animals relative to wildtype (Figure 5A). To investigate how JH titers could be increased in *Met*, *gce* mutant animals, we measured *jhamt* mRNA levels by real time-qPCR. We found increased *jhamt* mRNA levels in *Met*, *gce* mutants in several life stages, including during mid-stage embryo embryogenesis (Figures 5B, S5A). Consistent with compromised JH-dependent transcriptional signaling, expression of the target gene, *Kr-h1*, was not increased in *Met*, *gce* mutant animals (Figure 5B). These data uncovered a compensatory feedback mechanism in *Drosophila melanogaster* by which JH receptors mediate JH titer homeostasis. In addition, these data suggest that PGC migration to the gonad is compromised by either decreased or increased JH levels.

Isoprenoids in PGC migration

JHs belong to a broader class of secreted isoprenoids that have functions in reproduction throughout the animal kingdom. Such molecules include methyl farnesoate, which has potent impacts on sex differentiation in the crustaceans⁷, and retinoic acids (RAs), which have pleiotropic effects at many stages of vertebrate reproductive development and gametogenesis^{36–38}. Although the transcriptional receptors differ, they are promiscuous enough to bind to a broad class of exogenous secreted isoprenoids and other small molecules³⁹. To determine whether *in vitro* cross-activation extends to PGC migration, we enriched *Drosophila* PGCs by FACS and subjected them to JH III or two RA species, all-trans retinoic acid (ATRA) or 9-cis RA. Notably, 9-cis RA induced *Drosophila* germ cell translocation to levels comparable of JH III (Figure 6A). These data show that RAs can induce *Drosophila* PGC migration, suggesting commonality in response to this class of secreted isoprenoids.

Our studies revealed that the JH biosynthesis enzyme was present in the mesoderm near PGCs as they migrated toward the developing gonad during *Drosophila* embryogenesis. Interestingly, the enzymes critical for RA biosynthesis are also expressed in the developing bipotential gonadal ridge of mouse embryos as germ cells colonize this tissue²¹. To determine whether RAs may have a similar role in vertebrate germ cell development, we adapted the *in vitro* transwell migration assay developed for *Drosophila* PGCs to mouse germ cells. We obtained mouse germ cells by FACS from embryonic day E10.5 mice carrying a germ cell-specific transgene (*Oct4ΔPE:GFP*) (Figure S6A). At this stage in reproductive development, mouse PGCs are completing their migration and beginning to populate the bipotential gonad. We exposed germ cells to fetal bovine serum in PGC media as a positive control or serum-free PGC media as a negative control. Supplementing serum-free medium with ATRA or 9-cis RA induced translocation of mouse embryonic germ cells to levels comparable to the known mouse and zebrafish PGC chemoattractant, C-X-C Motif Chemokine Ligand (CXCL12) (also called SDF1a) (Figure 6B)^{40–42}. Although RAs have a proliferative effect on migratory mouse PGCs over 24–72 hours⁴³, we did not detect changes in total PGC number during our two-hour assay (Figure S6B). Therefore, the increased number of translocated PGCs was not due to RA-induced proliferation. Together, these data suggest that the newly uncovered role of JHs in early PGC migration to the gonad may be broadly shared among isoprenoids in invertebrate and vertebrate species.

Discussion

Somatic cells provide guidance cues that shepherd germ cells during development and differentiation. Here, we demonstrated that Juvenile hormones, key gonadotropins in adults, function in PGC guidance to the embryonic gonad. In contrast to its known endocrine role in juvenile and adult animals, we observed spatially restricted JH signaling enriched around PGCs as they migrated to the gonad (Figure 6C). We found that JHs were necessary for robust PGC migration in the *Drosophila* embryo and sufficient for PGC attraction in an *in vitro* assay. We expanded these studies to include the vertebrate isoprenoid, RA, finding that RA was sufficient for mouse and fly germ cell migration in the *in vitro* assay. Together, these studies strongly argue for paracrine functions by isoprenoids, and specifically JHs, in supporting embryonic germline development.

Investigations into JH biology have largely focused on endocrine signaling in juvenile and adult animals, particularly for insects that undergo a complete metamorphosis, like *Drosophila*. In juvenile and adult animals, JHs are produced in the CA, released into the circulatory system, and act systemically throughout the animal^{44,45}. Surprisingly, we found *jhamt* expressed in the mesoderm prior to CA development. This is consistent with previous reports of a burst of *Kr-h1* expression in 8–12 hour old embryos^{46,47} and of non-CA *jhamt* expression and local JH function in gut cells⁴⁸. Consistent with paracrine signaling, the JH biosensor was active within the mesoderm and juxtaposed to *jhamt* expression in the *Drosophila* embryo. How JH signaling is restricted is unclear as JH transcription factors, *Met*, *gce* and *taiwan* mRNAs are maternally loaded and potentially present in all cells of the early embryo, including PGCs^{49–52}. JH signaling may be restricted by expression patterns of JH synthetic and degradation enzymes, such as HMGCR. As a classic rate-limiting enzyme, HMGCR restricts precursor availability, first to the germband mesoderm and then

to the developing gonad¹⁹. Restricted availability of JH precursors can explain why ectopic *Hmgcr*, but not ectopic *jhamt*, mis-directs PGCs in all tissues tested. JH production may be further spatiotemporally restricted due to post-transcriptional regulation of *jhamt* by miRNAs^{53,54}.

Restriction of JH production is consistent with JH's function as a PGC attractant downstream of HMGCR¹⁹. Indeed, ectopic HMGCR directs PGC migration within a ~50µm zone²⁸. We found that loss of HMGCR compromises the final stage of PGC migration, a phenotype similar to that of *Jhamt* loss but more severe. The observed difference in phenotypic severity could be due to HMGCR-mediated production or secretion of pro-migratory factors independent of JH signaling, however a simpler explanation is that *Jhamt* loss does not completely abolish JH titers, as we and others observed²⁵. JHs are still present in *jhamt* mutant animals due to a long postulated, still elusive, second JH methyltransferase that could also contribute migratory activity^{26,27}. The second JH methyltransferase may be maternally provided like Farnesol-Diphosphate Synthase (FPPS), the enzyme that synthesizes Farnesol for JH production that is also required for PGC migration²⁹. One way to clarify the relationship between JH and HMGCR in PGC migration is to elucidate the many currently unknown intermediate enzymes.

Our findings show that JHs facilitate PGC migration. How migrating PGCs respond to JHs remains unclear. Classic JH signaling involves receptor-mediated transcription. In contrast, we find that transcription is not required in PGCs for their migrational response to JHs *in vitro*. *In vivo*, PGCs respond to ectopic HMGCR while still in the gut, which is prior to full activation of their zygotic genome. A non-transcriptional reaction to JH exposure is consistent with the need of PGCs to rapidly respond to environmental cues. Rapid responses to JHs during ovarian follicle development has been observed for decades^{55–58}, however, the mechanisms remain elusive. While receptor tyrosine kinases have been implicated in response to JHs³², several studies report G protein-coupled receptor (GPCR) involvement in rapid responses to JHs^{58–62}. A GPCR-mediated JH response could explain our observations that *Drosophila* PGC migration is attenuated at the highest dose of JHs *in vitro* and is compromised when JH titers are elevated *in vivo*, which are signs of GPCR desensitization. While GPCR signaling in PGCs via the Tre1 receptor is responsible for PGC polarization and individualization within the gut, these processes and gut exit are unaffected in embryos lacking *Jhamt* or HMGCR⁶³. We speculate that a still elusive plasma membrane receptor mediates JH signaling in PGCs and thereby provides not only a rapid but also a directional response as PGCs approach the gonad.

While PGC response to JHs do not seem to require transcription, the role of JH receptors, *Met* and *Gce*, remains unclear. The JH biosensor, which requires *Met/Gce* transcription factors¹⁶, is weakly detected in migrating PGCs *in vivo*, and PGCs contain *Met/gce* RNA, suggesting that PGCs are equipped to activate JH-mediated transcription. The similar, albeit weak, phenotype when *Met/Gce* are maternally or zygotically lost, as well as suppression of HMGCR-mediated gut retention by maternal *Met/Gce* loss, is consistent with a requirement of *Met/Gce* in PGCs. Yet, the location of extragonadal PGCs in *Met, gce* mutants differs from JH biosynthesis enzyme mutants. In the absence of *Jhamt* and HMGCR, extragonadal PGCs were often found lateral and posterior to the gonads, whereas extragonadal *Met, gce*

PGCs were found medial and anterior to the gonads. This location and frequent endodermal association suggests that migration is compromised in *Met*, *gce* mutants earlier than in *jhamt* mutants. There are several explanations for this unexpected observation. It is possible that JH signaling is compromised earlier and stronger in *Met*, *gce* mutants, as JH titers persist in *jhamt* mutants²⁵. Alternatively, migration may be compromised in *Met*, *gce* mutant animals because JH titers are increased in surrounding somatic cells such that the effect on PGC migration could be indirect. Future work is needed to elucidate precisely how *Met* and/or *Gce* are required for PGC development.

Animals mutant for *Met* and *gce* have compromised PGC migration and increased JHs. These findings support a model in which JH receptors play a role in tuning JH titers and spatial availability to guide PGCs to the gonad. Increased JH titers observed in *Met*, *gce* mutants are coincident with increased *jhamt* mRNA levels at multiple life stages, suggesting that constitutive feedback ensures stage-appropriate JH titers upon genetic perturbations in *Drosophila*. In *Rhodnius prolixus*, knockdown of *Met* or *taiman* increased *jhamt* mRNA levels, while ectopic JH decreased *jhamt* mRNA levels, suggesting that the compensatory feedback is conserved^{64,65}. The mechanism of compensatory feedback is unknown but may involve either *Met*/*Gce*-*Tai* repression or Ecdysone Receptor (*EcR*)-*Tai* activation, as *Met*, *Taiman*, and *EcR* binding have been detected ~1kb upstream of the *jhamt* transcription start site⁶⁶. Alternatively, feedback may involve *Kr-h1*, as ecdysone biosynthesis genes are repressed by *Kr-h1*^{67,68}. This intriguing regulatory loop may further expand our mechanistic understanding of how JH production is robustly tuned.

Our findings indicate *Drosophila* PGC migration is directed by local JH signaling. Local, rather than systemic, JH signaling is similar to retinoic acid (RA) action within vertebrate species. While reports of JH function in cell migration are limited⁶⁹, RAs impact the migration of many vertebrate cell types. Most investigations into the migratory role of RAs have focused on transcriptional upregulation of chemokines or their receptors^{70,71}. Indeed, RA was recently shown to facilitate the transition of human PGCLCs from a pre-migratory to a migratory transcriptional signature⁷². Evidence for transcription-independent functions of RAs is emerging⁷³. RAs facilitate axon guidance in *Xenopus laevis*⁷⁴ and snails, which is characterized by non-transcriptional mechanisms involving calcium flux, Rho GTPases and G proteins^{75–78}. We speculate that non-transcriptional signaling responses underlie JHs and RAs cross-reactivity despite using different classes of transcriptional receptors.

JH and RA isoprenoids share essential requirements for gametogenesis. Notably, the bioavailability of RAs and JHs during analogous stages of germ cell development is strikingly similar between mice and *Drosophila*, respectively^{20–22,79}. We envision *Drosophila* as a powerful genetic model to understand the primary functions and molecular mechanisms by which environmentally pervasive isoprenoids impact reproductive development.

STAR Methods

Resource Availability

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ruth Lehmann (Lehmann@wi.mit.edu)

Materials Availability—All fly stocks and plasmids generated in this study will be distributed by the Lead Contact without restrictions or deposited to repositories such as Bloomington Drosophila Stock Center and Addgene.

Data and Code Availability

- Microscopy data reported in this paper will be shared by the lead contact upon request.
- This study did not generate or analyze datasets, nor generate custom code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Drosophila Strains, Genetics, and Maintenance—All *Drosophila melanogaster* stocks and crosses were raised on medium containing 1.5% yeast, 3.6% molasses, 3.6% cornmeal, 0.112% Tegosept, 1.12% alcohol and 0.38% propionic acid. For embryo collections, apple juice plates contained 25% apple juice, 2.5% sucrose, 2.25% bactoagar, 0.15% Tegosept. Animals were kept at 25°C for all experiments. Male and female embryos were analyzed in their natural ratios from Stages 10–14. Fly strains used in this study are listed in Key Resources Table. The following flies were from Bloomington *Drosophila* Stock Center (BDSC): *y¹, w^{*}*, *gce^{mi02742}* (BDSC #60189), *Met¹* (BDSC #3472), *gce^{mi0274}* (BDSC #35131), *jhamt^{Df(2L)BSC781/CyO}* (BDSC #27353), and *w⁻; 48Y-GAL4* (BDSC #4935). Several strains were generated in the Lehmann previously: the *Hmgcr^{26.31}* and *Hmgcr^{11.54}* strains are described in⁸² the *Hmgcr⁰¹¹⁵²* and *UAS-Hmgcr* strains are described in¹⁹; and the *P[nos-moesin::egfp::nos 3'UTR]* strain is described in⁸³. The stock *Met²⁷*, *gce^{2.5k}* was a gift from Rebecca Spokony and Lynn Riddiford⁸⁴. The stock *UAS-jhamt* was a gift from Ryusuke Niwa¹⁸. The stock *twist-GAL4*; *24B-GAL4* was a gift from Michael Akam.

To generate the P [*JHRE-GFP, w+*] transgenic biosensor, eight copies of the wildtype JH response element (JHRE) from the *early trypsin* gene from *Aedes aegypti*²³, or eight tandem copies of the mutant JHRE (Figure S1), were excised from *pGL4.17* vectors provided by Marek Jindra. JHREs were inserted upstream of the minimal *hsp70b* promoter and *eGFP* coding sequence of pS3aG transformation vector (Addgene #31171) containing an *attb* for site-specific integration. JHRE::GFP sensor constructs were integrated into *y+*, *attP2* at cytological position 68A4 sites by Bestgene. To validate the JH sensor, transgenes were first placed in *met¹*, *gce^{MI}* mutant background. Embryonic GFP signal was measured by immunohistochemistry. To measure JH responsiveness, first instar larvae were placed in food containing 1.6µg of Methoprene per gram of food⁸⁵, or ethanol as a control for 24

hours. *GFP* and *Kr-h1* mRNA was measured in whole second instar larvae by qPCR using primers in the Key Resources Table (Figures 1, S1).

To generate new *jhamt* mutant alleles, the CRISPR guide CCTGGATGTGGTTCAGGATCT was cloned into pU6-2 gRNA vector (DGRC #1363) and injected into embryos into a stock derived from BL51324 carrying *w¹¹¹⁸*; *PBac{mDint2}=vas-cas9VK00027* by Bestgene. Of 29 fertile founders, 87 F1 crosses were set up and screened for *jhamt* mutations by PCR amplification and Sanger sequencing using primers listed in Table S1. Of 48 F1 lines screened, 13 lines were obtained wherein small indels created a frame shift just before the methyltransferase domain, which starts at amino acid 40, leading to a stop codon just downstream, two lines at amino acid 46 (12F1 and 12F2) and the remaining lines at amino acid 54 (1F1, 1F2, 4F1, 4F3, 6F3, 7F2, 7F3, 8F1, 8F2, 12F3, 14M2, 14M3). The effect of new *jhamt* mutations on JH III and JHB₃ titers were quantified in third instar larval hemolymph.

These studies used heteroallelic *Met^{1/27}*, *gce^{Mi/2.5k}* mutant alleles. *Met¹*, which encodes a point mutation in a highly conserved position of the PAS-A domain, and the *Met²⁷* allele, which strongly reduces *Met* mRNA, but the open reading frame appears wildtype (Marek Jindra, personal communication). Both *Met¹* and *Met²⁷* mutant animals have dramatically increased tolerance to methoprene³³. However, in our hands, adult escapers could only be obtained in heteroallelic *Met^{1/27}*, *gce^{Mi/2.5k}* combination. Even under sparse conditions, homoallelic *Met^{27/27}*, *gce^{2.5k/2.5k}* were not observed, suggesting either the presence of a second site lethal mutation in the *Met²⁷*, *gce^{2.5k}* genetic background or that *Met^{1/27}*, *gce^{Mi/2.5k}* animals have enough activity to support development through increased JH titers. The latter explanation is more likely because UAS-driven *Met* or *gce* can rescue *Met^{27/27}*, *gce^{2.5k/2.5k}* lethality¹⁶.

***Drosophila* Cell Lines and Maintenance:** *Drosophila* cell lines were cultured at 25°C at atmospheric oxygen and carbon dioxide levels. Cell lines were obtained from the *Drosophila* Genomics Resource Center (DRSC) were male Schneider's line 2 (S2, DGRC #181) from late stage Oregon R strain embryos and the female Kc-167 (DRSC #1) from dorsal closure stage *e/se* strain embryos. Each cell line has distinct morphologies, which were used to monitor for contamination.

***Mus Musculus* Stains and Maintenance:** Mice expressing a germ cell-specific *Oct4ΔPE:GFP* transgene⁸⁶ (B6; CBA-Tg(Pou5f1-EGFP)2Mnn/J) were obtained from Jackson Laboratory (#004654, Bar Harbor, ME, USA). Natural ratios of male and female Embryonic Day 10.5 embryos were used in this study. All protocols were approved by the NYU Institutional Animal Care and Use Committee.

Method Details

Embryo Immunofluorescence—Prior to embryo collection, young parental strains (less than 5 days old) were mated in vials for one day before transferring to embryo collection cages containing apple juice plates and wet yeast. Embryos were dechorionated in 50% bleach for three minutes, rinsed with water and fixed in a scintillation vial containing 2ml of 4% formaldehyde in PBS and 8ml of heptane. Embryos were fixed for 20 minutes on a

shaker. To remove the vitelline membrane, formaldehyde was removed from the scintillation vial and 10ml methanol was added, followed by one minute of vigorous shaking by hand. Embryos were washed with fresh methanol three times before storage at -20°C or future processing.

For immunohistochemistry, embryos were sequentially rehydrated with increasing percentage of PBSTx (PBS with 0.1% TritonX100) and then washed six times with PBSTx before a one-hour block using 1% BSA in PBSTx at room temperature. Primary antibodies were incubated overnight at 4°C and then washed six times in PBSTx. Secondary antibodies in 1% BSA in PBSTx were incubated for two hours at room temperature, washed six times in PBSTx. Embryos were then equilibrated in Vectashield (Vector Laboratories, H-1200) overnight at 4°C before mounting on coverslips.

Fluorescent *in situ* hybridization was done as previously shown⁸⁷. For *jhamt* and *Nanos* mRNA dual labeling, probes were generated using *Drosophila* clones from DGRC (*jhamt* #11840, clone AT13581) and (*Nanos* #1124533, clone RE53469). For *jhamt* and *GFP* dual mRNA labeling, probes were generated from genomic DNA isolated from *Drosophila* adults carrying the *JHRE-eGFP* transgene. All primers used for template generation are listed in Table S1.

mRNA quantification by qPCR—RNA was obtained by TRIzol (Invitrogen #15596018) extraction. DNA was removed using RQ1 RNase-Free DNase (Promega #M6101). cDNA was generated using SuperScript III Reverse Transcriptase (Fisher Scientific #18080–044) using both oligo dT and random hexamers. Primers used are listed in Table S1. qPCR was carried out using LightCycler 480 SYBR Green I Master 2X (Roche #04887352001) and a Roche LightCycler 480.

Evaluation of JH titers by quantitative mass spectrometry—The following genetic backgrounds were used to measure JH III and JHB₃ titers: *jhamt*^{8F2/12F1} paired with the *w*¹¹¹⁸; *CyO*, *ft-lacZ* genotype, which was the genetic background the CRISPR/Cas9-generated alleles were extensively crossed into and *met*¹, *gce*^{Mi} paired with *w*¹¹¹⁸. Third instar larvae per biological replicate were collected from sparsely populated vials, washed in PBS before placing in 50 μl of pre-chilled PBS for hemolymph collection. To collect hemolymph, the cuticle was pinched and pulled apart down the length of the animal. Care was taken not to rupture fat body or intestines. Hemolymph was transferred to pre-chilled silanized glass vials (Fisher Scientific #C4011S5 and #C401154B) using silanized glass pipettes (Brain Bits #FPP). The glass collection well was rinsed with 50 μl additional, pre-chilled PBS to obtain any remaining hemolymph and additional PBS was added to silanized glass vials for a total volume of 150 μl . Deuterated JH III (62.5pg) dissolved in acetonitrile (Thermo Fisher #51101) was added to each replicate, then 600 μl hexane (Sigma Aldrich #1037011000). Samples were vortexed for 1 minute and centrifuged for 5 min at 2000g at 4°C . Organic phase was transferred to new silanized vial for analysis by mass spectrometry.

For mass spectrometry analysis, the JH III and JH III bisepoxide (JHB₃) amounts present in hemolymph were quantified by liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS)²⁴. The identification and quantification of JH III and JHB₃

in hemolymph samples was based on multiple reaction monitoring (MRM), using the two most abundant fragmentation transitions: for JH III: 267->235 (primary) and 267->147 (secondary), and for JHB₃: 283->233 (primary) and 283->251 (secondary)²⁴.

Knockdown of *jhamt* in Kc167 Cells—To effectively knockdown *jhamt* mRNA, genomic DNA obtained from Kc167 cells using Qiagen's Blood and Tissue Kit (#69506) was used as a template. To target *lacZ*, pBluescript SK plasmid used as a template. dsDNA was generated by Choice Taq Mastermix (Denville Scientific #CB4070–8). Gradient PCR was used to obtain optimal dsDNA templates. dsRNAs were generated from dsDNA templates using T7 MEGAScript (Invitrogen #am1334) according to standard protocol. dsRNAs were DNase treated (Qiagen #79254) and purified using Qiagen RNAeasy Mini Kit (Qiagen #74104) according to instructions. For transfection, Kc167 cells were grown to confluency, resuspended in fresh Schneider's media (Fisher #21720–001) supplemented with Penn/Strep (Fisher, #15140122) and 10% heat inactivated FBS (Thermo Fisher #16140071) to 1×10⁶ cells/ml and allowed to settle overnight. 2µg of dsRNAs were used in conjunction with Effectene Transfection Reagent (Qiagen #301425) into each 6-well dish. After 48 hours at 25°C, media was removed, transfected Kc167 cells were washed in PBS and resuspended in serum-free Schneider's Media (with Penn/Strep) for three additional days at 25°C prior to collection of Kc167-conditioned media. To determine whether *jhamt* knockdown compromised Kc167 viability, the number of Kc167 cells was counted on the day conditioned media was collected. Knockdown efficiency was determined in Kc167 cells by qPCR when conditioned media was collected for the transwell assay. Primers used to measure knockdown efficiency were chosen outside of region targeted by dsRNAs.

Isolation of PGCs—*Drosophila* embryos carrying the *P[nos-Moe::eGFP.nos 3'UTR]* transgene from an overnight collection were dechorionated in 50% bleach for two minutes, rinsed and placed in filter-sterilized Chan and Gehring's Balanced Saline, PGC Sort Buffer (3.2g/L NaCl, 3.0g/L KCl, 0.69g/L CaCl₂·2H₂O, 3.7g/L MgSO₄·7H₂O, 1.79g/L Tricine buffer (pH 7), 3.6g/L glucose, 17.1g/L sucrose, 1g/L BSA, fraction V). Dechorionated embryos were manually homogenized in PGC Sort Buffer with Dounce homogenizer (Pyrex #7727–15) and passed through a 100µm filter and 20µm filter and placed in pre-chilled polypropylene falcon tubes (#352063). Live, GFP-positive *Drosophila* PGCs were sorted using a MoFlo XDP cell sorter fitted with a 70µm nozzle into Maxymum recovery microtubes (Axygen #311–05–051) containing 200µl Schneider's Media.

To enrich for mouse PGCs by FACS, B6; CBA-Tg(Pou5f1-EGFP)2Mnn/J (Jax #004654) mice were bred to produce embryos homozygous for the *Oct4PE::GFP* transgene. Embryonic day 0.5 (E0.5) was assumed to be noon of the day the vaginal plug was observed. Pregnant females were euthanized by carbon dioxide followed by cervical dislocation. Trunks were dissected from E10.5 embryos in chilled PBS. For each two embryos, tissue was dissociated in 300µl of 0.5% trypsin (0.25%, Life Technologies #25200056) for 15 min at 37°C, with occasional manual disruption by pipette. Trypsin was inactivated with 200µl of mouse PGC culture media [DMEM/F-12 (Thermo Fisher #11–320–033), 250µM sodium pyruvate (Thermo Fisher #11360070), 2mM L-glutamate (Gibco #25030081), 1X MEM Non-essential amino acids (Thermo Fisher #11–140–050) containing 25% Bovine Albumin

Fraction V (Thermo Fisher #15260037). Dissociated tissue was centrifuged at 200g for 7 min and cells were resuspended in 2mL of PGC media. Cell suspension was filtered through a 35µm cell strainer into a 5 ml falcon round bottom tube (VWR #21008–948) for sorting using a MoFlo XDP cell sorter fitted with a 100µm nozzle. Live, GFP+ germ cells were sorted into Maxymum recovery microtubes (Axygen #311–05-051) containing mouse PGC media.

***In vitro* Migration Assays**—For *in vitro* transwell assays, all plastic ware, tips, tubes and transwell plates used throughout the assay were pre-coated with PBS containing 1% BSA to prevent hydrophobic molecules from sticking to the surfaces. Assays with light sensitive isoprenoids were done in dark conditions as much as possible. For Kc167-conditioned media, serum-free media was added to washed, confluent Kc167 cells and collected three days later. Conditioned media was collected and centrifuged 4,000rpm for 20 min to remove all cellular debris and placed on ice. All isoprenoids (Key Resources Table) were prepared by dilution series in either serum-free Schneider's media for *Drosophila* PGC assays or mouse PGC media. Transwell assays were done using the 96-well ChemoTx Disposable Chemotaxis System (NeuroProbe #116–10) which had a PCTE filter membrane with 10µm pore size, 300µl well capacity and 5.7mm cell site diameter. Sorted germ cells recovered on ice for 30 min while the transwell system was set up and then diluted 10–15-fold in serum-free Schneider's media for *Drosophila* PGC assays or mouse PGC media so that 60µl diluted PGCs were placed above each well. Transwell plates with *Drosophila* PGCs were incubated 25°C for 1.5 hours and those with mouse PGCs were incubated at 37°C for 2 hours. After the incubation period, PGCs were removed from above membrane, the plate was centrifuged at 460rpm for 10min to dislodge cells from the well side of the membrane. Translocated germ cells were removed from the 96-well plate by pipetting up and down and transferring to Nunc Lab-Tek II Chambered Coverglass (Thermo Fisher #155409) for manual quantification by fluorescent microscopy.

Verification of Hormone Activity *in vitro*—To verify the activity of JH III and 20-hydroxyecdysone (Key Resources Table), hormones were added to 50% confluent Schneider 2 (S2) cells at the concentrations indicated in Supp Fig 3c. After a six-hour incubation at 25°C, S2 cells were harvested, and mRNA was extracted and analyzed using protocols described in the 'mRNA quantification by qPCR' Methods subsection.

Transcriptional Inhibition in *Drosophila* PGCs—Efficient inhibition of transcription in *Drosophila* PGCs isolated by FACS was determined by testing different concentrations of Actinomycin D and Flavopiridol (Key Resources Table). To visualize transcription by immunohistochemistry, Lab-Tek II 8-chambered coverglass were incubated with poly-L-Lysine solution (Sigma-Aldrich #P4707) for 30 min at room temperature and then dried for 1.5 hours. To ensure transcriptional elongation was inhibited quickly and throughout the duration of the assay, sorted *Drosophila* PGCs were added to incubation chambers with Flavopiridol for either 15 min and 3 hours, fixed with 4% paraformaldehyde for 15 minutes, washed in PBS with 0.05% TritonX100, blocked in 1% BSA for 30 min and stained for Vasa, RNA Pol II Ser2CTD and RNA Pol II Ser5 CTD (Key Resources Table). As expected³⁵, Flavopiridol rapidly inhibited transcription in *Drosophila* PGC while

not compromising cell viability. For transwell assays, sorted *Drosophila* PGCs were first incubated with 1 μ M Flavopiridol for 30 min prior to placing on transwell membrane and solutions in the bottom well chambers also contained 1 μ M Flavopiridol to ensure PGC transcription was inhibited throughout the assay.

Microscopy and Image Processing—Most embryos and other samples were imaged with a Zeiss LSM800, an upright laser scanning confocal microscope using Zen Blue 2.3 software and a Plan-Apochromat 20 $\times$ 0.8 NA air objective and 40 $\times$ 1.3NA oil objectives with pinhole size for all imaging was set to 1AU. Some images were collected using a Zeiss LSM 510 using Zen Blue software. Images were processed using ImageJ/FIJI.

Quantification and Statistical Analysis

Sample sizes and quantification methods—Sample sizes are noted within each appropriate Figure Legend. Briefly, studies involving the quantification of extragonadal PGCs *Drosophila* involved two to four biological replicates done on different days from different parents. Each biological replicate included 25–50 embryos. Precise sample sizes are encoded in these data as each dot in Figures 2E, 2F, 3B, 4D, 4E, 4F, and S3A represents a single embryo.

For JH titer measurements, twenty-five larvae per each of five biological replicate was used. Fecundity was measured as previously described⁸⁸ and reported as the average number of eggs laid in three- and four-day old females at 25°C. In each biological replicate, 10–12 females were crossed to 5–6 *w¹¹¹⁸* males to mitigate male fertility defects upon *jhamt* or *met*, *gce* loss. Two to four biological replicates, each including two to three technical replicates were used for qPCR and mRNA knockdown experiments. For mRNA expression experiments, fold change was calculated as previously described⁸¹ and normalized to one or two of three reference genes found to have invariant expression across tissues and developmental stages: *DCTN2-p50*, *und*, *DCTN5-p25*⁸⁹.

For transwell migration assays using *Drosophila* PGCs, each biological replicate started from grams of embryos were obtained large population cages. For transwell migration assays using mouse PGCs, each biological replicate included 10–30 embryos were obtained 3–6 pregnant dams. For *Drosophila* and mouse PGC *in vitro* migration assays, the number of PGCs put above each well was estimated by setting aside 10% input (6 μ l) in duplicate into Lab-Tek wells, each with 300 μ l serum-free media at the beginning of the transwell assay and quantifying the number of germ cells when the experimental translocated germ cells were quantified. To compare biological replicates done on different days, Specific Migration was calculated by first subtracting the number of germ cells that translocated in the negative control wells (serum-free media) and then calculating the percentage of normalized experimental condition to the respective positive control. Each transwell assay includes two to four biological replicates, with each biological replicate consisting of two technical replicates done on the same day.

Statistics—Statistical analysis was done as described in the Figure Legends. Briefly, for quantification of phenotypic data, such as extragonadal PGC quantification, data was not always normally distributed and thus median $\pm$ IQR are reported, and statistical

significance was assessed by Mann-Whitney U test. For mRNA Fold Change, the geometric mean (bar) of normalized expression per sample (dots) is reported with error bars indicating $\pm$ standard error of the mean Log_2 of normalized expression as previously described⁸¹. For *in vitro* Specific Migration results, mean $\pm$ standard deviation is reported and statistical significance was tested by unpaired t-test. For *in vivo* analysis, sample size per biological replicate was influenced by power analysis based on previously observed phenotypic variation. Significance is either noted precisely or as either ns (ns >0.05) or <0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Inclusion and diversity statement

We support inclusive, diverse, and equitable conduct of research.

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Highlights

- Juvenile hormones are necessary & sufficient for Primordial Germ Cell migration.
- JH signaling is active in and around migrating PGCs prior to endocrine function.
- Compensatory feedback sensitive to JH receptor function maintains JH homeostasis.
- JH-like retinoic acids may have similar roles during mammalian germ cell migration.

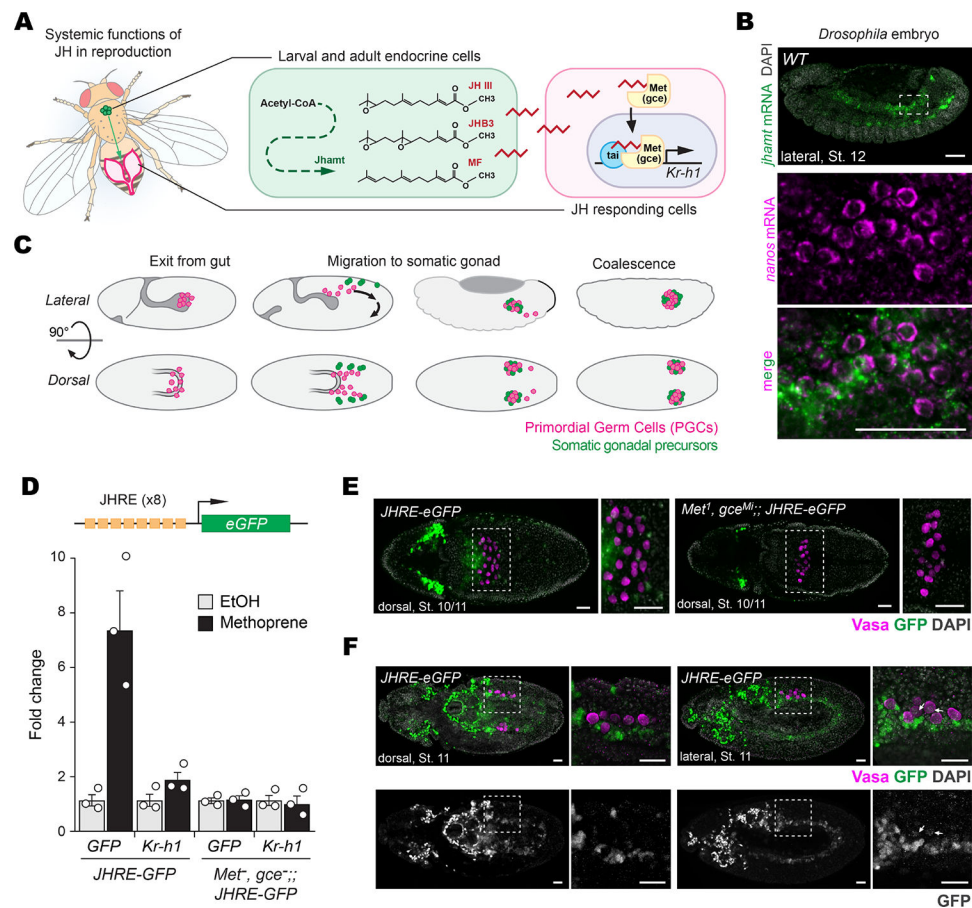


Figure 1: Juvenile hormone (JH) signaling is active in the embryonic mesoderm during PGC migration

A Left: Schematic summarizing gonadotropic effect of JH in insects, whereby JH is produced in an endocrine gland and acts systemically to promote oogenesis. **Right:** Summary of established JH signaling pathways wherein endocrine cells produce JHs downstream of Acetyl-CoA and a biosynthetic pathway that culminates in JH acid methyltransferase (Jhamt)-mediated JH production. JHs (red jagged lines) are then secreted in the hemolymph and enter JH-responding cells. Once in JH-responding cells, JH binds to one of two JH transcriptional receptors, Methoprene tolerant (Met) or Germ cell expressed (Gce). JH-bound Met/Gce translocates to the nucleus to regulate target genes. **B Top:** Lateral view image of a late Stage 12 embryo stained by Fluorescent *in situ* hybridization (FISH) for *jhamt* (green), and *nanos* (magenta) mRNA. Below are zoomed in images of the dashed boxed area from the top image, with *nanos* mRNA (magenta) in the middle panel and the merge of *nanos* (magenta) and *jhamt* (green) mRNAs in the bottom panel. Primers used to generate *in situ* probes are listed in Table S1. **C** Schematic of germ cell development during Stage 10–14 of *Drosophila* embryogenesis. **D Top:** schematic of a new transgenic JH sensor (JHRE-GFP) in which eight copies of the JH Response Element from the *early trypsin* gene from *A. aegypti* were placed upstream of eGFP (See Materials and Methods for construct design). **Bottom:** Induction of *Kr-h1* mRNA, a known JH transcriptional target, and *GFP* mRNA. deltaCt was calculated relative to the average Ct of two housekeeping

genes, *DCTN5-p25* and *Und*, and fold change was calculated for whole second instar larvae fed cornmeal food containing 1.6µg/mg of JH analogue, methoprene normalized to age and genotype-matched larvae fed ethanol. Animals were fed methoprene or solvent control for 24 hours prior to RNA isolation. Bars represent the geometric mean of normalized expression per biological replicate (dot), each consisting of ten second instar larvae. Error bars represent $\pm$ standard error of the mean as previously described⁸¹. **E** Dorsal (d) images of Stage 10 *JHRE-GFP* embryos that are wild type (left) or mutant for Met and Gce (right). **F Left:** dorsal view of a Stage 11 *JHRE-GFP* embryo stained for GFP (green) protein, Vasa (magenta) protein, and DNA using DAPI (gray). **Right:** lateral view of a Stage 11 *JHRE-GFP* embryo. GFP channel is shown below each merged image. For all images, magnified images in the boxed area outlined by a dashed, white square are shown to the right of each whole-embryo image. Arrows point to PGCs which are GFP-positive. Genotypes are noted in the top left corner, embryo orientation in the bottom, and scale bars represent 25µm. All images are maximum projections of two to three 2µm confocal slices. See Figure S1 for supplemental data relating to data within this figure.

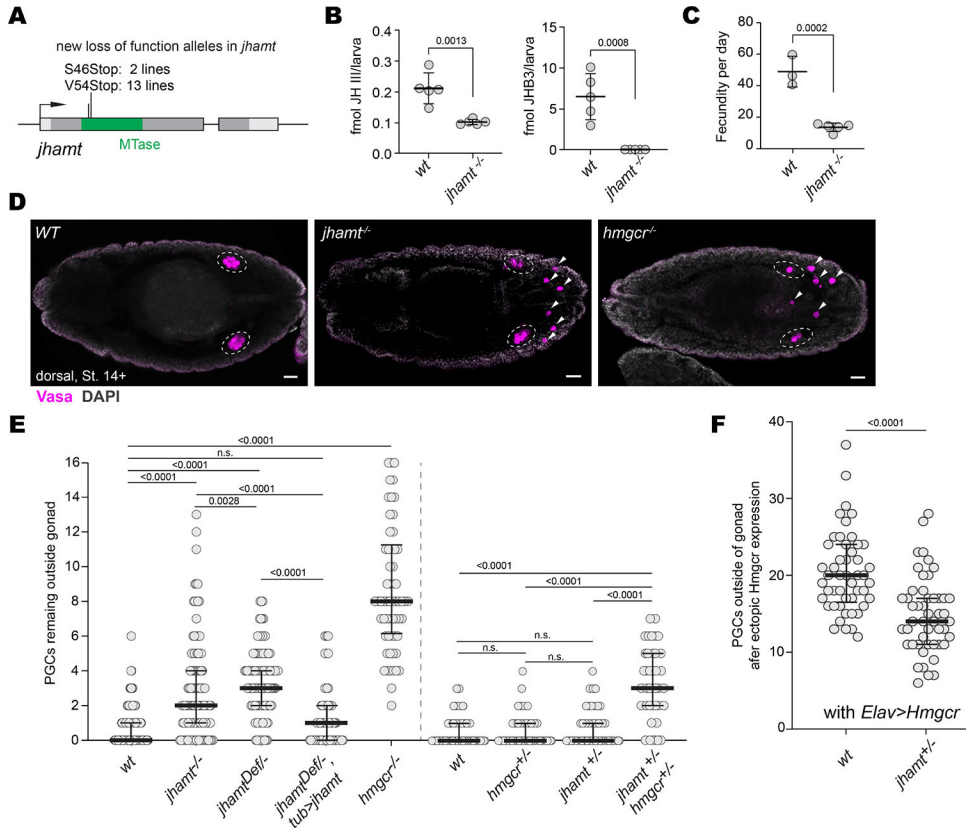


Figure 2: Key JH synthesis enzyme, Jhamt, facilitates PGC migration to the gonad.

A Schematic of the *jhamt* locus noting the location of premature stop codons within the methyltransferase domain (shown in green), which were introduced by CRISPR-mediated editing. Light gray denotes 5' and 3' UTR. **B** Titters of two JHs, JH III and JH bisepoxide (JHB₃), in hemolymph of third instar *Drosophila* larvae measured by quantitative mass spectrometry using deuterated standards. Each dot represents a biological replicate of hemolymph bled from 25 larvae. For titers, *w*⁻, *Sp/CyO,ftz-lacZ* was used as a wildtype strain as the CRISPR-induced alleles in a *w*⁻ background were initially crossed into this background. **C** Fecundity of young females. Each dot represents the average number of eggs laid per biological replicate, which was obtained by dividing the number of eggs laid over a 24-hour period by the number of females (10–12) in each cohort three- and four-days after eclosion. To avoid confounding male infertility effects, all virgin females were mated to wildtype (*w*¹¹¹⁸) males. For **B** and **C**, bars and whiskers represent mean $\pm$ standard deviation and statistical significance was tested by unpaired student t test. **D** Dorsal view images of Stage 14 *Drosophila* embryos stained for Vasa (magenta) and DNA (DAPI, gray). Zygotic genotypes are noted in the top left corner. Gonads are circled with a white dashed circle. Arrowheads highlight extragonadal germ cells. All embryo images are maximum projections of two to three 2 μ m confocal slices. Scale bars represent 25 μ m. **E** Quantification of extragonadal PGCs in Stage 14–16 embryos. Each dot represents one embryo. To yield *jhamt*^{-/-} embryos, with heteroallelic combinations of independently derived indels, *jhamt*^{8F1}/*CyO,ftz-lacZ* females were mated to *jhamt*^{12F1}/*CyO,ftz-lacZ*. To generate *jhamt*^{-/Def} embryos, *jhamt*^{8F1}/*CyO,ftz-lacZ* animals were mated to *Df(2L)BSC781/*

CyO, *ft-lacZ* animals. **F** Quantification of extragonadal PGCs in Stage 14–16 embryos when *Hmgcr* is ectopically expressed in the nervous system using *Elav-GAL4* and *UAS-Hmgcr*. Each dot represents an embryo. For panels E and F, bars and whiskers represent median $\pm$ interquartile range (IQR), statistical significance was tested by Mann-Whitney U test, with ns=not significant. The reference strain for *jhamt* studies was *w¹¹¹⁸*, as *jhamt* alleles were backcrossed into this background, crossed to Oregon R. See Figure S2 for supplemental data relating to data within this figure.

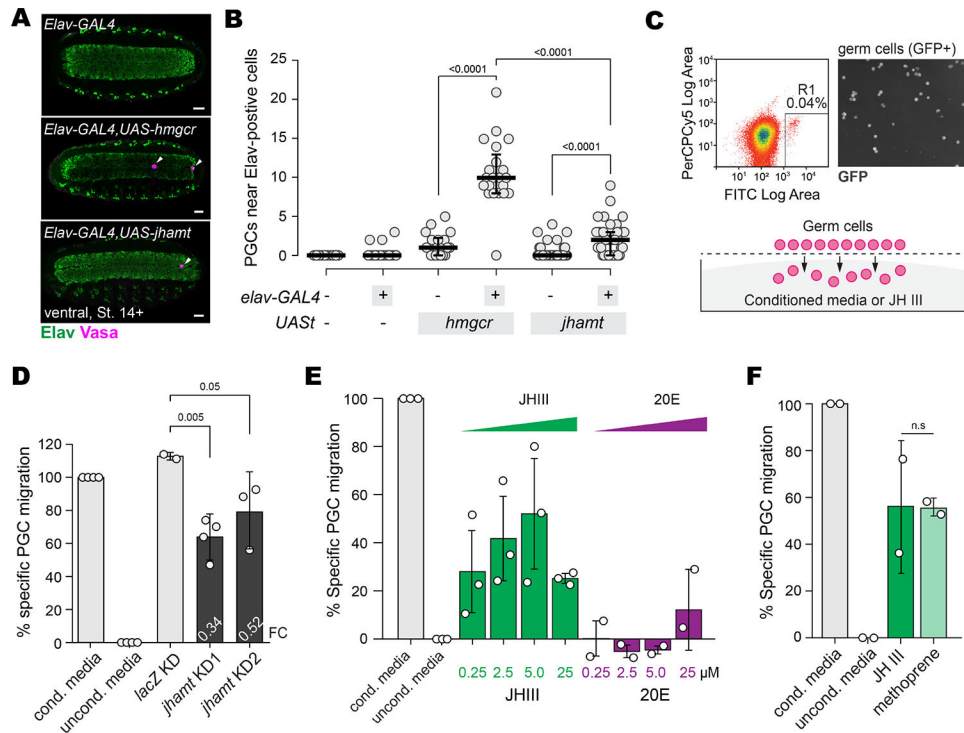


Figure 3: Juvenile hormone is sufficient for PGC migration.

A Ventral view images of Stage 14 embryos stained for Vasa (magenta) and Elav (green). Genotypes are noted in the top left corner of each image. Arrowheads highlight germ cells in contact with Elav-positive central nervous system. All embryo images are maximum projections of two to three 2 μ m confocal slices. Scale bars represent 25 μ m. **B** Quantification of the number of PGCs in Stage 14–16 embryos contacting Elav-positive cells upon ectopic expression of either *Hmgcr* or *jhamt* using the *Elav-GAL4* driver. Bar and whiskers represent mean $\pm$ standard deviation and statistical significance was tested by unpaired t-test. **C** **Top Left:** Scatter plots from FACS of GFP-positive PGCs from *nos-moesin::GFP* transgenic embryos. Region 1 (R1) denotes collection cutoff. **Top Right:** Image of isolated GFP-positive PGCs (gray). **Bottom:** Schematic of the *in vitro* transwell assay in which FACS-enriched PGCs are placed on a 10 μ m filter above either serum-free media conditioned with Kc167 cells as a positive control, serum-free unconditioned media as a negative control, or experimental conditions, such as serum-free media with JH III. The number of PGCs that translocated to the bottom well was quantified visually after 1.5 hours. **D** Quantification of Specific PGC migration, which is the percentage of experimental translocated PGCs relative to positive (serum-free Kc167-conditioned media) and negative (serum-free media) controls. Such normalization allows comparison of biological replicates done on different days, with each dot representing the average of two technical replicates done on the same day. See Materials and Methods for further details. Numbers shown in white within each *jhamt* knockdown bar represents the Fold Change (FC) of *jhamt* mRNA at the time when conditioned media was collected. Bar height represents mean and error bars represent standard deviation from two to four biological replicates and statistical significance was tested by unpaired t-test. **E** Quantification of specific migration in transwell assays using isolated PGCs placed above serum-free media containing various concentrations of

commercially available JH III (green bars) or 20-hydroxyecdysone (20E, magenta bars) with 1% BSA as a non-specific carrier protein. Bars represent means and error bars represent standard deviation from three biological replicates, which were each obtained by averaging two to three technical replicates. **F** Percentage Specific PGC migration wherein isolated PGCs were placed across either serum-free media conditioned (gray), serum-free media with 1% BSA (black) and either JH III (dark green, 2.5 μ M) or the JH analogue, methoprene, (light green, 2.5 μ M) with 1% BSA as a non-specific carrier protein. Bars represent means and error bars represent standard deviation from two biological replicates, which were each obtained by averaging two to three technical replicates. ns=not significant. See Figure S3 for supplemental data relating to data within this figure.

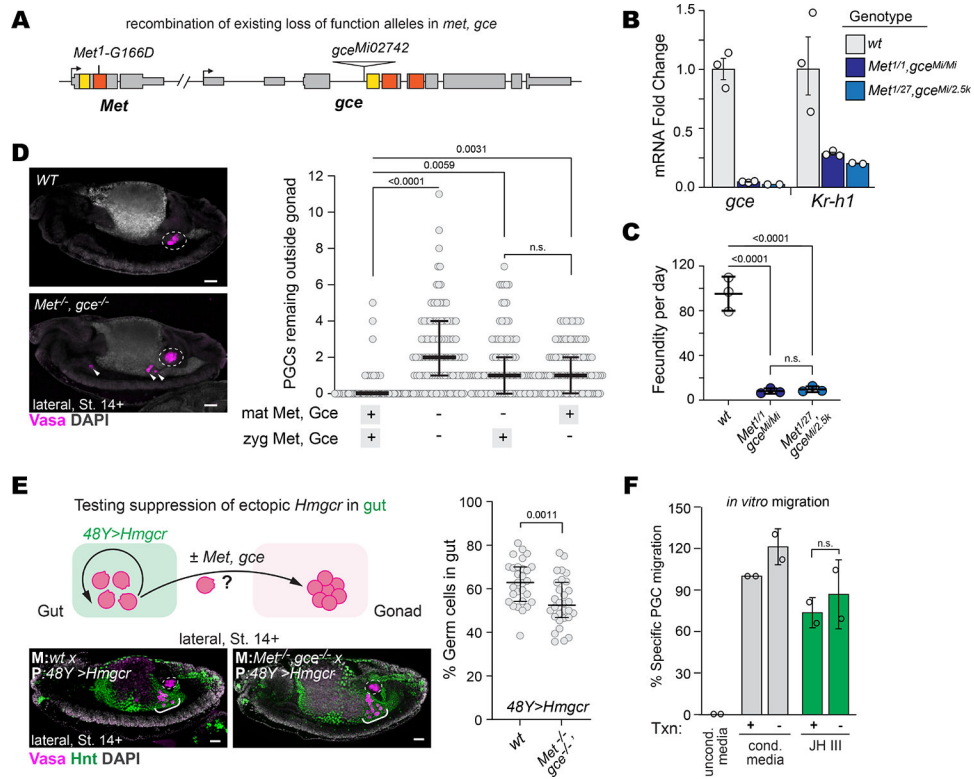


Figure 4: JH transcriptional receptor loss compromises PGC migration to the gonad

A Schematic of genetic loci encoding the two redundant JH transcriptional receptors, *Met* and *Gce* with location of extant mutations recombined for this study noted. **B** Fold change of *gce* and *Kr-h1* mRNAs in *Met*, *gce* mutant one-day old adult females relative to wildtype control animals normalized using housekeeping genes, *DCTN5-p25* and *Und*. Shown are Fold Change in homoallelic combination for the newly recombined mutant alleles *Met¹*, *gce^{Mi}* (dark blue) and heteroallelic combination of *Met¹*, *gce^{Mi}* with existing or *Met²⁷*, *gce^{2.5k}* alleles (light blue). Bars represent the geometric mean of normalized expression per biological replicate (dot), each containing pooled mRNA from five females. Error bars represent $\pm$ standard error of the mean as previously described⁸¹. **C** Fecundity of young females. Each dot represents the average number of eggs laid per biological replicate, which were obtained by dividing the number of eggs laid over a 24-hour period by the number of females (10–12) in each cohort three- and four-days after eclosion. To avoid any confounding male infertility effects, all virgin females were mated to wildtype (*w¹¹¹⁸*) males. Bars and whiskers represent mean $\pm$ standard deviation and statistical significance was tested by unpaired student t test. **D Left:** Lateral view images of Stage 14 embryos stained for Vasa (magenta) and DAPI (gray). Maternal and zygotic genotypes are noted in the top left corner. **Right:** Quantification of extragonadal PGCs in Stage 14–16 embryos. Each dot represents one embryo. To generate maternal and zygotic mutant embryos, mutant *Met^{1/27}*, *gce^{Mi/2.5k}* mothers were crossed to *Met¹, gce^{Mi}/Y* males. Arrowheads point to extragonadal germ cells. **E Top panel:** schematic of genetic suppression experiment. In otherwise wildtype embryos, ectopic expression of *Hmgcr* in the gut/endoderm (green) with *48Y-GAL4*; *UAS-Hmgcr* transgenes traps ~65% of PGCs (pink) in the gut (*left*

schematic). To determine if Met and Gce is required downstream of HMGCR, *Met*^{1/27}, *gce*^{Mi/2.5k} females were crossed *48Y-GAL4; UAS-Hmgcr* males to generate PGCs without maternal loading of Met/gce. *Bottom panels*: Lateral view of Stage 14 embryos stained for Vasa (magenta), the endodermal marker, Hnt (green) and DAPI (gray). Bold M: designates the maternal genotype that PGCs primarily rely on when exiting the endoderm. Bold P: designates the paternal genotype. White brackets indicate germ cells that remain in gut. *Right*: Percentage of PGCs remaining in the gut in Stage 14+ embryos upon expression of *UAS-Hmgcr* using the *48Y-GAL4* driver. Each dot represents one embryo. **F** Quantification of Specific *in vitro* migration when isolated PGCs were treated with 1μM flavopiridol to inhibit transcription (Txn) 30 minutes prior to placing PGCs in the transwell plate. Flavopiridol (1μM) was also added to the bottom chambers of the transwell plates for transcriptional inhibition experimental conditions. Specific migration was calculated relative to vehicle control treated PGCs subjected to serum-free media (uncond media, black) and serum-free media conditioned with Kc167 (Cond media, gray). The effect of transcriptional inhibition (–) was measured in PGCs subjected to cond media or containing 5μM JH III (green). Bars represent means and error bars represent standard deviation from two biological replicates, which were each obtained by averaging two technical replicates. Bars and whiskers within each graph represent median +/- interquartile range (IQR). Statistical significance was tested by Mann-Whitney U test, with ns=not significant. Dashed white circles outline the gonad and scale bars represent 25μm in all images. See Figure S4 for supplemental data relating to data within this figure.

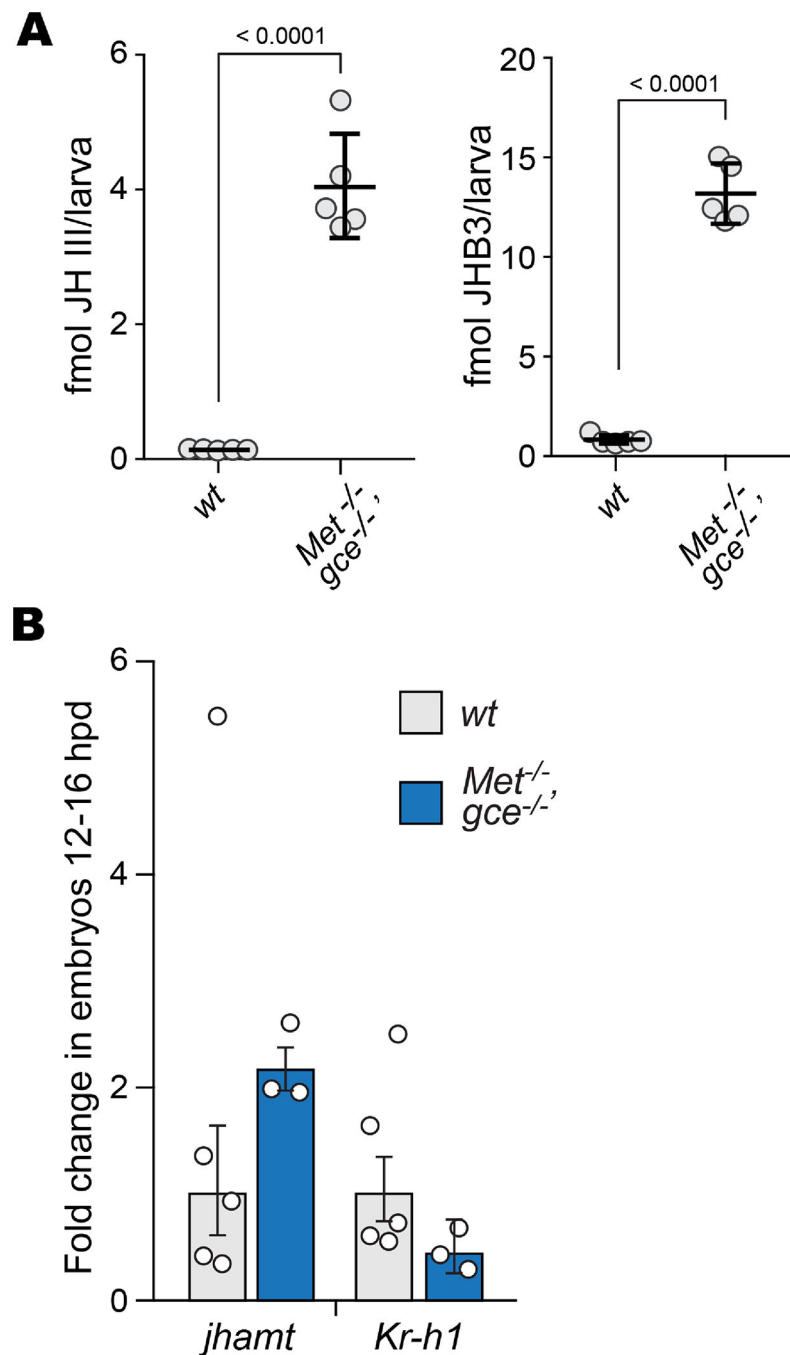


Figure 5: Compensatory feedback loop maintains JH homeostasis in *Drosophila*.

A Titters of two JHs, JH III and JH bisepoxide (JHB₃), in hemolymph of third instar *Drosophila* larvae measured by quantitative mass spectrometry using deuterated standards. Each dot represents a biological replicate of hemolymph bled from 25 *Met^{1/1}, gce^{Mi/Mi}* or *w¹¹¹⁸* third instar larvae. **B** Fold change of *jhamt* and *Kr-h1* mRNA in *Met^{1/27}, gce^{Mi/2.5k}* embryos 12–16 hours post deposition (hpd) relative to the wildtype strain obtained by crossing *w¹¹¹⁸* to OregonR and normalized using two housekeeping genes, *DCTN5-p25* and *Und*. Bars represent the geometric mean of normalized expression per biological replicate

(dot). Error bars represent $\pm$ standard error of the mean as previously described⁸¹. See Figure S5 for supplemental data relating to data within this figure.

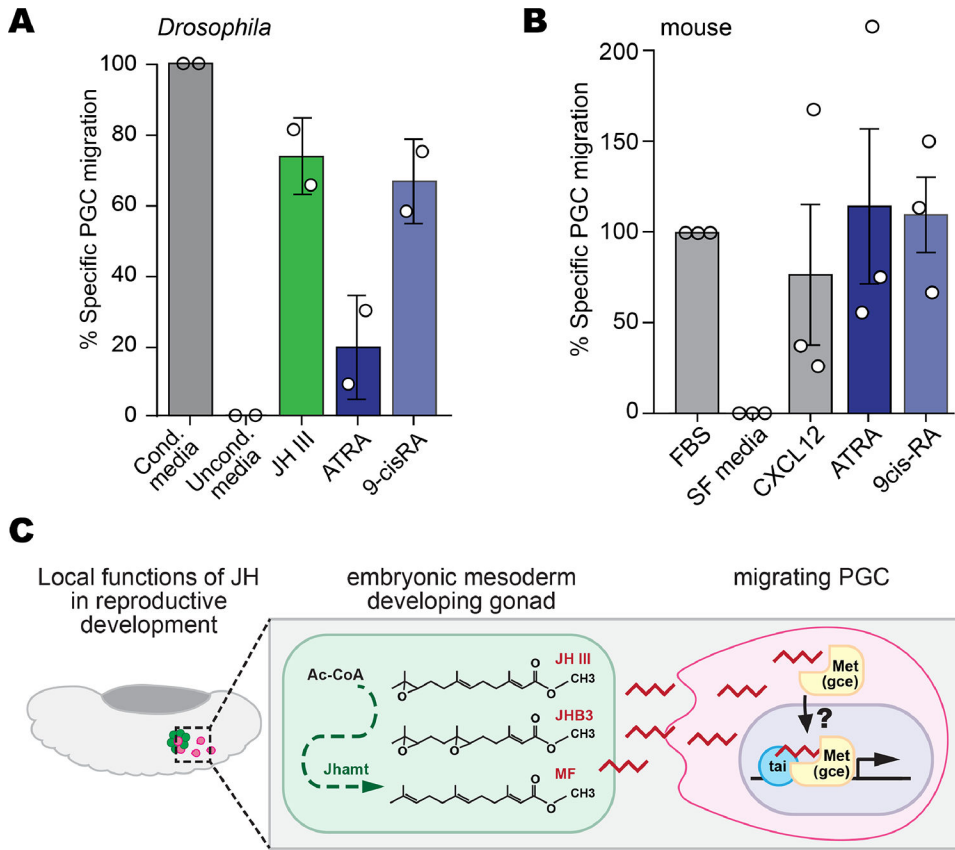


Figure 6: Model of JH function in PGC migration.

A Quantification of specific *in vitro* migration wherein isolated *Drosophila* PGCs are placed in a transwell migration system across Kc167-conditioned media (gray), serum-free media (black), JH III (green, 5 μ M), all trans retinoic acid (ATRA, dark blue, 5 μ M) or 9-cis RA (light blue 5 μ M). **B** Quantification of specific *in vitro* migration wherein mouse PGCs were FACS-enriched from trunk tissues dissected from E10.5 *Oct4-GFP* embryos and placed across two positive controls: Fetal Bovine Serum (FBS) or CXCL12 (50 ng/mL) (gray), serum-free media as a negative control (black) or ATRA (dark blue, 1 μ M) or 9cis-RA (light blue, 1 μ M). For A and B, bars and error bars represent mean $\pm$ standard deviation from at least two biological replicates, which were obtained by averaging two to three technical replicates. **C** Schematic of model for how JHs ensure migrating PGCs (pink) colonize the gonad (green) in the *Drosophila* embryo. See Figure S6 for supplemental data relating to data within this figure.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Vasa (1:5,000)	Moore et al. ⁸⁹	Flybase: FBrf0100750
Chicken polyclonal anti-GFP (1:500)	AVES	Cat #1020
Rabbit polyclonal anti-GFP (1:1,000)	Thermo Fisher Scientific	Cat #A11122
Mouse monoclonal anti-Singed (fascin) (1:100)	Developmental Studies Hybridoma Bank	Cat #sn7c
Mouse monoclonal anti-Elav (1:500)	Developmental Studies Hybridoma Bank	Cat #9F8A9
Chicken polyclonal anti-beta-galactosidase (1:500)	Abcam	Cat #ab9361
Mouse monoclonal anti-Hindsight (1:20)	Developmental Studies Hybridoma Bank	Cat #1G9
Rat monoclonal anti-RNA Pol II Ser5 (1:100)	Chromotek	Cat #3e8-1
Rabbit polyclonal anti-RNA Pol II Ser2 (1:500)	Abcam	Cat #ab5095
Bacterial and virus strains		
None		
Biological samples		
none		
Chemicals, peptides, and recombinant proteins		
JH III	Sigma-Aldrich	Cat #J2000-10MG
Methoprene	Sigma-Aldrich	Cat #33375-100MG
20-hydroxecdysone	Sigma-Aldrich	Cat #H5142-5MG
ATRA	Sigma-Aldrich	Cat #R2625-50MG
9cis-RA	Sigma-Aldrich	Cat #R4643
Flavopiridol	Selleck	Cat #S1230
Actinomycin D	Sigma-Aldrich	Cat #A1410-5mg
CXCL12/Sdf-1a	R&D Systems	Cat #460-SD-010
Critical commercial assays		
ChemoTx Disposable Chemotaxis System	NeuroProbe	Cat #116-10, PCTE filter, 10µm pore size
Deposited data		
none		
Experimental models: Cell lines		
<i>D. melanogaster</i> : Cell line S2: S2-DGRC	Drosophila Genomics Resource Center, Laboratory of Perrimon	Cat #181; FlyBase: FBtc0000181
<i>D. melanogaster</i> : Cell line Kc167-DGRC	Drosophila Genomics Resource Center, Laboratory of Goldschmidt-Clermont	Cat #1; FlyBase: FBtc0000001
Experimental models: Organisms/strains		
<i>D. melanogaster</i> : <i>P[JHREWt8x-eGFP]attp2</i>	This study	N/A
<i>D. melanogaster</i> : <i>P[JHREMut8x-eGFP]attp2</i>	This study	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : $y^1, w^*, gce^{mi02742}$	Bloomington Drosophila Stock Center	BDSC: 60189; FlyBase: FBti0178654
<i>D. melanogaster</i> : <i>Met</i> ¹	Bloomington Drosophila Stock Center	BDSC: 3472; FlyBase: FBal0014802
<i>D. melanogaster</i> : $y^+, w^+, v^+, Met^1, gce^{mi02742}/Fm7ai$	This study	BDSC: 3472 and 35131; FlyBase: FBst0003472 and FBst0035131
<i>D. melanogaster</i> : $w^-, v^-, Met^{27}, gce^{2.5k}/Fm7ai$	Rebecca Spokony and Lynn Riddiford ⁸⁴	FlyBase: FBfr0235602
<i>D. melanogaster</i> : $w^{1118}, jhamt^{8F2} or^{12F1}/CyO, ft-lacZ$	This study	N/A
<i>D. melanogaster</i> : $jhamt^{DR(2L)BSC781}/CyO, ft-lacZ$	Bloomington Drosophila Stock Center	BDSC: 27353; FlyBase: FBab0045870
<i>D. melanogaster</i> : $Hmgcr^{26.31}/Tm3, Sb, Ubx-lacZ$	Moore et al. ⁸²	FlyBase: FBfr0100750
<i>D. melanogaster</i> : $Hmgcr^{11.54}/Tm3, Sb, Ubx-lacZ$	Moore et al. ⁸²	FlyBase: FBfr0100750
<i>D. melanogaster</i> : $Hmgcr^{01152}/Tm3, Sb, Ubx-lacZ$	Van Doren et al. ¹⁹	FlyBase: FBfr0105951
<i>D. melanogaster</i> : <i>UAS-Hmgcr</i>	Van Doren et al. ¹⁹	FlyBase: FBfr0105951
<i>D. melanogaster</i> : $y, w; ; UAS-jhamt$	Ryusuke Niwa, Niwa et al. ¹⁸	FlyBase: FBfr0205213
<i>D. melanogaster</i> : $w^-; elav-GAL4$	Jessica Treisman	FlyBase: FBfr0237920
<i>D. melanogaster</i> : $w^-; 48Y-GAL4$	Bloomington Drosophila Stock Center	BDSC: 4935; FlyBase: FBst0004935
<i>D. melanogaster</i> : $w^-; twi-GAL4; 24B-GAL4$	Michael Akam	FlyBase: FBst0099029
<i>D. melanogaster</i> : $y, w; ; Tub-GAL4$		FlyBase: FBtp0020111
<i>D. melanogaster</i> : $w^-, P[nos-moesin::egfp::nos 3'UTR]$	Sano et al. ⁸³	FlyBase: FBfr0190943
<i>M. musculus</i> : B6; CBA-Tg(Pou5f1-EGFP)2Mnn/J	The Jackson Laboratory	Jax #004654
Oligonucleotides		
See Table S1 in Supplemental Information for list of oligonucleotides used in this study		
Recombinant DNA		
pU6–2 gRNA vector	DGRC	Cat #1363
pS3aG	Addgene	Cat #31171
<i>pGL4.17</i>	Marek Jindra, Promega	Cat #E6721
<i>D. melanogaster</i> : <i>Jhamt</i> clone	DGRC	Cat #11840, clone AT13581
<i>D. melanogaster</i> : <i>Nanos</i> cDNA	DGRC	Cat #1124533, clone RE53469
Software and algorithms		
ImageJ/FIJI	Johannes Schindelin, Ignacio Arganda-Carreras, Albert Cardona, Mark Longair, Benjamin Schmid	Fiji.sc
Zen Blue 2.3	Zeiss	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html#packages
Other		
none		