1 **Juvenile hormones direct primordial germ cell migration to the embryonic gonad**

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22 **Highlights**

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24 • Juvenile hormones (JH) are necessary & sufficient for Primordial Germ Cell (PGC)

25 migration.

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27 • JH signaling acts directly in and around migrating PGCs prior to its endocrine function.

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29 • Compensatory feedback sensitive to JH receptor function maintains JH homeostasis.

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31 • JH-like retinoic acids may have similar roles during mammalian germ cell migration.

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36 **Summary**

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38 Germ cells are essential to sexual reproduction. Across the animal kingdom, extracellular

39 signaling isoprenoids, such as retinoic acids (RAs) in vertebrates and juvenile hormones (JHs)

40 in invertebrates, facilitate multiple processes in the germline lifecycle. Here we investigated the

41 role of these potent signaling molecules in embryonic germ cell migration, using JHs in

42 *Drosophila melanogaster* as a model system. In contrast to their established endocrine roles

43 during larval and adult germline development, we found that JH signaling acts locally during

44 embryonic development. Using an *in vivo* biosensor, we found JH signaling is first active near

45 primordial germ cells (PGCs) as they migrate to the developing somatic gonad. Through *in vivo*

46 and *in vitro* assays, we found that JHs are both necessary and sufficient for PGC migration.

47 Analysis into the mechanisms of this newly uncovered paracrine JH function revealed that PGC

48 migration was compromised when JHs were reduced or increased, suggesting that specific

49 titers or spatiotemporal JH dynamics are required for robust PGC colonization to the gonad.

50 Compromised PGC migration can impair fertility and cause germ cell tumors in many species,

51 including humans. In mammals, retinoids, a JH-related family of signaling isoprenoids, has

52 many roles in development and reproduction. We found that retinoic acid, like JH, was sufficient

53 to impact PGC migration *in vitro*. Together, our study reveals a previously unanticipated role of

54 isoprenoids as local effectors of pre-gonadal PGC development and suggests a broadly shared

55 mechanism in PGC migration.

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68 **Key words**: primordial germ cells, cell migration, gonad, germline, mevalonate pathway,

69 juvenile hormone, retinoids

70 **Introduction**

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72 In sexually reproducing species, the germline links one generation to the next. Given the

73 importance to individuals, societies, and species, understanding how germ cells are set aside,

74 develop, and protected until undergoing gametogenesis to yield sperm and egg has been the

75 focus of intense efforts. Through recent advances in single-cell 'omics and *in vitro* derivation of

76 primordial germ cell (PGC)-like cells from induced pluripotent stem cells, we have gained

77 substantial insights into the molecular programs intrinsically required for germ cell specification

78 and development. However, the yield and gamete quality of *in vitro* derivation approaches falls

79 far short of what is observed *in vivo*[1]. This quality gap suggests that we are failing to recapitulate

80 key aspects of germline development. One critical component not yet replicated *in vitro*, nor fully

81 understood *in vivo*, is how germline development is exquisitely coordinated in space and time

82 during embryogenesis by surrounding somatic cells.

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84 Somatic cells coordinate germ cell development and gametogenesis through small, secreted

85 molecules such as steroid hormones and signaling lipids. These potent molecules have

86 multifaceted impacts on reproduction across the animal kingdom. In vertebrate animals, key

87 reproductive small molecules include steroid hormones (i.e., estrogen and testosterone) that are

88 synthesized from cholesterol, and paracrine retinoic acids (RAs), that are derived from

89 carotenoids, a subfamily of isoprenoids. Animals lack the ability to synthesize carotenoids and

90 thus obtain these critical molecules from their diet. Despite differences in biosynthetic origins

91 and structures, invertebrate animals also use steroids and isoprenoids to facilitate many aspects

92 of germline development and reproduction. In *Drosophila*, the steroid hormone, ecdysone,

93 regulates germline stem cell homeostasis, niche development, yolk uptake, and the migratory

94 behavior of certain ovarian somatic cell types[2-5]. The RA-like isoprenoids, Juvenile hormones,

95 have profound impacts on reproduction. For example, methyl farnesoate is a key sex

96 determination molecule in some crustaceans[6]. In many insects, JHs act as gonadotropins, with

97 essential roles in vitellogenesis[7,8]. Recent work in *Drosophila* have revealed that JHs also

98 regulate germline stem cell number and reproductive diapause[9,10].

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100 While much is known about how steroid hormones mechanistically shepherd the germline

101 lifecycle, how signaling isoprenoids regulate the germline lifecycle is much less understood.

102 Given the complexity surrounding RAs in germline biology, insights can be gleaned from JHs.

103 These potent sesquiterpenoids, derived from the mevalonate pathway[11], are best known for

104 their influence on juvenile development[12]. They include, among others, JH III, JH III bisepoxide

105   (JHB$_3$), and methyl farnesoate (**Fig 1a**). As signaling molecules, JHs bind to a basic helix-loop

106   helix/Per-ARNT-SIM (bHLH-PAS) receptor, which in *Drosophila* is encoded by two paralogous

107   genes, *Methoprene-tolerant* (*Met*) and *germ cell-expressed bHLH-PAS* (*gce*)[13-15]. Upon ligand

108   binding, these receptors regulate gene expression together with the co-activator Taiman[16]. In

109   contrast to paracrine acting RAs, JHs act hormonally such that JH is synthesized in the corpus

110   allatum (CA), a neuroendocrine gland, and released into the circulatory system (**Fig 1a**). JH

111   production is driven by Juvenile hormone acid methyltransferase (Jhamt), which is expressed in

112   the CA as soon as this gland forms at the end of embryogenesis[17]. Curiously, a burst of *jhamt*

113   expression has also been observed in the trunk mesoderm during mid-embryogenesis, well

114   before the specification of the CA precursors[17], leaving open the possibility that JHs have

115   additional, non-endocrine roles.

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117   Mesodermal *jhamt* expression coincides with the PGC migration stage of reproductive

118   development. In many species, newly specified PGCs are internalized during gastrulation and

119   then must migrate within the mesoderm toward the developing gonad to generate the germline

120   pool. In some mammals, including humans, extragonadal PGCs that fail to reach the gonad can

121   cause germ cell tumors[18]. To investigate the role of JHs during PGC migration, we first

122   generated an *in vivo* JH sensor and found that JH signaling was first active proximal to *jhamt*

123   expression and surrounding migratory PGCs. New *jhamt* mutants lacking the methyltransferase

124   domain compromised JH synthesis and caused a failure of PGCs reaching the gonad. A similar

125   defect in gonad colonization had previously been described in animals lacking HMG Coenzyme

126   A reductase (Hmgcr)[19], the rate-limiting enzyme of the mevalonate pathway upstream of Jhamt

127   in JH synthesis[11]. Consistent with Hmgcr and Jhamt being part of the same synthetic cascade,

128   we found that *jhamt* and *Hmgcr* genetically interact to facilitate PGC migration and that JH, like

129   Hmgcr, is both necessary and sufficient for PGC migration. Our further investigations into the

130   function of JH receptors in the embryo revealed a feedback mechanism necessary to maintain

131   JH homeostasis critical for PGC migration. Together, our studies uncovered a new requirement

132   for JH signaling in embryonic reproductive development. Studies in mice demonstrated that, like

133   JHs, genes critical to RA synthesis are expressed in the bipotential gonad during PGC

134   development PGC development[20-22]. However, despite shared expression of genes involved in

135   reproductive isoprenoids in vertebrate and invertebrate species, a role for isoprenoid signaling

136   during the PGC migration has not been described. Here we demonstrate that RA is sufficient to

137   induce migration of both *Drosophila* and mouse embryonic germ cells, suggesting this newly

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138   identified role for secreted isoprenoids on early germline development may be broadly shared

139   across invertebrate and vertebrate species.

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143   **Results**

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145   <u>Juvenile hormone signaling is active in the embryonic mesoderm during PGC development</u>

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147   Juvenile hormone production is driven by Juvenile hormone acid methyltransferase (Jhamt).

148   Jhamt is specifically expressed in the corpus allatum (CA), as soon as this JH-producing

149   endocrine gland develops in late embryogenesis[17]. Curiously, in *Drosophila jhamt* mRNA is also

150   detected during mid-embryogenesis in the trunk mesoderm, before specification and migration

151   of CA precursors[17]. Using dual-label fluorescent *in situ* hybridization for mRNAs encoding Jhamt

152   and the germ cell factor, Nanos, we investigated where *jhamt* is expressed relative to PGCs.

153   Consistent with previous work[17], we observed *jhamt* expression in the mesoderm from

154   embryonic Stage 10 (~4 hours after fertilization), continuing through Stage 13 (~11 hours after

155   fertilization) (**Fig 1b**). At this stage in embryogenesis, PGCs exit the endoderm and sort into two

156   lateral populations as they migrate within the mesoderm toward the somatic gonadal precursors

157   (**Fig 1c**). This *jhamt* expression pattern suggested that JH synthesis may occur prior to CA

158   formation and be involved in embryonic germline development.

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160   To determine whether mesodermal *jhamt* mRNA leads to functional JH production during

161   *Drosophila* embryogenesis, we generated an *in vivo* JH sensor. We placed *eGFP* downstream

162   of eight tandem copies of a JH response element (JHRE) from the *early trypsin* gene of *Aedes*

163   *aegypti*[15,23], which was previously shown to robustly and specifically responds to JH *in vitro*[15]

164   (**Fig 1d** and **Supp Fig 1a**). To test the responsiveness of this biosensor to JH *in vivo*, we fed

165   second instar larvae the JH mimic, methoprene, and measured mRNA levels of *GFP* and the

166   canonical JH signaling transcriptional target, *Krüppel homolog 1* (*Kr-h1*), by real-time-qPCR 24

167   hours later. We found that *Kr-h1* and *GFP* mRNA levels increased ~two-fold and seven-fold

168   respectively in *JHRE-GFP* larvae fed methoprene (**Fig 1d**). To determine whether this induction

169   by methoprene required JH signaling, we generated animals carrying the JHRE sensor but

170   lacking the JH receptors, Methoprene-tolerant (Met) and Germ cell-expressed bHLH-PAS (gce).

171   We found that methoprene did not increase expression of either *Kr-h1* or *GFP* mRNA in *Met,*

172   *gce; JHRE-eGFP* mutant animals (**Fig 1d**). Together, these data indicate that this new JH

173   sensor faithfully responds to JH signaling *in vivo*.

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175   With this *in vivo* JH sensor, we next investigated if and where JH signaling is active during

176   embryonic germline development. We stained *JHRE-GFP* embryos with antibodies against GFP

177   and the germ cell-specific RNA helicase, Vasa. GFP signal was first detected in Stage 10

178   embryos in two locations: in bilateral clusters in the anterior portion of the embryo and in

179   bilateral strips of the mesoderm of the posterior germband (**Fig 1e, f**). The anterior signal was

180   still detected in *Met, gce* mutant embryos carrying the JH biosensor, but the germband signal

181   was absent (**Fig 1e**). To explore whether this difference is due to non-specific, receptor-

182   independent transgene activation, we analyzed animals carrying a JH sensor transgene in

183   which the Met/gce binding motif was mutated (**Supp Fig 1a**)[15]. Anterior GFP was still present in

184   animals with the mutated biosensor; further investigation revealed that this non-JH-specific GFP

185   signal localized to hemocytes (**Supp Fig 1b-c**). Importantly, the mesodermal signal was not

186   detected in embryos carrying the JH biosensor with mutated receptor binding motifs, nor when

187   the functional biosensor JHRE-eGFP was tested in *Met, gce* receptor mutants. Thus, the

188   mesodermal signal is due to JH-mediated receptor activation and reflects JH signaling. We

189   found that this specific JH sensitive response began at Stage 10 in the mesoderm, when PGCs

190   enter this tissue from the endoderm. JH signaling continued to localize in and near PGCs as

191   they colonized the gonad (**Fig 1e, Supp 1c**). Together, these data demonstrate that JH

192   signaling is active near the mesodermal *jhamt* expression zone and surrounds migratory PGCs

193   during mid-embryogenesis.

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196   <u>Juvenile hormones are required for migrating PGCs to reach the somatic gonad</u>

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198   To determine whether the newly identified embryonic JH signaling supports early PGC

199   development, we generated new mutant *jhamt* alleles. Using CRISPR editing, 15 *jhamt* alleles

200   were generated, two of which carried a premature stop codon at amino acid 46, whereas the

201   remaining 13 carried a premature stop codon at amino acid 54 (**Supp Fig 2**). All new alleles

202   caused a deletion of the methyl transferase domain central to Jhamt-mediated JH biosynthesis

203   (**Fig 2a**). To avoid confounding effects from second site mutations, phenotypic analyses carried

204   out in this study use the newly generated *jhamt* lines in heteroallelic combinations or *in trans* to

205   an independently generated small deficiency lacking the entire *jhamt* locus (**Supp Table 1**). To

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206   verify that Jhamt function was compromised, titers of two JHs in *Drosophila*, JH III and JHB$_3$,

207   were directly quantified by liquid chromatography-tandem mass spectrometry[24]. We found that

208   JH III titers were reduced 50% and JHB$_3$ titers were reduced >90% in larvae possessing our

209   newly generated alleles (**Fig 2b**). These results are consistent with previous observations

210   showing that JHs were still detected in animals lacking the entire *jhamt* locus[25], suggesting the

211   *Drosophila* genome may contain a second, yet obscure, methyltransferase capable of JH

212   synthesis[26,27]. To attest the degree by which JH function was compromised, we measured

213   female fecundity. Consistent with previous reports[25], the number of eggs laid per day was

214   significantly reduced in females carrying our new *jhamt* mutant alleles (**Fig 2c**). Together, these

215   data suggest that the newly generated *jhamt* mutants significantly affected JH production and

216   display JH loss of function phenotypes.

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218   With new genetic tools, we next compared PGC development in wildtype and *jhamt* mutant

219   embryos. The first detectable germline defect in *jhamt* mutant embryos was an increased

220   number of PGCs that failed to reach the gonad at Stage 14, when PGC normally have

221   completed their migration to the somatic gonad (**Fig 2d)**. Animals lacking zygotic Jhamt had

222   significantly more extragonadal PGCs than wildtype animals (**Fig 2e**). This phenotype was

223   rescued when *jhamt* was zygotically expressed with a broad *Tub-GAL4* driver (**Fig 2e**). These

224   data indicate that *jhamt* expressed in somatic cells facilitates PGC migration to the developing

225   somatic gonad.

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227   The *jhamt* mutant PGC phenotype was similar to that of embryos lacking HMG-CoA reductase

228   (Hmgcr), the rate-limiting enzyme in the mevalonate pathway (**Fig 2d** and **e**)[19]. In *Drosophila*,

229   Hmgcr was shown to have a striking, instructive effect on PGC migration: in *Hmgcr* mutant

230   embryos PGCs failed to reach the gonad and when mis-expressed, elevated *Hmgcr* expression

231   alone mislocalized PGCs to the location of ectopic expression (**Fig 2f** and **Supp Fig 2a,**

232   **b)**[19,28,29]. Our observation that *jhamt* mutants phenocopied *Hmgcr* mutants is consistent with

233   previous reports that Hmgcr is required for JH synthesis and genetically interacts with *jhamt* in

234   the context of animal development[25]. To determine whether Jhamt functions downstream of

235   Hmgcr in embryonic PGC migration, we tested for genetic interaction in two ways. First, we

236   measured the effect of genetic *Hmgcr* reduction in sensitized *jhamt* heterozygous animals and

237   found that significantly more PGCs failed to reach the gonad in *jhamt*$^{+/-}$*; Hmgcr*$^{+/-}$ heterozygous

238   embryos than embryos heterozygous for *jhamt* alone (**Fig 2e**). Second, we tested whether

239   genetic reduction of Jhamt can suppress the ability of ectopic *Hmgcr* expression to misdirect

240   PGCs. Indeed, loss of one wildtype *jhamt* copy significantly reduced the number of PGCs

241   misdirected by GAL4/UAS-mediated expression of *Hmgcr* (**Fig 2f**). Together, these data

242   suggest that Jhamt functions downstream of Hmgcr to facilitate PGC migration to the somatic

243   gonad.

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246   <u>Juvenile hormone is sufficient for PGC migration</u>

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248   Hmgcr is sufficient to misdirect migrating PGC to many tissues, even tissues far from the normal

249   migratory path such as the central nervous system (**Fig 3a** and **Supp Fig 3a**)[19,28,29]. To

250   determine whether, like *Hmgcr*, *jhamt* expression is sufficient for PGC migration *in vivo*, we

251   ectopically expressed *jhamt* mRNA using the GAL4/UAS system. We found that neuronal *Elav-*

252   *GAL4* driven *jhamt* expression led to modest, yet significant, increase in PGCs inappropriately

253   near neuronal Elav-positive cells (**Fig 3b**). We even found some PGCs localized in the central

254   nervous system after neuronal *jhamt* expression (**Fig 3a**). Yet overall, significantly fewer PGCs

255   were misdirected by ectopic *jhamt* compared to ectopic *Hmgcr* driven by the same *Elav-GAL4*

256   transgene (**Fig 3b**). Moreover, ectopic *jhamt* expression was insufficient to drive PGCs to

257   inappropriate locations when *UAS-jhamt* was induced by the endodermal driver, *48Y-GAL4*, or

258   the pan-mesodermal drivers, *twist-GAL4* and *24B-GAL4*, even though ectopic *Hmgcr*

259   expression with these GAL4 drivers significantly mislocalized PGCs (**Supp Fig 3b**). These data

260   show that Jhamt was sufficient to impact PGC migration *in vivo* in at least one, but not all,

261   tissues, suggesting that other pathway components may be limiting.

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263   Hmgcr and Jhamt are both required for JH biosynthesis. There are at least two possible

264   explanations for the difference by which ectopic expression of *jhamt* and Hmgcr was sufficient to

265   attract PGCs *in vivo* (**Supp Fig 3b**). Hmgcr may direct PGCs through production of JH and non-

266   JH signals. Indeed, Hmgcr is required for the synthesis of many molecules and post-

267   translational protein modifications[29]. Alternatively, *jhamt* expression may not be sufficient in

268   every tissue due to a lack of precursors derived from the upstream mevalonate pathway.

269   Indeed, Hmgcr is the rate-limiting enzyme in the mevalonate pathway and it's expression is

270   spatially restricted during mid-embryogenesis[19]. To assess the role of JH directly, we turned to

271   an *in vitro* transwell migration assay, in which *Drosophila* PGCs were obtained from whole

272   embryos, enriched by fluorescence-activated cell sorting (FACS), and placed on a porous filter

273   above media (**Fig 3c**). Previous work showed that PGCs will translocate in response to serum-

274   free culture media conditioned with Kc167 or S2 embryonic *Drosophila* cells[30] (**Fig 3d**). To

275   explore the impact of JH on PGC migration in this *in vitro* migration assay, we knocked down

276   *jhamt* mRNA in Kc167 cells using two, independent dsRNAs (**Supp Table 3**), collected

277   conditioned media, and measured PGC translocation. Like Hmgcr depletion[30], significantly

278   fewer PGCs translocated in response to media that was conditioned by *jhamt*-depleted Kc167

279   cells compared to control conditioned media (**Fig 3d**). To verify that reduced PGC translocation

280   was not due to fewer Kc167 cells conditioning the media, we quantified Kc167 cell number and

281   found that *jhamt* depletion had no effect (**Supp Fig 3b**). These data indicated that Jhamt,

282   produced in somatic cells, facilitated PGC migration *in vitro*.

283

284   We next asked whether JHs are sufficient for PGC migration. To this end, FACS-enriched PGCs

285   were placed on the transwell filter above serum-free, unconditioned culture media

286   supplemented with commercially available JH III with 1% BSA as a non-specific carrier protein.

287   We found that PGCs translocated in response to JH III, with a positive correlation with JH

288   concentration except at the highest JH concentration tested (**Fig 3e**). To test the specificity of

289   this response, we subjected PGCs to the JH III analogue and insecticide, Methoprene, and

290   found that PGCs translocated in response to Methoprene at levels comparable to JH III (**Fig 3f**).

291   Together, these data demonstrated that JHs were sufficient to induce or direct PGC migration.

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294   <u>Compensatory feedback maintains JH homeostasis in *Drosophila melanogaster*</u>

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296   Our findings suggested that JHs originating from the somatic mesoderm direct PGC migration to

297   the somatic gonad. We next investigated the mechanisms by which PGCs respond to JHs. JH

298   signaling response canonically involves JHs binding to the Met/Gce receptor, which then

299   associates with a co-activator Taiman to influence transcription of target genes (**Fig 1a**)[15]. To

300   determine whether the JH receptors are required for PGC migration, we analyzed embryos

301   mutant for the two, partially redundant, JH receptors[14,15]. As existing *Met, gce* double mutant

302   strains die during metamorphosis[14], to test the maternal and zygotic contributions of Met/Gce

303   we generated new *Met[1], gce[Mi02742]* double mutants. In *gce[Mi02742], gce* mRNA is reduced by more

304   than 90% (**Fig 4a** and **b**) and *Met[1]* carries a point mutation that renders *Drosophila* 33-fold more

305   resistant to lethality inflicted by methoprene[31]. This strain was crossed with *Met[27], gce[2.5k]*

306   heterozygous females. Even under non-crowding conditions, only ~5% of the expected class of

307   heteroallelic *Met, gce* double mutant animals eclosed. As expected[14,32], the fecundity of these

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308   escaper *Met, gce* females was significantly reduced (**Fig 4c**), but nevertheless, allowed us to

309   analyze Met and Gce requirements in PGC development.

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311   To investigate PGC response requirements, we stained maternally and zygotically *Met, gce*

312   mutant embryos for Vasa and DAPI. Embryos lacking either maternal or zygotic Met and gce

313   showed a moderate loss of PGCs relative to wildtype controls at the end of migration (**Fig 4d**).

314   However, we observed a significant increase in the number of extragonadal PGCs in embryos

315   lacking both maternal and zygotic Met and gce (**Fig 4d**). Because PGCs primarily rely on

316   maternally deposited mRNAs during migration[33], the additive effect of maternal and zygotic loss

317   suggests either an early somatic requirement or a late PGC requirement for Met/gce. To test for

318   a link between JH receptors and Hmgcr, we next asked whether Met/Gce suppressed the ability

319   of PGCs to respond to Hmgcr. For this experiment, we took advantage of our observation that

320   ectopic *Hmgcr* expression in the endoderm trapped 50-80% of PGCs within the gut at Stage 10

321   (**Fig 4e**). To test whether loss of Met and Gce suppressed the ability of ectopic *Hmgcr*

322   expression to trap PGCs, we crossed wildtype or *Met, gce* double mutant females to males

323   containing *48Y-GAL4* and *UASt-Hmgcr* transgenes and quantified the percentage of PGCs

324   remaining in the gut after gonad colonization (**Fig 4e**). This experiment revealed a small, but

325   statistically significant, suppression of gut trapping in maternally *Met, gce* mutant PGCs (**Fig**

326   **4e**). Together, these data are consistent with a requirement for Met and Gce in PGC migration

327   to the gonad downstream of Hmgcr/JH signaling.

328

329   Canonical JH signaling leads to transcriptional changes of target genes. To determine whether

330   transcription is required for PGCs to respond to JHs, we returned to the *in vitro* migration assay,

331   where we inhibited transcription specifically in PGCs using the P-TEFb-associated CDK9

332   inhibitor, flavopiridol. As expected[34], flavopiridol rapidly and robustly inhibited transcription in

333   PGCs enriched by FACS (**Supp Fig 4**). We then subjected control and transcriptionally deficient

334   PGCs to conditioned media, serum-free media, or serum-free media containing JH III and found

335   that inhibition of transcription did not alter PGC translocation in response to any condition tested

336   (**Fig 4f**). These data suggest that the migratory response of PGCs to JH does not depend on

337   classical transcription-dependent JH receptor signaling.

338

339   We were surprised that PGC migration to the gonad was compromised in *Met, gce* mutant

340   animals *in vivo*, yet transcription was not required for JH-mediated PGC migration JHs *in vitro*.

341   However, we noticed that extragonadal PGCs in *Met, gce* mutant embryos were often located

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342   centrally and anterior to the gonad, rather than posterior to the gonad as observed in *jhamt* and

343   *Hmgcr* mutant embryos (**Fig 2d** and **Fig 4d**). The central, anterior location of extragonadal

344   PGCs in *Met, gce* mutant embryos suggests that migration to the gonad may be compromised

345   at an earlier stage than in *jhamt* mutant embryos. One possible explanation is that altered JH

346   homeostasis within the embryos, rather than loss of JH signaling response in migrating PGCs,

347   were the cause of extragonadal PGCs in *Met, gce* mutants. To test this possibility, we measured

348   JH titers by quantitative mass spectrometry. Interestingly, we found that both JH III and JHB$_3$

349   titers were dramatically increased in *Met, gce* mutant animals relative to wildtype (**Fig 5a**). To

350   investigate how JH titers could be increased in *Met, gce* mutant animals, we measured *jhamt*

351   mRNA levels by real time-qPCR. We found increased *jhamt* mRNA levels in *Met, gce* mutants in

352   several life stages, including during mid-stage embryo embryogenesis (**Fig 5b** and **Supp Fig**

353   **5a**). Consistent with compromised JH-dependent transcriptional signaling, expression of the

354   target gene, *Kr-h1*, was not increased in *Met, gce* mutant animals (**Fig 5b**). These data

355   uncovered a compensatory feedback mechanism in *Drosophila melanogaster* by which JH

356   receptors mediate JH titer homeostasis. Importantly, this study suggests that PGC migration to

357   the gonad is compromised by either decreased or increased JH levels.

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360   <u>Isoprenoids in PGC migration</u>

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362   JHs belong to a broader class of secreted isoprenoids that have functions in reproduction

363   throughout the animal kingdom. Such molecules include methyl farnesoate, which has potent

364   impacts on sex differentiation in the crustaceans[6], and retinoic acids (RAs), which have

365   pleiotropic effects at many stages of vertebrate reproductive development and gametogenesis[35-

366   37]. Although the transcriptional receptors differ, they are promiscuous enough to bind to a broad

367   class of exogenous secreted isoprenoids and other small molecules[38]. To determine whether *in*

368   *vitro* cross-activation extends to PGC migration, we enriched *Drosophila* PGCs by FACS and

369   subjected them to JH III or two RA species, all-trans retinoic acid (ATRA) or 9-cis RA. Notably,

370   9-cis RA induced *Drosophila* germ cell translocation to levels comparable of JH III (**Fig 6a**).

371   These data show that RAs can induce *Drosophila* PGC migration, suggesting commonality in

372   response to this class of secreted isoprenoids.

373

374   Our studies revealed that the JH biosynthesis enzyme was present in the mesoderm near PGCs

375   as they migrated toward the developing somatic gonad during *Drosophila* embryogenesis.

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376   Interestingly, the enzymes critical for RA biosynthesis are also expressed in the developing

377   bipotential somatic genital ridge of mouse embryos as germ cells are colonizing this tissue[21]. To

378   determine whether RAs may have a similar role in vertebrate germ cell development, we

379   adapted the *in vitro* transwell migration assay developed for *Drosophila* PGCs to mouse germ

380   cells. To this end, we obtained mouse germ cells by FACS from embryonic day E10.5 mice

381   carrying a germ cell-specific transgene (*Oct4ΔPE:GFP*) (**Supp Fig 6a**). At this stage in

382   reproductive development, mouse PGCs are completing their migration and beginning to

383   populate the bipotential somatic gonad. We exposed germ cells to fetal bovine serum in PGC

384   media as a positive control or serum-free PGC media as a negative control. Both ATRA and 9-

385   cis RA induced translocation of mouse embryonic germ cells to levels comparable to the known

386   mouse and zebrafish PGC chemoattractant, C-X-C Motif Chemokine Ligand (CXCL12) (also

387   called SDF1a) (**Fig 6b**)[39-41]. Although RAs can have a proliferative effect on mouse PGCs over

388   24-72 hours[42], we did not detect changes in total PGC number during our two-hour assay (**Supp**

389   **Fig 6b**). Therefore, the increased number of translocated PGCs was not due to RA-induced

390   proliferation. Together, these data suggest that the newly uncovered role of secreted

391   isoprenoids in early PGC migration and colonization to the somatic gonad may be broadly

392   shared among invertebrate and vertebrate species.

393

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395

396   **Discussion**

397

398   Germ cells are shepherded through development and differentiation by surrounding somatic

399   cells, which provide hormones and other signaling molecules. Here, we demonstrated that

400   Juvenile Hormones (JHs), key gonadotropins in adult gametogenesis, functioned in embryonic

401   reproductive development. In the embryo, JH signaling was spatially restricted and enriched

402   around PGCs as they migrated to the somatic gonad. We found that JHs were necessary *in vivo*

403   and sufficient *in vitro* for PGC migration. Together, these studies significantly expanded our

404   understanding of the scope, functions, and mechanisms by which JHs support the germline

405   lifecycle.

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410   <u>JH signaling is active during embryogenesis prior to endocrine gland formation in *Drosophila*</u>

411

412   The study of JH biology has largely focused on endocrine production in juvenile and adult

413   animals, particularly for insects that undergo a complete metamorphosis, like *Drosophila*. In

414   juvenile and adult animals, JHs are produced in the CA endocrine gland. CA precursors are first

415   distinguishable at Stage 13 and migrate dorsally to form the ring gland at Stage 15[43,44]. Through

416   the generation of an *in vivo* JH sensor, we provide evidence that JH signaling is active in

417   *Drosophila* embryos starting at Stage 10 (**Fig 1e** and **f**). Our data are consistent with a burst of

418   *Kr-h1* expression in 8-12 hr old embryos[45,46] and with recent reports of non-CA *jhamt* expression

419   in gut cells[47]. Combined, these studies show that sources of these important hormones exist

420   outside of the CA. Three species of biologically active JHs detected in *Drosophila* are proven

421   agonist ligands of Gce[25,48], yet whether these forms are temporally and functionally distinct *in*

422   *vivo* are unknown. The discovery of embryonic JH activity in the genetically tractable *Drosophila*

423   embryo and in an *in vitro* migration assay provide a unique opportunity to explore JH species-

424   specific functions.

425

426

427   <u>JH signaling is paracrine during mid-embryogenesis</u>

428

429   We observed JH activity near mesodermal expression of JH-producing enzyme Jhamt (**Fig 1b,**

430   **e, f**). This observation suggests that JH signaling is paracrine during embryogenesis. Paracrine

431   signaling mechanisms are consistent with reports that adult gut cells exhibit local JH signaling[49]

432   and that ectopic *Hmgcr* expression influences PGC migration within a ~50um zone[28]. How JH

433   signaling response is spatially restricted has yet to be understood, but one possibility is that JH

434   transport or diffusion is limited by the dense extracellular environment of the embryo.

435   Alternatively, JH signaling may be restricted by the spatio-temporal expression of enzymes that

436   synthesize or degrade JHs or be restricted to cells that possess all required factors in the

437   transcriptional signaling cascade. Whether all co-factors are present in all somatic cells during

438   mid-embryogenesis is unknown, however the core JH transcription factors, *Met, gce* and *taiman*

439   mRNAs are maternally loaded into the early embryo[50-53]. Whether JH binding proteins, which

440   facilitate JH circulation in whole juvenile and adult insects, are required for embryonic JH

441   signaling is unknown. The highly dynamic, well-characterized, and pre-circulatory features of

442   mid-embryogenesis can be leveraged to understand the mechanisms of paracrine JH signaling.

443

444

JH signaling ensures migrating PGCs reach the somatic gonad

446

447 Embryonic JH signaling is enriched in the trunk mesoderm as PGCs migrate in this tissue

448 toward the somatic gonad (**Fig 1c, e, f**). We found that JH biosynthesis enzymes are necessary

449 for efficient gonad colonization *in vivo* and JHs are sufficient for PGC migration *in vitro* (**Fig 2**

450 and **Fig 3**). While JHs have many functions in reproduction, our findings demonstrate that JH

451 activity impacts germline development far earlier than previously appreciated. Moreover, a role

452 for JHs in cell migration had not been reported to our knowledge. In contrast, ecdysone, the

453 steroid hormone controlled, in part, by JHs[54], regulates the migration of many somatic ovarian

454 cell types, including swarm cells during juvenile ovarian development[3] and border cells during

455 adult gametogenesis[4]. Given the antagonistic relationship between the steroid ecdysone and

456 the isoprenoid JHs[54], it remains possible that JHs also prevent ecdysone-mediated processes

457 while germ cells colonize the gonad. Such regulation by JHs may have been obscured by the

458 relatively high degree of genetic redundancy throughout the JH biosynthetic, degradative, and

459 signaling response cascades in the *Drosophila* genome[15,25,26]. The findings described here

460 provide an opportunity to understand the relationship between steroid hormones and JH/retinoid

461 signaling molecules. Indeed, antagonism between these two classes of signaling molecules are

462 observed in multiple tissue contexts throughout the animal kingdom[54,55].

463

464 Our findings that JHs impact PGC migration opens many questions. Do JHs regulate PGC

465 directionality or motility? What signaling events are required for migrating PGCs to respond to

466 JHs? Classic JH signaling involves receptor-mediated transcription. In contrast, we find that

467 transcription is not required in PGCs for migrational response to JHs *in vitro* (**Fig 4f**). Our finding

468 is consistent with the observation that PGCs respond to ectopic Hmgcr prior to full activation of

469 their zygotic genome (**Fig 4e**) and that migrating PGCs must rapidly respond to environmental

470 changes. Rapid responses to JHs during ovarian follicle development has been observed for

471 decades[56-59], however the mechanisms remain elusive. Both receptor tyrosine kinases and G

472 protein-coupled receptors (GPCRs) have been implicated in rapid responses to JHs[60,61]. A

473 requirement for GPCRs is consistent with our observations that migratory response of

474 *Drosophila* PGCs is attenuated at the highest dose of JHs *in vitro* (**Fig 3e**) and compromised

475 when JH titers are elevated *in vivo* (**Fig 4d** and **5a**), a characteristic feature of GPCR

476 desensitization. Identification of a plasma membrane receptor capable of mediating a rapid

477   response to JHs and related molecules during early PGC development will be an exciting area

478   of future exploration.

479

480   Signaling responses to JHs usually involve DNA-binding receptors that induce transcription. We

481   found that the *Drosophila melanogaster* JH receptors, Met/Gce, are required for PGCs to

482   efficiently migrate to the gonad (**Fig 4d**) downstream of Hmgcr (**Fig 4e**). However, genetic

483   suppression of Hmgcr-mediated trapping was mild and the location of extragonadal *Met, gce*

484   PGCs differs compared to embryos lacking the JH biosynthesis enzymes. In the absence of

485   Jhamt and Hmgcr, extragonadal PGCs are often found lateral and posterior to the gonads,

486   whereas extragonadal *Met, gce* PGCs are found medially and anterior to the gonads (**Fig 2d**

487   and **Fig 4d**). While anterior extragonadal PGCs could result from failing to stop migrating at the

488   target gonad, the medial localization and frequent endodermal association of *Met, gce* PGCs

489   suggests that migration is compromised earlier than in *jhamt* mutants. There are a few possible

490   explanations for this surprising observation. First, JH signaling may be more compromised

491   earlier and stronger in *Met, gce* mutants than in *jhamt* mutants. Indeed, loss of *jhamt* does not

492   completely abolish JH titers (**Fig 2b**), as has been reported previously[25]. Second, JH signaling

493   may be required maternally as JHs are maternally provided into eggs in some species[62].

494   However, it should be noted that zygotic loss of Jhamt and Hmgcr is sufficient to compromise

495   PGC migration (**Fig 2d**)[19]. Third, migration may be compromised in *Met, gce* mutant animals

496   because JH titers are increased (**Fig 5a**). Future work will be needed to elucidate precisely how

497   Met and/or Gce are required for PGC development. Yet, our findings suggest that either a

498   specific titer or spatial arrangement of JHs are required for PGCs to efficiently populate the

499   somatic gonad. How PGCs respond to finely tuned JH availability will be an interesting area of

500   future exploration.

501

502

503   Discovery of a compensatory feedback loop that maintains JH homeostasis

504

505   Animals carrying mutations in the JH receptors exhibit dramatically high JH titers (**Fig 5a**).

506   Elevated JH titers coincided with increased *jhamt* mRNA levels at multiple *Drosophila* life stages

507   (**Fig 5a**, **b** and **Supp Fig 5a**), suggesting that constitutive feedback ensures stage-appropriate

508   JH titers upon genetic perturbations. The mechanism of this compensatory loop is not known,

509   however Met, Ecdysone Receptor (EcR), and their shared co-factor, Taiman, binding has been

510   detected ~1kb upstream of the *jhamt* transcription start site[63] (**Supp Fig 5b**). Thus, a simple

15

511   feedback mechanism may involve either Met/Gce-Tai repression or EcR-Tai activation of *jhamt*

512   transcription. Indeed, reduced *Kr-h1* mRNA observed in *Met, gce* animals (**Fig 5b**) may lead to

513   the upregulation of EcR-dependent signaling as ecdysone biosynthesis genes are repressed by

514   Kr-h1[64,65]. For these studies, we used the *Met^1* allele, which encodes a point mutation in a highly

515   conserved position of the PAS-A domain and the *Met^{27}* allele, which strongly reduces *Met*

516   mRNA but the open reading frame appears wildtype (Marek Jindra, personal communication).

517   Both *Met^1* and *Met^{27}* mutant animals have dramatically increased tolerance to methoprene[31].

518   However, in our hands, adult escapers could only be obtained in heteroallelic *Met$^{1/27}$, gce$^{Mi/2.5k}$*

519   combination. Even under sparse conditions, homoallelic *Met$^{27/27}$, gce$^{2.5k/2.5k}$* were not observed,

520   suggesting either the presence of a second site lethal mutation in the *Met^{27}, gce$^{2.5k}$* genetic

521   background or that *Met$^{1/27}$, gce$^{Mi/2.5k}$* animals have enough JH signaling to support development

522   through increased JH titers. The later explanation is more likely because UAS-driven *Met* or *gce*

523   can rescue *Met$^{27/27}$, gce$^{2.5k/2.5k}$* lethality[15]. Moving forward, it will be important to expand the

524   experimental tools available to dissect this robust signaling pathway.

525

526

527   <u>New animal model to understand isoprenoid signaling in embryonic reproductive development</u>

528

529   We found that JH ensures that migrating PGCs reach the somatic gonad of the *Drosophila*

530   embryo. In contrast to systemic endocrine signaling in other life stages, our findings

531   demonstrate that during mid-embryogenesis, JH signaling is active near mesodermal

532   expression of the key JH biosynthesis enzyme, Jhamt. This local, paracrine signaling is similar

533   to how retinoic acids (RAs) act within vertebrate species. Indeed, the bioavailability of RAs and

534   JHs during analogous stages of germ cell development is strikingly similar between mice and

535   *Drosophila*, respectively[20-22,66]. RAs impact the migration of many cell types. Although most

536   investigations into the role of RAs in cell migration have focused on transcriptional upregulation

537   of chemokines or their receptors[67,68], evidence for transcription-independent functions of RAs is

538   an emerging field[69]. RAs facilitate axon guidance in *Xenopus laevis*[70], and in snails through non-

539   transcriptional mechanisms involving calcium flux, Rho GTPases and G proteins[71-74]. The many

540   similarities in structure, function, and periodic presence throughout the germline lifecycle

541   support the hypothesis that the isoprenoid signaling molecules, JHs and RAs, are functionally

542   analogous in reproductive development across the animal kingdom. We assert that *Drosophila*

543   can be a powerful genetic model to understand the primary functions and molecular

544   mechanisms by which environmentally pervasive isoprenoids impact reproductive development.

545 Such investigations will increase our understanding of how germ cell development is supported

546 by surrounding somatic cells, both endogenously and through *in vitro* gametogenesis

547 technologies.

548

549 **Acknowledgements**

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572 **Author Contributions**

573

574 Conceptualization: LJB, RL; Experimentation: LJB, JS, EPD MN, FGN, MS; Analysis: LJB, MN,

575 FGN; Writing: LJB, RL; Funding Acquisition: LJB, RL; Supervision: LJB, MS, RL

576

577 **Declaration of Interest**

578

579   The authors declare no competing interest

580   **STAR Methods**

581

582   Animal Maintenance

583   *Drosophila melanogaster* were raised on medium containing 1.5% yeast, 3.6% molasses, 3.6%
584   cornmeal, 0.112% Tegosept, 1.12% alcohol and 0.38% propionic acid. For embryo collections,
585   apple juice plates contained 25% apple juice, 2.5% sucrose, 2.25% bactoagar, 0.15% Tegosept.
586   Animals were kept at 25°C for all experiments. Fly strains used in this study are listed in
587   Supplementary Table 1.

588

589   Mice expressing a germ cell-specific *Oct4ΔPE:GFP* transgene[76] (B6; CBA-Tg(Pou5f1-
590   EGFP)2Mnn/J) were obtained from Jackson Laboratory (#004654, Bar Harbor, ME, USA). All
591   protocols were approved by the NYU Institutional Animal Care and Use Committee.

592

593

594   Generation and validation of the P [*JHRE-GFP, w+]* transgenic biosensor
595   Eight copies of the wildtype JH response element (JHRE) from the *early trypsin* gene from
596   *Aedes aegypti*[23], or eight tandem copies of the mutant JHRE (Supp Fig 1), were excised from
597   *pGL4.17* vectors provided by Jindra Marek. JHREs were inserted upstream of the minimal
598   *hsp70b* promoter and *eGFP* coding sequence of pS3aG transformation vector (Addgene
599   #31171) containing an *attb* for site-specific integration. JHRE::GFP sensor constructs were
600   integrated into *y+, attP2* at cytological position 68A4 sites by Bestgene.

601

602   To validate the JH sensor, transgenes were placed in *met^1, gce^{MI}* mutant background.
603   Embryonic GFP signal was measured by immunohistochemistry. To measure JH
604   responsiveness, first instar larvae were placed in food containing 1.6μg of Methoprene per gram
605   of food [77], or ethanol as a control for 24 hours. *GFP* and *Kr-h1* mRNA was measured in whole
606   second instar larvae by qPCR using primers in Table 3 (Fig 1 and Supp Fig 1).

607

608

609   Generation of *jhamt* alleles
610   The CRISPR guide CCTGGATGTGGTTCAGGATCT was cloned into pU6-2 gRNA vector
611   (DGRC #1363) and injected into embryos into a stock derived from BL51324 carrying *w^{1118}* ;;
612   *PBacy[mDint2]=vas-cas9VK00027* by Bestgene. Of 29 fertile founders, 87 F1 crosses were set
613   up and screened for *jhamt* mutations by PCR amplification and Sanger sequencing using
614   primers listed in Table 3. Of 48 F1 lines screened, 13 lines were obtained wherein small indels
615   created a frame shift just before the methyltransferase domain, which starts at amino acid 40,
616   leading to a stop codon just downstream, two lines at amino acid 46 (12F1 and 12F2) and the
617   remaining lines at amino acid 54 (1F1, 1F2, 4F1, 4F3, 6F3, 7F2, 7F3, 8F1, 8F2, 12F3, 14M2,
618   14M3). The effect of new *jhamt* mutations on JH III and JHB$_3$ titers were quantified in third instar
619   larval hemolymph.

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623
624 <u>Embryo Immunofluorescence</u>
625 Prior to embryo collection, young parental strains (less than 5 days old) were mated in vials for
626 one day before transferring to embryo collection cages containing apple juice plates and wet
627 yeast. Embryos were dechorionated in 50% bleach for three minutes, rinsed with water and
628 fixed in a scintillation vial containing 2ml of 4% formaldehyde in PBS and 8ml of heptane.
629 Embryos were fixed for 20 minutes on a shaker. To remove the vitelline membrane,
630 formaldehyde was removed from the scintillation vial and 10ml methanol was added, followed
631 by one minute of vigorous shaking by hand. Embryos were washed with fresh methanol three
632 times before storage at -20°C or future processing.
633
634 For immunohistochemistry, embryos were sequentially rehydrated with increasing percentage of
635 PBSTx (PBS with 0.1% TritonX100) and then washed six times with PBSTx before a one-hour
636 block using 1% BSA in PBSTx at room temperature. Primary antibodies were incubated
637 overnight at 4C and then washed six times in PBSTx. Secondary antibodies in 1% BSA in
638 PBSTx were incubated for two hours at room temperature, washed six times in PBSTx.
639 Embryos were then equilibrated in Vectashield (Vector Laboratories, H-1200) overnight at 4°C
640 before mounting on coverslips.
641
642 Fluorescent *in situ* hybridization was done as previously shown[78]. Probes were generated using
643 *Drosophila* clones from DGRC (*jhamt* #11840, clone AT13581) and (*Nano*s #1124533, clone
644 RE53469).
645
646
647 <u>mRNA quantification by qPCR</u>
648 RNA was obtained by TRIzol (Invitrogen #15596018) extraction. DNA was removed using RQ1
649 RNase-Free Dnase (Promega #M6101). cDNA was generated using SuperScript III Reverse
650 Transcriptase (Fisher Scientific #18080-044) using both oligo dT and random hexamers.
651 Primers used are listed in Table 3. qPCR was carried out using LightCycler 480 SYBR Green I
652 Master 2X (Roche #04887352001) and a Roche LightCycler 480. Results were calculated as
653 previously described[79] from crossing points and normalized to one of three reference genes
654 found to have invariant expression across tissues and developmental stages: *DCTN2-p50*, *und*,
655 *DCTN5-p25*[80].
656
657
658 <u>Evaluation of JH titers by quantitative mass spectrometry</u>
659 The following genetic backgrounds were used to measure JH III and JHB$_3$ titers:
660 *jhamt*$^{8F2/12F1}$ paired with the *w*^{1118}; *CyO, ft-lacZ* genotype, which was the genetic background the
661 crispr alleles were extensively crossed into and *met*^{1}, *gce*^{Mi} paired with *w*^{1118}. Twenty-five third
662 instar larvae per biological replicate were collected from sparsely populated vials, washed in
663 PBS before placing in 50µl of pre-chilled PBS for hemolymph collection. To collect hemolymph,
664 the cuticle was pinched and pulled apart down the length of the animal. Care was taken not to
665 rupture fat body or intestines. Hemolymph was transferred to pre-chilled silanized glass vials
666 (Fisher Scientific #C4011S5 and #C401154B) using silanized glass pipettes (Brain Bits #FPP).

19

667 The glass collection well was rinsed with 50µl additional, pre-chilled PBS to obtain any
668 remaining hemolymph and additional PBS was added to silanized glass vials for a total volume
669 of 150µl. Deuterated JH III (62.5pg) dissolved in acetonitrile (Thermo Fisher #51101) was added
670 to each replicate, then 600µl hexane (Sigma Aldrich #1037011000). Samples were vortexed for
671 1 minute and centrifuged for 5 min at 2000g at 4°C. Organic phase was transferred to new
672 silanized vial for analysis by mass spectrometry.
673
674 For mass spectrometry analysis, the JH III and JH III bisepoxide (JHB$_3$) amounts present in
675 hemolymph were quantified by liquid chromatography coupled to tandem mass spectrometry
676 (HPLC-MS/MS)[24]. The identification and quantification of JH III and JHB$_3$ in hemolymph
677 samples was based on multiple reaction monitoring (MRM), using the two most abundant
678 fragmentation transitions: for JH III: 267->235 (primary) and 267->147 (secondary), and for
679 JHB$_3$: 283->233 (primary) and 283->251 (secondary)[24].
680
681
682 Fecundity measurements
683 Fecundity was measured as previously described[81] and reported as the average number of
684 eggs laid in three- and four-day old females at 25ºC. In each biological replicate, 10-12 females
685 were crossed to 5-6 w^{1118} males to mitigate male fertility defects upon *jhamt* or *met, gce* loss.
686
687
688 Knockdown of *jhamt* in Kc167 Cells
689 To effectively knockdown *jhamt* mRNA, genomic DNA obtained from Kc167 cells using Qiagen's
690 Blood and Tissue Kit (#69506) was used as a template. To target *lacZ*, pBluescript SK plasmid
691 used as a template. dsDNA was generated by Choice Taq Mastermix (Denville Scientific
692 #CB4070-8). Gradient PCR was used to obtain optimal dsDNA templates. dsRNAs were
693 generated from dsDNA templates using T7 MEGAScript (Invitrogen #am1334) according to
694 standard protocol. dsRNAs were DNAse treated (Qiagen #79254) and purified using Qiagen
695 RNAeasy Mini Kit (Qiagen #74104) according to instructions. For transfection, Kc167 cells were
696 grown to confluency, resuspended in fresh Schneider's media (Fisher #21720-001)
697 supplemented with Penn/Strep (Fisher, #15140122) and 10% heat inactivated FBS (Thermo
698 Fisher #16140071) to 1x10^6 cells/ml and allowed to settle overnight. 2µg of dsRNAs were
699 used in conjunction with Effectene Transfection Reagent (Qiagen #301425) into each 6-well
700 dish. After 48 hours at 25ºC, media was removed, transfected Kc167 cells were washed in PBS
701 and resuspended in serum-free Schneider's Media (with Penn/Strep) for three additional days at
702 25ºC prior to collection of Kc167-conditioned media. To determine whether *jhamt* knockdown
703 compromised Kc167 viability, the number of Kc167 cells was counted on the day conditioned
704 media was collected. Knockdown efficiency was determined in Kc167 cells by qPCR when
705 conditioned media was collected for the transwell assay. Primers used to measure knockdown
706 efficiency were chosen outside of region targeted by dsRNAs.
707
708
709 Isolation of PGCs

710   *Drosophila* embryos carrying the *P[nos-Moe::eGFP.nos 3'UTR]* transgene from an overnight

711   collection were dechorionated in 50% bleach for two minutes, rinsed and placed in filter-

712   sterilized Chan and Gehring's Balanced Saline, PGC Sort Buffer (3.2g/L NaCl, 3.0g/L KCl,

713   0.69g/L CaCl2.2H2O, 3.7g/L MgSO4.7H2O, 1.79g/L Tricine buffer (pH 7), 3.6g/L glucose,

714   17.1g/L sucrose, 1g/L BSA, fraction V). Dechorionated embryos were manually homogenized in

715   PGC Sort Buffer with Dounce homogenizer (Pyrex #7727-15) and passed through a 100μm filter

716   and 20μm filter and placed in pre-chilled polypropylene falcon tubes (#352063). Live, GFP-

717   positive *Drosophila* PGCs were sorted using a MoFlo XDP cell sorter fitted with a 70μm nozzle

718   into Maxymum recovery microtubes (Axygen #311-05-051) containing 200μl Schneider's Media.

719

720   To enrich for mouse PGCs by FACS, B6; CBA-Tg(Pou5f1-EGFP)2Mnn/J (Jax #004654) mice

721   were bred to produce embryos homozygous for the *Oct⊿4PE::GFP* transgene. Embryonic day

722   0.5 (E0.5) was assumed to be noon of the day the vaginal plug was observed. Pregnant

723   females were euthanized by carbon dioxide followed by cervical dislocation. Trunks were

724   dissected from E10.5 embryos in chilled PBS. For each two embryos, tissue was dissociated in

725   300μl of 0.5% trypsin (0.25%, Life Technologies #25200056) for 15 min at 37°C, with occasional

726   manual disruption by pipette. Trypsin was inactivated with 200μl of mouse PGC culture media

727   [DMEM/F-12 (Thermo Fisher #11-320-033), 250μM sodium pyruvate (Thermo Fisher

728   #11360070), 2mM L-glutamate (Gibco #25030081), 1X MEM Non-essential amino acids

729   (Thermo Fisher #11-140-050) containing 25% Bovine Albumin Fraction V (Thermo Fisher

730   #15260037). Dissociated tissue was centrifuged at 200g for 7 min and cells were resuspended

731   in 2mL of PGC media. Cell suspension was filtered through a 35μm cell strainer into a 5 ml

732   falcon round bottom tube (VWR #21008-948) for sorting using a MoFlo XDP cell sorter fitted

733   with a 100μm nozzle. Live, GFP+ germ cells were sorted into Maxymum recovery microtubes

734   (Axygen #311-05-051) containing mouse PGC media.

735

736

737   *in vitro* Migration Assays

738   For *in vitro* transwell assays, all plastic ware, tips, tubes and transwell plates used throughout

739   the assay were pre-coated with PBS containing 1% BSA to prevent hydrophobic molecules from

740   sticking to the surfaces. Assays with light sensitive isoprenoids were done in dark conditions as

741   much as possible. For Kc167-conditioned media, serum-free media was added to washed,

742   confluent Kc167 cells and collected three days later. Conditioned media was collected and

743   centrifuged 4,000rpm for 20 min to remove all cellular debris and placed on ice. All isoprenoids

744   (Supplementary Table 4) were prepared by dilution series in either serum-free Schneider's

745   media for *Drosophila* PGC assays or mouse PGC media. Transwell assays were done using the

746   96-well ChemoTx Disposable Chemotaxis System (NeuroProbe #116-10) which had a standard

747   PCTE filter membrane with 10μm pore size, 300μl well capacity and 5.7mm cell site diameter.

748   Sorted germ cells recovered on ice for 30 min while the transwell system was set up. Sorted

749   germ cells were then diluted 10-15-fold in serum-free Schneider's media for *Drosophila* PGC

750   assays or mouse PGC media so that 60μl diluted PGCs were placed above each well.

751   Transwell plates with *Drosophila* PGCs were incubated 25°C for 1.5 hours and those with

752   mouse PGCs were incubated at 37°C for 2 hours. After the incubation period, PGCs were

753   removed from above membrane, the plate was centrifuged at 460rpm for 10min to dislodge cells

21

754   from the well side of the membrane. Translocated germ cells were removed from the 96-well
755   plate by pipetting up and down and transferring to Nunc Lab-Tek II Chambered Coverglass
756   (Thermo Fisher #155409) for manual quantification by fluorescent microscopy.
757
758   The number of PGCs in each top well was calculated by setting aside 10% input (6µl) in
759   duplicate into Lab-Tek wells, each with 300µl serum-free media at the beginning of the transwell
760   assay and quantifying the number of germ cells when the experimental translocated germ cells
761   were quantified. To compare biological replicates done on different days, Specific Migration was
762   calculated by first subtracting the number of germ cells that translocated in the negative control
763   wells (serum-free media) and then calculating the percentage of normalized experimental
764   condition to the respective positive control. Each transwell assay is the result of two to four
765   biological replicates, each consisting of two technical replicates.
766
767
768   <u>Verification of Hormone Activity *in vitro*</u>
769   To verify the activity of JH III and 20-hydroxecdysone (Supplemental Table 4), hormones were
770   added to 50% confluent Schneider 2 (S2) cells at the concentrations indicated in Supp Fig 3c.
771   After a six-hour incubation at 25ºC, S2 cells were harvested, and mRNA was extracted and
772   analyzed using protocols described in the '*mRNA quantification by qPCR*' Methods subsection.
773
774
775   <u>Transcriptional Inhibition in *Drosophila* PGCs</u>
776   Efficient inhibition of transcription in *Drosophila* PGCs isolated by FACS was determined by
777   testing different concentrations of Actinomycin D and Flavopiridol (See Supplemental Table 4).
778   To visualize transcription by immunohistochemistry, Lab-Tek II 8-chambered coverglass were
779   incubated with poly-L-Lysine solution (Sigma-Aldrich #P4707) for 30 min at room temperature
780   and then dried for 1.5 hours. Sorted *Drosophila* PGCs were added to chambers and incubated
781   with Actinomycin D or Flavopiridol for either 15 min or 3 hours, then fixed with 4%
782   paraformaldehyde for 15 minutes, washed in 0.05% PBS with TritonX100, blocked in 1% BSA
783   for 30 min and stained for Vasa, RNA Pol II Ser2CTD and RNA Pol II Ser5 CTD (See
784   Supplemental Table 2 for antibody information). As expected[34], Flavopiridol rapidly inhibited
785   transcription in *Drosophila* PGC while not compromising cell viability. For transwell assays,
786   sorted *Drosophila* PGCs were first incubated with 1µM Flavopiridol for 30 min prior to placing on
787   transwell membrane and solutions in the bottom well chambers also contained 1µM Flavopiridol
788   to ensure PGC transcription was inhibited throughout the assay.
789
790
791   <u>Microscopy and Image Processing</u>
792   Most embryos and other samples were imaged with a Zeiss LSM800, an upright laser scanning
793   confocal microscope using Zen Blue 2.3 software and a Plan-Apochromat 20x 0.8 NA air
794   objective and 40x 1.3NA oil objectives with pinhole size for all imagine was set to 1AU. Images
795   were processed using ImageJ/FIJI.
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799 Statistics

800 Statistical analysis was done as described in the Figure Legends. Briefly, for quantification of

801 phenotypic data, such as extragonadal PGC quantification, data was not always normally

802 distributed and thus median +/- IQR are reported, and statistical significance was assessed by

803 Mann-Whitney U test. For mRNA Fold Change and *in vitro* Specific Migration results, mean +/-

804 standard deviation is reported and statistical significance was tested by unpaired t-test. Sample

805 size per biological replicate was influenced by power analysis based on previously observed

806 phenotypic variation.

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815 **Supplemental Table 1:** Animal lines used in this study
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Name	Species	Source	Notes
P[JHREWt8x-eGFP]attp2	*Drosophila*	This study	JH responsive reporter, with non-specific activity in hemocytes
P[JHREMut8x-eGFP]attp2	*Drosophila*	This study	Negative control reporter transgene with non-specific activity in hemocytes
y¹,w, gce^{mi02742}*	*Drosophila*	BL60189	Insertion that leads to RNA null
Met¹	*Drosophila*	BL3472	EMS-generated from OregonR-C causing G1161736A, DNA mutation and G166D protein change, genetically amorphic allele [82] [31]
y⁺, w⁺, v⁺, Met¹, gce^{mi02742}/Fm7ai	*Drosophila*	This study	Recombinant between existing mutants listed above
w-, v-, Met²⁷, gce^{2.5k}/Fm7ai	*Drosophila*	Rebecca Spokony and Lynn Riddiford [83]	Both *Met²⁷* and *gce^{2.5k}* are large deletions that lead to full loss of RNA
w¹¹¹⁸; jhamt^{8F2} or ^{12F1}/CyO, ft-lacZ	*Drosophila*	This study	CRISPR-generated small deletions
jhamt^{Df(2L)BSC781}/CyO, ft-lacZ	*Drosophila*	BL27353	2L:16325113;16417726, including *jhamt* and 13 other genes
Hmgcr^{26.31}/Tm3, Sb, Ubx-lacZ	*Drosophila*	[84]	Also known as *clb²*
Hmgcr^{11.54}/Tm3, Sb, Ubx-lacZ	*Drosophila*	[84]	Also known as *clb¹*
Hmgcr^{01152}/Tm3,Sb, Ubx-lacZ	*Drosophila*	[19]	L(3)01152 source: Transposon insertion 179 nucleotides into *Hmgcr* locus
UAS-Hmgcr	*Drosophila*	[19]	pNB36C *Hmgcr* cDNA downstream of UASt
y,w; ; UAS-jhamt	*Drosophila*	Ryusuke Niwa [17]	RN286 and RN287, both on the third chromosome, containing two point mutations also found in w¹¹¹⁸ (F166Y and S182N)
w-; elav-GAL4	*Drosophila*	J Treisman	Second chromosome
w-; 48Y-GAL4	*Drosophila*		Second chromosome
w-; twi-GAL4; 24B-GAL4	*Drosophila*	M. Akam	Second and third chromosome
y,w;; Tub-GAL4	*Drosophila*		Third chromosome
w-, P[nos-moesin::egfp::nos 3'UTR]	*Drosophila*	[85]	
Oct4ΔPE::GFP	Mouse	Jax #004654	B6; CBA-Tg(Pou5f1-EGFP)2Mnn/J

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821 **Supplemental Table 2:** Antibodies used in this study

Name	Source	Species	Concentration
Vasa	Lehmann lab	Rabbit	1:5,000
GFP	AVES #1020	Chicken	1:500
GFP	Thermo Fisher Scientific #A11122	Rabbit	1:1,000
Singed (fascin)	DSHB #sn 7c	Mouse	1:100
Elav	DSHB #9F8A9	Mouse	1:500
b-galactosidase	Abcam #ab9361	Chicken	1:500
Hindsight	DSHB #1G9	Mouse	1:20
RNA Pol II Ser5	Chromotek #3e8-1	Rat	1:100
RNA Pol II Ser2	Abcam #ab5095	Rabbit	1:500

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826 **Supplemental Table 3:** Primers used in this study

Element	Purpose	Forward Primer	Reverse Primer
jhamt	Genotyping deletions	AGGTGCAAAGGCACGAT	AGGTGCAAAGGCACGAT
jhamt	Crispr guide	CTTCGAGATCCTGAACCCACATCC	AAACGGATGTGGGTTCAGGATCTC
jhamt	*in situ* probe	GGCACGATGCCAAATTGAT	GATAATACGACTCACTATAGGGGATGTGTAGGCTCCAGGAAA
Nanos	*in situ* probe	AGATTGCGCGATCCTTGAA	TAATACGACTCACTATAGGGCTGGATTCGCCCTCTCTAAAC
und	reference for qPCR	GCAAGAAAAGCGGTCAGACT	CGTGTTGATACGGTCCAGAG
Dnm/DCTN2-p50	reference for qPCR	AGACGCCTGGAAGTAAGCAG	GTAAGGCGGCTCAACTTGTC
DCTN5-p25	reference gene for qPCR	ACAAAGTTAGTCGCCACACG	ATGACGGAGTTCTTGCCAAT
twinstar	Detect DNA contamination	TCAGAGGTGCCTTGACACTG	GATGTGGAGACTGTGGCAGA
eGFP	mRNA quantification	GAACCGCATCGAGCTGAA	TGCTTGTCGGCCATGATATAG
gce	Genotyping and mRNA quant	GGATGCCATCGATCGCAAGT	GCTTCGTCACTACGCCGAAA
Met	Genotyping *met[1]* allele	CTGCTTCCTCACCCTAACCT	CTGCTTCCTCACCCTAACCT
jhamt	mRNA quantification	TGACCATGTCACCTCGTTCTACTGC	GAAGTCATCCAGGAACTGTTCATGC
jhamt	dsRNA 1 for knockdown in Kc167 cells	TAATACGACTCACTATAGGGCCTCACGCCTTCATAAACAA	TAATACGACTCACTATAGGGGGTAACGTGCTCATGGACT
jhamt	dsRNA 2 for knockdown in Kc167 cells	TAATACGACTCACTATAGGGCTTCGTAAAGCCACTGCTCC	TAATACGACTCACTATAGGGCCTCGCCAGGACTTAGACTG
lacZ	dsRNA knockdown DRSC42733	TAATACGACTCACTATAGGGCTGGCGTAATAGCGAAGAGG	TAATACGACTCACTATAGGGCATTAAAGCGAGTGGCAACA
jhamt	Knockdown efficiency	CAGTTCCTGGATGACTTCATAGAC	CCACCACCAATTTATAGGGAGATAG
Kr-h1	JH responsiveness	GAATACGACATAACAGCC	CGATTTCCGTGAATATGTTCT
Eip74b	20E responsiveness	GTACGCGTATTGGGACTATCAG	CACGCGAGATAGGGATTTCTT
Eip75	20E responsiveness	GTCCATCAGCGAGTTGGTATC	CACCAGCAGCCATCTGAA

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828 **Table 4:** Reagents used in this study
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Name	Source	Product Number	Stock solvent	Final Concentration
JH III	Sigma-Aldrich	J2000-10MG	Ethanol	0.25-25μM
Methoprene	Sigma-Aldrich	33375-100MG	Ethanol	2.5μM
20-hydroxecdysone	Sigma-Aldrich	H5142-5MG	Ethanol	0.25-25μM
ATRA	Sigma-Aldrich	R2625-50MG	DMSO	2.5μM
9cis-RA	Sigma-Aldrich	R4643	DMSO	2.5μM
Flavopiridol	Selleck	S1230	PBS with 0.1% BSA	1μM
Actinomycin D	Sigma-Aldrich	A1410-Smg	DMSO	1-5μg/mL
CXCL12/Sdf-1a	R&D Systems	460-SD-010	PBS with 0.1% BSA	50ng/mL

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856   **Figure Legends**

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

859   **PGC migration A** *Left*: Schematic summarizing gonadotropic effect of JH in insects, whereby

860   JH is produced in an endocrine gland and acts systemically to promote oogenesis. *Right*:

861   Summary of established JH signaling pathways wherein endocrine cells produce JHs

862   downstream of Acetyl-CoA and a biosynthetic pathway that culminates in JH acid

863   methyltransferase (Jhamt)-mediated JH production. JHs (red jagged lines) are then secreted in

864   the hemolymph and enter JH-responding cells. Once in JH-responding cells, JH binds to one of

865   two JH transcriptional receptors, Methoprene tolerant (Met) or Germ cell expressed (Gce). JH-

866   bound Met/Gce translocates to the nucleus to regulate target genes. **B** Lateral view images of a

867   late Stage 12 embryo stained by Fluorescent *in situ* hybridization (FISH) for *jhamt* (green) and

868   *nanos* (magenta) mRNA. Primers used to generate *in situ* probes are listed in Supplementary

869   Table 3. *Right:* Magnified image of *jhamt* and *nanos* mRNA in the boxed area outlined by a

870   dashed, white square. **C** Schematic of germ cell development during Stage 10-14 of *Drosophila*

871   embryogenesis. **D** *Top*: schematic of a new transgenic JH sensor (JHRE-GFP) in which eight

872   copies of the JH Response Element from the *early trypsin* gene from *A. aegypti* were placed

873   upstream of eGFP (See Materials and Methods for construct design). *Bottom*: Induction of *Kr-h1*

874   mRNA, a known JH transcriptional target, and *GFP* mRNA. deltaCt was calculated relative to

875   the average Ct of two housekeeping genes, *DCTN5-p25* and *Und*, and fold change was

876   calculated for whole second instar larvae fed cornmeal food containing 1.6μg/mg of JH

877   analogue, methoprene normalized to age and genotype-matched larvae fed ethanol. Animals

878   were fed methoprene or solvent control for 24 hours prior to RNA isolation. Bars and error bars

879   represent average fold change and standard deviation from three biological replicates, each

880   consisting of ten second instar larvae. **E** Dorsal (d) images of Stage 10 *JHRE-GFP* embryos

881   that are wild type (left) or mutant for Met and Gce (right). **F** *Left:* dorsal view of a Stage 11

882   *JHRE-GFP* embryo stained for GFP (green) protein, Vasa (magenta) protein, and DNA using

883   DAPI (gray). *Right*: lateral view of a Stage 11 JHRE-GFP embryo. GFP channel is shown below

884   each merged image. For all images, magnified images in the boxed area outlined by a dashed,

885   white square are shown to the right of each whole-embryo image. Genotypes are noted in the

886   top left corner, embryo orientation in the bottom, and scale bars represent 25μm. All images are

887   maximum projections of two to three 2μm confocal slices.

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

891 **A** Schematic of the *jhamt* locus noting the location of premature stop codons within the

892 methyltransferase domain (shown in green), which were introduced by CRISPR-mediated

893 editing. Light gray denotes 5' and 3' UTR. **B** Titers of two JHs, JH III and JH bisepoxide (JHB$_3$),

894 in hemolymph of third instar *Drosophila* larvae measured by quantitative mass spectrometry

895 using deuterated standards. Each dot represents a biological replicate of hemolymph bled from

896 25 larvae. For titers, w^-, *Sp/CyO,ft-lacZ* was used as a wildtype strain as the CRISPR-induced

897 alleles in a w^- background were initially crossed into this background. **C** Fecundity of young

898 females. Each dot represents the average number of eggs laid per biological replicate, which

899 was obtained by dividing the number of eggs laid over a 24-hour period by the number of

900 females (10-12) in each cohort three- and four-days after eclosion. To avoid confounding male

901 infertility effects, all virgin females were mated to wildtype (w^{1118}) males. For B and C, bars and

902 whiskers represent mean +/- standard deviation and statistical significance was tested by

903 unpaired student t test. **D** Dorsal view images of Stage 14 *Drosophila* embryos stained for Vasa

904 (magenta) and DNA (DAPI, gray). Zygotic genotypes are noted in the top left corner. Gonads

905 are circled with a white dashed circle. Arrowheads highlight extragonadal germ cells. All embryo

906 images are maximum projections of two to three 2μm confocal slices. Scale bars represent

907 25μm. **E** Quantification of extragonadal PGCs in Stage 14-16 embryos. Each dot represents

908 one embryo. To yield *jhamt$^{-/-}$* embryos, with heteroallelic combinations of independently derived

909 indels, *jhamt^{8F1}/CyO,ftz-lacZ* females were mated to *jhamt^{12F1}/CyO,ftz-lacZ*. To generate *jhamt$^-$*

910 *$^{/Def}$* embryos, *jhamt^{8F1}/CyO,ftz-lacZ* animals were mated to *Df(2L)BSC781/CyO, ft-lacZ* animals.

911 **F** Quantification of extragonadal PGCs in Stage 14-16 embryos when Hmgcr is ectopically

912 expressed in the nervous system using *Elav-GAL4* and *UAS-Hmgcr*. Each dot represents an

913 embryo. For panels E and F, bars and whiskers represent median +/- interquartile range (IQR),

914 statistical significance was tested by Mann-Whitney U test, with ns=not significant. The

915 reference strain for *jhamt* studies was w^{1118} as *jhamt* alleles were backcrossed into this

916 background.

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924   **Figure 3: Juvenile hormone is sufficient for PGC migration**. **A** Ventral view images of Stage
925   14 embryos stained for Vasa (magenta) and Elav (green). Genotypes are noted in the top left
926   corner of each image. Arrowheads highlight germ cells in contact with Elav-positive central
927   nervous system. All embryo images are maximum projections of two to three 2μm confocal
928   slices. Scale bars represent 25μm. **B** Quantification of the number of PGCs in Stage 14-16
929   embryos contacting Elav-positive cells upon ectopic expression of either *Hmgcr* or *Jhamt* using
930   the *Elav-GAL4* driver. Bar and whiskers represent mean +/- standard deviation and statistical
931   significance was tested by unpaired t-test. **C** *Top Left*: Scatter plots from FACS of GFP-positive
932   PGCs from *nos-moesin::GFP* transgenic embryos. Region 1 (R1) denotes collection cutoff. *Top*
933   *Right*: Image of isolated GFP-positive PGCs (gray). *Bottom*: Schematic of the *in vitro* transwell
934   assay in which FACS-enriched PGCs are placed on a 10μm filter above either serum-free
935   media conditioned with Kc167 cells as a positive control, serum-free unconditioned media as a
936   negative control, or experimental conditions, such as serum-free media with JH III. The number
937   of PGCs that translocated to the bottom well was quantified visually after 1.5 hours. **D**
938   Quantification of Specific PGC migration, which is the percentage of experimental translocated
939   PGCs relative to positive (serum-free Kc167-conditioned media) and negative (serum-free
940   media) controls. Such normalization is required to compare biological replicates done on
941   different days. See Materials and Methods for further details. Numbers shown in white within
942   each *jhamt* knockdown bar represents the Fold Change (FC) of *jhamt* mRNA at the time when
943   conditioned media was collected. Bar height represents mean and error bars represent standard
944   deviation from two to four biological replicates and statistical significance was tested by
945   unpaired t-test. **E** Quantification of specific migration in transwell assays using isolated PGCs
946   placed above serum-free media containing various concentrations of commercially available JH
947   III (green bars) or 20-hydroxyecdysone (20E, magenta bars) with 1% BSA as a non-specific
948   carrier protein. Bars represent means and error bars represent standard deviation from three
949   biological replicates, which were each obtained by averaging two to three technical replicates. **F**
950   Percentage Specific PGC migration wherein isolated PGCs were placed across either serum-
951   free media conditioned (gray), serum-free media with 1% BSA (black) and either JH III (dark
952   green, 2.5μM) or the JH analogue, methoprene, (light green, 2.5μm) with 1% BSA as a non-
953   specific carrier protein. Bars represent means and error bars represent standard deviation from
954   two biological replicates, which were each obtained by averaging two to three technical
955   replicates. ns=not significant.

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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. deltaCt was calculated relative to the average Ct of *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 and error bars represent mean +/- standard deviation from three biological replicates, each containing pooled mRNA from five females. **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 (*w^{1118}*) males. Bars and whiskers represent mean +/- 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; UASt-Hmgcr* transgenes traps ~65% of PGCs (pink) in the gut (*left schematic*). To determine if Met and Gce is required downstream of Hmgcr/JH, *Met^{1/27}, gce^{Mi/2.5k}* females were crossed *48Y-GAL4; UASt-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) during and 30 minutes prior to and during the same transwell migration assay described in Fig 3c-f. 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 (-)

992   was measured in PGCs subjected to cond media or containing 5µM JH III (green). Bars

993   represent means and error bars represent standard deviation from two biological replicates,

994   which were each obtained by averaging two technical replicates. Bars and whiskers within each

995   graph represent median +/- interquartile range (IQR). Statistical significance was tested by

996   Mann-Whitney U test, with ns=not significant. Dashed white circles outline the gonad and scale

997   bars represent 25µm in all images.

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1000   **Figure 5: Compensatory feedback loop maintains JH homeostasis in *Drosophila*. A** Titers

1001   of two JHs, JH III and JH bisepoxide (JHB$_3$), in hemolymph of third instar *Drosophila* larvae

1002   measured by quantitative mass spectrometry using deuterated standards. Each dot represents

1003   a biological replicate of hemolymph bled from 25 *Met$^{1/1}$, gce$^{Mi/Mi}$* or *w^{1118}* third instar larvae. **B**

1004   Fold change of *jhamt* and *Kr-h1* mRNA in *Met$^{1/27}$, gce$^{Mi/2.5k}$* embryos 12-16 hours post deposition

1005   (hpd) relative to the wildtype strain obtained by crossing *w^{1118}* to OregonR. deltaCt was

1006   calculated relative to the average Ct of two housekeeping genes, *DCTN5-p25* and *Und*. Bars

1007   and error bars represent the average fold change and standard deviation from three biological

1008   replicates.

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1011   **Figure 6: Model of JH function in PGC migration. A** Quantification of specific *in vitro*

1012   migration wherein isolated *Drosophila* PGCs are placed in a transwell migration system across

1013   Kc167-conditioned media (gray), serum-free media (black), JH III (green, 5µM), all trans retinoic

1014   acid (ATRA, dark blue, 5µM) or 9-cis RA (light blue 5µM). **B** Quantification of specific *in vitro*

1015   migration wherein mouse PGCs were FACS-enriched from trunk tissues dissected from E10.5

1016   *Oct4-GFP* embryos and placed across two positive controls: Fetal Bovine Serum (FBS) or

1017   CXCL12 (50 ng/mL) (gray), serum-free media as a negative control (black) or ATRA (dark blue,

1018   1µM) or 9cis-RA (light blue, 1µM). For A and B, bars and error bars represent mean +/-

1019   standard deviation from at least two biological replicates, which were obtained by averaging two

1020   to three technical replicates. **C** Schematic of model for how JHs ensure migrating PGCs (pink)

1021   colonize the somatic gonad (green) in the *Drosophila* embryo.

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Supplemental Data for Figure 1

A Sequence of the JH response element (JHRE) from the *early trypsin* gene of *A. aegypti* that contains the JH transcription factor, Met/Gce binding motif highlighted in bold[23]. The six base pair wildtype JHRE sequence is on top and the JHRE in which the Met/Gce-binding motif was mutated is on the bottom. Eight copies of each sequence were provided by Marek Jindra[15] and placed upstream of *eGFP*. **B** Ventral view of Stage 14 embryos carrying a mutated JH biosensor (*JHRE Mut8x-GFP*) in which the JHRE has been mutated. Embryos were stained for GFP (green) and the hemocyte marker, Fascin (gray). Right panel is the same image but with the Fascin signal alone in grayscale. **C** Lateral view of embryos stained for Vasa (magenta), GFP (green) and DAPI (gray). Genotype is noted above each early Stage 12 and Stage 14 pair. To the right of each whole-embryo image is the area within the white, dashed box magnified. Scale bars represent 25µm in all images.

Supplemental Data for Figure 2

Nature of the small deletions in *jhamt* created by CRISPR-mediated cutting. Seven founders generated fifteen lines which possessed small insertions or deletions at the cut site that created premature stop codons at the beginning of methyl transferase domain of *jhamt* (amino acids 40-137). Class 1 lines (12F1, 12F2) cause a premature stop codon at amino acid 46, while Class II mutations (1F1, 1F2, 4F1, 4F3, 6F2, 6F3, 7F2, 7F3, 8F1, 8F2, 12F3, 14M2, 14M3) cause a premature stop codon at amino acid 54.

Supplemental Data for Figure 3

A *Left:* Quantification of the number of PGCs in Stage 14-16 embryos that remained in the gut upon ectopic expression of either *Hmgcr* or *jhamt* using the *48Y-GAL4* driver. *Right:* Quantification of the number of germ cells in Stage 14-16 embryos that contact Zfh-1-positive cells upon the expression of either *Hmgcr* or *jhamt* using the *twist(twi)-GAL4* and *24B-GAL4* drivers. Each dot represents one embryo. Bars and whiskers represent median +/- IQR and statistical significance was analyzed by the Mann-Whitney U test. **B** Quantification of percent Kc167 cells, used to condition serum-free media, upon *jhamt* mRNA knocked down relative to transfection control. Bars and error bars represent mean +/- standard deviation from two biological replicates, each including at least two technical replicates. **C** Quantification of the fold change in mRNAs in S2 cells upon incubation with varying concentrations of 20-Ecdysone (20-

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1060   E) or JH III for six hours prior to mRNA isolation. mRNAs from two verified ecdysone-responsive

1061   genes were measured: *Eip74b* (lighter purple) and *Eip75* (darker purple) and one JH-responsive

1062   gene was measured, *Kr-h1* (green) and normalized to the housekeeping gene, *DCTN2-p50*.

1063   Error bars represent standard deviation from two biological replicates, each consisting of at

1064   least two technical replicates.

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1067   **Supplemental Data for Figure 4**

1068   Confocal images of *Drosophila* PGCs enriched by FACS and stained for Vasa (magenta), RNA

1069   Pol II, Ser5p (green), RNA Pol II, Ser2p (yellow) and DAPI (gray). Bottom panel shows a germ

1070   cell treated with 1µM Flavopiridol for 3 hours. Scale bars represent 5µm.

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1073   **Supplemental Data for Figure 5**

1074   **A** Fold change of *jhamt* mRNAs in *Met, gce* mutant one-day old adult females relative to

1075   wildtype control animals. deltaCt was calculated relative to the average Ct of *DCTN5-p25* and

1076   *Und*. Shown are Fold Change of *jhamt* mRNA in adults homozygous for the newly recombined

1077   mutant alleles Met^1, gce^{Mi} (dark blue) and heteroallelic combination of Met^1, gce^{Mi} with existing

1078   or Met^{27}, $gce^{2.5k}$ alleles (light blue). Bars and error bars represent mean +/- standard deviation

1079   from three biological replicates, each containing pooled mRNA from five females. **B** Schematic

1080   of the Ecdysone Receptor (EcR), Taiman (Tai) and Met binding sites near the *jhamt* locus.

1081   Binding sites were obtained from ModEncode[63].

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1084   **Supplemental Data for Figure 6**

1085   **A** Scatter plots from FACS of GFP-positive PGCs enriched from trunks of E10.5 mouse

1086   embryos carrying the *Oct4ΔPE:GFP* transgene. The number inside the gate lines denotes the

1087   percentage of collected events in a typical biological replicate. **B** Quantification of the percent

1088   change in the number of mouse germ cells before and two hours after application of 1µm ATRA

1089   or 9cis-RA. Two hours was chosen because this is the duration of the *in vitro* migration assay.

1090   Graphed is the mean and standard deviation from three biological replicated. Each dot

1091   represents the averaged technical replicates of each biological replicate.

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Figure 1
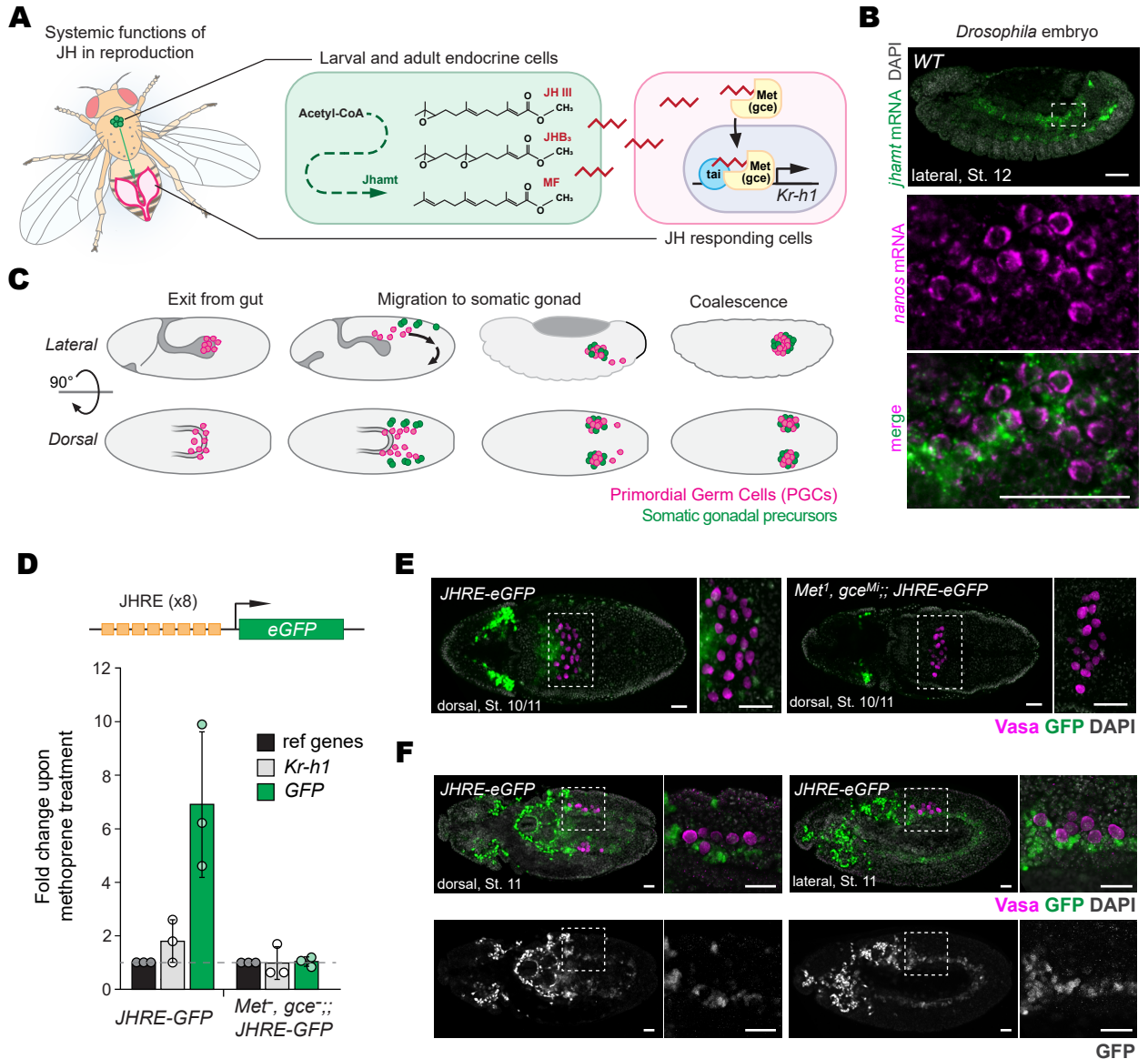# JH signaling is active in the embryonic mesoderm during PGC migration

A Systemic functions of JH in reproduction

B *Drosophila* embryo

C

Primordial Germ Cells (PGCs)
Somatic gonadal precursors

D JHRE (x8)

E JHRE-eGFP

F JHRE-eGFP

Figure 2
Key JH biosynthesis enzyme, Jhamt, facilitates PGC migration to the gonad

A

new loss of function alleles in *jhamt*

S46Stop: 2 lines
V54Stop: 13 lines

jhamt MTase

B

fmol JH III/larva — 0.0013

fmol JHB3/larva — 0.0008

wt, jhamt⁻/⁻

C

Fecundity per day — 0.0002

wt, jhamt⁻/⁻

D

WT | *jhamt⁻/⁻* | *hmgcr⁻/⁻*

dorsal, St. 14+

Vasa DAPI

E

PGCs remaining outside gonad

<0.0001
n.s.
<0.0001
<0.0001
<0.0001
0.0028
<0.0001
<0.0001

wt, jhamt⁻/⁻, jhamtDef/-, jhamtDef/-; tub>jhamt, hmgcr⁻/⁻, jhamt⁺/⁻, jhamt⁺/⁻; hmgcr⁺/⁻

F

PGCs outside of gonad afer ectopic Hmgcr expression

<0.0001

with *Elav>Hmgcr*

wt, jhamt⁺/⁻

Figure 3
Juvenile hormone is sufficient for PGC migration

A

Elav-GAL4

Elav-GAL4,UAS-hmgcr

Elav-GAL4,UAS-jhamt

ventral, St. 14+

Elav Vasa

B

PGCs near Elav-postive cells

elav-GAL4	-	+	-	+	-	+
UASt	-	-	hmgcr		jhamt	

<0.0001
<0.0001
<0.0001

C

PerCPCy5 Log Area / FITC Log Area

R1 0.04%

germ cells (GFP+)

GFP

Germ cells

Conditioned media or JH III

D

% specific PGC migration

0.05
0.005

0.34   0.52   FC

cond. media, uncond. media, lacZ KD, jhamt KD1, jhamt KD2

E

% Specific PGC migration

JHIII   20E

cond. media, uncond. media

0.25 2.5 5.0 25   0.25 2.5 5.0 25 μM

JHIII   20E

F

% specific PGC migration

n.s

cond. media, uncond. media, JH III, methoprene

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

A recombination of existing loss of function alleles in *met, gce*

B

C

D Vasa DAPI

| mat Met, Gce | + | - | - | + |
| zyg Met, Gce | + | - | + | - |

E Testing suppression of ectopic *Hmgcr* in gut

48Y>Hmgcr

± *Met, gce*

Gut   Gonad

M:*wt* x
P:*48Y >Hmgcr*

M:*Met⁻/⁻,gce⁻/⁻* x
P:*48Y >Hmgcr*

lateral, St. 14+

Vasa Hnt DAPI

F *in vitro* migration

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

Figure 6
Model of JH function in PGC migration

A

B

C

Local functions of JH in reproductive development

embryonic mesoderm developing gonad

migrating PGC
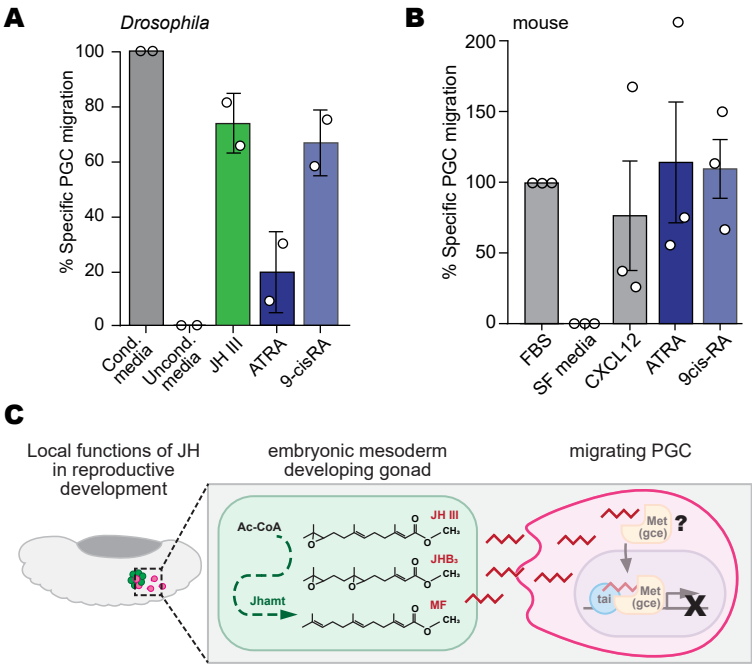
Supplementary Figure 1

A

```
JHRE Wt8x  -  5' ccatcccaCACGCGaagacgataaaacca 3'
JHRE Mut8x -  5' ccatcccaGTGCGCaagacgataaaacca 3'
```

B

ventral, St. 14

JHRE Mut8x-GFP

GFP Fascin
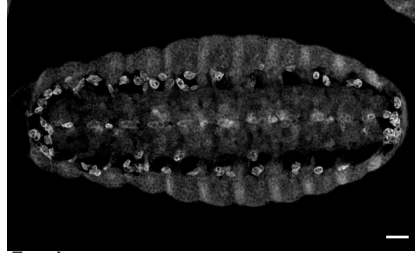
Fascin

C

JHRE WT8x-GFP
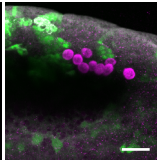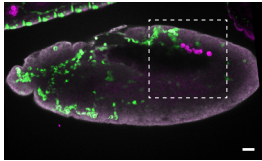
lateral, St. 12   lateral, St. 14
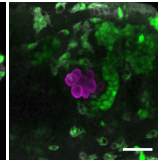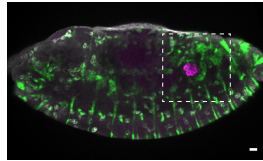
GFP Vasa Fascin

JHRE Mut8x-eGFP
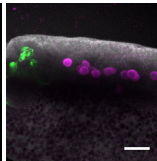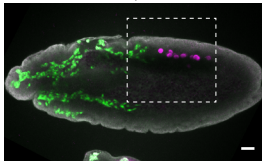
lateral, St. 12   lateral, St. 14
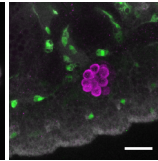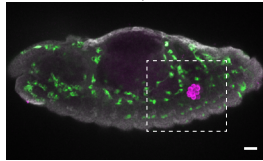
Supplementary Figure 2

Deletions in *jhamt* caused by guide: CCTGGA | TGTGGGTTCAGGATCT

Class I: frameshift leading to premature stop codon at a.a. 46

Lines with 5 bp deletion to generate TC | TGT: 12F1, 12F2
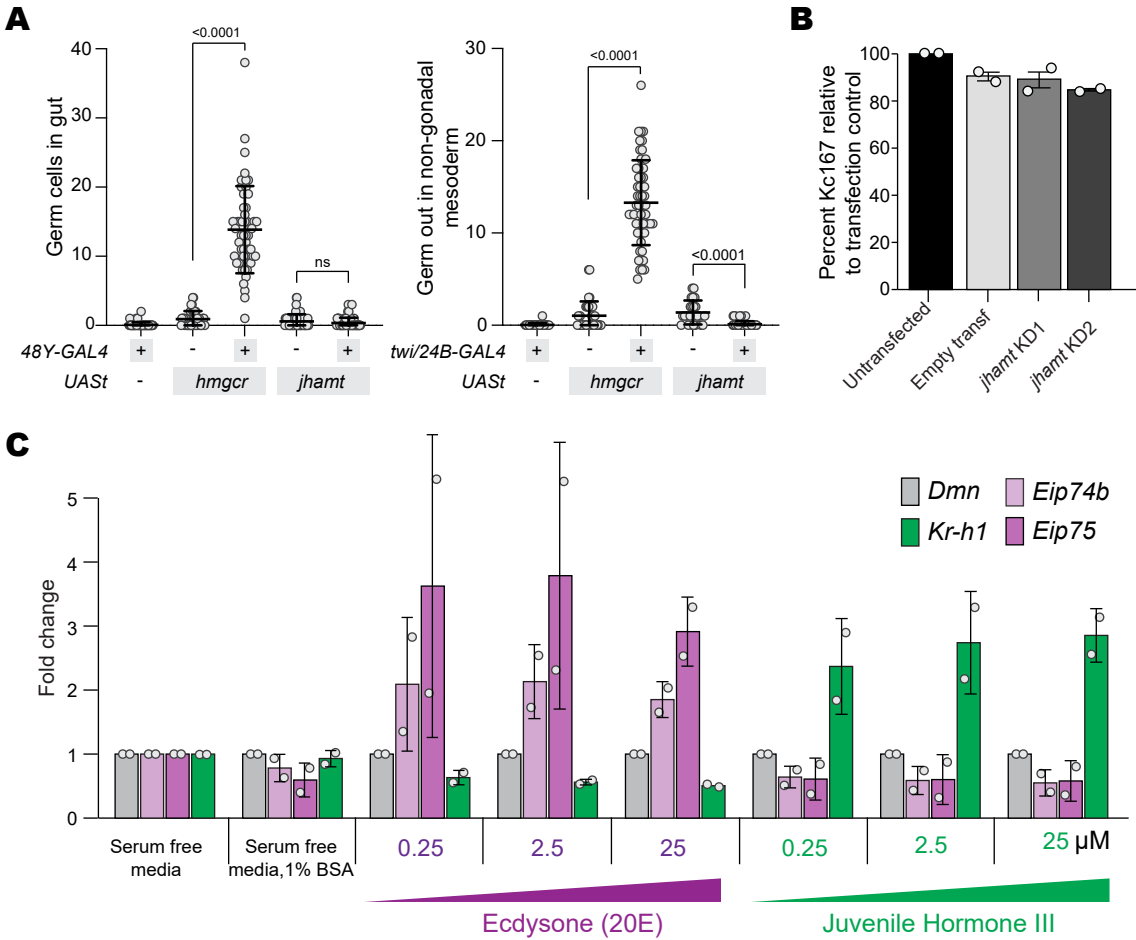
Class II: frameshift leading to premature stop codon at a.a. 54

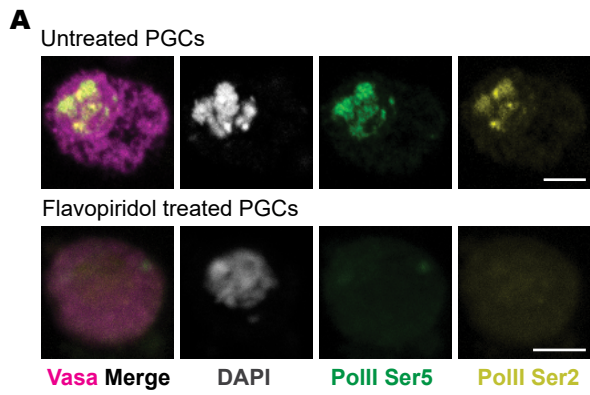Lines with T deletion to generate GGA | GT: 1F1, 1F2, 4F3, 6F2, 6F3, 7F2, 7F3, 8F1, 8F2, 12F3

Lines with A deletion to generate GG | TGT: 14M2, 14M3

Lines with TCC insertion and G deletion to generate GGA | TTCCGT: 4F1
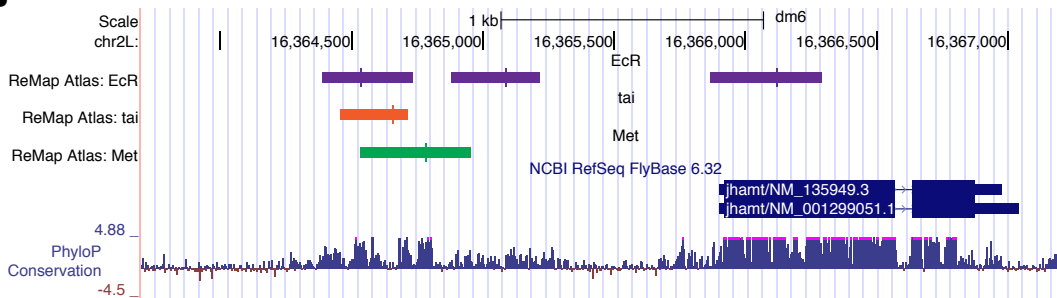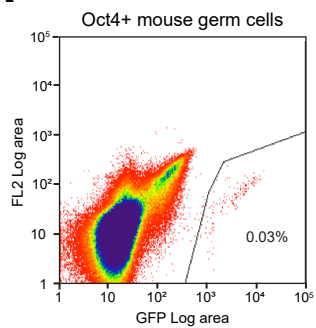
Supplementary Figure 3

Supplementary Figure 4

A

Untreated PGCs

Flavopiridol treated PGCs

Vasa Merge   DAPI   PolII Ser5   PolII Ser2

Supplementary Figure 5

A

B

Supplementary Figure 6

A

Oct4+ mouse germ cells

0.03%

FL2 Log area

GFP Log area

B

% change in mouse PGC number

SF media
ATRA
9cis-RA