The Hedgehog pathway and germ cell migration in Drosophila: Res Ipsa Loquitur

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Abstract

In addition to inducing the non-autonomous specification of cell fate, the *hedgehog* pathway has been shown to function in guiding cell migration in a number of different biological contexts including the nervous system and PGCs (primordial germ cells). While a role in axon guidance in the vertebrate nervous system is now widely recognized, a function in guiding the migratory path of PGCs from the outside surface of the *Drosophila* embryo, through the midgut and mesoderm to the SGPs (somatic gonadal precursors) has been deemed "controversial" since it was first proposed by Deshpande et al., 2001. In particular, it has been suggested that experiments documenting a function for this signaling pathway in guiding PGC migration in *Drosophila* cannot be reproduced. Here we present the conflicting data sets side-by-side from four different laboratories that have studied a possible role for *hh* in guiding PGC migration. We also present new experiments demonstrating a) that *hh* ligands produced by mesodermal cells function to guide PGC migration, b) that a key factor potentiating guidance signals emanating from the SGCs, *hmgcr*, functions upstream not only of *hh* but also of two *hh* pathway genes important for the formation of Hh containing cytonemes, and c) that factors required in *hh* receiving cells function in PGCs to help direct migration towards the SGPs.

Introduction

The *hedgehog* (*hh*) signaling plays a central role in the development and differentiation of multicellular animals. The pathway was first discovered in *Drosophila* where it was shown to function in the establishment of parasegmental polarity during embryogenesis (Ingham and McMahon, 2001; Beachy et al., 2010; Briscoe and Therond, 2013; Ingham 2022). Subsequent studies showed that it is required throughout much of development for pattern formation in among other contexts the larval discs, the CNS and stem cell niches. While flies have only a single gene encoding the Hedgehog (Hh) ligand, there are three homologs in mammals, Sonic Hedgehog, Desert Hedgehog and Indian Hedgehog (Echelard et al., 1993). As is the case in flies, the *hh* pathway in mammals is required for the proper patterning of a wide range of tissues and organs including the neural tube, cerebellum, eyes, limbs, muscles, and the gut. In both flies and mammals, signaling from *hh* expressing cells functions to induce cell fate specification in neighboring *hh* receiving cells. In the canonical pathway, reception of the Hh ligand by the *patched* (*ptc*) receptor results in the relocalization of *smoothen* (*smo*), to the cell membrane in flies and to the cilia in mammals. In flies, downstream kinases, *protein kinase A* (*pka*), *glycogen synthase kinase 3* (*GSK3*) and *casein kinase 1* (*ck1*) and *fused* (*fus*) control the activity of the fly Cubitus interruptus (Ci) transcription factor by regulating its proteolysis (Johnson et al., 1995; Price and Kalderon, 2002; Semelkinson and Kalderon, 2006). In the absence of *hh* signals, *pka*, *GSK3* and *ck1* promote the proteolytic cleavage of Ci to generate a protein that represses *hh* target genes, while in the presence of *hh* signals *fu* phosphorylation stabilizes the full-length Ci protein, which activates *hh* target genes. In mammals, there are two *hh* transcription factors that function as activators, *Gli1*, *Gli2* while a third, *Gli3* acts as a repressor (Ingham, 2020).

While the canonical *hh* signaling pathway is deployed in fate specification and morphogenesis, transcriptional regulation is not the only output of this signaling pathway. Two publications in 2001 implicated the *hh* signaling pathway in cell migration. One of these was a paper by Trousse et al. (2001) who reported that ectopic Shh suppresses neurite outgrowth from the chick retina. Based on this finding the authors suggested that this inhibition might help direct retinal ganglion cell migration. Subsequent studies on the vertebrate nervous system identified other contexts in which Shh has functions in guiding migration. However, instead of only repelling migrating neurons, Shh was found to act in conjunction with netrin to attract commissural axons to migrate towards the ventral midline (Charron et al., 2003; Yam and Charron, 2013). Unlike cell fate determination, neuronal guidance does not appear to depend directly on transcriptional regulation of *Shh* target genes. In the retinal ganglion, where Shh functions as a repellant, activation of protein kinase Cα (PKCα) and Integrin-linked kinase (ILK) induces growth cone collapse. In the case of the commissural axons, a non-canonical pathway mediated by Smo dependent phosphorylation of a Src family kinase helps remodel the cytoskeleton in the axon growth cones so that they extend towards the source of the Shh ligand (Yam et al., 2009). Once the commissural axon growth cones cross the midline, Shh begins to function as a repellant. The heparin proteoglycan Glypican1 (GPC1) mediates this switch by inducing a mammalian specific protein, Hhip (Hedgehog interacting protein).

Unlike the canonical pathway, reception of the Shh signal by the neurons does not appear to involve cilia. Reception does, however, depend upon Ptc and a conserved Shh co-receptor BOC (Brother of Cdon). Two proteins related to BOC and Cdon (Cell adhesion molecule-related/downregulated by oncogenes), Boi (Brother of Ihog) and Ihog (Interference hh), respectively, are found in flies. Though the fly and mammalian proteins share a similar set of repeated sequences (immunoglobulin and fibronectin type III repeats), they appear to interact with Hh/Shh by different mechanisms. Hh binding by the fly proteins depends upon heparin

sulfate modifications, while Shh binding by the mammalian proteins depends upon Ca^{++}. Studies in flies indicate that Ihog and Boi, together with Dispatched (Disp) and two heparin and glycosaminoglycan modified proteins, Dally and Dlp (Dally-like) play critical roles in the cytoneme dependent transmission and reception of the Hh ligand (Ramirez-Weber and Kornberg, 1999; Kornberg and Roy, 2014; Bischoff et al., 2013; Bodeen et al., 2017; Chen et al 2017; Gradilla et al 2014; Sanders et al., 2013; Simon et al., 2021) Cytonemes are thin actin based cellular protrusions that can extend as much as 300 uM from Hh sending or receiving cell (Kornberg, 2014). While reception of Shh by migrating axons requires at least one cytoneme component, BOC, the requirements for Shh transmission from its source in the neural tube has not been directly examined. However, studies by Hall et al., (2021) have implicated a Disp/Boc/Cdon co-receptor complex in generating cytonemes from Shh producing cells in vertebrates.

In the other 2001 publication, we reported that the *hh* pathway helps guide the migration of primordial germ cells (PGCs) towards the somatic components of the embryonic gonad, the SGPs in *Drosophila* (Deshpande et al., 2001). However, instead of functioning to repel migrating cells, we showed that the Hh ligand functions as an attractant, directing the migrating PGCs towards the source of the Hh signal, the SPGs. The primitive embryonic gonad in flies is composed of the PGCs and the somatic gonadal precursor cells (SGPs). SGPs are mesodermal in origin and arise in two bilateral clusters in parasegments (PS) 10-13. They are specified during mid-embryogenesis under the control of zygotic patterning genes as well as inductive signaling from the dorsal ectoderm (Boyle and DiNardo, 1995; Boyle et al., 1997). In contrast, the PGCs arise by precocious cellularization of nuclei which enter the posterior pole of the embryo during nuclear cycle 9/10. When the blastoderm cellularizes at the end of nuclear cycle 14, the PGCs lie on the outside surface of the embryo at the posterior pole. In order to form the embryonic gonad, the PGCs must undergo a series of migratory steps. PGC migration begins at the onset of gastrulation, when germ cells move away from the posterior pole along the dorsal surface of the embryo. The PGCs are carried into the embryo by the midgut invagination. The PGCs then move through the midgut to the posterior end and cluster on the dorsal side of the midgut. During stage 9/10 the PGC migrate through the midgut epithelium and enter the overlaying mesoderm. The PGCs then split into two clusters and migrate laterally through the mesoderm towards the SGPs. During stage 12 the PGCs align themselves with the SGPs, which are spread out in PS10-13 on both sides of the embryo. After the PGCs align with SGPs, the two cell types coalesce together to form the primitive embryonic gonad at stage 14.

In Deshpande et al., we presented several lines of evidence supporting the idea that the Hh ligand produced by SGPs acts as an attractant and that reception of Hh by the PGCs results in their directed migration towards the SGPs. First, beginning as early as stage 10, which is before the SGPs are thought to be specified, Hh is expressed in a segmentally repeating pattern in the mesoderm that partially overlaps the expression of Eyes absent (Eya or Clift) (Boyle et al., 1997). Eya expression in the mesoderm in PS10-13 subsequently becomes restricted to the SGPs (mSGPs), and these same cells express Hh. Second, ectopic expression of Hh from a *UAS-hh* transgene using mesodermal and ectodermal *Gal4* drivers induces PGC mismigration. Third, partially compromising genes required for receiving Hh signals (*ptc*, *smo*, *fu* and *pka*) in the PGCs by mating germline clone mutant mothers to wild type fathers perturbs PGC migration.

While a role for Shh in guiding neuronal migration in mammals is now widely accepted, the notion that the Hh signaling pathway might orchestrate PGC migration towards the SGPs has been controversial for two decades (cf., Santos and Lehmann, 2004a; Kunwar et al., 2006; Renault et al., 2009; Barton et al., 2016; Kenwick et al., 2019). The “controversy” does not stem

from differences in how the results reported in Deshpande et al. (2001) might be interpreted. Rather, it is claimed that the results in Deshpande et al (2001) and in subsequent publications from our lab cannot be reproduced. This claim is summarized by Barton et al (2016):

> *"Some studies suggest that Hedgehog is the hmgcr dependent germ cell attractant (39{Deshpande et al., 2013}, 71 {Deshpande and Schedl, 2004}; however intensive study of Hedgehog lipid modifications has not revealed prenylation (72 {Eaton, 2008}). Furthermore, the attracting capabilities of ectopic hedgehog expression has not proven reproducible and using several methods to alter Hedgehog signaling within germ cells failed to compromise migration (73 {Renault et al., 2009}).*"

Not surprisingly, claims that our findings are not reproducible have had a dampening effect on our research. For example, a 2014 NIH Summary Statement noted this problem:

> Significance (7): Weaknesses. *There has been significant controversy regarding the role of hedgehog in Drosophila germ cell migration, with different (experienced) labs supporting different v*iews.

Here we address this two-decade old "controversy" by comparing the conflicting data sets from four different laboratories that have investigated a connection between the *hh* signaling pathway and PGC migration. We also provide several new lines of evidence showing that most of the key genes implicated in signaling to the migrating PGCs by the mesodermal SGPs and in the response of the PGCs to these signals are components of the *hh* pathway and have important functions in either *hh* sending or receiving cells.

Results

Ectopic hh expression induces PGC mismigration

The "controversy" officially began some five months after Deshpande et al (2001) was published with the submission in Feb. 2002 of a "Commentary" to *Cell* by the Lehmann laboratory titled "*Hh does not guide migrating germ cells*." In this "Commentary" manuscript, the authors reported that they could not repeat any of the findings we presented in Deshpande et al. (Appendix 1). After having the manuscript reviewed by experts in the field, the editor of *Cell* sent a copy to us and asked whether we wished to withdraw our 2001 publication. We indicated that before making any decision, we would repeat our experiments to determine if we could reproduce them.

Of all of the findings in our paper that were questioned in the "Commentary," the most damning were our experiments showing that ectopically expressed *hh* perturbs PGC migration. According to the "Commentary" ectopically expressed *hh* had no discernable effect on PGC migration whatsoever (Fig. 1A: see Fig.2 in Appendix 1). For these ectopic expression experiments the "Commentary" authors used several of the stocks that we provided. Consequently, this discrepancy could not be explained by any sort of run of the mill experimental error on our part. For this reason, we also indicated to the *Cell* editor that we had already asked an independent scientist, Dr. DiNardo, to repeat this particular set of experiments (see explanation in Appendix 3). The editor agreed with this plan; however, we were told not to communicate further with Dr. DiNardo. Dr. DiNardo sent a final report of his findings to *Cell* in March and the editor forward a copy of his report to us (Appendix 2). After receiving the DiNardo report, we submitted a report describing the results of the experiments from Deshpande et al that we had repeated (Appendix 3).

Shown in Fig. 1B are the results of Dr. DiNardo's 2002 experiments (Appendix 2), while Fig. 1C shows the results from the experiments we repeated in 2002 and submitted to *Cell* (Appendix 3). Fig. 1D is an experiment done in 2022, while Sup Fig 1A shows results from experiments done in Deshpande et

al., (2013). In our 2002 experiments (Fig.1C), only half of the embryos have both the *Gal4* driver and *UAS-hh*, while a mixed population of homozygous and heterozygous *Gal4-Hairy* were used in Dr. DiNardo's experiments. Thus, in these cases the effects, if any, of ectopic *hh* will be underestimated.

As is evident from inspection of the different panels in Fig.1 and Sup Fig. 1A the outlier in all of these experiments are not our results. Instead, the results that were not reproducible were those reported in the 2002 "Commentary" manuscript which purported to show that *hh* misexpression has no effect on PGC migration. All of the other experiments done by us or by Dr. DiNardo in 2002 gave similar results: *hh* misexpression perturbs PGC migration. Likewise, when these experiments were repeated in Deshpande et al (2013) as illustrated in Sup Fig. 1A or in 2022 as indicated in Fig. 1E, ectopic *hh* expression was again found to induce PGC migration defects. The extent of the effects on migration vary between drivers and *UAS-hh* transgenes. They are also not as severe as those observed when *hmgcr* ((3-hydroxy-3-methylglutaryl-CoA reductase: Van Doren et al., 1997; see Sup Fig. 1A and below) is misexpressed. Nevertheless, PGC migration defects were observed in all of the combinations between the *UAS-hh* transgenes and the *Gal4* driver that were tested. While the "Commentary" manuscript was not published in *Cell* in 2002, the results in this manuscript, including the *hh* misexpression experiments shown in Fig. 1A were published *as is* seven years later in *Developmental Biology* by Renault et al. (2009). The results presented in the "Commentary" manuscript can be found in Fig. 2 of Renault et al.

We and Dr. DiNardo are not the only researchers who have found that ectopic *hh* impacts PGC migration. Further evidence that *hh* misexpression induces PGC mismigration comes from a recent publication by Kim et al. (2021). They showed that embryos heterozygous for a gain-of-function mutation in one of the *hh* enhancers, hh^{Mrt}, exhibit a significant frequency of PGC migration defects (~50% >6 mismigrated PGCs). Their results are presented in Sup Fig. 1B.

PGC migration defects are induced by knocking down hh expression in the mesoderm

Mutations in *hh* have widespread effects on embryonic development and are known to perturb PGC migration due defects in the specification of the SGPs (Moore et al., 1998a; Moore et al., 1998b). The available evidence indicates that SGP specification requires *hh* signaling from the overlying ectoderm to the mesoderm in PS10-13. Consistent with this idea, Sup Fig. 2 shows that RNAi knockdown of *hh* using a *UAS-hhRNAi*^{1} transgene and a mesodermal *twist-Gal4* driver does not appear to impact SGP specification as evident by the WT pattern of expression of the SGP marker Eyes Absent (Eya).

While SGP specification appears to be normal, we find that PGC migration defects are induced by RNAi knockdown of *hh* expression in the mesoderm using a *twist-Gal4* driver. Shown in Fig. 2A-F are stage 10-11 embryos in which *UAS* transgenes expressing RNAi directed against either *egfp* or *hh* were expressed using a *twist-Gal4* driver. The stage 10 *egfp* RNAi embryo resembles WT (Fig. 2A). At this point the PGCs have traversed the midgut epithelium and are beginning to migrate along the outside ventral surface of the midgut. In the stage 10 *hh* RNAi knockdown embryos, the PGCs have also exited the midgut; however, unlike WT, several have traversed the midgut on the dorsal rather than the ventral side and appear to be migrating towards the ventral CNS (arrows Fig. 2B & C). In the stage 11 *egfp* RNAi embryo (Fig. 3D), the PGCs have begun to migrate into the mesoderm towards the SGPs. Unlike WT, a subset of PGCs in the *hh* RNAi embryos remain associated with the midgut, while other PGCs have begun migrating in a ventral direction away from the overlying mesoderm (arrows Fig. 2F). We also quantitated the number of mismigrated PGCs in stage 13-15 *twist-Gal4 X UAS-hhRNAi* embryos (Sup Fig. 3) and these results are shown for three different *UAS-hhRNAi* lines in Fig. 2G. In all three cases there is an increase in the number of mismigrated or lost PGCs compared to the *UAS-egfpRNAi* control.

PGC migration defects induced by hmgcr expression in CNS are suppressed by knocking down hh, disp and ttv

i) *hmgcr, prenylation and hh:* Amongst the genes implicated in PGC migration thus far, *hmgcr* has the most profound effect on migration when ectopically expressed. Likewise, there are substantial PGC migration defects in *hmgcr* mutants. Consistent with a key role in generating the PGC attractant the *hmgcr* gene exhibits a dynamic transcription pattern during embryogenesis that parallels the steps in migration of the PGCs. It is initially broadly expressed in the mesoderm and then becomes progressively restricted to cells in the PS10-13 that correspond to the SGPs. This pattern of expression is consistent with it having a key role in guiding PGC migration.

In mammals, *hmgcr* is the rate limiting enzyme for the biosynthesis of sterols including cholesterol and for the farnesyl/geranylgeranyl prenylation of membrane associated polypeptides. Flies do not encode the enzymes downstream of *hmgcr* that are needed for cholesterol biosynthesis (Santos and Lehmann, 2004b), but do have genes encoding the enzymes required to synthesize farnesyl-PP and geranylgeranyl-PP. For this reason, flies depend upon dietary cholesterol.

Santos and Lehmann (2004b) showed that the enzymes involved in the biosynthesis of gernylgeranyl-PP (Quemao: Qm) functions downstream of *hmgcr* in orchestrating PGC migration. Based on this observation it was suggested that the PGC attractant is a prenylated polypeptide potentially similar to the yeast mating pheromone a-factor (Santos and Lehmann, 2004a; Ricardo et al., 2009; Barton et al., 2016). Consistent with this idea, they found that the Geranylgeranyl Transferase type 1 (GTT1) which prenylates polypeptides and proteins is also required for proper PGC migration. This prenylated polypeptide/protein would itself need to be broadly expressed in the endoderm and ectoderm since ectopic expression of *hmgcr* with many different *Gal4* drives induces PGC migration defects. However, according to this model, specificity for a signal coming from the SGPs would be generated because the attractant would only be functional when prenylated by a pathway whose activity depends upon high levels of *hmgcr* (Ricardo and Lehmann, 2009; Barton et al., 2016).

A similar problem exists for a model in which *hh* functions as a PGC attractant. *hh* expression in the mesoderm is not restricted to cells (SGPs) in PS10-13; it is also expressed in a repeating pattern in mesodermal cells anterior to PS10, that most likely correspond to fat body precursor cells (Deshpande et al., 2001). In the ectoderm it functions in segment polarity and is expressed in a two-cell wide stripe in each parasegment. Thus, a critical question is what, if anything, distinguishes *hh* signals from the SGP from *hh* expressed elsewhere in the embryo. One of several factors turns out to be *hmgcr*. We found that *hmgcr* is required for the release and/or transmission of the Hh ligand from *hh* expressing cells (Deshpande and Schedl, 2004). In *hmgcr* mutant embryos, Hh is inappropriately retained in *hh* expressing cells in the ectoderm, instead of spreading to neighboring *hh* receiving cells. We also found that embryos *trans*-heterozygous for mutations in *hmgcr* and either *hh* or *disp* displayed PGC migration defects not evident in single heterozygous mutants.

While it is known that the autoprocessing of the Hh ligand in *hh* expressing cells requires cholesterol, and that cholesterol plays a critical role in *hh* signaling (Ingham, 2022), this could not explain why *hmgcr* functions in PGCs migration since flies do not synthesize cholesterol *de novo*. Likewise, though the *hh* ligand has lipid modifications, it is not prenylated (Eaton 2008). However, we found that like *hmgcr*, *qm* which is required for the biosynthesis of gernylgeranyl-PP is also required for the efficient release and/or transmission of the *hh* ligand (Deshpande et al., 2009). We also identified a protein, the Gγ1 subunit of the heterotrimeric G protein complex, that not only requires geranylation for its activity (membrane association) but is also needed for

the efficient release and/or transmission of the *hh* ligand just like *hmgcr* (Deshpande et al., 2009). Consistent with a role in PGC migration, embryos homozygous for a *gγ1* mutation exhibited PGC migration defects, while ectopic expression using a *hh-Gal4* driver induced PGC migration defects. PGC migration defects are also observed in embryos *trans*-heterozygous for *gγ1* and either *hmgcr* or *hh* mutations, while no defects are evident in single heterozygous mutants (Deshpande et al., 2009).

ii) *hmgcr knockdown in the mesoderm induces unusual PGC migration defects:* As expected from its dynamic expression pattern in the mesoderm during mid-embryogenesis, PGC defects are observed when *hmgcr* is knockdown in the mesoderm using a *twist-Gal4* driver to express a *UAS-hmgcr* RNAi transgene. The PGC migration defects in the knockdown are quantitated in Fig. 2G, while Fig. 3 C and D shows examples of an unusual mismigration phenotypes in which PGCs are found on the outside surface of stage 15 embryos. This phenotype is similar to a phenotype reported by Kim et al. (2021) when they used RNAi to knockdown *smo*. In their experiments, they also examined early stage 9/10 embryos and observed PGCs that failed to enter the midgut during the midgut invagination and instead remained on the surface of the embryo (see below). Based on these observations, they suggested that instead of being passively internalized by the midgut invagination, PGCs might actually be migrating in response to *hmgcr* dependent *hh* signals emanating from mesodermal cells in stage 9/10 embryos.

iii) *The hh pathway is epistatic to hmgcr in PGC migration;* It has been argued that whatever role *hmgcr* might have in promoting the release and/or transmission of the Hh ligand, this function is not relevant to its role in the synthesis of the prenylated PGC attractant (Barton et al., 2016). If this model is correct, then the PGC migration defects induced by ectopic *hmgcr* expression should not depend on either *hh* or on genes important for the release and/or transmission of the *hh* ligand. To test these predictions, we mated homozygous *elav-Gal4 UAS-hmgcr* females to males carrying three different *UAS-hh* RNAi transgenes. Fig. 4 shows that the PGC migration defects induced by expressing *hmgcr* in the CNS/PNS are suppressed when *hh* RNAi is co-expressed in the same cells (compare Fig. 4A with B and C). Only about 20% of the *elav-Gal4 UAS-hmgcr*/+ embryos have 3 or fewer lost PGCs (Fig. 5E). In contrast, in *elav-Gal4 UAS-hmgcr* embryos that also have a copy of one of the *UAS-hh* RNAi transgenes, about 40% of the embryos have 3 or fewer lost PGCs. Conversely, the number of embryos with 10 or more lost PGCs in *elav-Gal4 UAS-hmgcr/+* embryos is reduced about two-fold when the embryos also carry a copy of one of the *UAS-hh* RNAi transgenes. (These findings are also inconsistent with a recent report from Kenwrick et al., 2019: see legend Fig. 4).

We also tested two genes that function downstream of *hh* and are required for the efficient transmission of the Hh ligand, *disp* and *toutvelu* (*ttv*). Disp has a sterol sensing domain like Ptc and Npc1 (Nieemann-Pick disease protein 1) and is a member of the RND (Resistance-Nodulation-Division) family of membrane transporters (Kuwabara and Labouesse (2002). Disp binds Hh and is required for the cytoneme dependent transmission of Hh from the sending cells to the Hh receiving cells (Ingham, 2022; Cannac et al., 2021; Hall et al., 2021). *ttv* encodes a Glucuronosyltransferase and together with Sister-of-toutvelu (Sotv) it is responsible for the addition of GlcA and GcNAC to nascent heparin sulfate chains. Two heparin sulfate proteoglycans Dally and Dally-like are required for the cytoneme dependent transmission of Hh, and their activity depends upon *ttv* and *sotv*. As was observed when *UAS-hh* RNAi transgenes are co-expressed with *UAS-hmgcr* in the CNS, RNAi knockdown of either *disp* or *ttv* suppresses the PGC migration defects induced by ectopic *hmgcr*. This is shown for *disp* and *ttv* in panels C and D of Fig. 5, while the number of lost PGCs is quantitated in Fig. 4E. Taken together these

findings indicate that *hh* and two factors required for the cytoneme dependent transmission of the Hh ligand act downstream of *hmgcr* in PGC migration.

The ABC transporter Mdr49

The yeast mating type pheromone, the a-mating factor, is a prenylated polypeptide that depends upon an ABC transporter, Ste6, for its release from a-factor yeast cells. Studies by Ricardo and Lehmann (2009) identified a fly ABC transporter, *Mdr49*, that unlike other ABC transporters tested, is required for PGC migration. Based on sequence similarity to Ste6, the authors suggested that Mdr49 is responsible for the release of an unknown prenylated polypeptide from the SGPs that functions to guide PGC migration. However, Mdr49 only shows a ~27% identity to Ste6, while it is more closely related to the mouse proteins ABCB4 (~45%) and ABCB1/MDR1 (~42%). ABC4 is reported to be phosphatidylcholine transporter, while ABCB1 has been implicated in the transport of cholesterol and vitamin A among other compounds (Kimura et al., 2007; Liu, 2019).

Since Mdr49 shows significant homology to a mammalian cholesterol ABC transporter and flies are unable to synthesize cholesterol, a plausible alternative model is that Mdr49 is needed for the transport of cholesterol. Moreover, as cholesterol is required for the autoprocessing of the Hh ligand, a role in cholesterol transport could explain why there are PGC migration defects in *mdr49* mutants. Consistent with this possibility, we found that the PGC migration defects in *mdr49* could be partially rescued by feeding mothers a high cholesterol diet (Deshpande et al., 2016). We also observed PGC migration defects in embryos *trans*-heterozygous for *mdr49* and *gγ1* mutations. Further supporting the idea that cholesterol transport is important for PGC migration, we found that there are PGC migration defects in embryos mutant for a known cholesterol transporter, *npc1* (Bialistoky et al., 2019).

mdr49 is reported to have a distinctive pattern of expression (Flybase Mdr49: FBgn0004512). It is not expressed in ovaries and *mdr49* mRNAs are not maternally deposited in the egg (Sup Fig. 4). *mdr49* mRNAs are first detected at the blastoderm stage. At this stage it is expressed along the entire anterior-posterior axis in cells destined to give rise to the endoderm and mesoderm (Sup Fig. 4). After invagination, expression is largely limited to the mesoderm and as the embryo develops it becomes progressively restricted to mesodermal cells located in the head of the embryo. By stage 9-10 it is no longer expressed. However, it is not reported to be expressed in the ectoderm, CNS or PNS (Flybase Mdr49: FBgn0004512).

Since *mdr49* mRNA is not detected in the nervous system and is not maternally deposited, it was surprising that Ricardo and Lehmann (2009) found that the PGC migration defects induced by expression of *hmgcr* in the CNS/PNS using an *elav-Gal4* driver could be suppressed by heterozygosity for *mdr49* mutations. Their model postulates that *mdr48* has a *cell autonomous* function and is needed for the release of an *hmgcr* dependent prenylated PGC attractant from the SGPs in the mesoderm. However, if *mdr49* is not expressed in the cells in the nervous system in which the *elav-Gal4* driver is active, then it cannot be functioning in these cells to help promote the release of a prenylated PGC attractant. In this case, reducing the *mdr49* gene dose would not be expected to suppress the *elav-Gal4 UAS-hmgcr* induced PGC migration defects. Instead, one would expect that a reduction in *mdr49* gene dose would diminish the guidance signal produced by the SGPs. In this case, it should, if anything, enhance the migration defects induced by *elav-Gal4 UAS-hmgcr*. The only explanation for their result is that *mdr49* has a *non-autonomous* function in the mesoderm that is somehow able to *potentiate* the action of an *hmgcr* induced

PGC attractant produced by *elav* expressing CNS/PNS cells. In this case, mesodermal potentiation would be partially impaired in heterozygous *mdr49* embryos, and this would reduce the ability of CNS/PNS cells expressing *hmgcr* to disrupt PGC migration.

As for a role in cholesterol transport, a reduction in *mdr49* gene dose could either enhance or suppress the PGC migration defects induced by *elav* driven expression of *hmgcr* in the nervous system. To test which of these possibilities is correct, we mated *mdr49* males to females carrying the *elav-Gal4* and *UAS-hmgcr* transgenes. The results of this analysis are presented in Fig. 6. In the control, *elav-Gal4, UAS-hmgcr/+*, about 20% of the embryos have more than 13 lost PGCs, while about 15% have 7-8 lost PGCs. When *elav-Gal4, UAS-hmgcr* is *trans* to the P-element excision allele (Ricardo and Lehmann, 2009), $mdr49^{\Delta 3.16}$, there is a general shift towards a more severe phenotype: nearly 40% of the embryos have 13 or more lost PGCs, while about 25% of the embryos have 7-8 lost PGCs. An even greater shift in the severity of the phenotype is observed when *elav-Gal4, UAS-hmgcr* is *trans* to a deficiency that uncovers *mdr49*,or when there is a second copy of the *UAS-hmgcr* transgene. These results would fit with a model in which reducing *mdr49* activity in the mesoderm has a greater effect on the guidance molecule (either a prenylated polypeptide or Hh) produced by the SGPs than it does on the competing guidance molecule generated by ectopic *hmgcr* expression in the nervous system.

Reception of the guidance molecule hh by the migrating PGCs

i) The GPCR superfamily protein Smo is required in PGCs: In Deshpande et al., 2001, we used germline clone females mated to WT males to investigate a possible role for *smo* and *ptc* in PGCs to direct their migration. In these experiments we took advantage of the fact that transcription is, with a few notable exceptions, turned off in PGCs when they precociously cellularize in pre-cellular blastoderm embryos and is not turned on until later in development as they begin to migrate. As a consequence, the PGCs in progeny of germline clone mothers will lack the corresponding gene product until transcription resumes. For smo^{m-z+}, we found that PGCs failed to establish normal contacts with the SGPs in stage 12-13 embryos; however, by stage 15/16 these defects disappeared and the number of lost PGCs was not much different from WT. We assumed at the time that the late rescue took place because zygotic expression of *smo* in the PGCs had been activated and a sufficient amount of the protein was produced to direct PGC migration. A similar experiment was done in the 2001 "Commentary" (and reappeared with the same data in Renault et al., 2009). As we found, they reported that there were no defects in stage 15 embryos; however, it is not clear from the text of either the "Commentary" or Renault et al., (2009) whether the authors examined earlier stages and detected defects similar to those we observed.

Further evidence that *smo* is required for proper PGC migration was reported by Kim et al., (2021). They used *mat-Gal4* to drive expression of two different *UAS-smo* RNAi transgenes in the mother. In their experiments, they detected PGC migration defects with both RNAi transgenes in stage 9/10 embryos, when the PGCs are still in the midgut, and unlike our experiments these defects persisted though stage 15/16 (Sup Fig. 5). They also observed PGCs that remained on the surface of the embryo instead of being internalized during the midgut invagination. Without the *mat-Gal4* driver, background levels of lost PGCs were observed in the progeny of the two *UAS-smo* RNAi transgenes. In contrast, in the progeny of mothers carrying both the driver and the RNAi transgene, more than 60% of stage 15/16 embryos had 6 or more lost PGCs (Sup Fig. 5).

One potential problem with the experiments in Kim et al. is that the siRNAs synthesized during oogenesis might be deposited in the egg and still be active in the zygote. In this case, the PGC migration defects might arise, at least in part, from the loss of Smo in somatic tissues, and not in the PGCs. To address this issue, we used *nos-Gal4* to drive expression of a *UAS-smo* RNAi transgene in PGCs. As shown in Fig. 7A-D, RNAi knockdown of *smo* in PGCs induces PGC migration defects and about 25% of the embryos have 5 or more lost PGCs. While the effects are not as strong as those observed when *smo* is knocked down in the mother, they are completely consistent with the findings reported in Kim et al., (2021). Moreover, as was reported by Kim et al. (2021) we observed embryos in which PGCs are found on the outside surface of the embryo (Sup Fig. 6).

ii) The Hh receptor ptc is required for PGC migration: According to the "Commentary" (Appendix 1: see also Renault et al., 2009) *ptc* germline clones were female sterile and the *ptc* germline clone experiments we reported in Deshpande et al. (2001) could not be reproduced. However, the authors of "Commentary" were able to test the role of *ptc* by expressing a dominant negative protein $ptc^{\Delta loop2}$ in PGCs using the *nos-Gal4* driver. Fig. 5 in "Commentary" (see also Fig. 3 in Renault et al 2009) shows the results of this experiment. As illustrated in Sup Fig. 7, a total of 14 embryos were examined. Of these, 12 had 0-2 lost PGCs, while 2 had 3- 4 lost PGCs. According to Renault et al (2009):

> "*...none of the components of the Hh signaling pathway that we tested had an effect on germ cell migration or survival. This is in contrast to findings in Deshpande et al (2007) who found that germ cell expression of* $ptc^{\Delta loop2}$ *did lead to germ cell migration defects in a portion of the embryos.*"

While we did not test the effects of $ptc^{\Delta loop2}$ expression in Deshpande et al (2001), we included an experiment in a 2007 publication using the same *UAS-*$ptc^{\Delta loop2}$ and *nos-Gal4* driver as was used in the "Commentary" manuscript (and in Renault et al., 2009). In our experiment we examined 222 embryos of which 122 were identified as male and 100 were identified as female (Sup Fig. 7). The reason for sexing the embryos is that the *UAS-*$ptc^{\Delta loop2}$ is on the X chromosome and in our experiment and in the experiment described in the "Commentary", males carrying the *UAS* transgene were mated to females carrying the *nos-Gal4* driver. Consequently, only the female embryos will have both the *UAS* transgene and the *Gal4* driver. We found that about 30% of the female embryos had 5 or more lost PGCs, while this was true for only 8% of the control male embryos. Note that the sex of the 14 embryos examined in the "Commentary" (and in Renault et al., 2009) was not determined; however, all 14 were counted in the graph as if they had both transgenes (see Appendix 1 Fig.5; Renault et al., 2009 Fig. 3; Sup Fig. 7A). In addition, Sup Fig. 7D shows that PGCs are found on the outside surface of embryos from a *UAS-*$ptc^{\Delta loop2}$*x nos-Gal4* cross as is observed when *smo* activity is knocked down with RNAi.

Kim et al., (2021) also tested the effects of *ptc* misexpression; however, instead of using the *UAS-*$ptc^{\Delta loop2}$ transgene, they used a *UAS-GFP-Ptc* transgene that expresses a wild type GFP-Ptc fusion protein. The authors reasoned that over production of the Ptc receptor would depress the response to Hh signals. In their experiment they found that about 30% of the *nos-Gal4xUAS-GFP-Ptc* embryos had 6 or more lost PGCs, while PGC migration defects were not observed in *nos-Gal4* or *UAS-GFP-ptc* embryos (see Sup Fig. 5).

Discussion

Twenty years of controversy:

Here we have addressed a controversy that dates back to 2002. In this controversy, it has been claimed that the results we have published in multiple papers documenting a role for the *hh* pathway in guiding PGC migration are not reproducible (Barton et al., 2016; Renault et al., 2009). However, this "controversy" is a fiction. The problem with "reproducibility" is not ours. Instead, the results and conclusions that cannot be reproduced are in the "Commentary" manuscript that was submitted to *Cell* in 2002 (Appendix 1) and in the Renault et al paper that was published in *Developmental Biology* in 2009.

According to the "Commentary" manuscript (and Renault et al. 2009), the key findings in our 2001 *Cell* paper, namely that ectopic *hh* expression induces PGC migration, are not reproducible. However, as described here an independent arbitrator, Dr. DiNardo, was engaged by the *Cell* editor to determine which of the conflicting data sets were reproducible. Importantly, Dr. DiNardo reproduced our findings in the spring of 2002, not those reported in the "Commentary" manuscript (Fig. 1 and Appendix 2). At the same time, we also redid several of the *hh* misexpression experiments in Deshpande et al., 2001 and found that we could reproduce the effects of *hh* expression on PGC migration that we had previously reported (Appendix 3). *hh* misexpression experiments over the intervening twenty have yield exactly the same results: ectopically expressed *hh* induces PGC mismigration (Fig. 1D. and Sup Fig. 1A from Deshpande et al., 2013). In addition, Kim et al (2021) reported that there are PGC migration defects in embryos heterozygous for a *hh* gain-of-function mutant hh^{Mrt} (Sup Fig. 1B).

It was also claimed that our experiments showing that the cell autonomous factors *ptc* and *smo* are required for proper PGC migration are not reproducible (Renault et al., 2009; Barton et al (2016). This claim dates back to the "Commentary" manuscript and is based on inclusive or poorly done experiments (e.g., the 14 embryos of unknown genotype used to demonstrate that ectopic expression of $ptc^{\Delta loop2}$ in PGCs has no effect on their migration) or misrepresentations of our results (*smo*). As noted above, our findings have been confirmed by Kim et al. (2021) who showed that both *ptc* and *smo* play an important role in PGC migration using completely different experimental approaches from those that we used in 2001. Here we show that *smo* knockdown in migrating PGCs results in mismigration phenotypes similar to those reported by Kim et al. (2021) using a maternal knockdown strategy.

Functioning of the hh pathway in PGC migration:

--*hh signals to migrating PGCs:* The evidence currently available indicates that the *hh* signaling pathway plays a central role in PGC migration. Our finding that *hh* is expressed in SGPs (Deshpande et al., 2001) has been confirmed by both Renault et al., (2009) and Kim et al., (2021). The experiments reported here which show that RNAi knockdown of *hh* (and *hmgcr*) using a *twist-Gal4* driver perturbs PGC migration provide strong evidence that *hh* expression in the mesoderm, likely from the SGPs and their progenitors, is important for proper PGC migration. That the Hh ligand is received by the migrating PGCs is supported by direct visualization of Hh tagged with GFP. We showed in Deshpande et al., (2013) that Hh-GFP ectopically expressed in the CNS/PNS using an *elav-Gal4* driver decorates the surface of migrating PGCs. More recently, Kim et al. (2021) showed that Hh-GFP expressed from a BAC rescue construct not only associates with PGCs, but is also internalized, which is a key step in signal transduction.

--*Factors generating specificity:* A major caveat with a model invoking Hh as a PGC attractant is that of specificity: how can Hh signals emanating from SGPs or their progenitors be distinguished from Hh expressed elsewhere in the embryo. Thus far, three genes, *hmgcr*, *shifted*

and *mdr49*, that help to potentiate Hh signaling in the mesoderm have been identified (Deshpande and Schedl 2004; Deshpande et al., 2013; Deshpande et al 2016). Of these, the most important is *hmgcr* (Van Doren et al., 1998). While all three are preferentially expressed in the developing mesoderm, the *hmgcr* expression pattern most closely parallels the migration of PGC from the surface of the embryo to their final association with the SGPs. We've shown previously that *hmgcr* is required for the release/transmission of the Hh ligand from *hh* expressing cells and that one of its important roles in the *hh* pathway is the prenylation of the heterotrimeric G protein subunit, Gγ1 (Deshpande and Schedl, 2004; Deshpande et al.,2009).

Here we have used genetic epistasis experiments to show that *hh* functions downstream of *hmgcr* in guiding PGC migration (Fig.4). In addition to *hh*, we've shown that two genes critical for the cytoneme dependent transmission of the Hh ligand from *hh* expressing cells, *disp* and *ttv* (Chen et al., 2017; Simon et al., 2021), are also epistatic to *hmgcr* in guiding PGC migration (Fig.4 and 5). These findings support our previous studies implicating both *disp* and *ttv* in PGC migration (Deshpande and Schedl, 2004; Deshpande et al., 2007). While not conclusive, the epistatic relationship between *hmgcr* and both *disp* and *ttv* suggests that a cytoneme based mechanism is used for communication between *hh* expressing cells in the mesoderm and the migrating PGCs. In this model Hh containing cytonemes extending from somatic cells in the mesoderm would establish contacts with cytonemes protruding from the migrating PGCs. These direct interactions would help guide the movement of the PGCs in the direction of the Hh expressing cells. This model is considerably more appealing than one in which a gradient of freely diffusing Hh ligand (or a prenylated polypeptide) produced from multiple mesodermal cells spread over PS10-13 is established and is then somehow sensed and followed by migrating PGCs. Further support for a cytoneme based mechanism comes from the finding that the Shifted protein, which interacts with Ihog and Boi, functions in PGC migration (Avanesov and Blair, 2013; Deshpande et al., 2013).

--*Reception of the Hh signal.* As noted above, our previous studies (Deshpande et al., 2001) as well as those of Kim et al., (2021) indicate that both *ptc* and *smo* are required for PGC migration. Here we have confirmed these findings using RNAi to specifically knockdown *smo* in migrating PGCs. However, the most important advance in terms of understanding how the reception of the Hh ligand by PGCs is able to direct their migration comes from recent experiments showing that a GPCR protein Trapped in endoderm (Tre1) functions downstream of Smo to promote the local remodeling of the actin cytoskeleton (Kim et al., 2021). Tre1 was identified in genetic screens as a factor essential for PGC migration (Coffman et al., 2992; Kunwar et al., 2003). It was initially thought to mediate PGC migration through the mid-gut epithelium by regulating E-cadherin activity (Kunwar et al. 2007); however, more recent studies showed that the mismigration phenotypes in *tre1* mutants are due to a failure to sense and respond to the guidance molecule produced by the SGPs (Lin et al. 2020; Kim et al. 2021). Kim et al. (2021) found that Tre1 is regulated by the *hh* pathway. They showed that reception of the Hh ligand by the PGCs induces the accumulation of PIAP 5 kinase (dPIP5K) at the leading edge of migrating PGCs. dPIP5k generates a localized source of the signaling molecule PI(4,5)P2 which in turn activates a WASP (Wiskott-Aldrich syndrome protein) and Arp2/3 dependent actin polymerization. It is this local alteration in the cytoskeleton that couples PGC migration to the reception of the *hh* signal. As observed when PGCs are compromised for *ptc* or *smo*, the defects in PGC migration in *tre1* mutants are first detected while the PGCs are being internalized during the midgut invagination.

--*Are there other guidance molecules?* While our studies and those reported by Kim et al. (2021) provide compelling evidence that the *hh* signaling pathway is deployed in flies to guide

PGC migration to the SGP, this does not exclude the possibility that other signaling pathways have functions in attracting or repelling migrating PGCs. In other contexts, like the mammalian nervous system, in which the *hh* signaling pathway provides migratory cues, it functions in conjunction with a combination of other attractants and repellants. Consequently, it would be reasonable to expect that signals beside Hh will be utilized to direct PGC migration. Indeed, a repulsive signal is provided by the lipid phosphate phosphatases, Wunen and Wunen-2 (Zhang et al., 1997; Starz-Gaiano et al., 2001; Hanyu-Nakamura et al., 2004; Renault et al., 2004).

Supporting the idea that yet other pathways might function in directing PGC migration, at least some PGCs manage to find the SGPs in embryos null for *tre1*. Likewise, in the *hh*, *hmgcr* and *smo* knockdown experiments reported here, a substantial fraction of the embryos have only a small number of lost PGCs. The same is true for our *hmgcr* epistasis experiments; knocking down *hh* and the two *hh* pathway genes in the cells in the CNS/PNS that express *hmgcr* does not fully rescue the PGC migration defects induced by ectopic *hmgcr*. Though the incomplete effects in the RNAi knockdown experiments are likely due, at least in part, to residual gene activity, they also leave open the possibility that another signaling pathway(s)/signaling molecule(s) is deployed to help guide the migrating PGCs. Whether this signaling pathway(s) will utilized a prenylated polypeptide like the yeast a-factor is not clear at this point as no such molecule has been identified. Likewise, while our experiments suggest that *mdr49* functions in cholesterol transport and is important for *hh* autoprocessing, we cannot exclude the possibility that it could also transport a prenylated polypeptide or some other molecule derived from mevalonate. Further, studies will clearly be required to identify new players and pathways that help guide PGC migration.

Materials and Methods

Immunohistochemistry

Embryos (0-12 or 0-16 hours old) were fixed using 4% paraformaldehyde and heptane essentially as described before. Vasa (from Paul Lasko) antibody is a rabbit polyclonal. It was used at a 1:1000 dilution. Eyes absent antibody is a mouse monoclonal and was used at 1:20 dilution. ß-Galactosidase antibody was either a rabbit polyclonal purchased from Cappel (used at 1:1000 dilution) or a mouse monoclonal antibody from Developmental Studies Hybridoma Bank (used at 1:10 dilution). GFP antibody is a rabbit polyclonal purchased from Torrey Pines Biolabs (used at 1:1000 dilution). For immunohistochemical analysis a magnification of 40X or 60X was used in all the instances, and images were collected using identical settings for the control and experimental samples.

Mutant and misexpression analysis

UAS-Hh (two different stocks on the second and third chromosome respectively; from Phil Beachy), *UAS-Hh-N*: Stock on the third chromosome (from Phil Beachy) respectively. *elavGAl4UAS hmgcr* recombinant, $mdr49^{3.16}$/*Cy0* allele, and the deficiency stock #7970/ Cy0 were obtained from Ruth Lehmann. *UAS-Ptc-delta-loop2* stock was a X-linked insert and was a gift from Gary Struhl.

Both *elav-Gal4*, *nanos-Gal4* stocks were obtained from Liz Gavis. *hh-Gal4*/*TM6 Ubx-LacZ* stock was obtained from Trudi Schupbach.

The other *UAS* and *Gal4* stocks used for the misexpression studies were from the Bloomington stock center. *UAS-egfp-RNAi* (#41552), *UAS-hh-RNAi* (three different transgenic inserts #31042, #25794, #31475), *UAS-smo-RNAi* (#27037), *UAS-hmgcr-RNAi* (#50652), *UAS-disp-RNAi* (# 27247), *UAS-ttv-RNAi* (# 51480), *twist-Gal4* (#2517).

Unless otherwise mentioned, in most experiments, males carrying two copies of the *UAS* transgene were mated with virgin females carrying two copies of the *Gal4* transgene. The resulting progeny embryos were fixed and stained for subsequent analysis. When necessary, the genotypes of the progeny embryos were unambiguously determined by using balancer chromosomes marked with either GFP or ß-galactosidase and double labelling using either with anti- ß-galactosidase or anti-GFP antibodies with anti-Vasa antibody.

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Figure legends:

Figure 1 Does *hh* misexpression induce PGC mismigration: A comparison of three data sets from 2002 and an experiment in 2022. Panel A: The graph in this panel is adapted from data presented in Fig. 2 of the 2002 "Commentary" manuscript (Appendix1). Precisely the same data can also be found in Fig. 2 of Renault et al. *Gal4* drivers and *UAS* transgene as indicated below the graph. Panel B: Adapted from data in Dr. DiNardo's March 2002 report to the editors of *Cell* (Appendix 2). Note that Dr. DiNardo used a mixed population of homozygotes and heterozygotes in his *hairy-Gal4* experiments. As result, not all of the progeny will have the *hairy-Gal4* driver. *Gal4* drivers and *UAS* transgene as indicated below the graph. Panel C: Adapted from our 2002 report to the editors of *Cell* (Appendix 3). Note that in these experiments only half of the embryos will have both the driver and the UAS transgene, either because heterozygotes were used or because the transgene from the father is on the X chromosome. *Gal4* drivers and *UAS* transgene as indicated below the graph. Panel D. An experiment done in 2022 using an *elav-Gal4* driver (CNS expression) and a *UAS-hh* transgene. Panels E and F. *UAS-hh/+* and *UAS-hh/elav-Gal4* embryos probed with Vasa antibody. "carrot" indicates a collection of

PGC associated with the ventral CNS. The percentage of embryos with 0-2 (blue), 3-4 (orange), 5-6 (grey) and 7 or more (yellow) is indicated in the graph. Bar represents 10 microns.

Figure 2: Early PGC migration phenotypes (stage 9-11) in mesodermal *hh-RNAi* knockdown embryos and quantitation of number of lost PGCs in stage 13-15 embryos. A *twi-Gal4* transgene was used to drive expression of three different *UAS-hhRNAi* transgenes specifically in the mesoderm. Embryos were stained with antibodies against Vasa, a PGC-specific marker. Panels A, B, C: stage 9/10 embryos Panels D, E. F: stage 11 embryos. Panels A, D: *twi-Gal4*/*UAS-egfpi*. Panels B, C, E. F: *twi-Gal4*/*UAS-hhRNAi* with two different *UAS-hhRNAi* transgenes as indicated. Abnormal phenotypes in *hh-RNAi* embryos relative to the controls can be seen by comparing *egfpi* and *hhRNAi* embryos. Arrows in panels B, C, and F: see text. Panel G: Quantitation of the PGC migration defects in stage 13-15 *egfi* and *hh-RNAi* embryos. Embryos were classified based on the total number of mismigrated PGCs. *twi-Gal4/UAS-hmgcrRNAi* show similar defects in SGP association: see Fig. 3. The percentage of embryos with 0-2 (blue), 3-4 (orange), 5-6 (grey) and 7 or more (yellow) is indicated in the graph. Bar represents 10 microns.

Figure 3: PGC migration phenotypes are induced by *RNAi* knockdown of *hmgcr* in the mesoderm. Embryos were stained with VASA, a PGC-specific marker. Panels A and C: stage 13 embryos. Panels B and D: stage 14 embryos. Panels A and B: *twi-Gal4/+* control embryos. Panels C and D: *twi-Gal4/UAS-hmgcr-RNAi* embryos *UAShmgcr-RNAi* embryos display abnormal PGC migration and gonad coalescence compared to the control. The phenotypes include a failure of PGCs to align with the SGPs or coalesce properly. A subset of PGCs also remain attached to the outer surface of the dorsal side of the embryo. (For quantitation of the migration defects refer to Fig.2 and corresponding legend). Bar represents 10 microns.

Figure 4: PGC migration defects induced by ectopic expression of *hmgcr* using an *elav-Gal4* driver can be suppressed by simultaneous expression of three different *UAS-hhRNAi* transgene. Females homozygous for a recombinant *elav-Gal4, UAS-hmgcr* chromosome were mated with males carrying either *UAS-hhRNAi* (experimental) or *UAS-egfp*i (control) transgene inserts respectively. Stage 13-15 embryos were stained with Vasa. Panel A: *elav-Gal4*UAS-hmgcr/*UASegfpi*. Panel B: *elav-Gal4UAS-hmgcr*/*UAShhRNAi*^{1}. Panel C: *elav-Gal4UAS-hmgcr*/*UAShhRNAi*^{2}. Panel A: Nervous system specific ectopic expression of *hmgcr* induces mis-migration of PGCs with a substantial number of PGCs diverted towards the CNS/PNS. Panel B and C: Embryos simultaneously expressing *hh-RNAi* and *hmgcr* in the same cells show variable suppression of the PGC migration defects induced by ectopic *hmgcr* expression in the CNS/PNS. Panel D: Quantitation of migration defects in different genetic induced by *elav-Gal4, UAS-hmgcr* with and without *UAS* transgenes expressing dsRNAs directed against *hh*, *ttv* and *disp*. These findings are also inconsistent with studies recently reported by Kenwrick et al., (2019). In their experiments they used a Gal4 driver, *NP5141*, that is active in parasegment 14 to drive *hmgcr* expression. To test for epistasis, the authors generated embryos that were also homozygous for a *hh* null allele, hh^{Ac}. They reported that the mislocalization of PGCs induced by *hmgcr* was unaltered in the *hh* null background. This conclusion was based on the claim that PGC migration was completely WT in hh^{Ac} null allele until at least stage 12. However, this does not fit with the studies of Kim et al., 2002 (or with earlier work: Monroe et al., 1998a, Monroe et al., 1998b) who showed that hh^{Ac} mutant embryos display PGC migration defects from stage 10 onwards.

Figure 5: PGC migration defects induced by ectopic expression of *hmgcr* using an *elav-Gal4* driver can be suppressed by simultaneous expression of *dispRNAi* and *ttvRNAi*. All embryos

were stained with Vasa and are at stage 14. Panel A: *elavGal4 UAS-hmgcr*/ *UAS-egfpi* control. Ectopic expression of *hmgcr* in the nervous system results induces PGCs to migrate towards the CNS/PNS. Panel B: *elavGal4 UAS-hmgcr*/ *UAS-dispRNAi* embryo. Panel C: *elav-Gal4 UAS-hmgcr*/ *UAS-ttvRNAi* embryo. Simultaneous expression of *dispRNAi* or *ttvRNAi* (Panels B and C) suppresses the migration defects induced by expressing *hmgcr* in the CNS/PNS. The migration defects in these three genetic backgrounds are quantitated in Fig.4D. Bar represents 10 microns.

Figure 6: Reducing the dose of the ABC transporter *mdr49* enhances the migration defects induced by *elav-Gal4* driven expression of *hmgcr* in the CNS/PNS. All embryos were stained with Vasa. All embryos are at stage 13-14. Panel A-E: *elav-Gal4UAS-hmgcr*/ + control embryos. Ectopic expression of *hmgcr* in the nervous system induces PGCs to migrat towards the CNS/PNS. Panel F-J: $mdr49^{del3.16}$/+; *elav-Gal4 UAS-hmgcr*/ + experimental embryos. A reduction in the dose of the *mdr49* enhances PGC migration defects induced by ectopic expression of *hmgcr* (compare Panels A-E with panels F-J). Quantitation of the PGC migration defects in the genetic backgrounds as indicated.

Figure 7: *RNAi* knockdown of *smo* in PGCs induces migration defects. Females carrying the PGC specific *nos-Gal4* driver were mated to males carrying a *UAS-smoRNAi* transgene. Panel A: *nos-Gal4*/+ control embryo (stage 13). Panel B: *nos-Gal4*/*UAS-smoRNAi* at early stage 13. Panel C: *nos-Gal4*/*UAS-smoRNAi* at late stage 13. Compared to control embryos, *smo-RNAi* embryos display abnormal PGC migration. The phenotypes include a failure of germ cells to align with the SGPs, and a subset of PGCs scattered throughout the posterior end of the embryo. Panel D: >55% of smo-RNAi embryos had 3 or more mismigrating germ cells as opposed to <10% in control embryos. All embryos were stained with Vasa. Bar represents 10 microns.

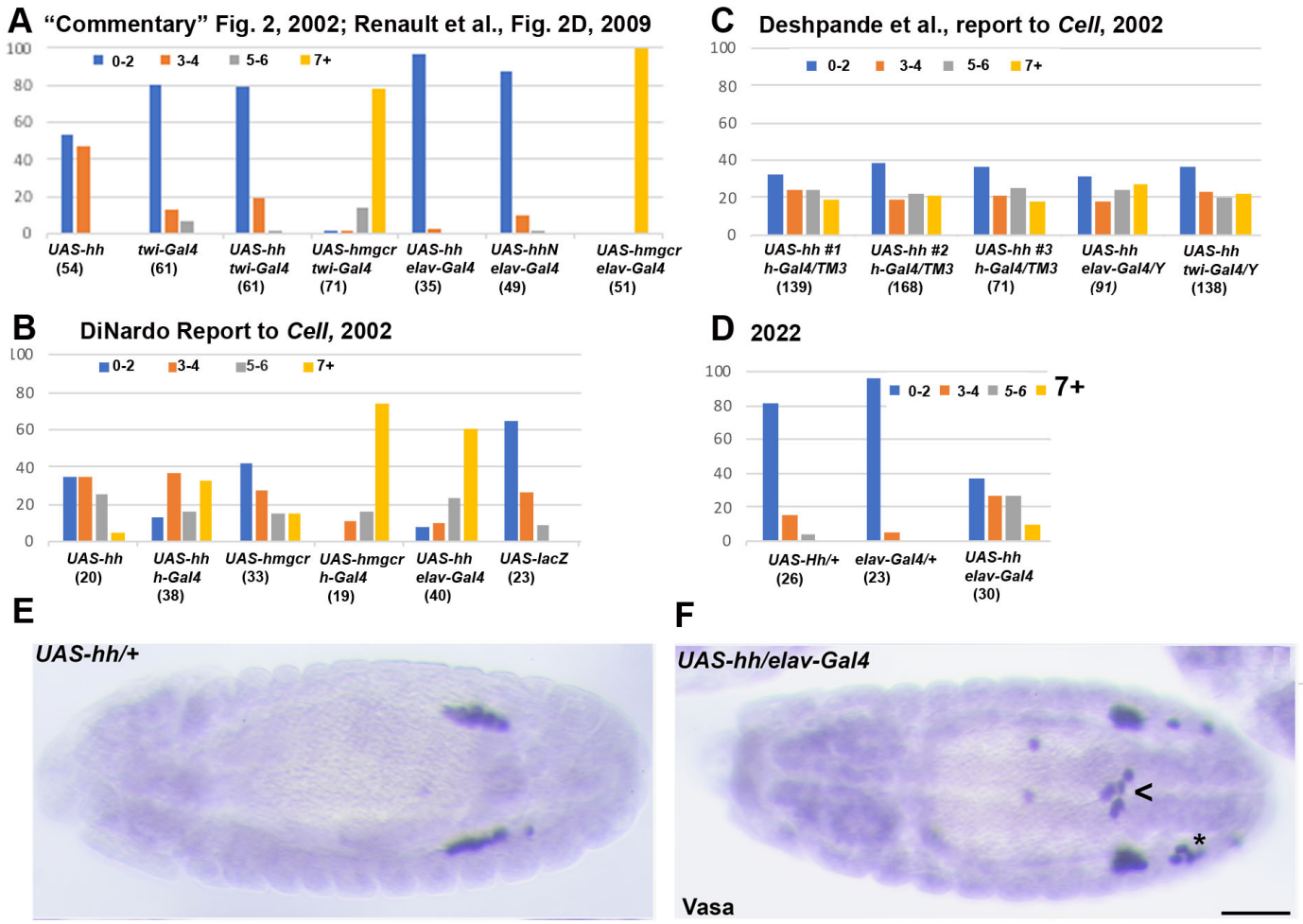

A
"Commentary" Fig. 2, 2002; Renault et al., Fig. 2D, 2009
0-2
3-4
5-6
7+
UAS-hh (54)
twi-Gal4 (61)
UAS-hh twi-Gal4 (61)
UAS-hmgcr twi-Gal4 (71)
UAS-hh elav-Gal4 (35)
UAS-hhN elav-Gal4 (49)
UAS-hmgcr elav-Gal4 (51)
C
Deshpande et al., report to Cell, 2002
UAS-hh #1 h-Gal4/TM3 (139)
UAS-hh #2 h-Gal4/TM3 (168)
UAS-hh #3 h-Gal4/TM3 (71)
UAS-hh elav-Gal4/Y (91)
UAS-hh twi-Gal4/Y (138)
B
DiNardo Report to Cell, 2002
UAS-hh (20)
UAS-hh h-Gal4 (38)
UAS-hmgcr (33)
UAS-hmgcr h-Gal4 (19)
UAS-hh elav-Gal4 (40)
UAS-lacZ (23)
D
2022
UAS-Hh/+ (26)
elav-Gal4/+ (23)
UAS-hh elav-Gal4 (30)
E
UAS-hh/+
F
UAS-hh/elav-Gal4
Vasa

Figure 1

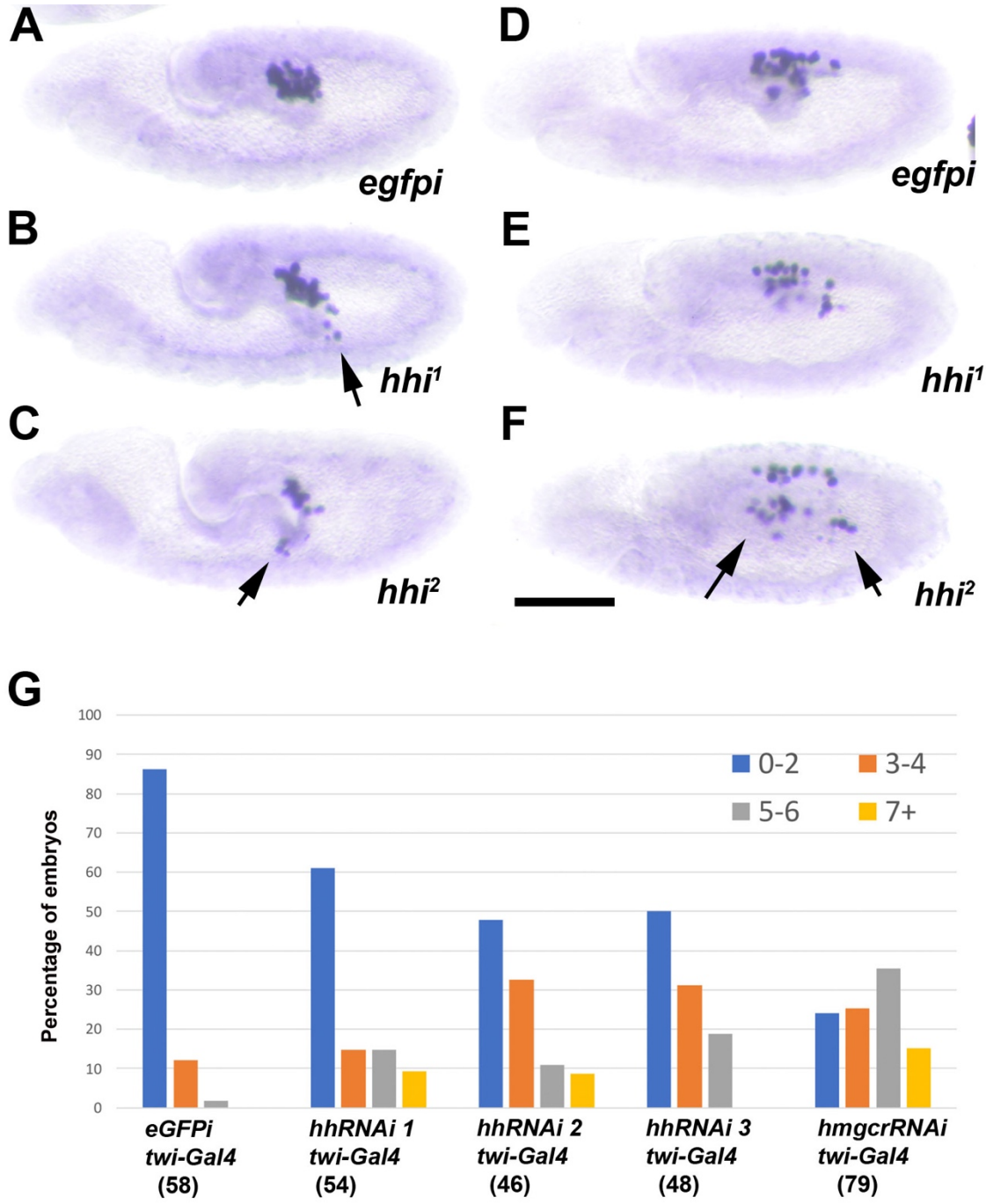

A
egfpi
B
hhi1
C
hhi2
D
egfpi
E
hhi1
F
hhi2
G
Percentage of embryos
100
90
80
70
60
50
40
30
20
10
0
0-2
3-4
5-6
7+
eGFPi twi-Gal4 (58)
hhRNAi 1 twi-Gal4 (54)
hhRNAi 2 twi-Gal4 (46)
hhRNAi 3 twi-Gal4 (48)
hmgcrRNAi twi-Gal4 (79)

Figure 2

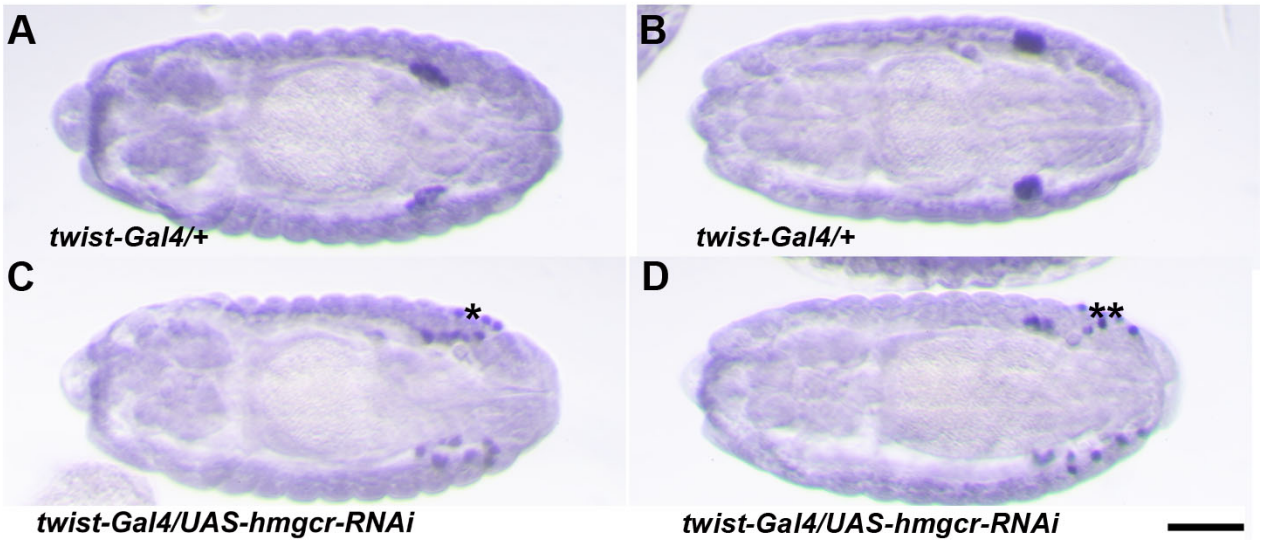

A
twist-Gal4/+
B
twist-Gal4/+
C
*
twist-Gal4/UAS-hmgcr-RNAi
D
**
twist-Gal4/UAS-hmgcr-RNAi

Figure 3

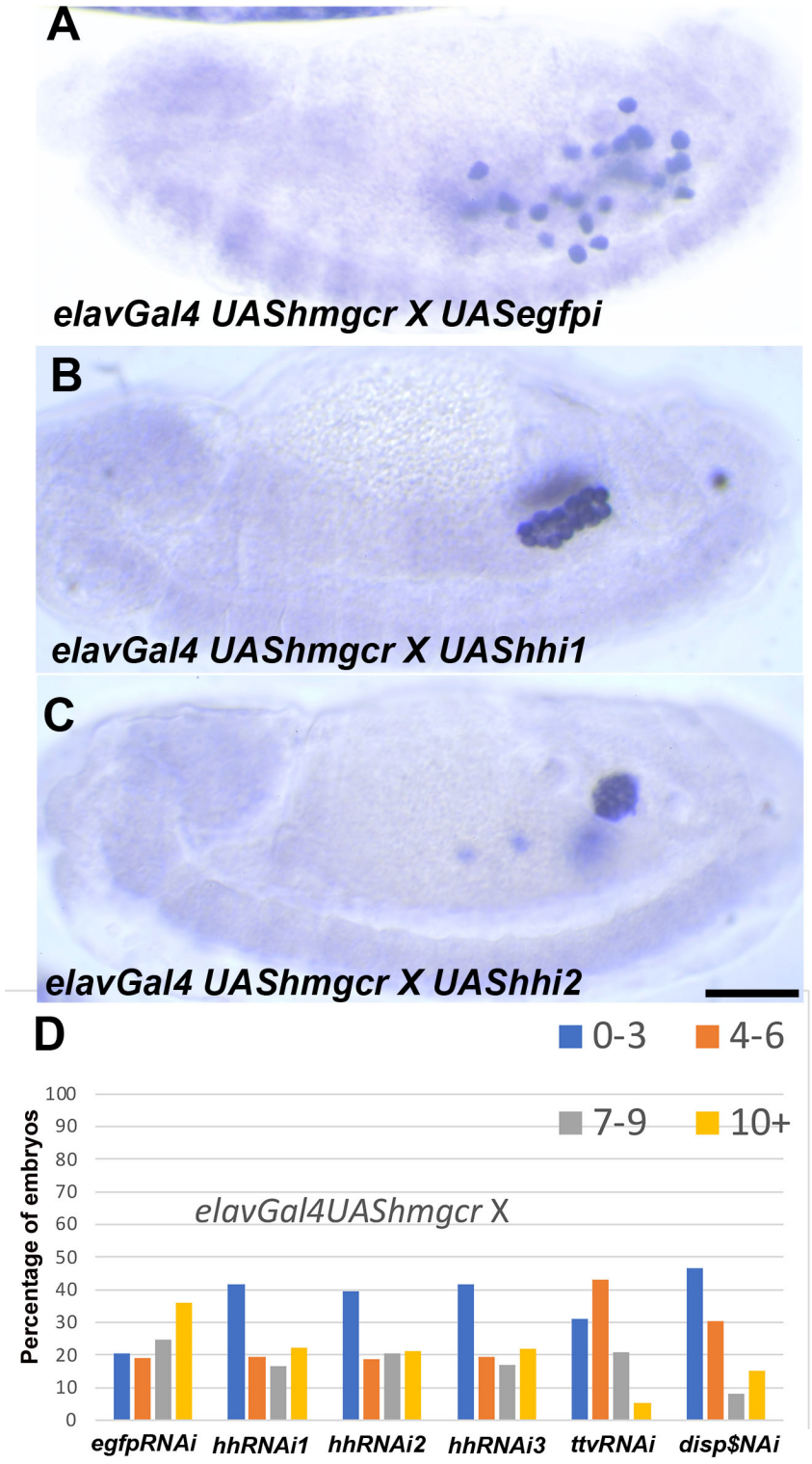

A
elavGal4 UAShmgcr X UASegfpi
B
elavGal4 UAShmgcr X UAShhi1
C
elavGal4 UAShmgcr X UAShhi2
D
0-3
4-6
7-9
10+
Percentage of embryos
100
90
80
70
60
50
40
30
20
10
0
elavGal4UAShmgcr X
egfpRNAi
hhRNAi1
hhRNAi2
hhRNAi3
ttvRNAi
disp$NAi

Figure 4

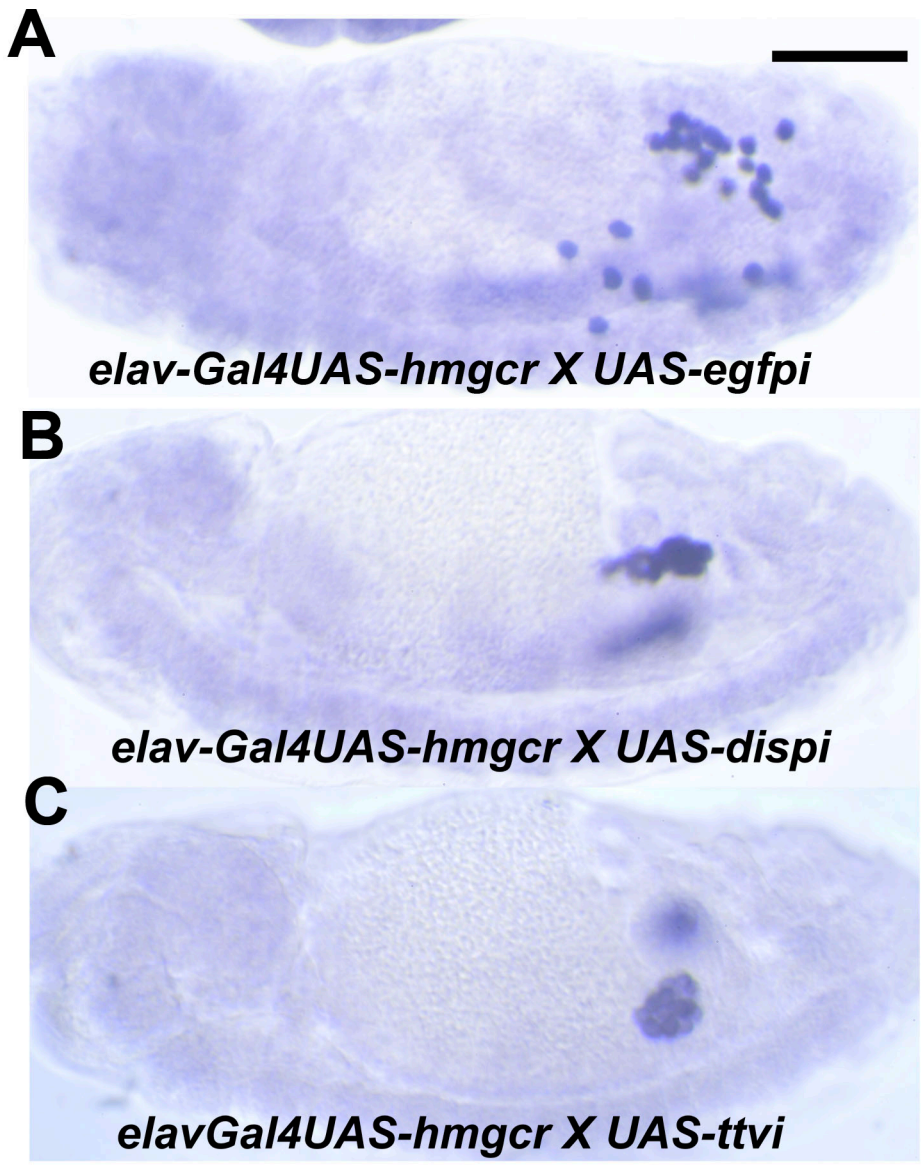

A
elav-Gal4UAS-hmgcr X UAS-egfpi
B
elav-Gal4UAS-hmgcr X UAS-dispi
C
elavGal4UAS-hmgcr X UAS-ttvi

Figure 5

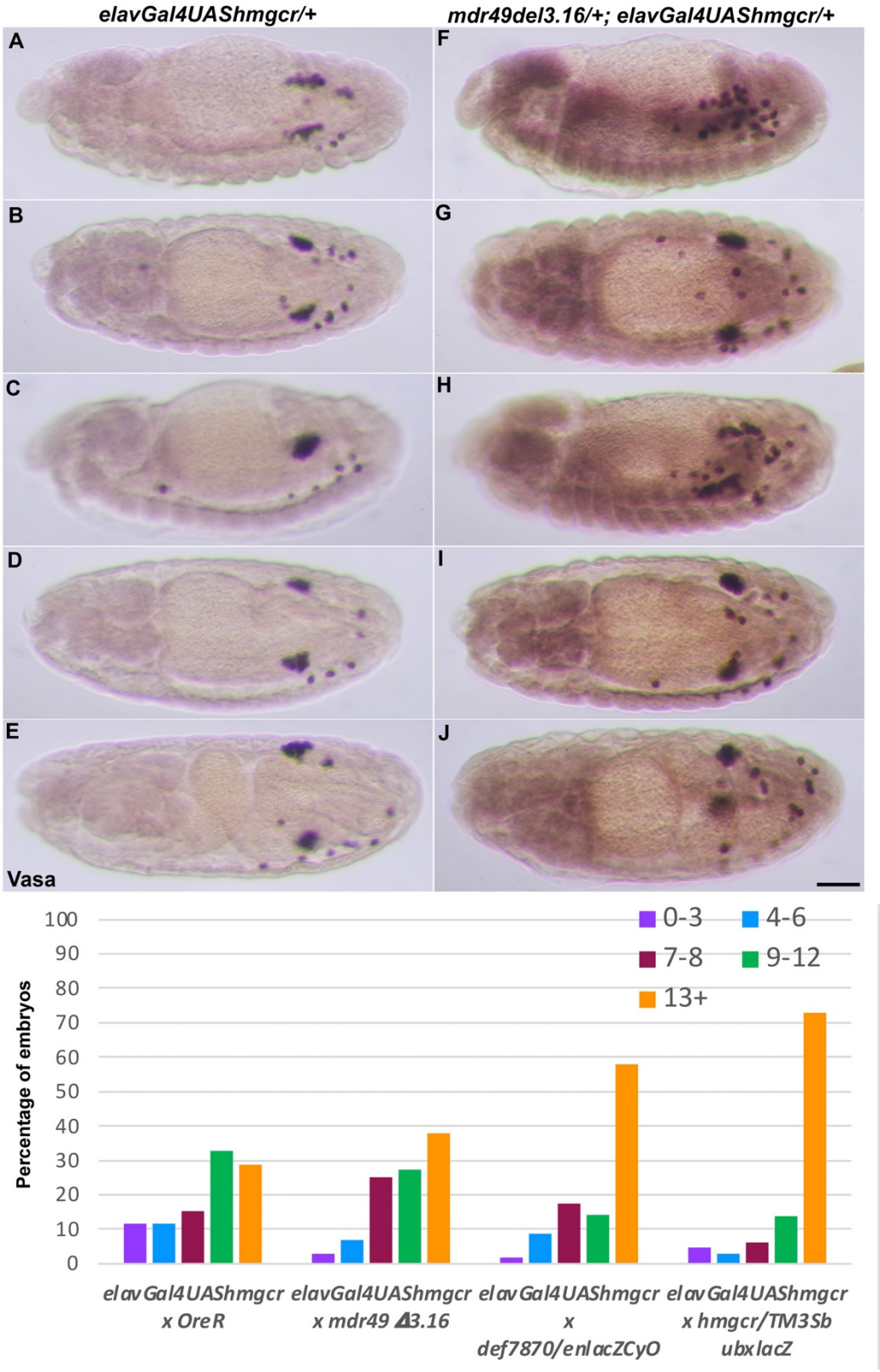

elavGal4UAShmgcr/+
mdr49del3.16/+; elavGal4UAShmgcr/+
A
B
C
D
E
F
G
H
I
J
Vasa
Percentage of embryos
100
90
80
70
60
50
40
30
20
10
0
0-3
4-6
7-8
9-12
13+
elavGal4UAShmgcr x OreR
elavGal4UAShmgcr x mdr49 Δ3.16
elavGal4UAShmgcr x def7870/enlacZCyO
elavGal4UAShmgcr x hmgcr/TM3Sb ubxlacZ

Figure 6

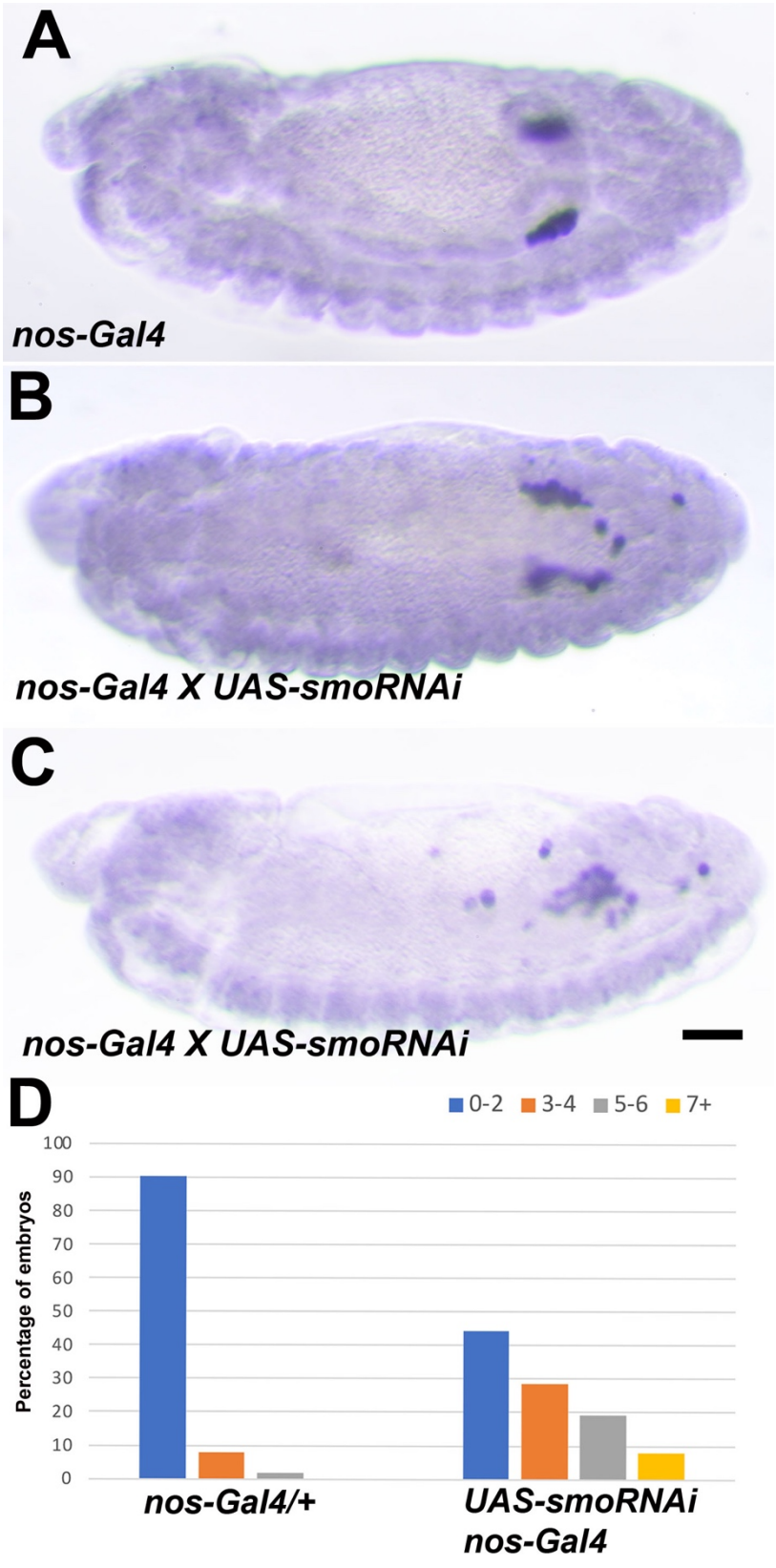
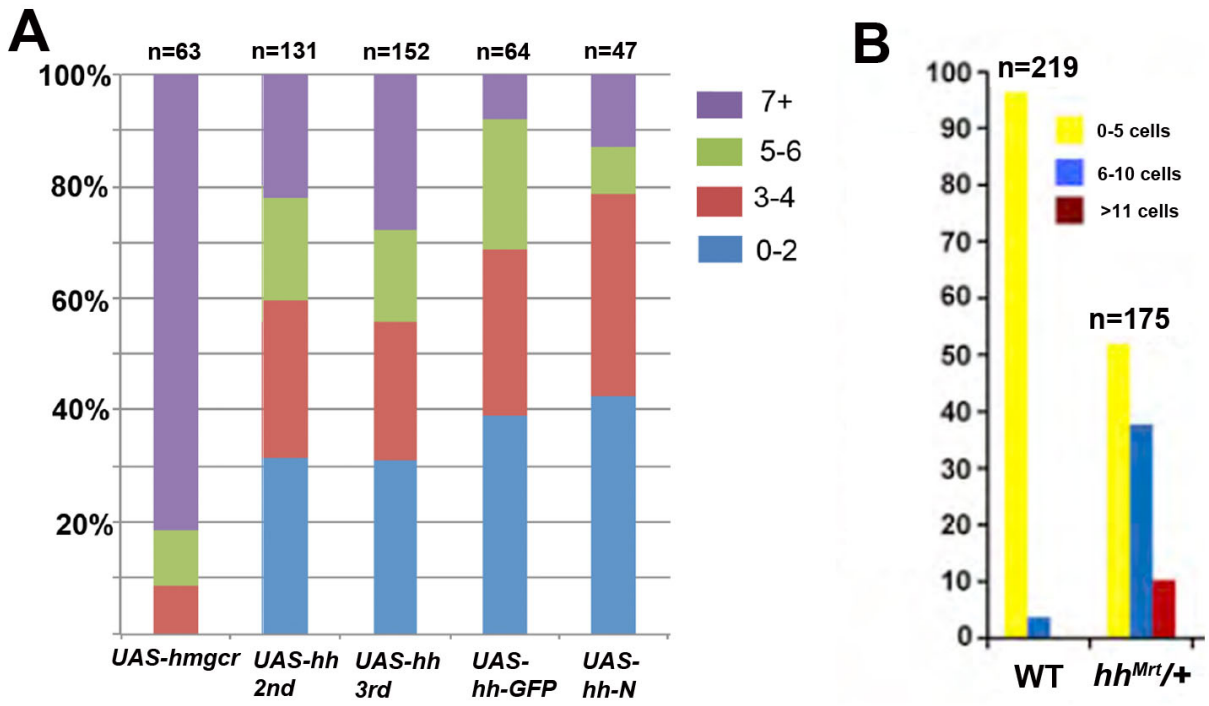
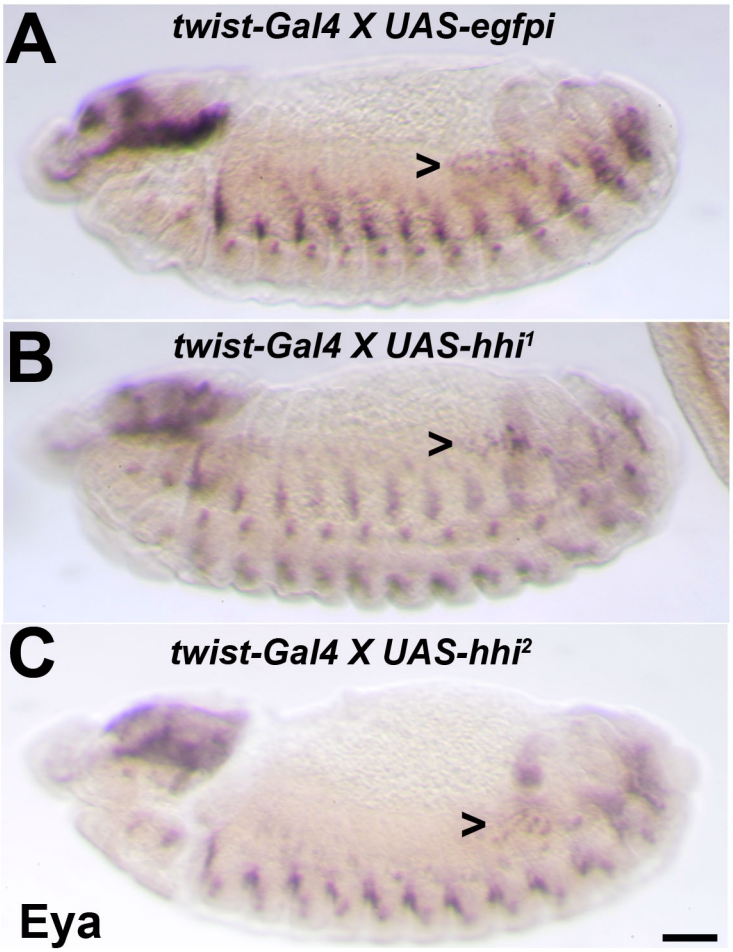

A
nos-Gal4
B
nos-Gal4 X UAS-smoRNAi
C
nos-Gal4 X UAS-smoRNAi
D
0-2
3-4
5-6
7+
100
90
80
70
60
50
40
30
20
10
0
Percentage of embryos
nos-Gal4/+
UAS-smoRNAi
nos-Gal4

Figure 7

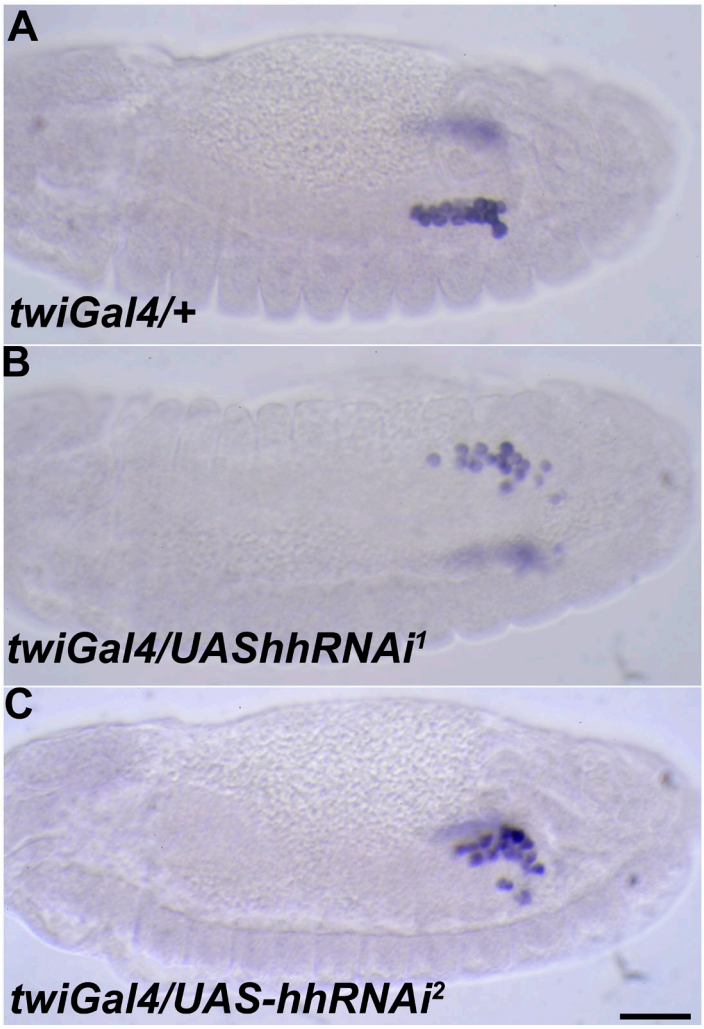
Supplemental Figures



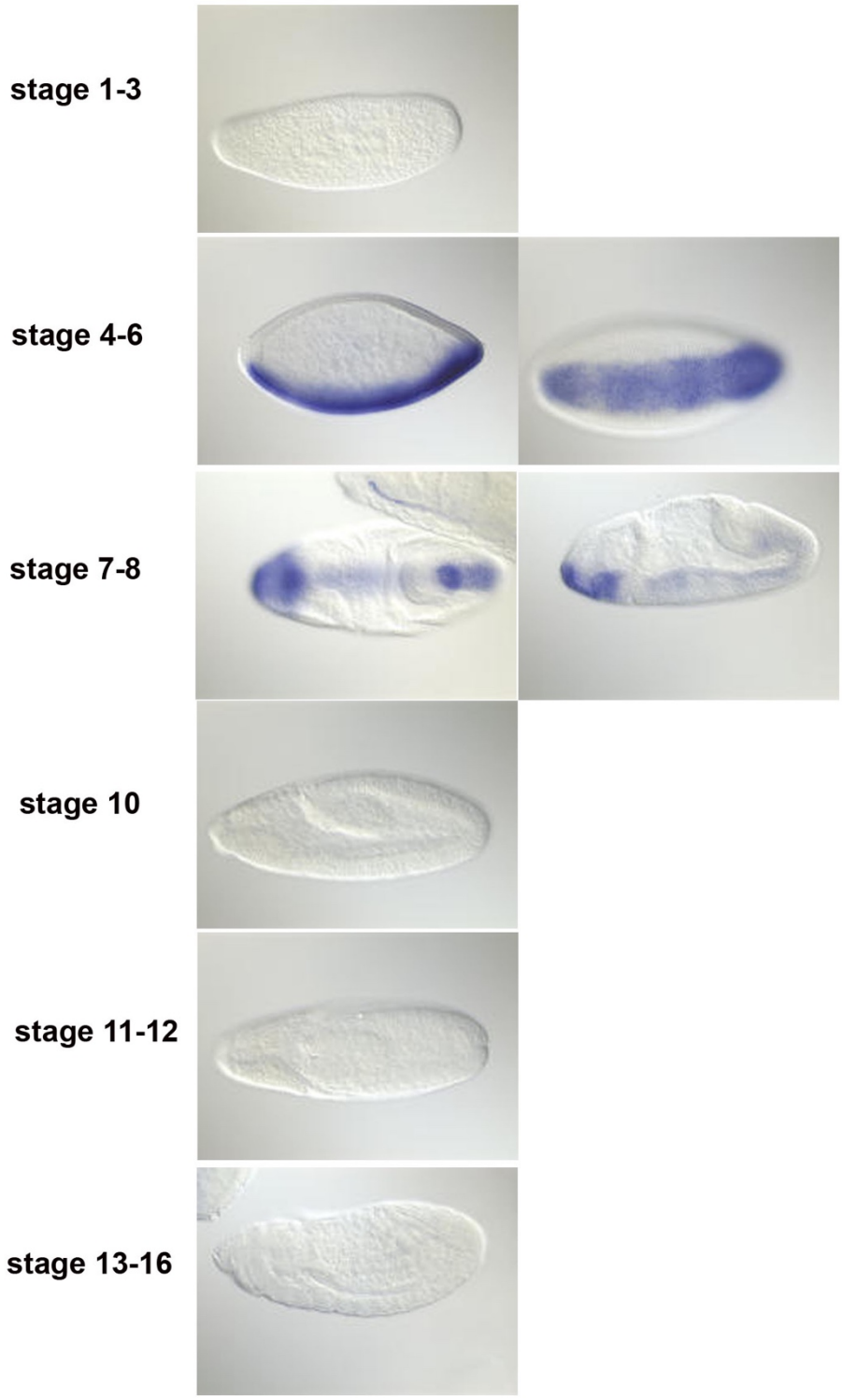
Supplemental Fig. 1 PGC migration defects induced by *hh* misexpression. Panel A. Adapted from Deshpande et al., (2013) Fig. 5. Homozygous *twi-GAL4* females were mated with males homozygous for the following *UAS* transgenes: *hmgcr*, *hh* (on 2nd and 3rd chromosomes), *hh-GFP*, and *hh-N* (N terminal truncation lack cholesterol modification). Purple: 7+ scattered PGCs; Green: 5–6 scattered PGCs; Red: 3–4 scattered PGCs; Blue: 0–2 scattered PGCs. Number of embryos examined in each case indicated above. In the wild type controls fewer than 2 embryos is less than shown in Figure 3. Bars show percentage of embryos in each category as indicated. Panel B. Adapted from Kim et al., (2021). Migration defects were compared in wild type and in embryos heterozygous for the *hh* enhancer mutant hh^{Mrt} (see text). Yellow: 0-5; blue 6-10 and red 11 or greater. Number of embryos examined in each sample is indicated above.



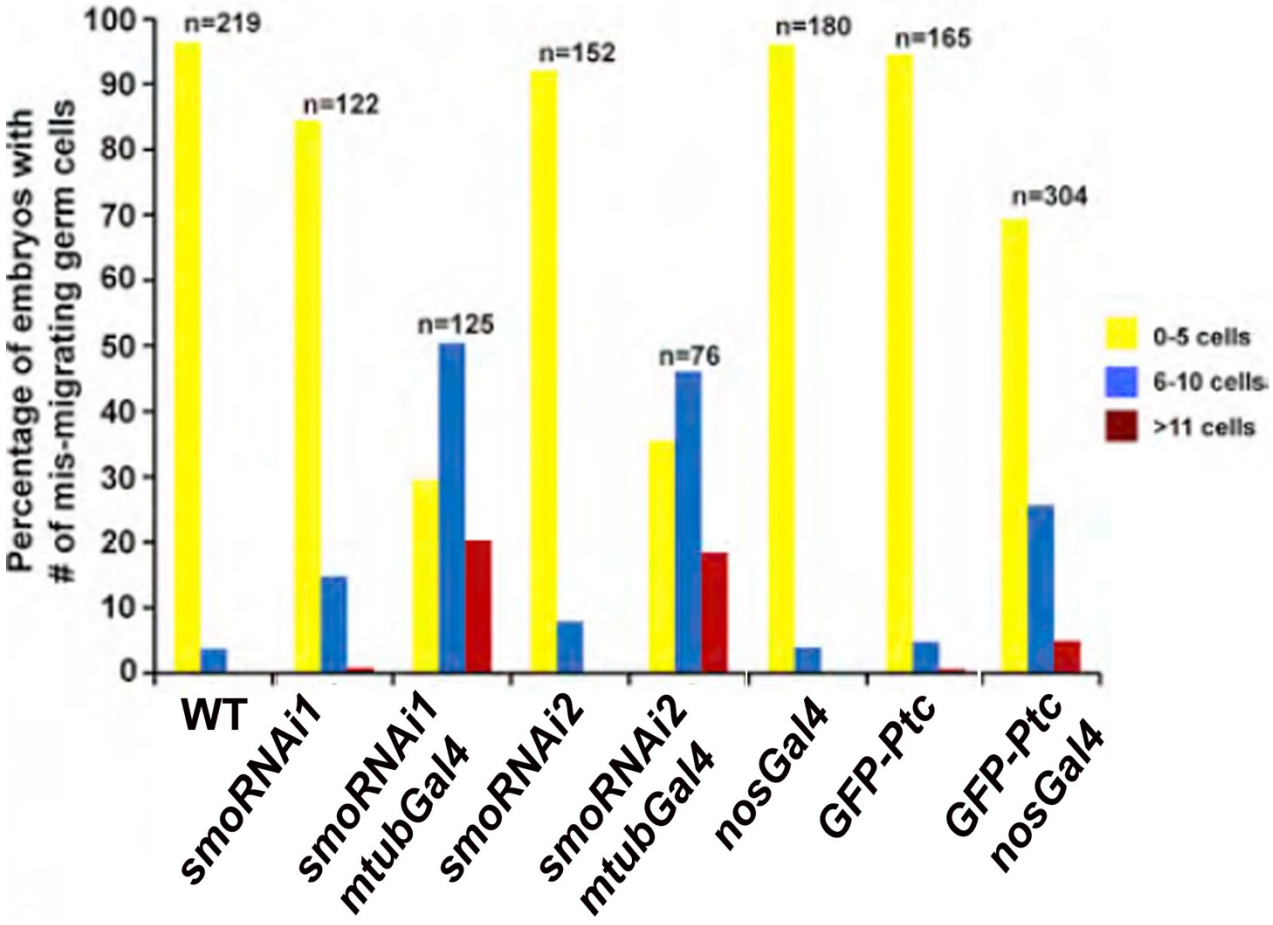
Supplemental Fig. 2 Mesodermal loss of *hh* does not affect specification of SGPs. Antibodies against the mesodermal marker protein, Eyes absent (Eya) were used to mark the somatic gonadal precursor cells (SGPs) in embryos at stage 13-14. Panel A: *twist-Gal4*/*UAS-egfpi* control embryo. Panel B and C: *twist-Gal4*/*UAS-hhRNAi*^{1} (Two different transgenic insert lines were tested and both gave comparable results). Comparison showed no significant difference in the total number of SGPs. N=12. μ≈31 SGPs in control and μ≈33 in experimental. Bar: 10 microns



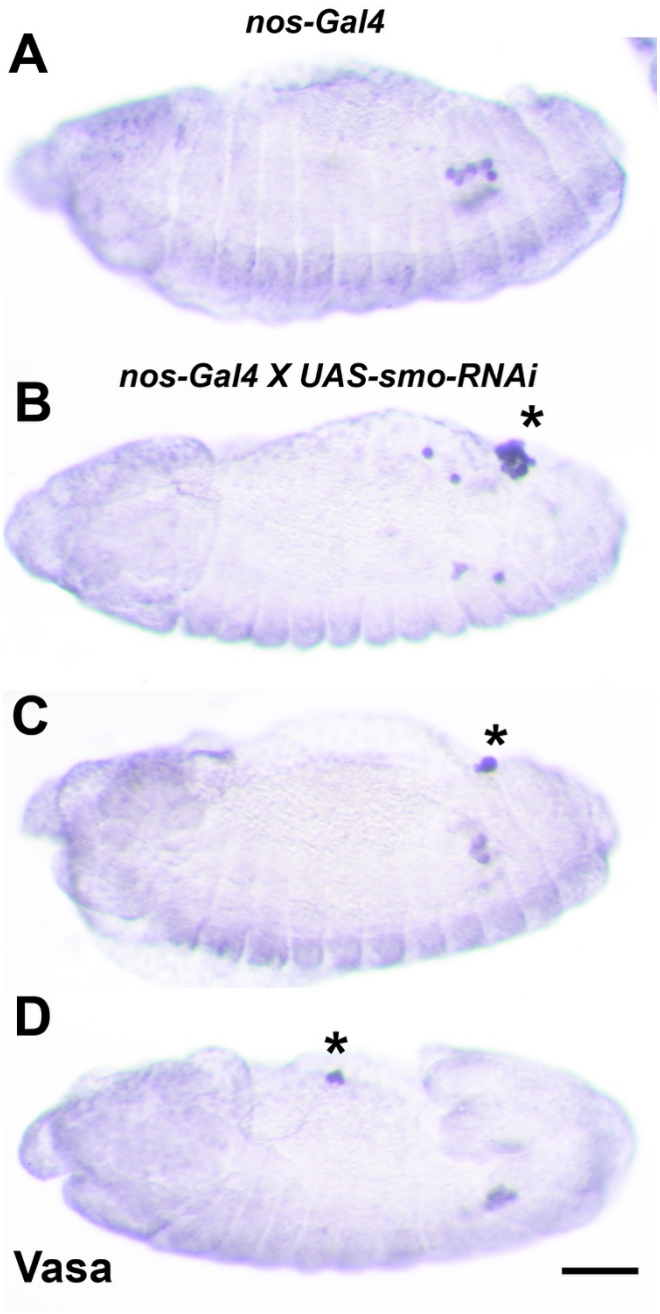
Supplemental Fig. 3 PGC migration phenotypes due to mesodermal loss of *hh* persist during later stages of embryogenesis. *twiGal4* driver was used to drive expression of two different *hh* RNAi lines specifically in the mesoderm. Embryos were stained with Vasa, a PGC-specific marker. Panels A-C: stage 13 embryos. Panel A: *twi-Gal4*/*UAS-egfpi* control embryo. Panels B and C: *twi-Gal4*/*UAS-hhRNAi* I and *twi-Gal4*/*UAS-hhRNAi* II embryos at comparable stage. Bar: 10 microns



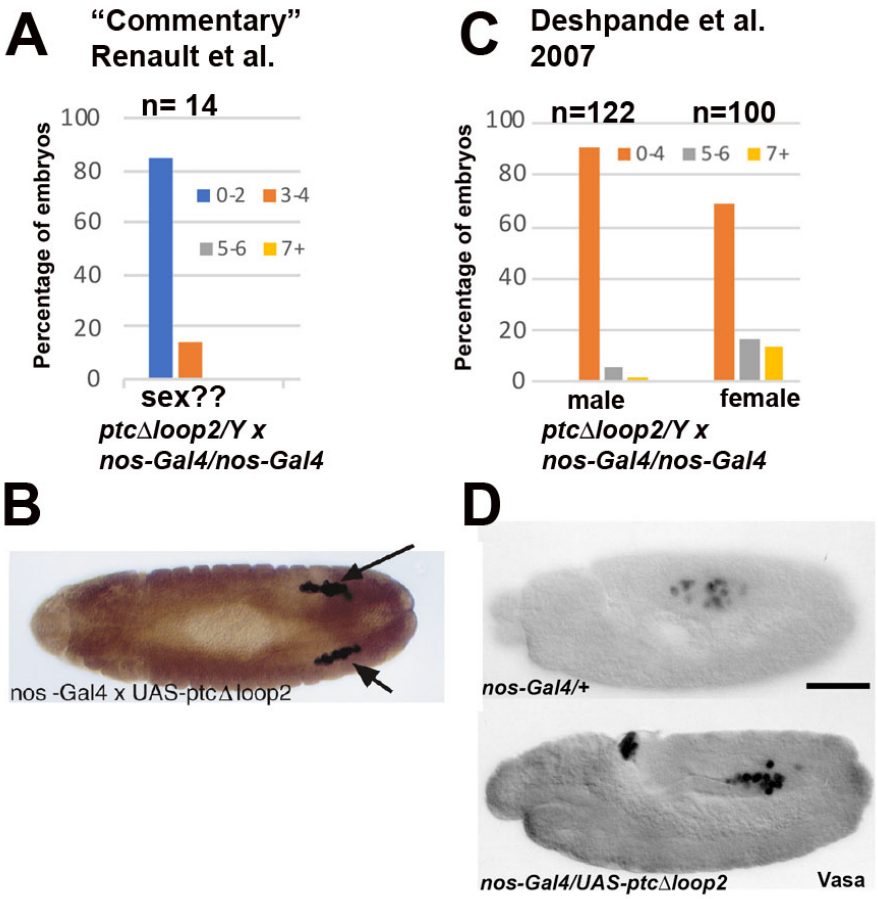
Supplemental Fig. 4 *mdr49* pattern of expression during embryogenesis. Images of *in situ* hybridization to *mdr49* mRNAs during the indicated stages of embryogenesis. Images were generated by BDGP and were taken from the Flybase link number: FBgn0004512



Supplemental Fig. 5. *smo* and *ptc* are required for PGC migration. Adapted from Kim et al., 2021. This figure shows PGC migration defects in embryos compromised for either *smo* or *ptc*. For *smo*, stage 15/16 embryos produced by females carrying *matTub-Gal4* and one of two different *UAS-smo* RNAi transgenes (#1: HMC03577 and #2 GL01472) as well as control females carrying only the *UAS-smoRNAi* transgenes were examined for PGC migration defects. For *GFP-Ptc*, parents homozygous for either the *nos-Gal4* driver of the *UAS-GFP-Ptc* transgenes were mated to either each other to generate *UAS-GFP-Ptc/nos-Gal4* progeny. *nos-Gal4* and *GFP-Ptc* are the control lines. For each genotype, the percentage of embryos with the indicated number of mismigrated PGCs was calculated.



Supplemental Figure 6. Left behind PGCs in "zygotic" *smoRNAi* knockdowns. In a subset of *smoRNAi* knockdowns PGCs are not internalized, but instead are found on the outsie of the embryo. This phenotype was observed by Kim et al., (2021) in *tre1* mutants and in their maternal *smoRNAi* knockdowns (see text). Based on their studies they suggested that the PGCs failed to migrate into the midgut during the midgut invagination. We observe the same phenotype when we used a *nos-Gal4* to drive expression of *UAS-smoRNAi* transgenes in PGCs. All embryos were stained with Vasa at stage 12/13. * indicates PGCs that remain on the outside surface of the embryo. Panel A: *nos-Gal4* control embryo. Panel B-D: *nos-Gal4*/*UAS-smoRNAi* embryos. *smo-RNAi* embryos display PGC migration defects as compared to control embryos. Bar: 10 microns



Sup Fig. 7. *ptcΔloop2* expression in PGCs disrupts their migration. Panels A and B are adapted from Fig. 4 in "Commentary" Fig.4 or Fig. 3 in Renault et al., 2009. Panel A shows the quantitation of PGC migration defects embryos collected from a cross in which males carrying an X-linked *UAS- Ptc$^{\Delta loop}$* transgene were mated to females homozygous for a *nos-Gal4* transgene. A total of 14 embryos (of unknown sex) were scored and quantitated in Panel A. Panel B shows one of these 14 embryos probed with Vasa antibodies. It has a wild type distribution of PGCs. Panel C is adapted from Table 1 in Deshpande et al., 2007. In this experiment 222 embryos were stained with antibodies against Vasa and Sxl. Vasa labels the PGCs, while Sxl antibodies label female embryos. The embryos were visualized using confocal microscopy. Of these 222 embryos, only the 100 female embryos have both the *nos-Gal4* driver and the *UAS- Pt$^{c\Delta loop}$* transgene. In this case the 122 male embryos serve as an internal control. The number of mismigrated PGCs were then quantitated as indicated in Panel C. Panel D shows the distribution of PGCS in two stage 11 embryos. The embryo at the top is from a control cross in which *nos-Gal4* males were mated to WT females. The embryo at the bottom is from a cross in which homozygous *nos-Gal4* males were mated to (homozygous) *UAS- Pt$^{c\Delta loop}$* females. When the cross is in this direction all of the embryos carry both *nos-Gal4* and the *UAS- Pt$^{c\Delta loop}$* transgene. In the embryo shown in the bottom panel, a subset of PGCs are not properly internalized during the midgut invagination and remain on the outside surface of the embryo.