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Isoprenoids Control Germ Cell Migration Downstream of HMGCoA Reductase

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Summary

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAr) provides attractive cues to *Drosophila* germ cells, guiding them toward the embryonic gonad. However, it remains unclear how HMGCoAr mediates this attraction. In a genomic analysis of the HMGCoAr pathway, we found that the fly genome lacks several enzymes required for cholesterol biosynthesis, ruling out cholesterol and cholesterol-derived proteins as mediators of PGC migration. Genetic analysis of the pathway revealed that two enzymes, farnesyl-diphosphate synthase and geranylgeranyl-diphosphate synthase, required for the production of isoprenoids, act downstream of HMGCoAr in germ cell migration. Consistent with a role in geranylgeranylation, embryos deficient in geranylgeranyl transferase type I show germ cell migration defects. Our data, together with similar findings in zebrafish, implicate an isoprenylated protein in germ cell attraction. The specificity and evolutionary conservation of the HMGCoAr pathway for germ cells suggest that an attractant common to invertebrates and vertebrates guides germ cells in early embryos.

Introduction

In many species, primordial germ cells (PGCs) originate at a location separate from that of the somatic gonad. Thus, in order to form functional gametes, germ cells often migrate through and along embryonic tissues to reach the somatic part of the gonad. *Drosophila* germ cell migration provides an excellent system to study the influence of multiple guidance cues on the migratory behavior of a single group of cells (reviewed in Starz-Gaiano and Lehmann, 2001). *Drosophila* germ cells form at the posterior pole of the embryo in close apposition to the primordium of the posterior midgut (PMG). At gastrulation, as the germ band extends dorsally and the PMG invaginates, germ cells are carried to the inside of the embryo. Once in the lumen of the newly formed PMG, germ cells start to actively migrate. Initially, at early stage 10 of embryogenesis, they migrate across the PMG. They then migrate dorsally along the basal side of the PMG at late stage 10 and subsequently migrate away from the midgut toward the adjacent mesoderm at stage 11. In the mesoderm, germ cells associate with somatic gonadal precursor cells (SGPs), three bilateral clusters of mesodermal cells in parasegments ten to twelve (Boyle and DiNardo, 1995). During germ band retraction, starting at stage 12, germ cells and the associated SGPs migrate anteriorly until the gonadal cells round up to coalesce into the embryonic gonad (Starz-Gaiano and Lehmann, 2001; Van Doren et al., 2003).

Genetic analysis of *Drosophila* germ cell migration has shown that lipid metabolism plays an important role during multiple stages of germ cell migration. *wunen* and *wunen 2*, encoding phospholipid phosphatases, have been shown to repel germ cells away from the most ventral region of the PMG into more dorsal regions (Burnett and Howard, 2003; Starz-Gaiano et al., 2001; Zhang et al., 1997). *Drosophila* 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAr), the enzyme responsible for the biosynthesis of mevalonate, provides attractive cues to germ cells, guiding them toward the SGPs (Van Doren et al., 1998). *Hmgcr* (the gene encoding HMGCoAr also referred to as *columbus* [*clb*]) is initially broadly expressed in the mesoderm, and as development proceeds its expression becomes restricted to the SGPs. Analysis of *Hmgcr/columbus* mutant embryos suggests that this tissue-specific expression pattern is required to attract germ cells to the mesoderm and later for the association of germ cells with the SGPs. Indeed, expression of *Hmgcr* is sufficient to attract germ cells as shown by the movement of germ cells toward places of ectopic expression of *Hmgcr* (Van Doren et al., 1998). In *Hmgcr* mutants, most germ cells fail to migrate into the mesoderm and as a result remain associated with the dorsal surface of the PMG. Besides germ cell migration defects, these mutant embryos die at the end of embryogenesis with no obvious patterning defects. Indeed, it is quite striking that mutations in HMGCoAr, an enzyme required for the biosynthesis of products such as ubiquinones, carotenoids, isoprenoids, and cholesterol (Figure 1) has a phenotype so specific for germ cell migration. Also, it remains unclear what molecule downstream of HMGCoAr functions to attract germ cells. One possibility is that HMGCoAr is limiting for the biosynthesis of a product that directly attracts germ cells. Indeed, HMGCoAr has been described as the rate-limiting step in the mevalonate pathway. Another possibility is that HMGCoAr regulates protein modifications that are more indirectly required to regulate germ cell attraction (Deshpande et al., 2001).

To determine how HMGCoAr may mediate germ cell attraction, we analyzed the biosynthetic pathway downstream of HMGCoAr. We show that several enzymes required for cholesterol biosynthesis are not encoded in the fly genome, ruling out cholesterol and cholesterol modified proteins as mediators of PGC migration downstream of HMGCoAr. We further show that farnesyl-diphosphate synthase and geranylgeranyl-diphosphate synthase, the enzymes required for the biosynthesis of

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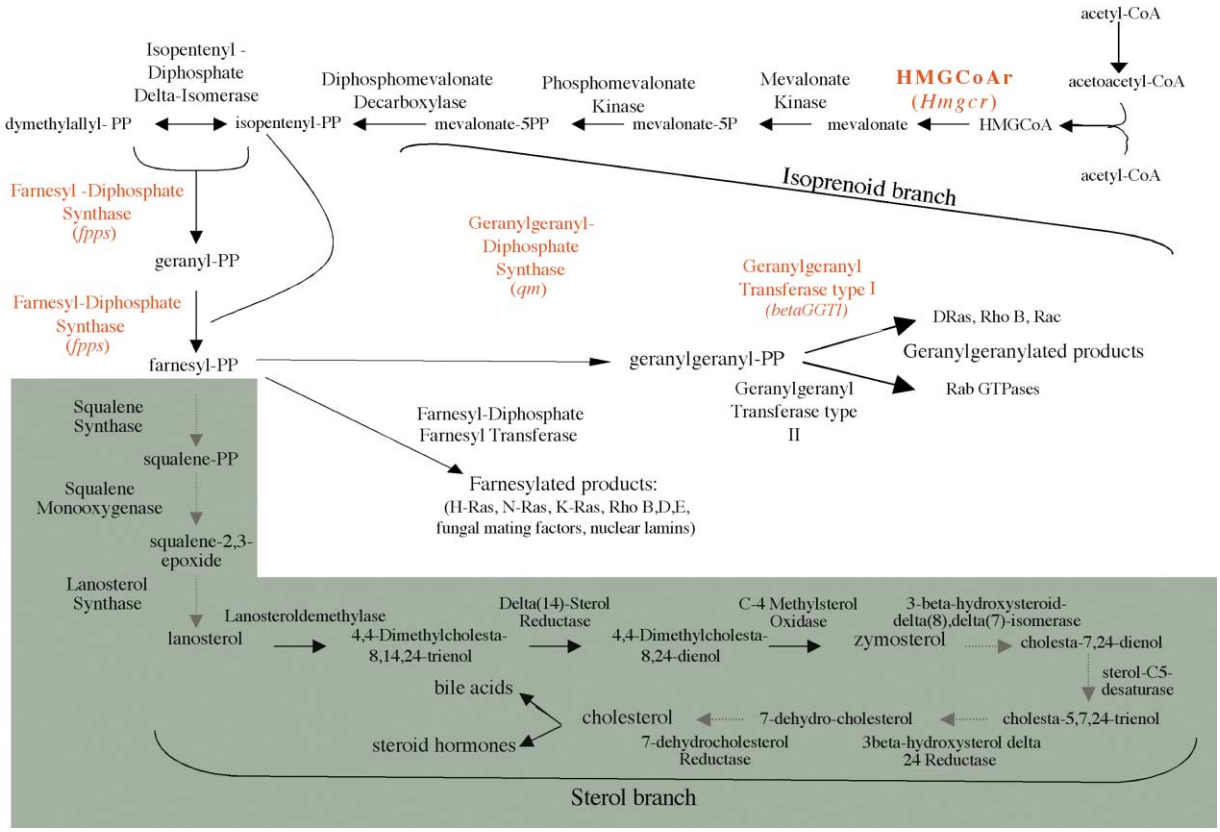


Figure 1. The Isoprenoid/Cholesterol Biosynthetic Pathway in the Fly

There are two main branches downstream of HMGCoAr, the cholesterol biosynthetic branch (shaded in green) and the isoprenoid biosynthetic branch (Moebius et al., 2000). The isoprenoids/cholesterol pathway (also called mevalonate pathway) produces many other molecules (terpenoids, Vitamins K and E, ubiquinone) that are not shown for simplicity. Dashed, gray arrows represent enzymes absent in the fly genome (see Results). Genes with germ cell migration defects are indicated in red.

isoprenoids downstream of HMGCoAr, are expressed in the mesoderm and are required for germ cell migration. Mutant embryos for either of these proteins show germ cell migration defects that are similar to those observed in *Hmgcr* mutant embryos. Furthermore, overexpression of either of these proteins in ectopic locations is sufficient to attract germ cells. Also, we show that mutants for geranylgeranyl transferase type I, an enzyme required for transferring geranylgeranyl pyrophosphate to target proteins, have germ cell migration defects similar to *Hmgcr*. In parallel to our study, Thorpe et al. (2004), using inhibitors of the HMGCoAr pathway, also identified geranylgeranylation as a critical step in zebrafish germ cell migration. Our data strongly suggest that a geranylgeranylated protein common to vertebrates and invertebrates mediates germ cell attraction downstream of HMGCoAr. The striking conservation of a requirement for isoprenoids in *Drosophila* and zebrafish germ cell migration suggests that evolutionary conserved signals may guide migrating germ cells.

Results

Cholesterol and Cholesterol-Modified Proteins Do Not Mediate Germ Cell Attraction Downstream of HMGCoAr

In mammals, HMGCoAr is the enzyme required for the conversion of 3-hydroxy-3-methylglutaryl coenzyme A into mevalonate. Mevalonate is required for the biosynthesis of many different compounds such as ubiquinones, carotenoids, and, as shown in Figure 1, isoprenoids and cholesterol. In order to identify the components of the HMGCoAr biosynthetic pathway (also called mevalonate pathway) in the fly, sequences of the human proteins involved in this pathway (Moebius et al., 2000) were used to identify the corresponding fly homologs. Table 1 lists the human proteins used to search for fly homologs as well as those fly genes that encode homologs. We found a single fly gene encoding each enzyme required for the biosynthesis of isoprenoids, from mevalonate kinase to farnesyl-diphosphate synthase and geranylgeranyl-diphosphate synthase. Isoprenoids are a family of lipids well known as posttranslational modifiers of proteins. This posttranslational modification of proteins consists of the covalent attachment of farnesyl-pyrophosphate (FPP) or geranylgeranyl-pyrophosphate (GGPP) to specific motifs on the C terminus of target proteins by their respective transferases, farnesyl transferase (FNT) and geranylgeranyl transferase type I and type II (GGTI and GGTII). Both FNT, GGTI, and GGTII are heterodimeric proteins composed of one α and one β subunit. All of these prenyltransferases are encoded in the fly genome by a single α and β subunit. However, quite strikingly we failed to identify homologs of several enzymes required for the biosynthesis of cholesterol from FPP (shaded in green in Figure 1). These

Table 1. The Proteins Required for the Biosynthesis of Cholesterol Are Not Encoded in the Fly Genome

Human Gene/Protein	Fly Gene	Identity[a]	Cytological Location in Flybase
Isoprenoid Branch			
HMGCR/HMGCoAr	*Hmgc*	57%	95B1
MVK/mevalonate kinase	*CG33009*	31%	49B10
PMVK/phosphomevalonate kinase	*CG10268*	43%	37F2
MVD/diphosphomevalonate decarboxilase	*CG8239*	45%	13E14
IDI1/isopentenyl-diphosphate delta-isomerase 1	*CG5919*	49%	93D2
IDI2/isopentenyl-diphosphate delta-isomerase 2	*CG5919*	49%	93D2
FDPS/farnesyl-diphosphate synthase	*fpps*	46%	47F1
FNTA/farnesyl-diphosphate farnesyl transferase α subunit	*CG2976*	47%	25A8
FNTB/farnesyl-diphosphate farnesyl transferase β subunit	*CG17565*	50%	89C5-6
GGPS1/geranylgeranyl-diphosphate synthase	*quemao(qm)*	59%	65F5-6
PGGT1B/geranylgeranyl transferase type I, β subunit	*CG3469*	44%	25B4
RABGGTA/geranylgeranyl transferase type II, α subunit	*CG12007*	37%	82F1
RABGGTB/geranylgeranyl transferase type II, β subunit	*CG18627*	64%	23B7
Sterol Branch			
FDFT1/squalene synthase	No relevant homology found		
SQLE/squalene monooxygenase	No relevant homology found		
LSS/lanosterol synthase	No relevant homology found		
CYP51/lanosteroldemethylase	*Cyp4d1*	31%	2D5-6
TM7SF2/delta(14)-sterol-reductase	*CG17952*	30%	57F10-11
SC4MOL/C-4 methylsterol-oxidase	*CG11162, CG1998*	29%, 30%	12B2, 11F4
EBP/3-β-hydroxysteroid-delta(8),delta(7)-isomerase	No relevant homology found		
SC5DL/sterol-C5-desaturase	No relevant homology found[b]		
DHCR24/3β-hydroxysterol-delta(24)-reductase	No relevant homology found		
DHCR7/7-dehydrocholesterol-reductase	No relevant homology found[c]		

[a] Identity between human and fly proteins according to fly BLAST.
[b] SC5DL shows some identity with *CG11162* and *CG1998*; however, *CG11162* and *CG1998* are the most likely homologs of SC4MOL.
[c] DHCR7 shows some identity with *CG17952*; however, *CG17952* is the most likely homolog of TM7SF2
Human gene and protein sequences for each enzyme involved in the mevalonate pathway (NCBI PubMed, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) were used in fly BLAST (http://www.fruitfly.org/blast) to identify fly homologs. (First column) Human genes and proteins used to identify fly homologs. (Second column) Fly homologs. (Third column) Percent identity between homologs at the protein level according to fly BLAST. (Fourth column) Cytological location of fly homologs, according to flyBase (http://flybase.bio.indiana.edu). All enzymes required for the biosynthesis of isoprenoids are encoded in the fly genome as well as three of the enzymes required for the biosynthesis of cholesterol: lanosteroldemethylase, delta(14)-sterol-reductase, and C-4 methylsterol oxidase. All other enzymes required for the biosynthesis of cholesterol are not encoded in the fly genome: squalene synthase, squalene monooxygenase, lanosterol synthase, as well as 3β-hydroxysteroid-delta(8), delta(7)-isomerase and 3β-hydroxysterol-delta(24)-reductase. Lanosteroldemethylase is a member of the cytochrome P450 family with homology to several fly cytochrome P450, including *Cyp4d1* with which it shares the highest identity. delta(14)-sterol-reductase has one fly homolog, *CG17952*, whereas C-4 methylsterol oxidase, a protein with a sterol desaturase domain shows homology to two fly genes with sterol desaturase domains: *CG1998* and *CG11162*. Finally, sterol-C5-desaturase has no fly homolog except for a low homology with fly *CG1998* and *CG11162* as all these proteins have a sterol desaturase domain. Also, 7-dehydrocholesterol reductase has no fly homolog except for some degree of homology with delta(14)-sterol-reductase and therefore with fly *CG17952* as they all have an ERG4 domain.

include: squalene synthase, squalene monooxygenase, and lanosterol synthase, which catalyze the first three steps in the biosynthesis of cholesterol as well as 3β-hydroxysteroid-delta(8)-delta(7)-isomerase, sterol-C5-desaturase, 3β-hydroxy-sterol-delta(24)-reductase, and 7-dehydrocholesterol-reductase. While some fly proteins contained small regions of homology with these human proteins, they did not contain any of the conserved signature domains used to identify these specific classes of proteins, and were therefore not considered to be homologs. The lack of homology to enzymes of the cholesterol branch was confirmed at the nucleotide level. We did identify clear fly homologs for three of the enzymes required for the biosynthesis of cholesterol: lanosterol-demethylase, delta(14)-sterol-reductase, and C-4 methylsterol oxidase. Their fly homologs are shown in Table 1. Our data provide the molecular basis for earlier findings that indicated that insects lack the enzymes to synthesize sterols from mevalonate (reviewed by Clayton, 1964). To extend this analysis to other insects, we analyzed the genome of *Anopheles gambiae* for the presence of cholesterol biosynthetic enzymes. We found that, as in the case of *Drosophila melanogaster*, several of the enzymes critical for cholesterol biosynthesis are also not encoded in this species of insects (data not shown). Thus, the observed widespread inability of insects to survive in sterol free medium could be based on genomic deletion of the enzymes needed to synthesize sterols from mevalonate. Most importantly, with regard to germ cell attraction, our results unequivocally rule out cholesterol or any cholesterol-derived proteins, such as Hedgehog as mediators downstream of HMGCoAr (Deshpande et al., 2001).

fpps and *quemao*, Genes Required for the Biosynthesis of Isoprenoids, Are Coexpressed with *Hmgcr*

Since our genomic analysis eliminated cholesterol as a candidate effector in germ cell migration downstream of HMGCoAr, we focused on other branches of the mevalonate biosynthetic pathway to determine how *Hmgcr* attracts germ cells. It is possible that a product of the

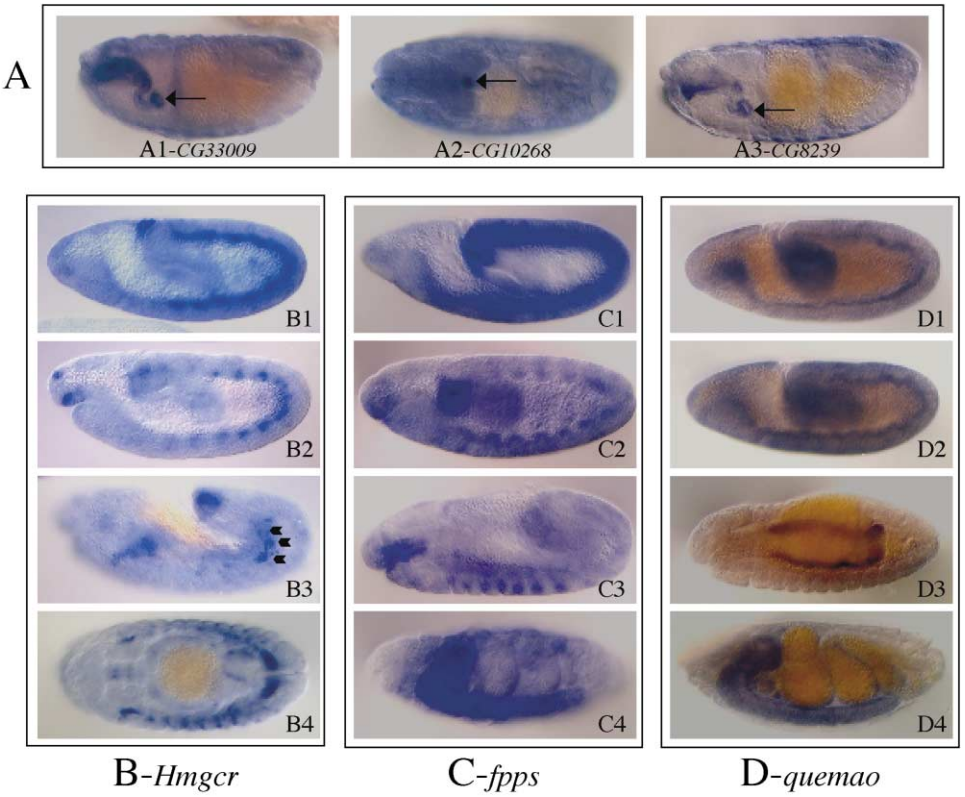


Figure 2. *fpps* and *qm* Are Highly Expressed in the Mesoderm

RNA expression profile of several genes in the mevalonate pathway. Anterior is to the left and dorsal is up in all panels (except for B4 which is a frontal view). All embryos are lateral views except (A2), which is a top view. (A) Most genes in the pathway are broadly expressed in the embryo throughout development but show increased expression in tissues such as (A1) pharynx and esophagus (arrow) (*CG33009*/mevalonate kinase), (A2) ring gland (arrow) (*CG10268*/phosphomevalonate kinase), and (A3) esophagus (arrow) (*CG8239*/diphosphomevalonate decarboxylase).

(B) *Hmgcr* RNA expression. *Hmgcr* is expressed in the mesoderm at stage 10 (B1) and 11 (B2) and gets restricted to the SGPs (arrowheads) at stage 12 (B3) and in coalesced gonads (B4).

(C) *fpps* RNA expression profile. *fpps* is expressed throughout the mesoderm at stage 10 and 11 (C1 and C2, respectively). As the germ band retracts (C3) mesodermal expression is no longer predominant and after germ band retraction *fpps* is mostly expressed in the CNS (C4) and ring gland (not shown).

(D) *qm* RNA expression profile. *qm* is expressed in the visceral mesoderm throughout development, from stage 10 (D1) and 11 (D2) to stage 13 (D3). At the end of embryogenesis, *qm* is highly expressed in the CNS (D4).

pathway, such as mevalonate, directly attracts germ cells. Alternatively, products of the pathway, such as the isoprenoids farnesyl-pyrophosphate or geranylgeranyl-pyrophosphate, could be needed for the posttranslational modification of a protein that only upon modification can attract germ cells. In either case, we expect the enzymes required for the biosynthesis of the attractant to colocalize with *Hmgcr* in the mesoderm, the attractive tissue, at stage 11, when germ cells are migrating away from the PMG toward the mesoderm. We therefore systematically analyzed the RNA expression profile of each fly gene we had identified in the mevalonate pathway. Our expression pattern analysis showed that most genes involved in this pathway are broadly expressed in the embryo. While expression of several genes was slightly increased in tissues such as the pharynx, the esophagus, and the ring gland (Figure 2A, arrows), their predominant pattern of expression was uniform in all cells of the developing embryo. This broad expression pattern points to a general need of the mevalonate pathway in all cells of the fly embryo. Only two genes, *fpps* (Figure 2C), the fly gene encoding farnesyl-diphosphate synthase, and *quemao (qm)* (Figure 2D), the fly gene encoding geranylgeranyl-diphosphate synthase, were expressed in a pattern consistent with a more specific role in germ cell attraction. *fpps* RNA expression in the mesoderm resembles *Hmgcr* expression: both genes are expressed initially uniformly in the mesoderm (Figures 2B1 and 2C1) and subsequently develop a segmental pattern (compare Figures 2B1–2 and 2C1–2). However, unlike *Hmgcr*, *fpps* expression does not become enriched in the SGPs (arrowheads, compare Figures 2B3 and 2C3). Besides being expressed in the mesoderm, *fpps* is also expressed in the nervous system (Figure 2C4), foregut, and midgut, as well as in the ring gland later during embryogenesis. *qm* RNA is also highly expressed in the mesoderm, with elevated expression in the visceral mesoderm (Figures 2D1–3). Besides being enriched in the mesoderm, *qm* is also expressed in the PMG and in the nervous system at high levels in late embryonic development (Figure 2D4). Both *fpps* and *qm* are required for the biosynthesis of isoprenoids, a

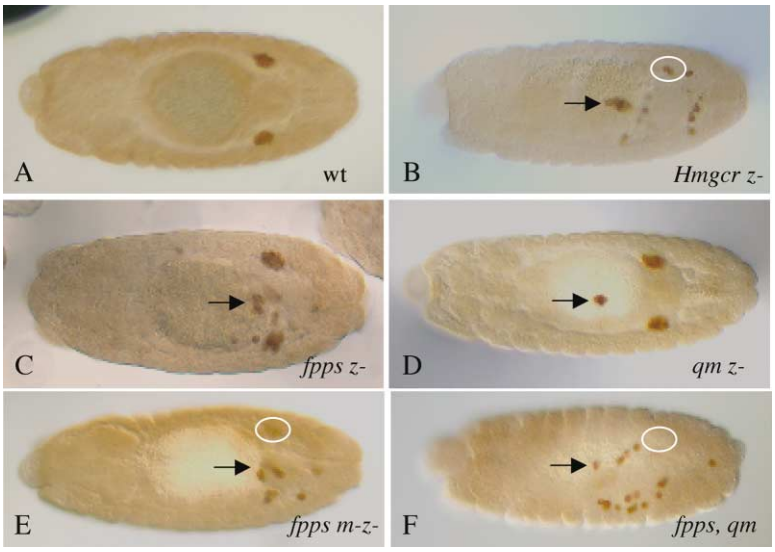


Figure 3. *fpps* and *qm* Mutant Embryos Display Germ Cell Migration Defects

Embryos of the indicated genotypes were labeled with α-Vasa to mark the germline. Dorsal view, anterior to the left in all panels. All embryos are at stage 14.

(A) Wild-type embryo, (B) $Hmgcr^{clb18}$ embryo showing several germ cells that failed to migrate into the gonad (arrow) and few germ cells that correctly migrated and formed gonads (white oval), (C) $fpps^{k06103}$embryo showing several lost germ cells (arrow), (D) $qm^{L14.4}$ embryo showing some lost germ cells (arrow), (E) $fpps^{M-Z-}$ showing many lost germ cells (arrow) and very few germ cells in gonads (white oval), and (F) *fpps;qm* embryo showing many lost germ cells (arrow) and no germ cells in the gonads (white oval).

special group of lipids involved in the posttranslational modification of many proteins, suggesting that isoprenoids may regulate germ cell migration.

fpps and *qm* Mutant Embryos Display Germ Cell Migration Defects

The colocalization of *fpps* and *qm* with *Hmgcr* in the attractive tissue, at the time when germ cells are being attracted toward it, makes these genes good candidates to play a role in germ cell migration downstream of *Hmgcr*. To test their function in germ cell migration, we analyzed the phenotype of strong, possibly null alleles of *fpps* and *qm* mutants (Figure 3). In embryos homozygous for the P-element-induced allele, $fpps^{k06103}$, a small number of germ cells fail to migrate correctly toward the mesoderm and instead remain associated with the dorsal side of the PMG (Figure 3C and Supplemental Table S1 [http://www.developmentalcell.com/cgi/content/full/6/2/283/DC1]). Similarly, in embryos homozygous for $qm^{L14.4}$, a nonsense mutation (Lai et al., 1998), some germ cells fail to migrate into the mesoderm (Figure 3D and Supplemental Table S1). While the general nature of the germ cell migration phenotype is very similar to that observed in *Hmgcr* mutant embryos, the phenotype of either mutant is weaker than that observed in *Hmgcr* mutants. In strong *Hmgcr* alleles (Figure 3B), 10 germ cells on average fail to migrate into the mesoderm (Supplemental Table S1), while 4 germ cells on average fail to migrate toward the mesoderm in $fpps^{k06103}$ or in $qm^{L14.4}$ alleles. Although weak, these phenotypes are significantly different ($p < 0.0001$) from wild-type in which only 1.5 germ cells on average fail to migrate into the mesoderm (Supplemental Table S1).

To determine whether the difference in phenotypic strength is due to a difference in the relative maternal and zygotic contributions of *fpps* and *qm* compared to *Hmgcr*, we generated *fpps* and *qm* homozygous mutant germline clones using the flp-FRT-ovoD system to test the maternal contribution. While embryos lacking either the maternal ($fpps^{M-Z+/-}$) (data not shown), or zygotic ($fpps^{M+/-Z-}$) *fpps* function display a weak germ cell migration phenotype, embryos that lack both maternal and zygotic *fpps* ($fpps^{M-Z-}$), display a very strong germ cell migration phenotype (Figure 3E and Supplemental Table S1). In $fpps^{M-Z-}$ embryos, 13 germ cells, on average, fail to migrate into the mesoderm. To rule out the possibility that the germ cell migration phenotype observed in the $fpps^{M-Z-}$ mutant embryos is due to patterning defects, we stained these embryos with a probe against the retrotransposon 412, which specifically marks the mesoderm and the SGPs. Although 20% of the $fpps^{M-Z-}$ embryos showed patterning defects, 80% of the embryos were correctly patterned and both the mesoderm and the SGPs were properly specified (data not shown).

We also generated females carrying germline clones mutant for *qm*; however, these females are sterile and therefore we could not analyze germ cell migration. Similar to *qm*, germline clones of strong *Hmgcr* alleles also fail to produce mature eggs (Jason Morris, Monique Samuels, Mark Van Doren, and R.L., unpublished data).

Our results suggest that disruption of the mevalonate pathway at the step of isoprenoid biosynthesis disrupts germ cell migration. Within the isoprenoid branch, farnesyl-diphosphate synthase (*fpps*) and geranylgeranyl-diphosphate synthase (*qm*) cooperate in the synthesis of geranylgeranyl pyrophosphate (Figure 1). Thus if geranylgeranylation was a critical step in the production of the germ cell attractant, we would expect that the strength of the germ cell migration phenotype may increase if we simultaneously removed the zygotic contributions of *fpps* and *qm*. Indeed, we observe that *fpps; qm* double mutant embryos show a very severe germ cell migration phenotype (Figure 3F). These results suggest that production of geranylgeranyl pyrophosphate may be a critical step in the production of the germ cell attractant downstream of *Hmgcr*.

Mutations in *fpps* and *qm* Enhance Weak *Hmgcr* Phenotype

Disruption of isoprenoid biosynthesis causes germ cell migration defects that are similar to those of *Hmgcr*. To determine if depletion of isoprenoids contributes directly to the *Hmgcr* germ cell migration phenotype, we

Figure 4. Mutations in Either *fpps* or *qm* Enhance Weak *Hmgcr* Phenotype

Embryos of the indicated genotypes were labeled with α-Vasa to mark the germline. Dorsal view, anterior to the left in all panels. All embryos are at stage 13–14. (A) *Hmgcr*^{01152} embryo showing few lost germ cells. (B) *fpps;Hmgcr* embryo showing many germ cells that did not undergo normal germ cell migration and (C) *qm;Hmgcr* embryo showing several lost germ cells.

tested whether reduction in isoprenoid levels could enhance the phenotype of a weak *Hmgcr* allele (*Hmgcr*^{01152}). About 40% of the *fpps; Hmgcr* double mutant embryos show severe patterning defects; in the remaining 60% of double mutant embryos the mesoderm was patterned correctly according to 412 staining. These well-patterned embryos show very severe germ cell migration defects (Figure 4B and Supplemental Table S2) in which on average 14 germ cells remain on the gut. This phenotype is significantly stronger ($p < 0.0001$) than both *fpps* (4 germ cells lost on average) and *Hmgcr*^{01152} (8 germ cells lost on average, Supplemental Table S2). *qm; Hmgcr* double mutants are well patterned and show on average 12 lost germ cells (Figure 4C and Supplemental Table S2). This phenotype is significantly stronger ($p = 0.0002$) than the ones observed for *qm* (4 germ cells lost on average) and *Hmgcr*^{01152} (8 germ cells lost on average). The enhanced germ cell migration phenotype observed in *fpps; Hmgcr* double mutant embryos points to isoprenoids as mediators of germ cell migration downstream of *Hmgcr*. The *qm; Hmgcr* double mutant phenotype further defines a role for geranylgeranylation in germ cell migration downstream of *Hmgcr*.

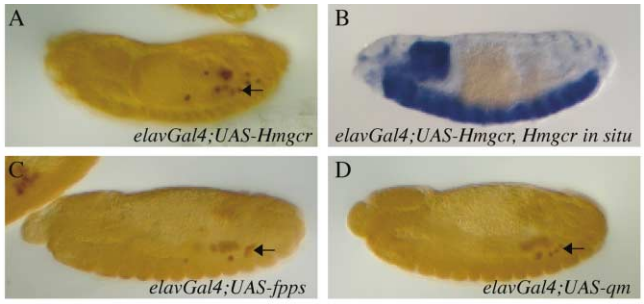
fpps and *qm* Are Sufficient to Attract Germ Cells to the Nervous System

Misexpression of *Hmgcr* in tissues other than the mesoderm is sufficient to attract germ cells to ectopic positions (Van Doren et al., 1998) (Figure 5A). To determine whether enzymes involved in isoprenoid synthesis are also sufficient to attract germ cells to new locations, we misexpressed *fpps* and *qm* in the nervous system using the *elav-GAL4* line (Figure 5B). Gal4-mediated expression of UAS-*fpps* (Figure 5C) and UAS-*qm* (Figure 5D) in the nervous system leads to weak but significant attraction of germ cells to the nervous system, compared to the control UAS-GFP ($p = 0.001$; Figure 5E). However, fewer germ cells are attracted by *fpps* or *qm* expression compared to misexpression of *Hmgcr* (Figure 5E). One plausible explanation is that the effect of overexpressing enzymes downstream in the pathway is limited by the level of HMGCoAr and mevalonate. Our results further suggest that isoprenylation and more specifically geranylgeranylation are limiting and necessary steps in the production of a germ cell attractant.

Geranylgeranyl Transferase Type I Mutant Embryos Display Germ Cell Migration Defects

Our experiments suggest that either geranylgeranyl-PP itself or geranylgeranylation of protein targets mediate the effects of HMGCoAr on germ cell migration. Protein geranylgeranylation requires geranylgeranyl transferases, type I or type II. Type I geranylgeranyl transferase (GGTI) is required for the geranylgeranylation of a wide range of target proteins and is composed of two subunits, α and β. The α subunit is shared with farnesyl transferase and the β subunit is specific for this transferase. GGTII more specifically attaches geranylgeranyl moieties to small RAB GTPases and thus plays an important role in the regulation of secretion.

To test for a function of geranylgeranyl transferase type I in germ cell migration, we analyzed mutants for the



E

Number of germ cells in nervous system	*elavGal4 xUASGFP n=97*	*elavGal4 xUASfpps n=109*	*elavGal4 xUASqm n=108*	*elavGal4 xUASHmgcr n=103*
0 to 2	80%	61%	54%	0%
3 to 4	16%	21%	31%	0%
5 or more	4%	18%	15%	100%

Figure 5. *fpps* and *qm* Are Sufficient to Attract Germ Cells to the Nervous System

Embryos of the indicated genotypes were labeled with α-Vasa to mark the germline except (B), which shows *Hmgcr* RNA expression. Dorsal view, anterior to the left in all panels. All embryos are at stage 13–14.

(A) *elav*Gal4;UAS-*Hmgcr* showing several germ cells being attracted to the nervous system (arrow). (B) High level of *Hmgcr* RNA expression in the nervous system in *elav*-Gal4;UAS-*Hmgcr* (compare to Figures 2B3 and 2B4). (C) *elav*Gal4;UAS-*fpps* and (D) *elav*-Gal4;UAS-*qm*. (C and D) Few germ cells close to the CNS (arrows). (E) Summary of *fpps*, *qm*, and *Hmgcr* misexpression phenotypes. Percentage of embryos having 0 to 2, 3 to 4, or more than 5 germ cells ectopically located in the nervous system. N = total number of embryos analyzed at stage 13 for each genotype.

Figure 6. Geranylgeranyl Transferase Type I Mutant Embryos Display Germ Cell Migration Defects

Embryos of the indicated genotypes were labeled with α-Vasa to mark the germline. Dorsal view, anterior to the left in all panels. All embryos are at stage 14. (A) β GGT-I^{s-483} and (B) β GGT-I$^{xs-2554}$ show some lost germ cells (arrow).

β subunit of geranylgeranyl transferase type I (βGGTI). βGGTI mutants show defects in germ cell migration that are similar to those observed in *Hmgcr*, *fpps*, and *qm* mutant embryos (Figure 6). In embryos mutant for the βGGTI^{s-483} allele, on average 6 germ cells fail to migrate into the mesoderm and remain associated with the PMG (Figure 6A and Supplemental Table S3) and 4 germ cells are lost on average in βGGTI$^{xs-2554}$ mutants (Figure 6B and Supplemental Table S3). This defect is specific to germ cell migration, as the mesoderm and the SGPs are properly specified in these mutants as judged by 412 staining (data not shown). These results identify geranylgeranylation as the necessary step for germ cell migration downstream of *Hmgcr*.

Discussion

Mutants affecting HMGCoAr activity show a dramatic and very specific germ cell migration defect. By catalyzing the synthesis of mevalonate, HMGCoAr is placed at the beginning of a series of enzymatic pathways. The nature of the *Hmgcr* germ cell migration phenotype and the fact that ectopic expression of *Hmgcr* is able to attract germ cells to a new location while not interfering with other aspects of development suggested that some aspect of the HMGCoAr/mevalonate pathway may lead to the production of a component that is both rate limiting and specific for germ cell migration. To identify these components, we have used genetic and genomic methods to determine which part of the pathway downstream of HMGCoAr mediates germ cell guidance activity. Our data establish (1) that squalene and its products such as cholesterol are not produced by the HMGCoAr pathway because the enzymes necessary to synthesize sterols are not present in the *Drosophila* genome, (2) that two enzymes in the HMGCoAr pathway, farnesyl-diphosphate synthase (*fpps*) and geranylgeranyl-diphosphate synthase (*qm*), are coexpressed with *Hmgcr* in the mesoderm, (3) that mutations in *fpps* and *qm* exhibit similar phenotypes to *Hmgcr* and enhance a weak *Hmgcr* phenotype, (4) that misexpression of *fpps* and *qm* can attract germ cells to new sites, and (5) that mutants in geranylgeranyl transferase type I exhibit germ cell migration defects similar to those of *fpps*, *qm*, and *Hmgcr*. Taken together, our data suggest that germ cell attraction is dependent upon geranylgeranyl protein modifications. Similar conclusions have been reached in a study on zebrafish germ cell migration using inhibitors of specific enzymatic steps in the HMGCoAr pathway (Thorpe et al., 2004). We conclude that the HMGCoAr pathway plays a specific and evolutionarily conserved role in germ cell attraction by limiting the production of a geranylgeranylated product.

Cholesterol and Cholesterol-Modified Proteins Are Ruled Out as Mediators of Germ Cell Attraction Downstream of HMGCoAr

We find that several enzymes required for the synthesis of cholesterol from mevalonate are missing from the fly and *Anopheles* genomes. This finding explains earlier observations that showed that insects require sterols in their diet, are unable to convert mevalonate to squalene or cholesterol, and lack the enzymatic activity to produce squalene or cholesterol (Clark, 1959; Clayton, 1964). While these experiments suggested that neither squalene nor cholesterol is synthesized by insects, the basis of the defect remained unclear until our study. Our genomic analysis in both *D. melanogaster* and *A. gambiae* shows that many of the enzymes required for the biosynthesis of cholesterol are not present in the genome of these species and provides an explanation for the need of insects to obtain cholesterol from their diet (Clark, 1959; Clayton, 1964). It is unclear when during evolution and how this deletion occurred as the enzymes encoding for the synthesis of cholesterol are not clustered in one region of the genome. Further systematic evolutionary experiments will be required to clarify this aspect.

In mammals, cholesterol regulates the transcription of HMGCoAr via negative feedback regulation mediated by Sterol Regulatory Element Binding Protein (SREBP) (Goldstein and Brown, 1990; Rawson, 2003; Shimano et al., 1996; Shimomura et al., 1997). Interestingly, while *D. melanogaster* HMGCoAr is insensitive to sterol regulation (Brown et al., 1983; Silberkang et al., 1983), Theopold et al. (1996) found a functional homolog of the Sterol Regulatory Element Binding Protein in *Drosophila* (DSREBP). However, *Drosophila* SREBPs regulate the transcription of genes involved in fatty acid biosynthesis and not in cholesterol or isoprenoid biosynthesis. Also, DSREBP cleavage, which activates the protein, is inhibited by palmitate in *Drosophila* and not by sterols, as in mammals (Rawson, 2003; Seegmiller et al., 2002). These findings, together with our observation that enzymes required for the production of squalene are missing from both the fly and the mosquito genome, suggest that the synthesis and regulation of sterols by the mevalonate pathway may have evolved separately in the vertebrate lineage. This evolutionary diversification is in striking contrast to the apparent conservation of the pathway that leads to production of a germ cell attractant produced by the mevalonate pathway (see below).

The Isoprenoid Branch of the Pathway Plays a Role in Germ Cell Migration

Our results demonstrate that two genes, *fpps* and *qm*, required for the biosynthesis of isoprenoids are coexpressed with HMGCoAr in the mesoderm and that

mutations in these genes have a germ cell migration phenotype very similar to that of *Hmgcr* mutants. Enhancement of a weak *Hmgcr* migration phenotype by *fpps* and *qm* mutants further supports the notion that *fpps* and *qm* act downstream of *Hmgcr* in germ cell migration. However, overexpression of either gene in the nervous system was less effective in attracting germ cells to the new site than overexpression of *Hmgcr*. This result may be expected if one considers that *Hmgcr* is normally only weakly expressed in the nervous system; thus the effect of overexpression of enzymes that rely on HMGCoAr activity for their substrate would be limited by this basal level. The notion that HMGCoAr activity is limiting in the nervous system for production of the germ cell attractant is further supported by the observation that the *Hmgcr* overexpression phenotype is gene dosage dependent: *elav-Gal4* driving one copy of UAS-*Hmgcr* was sufficient to attract on average 12 germ cells to the nervous system whereas two copies of UAS-*Hmgcr* were sufficient to attract up to 20 cells (data not shown). In the mesoderm, on the other hand, *Hmgcr* seems only limiting for germ cell attraction when its activity is reduced beyond 50%, as a germ cell migration phenotype is observed in the weak homozygous mutant but not in heterozygous animals.

A Conserved Role of Geranylgeranylation for Germ Cell Migration

We show that embryos mutant for the β subunit of geranylgeranyl transferase type I display germ cell migration defects that are similar to the ones observed for *Hmgcr*, *fpps*, or *qm* mutants. This suggests that geranylgeranylation of proteins rather than an intermediate of the isoprenoid pathway is necessary for proper germ cell migration in the fly. This conclusion is further supported by the findings of Thorpe et al. (2004) that show that zebrafish embryos treated with geranylgeranyl transferase inhibitors but not with farnesyl transferase inhibitors display strong germ cell migration defects that are similar to the defects observed in fish treated with statins, a family of potent HMGCoAr inhibitors. Taken together, these results favor the idea that geranylgeranyl-PP protein modification plays a specific and conserved role in the production of a germ cell attractant. In *Drosophila*, restriction of expression and consequently activity of the protein modification pathway governed by *Hmgcr*, *fpps*, and *qm* provides the spatial instruction for migrating germ cells. In contrast, enzymes of the isoprenoid pathway have not been found to be expressed specifically in cells that attract germ cells in the zebrafish embryo and uniformly provided mevalonate can rescue the germ cell migration defect after statin treatment. If indeed geranylgeranyl modification of the same protein is critical for germ cell attraction in fly and fish, this difference suggests that in *Drosophila* the protein substrate is expressed in most cells of the embryo and it is the tissue-specific protein modification that provides spatial information to the attractant, while in zebrafish the attractant may be restricted in its expression but remains inactive without geranylgeranyl modification.

The Nature of the Attractant

There are at least two possible scenarios as to how *Hmgcr* is attracting germ cells. In one, geranylgeranylation of a protein would indirectly result in the production of the attractant; in the other, a geranylgeranylated protein is directly attracting germ cells.

In the indirect model, geranylgeranylated proteins would promote the expression and/or secretion of the attractant molecule. Small G proteins such as Ras, Rac, and Rab are well-known prenylated signaling molecules that affect gene expression and vesicle trafficking as well as cell migration and are good candidates to play such an indirect role in germ cell migration (for a review on small G proteins, see Takai et al., 2001). To test whether *Hmgcr* may affect gene expression during imaginal disc development, we performed clonal analysis of *Hmgcr* using the *flp*-FRT system. We observed that whereas flies carrying small clones homozygous mutant for *Hmgcr* in the eye (*ey-flp*) or randomly in the body (*hs-flp*) were normal, flies carrying large mutant clones died as pupae and displayed major body patterning defects (data not shown). We analyzed the expression of markers such as *engrailed (en)* and *wingless (wg)* in the large *Hmgcr* mutant clones in wing discs and observed that the expression of these genes is altered. *en* expression appeared downregulated whereas *wg* was upregulated and patchy (data not shown). These results suggest that *Hmgcr* can indeed affect patterning, either by directly modulating the expression of target genes or perhaps by affecting the intracellular localization of factors required for gene expression. However, in *Hmgcr* mutant embryos, which display strong germ cell migration defects, we were unable to see any evident changes in the expression profile of either *en* or *wg* (data not shown). The same is true in embryos overexpressing *Hmgcr* in the nervous system (data not shown). In fact, zygotic *Hmgcr* seems not to be required for general gene expression or secretion as *Hmgcr* zygotic mutant embryos display no major patterning defects, while showing strong germ cell migration defects. This suggests that germ cell migration is more sensitive to *Hmgcr* levels than is gene expression and secretion and makes the indirect model less likely.

In the direct model, a geranylgeranyl-PP modified protein emanating from the mesoderm would be recognized by germ cells and attract their migration toward the source. This model seems at first rather unconventional as geranylgeranylation in general fosters membrane association of proteins rather than promoting modification of a secreted protein that acts at a distance, as described for other lipid modifications including cholesterol (Nusse, 2003). However, the fission yeast M-factor as well as the budding yeast a-mating factor provide well-characterized examples of prenylated proteins working directly as secreted attractants. Mating in yeast requires the reciprocal interaction of secreted pheromones with membrane bound receptors. The *S. cerevisiae* a-type pheromone is a farnesylated protein that is secreted by a-cells via a nonclassical export mechanism and binds the STE3 (seven-transmembrane G protein coupled receptor) on the surface of α cells (Dawe et al., 1997; Hagen et al., 1986; Hagen and Sprague, 1984; McGrath and Varshavsky, 1989). The binding of the

a-factor to its receptor in the α cell promotes cytoskeletal rearrangements on this cell and the formation of a polarized center: the "shmoo." Farnesyl modification of the a-mating factor is essential for the presentation of the ligand to its receptor and replacement of the farnesyl group by a geranylgeranyl group does not alter significantly the functionality of a-factor (Caldwell et al., 1994; Dawe et al., 1997). In an analogous manner, one may hypothesize that a geranylgeranylated germ cell attractant may be secreted by the mesoderm and recognized by a G protein coupled receptor family member expressed in germ cells. Indeed, G protein coupled receptors have been shown to be required for germ cell migration in zebrafish, mouse, and flies (Ara et al., 2003; Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003, Kunwar et al., 2003). However, at this point genetic evidence in flies and fish suggests that the isoprenoid pathway is not involved in the production or modification of the respective ligands, SDF1 the CXCR4 ligand in zebrafish, or the as yet unknown ligand for the fly GPCR Tre1 (Prabhat Kunwar, A.C.S., and R.L., unpublished data). Another pathway involved in *Drosophila* germ cell migration is controlled by the Wunen proteins, which are homologs of mammalian phosphatidic acid phosphohydrolases, LPP3 (Burnett and Howard, 2003; Starz-Gaiano et al., 2001; Zhang et al., 1997). Biochemical and genetic arguments make a direct functional connection between the Wunen/LPP3 and HMGCoAr pathways unlikely, as the known phospholipid substrates for Wunen/LPP3 are not among the known products of the HMGCoAr pathway and as mutants in *Hmgcr* affect a different migratory step than *wunen* mutants (Burnett and Howard, 2003; Starz-Gaiano et al., 2001).

While the nature of the germ cell attractant downstream of HMCCoAr remains elusive, our findings have considerably narrowed the search and provide a working model that should ultimately lead to the identification of a germ cell attractant for *Drosophila* and possibly beyond.

Experimental Procedures

Fly Stocks

The following *fpps* (farnesyl-diphosphate synthase) and *quemao* (*qm*, geranylgeranyl-diphosphate synthase) alleles were analyzed for germ cell migration defects: $fpps^{k06103}$, a P allele (provided by the Bloomington stock center), and $qm^{L14.4}$, a nonsense mutation (provided by Charles Langley). $fpps^{k06103}$ and $qm^{L14.4}$ are likely null alleles. $fpps^{k06103}$ homozygous embryos do not express *fpps* by in situ hybridization (data not shown). The nonsense mutation in $qm^{L14.4}$ is located in the 12th codon of the putative coding region and is expected, if the protein is stable, to produce a truncated protein lacking all functional domains (Lai et al., 1998).

The following β geranylgeranyl transferase type I mutants were analyzed for germ cell migration: $\beta ggt\text{-}I^{S-483}$ and $\beta ggt\text{-}I^{xS-2554}$ (both provided by the Rubin lab). Both alleles are small deletions that remove the first exon and part of the 5′ regulatory sequences of β geranylgeranyl transferase type I.

fpps; qm double mutants were generated with $fpps^{k06103}$ and $qm^{L14.4.\ fpps}$; *Hmgcr* double mutants were generated with $fpps^{k0610}$ and $Hmgcr^{01152.\ qm}$; *Hmgcr* double mutants were generated with $qm^{L14.4}$ and $Hmgcr^{01152}$.

Since *qm*, *fpps*, *βggt-I*, and *Hmgcr* mutations cause homozygous lethality, all mutant lines were kept in *trans* to a balancer marked with a LacZ expressing transgene *CyO, P(ftz-lacZ)* and *TM3, P(Ubx-LacZ)* for second (*βggt-I, fpps*) and third (*qm*, *Hmgcr*) chromosome mutations, respectively. In parallel to antibody staining for Vasa to mark germ cells, embryos were also stained for β-galactosidase activity, to unequivocally distinguish homozygous mutant embryos from their heterozygous or homozygous balancer bearing siblings. The balancer chromosomes used in this study have no effect on germ cell migration. Around 100 embryos were analyzed for each genotype (see Supplemental Tables S1–S3). Fewer embryos were analyzed for *fpps; Hmgcr* double mutants because this stock produces only few eggs; the double mutant embryos analyzed had a strong and highly penetrant phenotype.

Germline clones of *fpps* were made by crossing $fpps^{k06103}$*FRT 42B/CyO* virgins to *hs-flp; ovo^D FRT42B/CyO* males and heat shocking the progeny at 37°C for 2 hr on 2 successive days during first and second larval instar. Germline clones of $qm^{L14.4}$ were made by crossing $qm^{L14.4}$ *FRT2A/TM3* virgins to *hs-flp; ovo^D FRT2A/TM3* males followed by heat shock as described for *fpps*. To test the phenotype of maternal and zygotic *fpps* mutants ($fpps^{M-Z-}$), *hs-flp*; $fpps^{k06103}$*FRT42B/ovo^D FRT42B* virgins that contained *fpps* germline clones were crossed to *fpp/CyO, P(ftz-lacZ)* males. To test the phenotype of qm^{M-Z-} mutants, *hs-flp*; $qm^{L14.4}$ *FRT 2A/ovo^D FRT 2A* virgins that contained *qm* germline clones were crossed to $qm^{L14.4}$*/TM3, P(Ubx-lacZ)* males. *elav*-Gal4 (CNS, received from Brad Jones) was the Gal4 line used for embryonic misexpression.

Generation of Transgenic Flies

To generate pUAS-*fpps* and pUAS-*qm*, full-length cDNA sequences for *fpps* and *qm* were generated by PCR from an embryonic cDNA library (provided by Chris Yohn). The following primers were used for the amplification: *fpps*-GCTCTAGAGATAGTTACCTGCCGATA ATC and CCCTCGAGGTAATTTAGGAGTCACGTTGG; *qm*-GCTCTA GAGCGACAACAATGGAAGAACTG and GGGGTACCGCCTCTTTG GCTGCTAATTGC.

The amplified cDNAs were digested with NotI and XbaI for *fpps* and KpnI and XbaI for *qm*, followed by ligation into digested pUAS vector (kindly provided by Andrea Brand and Norbert Perrimon). The resulting plasmids were introduced into the *Drosophila* genome using standard P-element-mediated transformation techniques.

In Situ Hybridization and Immunohistochemistry

In situ hybridization was performed as described in Lehmann and Tautz (1994). Embryos were incubated with DIG-labeled RNA probes at 55°C overnight. Probe hybridization was visualized with an alkaline phosphatase conjugated anti-DIG antibody (Roche), followed by treatment with NBT and BCIP. Embryos were mounted in Poly-Bed812 (Polysciences, Inc.) according to Ephrussi et al. (1991) and analyzed with a Zeiss Axiophot using Nomarski optics.

The following probes were made from subcloned cDNA fragments: *CG33009*, *CG10268*, *CG8239*, *CG5919*, *fpps*, *CG2976, CG17565*, *quemao*, *CG12007*, and *CG18627*. Geranylgeranyl transferase type I β subunit cDNA was a gift from the Rubin lab. RNA probes were transcribed with T3 polymerase, after linearization of the subcloned fragments with XbaI or NotI. T7 polymerase was used to transcribe probes after linearization with XhoI or SalI. Transcription reactions were carried out with the DIG RNA labeling kit (Roche).

Antibody staining was performed using the following polyclonal antibodies: rabbit α-Vasa (Helen Zinszner, 1/2500) and rabbit α-β-galactosidase (Cappel, 1/20,000). Antibody detection was performed using α-rabbit biotinylated secondary antibody (Jackson ImmunoResearch) and the ABC Elite Kit (Vector Labs). Embryos were fixed and devitellinized according to the method described in Gavis and Lehmann (1992) with the modification that 1 X PBS was used in place of PEMS during the fixation. The fixation was followed by devitellinization in 100% methanol. Embryos were rehydrated and subjected to antibody staining as described in Eldon and Pirotta (1991). Embryos were mounted as described for in situ hybridization.

For double labeling of embryos with an antibody and in situ hybridization probe, embryos were first antibody stained as described above (except washes were done in 1 X PBS, 0.1% Tween-20, 50 μg/ml heparin, and 250 μg/ml tRNA) and then subjected to in situ hybridization.

Statistical Tests

The significance of the difference between two groups of data was determined using t tests (http://faculty.vassar.edu/lowry/webtext.html).

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Note Added in Proof

In a screen for maternal effect mutations affecting germ cell migration, we identified three EMS-induced alleles of farnesyl-diphosphate synthase *(fpps)* (P.S. Kunwar, A. Renault, H. Sano, and R.L., unpublished data). We used the FRT-flp/OvoD system to induce germline clones in heterozygous females and analyzed germ cell migration in the offspring. As described for the P-element-induced mutation described in the text, homozygous mutant embryos derived from a homozygous mutant germline ($fpps^{M^-Z^-}$) showed a strong and fully penetrant germ cell migration phenotype. The $fpps^{M^-Z^-}$ embryos had no obvious defects in embryonic patterning. The new *fpps* alleles are homozygous lethal, consistent with vital roles during late embryonic and postembryonic development. These results further support the notion that *Drosophila* farnesyl-diphosphate synthase has a specific role in germ cell migration.