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RESEARCH ARTICLE

Role of the ABC transporter Mdr49 in Hedgehog signaling and germ cell migration

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ABSTRACT

Coalescence of the embryonic gonad in *Drosophila melanogaster* requires directed migration of primordial germ cells (PGCs) towards somatic gonadal precursor cells (SGPs). It was recently proposed that the ATP-binding cassette (ABC) transporter Mdr49 functions in the embryonic mesoderm to facilitate the transmission of the PGC attractant from the SGPs; however, the precise molecular identity of the *Mdr49*-dependent guidance signal remained elusive. Employing the loss- and gain-of-function strategies, we show that *Mdr49* is a component of the Hedgehog (*hh*) pathway and it potentiates the signaling activity. This function is direct because in *Mdr49* mutant embryos the Hh ligand is inappropriately sequestered in the *hh*-expressing cells. Our data also suggest that the role of Mdr49 is to provide cholesterol for the correct processing of the Hh precursor protein. Supporting this conclusion, PGC migration defects in *Mdr49* embryos are substantially ameliorated by a cholesterol-rich diet.

KEY WORDS: Cholesterol, *Drosophila melanogaster*, Gonad coalescence, Hedgehog, Primordial germ cells

INTRODUCTION

Proper morphogenesis of complex tissues and organs depends upon the carefully orchestrated migratory behavior of different cell types (Rørth, 2009; Friedl and Gilmour, 2009; Pocha and Montell, 2014). For example, directed cell migration plays a crucial role in the coalescence of the embryonic gonad in *Drosophila melanogaster* (Kunwar et al., 2006). The embryonic gonad consists of two different cell types: primordial germ cells (PGCs) and somatic gonadal precursor cells (SGPs) (Boyle and Dinardo, 1995; Boyle et al., 1997). SGPs and PGCs are formed at different locations and times in the fly embryo and their identity depends upon completely different mechanisms. SGPs are mesodermal in origin and are specified by zygotically active segmentation genes during mid-embryogenesis in parasegments 10-13. By contrast, PGCs are formed at the posterior pole on the outside surface of the embryo at the blastoderm stage and their specification depends upon maternal determinants deposited at the pole during oogenesis (Ephrussi and Lehmann, 1992; Smith et al., 1992).

At the onset of gastrulation, PGCs commence their journey towards the SGPs. This is a multistep process (reviewed by Kunwar et al., 2006; Pocha and Montell, 2014). PGCs are first carried into the interior of the embryo by the midgut invagination. The PGCs then undertake an invasive trans-epithelial migratory step across the mid-gut primordium, and subsequently move along the dorsal surface of the midgut until they split into two groups. Germ cells in each group migrate laterally until they reach the gonadal mesoderm on either side of the embryo and subsequently align with the SGPs in parasegments 10-13. The juxtaposed germline and somatic cells eventually coalesce into the primitive embryonic gonad. A combination of repulsive and attractive cues guides PGC migration through the midgut and toward the SGPs. Once the PGCs exit the midgut, repulsive clues, controlled by *wunen* (*wun*) and *wunen-2* (*wun2*), are thought to direct their bilateral movement away from midgut surface (reviewed by Santos and Lehmann, 2004a). Subsequently, attractive cues produced by the SGPs direct the PGCs towards the lateral mesoderm and then help to promote and maintain their association with the SGPs (Jaglarz and Howard, 1994; Van Doren et al., 1998).

Several lines of evidence indicate that the signaling molecule Hedgehog (Hh) (for a detailed review of Hh signaling, see Ingham and McMahon, 2001; Jiang and Hui, 2008; Gallet, 2011; Briscoe and Thérond, 2013) is one of the key attractants generated by the SGPs to orchestrate PGC migration (Deshpande et al., 2001, 2007, 2009; Deshpande and Schedl, 2005). Ectopic expression of Hh in other tissues like the nervous system induces a subset of PGCs to migrate away from the SGPs towards the source of the misexpressed Hh. Supporting the idea that Hh functions directly as a guidance cue for migrating PGCs is the finding that two cell-autonomous Hh 'receptors', Patched (Ptc) and Smoothened (Smo), are required in PGCs for proper migration. In the absence of the Hh ligand, the transmembrane receptor Ptc inhibits the seven-pass transmembrane protein Smo from mediating signal transduction. Hh binding to Ptc is thought to relieve the negative influence of Ptc, resulting in the relocalization of Smo to cell membranes, and activation of the signal transduction cascade downstream of the Hh signal. Consistent with their reciprocal functions in *hh* signaling, PGCs compromised for maternally derived *ptc* or *smo* activity behave differently. For *ptc*, PGCs clump prematurely on the external surface of the midgut presumably because of ligand-independent signal transduction. For *smo*, PGCs behave as if they are 'signal-blind' and scatter in the posterior of the embryo.

Since *hh* is expressed in a wide range of embryonic tissues and functions in patterning throughout much of development, a crucial question is what mechanism(s) distinguishes Hh produced by the SGPs from Hh produced by other cell types in the mesoderm and ectoderm. One important solution to this puzzle is the pivotal role played by the isoprenoid biosynthesis pathway in Hh signaling. The isoprenoid geranylgeranyl-pyrophosphate (GGPP) is used for geranylation of the Gγ1 subunit of the heterotrimeric G-protein complex GαGβGγ1 (Yi et al., 2006). In order for this complex to function it must be tethered to the membrane by the geranylation of

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the Gγ1 subunit. In *Gγ1* mutants Hh is not properly released from the sending cell and instead accumulates in large puncta distributed along the basolateral membrane (Deshpande et al., 2009). Because activity of the GαGβGγ1 complex depends upon geranylation of Gγ1, the same phenotype is observed in embryos mutant for enzymes in the isoprenoid biosynthetic pathway.

The rate-limiting step in this biosynthetic pathway (and as a consequence the extent of Gγ1 geranylation) is the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate by HMG-CoA reductase (Hmgcr). The activity of Hmgcr is regulated at many different levels, including transcription, translation, post-translational modification, and degradation (Ness, 2014; Sharpe et al., 2014). In mammals, these different regulatory inputs on *Hmgcr* activity are essential for setting the levels of cholesterol and isoprenoids (Edwards and Ericsson, 1999). Although flies lack enzymes needed for *de novo* cholesterol biosynthesis, regulating *Hmgcr* activity is important in flies for controlling isoprenoid production and, as a consequence, in modulating the long-distance basolateral signaling activity of the Hh ligand. That the *Hmgcr* activity normally functions to potentiate the basolateral transmission of Hh, is most clearly illustrated in experiments in which the Hh-GFP fusion protein was ectopically expressed in the embryonic CNS during mid-embryogenesis with or without co-expression of *Hmgcr*. When co-expressed with *Hmgcr*, Hh-GFP spreads into and through the mesoderm, making direct contact with the migrating PGCs. By contrast, in the absence of *Hmgcr*, the transmission of Hh-GFP is largely restricted to the CNS and immediately neighboring cells in the ectoderm (Deshpande et al., 2013). One reason that ectopic Hmgcr activity in the CNS is limiting at this stage of development is that transcription of the *Hmgcr* gene is subject to stage- and tissue-specific regulation. During early embryogenesis, *Hmgcr* is broadly expressed throughout the embryonic mesoderm and there is also a substantial maternal contribution. However, by mid-embryogenesis *Hmgcr* transcripts are restricted to the SGPs. Consistent with this developmental restriction in *Hmgcr* expression, there are severe PGC migration defects in *Hmgcr* mutant embryos (Van Doren et al., 1998; Santos and Lehmann, 2004b).

Although our experiments argue that the *Hmgcr* to isoprenoid biosynthetic pathway is responsible for potentiating the transmission of Hh expressed in SGPs, other mechanisms must also contribute to enhancing the production of this ligand and its long-distance signaling activity. In this regard, cholesterol and cholesterol-derived products deserve special attention. First, cholesterol is required for auto processing of the Hh ligand (Porter et al., 1996). Second, the resulting covalent cholesterol modification then plays a crucial role in the intracellular sorting of the processed Hh peptide, and its subsequent transmission and release from basolateral membranes (Gallet et al., 2003; Callejo et al., 2011). Since flies are cholesterol auxotrophs, they must obtain this sterol through their diet (Clayton, 1964; Santos and Lehmann, 2004a,b). For this reason *hh* signaling would be expected to be sensitive to levels of dietary cholesterol. It should also depend upon the factors that are responsible for uptake of cholesterol stored in the yolk by cells in the intestine, for movement of cholesterol from the intestine to tissues throughout the animal, and finally, for translocation of cholesterol from the surface of the *hh* signaling cell to the subcellular compartment where the Hh precursor protein is processed.

A tantalizing clue in this regard came in the form of a discovery of a new component of germ cell migration – the ABC transporter Mdr49. Ricardo and Lehmann (2009) screened embryos mutant in a small set of ABC transporters for PGC migration defects. Of the ABC genes tested, *Mdr49* was the only transporter that had a significant effect on PGC migration. They found that mutations in *Mdr49* resulted in PGC migration defects, whereas restoration of *Mdr49* activity by ectopic expression in the mesoderm (where it is normally expressed) was sufficient to rescue the migration defects. ABC transporters are conserved from bacteria to humans and they transport a wide range of compounds, including hydrophobic lipophilic molecules (reviewed by Higgins, 1992; Chen and Tiwari, 2011). *Mdr49* shares sequence similarity with Ste6p, another member of the ABC transporter family in budding yeast that is required for the export of the farnesylated a-mating type pheromone. Based on this connection and the known involvement of the *Hmgcr*-isoprenoid biosynthetic pathway in PGC migration, Ricardo and Lehmann proposed that migrating PGCs might be attracted by an equivalent isoprenoid-modified peptide that is exported from SGPs by Mdr49.

However, arguing against this simple model, Mdr49 is not the most closely related ABC transporter to Ste6p in flies (30% identity). The closest fly relative is CG1824, which has 40% identity to Ste6p. There are also many other fly ABC transporters, including MRP (33%), CG4562 (32%), CG31789 (32%) and White (32%) that share more homology with Ste6p than Mdr49. The only known PGC attractant, Hh, is probably far too large to be translocated through the SGP membrane via an ABC transporter (Germann and Chambers, 1998). Moreover, the two known lipid modifications of Hh, namely cholesterol and palmitate, are not isoprenoids. For this reason, we decided to explore other less direct functions for Mdr49 in PGC migration.

One of the closest mammalian relatives of Mdr49 is Mdr1/P-cg1 (42% identity). Like other ABC transporters it is relatively promiscuous and can translocate many seemingly unrelated small molecules such as glutamate, cortisol, aldosterone, methotrexate, colchicine and vinblastine (Choi, 2005; Orlowski et al., 2006; Chen and Tiwari, 2011). Mdr1 has also been reported to function in the translocation of cholesterol from the cytosyolic to the exoplasmic bilayer and from the plasma membrane to the endoplasmic reticulum (Garrigues et al., 2002; Metherall and Waugh, 1996). Moreover, Tessner and Stenson (2000) have shown that overexpression of Mdr1 in intestinal cells upregulates the import of cholesterol. Given the importance of cholesterol in the production of a functional Hh ligand, a compelling hypothesis is that during germ cell migration Mdr49 functions at some step in cholesterol translocation. In this model, this translocation function would be essential for ensuring that the levels of cholesterol in SGPs are high enough so that these cells can produce sufficient amounts of processed Hh to effectively signal the migrating PGCs. Here, we have directly tested this model.

RESULTS

Characterization of *Mdr49* alleles: germ cell migration defects in *Mdr49* mutant embryos

We first determined the frequency of PGC migration defects induced by three independent loss-of-function alleles of *Mdr49*, $Mdr49^{MB04959}$, $Mdr49^{KG08611}$ and $Mdr49^{\Delta 3.16}$ generated by transposable element insertions. Homozygous mutant embryos were screened for PGC migration defects by counting the number of mismigrated or 'lost' germ cells. As can be seen in Fig. S1, there were only minor effects on PGC migration in $Mdr49^{MB04959}$, with most embryos having only 0-3 lost germ cells. An intermediate phenotype was observed for $Mdr49^{KG08611}$. As reported previously, $Mdr49^{\Delta 3.16}$ displayed the most severe defects of the three mutants, with a substantial number of embryos (>50%) showing 5-7 lost PGCs (Ricardo and Lehmann, 2009). As the frequency of PGC

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migration defects was even greater in embryos homozygous for a deficiency, *Df(2F)Mdr49*, which uncovers *Mdr49* (not shown), it seems likely that none of these alleles is a null. However, *Df(2F)Mdr49* has an ~120 kb deletion that removes not only *Mdr49*, but also several other genes. For this reason it is difficult to attribute the strong phenotype solely to loss of *Mdr49*. Consequently, we used either $Mdr49^{\Delta 3.16}$ or $Mdr49^{\Delta 3.16}$/*Df(2R)Mdr49* embryos for most of our experiments.

Feeding excess cholesterol to *Mdr49* flies rescues germ cell migration defects in homozygous mutant *Mdr49* offspring

Since the production of mature Hh proceeds by an internal proteolytic cleavage and the concomitant addition of cholesterol to the C-terminal amino acid of the cleaved peptide, a plausible explanation for the PGC migration defects in *Mdr49* mutants is that *hh* signaling from SGPs is disrupted because there is an insufficient amount of cholesterol to fully process the Hh precursor peptide. Unlike mammals, flies are not able to synthesize cholesterol *de novo* (Clayton, 1964). Instead they depend upon exogenous sources of this lipid. All of the cholesterol in embryos is of maternal origin and is deposited in the yolk during oogenesis (reviewed by Welte, 2015). Following the cellular blastoderm stage, the yolk is incorporated into the midgut by the process of gastrulation. Thereafter, any requirements for exogenous cholesterol must be met by transporting it from the intestine into the mesoderm and through the mesoderm to the ectoderm. The cholesterol must then be imported into the cell and distributed to the appropriate subcellular compartment(s) for processing of the Hh precursor.

Because of the reported involvement of the mammalian Mdr1/P-cg1 transporter in cholesterol translocation, we wondered whether *Mdr49* might be required to ensure that there are sufficient levels of cholesterol in the SGPs for Hh processing. If so, we reasoned that it might be possible to augment the production of a functional Hh ligand in *Mdr49* mutants by increasing cholesterol levels in the embryo. For this purpose, we raised *Mdr49* flies from the embryonic to the adult stage on a cholesterol-rich diet (see Materials and Methods) and then collected progeny from the cholesterol-fed adults. Fig. 1 shows that raising flies on a cholesterol-rich diet partially suppresses the PGC migration defects evident in *Mdr49* embryos. The number of embryos with five or more lost PGCs was reduced by 50% in the progeny of *Mdr49* flies raised on a high-cholesterol diet. We also tested the effects of feeding adults on a cholesterol-rich yeast paste before collecting their progeny for analysis. In this instance, we observed a weaker amelioration (20% reduction in number of embryos with five or more lost PGCs; data not shown) of the PGC migration defects compared with the progeny of control adults that were fed yeast lacking the cholesterol supplement. Interestingly, in this experimental design the extent of rescue improved as the duration of feeding increased.

Genetic interactions between *Mdr49* and the *hh* pathway gene *Gγ1*

The dietary supplement experiments in the previous section support the idea that *Mdr49* functions to ensure that SGPs have sufficient levels of cholesterol for the processing of the Hh ligand. To explore this point further we tested for genetic interactions in embryos trans-heterozygous for the strongest *Mdr49* mutation, $Mdr49^{\Delta 3.16}$, and $G\gamma 1^{1}$, a gene involved in Hh signaling and germ cell migration. While heterozygosity for either $Mdr49^{\Delta 3.16}$ or $G\gamma 1^{1}$ alone had little effect on PGC migration (>90% have 0-3 lost PGCs), the frequency of lost PGCs increases in the trans-heterozygous combination. 20% of the trans-heterozygotes had 4-6 lost PGCs, whereas just over 10% had 7 or more lost PGCs (Fig. 2). Since *Gγ1* activity depends upon the *Hmgcr*-isoprenoid biosynthetic pathway, we also tested for genetic interactions between $Mdr49^{\Delta 3.16}$ and *Hmgcr*. Unlike either $Mdr49^{\Delta 3.16}$ or $G\gamma 1^{1}$, embryos heterozygous for a strong *Hmgcr* allele had a significant frequency of PGC migration defects (nearly equivalent to that seen in $Mdr49^{\Delta 3.16}/G\gamma 1^{1}$ trans-heterozygotes). Perhaps, for this reason there was only a modest increase in the number of lost PGCs in the *Mdr49/Hmgcr* trans-heterozygotes compared with the *Hmgcr*/+ control (not shown).

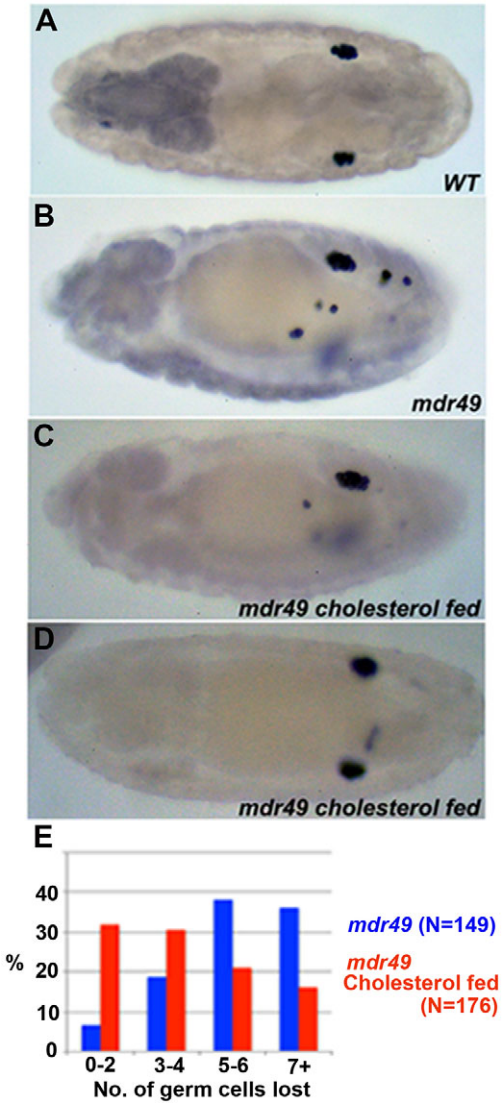

Fig. 1. Germ cell migration defects in *Mdr49* embryos are alleviated by a cholesterol-rich diet. WT and *Mdr49* (*Δ3.16*) adult flies were raised either on regular medium or medium supplemented with cholesterol (see Materials and Methods). Embryos (0-16 h old) derived from flies raised on a cholesterol-rich diet and on a regular diet were collected, fixed and stained with anti-Vasa antibodies. Germ cell migration defects in stage 13-15 embryos are quantified according to the categories in E. (A) Wild-type control. (B) *Mdr49* embryo derived from parents raised on regular food. (C,D) Two different *Mdr49* embryos derived from parents raised on a cholesterol-rich diet. (E) Graphical representation of rescue of germ cell migration defects upon feeding excess cholesterol.

Mdr49 mutations dominantly suppress wing abnormalities caused by gain-of-function allele $hh^{Moonrat}$

In *Drosophila* wing discs, Hh is expressed exclusively in the posterior compartment, and functions as a long-distance morphogen to

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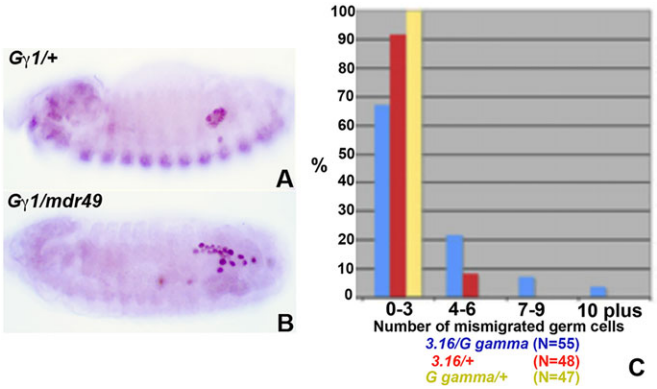

Fig. 2. Genetic interaction between *Mdr49* and components of germ cell migration also involved in Hh signaling. To test whether *Mdr49* exhibits genetic interactions with the *hh* pathway in the context of germ cell migration, we quantified germ cell migration defects in embryos trans-heterozygous for *Mdr49* and *Gγ1* a gene known to be involved in the release of Hh. Stage 13-15 embryos heterozygous for mutations in either gene alone exhibit negligible migration defects; however, germ cell migration defects are observed in embryos trans-heterozygous for *Mdr49* and *Gγ1*. Additionally, in trans-heterozygous embryos (*Δ3.16*/*Gγ1*) gonad size in a majority of stage 15 embryos is substantially reduced to 3-5 cells per gonad compared with wild type (where there are 10-12 cells per gonad). (A) *Gγ1*/+ control embryo. (B) *Gγ1*/*Mdr49* (*Δ3.16*). (C) Graphical quantitation of enhancement of germ cell migration defects. The blue, red and yellow bars represent *Gγ1*/*Mdr49* (*Δ3.16*), *Mdr49* (*Δ3.16*/+) and *Gγ1*/+ embryo counts, respectively.

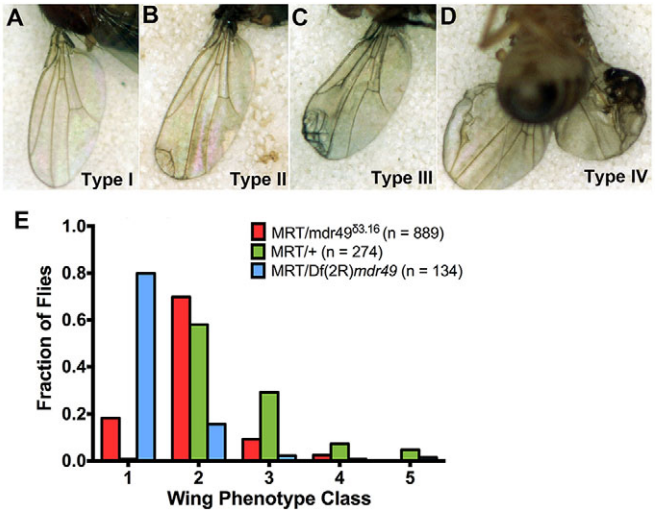

Fig. 3. Dose-sensitive suppression of wing duplication induced by *hh*^{Mrt} and *Mdr49*. In *hh*^{Mrt} flies *hh* is inappropriately expressed in the anterior compartment leading to partial duplication. (A-D) Different classes of *hh*^{Mrt} wing phenotypes. (A) An adult displaying two class 2 wings showing near-normal morphology with only minor defects including altered margins. (B) A mutant fly with a wing with relatively minor defect (class 2). (C) A fly with a wing blade showing substantial problems with wing veins and intermediate wing deformities. (D) A fly with one class III wing (left) and a severely deformed class IV wing. Virgin females carrying each of the four different *Mdr49* alleles were crossed with *hh*^{Mrt} males. The wing phenotypes of the resulting trans-heterozygous progeny were scored as above. (C) Distribution of wing classes observed in different trans-heterozygous mutant combinations as indicated. *hh*^{Mrt} heterozygotes were used as a control. With the exception of the weakest allele, *MB04959*, the *hh*^{Mrt} wing phenotype was suppressed in all trans-heterozygous combinations with *Mdr49*.

organize patterning of the wing blade (Basler and Struhl, 1994). Hh influences wing development by inducing the expression of downstream targets such as *patched* (*ptc*) and *decapentaplegic* (*dpp*) in the anterior compartment. In the absence of *hh* signaling, these target genes are not activated and there are patterning and growth defects along the anterior-posterior (A-P) axis. Conversely, when *hh* is inappropriately expressed in the anterior compartment in the dominant gain-of-function allele *hh*^{Moonrat} (*hh*^{MRT}) *dpp* is ectopically activated causing overgrowth of anterior tissues and partial duplication of distal wing structures (Felsenfeld and Kennison, 1995; Haines and Van den Heuvel, 2000). The severity of the wing patterning abnormalities in heterozygous *hh*^{MRT} flies was variable (see examples in Fig. 3A,B) and was classified into five categories, where class 1 is wild type and class 5 has the most extreme wing duplication/deformation. Since the severity of the wing phenotype depends upon excess Hh signaling in the anterior compartment, it can be dominantly suppressed by mutations in genes that promote *hh* signaling in either the sending or responding compartments.

To test whether *Mdr49* activity is needed to potentiate Hh signaling in the wing disc, we generated *hh*^{MRT} trans-heterozygous combinations with the strongest *Mdr49* allele, *Mdr49*$^{Δ3.16}$ and with *Mdr49* deficiency *Df(2R)Mdr49*. Slightly less than 60% of the control *hh*^{MRT}/+ flies had class 2 wings, 30% had class 3 wings and the remaining flies belonged to either class 4 or class 5 (Fig. 3). Although none of the control *hh*^{MRT}/+ flies had wild-type class 1 wings, about 20% of the trans-heterozygous *hh*^{MRT}/*Mdr49*$^{Δ3.16}$ flies had class 1 wings. There was also a clear shift towards a less severe phenotype in the remaining trans-heterozygotes. There were no class 5 trans-heterozygotes and the frequency of class 3 and class 4 flies was reduced from 30% to 7% in the case of class 3 wings and from 6% to 1% in the case of class 4 wings. An even greater suppression was observed in *hh*^{MRT}/*Df(2R)Mdr49*. In this combination, about 80% of the flies had wild-type wings, whereas the remaining flies were class 2 (Fig. 3). In other experiments, we compared the effectiveness of three *Mdr49* alleles. Consistent with their PGC migration phenotypes, *Mdr49*^{MB04959} was the weakest suppressor, *Mdr49*^{KG08611} was intermediate and *Mdr49*$^{Δ3.16}$ was the strongest (data not shown).

Overexpression of *Mdr49* in the wing pouch leads to enhancement of Ptc levels

While the dominant suppression of *hh*^{MRT} argues that *Mdr49* is needed to potentiate *hh* signaling, it is also possible that reducing *Mdr49* activity suppresses the wing defects in this gain-of-function allele because it functions in the downstream *dpp* signaling pathway. To address this issue, we used a wing pouch-specific driver *nubbin-Gal4* to overexpress *Mdr49* in the wing and then assayed the expression of Ptc, which is a direct target of the Hh signaling pathway. As evident from a comparison of Ptc expression in the control and the *UAS-Mdr49*/*nub-Gal4* wing discs, the total level Ptc protein accumulation increases in the presence of ectopic *Mdr49*. This increase in the level of Ptc protein is quantitated in Fig. 4C, which also shows that in addition to increasing the total amount of Ptc protein, Ptc expression is induced in cells in the anterior compartment that are located farther from the compartment border than in the wild type.

Mdr49 is required for *hh* signaling during wing development

The experiments in the previous section indicate that ectopic *Mdr49* enhances *hh* signaling in wing discs. To test whether *hh* signaling is sensitive to a reduction in *Mdr49* activity we used *nubbin-Gal4* to drive the expression of a *UAS-Mdr49RNAi*. Fig. 5 shows that the *Mdr49* RNAi knockdown indeed led to a reduction in the level of Ptc protein accumulation (compare panels A and C with B and D, respectively). However, the effect of the knockdown of *Mdr49* on

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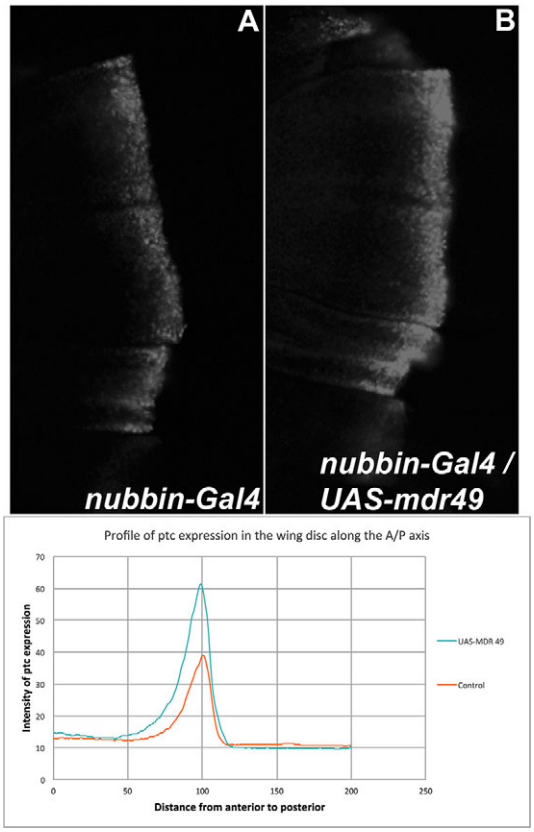

Fig. 4. Overexpression of *Mdr49* in the wing discs leads to expansion of the Hh target Ptc. *yw*; *nubbin-Gal4*; *UAS-GFP* flies were mated with *UAS-Mdr49* flies. (A,B) Wing discs from the third instar progeny larvae were fixed and stained using anti-Patched antibodies. The panels show representative wing discs of the indicated genotype. In both panels anterior is to the left. (A) *yw*; *nubbin-Gal4* (control). (B) *nubbin-Gal4*/*UAS-Mdr49*. (C) Graphical representation of the expansion of Ptc protein across the anterior-posterior axis of wing discs. Both the intensity and the spread of Ptc are enhanced in the experimental samples (*n*=20) compared with the control (*n*=20).

Ptc levels (Fig. 5E) was modest compared with the upregulation of Ptc observed when *Mdr49* is overexpressed.

To assess whether this reduction in Ptc levels leads to phenotypic consequences, we examined the wings of adult flies. Indeed, we found that the area between wing veins L3 and L4 was decreased in both female and male knockdown files (*nubbin-Gal4×UAS Mdr49RNAi*). In several instances, the anterior crossvein was completely missing (Fig. 5G). In addition, the total area of the adult wing was also reduced in the knockdown flies of both sexes (Fig. 5, compare F and G). To confirm the effects of the knockdown, we measured the wing area of d.f.*(2R)Mdr49*/*Mdr49*$^{\Delta 3.16}$ trans-heterozygous flies. As in the case of the knockdown, *Mdr49* mutant flies also displayed reduced wing size and area compared with the wild type (not shown). These wing phenotypes are characteristic of mutations in Hh pathway components such as *smo*, *fused* (*fu*) and *ci*.

Embryos zygotically compromised for *Mdr49* display reduced expression of *hh* pathway genes

The results of the loss- and gain-of-function analysis presented in the previous sections show that *Mdr49* functions to potentiate the *hh* signaling pathway in the wing disc. However, it could be argued that it does not play a similar role in *hh* signaling during embryonic development in the time frame when PGCs are migrating towards the SGPs. To address this possibility, we examined expression of two segment polarity genes, *wingless* (*wg*) and *engrailed* (*en*), that are 'downstream' of *hh* in germband extended embryos. During early embryogenesis, the expression of *wg*, *en* and also *hh* are controlled by gap and pair-rule genes and are independent of each other. However, later in development, during germband extension, their regulation switches to a positive autoregulatory loop. In this loop, the Hh ligand expressed by the anterior-most cells in each parasegment signals to the posterior-most cells in the neighboring parasegment, inducing *wg* expression. In turn, the Wg ligand signals back to the *hh*-expressing cells, promoting the expression of the En transcription factor. En then activates *hh* expression. The effects of this autoregulatory loop first become evident only by mid-embryogenesis (stage 11-12). As a result, in mutants such as *dispatched* or *Hmgcr* that disrupt the *hh* signaling pathway, the pattern of *wg* and *en* expression is relatively normal during earlier stages of embryonic development. However, their expression begins to decay during germband extension and clear differences from the wild type become evident in stages 11-13 embryos.

To determine whether *Mdr49* is required for optimal *hh* signaling during mid-embryogenesis, we probed stage 11-15 *Mdr49*$^{\Delta 3.16}$/*Df(2R)Mdr49* mutant embryos using antibodies against En and Wg. Fig. 6 shows that both En and Wg protein levels were reduced in stage 11 *Mdr49*$^{\Delta 3.16}$/*Df(2R)Mdr49* mutant embryos compared with the control. Similar results were observed in embryos homozygous for *Mdr49*$^{\Delta 3.16}$ and *Df(2R)Mdr49* mutant embryos (not shown). However, as was observed for PGC migration, the reduction in En and Wg expression was less pronounced in embryos homozygous for the *Mdr49*$^{\Delta 3.16}$ allele compared with the trans-heterozygous combination. Likewise, the reduction in En and Wg was greater in embryos homozygous for *Df(2R)Mdr49*.

Mutations in *Mdr49* result in inappropriate sequestration of Hh ligand in synthesizing cells in the embryonic epidermis

The results in the previous section support the idea that reducing *Mdr49* activity during embryogenesis compromises the range and/or strength of *hh* signaling. To confirm this suggestion, we examined the pattern of Hh protein accumulation in the ectoderm of germband extended *Mdr49*$^{\Delta 3.16}$/*Df(2R)Mdr49* and *Df(2R)Mdr49* embryos. Since the cholesterol modification is crucial for the processing and subsequent release/transmission of the Hh ligand, our hypothesis would predict that the distribution of Hh protein would be abnormal when *Mdr49* activity is reduced. The confocal images shown in Fig. 7A-D show that this is indeed the case. There are two striking differences in the pattern of Hh accumulation in the *Mdr49* mutant embryos. First, *hh*-expressing cells accumulate higher levels of Hh protein. Second, Hh protein is not properly transmitted from the expressing cells to the neighboring cells in the parasegment. To quantitate the differences in Hh protein accumulation, we generated a series of sequential scans across the parasegment (see Fig. S2). In *Mdr49* mutant embryos, Hh protein was retained in Hh-expressing cells, whereas only relatively low levels were transmitted into the parasegment (Fig. S2, right-hand side).

DISCUSSION

Mdr49 is a Hh pathway component

In the studies reported here, we investigated a possible connection between *Mdr49*, a recently discovered component of the germ cell migration pathway, and Hh function. The first indication that there is a close connection between *Mdr49* and *hh* signaling came from the discovery that the PGC migration defects evident in embryos compromised for *Mdr49* activity can be rescued by feeding their

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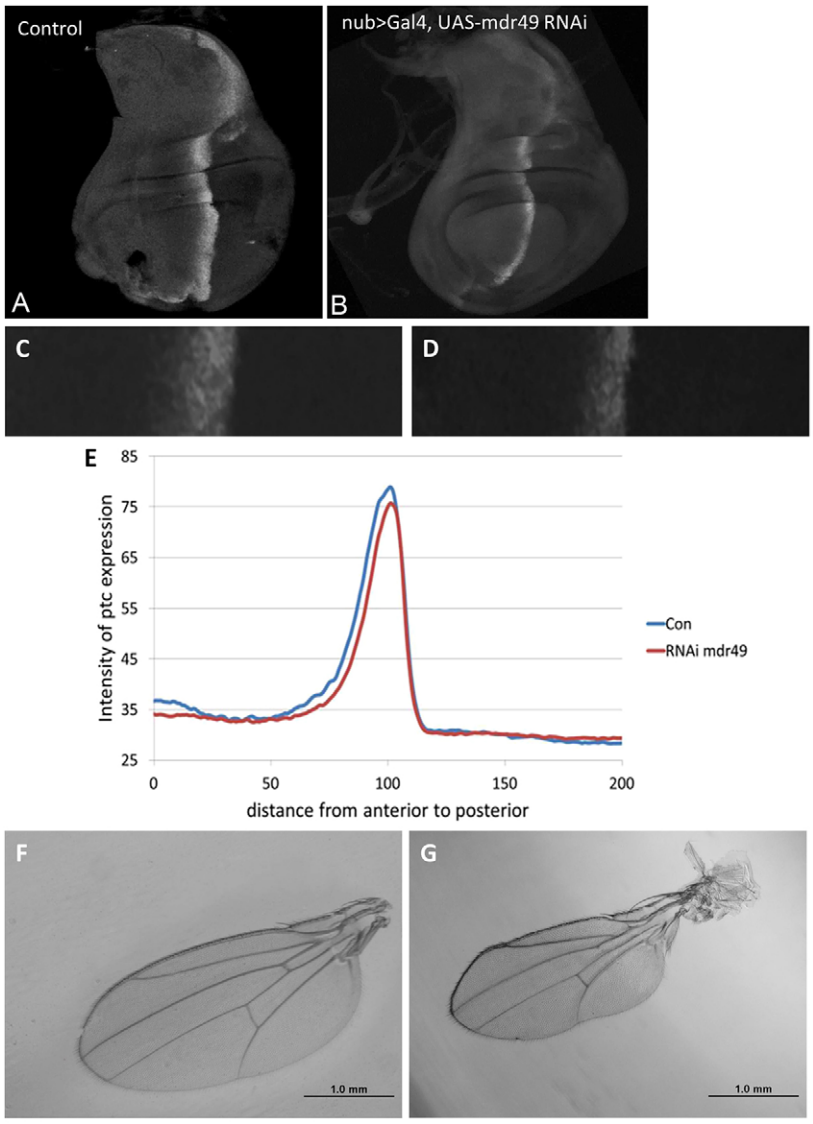

Fig. 5. Reduction in *Mdr49* activity decreases Ptc levels and results in corresponding adult wing abnormalities. *yw*; *nub-Gal4* flies were mated with *UAS-RNAi-Mdr49* flies. (A-D) Wing discs from the third instar larvae were analyzed after fixation and staining with anti-Ptc antibodies. The representative wing discs of the defined genotype are shown (anterior is to the left, 20×). (A) control wing disc (*nub-gal4*). (B) *Mdr49* knockdown using *UAS-Mdr49-RNAi*; *nub-gal4*/*UAS-Mdr49-RNAi*; *UAS-dicer*/*UAS-Mdr49-RNAi*. To improve the efficiency of the knockdown, two different transgenes of *UAS-Mdr49-RNAi*, inserted on the second and third chromosome, respectively, were introduced in a stock. (C) Magnified view (40×) of Ptc expression in control wing disc (*nub-gal4* only). (D) Magnified view (40×) of the Ptc expression from the experimental wing disc shown in B. (E) Quantitation of Ptc expression levels in the control (blue curve) and experimental *Mdr49* knockdown discs (red curve). Twenty discs of each genotype were analyzed. (F) Control adult wing (*nub-gal4*; *UAS-dicer*). (G) *Mdr49* knockdown adult wing: *nub-gal4*/*UAS-Mdr49-RNAi*; *UAS-dicer*/*UAS-Mdr49-RNAi*.

parents a high-cholesterol diet. Since the production of a functional Hh ligand requires the cleavage of the precursor polypeptide that is catalyzed in part by a cholesterol-dependent nucleophilic attack, the most plausible explanation for this finding is that the function of *Mdr49* in PGC migration is to ensure that the SGPs are able to generate sufficient levels of functional cholesterol-modified Hh. Consistent with this hypothesis, our experiments demonstrate that *Mdr49* is a component of the Hh signaling pathway in two independent contexts – the wing disc and the embryonic ectoderm. In the wing disc, ectopic expression of *Mdr49* potentiates Hh signaling, most notably by increasing not only the amplitude of the response but also the distance that Hh signal is able to travel effectively. *Mdr49* mutations dominantly suppress the phenotypic effects of the gain-of-function *hh* allele, hh^{MRT}, whereas *hh* signaling in a wild-type background is weakened by RNAi knockdown. In the embryo, *Mdr49* mutations disrupt the *en*→*hh*→*wg*→*en* positive autoregulatory loop during mid-embryogenesis. Finally, we have shown that the release of Hh from *hh*-expressing cells and its transmission to the neighboring cells in the ectoderm is compromised in *Mdr49* mutant embryos.

A question that remains to be answered is the precise function of *Mdr49* in cholesterol transport. Is it needed in the SGPs, in which case its function will be cell autonomous? Or does it function in the mesoderm, in which case its germ cell migration function would likely be cell non-autonomous? Based on what is known about the activity of the *Mdr49* homolog in mammals, Mdr1/P-cg, a cell-autonomous function, for example in the transport of cholesterol from the plasma membrane of SGPs to the ER, would be plausible. In either instance, yet other ABC transporters (e.g. ABCG1 and ABCA1) will probably be needed to ensure that the SGPs have a sufficient supply of cholesterol for the processing of the Hh precursor (Phillips, 2014). In this context, it will also be of interest to determine whether other proteins involved in the transport or sequestration of cholesterol, such as the fly Niemann-Pick type C proteins, Npc1a and Npc1b (Huang et al., 2005; Fluegel et al., 2006; Voght et al., 2007) are also required for both *hh* signaling and PGC migration.

Function of Mdr49 in PGC migration

Attractive and repulsive signals generated by somatic tissues guide the embryonic PGCs to their ultimate destination, the SGPs. One of the PGC attractants produced by the SGPs is the signaling molecule Hh (Deshpande et al., 2001). While it is known that Hh (or Sonic Hh) can direct cell migration in other biological contexts (Charron

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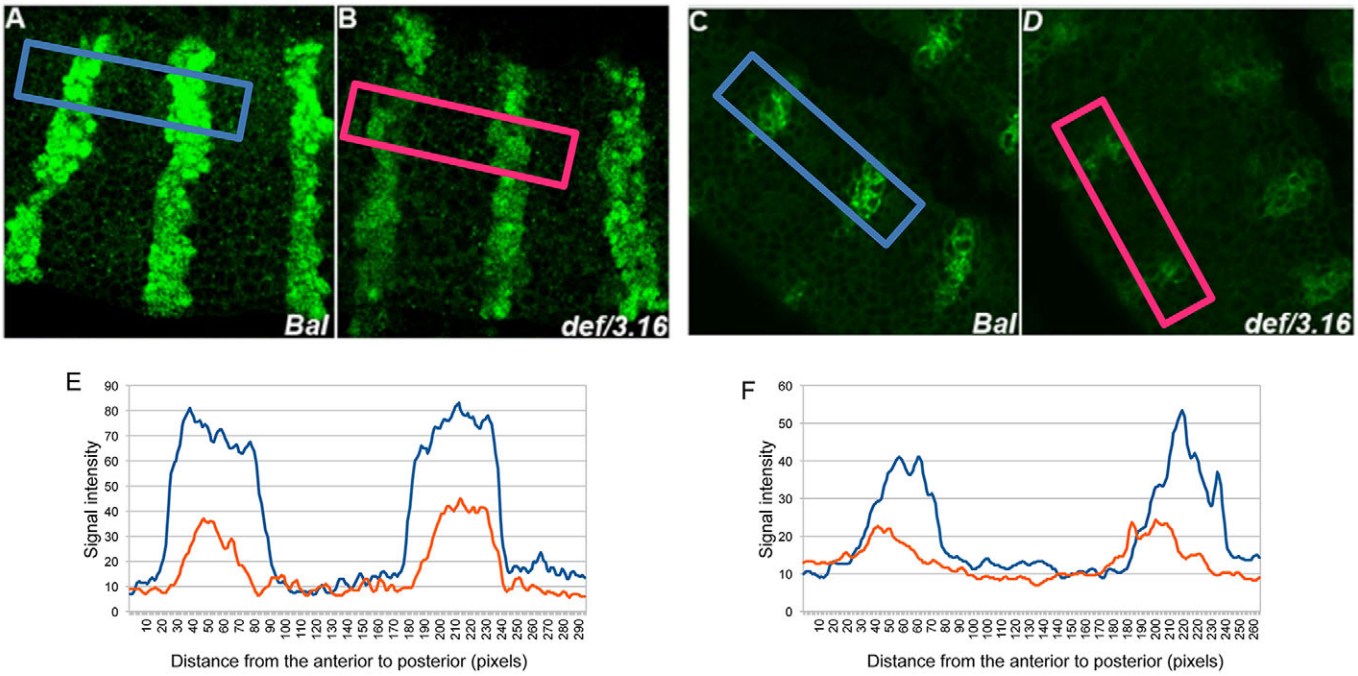

Fig. 6. Loss of *Mdr49* leads to diminished expression of *hh* target genes in the germband extended embryos. To test whether compromising *Mdr49* function downregulates *hh* signaling, fixed embryos of the following genotypes were stained using En and Wg antibodies: *Δ3.16*/Df(2R)*Mdr49*, Df(2R)*Mdr49*/*CyO en-lacZ* and *Δ3.16*/*Cyo en-lacZ*. Heterozygous embryos carrying the balancer chromosome were identified using β-galactosidase-specific staining. Clear reductions in En (A,B) and Wg (C,D) levels are observed in the *Δ3.16*/Df(2R)*Mdr49* (*n*=10) embryos (B,D) as opposed to balancer carrying heterozygous control (A,C) (*n*=10). The reduction in signal intensity was quantitated using ImageJ for En (E) and Wg (F). It should be noted that reduction in signal intensity for both the antigens was observed consistently in the mutant embryos (red) and the level of signal in the control embryos was always higher (blue).

et al., 2003; Yam et al., 2009), significant questions remain about how this ligand is able to function as a guidance cue.

For example, although Hh is expressed in the SGPs, this is not the only source of Hh in the embryo that could potentially signal to the migrating PGCs during mid-embryogenesis. In the mesoderm, the fat body precursor cells, which are located in more anterior parasegments (PS 5-9) than the SGPs, also express Hh (Deshpande et al., 2001; Renault et al., 2009). Moreover, in the overlying ectoderm, the cells that define the anterior border of each parasegment synthesize Hh (Tabata and Kornberg, 1994; Struhl et al., 1997). Unless there are mechanisms that effectively differentiate the Hh produced by the SGPs from these other sources, one would expect that that Hh expressed by the fat body precursor cells and cells in the ectoderm could potentially signal to the PGCs and direct their migration away from the SGPs. The same problem of distinguishing the bona fide source of Hh/Sonic Hh from sources that could provide misleading guidance cues exists in other contexts where this signaling molecule is thought to function in directed cell migration.

One mechanism that could help distinguish Hh produced by the SGPs from other sources in the embryo would be a ‘combinatorial’ migration signal consisting of Hh (or Sonic Hh) and one or more additional signaling molecules (Ricardo and Lehmann, 2009). A mechanism of this type for PGC migration was suggested by the discovery that the ABC transporter *Mdr49* is required for PGC migration. While a combinatorial code specific for SGP-PGC communication remains a very attractive possibility, no other PGC attractant besides Hh has been identified despite nearly two decades of research. However, several factors that are expected to specifically potentiate Hh signal emanating from the SGPs but not other *hh*-expressing cells in the embryo have been discovered.

As described above, the release of the lipidated Hh ligand from basolateral membranes is limited by geranylation of the Gγ1 subunit of the heterotrimeric G protein complex, GαGβGγ1. The extent of Gγ1 geranylation is, in turn, determined by the rate-limiting step in the isoprenoid biosynthetic pathway – the synthesis of mevalonate by Hmgcr. During mid-embryogenesis, when the SGPs are sending Hh signals to the migrating PGCs, the only cells in the embryo expressing *Hmgcr* are the SGPs. Though much less precise, a transcription-based mechanism for potentiating Hh signals produced by the SGPs is also in place for the *shifted* (*shf*) and *Mdr49* genes. *shf* encodes an extracellular protein that functions in Hh transmission through the basolateral heparan sulfate proteoglycan. While the transcription of *shf* is not restricted to SGPs, its expression is controlled by *Abdominal-B* and it is specifically upregulated in mesodermal cells in the posterior parasegments of the embryo during mid-embryogenesis (Zhai et al., 2010; Deshpande et al., 2013).

Although we can not formally exclude the possibility that *Mdr49* has a novel cholesterol-dependent but Hh-independent function in the mesodermal SGPs, the experiments that we have presented here would be most consistent with the idea that *Mdr49* functions to potentiate the Hh signals emanating from the SGPs. Because Ricardo and Lehmann (2009) have shown that *Mdr49* transcription is restricted to mesodermal cells at this stage as well, this would place *Mdr49* in the same category as two other components of the *hh* signaling pathway – *Hmgcr* and *shf*. A plausible inference from these observations is that the potentiation of the Hh signals produced by the SGPs depends primarily on the restricted expression of *Hmgcr*; however, the effects of *Hmgcr* are further augmented by the mesoderm-limited expression of *shf* and *Mdr49*. In this context, it seems likely there will be yet other genes that help to augment Hh signals emanating from the SGPs.

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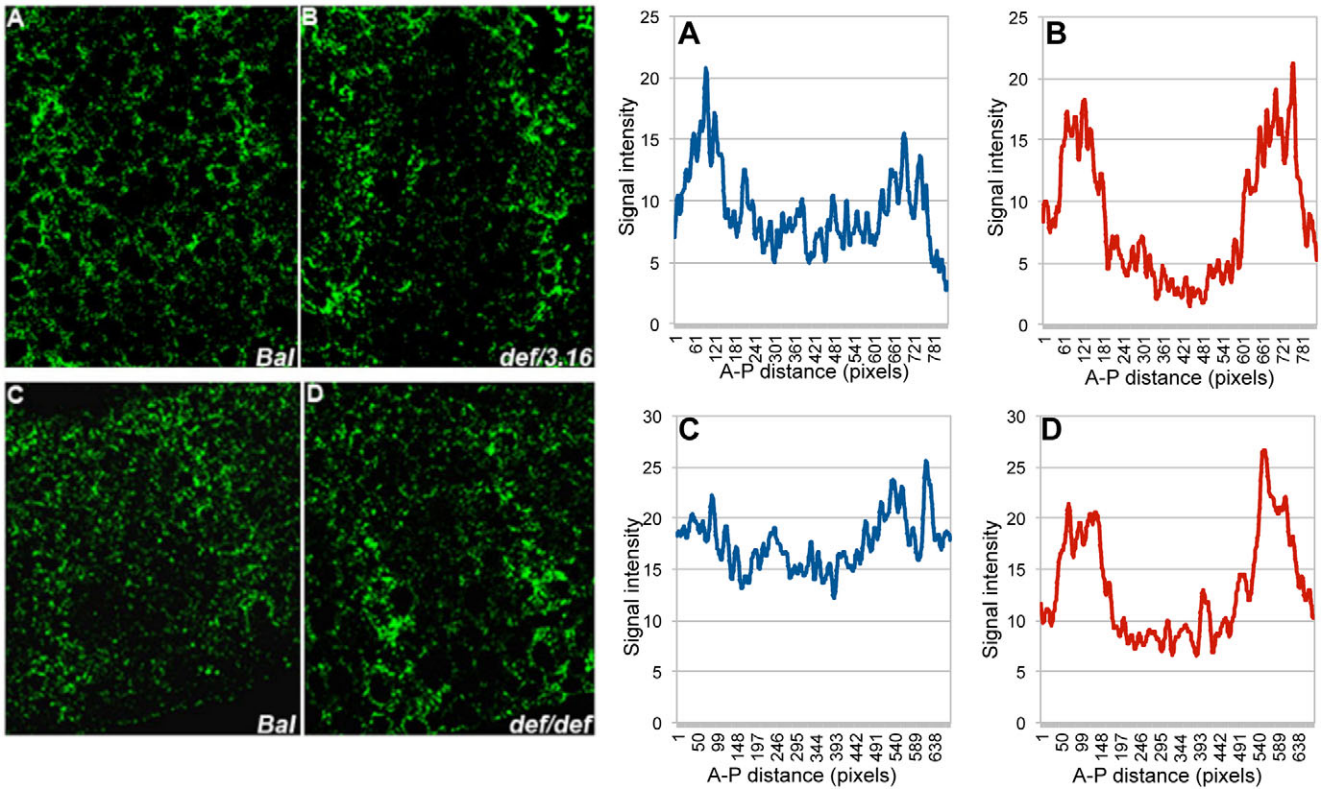

Fig. 7. Hh ligand is inappropriately sequestered upon reduction in *Mdr49* activity. Compromise of *Mdr49* function influences Hh protein distribution across the ectodermal segments in germband extended embryos. Fixed embryos of the following genotypes were stained using anti-Hh antibody (green): *Δ3.16*/Df(2R)*Mdr49*, Df(2R)*Mdr49*/*CyO en-lacz* and *Δ3.16*/*Cyo en-lacz*. Heterozygous embryos carrying the balancer chromosome were identified using β-galactosidase-specific staining (not shown). Several pairs of similarly aged embryos were analyzed using identical settings. Hh ligand transmission is clearly affected in the embryos compromised for *Mdr49* (B,D) as opposed to the heterozygous controls (A,C). In the mutant embryos, the inter-stripe staining is reduced and the Hh ligand seems to be accumulated in the synthesizing cells. (A′-D′) Graphical representation showing quantitation of the signal in A-D (see Fig. S2 for details of quantitation).

Since most of the genes that have been implicated in SGP-dependent migration of PGCs have turned out to function in the Hh signaling pathway, it is possible that the only guidance molecule produced by the SGPs is Hh. In this case, instead of a 'combinatorial' migration signal, guidance specificity would be achieved by combining multiple mechanisms for enhancing the Hh signals emanating from the SGPs. An important question is whether a similar combinatorial enhancement strategy is also used in other systems in which Hh/Sonic Hh has been implicated as a guidance cue. In mammals, at least some of the molecules that potentiate Hh signals from the SGPs in fly embryos have functions in other signaling pathways (e.g. Shifted: Glise et al., 2005; Gorfinkiel et al., 2005). This means that even if the same strategy is used to generate specificity in mammals, the precise mechanisms for enhancing Sonic Hh signaling from the bona fide source can not be the same as that used in flies. Another interesting question that has remained unresolved is how the Hh ligand influences the motility of PGCs. The transcription-based canonical signal transduction pathway is likely to be inactive in the migrating PGCs. Future experiments will thus explore the non-canonical pathway(s), downstream of Hh, that are specifically involved in cell migration.

MATERIALS AND METHODS

Immunohistochemistry

Embryos

Embryo collection and staining were performed essentially as described previously (Deshpande et al., 2013). Vasa (from Paul Lasko, Department of Biology, McGill University, Canada) and Hh (from Tom Kornberg, University of California, San Francisco, USA) antibodies are rabbit polyclonal antibodies. Both were used at 1:500 dilution. Engrailed and Wingless antibodies (Developmental Studies Hybridoma Bank) are mouse monoclonal antibodies and were used at 1:10 dilution. β-galactosidase antibody was either a rabbit polyclonal [MP Biomedicals (previously Cappel), 55976; 1:1000] or a mouse monoclonal antibody (Developmental Studies Hybridoma Bank; 1:10). GFP antibody is a rabbit polyclonal (Torrey Pines Biolabs, ABIN2562810; 1:1000). Secondary antibodies were purchased from Molecular Probes and used at 1:500. For confocal analysis 40× magnification was used in almost all the instances, and images were collected using identical settings for the control and experimental samples on a Nikon A1 microscope with GaAsP detectors. Multiple pairs of wild-type (sibs) and mutant embryos were imaged in each case and representative examples are presented.

Wing discs

Imaginal discs from third instar larvae were fixed and stained by standard techniques. The specific primary antibodies used were: mouse anti-ptc [1:100; DSHB *Drosophila* Ptc (Apa 1)-s]. Secondary antibodies were conjugated with Rhodamine Red-X or Cy5 (1:400; Jackson Labs). Images were taken on a TE2000-E confocal microscope (Nikon). Figures were edited using Adobe Photoshop 7.0.

Mutant and misexpression analysis

Gγ1 mutant stocks, $Gγ1^{N159}$ and $Gγ1^{k0817}$, were obtained from Fumio Matsuzaki (RIKEN Center for Developmental Biology, Kobe, Japan). The *Mdr49* mutants and overexpression stocks were obtained from Ruth Lehmann (Skirball Biomedical Institute, NYU, USA). The UAS-

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RNAi lines specific for *Mdr49* (Bloomington #32405) and *Gal4* stocks used for the misexpression studies were from the Bloomington Stock Center (*patched-Gal4*, *UAS-β-galactosidase*, *hh-Gal4*/*TM6 Ubx-lacZ*). In most experiments, males carrying two copies of the *UAS* transgene were mated with virgin females carrying two copies of the *Gal4* transgene. Embryos derived from the cross were fixed and stained for subsequent analysis. The genotypes of the embryos were unambiguously determined by using the balancer chromosomes marked with either GFP or β-galactosidase.

The following stocks were used for analysis of wing discs: *yw; nub-Gal4; UAS-GFP*, *UAS-dicer-2; nub-Gal4* (Bloomington #25754), *UAS-Mdr49* (a gift from Ruth Lehmann), *UAS-RNAi-Mdr49* (VDRC#108237, #VDRC42513, Bloomington #32405). Transgenes were expressed using the *Gal4-UAS* binary system.

Cholesterol feeding assay

Drosophila food containing cholesterol was prepared as described previously (Fluegel et al., 2006). Cholesterol (Sigma) was used to prepare a stock solution (30 mg/ml) in ethanol. The final concentration was 200 ng/ml. To achieve uniform dispersion of cholesterol, food was mixed thoroughly after cooling it down to 65°C before pouring into either vials or bottles.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization and methodology: G.D., O.G. and P.S. Formal analysis and investigation: all the authors. Writing of original draft: G.D. Review and editing: G.D., O.G. and P.S. Funding acquisition: G.D., P.S. and O.G.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.133587.supplemental

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