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EDITOR

Hedgehog does not guide migrating germ cells.
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Hedgehog (Hh) plays an important role as a secreted, diffusible signal that controls cell fate specification during Drosophila embryogenesis, oogenesis and imaginal disc development (Basler and Struhl, 1994; Heemskerk and DiNardo, 1994; Lane and Kalderon, 1994). Recently it has been proposed that in addition to its role as a morphogen, Hh may also act as a diffusible chemoattractant that guides primordial germ cells to the somatic gonad. In a study published by Deshpande et al. (2001), three different types of experiments were used to analyze Hh function in germ cell migration. First, they report that *hh-LacZ* is expressed in the somatic gonadal mesoderm, a tissue that attracts germ cells and aggregates with them to form the embryonic gonad (Boyle and DiNardo, 1995; Broihier et al., 1998). Second, Deshpande and colleagues report that ectopic expression of *hh* using different GAL4 drivers leads to germ cell migration defects and in the case of the *twi-GAL4* driver to attraction of germ cells to the site of ectopic *hh* expression. Third, the authors report that depletion of the maternal

components of the Hh signaling pathway (using germ line clones homozygous for mutations in the Hh receptors Patched (Ptc), the co-receptor Smoothened (Smo), or the downstream effectors Fused (Fu) and PKA) leads to germ cell migration defects in the progeny. As outlined in detail below, we have carried out similar experiments to those described by Deshpande et al. (2001). In contrast to the conclusions drawn in that paper, our experiments do not provide any evidence to support the notion that Hh has a direct role in the guidance of migrating germ cells.

Hh is not expressed in the early gonadal mesoderm.

We have used RNA in situ hybridization to analyze the expression of *hh* in the Drosophila embryo. As described previously, *hh* is expressed in the ectoderm and the hindgut (Mohler and Vani, 1992). We do detect weak *hh* RNA staining in the mesoderm in a segmental pattern during stages 10-12 (Fig. 1). We also analyzed the *hh-LacZ* enhancer trap line used by Deshpande et al. and found *hh-LacZ* expressed in a segmental pattern in the mesoderm. We detected co-expression of LacZ and the gonadal mesoderm marker Clift in the gonadal mesoderm during stage 11 and 12. However, while Clift is specifically expressed in parasegment 10-12 (Fig. 1B, arrowhead, Boyle et al., 1997), the segments that give rise to gonadal mesoderm, *hh-LacZ* is expressed at similar levels in every segment (Fig 1C lines). Deshpande et al. (their Fig. 1) report partial co-localization with Clift in the mesoderm, but fail to mention that while Clift is expressed in the mesoderm of only parasegments 10-12, Hh is expressed in each segment at similar levels.

Ectopic expression of *hh* does not lead to germ cell migration defects

To test whether misexpression of *hh* causes germ cells to move towards *hh* expressing cells, we ectopically expressed *hh* in three different tissues: the nervous system (using *elav-GAL4*), a segmentally repeated pattern in the ectoderm (*hairy-GAL4*), and in the mesoderm (*twist-GAL4* or *twi-GAL4*, *24B-GAL4* double driver). We also tested three different *UAS-hh* lines, a *UAS-hh* line from M. Frasch, a *UAS-hhN* line from P. Beachy and the *UAS-hh* line used in the experiments by Deshpande et al. As a positive control for the biological activity of these lines, we tested the UAS lines for their effectiveness in Hh signaling during disc development using a *pannier-GAL4* driver. The *UAS-hh* construct caused notum and thoracic bristle defects as expected, while the *UAS-hhN* construct caused lethality with the *pannier-GAL4* driver used (Lee and Treisman, personal communication; Heitzler et al., 1996; Porter et al 1996). As summarized in Fig. 2, for each experiment we counted the number of germ cells lost per embryos, and compared these results with a negative control (*twi-Gal4* driver alone, *UAS-hh* alone) and with a positive control *UAS-HMGCoA reductase* (*hmgcr*, Van Doren et al., 1998b). While we occasionally observe an embryo with migration defects, we see similar defects in the experimental and control embryos. Furthermore, we do not see any attraction of germ cells to the particular region/tissue in which *hh* is mis-expressed. The failure to observe any effect on germ cell migration after ectopic expression of Hh is in stark contrast to the results obtained with the same type of experiments using a *UAS-HMGCoA reductase* transgene (Van Doren et al., 1998b, see Fig. 3). We showed in these experiments that germ cells move to the region of high HMGCoA reductase expression. For the *hairy-GAL4* and *elav-GAL4* drivers, Deshpande et al. noted germ cell migration

defects but no specific colocalization of germ cells and ectopic *hh* expression. Deshpande et al. report an association of germ cells with the mesodermal "cells just to the right of the posterior midgut invagination" after pan-mesodermal expression (Fig. 3 of Deshpande et al., 2001). The authors conclude that these results suggest a direct attraction of germ cells to regions with ectopic *hh* expression. However, *hh* is normally expressed at high levels at the posterior of the embryo (see Fig. 1), thus one may expect germ cells to move there even without misexpression. Furthermore, pan-mesodermal expression using *twi-GAL4* causes broad defects in mesodermal segmentation (Azpiazu et al., 1996) and thus it seems unlikely that this pattern of *hh* misexpression would evoke attraction of germ cells to a specific site. Indeed, after global *hmgcr* expression in the mesoderm we observed broad germ cell migration defects, but no attraction to specific cell groups (Van Doren et al., 1998b).

Mutant analysis of the Hh pathway reveals no direct effect on germ cell migration

hh mutant embryos show patterning abnormalities which preclude the direct analysis of Hh as a germ cell attractant in these embryos (Moore et al., 1998b). To determine whether activation of the Hh signaling pathway in germ cells may be required for normal germ cell migration, Deshpande et al. analyzed mutants in the Hh receptors, *patched* and *smoothened*, and the effectors *fused* and *DCO,* which encodes Drosophila protein kinase A (PKA). Deshpande et al. observed germ cell migration defects in embryos derived from females that carried germ line clones homozygous mutant for the respective gene (*ptc*, *smo, DCO/PKA* and *fu)* and were fertilized with wild-type sperm. Since zygotic transcription cannot be detected in germ cells until shortly before germ cell migration is

initiated (Van Doren et al., 1998a; Zalokar, 1976), the rationale is that the Hh signaling pathway would have to be provided maternally to the germ cells.

So far members of the *hh* pathway have not been reported to be expressed in primordial germ cells. We conducted two types of experiments to analyze the effect of mutants in the Hh-signaling pathway on germ cell migration. First, we generated germ line clones for an allele of *smo* (smo^{X43}, referred to as smo^{2} in Deshpande et al.). Figure 4 and 5 show that we failed to find any significant deviation from normal germ cell migration in embryos derived from homozygous germ line clones, which had received a wild-type smo^{+} gene copy from the father. Within the same staining reaction we observed half of the embryos (n=104), which presumably received a smo^{+} allele from the father, develop into normally segmented larva with properly formed gonads. Half of the embryos (n=101), which presumably received the mutant smo^{-} allele from the father, developed abnormally and in cuticle preparations showed the "lawn" cuticle phenotype typical for *hh* or *smo* mutants (see Fig.4, van den Heuvel and Ingham, 1996). The strong patterning phenotype observed in embryos lacking both maternal and zygotic *smo* function demonstrated that the lines used are defective in *hh* signaling.

We were unable to analyze the progeny of germ line clones mutant for *DCO*/PKA since no eggs were produced over many days of collection. As reported previously by Lane et al. (1994), homozygous mutant PKA germ line clones show defects in the microtubule organization of the oocyte and fail to complete oogenesis. As a consequence of failed PKA signaling, *oskar* RNA, the germ cell determinant, is localized to the middle of the

oocyte and eggs are not produced. Despite these problems, Deshpande et al. were able to analyze germ cell migration defects in the progeny of PKA mutant germ line clones. Furthermore, they report an increase in the number of germ cells in embryos from PKA mutant germ line clones, although these embryos should have no or reduced Oskar protein levels and thus would be expected to be defective for germ cell formation. The results by Deshpande et al. (2001) contradict previously reported findings by Lane et al. (1994) and our own observations with the same strains as those used in the Deshpande study. We were also unable to obtain embryos for analysis of *ptc* mutant germ line clones. This finding is supported by a recent publication by King et al. (2001) where the authors show that germ line clones for *ptc* fail to develop past stage 9 of oogenesis. Thus females, which lack *ptc* activity in the germ line, produce no embryos. The allele used (ptc^{IIW}) in the Lin study and our studies is identical to the allele used by Deshpande et al.

To address the role of the Hh signaling pathway in germ cells more directly, we used a *GAL4* driver to specifically drive components of the signaling pathway in germ cells (Van Doren et al., 1998a). As described previously, the *nos-GAL4* driver contains the transcriptional regulator region and RNA localization and translation elements (5' and 3'UTR) of the *nanos* gene while the *nanos* coding region was replaced by GAL4-VP16 (Van Doren et al., 1998a). Consequently, GAL4 is maternally synthesized, localized to and translated at the posterior pole of the embryo. GAL4 activity persists during embryogenesis in the germ cells. We and others have used this construct successfully to drive gene expression in germ cells (Van Doren et al. 1998a, Starz-Gaiano et al. 2001) and to disrupt germ cell migration (i.e. UAS-rac^{V12}, EP-1, MS-G and RL, unpublished).

We used the following UAS lines in this experiment: 1) Patched$^{\Delta loop2}$ (Ptc$^{\Delta loop2}$) a deleted form of the *hh* receptor, which no longer binds Hh and thus constitutively blocks Hh signaling (Briscoe et al., 2001). 2) A dominant active and a negative form of Ci, the transcription factor regulated by Hh and required for Hh signal transduction; specifically we used *UAS-Ci76* (dominant negative, truncated repressor form) and *UAS-HACi* (M1-4) (the mutated, unprocessed activator form) (Aza-Blanc et al., 1997). As illustrated in Fig. 4 and the graph in Fig. 5, expression of these constructs in germ cells did not affect their migration.

As a negative control for these experiments, we expressed *UAS-GFP* in germ cells. Germ cells are initially transcriptionally silent and do not show transcriptional activation via the *nos-GAL4* driver until stage 9 of embryogenesis (Van Doren et al., 1998a). Critical for the validation of the experiments described in Figs. 4 and 5 was to demonstrate that germ cell migration could be disrupted using the *nos-GAL4* driver. Data in preparation show that activated RAC (UAS-Rac^{V12}) and an EP line, which was identified in our lab among the original collection from P. Rorth (1996), using the *nos-GAL4* driver, cause significant migration defects when expressed in germ cells (MS-G and RL in prep.). For comparison and as a positive control, we included results with EP1 in Figs. 4 and 5. Thus, while our experiments with positive controls clearly show that expression of particular genes in the germ cells can affect germ cell migration, none of the components of the Hh signaling pathway we tested had an effect on germ cell migration or survival.

In summary, our data provide no evidence for a role of Hh or its downstream signaling pathway in germ cell migration. In a previous study (Van Doren et al., 1998b), we showed that embryos homozygous mutant for the Drosophila HMGCoA reductase gene show germ cell migration defects with high penetrance and expressivity. In this study we also reported that HMGCoA reductase is expressed initially in the lateral mesoderm and subsequently in the gonadal mesoderm. Furthermore we showed that ectopic expression of HMGCoA reductase attract germ cells to regions of high levels of expression. In these experiments we used the same GAL4 drivers as used in the study by Deshpande et al. Our data clearly showed a colocalization of germ cells and the ectopically expressed HMGCoA reductase. For example, use of the *hairy-GAL-4* driver led to 'striped' expression of HMGCoA reductase and preferential accumulation of germ cells in the '*hmgcr* expressing stripe' compared to the intervening region. Using a nervous system driver (*elav-GAL4*) or an ectodermal driver (*69B-GAL4*), we observed germ cells moving to the region of high HMGCoA reductase expression in the CNS or ectoderm respectively. These experiments provided clear evidence for a role of HMGCoA reductase in germ cell attraction. By showing that high expression of HMGCoA reductase at ectopic sites attracts germ cells, we provided a paradigm for future identification of instructive germ cell guidance cues. We do not know the nature of the attractant that is dependent on HMGCoA reductase activity.

We do not consider Hh a good candidate as a downstream effector of HMGCoA reductase signaling as suggested by Deshpande et al. for the following reasons. The Deshpande et al. study fails to show direct evidence that germ cells colocalize with

tissues where *hh* is misexpressed. Furthermore, Deshpande et al. suggest in their discussion that HMGCoA reductase activity mediates cholesterol-modifications of secreted signals like Hh. However, several observations make this highly unlikely. First, it has been shown that insects are auxotroph for cholesterol and that radiolabeled mevalonate (the product of HMGCoA reductase enzymatic activity) will not be incorporated into cholesterol (Clayton, 1964; see also Seegmiller et al., 2002). Second, our genomic analysis of the HMGCoA reductase pathway suggests that the enzymatic steps required to synthesize cholesterol cannot be executed in Drosophila by this pathway since several enzymes that mediate these steps are not present in the Drosophila genome (AS and RL in prep.).

In summary, we have repeated the experiments carried out by Deshpande et al. as well as performed additional experiments to address the role of Hh as a germ cell attractant. Four different experimental approaches have failed to provide any convincing evidence regarding a direct role of Hh in germ cell migration. As previously reported, Hh does, however, play a very important role in the cell fate specification of the mesoderm including the gonadal mesoderm (Azpiazu et al., 1996; Moore et al., 1998a; Moore et al., 1998b; Riechmann et al., 1998).

Materials and Methods

Drosophila mutant strains:

FRT, DCO^{H2}	from D. Kalderon, (Lane and Kalderon, 1994), we also tested a line provided by G. Deshpande
FRT, smo^{X43} = FRT, smo^{2}	from Marek Mlodzik, we also tested a line provided by G. Deshpande
FRT, ptc^{IIW}	from Haifan Lin, (King et al., 2001) (same line as tested by Deshpande)

UAS and Gal4 lines

elav-GAL4:	gift from B. Jones, we also tested a line provided by G. Deshpande
h-Gal 4:	(Brand and Perrimon, 1993)
twi-GAL4:	(Brand and Perrimon, 1993)
twi, 24B GAL4:	(Greig and Akam, 1995)
nos-GAL4	(Van Doren et al., 1998a)
pannier-GAL4	gift from J. Treisman (Heitzler et al., 1996)
UAS-hh	from M. Frasch, (Azpiazu et al., 1996); we also tested a line provided by G. Deshpande
UAS-hhN	gift from J. Treisman (Porter et al., 1996)
UAS-Ci76	gift from J. Treisman (Aza-Blanc et al., 1997)
UAS- HACi(M1-4)	gift from J. Treisman (Porter et al., 1996)
UAS-hmgcr	(Van Doren et al., 1998b)
UAS- $Ptc^{\Delta loop2}$	gift from G. Struhl, (Briscoe et al., 2001)

Antibody staining

The following antibodies were used:
polyclonal anti β-Gal (Cappel)
monoclonal anti-Clift (N. Bonini, and monoclonal #eya 046 Hybridoma bank)
polyclonal anti-Vasa (Zinszner, Lehmann Lab)
hh cDNA was obtained from J. Treisman.
hh-LacZ enhancer trap line was obtained from G. Deshpande
Antibody detection was carried out with horseradish peroxidase using a biotinylated secondary antibody (Jackson Immuno research) and the Vectastain Elite ABC Kit. For fluorescent labeling we used CY3 anti-mouse (Jackson lab) and Alexa 488 anti rabbit (Molecular probes) secondary antibodies.
mRNA was detected according to Tautz and Pfeifle (1989).

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Figure Legends

Figure 1: *hh* RNA expression.

Panel A shows an embryo at stage 11 hybridized with *hh* antisense RNA. Note the segmental expression of the RNA in a similar expression pattern in each segment. A square in parasegments 10-12 indicates the position of the gonadal mesoderm.
Panel B shows a stage 11 embryo doubly stained to detect *hh* expression using a *hh-LacZ* enhancer line (green) and anti-Clift (red) antibody. In the mesoderm, *hh-LacZ* is expressed at low levels in every segment, while Clift is expressed only in the gonadal mesoderm in parasegments 10-12 (lines). Clift expression partially overlaps with Hh (arrows). Embryos are oriented anterior to left and dorsal up.

Figure 2: Graphic summary of *UAS-hh* misexpression experiments.

Flies homozygous for the respective *GAL4* transgene insertion were crossed with flies homozygous for the *UAS* transgene such that all embryos should express the *UAS* transgene. Germ cell migration was assayed by anti-Vasa antibody staining. The number of germ cells lost per embryo was recorded and tabulated in a color scheme, where the darker blue color indicates increased loss of germ cells.

Summary of *UAS-hh* misexpression experiments (see figure 2)

Number of lost germ cells	UAS-*hh*	*twist*-Gal4	*twist*-Gal4x UAS-*hh*	*twist*-Gal4x UAS-*hmgcr*	elav-Gal4x UAS-hh	elav-Gal4x UAS-hhN	*elav*-Gal4x UAS-*hmgcr*
0 TO 2	24	49	48	2	34	43	0
3 TO 4	21	8	12	3	1	5	0
5 TO 6	9	4	1	10	0	1	0
7+	0	0	0	56	0	0	51
Total number of embryos	54	61	61	71	35	49	51

Figure 3: Expression of *UAS-hmgcr* but not *UAS-hh* or *UAShhN* in the nervous system can attract germ cells.

Embryos of similar stage (stage 15) are stained with anti-Vasa antibody. The driver *elav-GAL4* activates UAS expression in the nervous system. Note that *UAS-hmgcr* (C) expression in the nervous system leads to attraction of germ cells, while expression of *UAS-hh* (A) or the more active *UAS-hhN* has no effect on germ cell migration. Embryos are oriented anterior to left, dorsal up.

Figure 4 Mutations in the *hh*-signaling pathway do not affect germ cell migration.

Upper panel: Embryos from females with germline clones homozygous mutant for *smo* (smo^{X43}, m^-). The two embryos are from the same egg collection, the embryo in (A) received a wild-type *smo* allele from the father (z^+) and the embryo in (B) received a mutant *smo* allele copy (z^-). Note the lack of a germ cell migration phenotype and formation of two gonads as attested by anti-Vasa staining in (A). The embryo in B has lost germ cells, but also patterning defects. It is visibly shorter than the wild-type embryo. Cuticle preparations of larva from this cross also showed a 50:50 distribution of

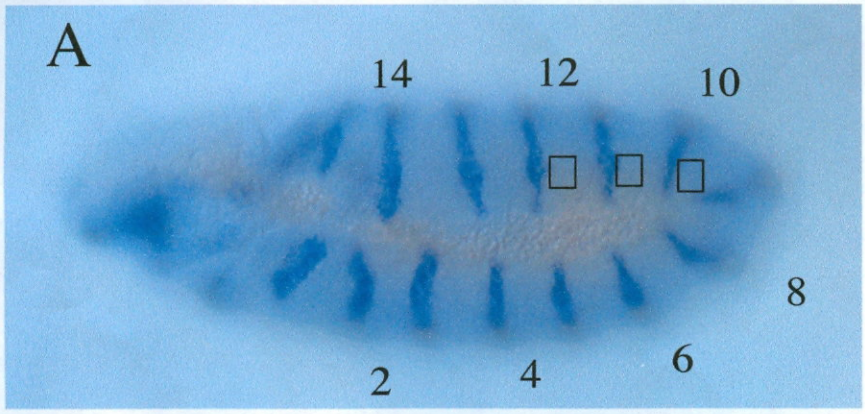
embryos which exhibited the lawn phenotype (m^-z^-) typical for mutants defective in *hh* signaling to those with normal patterning (m^-z^+). Large arrows in (A) point to the two gonads, small arrow points to a lost germ cells. Weak (1-3 germ cells/embryo) loss is observed in most 'wild-type' line.
Lower panel: Expression of an *UAS-ptc*$^{\Delta loop2}$ transgene in germ cells does not disrupt germ cell migration (C). (D) Positive control shows an embryo expressing the EP-1 line in the germ cells using the same *nos-GAL4* driver used in (C). A + B stage 14, C + D stage 16 embryos stained for Anti-vasa. Embryos are oriented anterior to the left in a frontal view. Large arrows in (C) points to germ cells aligned into two rows, typical for wild type migration.

Figure 5: Graphic summary of *hh* pathway mutant analysis
In each experiment homozygous *nos-GAL-4* females were crossed with males homozygous for a particular UAS transgene such that all embryos should express the *UAS* transgene in the germ cells. Germ cell migration was assayed by anti-Vasa antibody staining. The number of germ cells lost per embryo was recorded and tabulated in a color scheme, where the darker blue color indicate increased loss of germ cells.

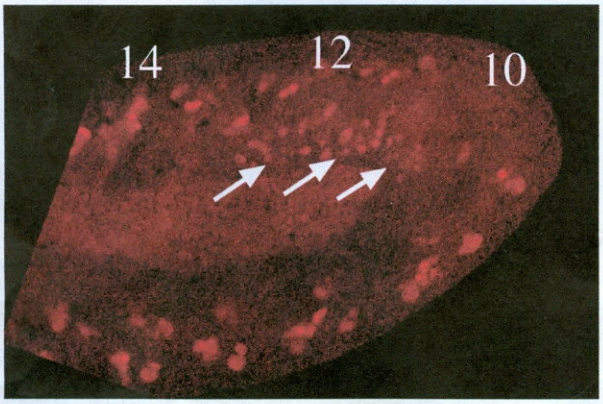
Summary of *hh* pathway mutants (see figure 5)

Number of germ cells lost	smo gl clones	nos Gal4 x UAS GFP	nos Gal4 x UAS ptcΔloop2	nos Gal 4x UAS CI	nos Gal4 x UAS HA CI	nos Gal4 x EP1
0 TO 2	73	23	12	27	26	1
3 TO 4	5	3	2	3	0	0
5 TO 6	2	1	0	0	0	0
7+	0	0	0	0	0	34
Total number of embryos	80	27	14	30	26	35

Fig. 1



hh RNA
stage 11



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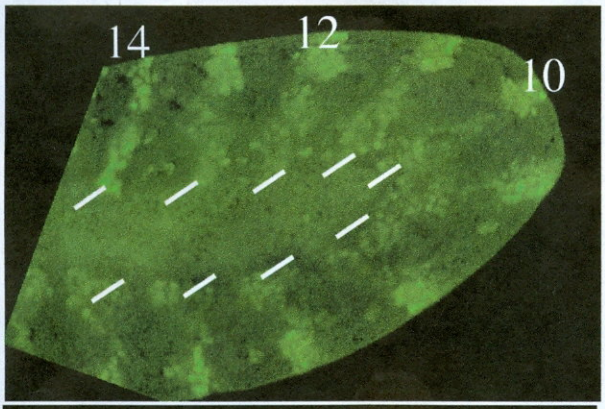

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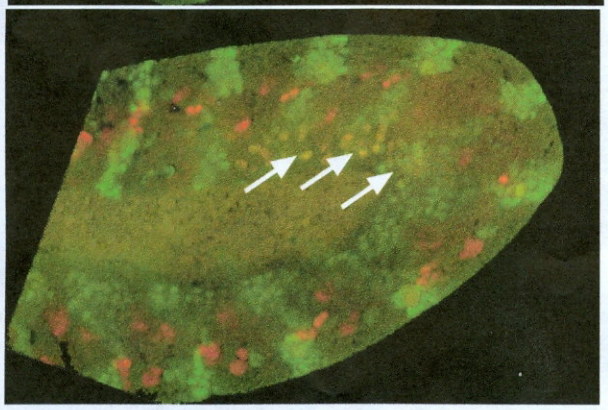


overlay
stage 11

Fig. 2

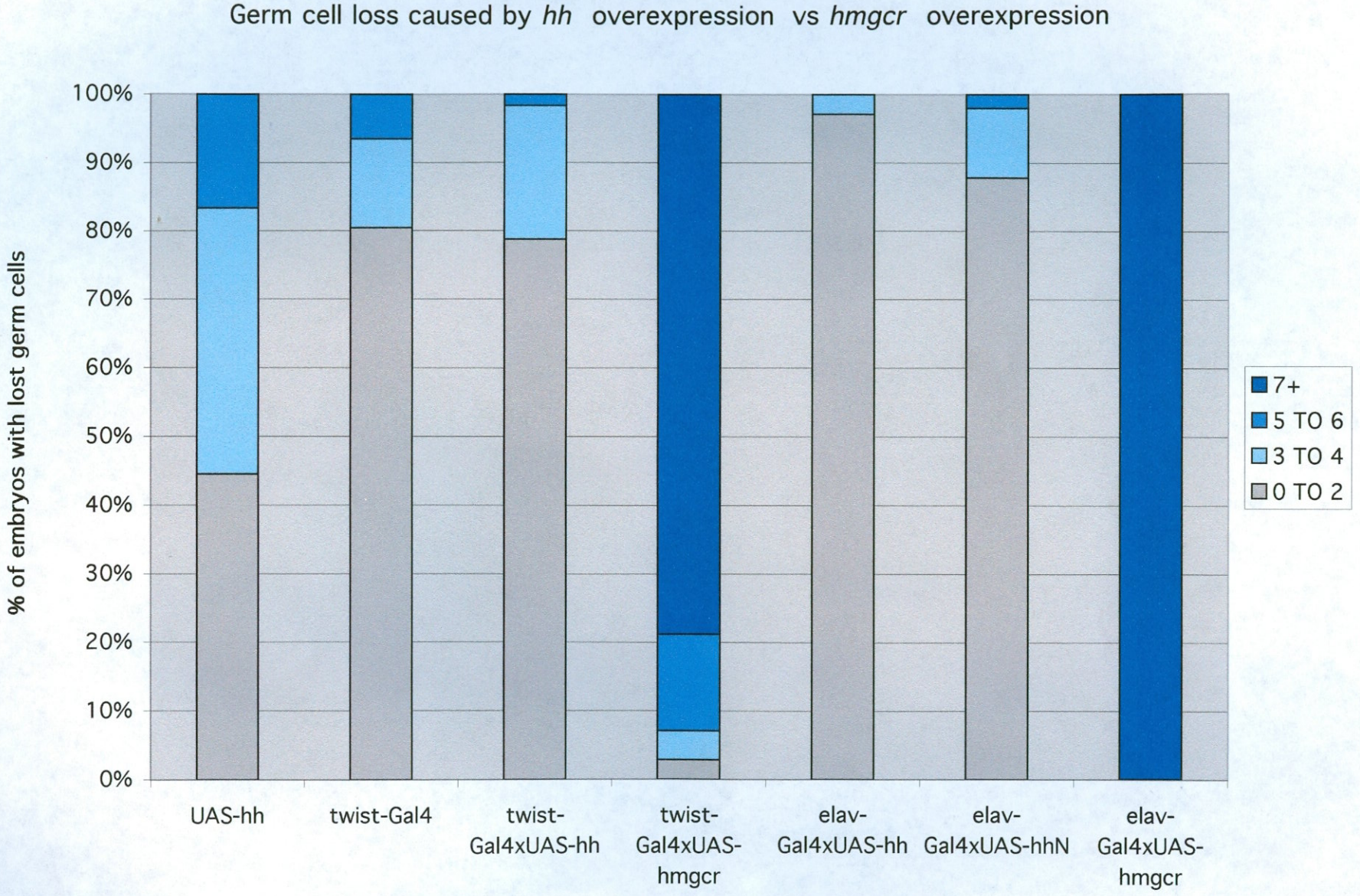
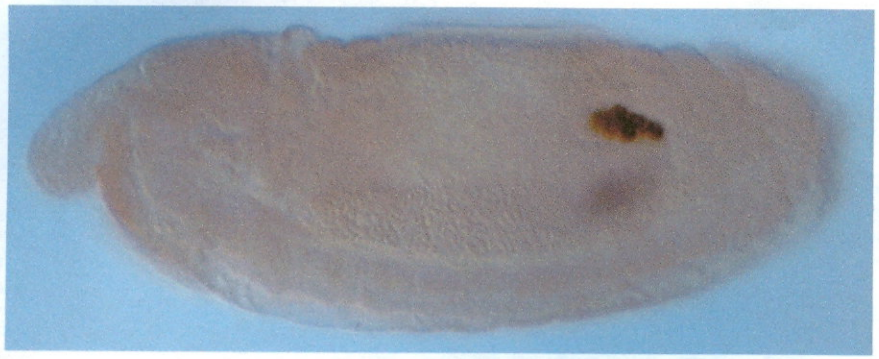

Germ cell loss caused by *hh* overexpression vs *hmgcr* overexpression
% of embryos with lost germ cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
UAS-hh
twist-Gal4
twist-Gal4xUAS-hh
twist-Gal4xUAS-hmgcr
elav-Gal4xUAS-hh
elav-Gal4xUAS-hhN
elav-Gal4xUAS-hmgcr
7+
5 TO 6
3 TO 4
0 TO 2

Fig. 3



Elav-Gal 4, UAS-hh

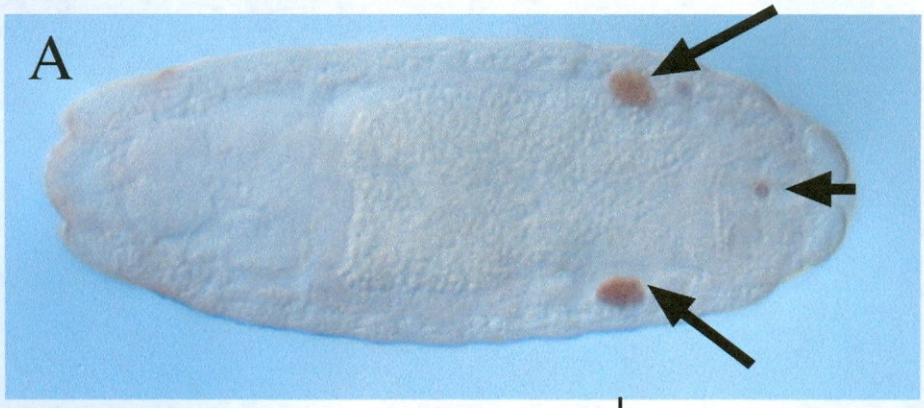
Elav-Gal 4, UAS-hh-N

Elav-Gal 4, UAS-hmgc

Figure 3

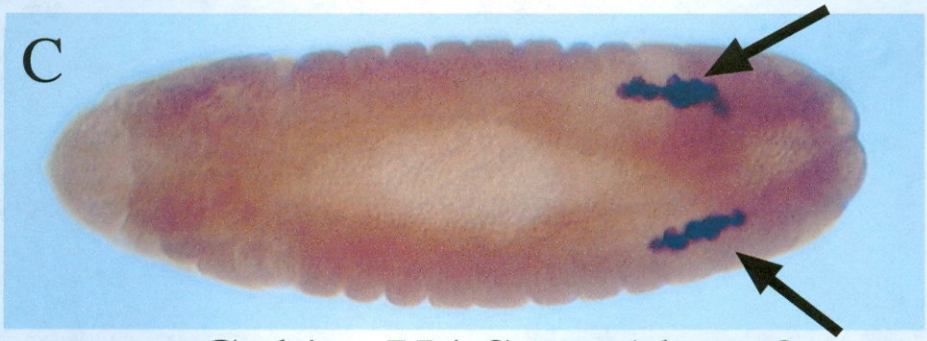
Figure 4

Fig. 4

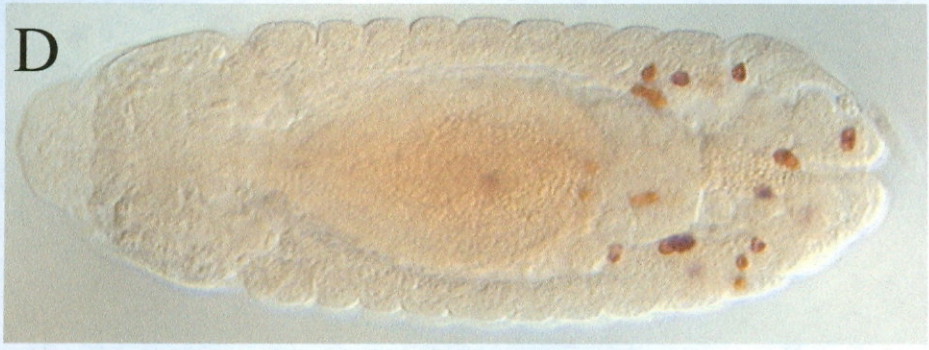


smo^{X43} *m*$^{-}$ *z*$^{+}$

smo^{X43} *m*$^{-}$ *z*$^{-}$

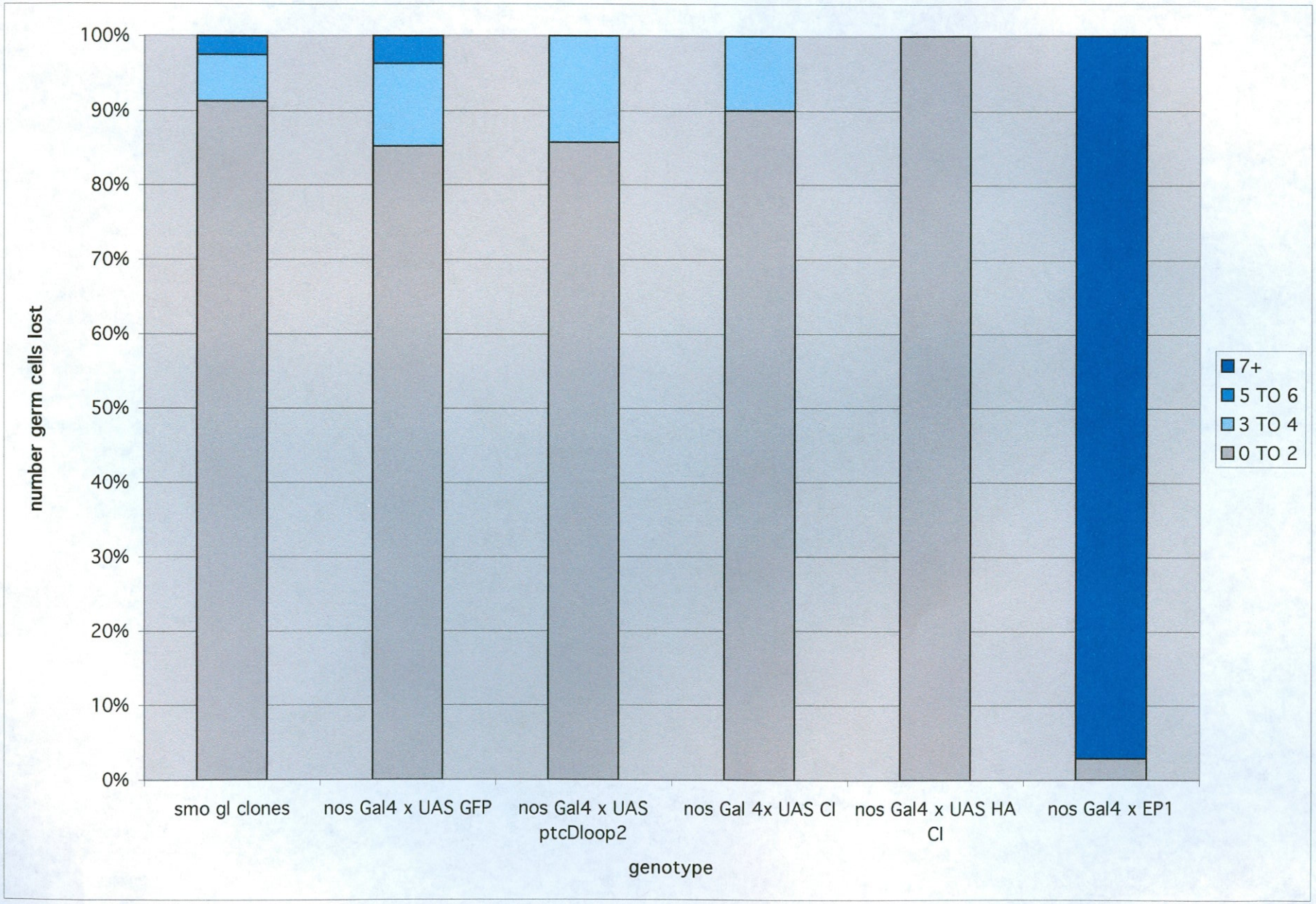


nos-Gal4 x UAS-ptcΔloop2



nos-Gal4 x EP1

Fig. 5


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
number germ cells lost
7+
5 TO 6
3 TO 4
0 TO 2
smo gl clones
nos Gal4 x UAS GFP
nos Gal4 x UAS ptcDloop2
nos Gal 4x UAS CI
nos Gal4 x UAS HA CI
nos Gal4 x EP1
genotype