Dear Vivian

Thanks for your e-mail and the copy of Steve's report.

When you first wrote to us on Feb. 21st to ask us to respond to Ruth Lehmann's manuscript, I indicated in my e-mail of Feb. 24th that it would be premature and inappropriate for us to do so at that juncture. However, I believe that we are now in a position to make a formal response to many of the points raised in Ruth's manuscript. Since this is a formal response, I will first review some of the key events.

As I mentioned in my Feb. 24th e-mail, Ruth called me in the early evening on Friday, Jan. 19th. I suspect that Ruth called because she had been invited to give a seminar in our Department on the following Wednesday, Jan.23rd by her former post-doctoral fellow Liz Gavis. In fact, earlier in the week of the 19th I had asked Liz to put Girish on Ruth's schedule so that he would be able to talk with her when she came to give her seminar (I was planning to go out to dinner with Ruth that evening). However, Ruth had unexpectedly refused to meet with Girish. The reasons became clear in the course of our phone conversation. She told me that her laboratory had been "unable" to reproduce any of the key experiments reported in our paper on the possible role of *hh* signaling in germ cell migration. She said that she was putting the finishing touches on a manuscript documenting her case, and that she would be submitting this manuscript to *Cell* very soon. Our conversation covered many of the topics in this manuscript including the question of whether *hh* is expressed in SGP cells, the effects of ectopic *hh* on germ cell migration, and the fact that our germ line clone results could not be reproduced. Ruth also indicated that she would bring some of her microscope slides of *hh* expression in embryos to Princeton so that I could see for myself. Since some of the specific points she made during this phone conversation are pertinent to my critique of her manuscript, they will be discussed further below.

Since I believe that experimental results obtained by one laboratory should be reproducible in another laboratory, I discussed the points Ruth mentioned in our phone conversation with Girish that same evening. Girish and I agreed that he should repeat some of the key experiments in the paper to make sure that he was able to reproduce his earlier findings.

After considering the matter further over the weekend, I decided that because of the nature of the accusations, the question of reproducibility was not the sole or ultimately the most important issue. Even if Girish was able to reproduce his earlier results, this would be insufficient as in the end it would only be our word against Ruth's. I knew from previous experience that Ruth would be much more effective in promoting her own point of view than we would irrespective of the truth. She is extremely well connected in the scientific community and her heavy seminar and meeting schedule would give her ample opportunity to convince others that none of the experiments in our paper could be reproduced no matter what we found or said. For this reason, I decided that we would be obliged to ask other scientists outside of my lab to repeat several of the key experiments. In retrospect this was good decision since it appears that our credibility and integrity had already been tarnished even before the manuscript was submitted. Amongst other things, the "fact" that the experiments in our paper were not reproducible has been circulating through the fly and broader scientific community since last fall. It would not be at all surprising if the reviewers of Ruth's

manuscript had heard of this "fact" either directly or indirectly before you sent it to them for their comments. It was also my impression that our credibility at *Cell* had been damaged as well.

Since redoing all of the key experiments in the paper would be a quite significant burden for any one individual, my initial plan was to ask several different scientist to repeat different experiments. Based on my conversation with Ruth on that Friday, by far the most important and pressing were our experiments showing that germ cell migration is altered by ectopic *hh* (the reasons are explained below). The following Monday, Jan. 21st, I asked Eric Wieschaus about doing these experiments. However, he was reluctant to get involved, partly because he is in the same institution and partly because he did not consider himself sufficiently expert in germ cell migration. I then decided to contact Steve DiNardo to ask him if he would be willing to redo the ectopic expression experiments. Fortunately, he agreed. I told Steve that Girish would first make sure that he could reproduce his earlier findings. Once we were convinced that our ectopic expression results could be repeated in own our hands, we would send the stocks on to Steve. Of course, if we could not repeat our experiments, we would not have asked Steve to try as this would simply be wasting his time. I had intended to follow the same plan for several of the other experiments that had been called into question. Girish would first try to repeat his earlier results, and assuming that he could, I would contact other individuals to ask them if they would be willing to redo these experiments as well. However, for reasons that will become clear below, this is no longer necessary.

I also decided that I would not see Ruth when she visited Princeton on the 23rd of Jan. I did so because I found her accusations and behavior inappropriate. Ruth has been after Girish for the last several years, and at every opportunity has severely criticized his work and questioned its validity. One of the most glaring public incidents occurred at the Cold Harbor Germ Cell meeting held a little over a year ago. Ruth attacked Girish in the question session after his talk, basing most of her claims as to why he was wrong on experiments that had purportedly been done in her laboratory. After a series of e-mail exchanges with her and her former post-doctoral fellow in the weeks following the meeting, she admitted that her lab had never done the experiments on which her claims had rested. Of course, the fact that these claims were false is not public knowledge. Instead, she succeeded in leaving the scientists who attended the meeting with a rather negative impression of Girish and his work. (I often wonder if this is why we have been unable to publish the studies he presented in his talk.) Given the rumors that have been spread about the *hh* paper, it seems likely that the attacks on Girish's work, and likely other work from my laboratory did not stop after the Cold Spring Harbor meeting but continued unabated. While this latest incident could be viewed as simply a continuation of an ongoing campaign, there was a clear and important difference. In the past, we were just accused of incompetence or stupidity; however, this time her claims called into question our integrity.

Girish has now repeated several of the key experiments described in our paper. In each case he has been able to reproduce the results that have been called into question. We did, however, discover a potential error in genotype (we may have used an allele different from that indicated in our paper) which will be described in the appropriate section below.

For the convenience of the reviewers, I will follow the subject order in Ruth's manuscript.

1) The first section of the result deals with the question of whether Hh is expressed in the somatic gonadal mesoderm. This section is titled "***Hh is <u>not</u> expressed in the early gonadal mesoderm***".

When Ruth called me on Jan 19th, one of the points we discussed at length was the fact that she ***could not detect*** any *hh* expression whatsoever in the gonadal mesoderm. She told me that two independent lines of evidence unequivocally demonstrated that we had made a mistake. The first came from experiments using the same *hh* promoter *LacZ* reporter as we did. She double stained transgene embryos for LacZ and Clift (a marker for the mesodermally derived somatic gonadal precursor cells—SGPs). Though she did observe some Clift positive cells that expressed LacZ (and hence should be expressing *hh*) she said that these cells were clearly not mesodermal cells. Rather these were cells in the ectoderm that also express the Clift marker. The second line of evidence came from *in situ* hybridization to *hh* mRNA. She said that she had obtained precisely the same results in the *in situ* experiments; *hh* mRNA could not be detected in cells of mesodermal origin, only in cells in the ectoderm. I suggested that it would be difficult to use whole mount *in situ* to conclude that *hh* is not expressed in the mesoderm as the resolution of this technique would make it exceedingly difficult to rule out (or rule in) mesodermal expression. However, when I questioned her on this, she indicated that some type of "tissue sectioning" had been done for the *in situ* hybridization. Based on the results of these two experiments, Ruth told me that we made a serious mistake in our paper, and misidentified what were clearly ectodermal cells as "mesodermal SGPs".

The "finding" that Hh is expressed in Clift positive SGP cells was one of the key observations in our paper. If Ruth claims were correct it would mean that the attractive signal emanating from the SGPs could not be Hh, nor could this signal—whatever it is-- depend upon a cell autonomous Hh function in the SGPs. (Though, of course, it would not rule out the less interesting possibility that ectopically expressed Hh could somehow influence migration.) For this reason, Girish and I re-examined his old confocal images of *hh:lacZ* that same evening, and just to be sure Girish began setting-up *hh:lacZ* embryo collections to repeat the LacZ antibody staining. As we expected, his new experiments confirmed our earlier findings: we could detect LacZ in the Clift positive SGPs. Since Girish's results seemed so clear cut, I decided to put off asking another scientist to repeat the *hh* expression experiments until we had seen the actual data in Ruth's manuscript. It turned out that this was the right decision.

Ruth gave her seminar in Princeton on Jan. 23rd, bringing along her *hh* expression slides so that she could show me that our paper was wrong. Though I did not see her, she met with faculty in our department including Trudi Schupbach. Ruth repeated much of what she had told me on the phone to Trudi. She indicated to Trudi that she had unequivocal evidence that *hh* is not expressed in the SGPs, and that we had misidentified ectodermal cells as mesodermal SGPs in our experiments with the *hh:LacZ* transgene. She indicated that her results with the *hh:LacZ* transgene were confirmed by *in situ* hybridization to *hh* mRNA in embryonic tissue sections.

Given the conviction and confidence with which Ruth described her "compelling and overwhelming evidence" that *hh* is not expressed in the gonadal mesoderm, I must admit that when the manuscript arrived from *Cell* and I turned to the first section of the results and read the title ***"Hh is not expressed in the early gonadal mesoderm"*** I felt more than a little uneasy. I kept thinking that we must have made some amateurish mistake in our analysis of the LacZ staining pattern that Ruth was going to expose for all to see. Consequently, I was more than surprised, I was shocked to

discover that Ruth had instead confirmed the results we presented in the first section of our paper. Although the title of this section of her text asserts that *hh* is not expressed in the gonadal mesoderm, this is not what the text actually says, nor does the title accurately reflect what is shown in Figure 1. At two points the text of her manuscript states exactly the opposite of what written in the title of this section. First with regard to the *in situ* hybridization (which is a whole mount *not* a tissue section) the text states "We **do** detect *hh* RNA staining in the mesoderm in a segmental pattern during stages 10-12". Second, with regard to the confocal analysis of *hh-lacZ* the text states "We **detected** co-expression of LacZ and the gonadal mesoderm marker Clift in the gonadal mesoderm during stage 11 and 12."

Having failed to demonstrate that ***"Hh is not expressed in the early gonadal mesoderm"***, our paper is attacked on other entirely spurious grounds. Thus, the text goes on to claim that Deshpande et al failed "to mention that while Clift is expressed in the mesoderm of only parasegments 10-12, Hh is expressed in each segment at similar levels". This is simply a misrepresentation. It should be clear from even a careless reading of our opening section of the Results (*hh-lacZ is transcribed in SGP cells*) that this assertion is simply false. We write… "Note that b-gal can also be detected in Clift negative mesodermal cells in parasegments anterior to PS10." In fact we go on to state… "Based on their homologous position in the mesoderm these cells are likely to be fat body precursor cells… etc."

From the stamp on the title page of Ruth's manuscript, it would appear that it was submitted on Feb 5th or 6th. Yet just two weeks earlier, on Jan. 23rd, she was telling colleagues in my department (and apparently elsewhere in the scientific community) that she had incontrovertible evidence that there was absolutely no *hh* expression in the gonadal mesoderm. In our phone conversation on Jan. 19th, Ruth indicated that she had just about finished writing her manuscript. Given what she said on phone that evening, the first section of results in this version of the manuscript must have had Figures and accompanying text which demonstrated that ***Hh is <u>not</u> expressed in the early gonadal mesoderm***". I find it difficult to imagine how such compelling and unequivocal evidence could have completely disappeared in the few days between her phone call / visit to Princeton and her submission of the paper. When in that two-to-three week period did Ruth discover that her results weren't as compelling as they had seemed? Did she repeat the *in situ* hybridization and *hh:LacZ* expression experiments, or did she look at the Figures that were already in her manuscript a bit more carefully? Why, in spite of this last minute revelation that her *hh in situ* and *hh:LacZ* expression data was either wrong or misinterpreted, did she hurriedly revise the first section of the results (leaving the original title in by mistake) and then submit her paper. Wouldn't it have been much more sensible to rethink the whole business and hold off until one could be sure of ones footing?

2) In the second section of the results Ruth presents data indicating that ectopic *hh* has absolutely no effect on germ cell migration. Of all of Ruth's charges in her phone call and subsequently in her paper, I found this to be by far the most troublesome. There were two reasons. First, the ectopic expression experiments were the centerpiece of our paper. If they were wrong, all of the rest of the data in the paper was largely meaningless. Second, Ruth claimed that she could detect absolutely no effects of *hh* misexpression on germ cell migration, and that the ectopic *hh* embryos were essentially indistinguishable from wild type. Unlike any of our other "errors", this discrepancy could not be readily explained by some type of amateurish mistake or incompetence on our part.

By implication, the only possible explanation for our results was that they had been fabricated in some fashion. I presumed that this was why Ruth refused to talk with Girish when she visited, and why she called to "warn" me that our results were false. It was the severity of these particular charges that made it imperative that we find some independent person to repeat our ectopic expression experiments.

Girish has now repeated the ectopic *hh* expression experiments described in our paper. He has also examined the effects of ectopic *clb* expression. (Girish had generated *UAS clb* /Driver-GAL4 embryos previously, but never carefully compared the migration defects in these embryos with those seen with ectopic *hh*.) In all cases, the results of his latest experiments match those reported in our paper. Girish's latest results also closely match those obtained by Steven DiNardo.

Contrary to the claims in Ruth's manuscript, Girish observes abnormalities in germ cell migration in *UAS hh*/Driver-GAL4 embryos. These abnormalities are seen in stages 10 through 15, though they are typically most "severe" in earlier stage embryos. The number of misplaced/lost germ cells for several different *UAS hh*/Driver GAL4 combinations is tabulated below. In each of these experiments only half of the embryos should receive both *UAS hh* and the driver, and it is these embryos that are expected to show a migration phenotype. In all cases, slightly less than 50% of the embryos exhibit unambiguous migration defects (5-6 or more lost germ cells).

UAShh /UAShh (II) X *hairy-GAL4 / TM3* (Trial 1)

Germ cells lost	no. of embryos
0-2	45
3-4	34
5-6	34
7+	26

UAS hh / UAS hh (II) X *hairy GAL4 /TM3* (Trial 2)

Germ cells lost	no. of embryos
0-2	64
3-4	31
5-6	37
7+	36

UAShh /UAShh (III) X *hairy-GAL4 / TM3*

Germ cells lost	no. of embryos
0-2	25
3-4	15
5-6	18
7+	13

UAShh /UAShh (II) X *elav-GAL4 /Y* (X-linked insert)

Germ cells lost	no. of embryos
0-2	28
3-4	16
5-6	22
7+	25

UAShh /UAShh (II) X *twist-GAL4* /Y (X-linked insert)

Germ cells lost	no. of embryos
0-2	48
3-4	30
5-6	26
7+	29

As we previously reported, each driver gives a different pattern of migration defects, and for each driver the germ cells migrate towards the source of ectopic *hh*. Steve DiNardo came to essentially the same conclusions for the drivers he examined.

In the case of ectopically expressed *clb*, the migration defects seen in stages 13 & 14 are quite similar to those observed for ectopic *hh*. However, at stage 15, there are usually a larger number of misplaced/lost germ cells in the ectopic *clb* embryos than in the ectopic *hh* embryos. (We never compared *clb* and *hh* in our paper, and the fact that the phenotypes are slightly stronger with ectopic *clb* does not in anyway detract from the main conclusions of our paper.)

UAS clb / UAS clb X *hairy GAL4 / TM3*

Germ cells lost	no of embryos
0-2	15
3-4	8
5-6	14
6+	38

One possible reason for the difference in number of "lost germ cells" particularly in later stages, is that the total number of germ cells in the ectopic *hh* embryos drops almost two-fold between stages 13 and 15, while it remains close to the same in the ectopic *clb* embryos. (It would also appear that the *UAS clb* transgene by itself has a somewhat higher background of germ cell migration defects than the *UAS hh* transgene.)

UAS hh Stage 13 average number of germ cells 31.4 (Counts done on 10 embryos).
Stage 15 average number of germ cells 17.7 (Counts done on 10 embryos).

UAS clb Stage 13 average number of germ cells 27.3 (Counts done on 10 embryos).
Stage 15 average number of germ cells 23.3 (Counts done on 10 embryos).

Since Steve has confirmed Girish's findings, Ruth's most serious charge, that germ cell migration is unaffected by ectopic *hh*, has clearly not been substantiated by an impartial and independent scientist. I find it extremely difficult to understand why Ruth failed to observe any misplaced/lost germ cells whatsoever in *UAS hh*/ Driver *GAL4* embryos, especially when the effects are so obvious.

3) The third section of Ruth's manuscript is titled **"Mutant analysis of the Hh pathway reveals no direct effect on germ cell migration".** Most of this section describes unsuccessful attempts to repeat our experiments on germ line clones for various *hh* pathway genes.

The "goal" of our germ line clone experiments was to remove components of the *hh* signalling pathway from germ cells in an other wild type soma, and then examine their migration behavior. Of the experiments presented in our paper, the germ line clones were the most problematic. The first problem was that in order to have a "wild type" soma, the clone carrying mothers were mated to wild type (or heterozygous mutant) fathers. Thus, though the germ cells would receive no maternally deposited *hh* pathway gene product, the germ cells themselves would be heterozyogous for the *hh* pathway mutation. Though zygotic transcription in germ cells doesn't begin until around the time the germ cells start exiting the gut, by the time migration is finished there could be sufficient zygotic expression of the *hh* pathway gene to rescue the maternal deficient germ cells. Thus it would be possible to miss defects in germ cell migration because the maternally deficient germ cells are rescued zygotically. The second problem is just the opposite. Embryos derived from clone mothers might have defects in somatic development because zygotic expression of the *hh* pathway gene is not quite sufficient to fully compensate for the lack of the maternal *hh* pathway gene product. These defects in somatic development could be rather subtle or transient, yet nevertheless they could perturb the process of germ cell migration. In this case, we might incorrectly attribute migration abnormalities seen in germ line clone embryos to cell autonomous defects in the germ cells when in fact the problems are non-autonomous and in the soma. For these reasons, we tried to be judicious in drawing conclusion from the germ line clones.

a) In the first part of this section, Ruth describes her "attempt" to repeat our germ line clone experiment with *smo*. In Fig. 4 she shows that stage 15 m-z+ *smo* embryos have near wild type coalesced gonads with a few scattered germ cells. She also reports analyzing about 100 m-z+ *smo* embryos at this same stage (since she states that the embryos have developed to the point where the gonads have coalesced and segmentation is complete). She says that all of these m-z+ *smo* embryos have coalesced gonads exactly like the embryo shown in Fig. 4. While Ruth asserts in this part of the paper that she has uncovered a "mistake" in our analysis, the results she describes for stage 15 m-z+ *smo* embryos are precisely the same as the results we reported in our paper. We found that the gonads of stage 15 m-z+ *smo* were roughly similar to that of wild type. Though we didn't show an embryo of this stage, this finding is stated explicitly in the last sentences in paragraph 2 on page 765 of our paper.

In addition to misrepresenting our results on stage 15 *smo*$^{m-z+}$, Ruth neglects to mention the fact that the migration defects we observed and reported in our paper are actually evident earlier, during stages 12 and 13. We also suggested in paragraph 2 of page 765 a possible reason why most of the defects seen at these earlier stages have disappeared by stage 15. If Ruth wishes to question our results, she should at least present data and pictures on the pattern of germ cell migration at the same earlier stages that we reported showed defects.

Since Ruth called our findings into question, Girish re-examined germ cell migration in embryos from *smo* clone mothers. As originally reported, he found "scattering" defects during stages 12 and 13, and "rescued" coalesced gonads at stage 15. At this point, even if Ruth had bothered to examine the appropriate stage 12-13 *smo*$^{m-z+}$ embryos, I would find it extremely difficult to believe any claims on her part that our *smo* experiments are incorrect.

b) Ruth's manuscript next turns to *pka* clones. It states " We were unable to analyze the progeny of gem line clones mutant for *pka* since no eggs were produced over many days of collection." She supports her inability to obtain any eggs whatsoever from *pka* germ line clones by citing the work of Lane *et al* (l994). "As reported previously by Lane *et al.*, (l994) 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."

While I can not be certain why Ruth failed to obtain any eggs at all, I suspect that the most likely explanation is that she carried out only a very small scale germ line clone experiment, and quit soon after she started. In each of his experiments, Girish collected eggs from >100 females carrying the *pka* germ line clones over a ~2 1/2 week period. Altogether he obtained only ~ 200-300 eggs total, or roughly 2-3 eggs per female. By contrast, a single wild type female usually produces between 20-30 eggs per day, or about 400-500 in a period of 2 1/2 weeks.

In addition to the paper cited in Ruth's manuscript, there is a subsequent paper from Lane and Kalderon in *Mechanisms of Development* (49, 191-200; 1995). In the '95 paper Lane and Kalderon report "We observe very little egg laying among populations that included females with PKA germline clones, although mature eggs were found in ovaries dissected from these females." Girish contacted Kalderon about a year ago to ask him about the embryos produced by *pka* germ line clones. Kalderon writes in an e-mail dated Jan 11, 2001: "….When Mary Ellen Lane looked at germline clones that was in pre-FRT days so the starting chromosome was different (possibly some background or EMS mutations that enfeebled a little) and the number of eggs we could look at was not very high. Later we did use the same stock I sent you and tried to look at whether embryos developed (early in Yan's graduate career, but may have not looked at enough or carefully enough. …" Thus Ruth is simply misrepresenting the facts when she implies that Lane and Kalderon never saw any eggs from their *pka* germ lines clones.

c) Ruth also asserts that she was unable to obtain any embryos from the *ptc* germ line clones using the *ptc* mutant we sent her. Girish has also now redone key parts of the *ptc* germ line clone experiment. The results of these new experiments are the same as we described in the paper. First, he can obtain *ptc*$^{m-z+}$ embryos from the *ptc* germ line clones (mothers carrying the clones were

mated to *w* males). Second, he observed the same spectrum of germ cell migration defects as reported in the paper as well as an increase in the number of germ cells compared to wild type around the time they beginning exiting the gut. Because of the time pressures to repeat our work this new experiment was on a much smaller scale that those described in our paper, and Girish did not have enough germ line clone mothers to mate to *ptc*/+ fathers and then stain for *wg*. However, the earlier *wg* expression experiments were internally controlled and the results were unambiguous. As expected 50% of the embryos showed an expansion of *wg* stripes. This would be the defect expected for embryos deficient in *ptc*, while the frequency of the *ptc* mutant phenotype is that predicted for germ line clones.

While it is clear from these latest *ptc* experiments that Ruth's assertions are simply incorrect, it is also the case that we may have made a mistake in the identification of the *ptc* allele used to generate the germ line clones. We received the *ptc* FRT chromosome about three years ago from Jackie Chang, who was in Haifan Lin's laboratory at Duke at the time. The vial arrived without a *ptc* allele designation, and was maintained in our stocks as "*ptc* FRT42". Instead of contacting Jackie again to ask about the allele when we finally wrote our paper (Jackie had by then left Haifan's lab), Girish looked up publications from Haifan's laboratory that used a *ptc* FRT chromosome and found King *et al* 2001. This publication had a null allele, ptc^{Iiw}, listed in the methods section, and he assumed that his *ptc* FRT42 stock must have been the same *ptc* allele. He did not read the rest of Haifan's article at that time. After we received Ruth's manuscript we read the King *et al* paper and realized that it reported that oogenesis arrests at stages 9-10, and that no eggs were produced. We contacted Jackie to ask if she could remember which *ptc* allele she sent us. She told us that she thought that it was ptc^{s2}. (Haifan also told us that he had a ptc^{s2} stock in the past; however, he no longer does.) ptc^{s2} is reported to be a hypomorphic allele by Rodriguez and Basler 1997, not a null. If we used a hypomorphic allele while King *et al* used a null this could explain why we obtained eggs, while King *et al* did not. At this point there is no way for us to readily confirm that we have ptc^{s2} not ptc^{IIw} short of.sequencing the mutant we have and both of the *ptc* alleles in question. I would also note that King *et al* did the experiment quite differently than we did, and that could also account for the difference between our results and theirs. In order to clear out any egg chambers in the ovaries that have residual *ptc* gene product (produced by the *ovo* chromosome prior to the time the clones were generated), King *et al* aged the females for 8-10 days before examining the ovaries (or collecting eggs). By this time the stem cells would have undergone many rounds of cell division and any residual *ptc* product would be diluted to nothing. In contrast, we started egg collections immediately after eclosion and mating. We have also found that egg production from the germ line clone mothers drops to nothing after a few days. Since Haifan could not remember whether they had checked if their ptc^{IIw} germ line clone females laid any eggs prior to day 8-10, we can not exclude the possibility that our allele is actually ptc^{Iiw}.

As indicated above, my original plan was to ask an independent scientist to confirm the germ line clone experiments once Girish had repeated the findings reported in our paper. However, it would unreasonable to do so. There is no good reason to believe any of Ruth's claims, and that person would be unnecessarily wasting his/her time. In fact, in each and every instance Ruth's claims about our paper have proven false. Her allegations were based on misinterpreted data and/or poorly done experiments and on the misrepresentation of our results and of the work of others.