Frazzled/Dcc acts independently of Netrin to promote germline survival during *Drosophila* oogenesis

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MS TITLE: Frazzled/Dcc acts independently of Netrin to promote germline survival during *Drosophila* oogenesis

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant concerns and recommend a substantial revision of your manuscript. Specifically, as reviewer 2 suggests (and reviewer 1 agrees with this) a detailed phenotypic analysis of the germ line in the mutants is necessary. Additionally, as reviewer 1 points out the conclusion that the C terminal domain of Frazzled which rescues the transcriptional deficits in the nervous system does so in the ovary is not supported by the data. This conclusion either needs to be altered or better substantiating data needs to be provided. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the manuscript “Frazzled/Dcc acts independently of Netrin to promote germline survival during Drosophila oogenesis”, Russell and colleagues determined the role of Frazzled in preventing

degeneration of mid-staged egg chambers. Their findings are interesting and worth publication after some revision as detailed below.

Comments for the author

In the manuscript “Frazzled/Dcc acts independently of Netrin to promote germline survival during Drosophila oogenesis”, Russell and colleagues determined the role of Frazzled in preventing degeneration of mid-staged egg chambers. Their findings are interesting and worth publication after some revision as detailed below.

Main concern: The authors claimed that they had found Frazzled function in a “Netrin-independent Fra transcriptional regulation”. The conclusion is not warranted for two reasons: 1) They only shew that a point mutation in the transcription activation domain identified in the nervous system is unable to rescue the loss-of-function of Frazzled. This is consistent with Fra’s involving in transcription control, however, alternative interpretations, such as protein interaction that involves the same protein domain but is unrelated to transcription, is not addressed. 2) The Frazzled c-terminal domain that is sufficient to rescue the transcription defect resulted from the lack of Frazzled in the nervous system did not rescue the oogenesis defect. Therefore, the conclusion is not substantiated by the experiments performed.

Minor concerns:

A. netrinAB mutant Is this a complete loss-of-function of netrin? If not, the conclusion that Frazzled functions independent of netrin during mid-staged egg chambers needs to be reconsidered because low level of netrin function might be sufficient in the process.

B. Line 164: “(Fig. 1B’, arrowhead) We also”: a period should be added before “We”.

C. Line 174: “flies by using the”: I think that either “by” or “using” should be deleted.

D. Line 204: “GFP (3A,C)”: “Fig.” should be added before “3A”.

E. Line 268: “ovarioles. (Fig. 4I)”: the period after “ovarioles” should be deleted.

F. Lines 322-325: The panels of Figure 6 C-E seem to be mislabeled.

G. Line 359: “clones. (Fig. 7B,C)”: the period after “clones” should be deleted.

H. Lines 432-434: “However, Orb and Armadillo localization in fra mutant clones indicates that neither follicle cell apical-basal polarity nor germline polarity are controlled by Fra”. I suggest to tune-down this conclusion since only two markers have been studied. How do you know other polarity markers are not affected?

I. Line 864: “Foxo (grey) (B)”: a period should be added before “(B)”.

J. Line 899: “driven by by”: one “by” should be deleted.

K. Table legends: I would add a “:” after “a” for both tables to reduce confusion.

L. Figure 1A: it would be helpful to label the vitellarium, a term referred to often in the article.

Reviewer 2

Advance summary and potential significance to field

Frazzled is the receptor for netrin in axon guidance, but also has netrin-independent roles. Fra can be cleaved and its ICD can directly act as a transcription factor in neurons. The mammalian ortholog DCC has also been shown to act as a tumor suppressor and promote cell death. This study follows up on data that Dcc/Fra may have roles in the female reproductive system. Indeed, Fra is

required in the germline for egg chamber survival through mid-oogenesis, and acts separately from the starvation pathway. Degenerating egg chambers show activation of Dcp-1, but Fra- egg chambers do not show changes in Diap1 expression. Egg chamber or follicle cell polarity is not affected, thus the reason Fra- egg chambers die is not clear. Rescue experiments suggest that the transcriptional activation domain is required, but the ICD alone cannot rescue, so this also remains unclear. NetrinA/B mutants do not show an egg chamber degeneration phenotype so the authors propose that this is a new netrin-independent role for Fra. The significance of the study is that a new role for Fra has been uncovered, and unlike previous studies it is found to promote cell survival. Overall the study is well done, but further details on the phenotype and form of cell death being activated would strongly improve the manuscript. Specific comments and details are listed below.

Comments for the author

Major points (essential):
1. The complete phenotype of the germline clones should be provided. Do the non-degenerating egg chambers progress to late oogenesis normally? Are the flies fertile? Addressing these points could give some indication as to why the egg chambers might be dying.
2. In Figure 2 B and B', the chromatin looks abnormal before and during degeneration. Is this consistently observed? Does the degeneration look like starvation-induced degeneration with engulfment by follicle cells or are there morphological differences? Morphological description beyond "pyknotic nuclei" would be very helpful in understanding the kind of cell death that is occurring.
3. The authors show that Dcp-1 is activated, but they don't provide any evidence that caspases are required. They should investigate if inhibition of caspases (UASp-p35 or UASp-Diap1) can suppress the cell death in Fra GLCs. Germline compatible RNAi lines of the individual caspase genes are also available. These experiments would confirm if the Fra GLCs activate the same caspase pathway as starvation, if apoptosis is being induced, or some other form of cell death. Without this analysis, the word "apoptosis" should be eliminated from the abstract and replaced with "cell death."
4. The authors conclude in the discussion, "Our results establish the ovary as a second in vivo tissue context where Fra regulates transcription." This seems overstated since the ICD doesn't rescue and it's possible the point mutation could affect other aspects of signaling. The sentence should be modified accordingly.

Minor points:
1. Are there engulfment defects in egg chambers with follicle cell clones, or do they have defects in Arm localization? Are there any phenotypes seen in the FC clones?
2. Figure 5 – Diap1 appears to localize differently in the FCs in the Fra GLCs – is that consistent? Is Diap1 expression maintained in all of the egg chambers? Only 60% die so it could be regulated differently in the ones destined to die.

First revision

Author response to reviewers' comments

We thank the reviewers for their constructive feedback on our manuscript. Based on their criticisms, we have included additional experiments to further support our main claims and have revised the text to address other concerns (these sections of the manuscript have been highlighted). We believe that our revised manuscript has been significantly strengthened and that we have addressed the initial concerns of the reviewers.

Reviewer 1
Reviewer 1 noted that our manuscript was "interesting and worth publication" but raised several points of concern, addressed below.

Major concern:
"The authors claimed that they had found Frazzled function in a 'Netrin-independent Fra

transcriptional regulation'. The conclusion is not warranted for two reasons: 1) They only shew that a point mutation in the transcription activation domain identified in the nervous system is unable to rescue the loss-of-function of Frazzled. This is consistent with Fra's involving in transcription control, however, alternative interpretations, such as protein interaction that involves the same protein domain but is unrelated to transcription, is not addressed. 2) The Frazzled c-terminal domain that is sufficient to rescue the transcription defect resulted from the lack of Frazzled in the nervous system did not rescue the oogenesis defect. Therefore, the conclusion is not substantiated by the experiments performed."

This is a concern shared by Reviewer 1, 2, and Dr. Arur. We agree with the reviewers that our initial experiment, in which germline-specific expression of the *UASp-FraE1354A* transgene failed to rescue egg chamber degeneration, falls short of conclusively demonstrating that the transcriptional activity of this domain is specifically required for progression through mid-oogenesis. To address this concern, we have included a new experiment in which we rescued our *fra* mosaic ovarioles with a version of this transgene including the VP16 activation domain (*UASp-FraE1354A-VP16*). In the nervous system, this transgene rescues the *fra* mutant phenotype (Neuhaus-Follini and Bashaw, 2015). In the ovary, germline-specific expression of *UASp-FraE1354A-VP16* is also able to rescue the block to mid-oogenesis in *fra* mosaic ovarioles (now included in Figure 7). We argue that this experiment demonstrates that it is the transcriptional activity, not another feature of the same domain, that is responsible for progression through mid-oogenesis (lines 368-375).

We are also eager to learn more about why the intracellular domain of Frazzled, which is sufficient to rescue Comm expression in the nervous system, does not restore progression through mid-oogenesis in this system. We have commented on possible reasons for this discrepancy in the manuscript (see lines 375-381 and, in the discussion, 505-515), including both biological and technical possibilities. Nevertheless, we believe that these new results strongly support our claim that Frazzled functions in Netrin-independent transcriptional regulation of gene expression, and we hope the reviewers will agree.

Minor concerns:

"Is this a complete loss-of-function of netrin? If not, the conclusion that Frazzled functions independent of netrin during mid-staged egg chambers needs to be reconsidered because low level of netrin function might be sufficient in the process."

The *netAB*$^{\Delta GN}$ allele we use in this study is a deletion spanning the *netA* and *netB* genomic loci (Bankatschk and Dickson, 2006; Newquist et al. 2013). We apologize for not making this clear in the manuscript and have revised the text (lines 331-334) to prevent confusion.

"'However, Orb and Armadillo localization in fra mutant clones indicates that neither follicle cell apical-basal polarity nor germline polarity are controlled by Fra'. I suggest to tune-down this conclusion since only two markers have been studied. How do you know other polarity markers are not affected?"

We thank the reviewer for pointing out that our language may have presented an undue level of certainty regarding the role of Frazzled in regulating cell polarity. To address this concern, we have softened our language to convey the difficulty of interpreting these negative results (lines 260-274). Furthermore, we have added an additional marker, Discs large, to evaluate lateral follicle cell polarity in ovarioles with *fra* mutant germline clones (now included in Figure 4). Like the other markers of polarity that we tested, Discs large expression and localization is unaffected in the follicle cells of ovarioles with *fra* mutant egg chambers and also in *fra* mutant follicle cells (included as new Supplemental Figure 1). We argue that given the lack of changes in localization of these polarity markers, any role Fra plays in regulating cell polarity is likely to be subtle; we hope the reviewers will agree.

We also thank the reviewer for bringing up typos and other errors in the body of the text, which have now been corrected. We have also added the label "vitellarium" to Figure 1A.

Reviewer 2
Reviewer 2 described our study as "well-done" but desired more characterization of the phenotype. Further, Reviewer 2 shared the concerns of Reviewer 1 and Dr. Arur with respect to our interpretation of our genetic rescue experiments (addressed as a "major concern" above).

Major concerns:

"The complete phenotype of the germline clones should be provided. Do the non- degenerating egg chambers progress to late oogenesis normally? Are the flies fertile? Addressing these points could give some indication as to why the egg chambers might be dying."

We agree with the reviewer that further description of this phenotype would provide insight into the role of Fra in oogenesis. While we completed our initial analysis with the dominant female sterile technique, we have recently encountered persistent egg chamber degeneration in control mock clones, preventing their use in this revision. In our negatively-marked clones, we do not detect late-stage germline clones with enough frequency to allow phenotypic analysis. (We suspect that because the GFP status of egg chambers cannot be determined once egg chamber degeneration is underway, our method of quantification undercounts degeneration events in this experiment.) Furthermore, in the process of mounting ovarioles, we remove late- stage egg chambers to allow smaller early egg chambers to be flattened; these removed egg chambers likely include degenerating tissue. We tried using RNAi to assess the physiological impacts of Fra knockdown; however, none of the RNAi lines gave sufficient knockdown to generate the degeneration phenotype that we observe in germline clones and ovoD clones.
Therefore, although we believe it is highly likely that the profound degeneration that we observe would result in decreased egg production, we cannot currently support that conclusion. We think the full exploration of this phenotype after mid-oogenesis is interesting and hope to address it in the future.

"In Figure 2 B and B', the chromatin looks abnormal before and during degeneration. Is this consistently observed? Does the degeneration look like starvation-induced degeneration with engulfment by follicle cells or are there morphological differences? Morphological description beyond "pyknotic nuclei" would be very helpful in understanding the kind of cell death that is occurring."

We thank the reviewer for suggesting this additional characterization. Based on the morphology of follicle cells in degenerating egg chambers (now highlighted in Fig. 2B''), we believe that follicle cells are engulfing *fra* germlines. To address this directly, we stained negatively-marked clones with anti-Draper, which should be activated as follicle cells engulf the germline (Etchegaray et al. 2012; new Supplemental Figure S2). In both control ovarioles and those containing *fra* mutant germline cysts, we detect Draper. This is consistent with a model in which follicle cells are engulfing the mutant germline, closely mirroring the process observed in starvation-induced degeneration.

"The authors show that Dcp-1 is activated, but they don't provide any evidence that caspases are required. They should investigate if inhibition of caspases (UASp-p35 or UASp-Diap1) can suppress the cell death in Fra GLCs. Germline compatible RNAi lines of the individual caspase genes are also available. These experiments would confirm if the Fra GLCs activate the same caspase pathway as starvation, if apoptosis is being induced, or some other form of cell death. Without this analysis, the word 'apoptosis' should be eliminated from the abstract and replaced with 'cell death.'"

Taking Reviewer 2's concerns into consideration, we have extended our analysis of cell death in our mutant mosaic ovarioles. We inhibited caspase activity with the suggested *UASp-p35* transgene and see that, indeed, cell death is suppressed in *fra* germline clones (now included in Figure 5). We have commented on our interpretation of these results in lines 292-299.

Minor concerns
Are there engulfment defects in egg chambers with follicle cell clones, or do they have defects in Arm localization? Are there any phenotypes seen in the FC clones?

While we share the reviewer's interest in the role of Fra in follicle cells, it does not appear to contribute to the progression of egg chambers through mid-oogenesis. We address the lack of phenotype in this respect in the text (Figure 3, lines 211-215). Further, we do not observe defects in Arm localization in follicle cell clones (lines 269-270; new Supplemental Figure S1).

"Diap1 appears to localize differently in the FCs in the Fra GLCs – is that consistent? Is Diap1 expression maintained in all of the egg chambers? Only 60% die so it could be regulated differently in the ones destined to die."

We thank the reviewer for this keen observation. We do not consistently observe this difference. We have changed the image for this figure to better represent our observations (Figure 5).

Second decision letter

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AUTHORS: Samantha A Russell, Kaitlin M Laws, and Greg Bashaw
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

The authors have demonstrated a new role for Frazzled/Dcc.

Comments for the author

The authors have addressed my concerns and added new experiments that strengthen the paper.