Multifunctional role of GPCR signaling in epithelial tube formation

Vishakha Vishwakarma, Thao Phuong Le and SeYeon Chung
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MS TITLE: Multifunctional role of GPCR signaling in epithelial tube formation

AUTHORS: Vishakha Vishwakarma, Thao Phuong Le, and SeYeon Chung

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

In this study, Vishwakarma et al. characterized the function of the G-protein coupled receptor (GPCR) Smog in Drosophila salivary gland (SG) invagination, a model for epithelial tube formation. SG invagination is mediated by apical actomyosin contractions. Three distinct myosin structures have been observed in invaginating SG, including a medioapical pool, a junctional pool and a supracellular myosin cable that surrounds the entire SG primordium. The upstream signals that activate these myosin structures have not been fully elucidated. Previous studies have shown that

Fog, a secreted ligand for GPCR signaling, mediates the activation of medioapical myosin in SG. Through epistasis analysis, Vishwakarma et al. show that Smog, a ubiquitously expressed GPCR, function downstream of Fog to activate medioapcial myosin during SG invagination. Interestingly, the authors also present evidence that Smog, unlike Fog, regulates junctional myosin and the supracellular myosin cable. Furthermore, the authors found that depletion of Smog results in abnormal apical junction organization in embryonic epithelium and defects in microtubules and apical F-actin in SG cells. The authors conclude that Smog regulates myosin activation and epithelial integrity through both Fog-dependent and Fog-independent mechanisms.

A role for Smog in SG formation has not been previously reported. This work is important and timely given the recent finding that Smog regulates myosin activation during Drosophila gastrulation and germband extension. The phenotypes described in this study should be of interest to those studying actomyosin contractility, GPCR-RhoA signaling and epithelial remodeling. The epistasis analysis between Fog and Smog and the analyses of the medioapical myosin phenotype were well performed, and the conclusions are solid. All phenotypes presented in the figures are carefully quantified with appropriate statistical test, which is another strength of the work. The reported phenotypes in junctional integrity, MT organization and apical bleb formation are also very interesting.

Comments for the author

There are several things that I think the authors should clarify in order to strengthen their conclusions, in particular regarding the proposed Fog-independent functions of Smog. My detailed comments are listed below.
1. The authors conclude that Smog regulates junctional myosin in a Fog-independent manner by comparing the results from the current work to the fog mutant phenotype presented in a previous study (Chung et al., 2017). However, in the current study, junctional myosin intensity was directly measured and compared between control and smog deficient embryos. In contrast, in Chung et al., 2017, only the ratio between junctional myosin and medioapical myosin was reported. The difference in the way how data are analyzed makes it difficult to compare the two mutant phenotypes, and this weakens the “Fog-independent” argument.
2. Only severe smog null embryos were included in the analysis of the supracellular myosin cables. Did the smog RNAi or mild smog null embryos show similar defects in the myosin cables? The fact that only a small fraction of the smog null embryos are categorized as “severe” raises the concern whether myosin cable defects are also present in a small portion of the fog mutant embryos that show the severest phenotype.
3. The wide spectrum of phenotypes in epithelial organization in severe smog null embryos raise the question whether the observed junctional and cytoskeletal defects are secondary consequences of some unknown earlier defects. For example, in regions of the embryo where there is no E-cad or Crb signals, is the cell membrane still intact in those regions? (Are they tissue tears?)
4. For the microtubule phenotype, the authors should show both mild and severe null mutants. Is the microtubule phenotype specific to the SG cells? What is the rationale for comparing the ratio of tyr-MTs inside and outside of SG? Why are MTs inside of SG expected to be more affected by loss of Smog?
5. Regarding the Dia phenotype, since Dia is a known downstream effector of RhoA, it is not surprising that apical Dia is reduced when GPCR-RhoGEF-RhoA signaling is disrupted. Since the other RhoA effector, Rok, showed defective apical localization in both mild and severe smog null embryos, one would expect that Dia also shows defects in both mild and severe embryos – is this the case? This is an important question since the formation of apical blebs was only observed in severe null embryos. It is also unclear whether apical F-actin or Dia is defective in fog mutant SGs. Without such data, it is hard to draw the conclusion that the role of Smog in organizing cortical actin is entirely Fog-independent.

Minor points:
1. The effect of smog RNAi treatment on Smog levels has not been demonstrated. This presumably could be examined by looking at the knockdown effect on Smog-GFP or by RT-PCR of early embryos with maternal KD.
2. Fig. 3G and H shows that there is an increase in the apical cell area in smog null embryos, which is different from the reported fog mutant phenotype (Chung et al., 2017). This difference between the two mutants should be discussed.

3. There appear to be a higher cell membrane signal for Rok-GFP in smog deficient embryos (e.g. Fig. 4G, Fig. S4E). Is this a true phenotype? If so, why would junctional myosin reduce in smog embryos?
4. When describing the SG phenotype in smog null embryos, the authors stated: “Notably, these embryos have relatively normal internalized SGs (Fig. S2F, G), except for rare cases of crooked SG morphology (Fig. S2D), confirming our previous finding that apical constriction is not required for SG internalization (Chung et al., 2017)”. – This is confusing since the supracellular myosin cable, which has been shown to be important for SG invagination (Chung et al., 2017), is also defective in smog mutant embryos.
5. When describing the medioapical myosin phenotype, the authors stated: “compared to clear web-like structures of apicomedial myosin in control SG cells (Fig. 4A-A’’), SG cells in smog knockdown or smog null mutants showed reduced areas of sqh-GFP puncta, suggesting dispersed myosin along the entire apical surface in SG cells (Fig. 4K).” However, this description appears to only fit the situation in smog RNAi (m/z), but not in smog RNAi (z) or mild smog null conditions. In these conditions, myosin appeared to be absent from the medioapical domain without showing clear puncta structures (Figure 4 A-D).
6. Figure 5A,C do not appear to be cited in the text.
7. Line 276: “we occasionally observed large areas in the epidermis and the SG where E-Cad (Fig. 5B, B’) and Crb (Fig. 5D, D’) signals were absent or significantly reduced.” Fig 5B, D only show epidermis but not SG.
8. Based on the images in Figure 6C and D, junctional Dia appears to be reduced in smog null embryos. It is surprising that the quantification shows there is no difference.
9. Figure 6H, I: the mild smog-null mutant should also be presented for comparison.

Reviewer 2

Advance summary and potential significance to field

Vishwakarma et al. investigate the roles of Smog GPCR signaling during epithelial tube formation of the Drosophila embryonic salivary gland (SG). Development of the salivary glands is driven by actomyosin contractility that constricts the apical surface of inner SG cells, convergent extension, and actomyosin cable formation surrounding the invaginating pit (RÃ¶per, 2012; Chung et al., 2017; Sanchez-Corrales et al., 2018).
The coordinated apical constriction and invagination of the salivary gland is dependent on the transduction of Folded gastrulation (Fog) ligand through an unknown G protein-coupled receptor (GPCR) to activate RhoA GTPase signaling (Kolesnikov and Beckendorf, 2007; Chung et al., 2017). Through a series of genetic perturbations in Drosophila embryos, the authors determined that the ubiquitous GPCR Smog receptor responds to and transduces Fog signal within SG cells. Specifically, Fog over-expression leads to over-accumulation of Smog, myosin, and Rho kinase on the medioapical surface of SG cells, and smog knock-down suppresses Fog over-expression defects. Furthermore, Smog is required for Fog’s downstream regulation of apical constriction, as disruption of smog resulted in larger apical areas and compromised epithelial morphology.
In addition to this Fog-dependent role, the authors suggest a Fog-independent role for Smog in regulating the junctional myosin organization, supracellular myosin cables, microtubule networks, and cortical F-actin. While some of the effects of Smog have been described for earlier morphogenetic events in the Drosophila embryo this manuscript describes a new developmental context for Smog signaling and its downstream effectors in regulating different subcellular domains, which will be of broad interest to researchers studying morphogenesis and tissue remodeling processes. There are a few points that I think should be strengthened and/or clarified.

Comments for the author

Main points:
1. Regarding the conclusion about Fog-independent Smog signaling: The authors conclude there is a novel role for Smog that is independent of the Fog ligand. Specifically, they describe defects in the junctional myosin pool and supracellular myosin cables as well as disruptions in the organization of microtubule and cortical actin networks that are unique to the various smog genetic perturbations. The authors claim that these phenotypes are specific to Smog compared to previously reported data for fog mutants (Chung et al., 2017), yet only compared their Smog data

to wild-type and Fog over-expression embryos. Including images and analysis of Fog mutant embryos and demonstrating a distinct phenotype would significantly strengthen the argument for Smog signaling independent of Fog.
2. In the authors' model, Smog signaling regulates epithelial integrity under the Fog-independent pathway.
However, in Supp Fig. S1 and S4, the authors state that "the embryo surface is often uneven in fog mutant embryos due to additional folding and grooves". In Fig 5 B', D' there are gaps in E-cadherin staining, however it is unclear if there are cell interfaces or even cells still in this region. The lack of staining could also be due an uneven surface and it would be more convincing to co-stain with a membrane marker to show that cells are present.
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First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
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Reviewer 1 Comments for the author

There are several things that I think the authors should clarify in order to strengthen their conclusions, in particular regarding the proposed Fog-independent functions of Smog. My detailed comments are listed below.

1. The authors conclude that Smog regulates junctional myosin in a Fog-independent manner by comparing the results from the current work to the fog mutant phenotype presented in a previous study (Chung et al., 2017). However, in the current study, junctional myosin intensity was directly measured and compared between control and smog deficient embryos. In contrast, in Chung et al., 2017, only the ratio between junctional myosin and medioapical myosin was reported. The difference in the way how data are analyzed makes it difficult to compare the two mutant phenotypes, and this weakens the “Fog-independent” argument.

We have included new quantifications for the medioapical and junctional myosin intensities and the ratio of the medioapical to junctional myosin in fog mutants (Fig. S3A-E). Consistent with Chung et al., 2017, medioapical myosin intensity was reduced in fog mutants, but junctional myosin intensity was not. It supports the Fog-independent role of Smog.

2. Only severe smog null embryos were included in the analysis of the supracellular myosin cables. Did the smog RNAi or mild smog null embryos show similar defects in the myosin cables? The fact that only a small fraction of the smog null embryos are categorized as “severe” raises the concern whether myosin cable defects are also present in a small portion of the fog mutant embryos that show the severest phenotype.

smog RNAi (Z) SGs and smog null [mild] embryos do not show defects in the myosin cable. We have added a quantification for the circularity of the SG boundary for smog null [mild] (Fig. 4O, R). We did not observe defects in the myosin cable in fog mutant embryos (n > 20). Given that > 25% of the smog null mutant embryos are categorized as “severe” and show defects in the myosin cable, we would expect to see at least a few fog mutant embryos with similar defects if they behave similarly to smog mutants. fog mutant embryos with the most severe phenotype are severely twisted and form extra grooves and folds here and there (Fig. S3B, G; also shown in Chung et al., 2017), which made it difficult to capture the whole SG boundary in these embryos. However, we still could see a smooth SG boundary as we go up and down the confocal stack (Fig. S3F-H). Using less twisted embryos, we quantified the circularity of the SG boundary for fog mutants and included it in Fig. S3F-H.

3. The wide spectrum of phenotypes in epithelial organization in severe smog null embryos raise the question whether the observed junctional and cytoskeletal defects are secondary consequences of some unknown earlier defects. For example, in regions of the embryo where there is no E-cad or Crb signals, is the cell membrane still intact in those regions? (Are they tissue tears?)

The cell membrane is still intact in those regions. We have included a new panel showing relatively normal signals of the membrane marker Gap43-mCherry in the region with no E-Cad or Crb signals (Fig. 5E-E’’).

4. For the microtubule phenotype, the authors should show both mild and severe null mutants. Is the microtubule phenotype specific to the SG cells? What is the rationale for comparing the ratio of tyr-MTs inside and outside of SG? Why are MTs inside of SG expected to be more affected by loss of Smog?

We compared the ratio of microtubule signals inside and outside because microtubule levels outside the SG appear to be comparable to WT (or reduced only a little), but the reduction of the intensity inside the SG is prominent. Representative images for Ace-Tub and Tyr-Tub signals have been added for smog null mutants (both mild and severe) and fog mutants (Fig. 5P-S, 5T-W; fog mutants were included in response to Reviewer 2’s Major Point 1). Quantifications of the ratio of inside to outside signals have been included in Fig. 5X (Ace-Tub) and Fig. 5Y (Tyr-Tub).

5. Regarding the Dia phenotype, since Dia is a known downstream effector of RhoA, it is not surprising that apical Dia is reduced when GPCR-RhoGEF-RhoA signaling is disrupted. Since the

other RhoA effector, Rok, showed defective apical localization in both mild and severe smog null embryos, one would expect that Dia also shows defects in both mild and severe embryos – is this the case? This is an important question since the formation of apical blebs was only observed in severe null embryos. It is also unclear whether apical F-actin or Dia is defective in fog mutant SGs. Without such data, it is hard to draw the conclusion that the role of Smog in organizing cortical actin is entirely Fog-independent.

Thank you for the suggestion. We quantified Dia levels in smog null [mild], smog null [severe], and fog mutants (Fig. 6G-K). Indeed, Dia levels were reduced in all three of them, with the most significant reduction in smog null [severe] embryos. We have included representative images for Dia (Fig. 6G-J) and apical F-actin (Fig. 6C-F) and the quantification of the total Dia intensity in smog null [mild], smog null [severe], and fog mutants (Fig. 6K). Despite the reduction of Dia levels in all three pools of embryos, only smog null [severe] embryos show the blebbing phenotype. A simple interpretation would be that subtle changes in Dia levels in smog [mild] or fog mutants may not be enough to cause membrane blebbing. Alternatively, reduced F-actin levels in fog mutants are due to a defective medioapical myosin pool. Based on these data, we have slightly modified our model and propose that the role of Smog in cortical actin organization may be both Fog-dependent and -independent (Fig. 7).

Minor points:

1. The effect of smog RNAi treatment on Smog levels has not been demonstrated. This presumably could be examined by looking at the knockdown effect on Smog-GFP or by RT-PCR of early embryos with maternal KD.

As suggested, we knocked down smog in SG cells using both RNAi lines we used in the study (TRiP. HMC03192, a stronger line; TRiP. GL01473, a weaker line) and fkh-Gal4 and quantified the intensity of Smog-GFP signals (Fig. S1A-D). Smog-GFP signals were reduced with both lines, with a stronger effect with TRiP. HMC03192, consistent with stronger phenotypes with it.

2. Fig. 3G and H shows that there is an increase in the apical cell area in smog null embryos, which is different from the reported fog mutant phenotype (Chung et al., 2017). This difference between the two mutants should be discussed.

We have added the discussion (Lines 190-197).

3. There appear to be a higher cell membrane signal for Rok-GFP in smog deficient embryos (e.g. Fig. 4G, Fig. S4E). Is this a true phenotype? If so, why would junctional myosin reduce in smog embryos?

We carefully compared all Rok-GFP images for smog knockdown or smog null [mild] and did not observe higher membrane signals for Rok-GFP. A better representative image has been chosen in Fig. 4G. In smog null [severe] embryos, Rok-GFP signals appear to be increased in the membrane in cells with a blebbing phenotype, consistent with the idea that Rok is recruited to the bleb for retraction.

4. When describing the SG phenotype in smog null embryos, the authors stated: “Notably, these embryos have relatively normal internalized SGs (Fig. S2F, G), except for rare cases of crooked SG morphology (Fig. S2D), confirming our previous finding that apical constriction is not required for SG internalization (Chung et al., 2017)”. – This is confusing since the supracellular myosin cable, which has been shown to be important for SG invagination (Chung et al., 2017), is also defective in smog mutant embryos.

We have removed the sentence to avoid any confusion.

5. When describing the medioapical myosin phenotype, the authors stated: “compared to clear web-like structures of apicomedial myosin in control SG cells (Fig. 4A-A’’), SG cells in smog knockdown or smog null mutants showed reduced areas of sqh-GFP puncta, suggesting dispersed myosin along the entire apical surface in SG cells (Fig. 4K).” However, this description appears to only fit the situation in smog RNAi (m/z), but not in smog RNAi (z) or mild smog null conditions. In

these conditions, myosin appeared to be absent from the medioapical domain without showing clear puncta structures (Figure 4 A-D).

Myosin intensity and areas of apicomedial myosin are reduced in smog knockdown and smog null [mild] SGs (Fig. 4A-D'', I-L). As the reviewer pointed out, however, the dispersed myosin phenotype along the entire apical surface in SG cells is most prominent in smog RNAi (M/Z). To make it clearer, we have rephrased the sentence: "compared to clear web-like structures of medioapical myosin in control SG cells (Fig. 4A-A''), SG cells in smog knockdown or smog null mutants showed reduced areas of sqh-GFP puncta (Fig. 4L), with dispersed myosin along the entire apical surface in smog M/Z knockdown SG cells (Fig. 4C-C'')." (Lines 237-240). We also have replaced sqh-GFP images for smog RNAi (Z) and smog null [mild] with better representatives (Fig. 4B-B'', D-D'').

6. Figure 5A,C do not appear to be cited in the text.

We have cited them.

7. Line 276: "we occasionally observed large areas in the epidermis and the SG where E-Cad (Fig. 5B, B') and Crb (Fig. 5D, D') signals were absent or significantly reduced." Fig 5B, D only show epidermis but not SG.

We have removed the SG from the text (Line 284).

8. Based on the images in Figure 6C and D, junctional Dia appears to be reduced in smog null embryos. It is surprising that the quantification shows there is no difference.

Yes, we agree that overall Dia levels appeared reduced in smog null embryos. In the previous quantification, however, the junctional Dia intensity in smog null mutant did not show a significant difference compared to control after background subtraction. As we have added new analyses of Dia levels in smog null [mild] and fog mutants (see the response to the main point 5 above), we have replaced Dia quantifications for separate domains with the total intensity of Dia in the whole SG placode (Fig. 6G-K).

9. Figure 6H, I: the mild smog-null mutant should also be presented for comparison.

A representative image for smog null [mild] has been added (new Fig. 6L-N).

Reviewer 2 Advance summary and potential significance to field
Vishwakarma et al. investigate the roles of Smog GPCR signaling during epithelial tube formation of the Drosophila embryonic salivary gland (SG). Development of the salivary glands is driven by actomyosin contractility that constricts the apical surface of inner SG cells, convergent extension, and actomyosin cable formation surrounding the invaginating pit (Röper, 2012; Chung et al., 2017; Sanchez-Corrales et al., 2018). The coordinated apical constriction and invagination of the salivary gland is dependent on the transduction of Folded gastrulation (Fog) ligand through an unknown G protein-coupled receptor (GPCR) to activate RhoA GTPase signaling (Kolesnikov and Beckendorf, 2007; Chung et al., 2017). Through a series of genetic perturbations in Drosophila embryos, the authors determined that the ubiquitous GPCR Smog receptor responds to and transduces Fog signal within SG cells. Specifically, Fog over-expression leads to over-accumulation of Smog, myosin, and Rho kinase on the medioapical surface of SG cells, and smog knock-down suppresses Fog over-expression defects. Furthermore, Smog is required for Fog's downstream regulation of apical constriction, as disruption of smog resulted in larger apical areas and compromised epithelial morphology.
In addition to this Fog-dependent role, the authors suggest a Fog-independent role for Smog in regulating the junctional myosin organization, supracellular myosin cables, microtubule networks, and cortical F-actin. While some of the effects of Smog have been described for earlier morphogenetic events in the Drosophila embryo, this manuscript describes a new developmental context for Smog signaling and its downstream effectors in regulating different subcellular domains, which will be of broad interest to researchers studying morphogenesis and tissue remodeling processes. There are a few points that I think should be strengthened and/or clarified.

Reviewer 2 Comments for the author
Main points:

1. Regarding the conclusion about Fog-independent Smog signaling: The authors conclude there is a novel role for Smog that is independent of the Fog ligand. Specifically, they describe defects in the junctional myosin pool and supracellular myosin cables as well as disruptions in the organization of microtubule and cortical actin networks that are unique to the various smog genetic perturbations. The authors claim that these phenotypes are specific to Smog compared to previously reported data for fog mutants (Chung et al., 2017), yet only compared their Smog data to wild-type and Fog over-expression embryos. Including images and analysis of Fog mutant embryos and demonstrating a distinct phenotype would significantly strengthen the argument for Smog signaling independent of Fog.

As suggested, fog mutants have been analyzed using the same method as smog mutants. Representative images and quantifications have been added for myosin (Fig. S3), acetylated and tyrosinated α-Tubulin (Fig. 5P-Y), Dia (Fig. 6G-K), and phalloidin (Fig. 6C-F). Junctional myosin and the supracellular myosin cable are not changed in fog mutants, supporting our model for the Fog-independent role of Smog in regulating junctional/supracellular myosin. Microtubules and F-actin/Dia are slightly changed in fog mutants, to a similar extent to smog null [mild] but significantly lesser than smog null [severe]. Based on these new data, we have slightly modified our model (Fig. 7) and added additional discussion.

2. In the authors' model, Smog signaling regulates epithelial integrity under the Fog-independent pathway. However, in Supp Fig. S1 and S4, the authors state that "the embryo surface is often uneven in fog mutant embryos due to additional folding and grooves". In Fig 5 B', D' there are gaps in E-cadherin staining, however, it is unclear if there are cell interfaces or even cells still in this region. The lack of staining could also be due an uneven surface and it would be more convincing to co-stain with a membrane marker to show that cells are present.

An extra thought (not asking for anything here): The gap in E-cadherin staining and the presence of blebs strikes me as hallmarks of the epithelial 'tearing' phenotypes in other contractile tissues, such as the mesoderm (Jodoin et al., 2015). A membrane marker would also determine if there is a similar phenotype in the salivary gland, especially if there are membrane tethers.

The cell membrane is still intact in the regions where E-Cad and Crb are gone in smog [severe] embryos (but these cells have enlarged apical areas compared to neighboring cells with strong E-Cad/Crb levels). We have included a new panel showing clear signals of the membrane marker Gap43-mCherry in the region with no E-Cad or Crb signals (Fig. 5E-E''). In fog mutants, the embryo surface is uneven due to the twisted embryonic morphology or extra foldings; we have never observed gaps in Crb and E-Cad signals in fog mutants.

Minor comments:

1. Fig. 2: Need to define what V5 tag labels in figure legend or text.
Figure labels have been changed to S2-Mist-V5 and S2-Smog-V5.

2. Lines 245-252: Does smog RNAi also affect junctional Rok-GFP?

In the invaginating SG, Rok-GFP signals mostly accumulate in the medial region and are not detected at AJs. The images shown in the manuscript are merged images of two z-sections of the apical region of SG cells with the highest Rok signals. (Weak Rok-GFP signals are detected along the lateral membrane basal to AJs, and we did not observe any changes for these signals in smog RNAi.)

3. Line 277: Crb not introduced before being discussed.
Thanks for catching it. Introduced (Line 285).

4.Fig. 5Q: The difference in microtubule staining in the smog null mutant could be a consequence of these cells failure to constrict and concentrate their apical microtubules. Should somehow normalize to apical area.

We used the mean gray value for acetylated and tyrosinated a-tubulin intensities, which takes the apical area into account. In new Figs. 5X and 5Y, we showed the ratio of the mean gray value of the entire SG placode to the average mean gray value of cells outside the placode. We also attempted to directly compare the mean gray values of acetylated α-tubulin signals in the SG placode between WT and smog null [severe] and observed a significant reduction in smog null [severe] SGs (data not shown).

Second decision letter

MS ID#: DEVELOP/2022/200519

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AUTHORS: Vishakha Vishwakarma, Thao Phuong Le, and SeYeon Chung

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The overall evaluation is very positive and we would like to publish your manuscript in Development. Before we can proceed with acceptance, please address the points by one of the referees?

Reviewer 1

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The authors have carefully addressed all my previous questions. The addition of new experimental results, data quantification and discussion have greatly improved the manuscript. I fully support the publication of this work.

Comments for the author

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Comments for the author

The paper has been much improved. I have a few minor comments that the authors may wish to address.
1) Now that I understand what is labeled in the S2 cell experiment I see that there appear to be transfected and untransfected cells. Did the authors look at the percentage of cells that are contracted the transfected cells? Might help to point this out.
2) For the enhancement of the smog phenotype by chic-RNAi, how are the authors distinguishing being smog[severe] from chic-RNAi enhancing the mild phenotype? Does chic-RNAi shift the distribution to having more of the [severe] class?
3) For statistics the authors use a Welch's t-test. This is a parametric test that assumes normality in the data. Did the authors test for normality?

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
The authors have carefully addressed all my previous questions. The addition of new experimental results, data quantification and discussion have greatly improved the manuscript. I fully support the publication of this work.

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Reviewer 2 Advance summary and potential significance to field
Vishwakarma et al. investigate the roles of Smog GPCR signaling during epithelial tube formation of the Drosophila embryonic salivary gland (SG). Development of the salivary glands is driven by actomyosin contractility that constricts the apical surface of inner SG cells, convergent extension, and actomyosin cable formation surrounding the invaginating pit (Röper, 2012; Chung et al., 2017; Sanchez-Corrales et al., 2018). The coordinated apical constriction and invagination of the salivary gland is dependent on the transduction of Folded gastrulation (Fog) ligand through an unknown G protein-coupled receptor (GPCR) to activate RhoA GTPase signaling (Kolesnikov and Beckendorf, 2007; Chung et al., 2017). Through a series of genetic perturbations in Drosophila embryos, the authors determined that the ubiquitous GPCR Smog receptor responds to and transduces Fog signal within SG cells. Specifically, Fog over-expression leads to over- accumulation of Smog, myosin, and Rho kinase on the medioapical surface of SG cells, and smog knock- down suppresses Fog over-expression defects. Furthermore, Smog is required for Fog’s downstream regulation of apical constriction, as disruption of smog resulted in larger apical areas and compromised epithelial morphology.
In addition to this Fog-dependent role, the authors suggest a Fog-independent role for Smog in regulating the junctional myosin organization, supracellular myosin cables, microtubule networks, and cortical F-actin. While some of the effects of Smog have been described for earlier morphogenetic events in the Drosophila embryo, this manuscript describes a new developmental context for Smog signaling and its downstream effectors in regulating different subcellular domains, which will be of broad interest to researchers studying morphogenesis and tissue remodeling processes.

Reviewer 2 Comments for the author
The paper has been much improved. I have a few minor comments that the authors may wish to address.
1) Now that I understand what is labeled in the S2 cell experiment I see that there appear to be transfected and untransfected cells. Did the authors look at the percentage of cells that are contracted the transfected cells? Might help to point this out.

Yes, the percentage of contracting cells among the transfected cells was calculated. We have added the description to the figure legend for Fig. 2G.

2) For the enhancement of the smog phenotype by chic-RNAi, how are the authors distinguishing being smog[severe] from chic-RNAi enhancing the mild phenotype? Does chic-RNAi shift the distribution to having more of the [severe] class?

smog [severe] embryos were categorized based on the disrupted embryonic morphology with SGs elongated along the dorsal/ventral axis. chic was knocked down using the SG-specific fkh-Gal4 and did not affect the distribution of smog mutant severity. In chic RNAi in smog null mutants, we observed blebs even in normal-looking embryos and circular SG placodes. To make it clearer, we have slightly modified the text: “we observed enhanced blebbing even in mildly defective SG cells” -> “we observed enhanced blebbing even in SG cells in smog null [mild] embryos” (lines 371-372).

3) For statistics the authors use a Welch's t-test. This is a parametric test that assumes normality in the data. Did the authors test for normality?

Thank you for pointing this out. We have performed the Jarque-Bera test in Excel and also tested for a normal bell-shape of the dataset by making histograms. The Jarque-Bera test is a goodness-of-fit test that determines whether sample data have skewness and kurtosis that matches a normal distribution. Medial and junctional myosin intensities showed a normal distribution, and we kept the original statistical analyses (Fig. 1H-J; Fig. 4I-K). We have added, “A normal distribution was tested using Jarque-Bera test (Microsoft Excel) or a bell-shape of data distribution by making a histogram.” in Materials & Methods (lines 573-575).

We noticed that the dataset was indeed skewed for Rok-GFP particle areas and sqh-GFP particle areas. We re-performed statistical analyses using the Mann-Whitney U test, a method to compare differences between two independent groups when the dependent variable is either ordinal or continuous but not normally distributed. The new analysis did not affect our conclusions. Figures have been replaced, and the figure legends were modified accordingly (Fig. 1N, O; Fig 4L, M).
We also re-performed statistical analyses for smog-GFP intensity, Dia intensity, Ace-tub and Tyr-tub intensities, and E-Cad/Crb gap length/numbers, using Mann-Whitney U test. Figures and legends have been replaced accordingly (Fig. 1E; Fig. S1D; Fig. 5L-O; Fig. 5X, Y; Fig. 6K). P values were similar to the previous values for all of them, except for the one for the comparison of Dia levels between control and fog mutants (p<0.05 to non-significant; Fig. 6K), which strengthens our conclusion that Dia/cortical F-actin levels were not significantly affected by fog loss. We modified the text to “SGs in fog mutants appeared to show a slight reduction of F-actin (Fig. 6F), but Dia levels in fog mutants were comparable to WT (Fig. 6J, K)” (lines 358-359). Based on these, we have removed red dotted lines for Dia and cortical actin from our model (Fig. 7).

Third decision letter

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MS TITLE: Multifunctional role of GPCR signaling in epithelial tube formation

AUTHORS: Vishakha Vishwakarma, Thao Phuong Le, and SeYeon Chung
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.