Early perturbation of Wnt signaling reveals patterning and invagination-evagination control points in molar tooth development

Rebecca Kim, Tingsheng Yu, Jingjing Li, Jan Prochazka, Amnon Sharir, Jeremy B.A. Green and Ophir D. Klein
DOI: 10.1242/dev.199685

Editor: Patrick Tam

Review timeline

First submission:	8 April 2020
Reject with Hope:	21 May 2020
Resubmission:	22 April 2021
Editorial decision:	7 June 2021
First revision received:	22 June 2021
Accepted:	28 June 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/191353

MS TITLE: Early epithelium-specific blockade of Wnt/?-catenin deletes the molar tooth whereas hyperactivation produces mesenchyme-driven evaginations

AUTHORS: Rebecca Kim, Tingsheng Yu, Jingjing Li, Jan Prochazka, Amnon Sharir, Jeremy B.A. Green, and Ophir D Klein ARTICLE TYPE: Research Report

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 from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it (please see Editor's note appended below). Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential merit of the work on the role of Collagen VI. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

Editor's note:

The consensus of the referees is that the findings on the impact of disruption of beta-catenin activity on epithelial evagination have supported the concept that Wnt/bCat activity is essential for placode induction at early stages of the formation of molar tooth. Apart from confirming previous findings that loss and gain of bCat activity affect molar formation differently, the study stopped short of elucidating the factors and molecular mechanisms downstream of beta-catenin

activity in the epithelial cells for the regulation of epithelial-mesenchymal interaction. The reliance on directly disrupting the beta-catenin activity to investigate the functions of Wnt signalling could not separate the structural role of beta-catenin in epithelial integrity from its role in Wnt signalling. The novel finding about the role of Collagen VI in the mesenchymal condensation is not fully explored. Providing a synopsis of what is known about this collagen and whether it is associated (directly or indirectly) with tooth pathologies would be useful. The non-specific inhibition of collagen biosynthesis was too disruptive to pinpoint any specific role of this collagen in the formation of evaginations in the diastema region. The assertions that the evagination by local accumulation of underlying mesenchyme represents a novel morphogenetic mechanism and this study has revealed new aspects of Wnt/beta-catenin signalling in tooth formation were not warranted. Overall, the descriptive study did not achieve this goal and was short of revealing new mechanisms regarding Wnt signalling in tooth development.

Reviewer 1

Advance Summary and Potential Significance to Field

This is a very well written paper where the authors using an early expressed FGF8 driven promoter confirmed previously published work at an earlier stage of development.

Comments for the Author

This is a manuscript where the authors using an Fgf8-promoter-driven dominant-negative beta-catenin transgene, found that (i) loss of Wnt/beta-catenin signaling completely deletes the molar tooth, confirming that this pathway is important to the early stages of tooth formation and (ii) that Fgf8-promoter-driven dominant-active beta-catenin protein produces evaginations that lead to the formation of supernumerary teeth later on. The role of Wnt/beta-catenin signaling in tooth development has been previously studied. Hyperactivation of Wnt signaling led to formation of supernumerary teeth (Jarvinen et al., 2006; Wang et al., 2009; Liu et al., 2008). Hypoactivation of the Wnt pathway disrupted tooth development at different stages (Andl et al., 2002; Liu et al., 2008; Sasaki et al., 2005). Hyperactivation and hypoactivation of Wnt/beta-catenin signaling in the mesenchyme resulted in a decrease in tooth number (Chen et al., 2009; Jarvinen et al., 2018). In humans, loss-of-function mutations of the Wnt inhibitors APC and AXIN2 cause hyperdontia and hypo/oligodontia(Lammi et al., 2004). Thus, the role of the Wnt/beta-catenin pathway is not unclear. The existence of different phenotypes obtained with distinct genetic approaches suggest rather the presence of a fine tuned process that requires the coordination of different pathways at different stages of development. The authors using an early expressed FGF8 driven promoter confirmed previously published work at an earlier stage of development.

The conclusion that the existence of early evaginations that are associated with premature mesenchymal accumulation of condensation-associated collagen VI represents a novel morphogenetic mechanism is premature, especially when we know from previous studies and from this study that these evaginations lead to supernumerary teeth in the diastema region.

Reviewer 2

Advance Summary and Potential Significance to Field

This study provides a temporo-spatially specific approach to studying the role of Wnt signaling in molar development. Authors use the FGF8 promoter to genetically manipulate the beta-catenin and modulate the Wnt signaling up and down at early stages of molar development. Their data show that the Wnt/? -catenin pathway plays an early, critical role in determining the invagination versus evagination of the molar epithelium.

Comments for the Author

This is an excellent, well conducted manuscript with a logical flow and high data of high quality. I sincerely have no additional comment or criticism to make. The scientific interest is obvious and the evigination formation process with accumulation of collagen VI opens new avenues. May be in

the discussion section, a bit more need to be said about what is known about this collagen and whether it is associated (directly or indirectly) with human pathologies.

Reviewer 3

Advance Summary and Potential Significance to Field

In this manuscript, Kim et al. investigated effects of early epithelium-specific inactivation or constitutive activation of beta-catenin in molar tooth development. Although the roles of Wnt signaling in tooth development have been studied in several previous publications, the authors reasoned that the precise role of the pathway is still unclear due in part to the different phenotypes obtained with distinct genetic approaches. In this study, the authors used Fgf8-CreER in combination with intraperitoneal tamoxifen administration to achieve molar epithelial specific inactivation or constitutive activation during the molar tooth germ initiation stage. They termed the beta-catenin deletion mutants as WntLOF mutants and the constitutively active beta-catenin mutant as WntGOF mutants. The WntLOF mutants showed lack of molar tooth epithelial invagination whereas the WntGOF mutants showed aberrant evagination of the molar tooth epithelium by E12.5. Using Rosa26-mTmG reporter, they demonstrated that the WntLOF mutant had defects in suprabasal cell convergence and lacked the normal F-actin enrichment in the suprabasal cells during molar tooth bud formation. In the WntGOF mutants, they observed premature accumulation of collagen VI, an extracellular matrix component enriched in condensed dental mesenchyme cells. Inhibition of collagen biosynthesis using Mithramycin A inhibited evagination of molar epithelium in WntGOF mutants as well as normal molar epithelial invagination in wildtype molar tooth germs. They authors concluded that the evagination by local accumulation of underlying mesenchyme represents a novel morphogenetic mechanism and their studies reveal new aspects of Wnt/beta-catenin signaling in tooth formation.

Comments for the Author

1. Overall, the manuscript provides phenotypic description of the WntLOF and WntGOF mutants with disruption of tooth development at an earlier developmental stage than previously described from using other Cre drivers. However, the manuscript, as written, does not provide clearer understanding than before regarding the roles of Wnt signaling in tooth development. The WntLOF results are suggestive that Wnt signaling in the dental placode epithelium might play an important role in tooth bud invagination, but the results are preliminary and requires further validation using Wnt signaling specific reagents (see next two points).

2. Both the WntLOF and WntGOF disrupted beta-catenin function and both mutants showed disruption of epithelial integrity, with the WntLOF mutants showed increased epithelial apoptosis and the WntGOF mutants showed reduction/disruption of E-cadherin expression. It is quite possible that the lack of tooth epithelial invagination in the WntLOF mutants was due to the increased epithelial cell death and disruption of epithelial integrity rather than a specific Wnt-induced suprabasal cell convergence. The authors showed that K14-CreERT2;Porcn>fl/fl embryos had a similar phenotype of shallower tooth bud invagination at E12.5, but did not show whether the tooth buds continue to form in the K14-CreERT2;Porcn>fl/fl embryos by E13.5 and whether these Porcn mutants also show similar dental epithelial apoptosis and lack of mesenchymal condensation. It is not known and this manuscript does not address the mechanism how Wnt signaling could be inducing the suprabasal epithelial convergence during tooth bud formation.

3. Figure 2M only quantified percent of EdU-labeled epithelial cells, but Fig. 2E clearly shows a dramatic reduction in nascent dental mesenchyme proliferation in the WntLOF embryo and Fig. 2F shows a likely increase in mesenchymal cell proliferation in the WntGOF embryo. Thus, both of the lack of epithelial invagination in the WntLOF and the evagination in the WntGOF mutants resulted from a combination of disruption of overlying epithelial integrity and secondary defects in the underlying mesenchyme. It is very difficult to tease apart the effects of Wnt signaling from the structural requirements of beta-catenin using those beta-catenin mutants. The authors should examine more Wnt pathway-specific mutants, such as tissue-specific deletion of Wntless to validate effects of Wnt signaling without directly disrupting beta-catenin.

4. While it is clear that the mesenchymal underlying the WntGOF mutant dental epithelium is affected in both proliferation and ECM production, these are cell non-autonomous effects resulting from disruption of the dental placode signaling center. The manuscript stops short of identifying the factors and molecular mechanisms downstream of beta-catenin in the epithelial cells in the regulation of epithelial-mesenchymal interaction.

5. The inhibition of collagen biosynthesis is too disruptive a method to reveal any mechanism of epithelial invagination/evagination and does not reveal any specific role of Collagen VI.

6. In the Abstract, the authors wrote "using an Fgf8-promoter driven and therefore early-expressed dominant-negative ?-catenin transgene, we found that loss of Wnt/?-catenin signaling completely deletes the molar tooth". There is no "dominant-negative ?-catenin transgene" used in any of the experiments described in the Results section.

Reviewer 4

Advance Summary and Potential Significance to Field

The paper titled Early epithelium-specific blockade of Wnt/?-catenin deletes the molar tooth whereas hyperactivation produces mesenchyme-driven evaginations, by Kim et al., uses complementary gain and loss of function genetic approaches with an Fgf8-promoter-cre to investigate mouse tooth development. The novelty of their findings relate to the promoter being turned on early in development in the LOF than previously published K14-Cre and so they observe less invagination and complete deletion of a molar. Additionally, with the GOF they also observe epithelial protrusions and supernumerary teeth, confirming previous studies. They show these protrusions do not involve increase in proliferation but occur due to increased Collagen VI expression, which is associated with mesenchymal condensation. The study provides additional genetic approaches and findings that confirm and extend the existing literature on B-catenin in tooth development.

Comments for the Author

Major comments:
1. A novel finding is the increased ColVI in the GOF, suggesting Wnt/B-catenin signaling regulates extracellular matrix expression. They then reduce the phenotype using Mithromycin, which is likely inhibiting other collagen or matrix synthesis. They provide no direct evidence Mithromycin specifically reduces ColVI in the experiment, this should be clarified.

2. Is the mesenchymal condensation involving a generalized increased in ECM or is it specific to ColVI?

3. The authors should comment in Fig 3 on the polarized columnar shape of the epithelium that clear occurs using f-actin staining during control molar development and that it seems to be reduced in the in WntGOF along with a reduced cell size. Is polarity of the basal epithelial cells reduced?

4. The authors claim "that such evagination by local accumulation of underlying mesenchyme represents a novel morphogenetic mechanism." Can they discuss whether this may be relevant to any other morphological process, or is it just relevant to the formation of supernumerary teeth?

Additional minor comments:
Clarify description of IWP and IWP2.
Supplementary Fig 3, should mention what anti-GM130 is labeling, why it was used and describe what the staining is showing in the text.
Images mislabeled in Fig4, K and L.
Duplicated reference Jarvinen 2006.

First revision

Author response to reviewers' comments

We are pleased to submit our paper **"Early perturbation of Wnt signaling reveals patterning and invagination-evagination control points in molar tooth development,"** which is a significantly revised version of our manuscript, entitled "Early epithelium-specific blockade of Wnt/ß-catenin deletes the molar tooth whereas hyperactivation produces mesenchyme-driven evaginations"; (manuscript ID DEVELOP/2020/191353). We have made amendments in response to all four reviewers' comments, which were helpful in improving the paper. Due to the need for additional experiments as well as pandemic-related delays, it has taken us longer than expected to complete the revisions, and we hope that the manuscript is now acceptable for publication.

Thanks to the extra experiments (detailed below), we have been able to clarify that the main novel finding in the paper is the role of canonical Wnt signalling levels in controlling the timing of mesenchymal condensation, which in turn determines whether epithelial invagination or evagination predominates. This is reflected in the title change and substantial re-writing in much of the text.

Below, we summarize the main changes that we have made:

Reviewer 1 Comments for the Author:
This is a manuscript where the authors using an Fgf8-promoter-driven dominant-negative beta-catenin transgene, found that (i) loss of Wnt/beta-catenin signaling completely deletes the molar tooth, confirming that this pathway is important to the early stages of tooth formation and (ii) that Fgf8-promoter-driven dominant-active beta-catenin protein produces evaginations that lead to the formation of supernumerary teeth later on. The role of Wnt/beta-catenin signaling in tooth development has been previously studied. Hyperactivation of Wnt signaling led to formation of supernumerary teeth (Jarvinen et al., 2006; Wang et al., 2009; Liu et al., 2008). Hypoactivation of the Wnt pathway disrupted tooth development at different stages (Andl et al., 2002; Liu et al., 2008; Sasaki et al., 2005). Hyperactivation and hypoactivation of Wnt/beta-catenin signaling in the mesenchyme resulted in a decrease in tooth number (Chen et al., 2009; Jarvinen et al., 2018). In humans, loss-of-function mutations of the Wnt inhibitors APC and AXIN2 cause hyperdontia and hypo/oligodontia(Lammi et al., 2004). Thus, the role of the Wnt/beta-catenin pathway is not unclear. The existence of different phenotypes obtained with distinct genetic approaches suggest rather the presence of a fine tuned process that requires the coordination of different pathways at different stages of development. The authors using an early expressed FGF8 driven promoter confirmed previously published work at an earlier stage of development.

The conclusion that the existence of early evaginations that are associated with premature mesenchymal accumulation of condensation-associated collagen VI represents a novel morphogenetic mechanism is premature, especially when we know from previous studies and from this study that these evaginations lead to supernumerary teeth in the diastema region.

We agree that there is indeed a substantial body of evidence that canonical Wnt signalling is odontogenic, but – as we have now pointed out more explicitly in the text – observing "disruption" upon loss of function and formation of supernumerary teeth with gain-of-function does not equal understanding of what Wnt does or how it controls tooth morphogenesis. Our loss-of-function experiments show that Wnt signaling is an early "first mover" in tooth formation, likely as part of a reaction-diffusion system, which we agree is not especially surprising, but is clearer than any previous study. We have therefore added further analyses of our gain-of-function phenotype, which the reviewer acknowledges are novel. In particular we have now measured actual premature condensation, while pointing out that collagen VI, like some other collagens, is a marker of condensation (and we have made sure that the revised text clearly indicates no claim that it is a "master molecule" for this process). We have further reinforced this point by referring to a range of other biological systems in which mesenchymal condensation leads to evagination. Our demonstration that the direction (invagination/evagination) of epithelial bending is determined by the relative timing of epithelial and mesenchymal cell convergence represents a novel perspective on the evolvability of the respective morphologies.

Reviewer 2 Advance Summary and Potential Significance to Field:
This study provides a temporo-spatially specific approach to studying the role of Wnt signaling in molar development. Authors use the FGF8 promoter to genetically manipulate the beta-catenin and modulate the Wnt signaling up and down at early stages of molar development. Their data show that the Wnt/ß -catenin pathway plays an early, critical role in determining the invagination versus evagination of the molar epithelium.

Reviewer 2 Comments for the Author:
This is an excellent, well conducted manuscript with a logical flow and high data of high quality. I sincerely have no additional comment or criticism to make. The scientific interest is obvious and the evagination formation process with accumulation of collagen VI opens new avenues. May be in the discussion section, a bit more need to be said about what is known about this collagen and whether it is associated (directly or indirectly) with human pathologies.

We appreciate the reviewer's suggestion that we expand on what is known about collagen VI and its association with human pathologies. We have added to our revised manuscript a discussion of reports of Collagen VI being associated with Bethlem myopathy and Ullrich congenital muscular dystrophy in humans (reviewed in Baker et al., 2005). These patients are reported to have irregular, crowded dentition.

Reviewer 3 Comments for the Author:
1. Overall, the manuscript provides phenotypic description of the WntLOF and WntGOF mutants with disruption of tooth development at an earlier developmental stage than previously described from using other Cre drivers. However, the manuscript, as written, does not provide clearer understanding than before regarding the roles of Wnt signaling in tooth development. The WntLOF results are suggestive that Wnt signaling in the dental placode epithelium might play an important role in tooth bud invagination, but the results are preliminary and requires further validation using Wnt signaling specific reagents (see next two points).

We appreciate the reviewer's comments, and below we detail how we have addressed these suggestions.

2. Both the WntLOF and WntGOF disrupted beta-catenin function and both mutants showed disruption of epithelial integrity, with the WntLOF mutants showed increased epithelial apoptosis and the WntGOF mutants showed reduction/disruption of E-cadherin expression. It is quite possible that the lack of tooth epithelial invagination in the WntLOF mutants was due to the increased epithelial cell death and disruption of epithelial integrity rather than a specific Wnt-induced suprabasal cell convergence. The authors showed that K14-CreERT2;Porcn>fl/fl embryos had a similar phenotype of shallower tooth bud invagination at E12.5, but did not show whether the tooth buds continue to form in the K14-CreERT2;Porcn>fl/fl embryos by E13.5 and whether these Porcn mutants also show similar dental epithelial apoptosis and lack of mesenchymal condensation. It is not known and this manuscript does not address the mechanism how Wnt signaling could be inducing the suprabasal epithelial convergence during tooth bud formation.

We agree with the reviewer that decreased proliferation and increased cell death likely contribute to the WntLOF phenotype, and this point is discussed further in the revised manuscript. We also agree with the reviewer that the lack of suprabasal cell convergence in WntLOF epithelium (Movies 1 and 2) could be secondary to a proliferative defect in generating the suprabasal cell population. However, since we also show loss of Shh expression (Fig. S2 panels H and CC), and we previously showed that Shh signaling is required for suprabasal cell convergence (Li et al, 2016), this too is also likely to be a significant factor in the phenotype. The conclusion is that the phenotype is the sum of these factors and shows that Wnt regulates them all. These points have now been incorporated into our revised text, and we appreciate the reviewer for prompting us to reconsider how we presented these findings.

Following the reviewer's suggestion, we have investigated the question of epithelial integrity, and we have now added to the result section an examination of γ-catenin, which could substitute for the adhesion function of b-catenin in the developing molar (Fig. 3A-F). The uniform expression of γ-catenin in the WntLOF molar epithelium at E12.5 suggests intact cell adhesion of the epithelium.

In response to this reviewer's comment about later development of the K14-Cr;Porcn-fl/fl mutants, we have now included data showing that these embryos develop an enamel organ at E18.5 (Fig. S1E). This is consistent with other K14-driven phenotypes (i.e. tooth "disruption" rather than complete loss), but the similarity to our early WntLOF at early stages also shows that that phenotype is likely to be due to loss of signaling in our WntLOF rather than loss of adhesion, as we now point out more clearly in the text.

3. Figure 2M only quantified percent of EdU-labeled epithelial cells, but Fig. 2E clearly shows a dramatic reduction in nascent dental mesenchyme proliferation in the WntLOF embryo and Fig. 2F shows a likely increase in mesenchymal cell proliferation in the WntGOF embryo. Thus, both of the lack of epithelial invagination in the WntLOF and the evagination in the WntGOF mutants resulted from a combination of disruption of overlying epithelial integrity and secondary defects in the underlying mesenchyme. It is very difficult to tease apart the effects of Wnt signaling from the structural requirements of beta-catenin using those beta-catenin mutants. The authors should examine more Wnt pathway-specific mutants, such as tissue-specific deletion of Wntless to validate effects of Wnt signaling without directly disrupting beta-catenin.

We appreciate the reviewer's suggestion and have now added mesenchymal cell proliferation and apoptosis data to the paper (Fig. 2P and Q). While the number of apoptotic cells remained comparable, there was in fact an increase in the proliferation rate in the mesenchyme of WntLOF and WntGOF.

Our Porcn conditional knockout does in fact address the point about the requirements for signaling versus the structural role of beta-catenin, essentially in the same way that the Wntless mutants that are suggested would do. We have also gone one step further and investigated the presence of γ-catenin. Other groups have shown that the intercellular adhesion role of β-catenin can be substituted by γ-catenin (Huelskenet et al., 2001; Huelskenet et al., 2000). Our data show that γ-catenin is expressed at least to the levels of control in the WntLOF molar epithelium at E12.5, suggesting intact cell adhesion of the epithelium (Fig. 3A-F).

Interestingly, E-cadherin showed reduced expression in the evaginating epithelium of the WntGOF mutants (Fig. 3C,F and I). We predict that this may lead to reduced epithelial integrity, which allows the mesenchyme to push the epithelium outward, and this is now discussed in more detail in our revised manuscript.

4. While it is clear that the mesenchymal underlying the WntGOF mutant dental epithelium is affected in both proliferation and ECM production, these are cell non-autonomous effects resulting from disruption of the dental placode signaling center. The manuscript stops short of identifying the factors and molecular mechanisms downstream of beta-catenin in the epithelial cells in the regulation of epithelial-mesenchymal interaction.

We have now added whole-mount in situ hybridization of *Barx1*, which is important in mesenchymal condensation. We have also added additional *in situ* hybridization data on *Fgf4* and *Bmp4* showing that they are downregulated in WntLOF and upregulated in WntGOF (Fig. S2P-PP), suggesting that the BMP and FGF pathways are involved in the epithelium-to- mesenchyme signaling. We do not include a more detailed analysis of the processes regulating mesenchymal condensation: condensation is, in any case, a poorly understood process, and this would take an additional paper's-worth of experiments (such as those in the Mikkola lab Biggs et al, eLife 2018 paper on condensation in the hair follicle mesenchyme) to investigate properly.

5. The inhibition of collagen biosynthesis is too disruptive a method to reveal any mechanism of epithelial invagination/evagination and does not reveal any specific role of Collagen VI.

We appreciate this comment and first would like to clarify that we used collagen VI as a proxy marker for mesenchymal condensation, as we show in Fig. 4 and based on a previous study by Mammoto et al. (2015), but we did not intend to conclude that collagen VI is solely responsible for the evagination phenotype in WntGOF. We agree with the reviewer that pan-inhibition of collagen synthesis is a very broad approach, and that we cannot conclude that collagen VI is the only ECM contributor to the evagination phenotype. This point has been clarified in the revision. Rather, we

explain that we used a broad-spectrum collagen inhibitor as there are several collagen types that are expressed and involved in dental mesenchymal condensation.

In addition, we have re-analyzed mesenchymal cell density with stricter parameters and found that there is increased cell density in the evaginating mesenchyme (*i.e.* 5-6 cell layers in the mesenchyme from the basement membrane surrounding the invaginating epithelium). This indicates that there is indeed premature compaction of mesenchymal cells upon perturbation of the Wnt pathway. These data have been added to the revised manuscript (Fig. 2M).

6. In the Abstract, the authors wrote "using an Fgf8-promoter driven and therefore early-expressed dominant-negative ß-catenin transgene, we found that loss of Wnt/ß-catenin signaling completely deletes the molar tooth". There is no "dominant-negative ß-catenin transgene" used in any of the experiments described in the Results section.

We appreciate the reviewer pointing this out and have now fixed this error.

Reviewer 4 Advance Summary and Potential Significance to Field:
The paper titled Early epithelium-specific blockade of Wnt/ß-catenin deletes the molar tooth whereas hyperactivation produces mesenchyme-driven evaginations, by Kim et al., uses complementary gain and loss of function genetic approaches with an Fgf8-promoter-cre to investigate mouse tooth development. The novelty of their findings relate to the promoter being turned on early in development in the LOF than previously published K14-Cre and so they observe less invagination and complete deletion of a molar. Additionally, with the GOF they also observe epithelial protrusions and supernumerary teeth, confirming previous studies. They show these protrusions do not involve increase in proliferation but occur due to increased Collagen VI expression, which is associated with mesenchymal condensation. The study provides additional genetic approaches and findings that confirm and extend the existing literature on B-catenin in tooth development.

Reviewer 4 Comments for the Author:

Major comments:
1. A novel finding is the increased ColVI in the GOF, suggesting Wnt/B-catenin signaling regulates extracellular matrix expression. They then reduce the phenotype using Mithromycin, which is likely inhibiting other collagen or matrix synthesis. They provide no direct evidence Mithromycin specifically reduces ColVI in the experiment, this should be clarified.

We appreciate the reviewer's concern that the control showing that Mithramycin inhibits at least collagen VI synthesis was missing. We have now added collagen VI staining of the explant upon treatment with DMSO (vehicle control) and Mithramycin A (Fig. S3I-L). The immunofluorescence of collagen VI on sectioned explant reveals a visible reduction of collagen VI, especially around the invaginating tooth germ.

2. Is the mesenchymal condensation involving a generalized increased in ECM or is it specific to Col VI?

It has been shown in other studies that mesenchymal condensation involves a suite of ECM proteins (Mammoto et al., 2016). We now clarify that we are using collagen VI as a molecular marker for mesenchymal condensation, but that we are not proposing it as the only driver or even the only collagen involved.

3. The authors should comment in Fig 3 on the polarized columnar shape of the epithelium that clear occurs using f-actin staining during control molar development and that it seems to be reduced in the in WntGOF along with a reduced cell size. Is polarity of the basal epithelial cells reduced?

Apical location of the Golgi, as marked by GM130, relative to the nucleus of the basal cells indicates that the apicobasal polarity as such is maintained in WntGOF. The factors that regulate cell height or columnarity (height/width ratio) are not the same as those that establish polarity, so it is not surprising that polarity as such is not affected even though the cells are not as columnar

in the mutants. Determining what is regulating the cell aspect ratios is indeed an interesting question that could be investigated in this system, but it is beyond the scope of this current manuscript.

4. The authors claim "that such evagination by local accumulation of underlying mesenchyme represents a novel morphogenetic mechanism." Can they discuss whether this may be relevant to any other morphological process, or is it just relevant to the formation of supernumerary teeth?

We appreciate this suggestion and have reflected quite deeply on this point. The reviewer is exactly right, that this morphogenesis is not novel and has been observed in other systems, although its remarkably close relationship to invagination has not. We now discuss in the revised manuscript that increased mesenchymal density has also been observed in organs that develop through evagination (*e.g.* feather and intestinal villi) and that the relationship between condensation-driven evagination and condensation around invaginations is remarkably close.

Additional minor comments:

Clarify description of IWP and IWP2.
IWP was a typo and has been fixed. Thank you for catching this.

Supplementary Fig 3, should mention what anti-GM130 is labeling, why it was used and describe what the staining is showing in the text.

We appreciate this point and have added to the revised manuscript text explaining that GM130 labels the Golgi apparatus and serves as a proxy marker for apicobasal polarity. The apical expression of GM130, relative to the location of nuclei of the basal cells, indicates that the apicobasal polarity is maintained within the basal layer.

Images mislabeled in Fig4, K and L. Duplicated reference Jarvinen 2006. These have been corrected.

Resubmission

Second decision letter

MS ID#: DEVELOP/2021/199685

MS TITLE: Early perturbation of Wnt signaling reveals patterning and invagination-evagination control points in molar tooth development

AUTHORS: Ophir D Klein, Rebecca Kim, Amnon Sharir, Jeremy B.A. Green, Tingsheng Yu Drennon, Jan Prochazka, and Jingjing Li

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 response to the previous review and the revision made to the MS are generally adequate. We would like to publish a revised manuscript in Development, provided that the referees' comments of Reviewer 2 can be satisfactorily addressed (please see Editor's note). Please attend to the comments in the revised manuscript and detail them in a 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.

Editor's note:

Please address, to your best ability, the issues raised by Reviewer 2: (i) relationship of "signalling centre" to molar progenitor and (ii) stage of tooth morphogenesis. Most of the specific comments can be addressed by modifying the presentation of data/results and revising the accompanying text.

Reviewer 1

Advance summary and potential significance to field

I already reviewed this manuscript in April 2020 and I was already in favor of publications. Clearly, the authors properly addressed my comments and in particular further discussed the role of Coll IV in the mesenchyme. I think this is an important and elegant study on the role of the WNT signaling pathway on the early stages of molar formation. Once again, this manuscript deserves publication.

Comments for the author

I already reviewed this manuscript in April 2020 and I was already in favor of publications. Clearly, the authors properly addressed my comments and in particular further discussed the role of Coll IV in the mesenchyme. I think this is an important and elegant study on the role of the WNT signaling pathway on the early stages of molar formation. Once again, this manuscript deserves publication.

Reviewer 2

Advance summary and potential significance to field

This study uses Fgf8-promoter driven loss- and gain-of-function approach to manipulate epithelial ß-catenin to assess its role in early molar development.
Their findings show an early arrest of molar tooth development when ß-catenin is deleted, and tissue evagination when ß-catenin is stabilized.

Comments for the author

In this Manuscript, Kim et al. study the role of ß-catenin, the mediator of the canonical Wnt pathway, in early molar tooth development by using genetic loss- and gain-of-function approach, as well as pharmacological manipulations. Their report confirms previous studies showing the early requirement for epithelial ß-catenin in tooth morphogenesis and suggest that when stabilized, it leads to supernumerary tooth formation, as also previously shown. Additionally, it is shown that early activation leads to epithelial evaginations rather than the typical invagination of the tooth bud – yet ultimately supernumerary teeth form in line with previous reports. Thus, in this respect the manuscript does not provide much advancement in the field.

Before going to the detailed comments, I have a couple of more general issues that should be clarified.

The first one is the authors' view on early molar development. The article depicts the early signaling center as a group of cells separate of the actual molar (e.g. Fig. 1A and Fig. S1A). Presumably this view stems from the authors' previous paper proposing that the molar forming cells are derived from a Fgf8+ 'rosette' (Prochazka et al., 2015) located posteriorly to the signaling center.
However, to my understanding there is no evidence showing that the molar would be situated there, only that cells posterior to the signaling center contribute to the growing molar.

Do the authors wish to suggest that the signaling center is a solitary group of cells that is not part of an epithelial thickening, and that the only epithelial thickening present at E11.5-E11.75 is what is depicted as “molar” in their schematic pictures? This is very confusing. Numerous previous studies have shown (based on in situ hybridization on tissue sections) that the early signaling center is part of an epithelial thickening, and the very recent paper by Mogollon et al. (published in Development) provides 3D data confirming that the early signaling center is part of the molar placode.

So are the authors proposing that there are two epithelial thickenings at the same time? If yes, this should be shown. And if the authors think that there are two thickenings of the molar at E11.5-E11.75, which one are they studying e.g. in their E11.5 slice tissue explants?

The second issue to be clarified is the staging of mouse embryos. Please, detail how the embryos were staged, because the staging used seems to deviate about half-a-day from what is commonly used in the field, i.e. the embryos appear older than what the labeling suggests. According to the authors, the early molar signaling center genes are no longer expressed at E12.5 (Fig. S2, right hand side of the panel) – the genes analyzed include Wnt10b and Shh. The latter in particular, has been studied by many research groups by the same method than here (whole mount ISH), and all report Shh expression in the molar placode at E12.5, while later on is known to wane (~E13.0) (e.g. Zhang et al., Dev Dyn 1999; Kangas et al., Nature 2004; Fujimori et al., 2010; Nakatomi et al, J Dent Res 2013; Ahtiainen et al., J Cell Biol 2016, Mogollon et al., Development 2021). Likewise, the authors call the E11.5 stage as placode stage while this seems to be the stage when there is only a very thin epithelial thickening, and e.g. the Shh expression domain has not yet split from the initial ‘whisker’ pattern (continuous between incisor and molar region) to two separate expression incisor and molar signaling centers (see e.g. Dassule and McMahon, Dev Biol 1998). This is an important issue as staging that differs from the conventional one will create misunderstandings. It would also be important to report the strain of the mouse models used in the study.

A third general issue relates to the sample sizes. Although these are clearly stated when cellular level results are reported (such as EdU+ or TUNEL+ cells),

I did not find this information when it comes to other phenomenon that were not quantified (which are quite numerous: SEM data, time-lapse data, F-actin and immunostaingings, in situ hybridizations).

Specific comments
1. The authors use Fgf8-CreERT as their Cre driver. It is said to be expressed in the “molar region” only, yet the pictures (Fig. S2, left hand side, e.g. Wnt10b Shh) suggest multiple small ectopic expression foci even anterior to the incisor at E11.75. I guess this must mean that there are Fgf8-expressing cells also elsewhere than posterior to the molar signaling center?

2. The authors use inducible K14-Cre line to delete epithelial porcupine, a gene essential for Wnt secretion. It is stated that the phenotype is similar to the ß-catenin deletion but to me this looks quite different as an enamel organ forms later on. Revise the text, please. Based on the phenotype the authors conclude:
“WntLOF phenotype at early stages confirms that the latter is likely due to a loss of canonical signaling and not primarily loss of ß-catenin-dependent cell adhesion”. I agree with the conclusion, but I disagree that it is the porcupine deletion that shows this. For that purpose, the same Fgf8-CreERT model should have been used, and if the same early phenotype was observed, this would be a strong argument.

3. Figure S2 shows always only one control for both WntLOF and WntGOF in situ hybridization, but apparently, it can represent littermate control only for one of them. Which one? Were all samples (control, GOF, LOF) for a given gene always in the same ISH experiment? How many samples were analyzed for each stage and gene?

4. Figure 2: There’s quite a lot of things that need to be clarified regarding these data, and therefore, in its current state, it is difficult to evaluate the findings.
Please, indicate what exactly was quantified – number of TUNEL+ cells (per what? per section, per ?). The quantification suggests that there is maybe 15x more TUNEL+ cells in LOF than in control or

GOF -- but this seems odd as it is not obvious from the images shown (2G-I). Overall, it remains unclear how the values were obtained – for example, how many sections from each embryo were quantified for the obtained values?

The EdU+ cells should be compared to littermate controls from the same injection, but the images suggest that control embryos were only analyzed either from GOF or LOF litters (but not clear which one). Please, provide proper controls. Furthermore, the text reads: "No significant difference was detected in EdU or TUNEL labeling in WntGOF mutants, in both epithelium and mesenchyme."

Yet, Figure 2P shows a significant increase in mesenchymal EdU+ cells.
The authors should also describe how they defined the mesenchyme, i.e. what was the region that was used to quantify EdU+ and TUNEL+ cells? Within a certain distance from the epithelial-mesenhcymal forder? A fixed area? I also find many graphs (Fig. 2M-Q) misleading as they Y scale does not start from 0. The mesenchymal cell density graph (2M) lacks information of the value – what do these numbers stand for?

Figure 2R-W shows snapshots of live imaging videos. The information content of these videos is low without quantifications. All statements on cell movements (in Results and Discussion) should be omitted without quantifications. ("movement of epithelial cells towards the center of the placode was observed within 9 hours as the width decreased and the depth increased (Fig. 2R-T), consistent with previous work (Panousopoulou and Green, 2016). This cell convergence was not observed in WntLOF.") If this is obvious to the authors then it should be possible to quantify these movements.

Finally, in Fig. 2X-CC and S3C-H, the authors analyze F-actin by phalloidin staining. The text reads: "At E11.5, prior to invagination, there was no F-actin enrichment in the molar placodes of the control and both mutants." However, I find that phalloidin staining is a lot more intense in WntGOF that in other samples. Were these specimens stained at the same time (again the same issue with controls – whose littermate controls are they?). The authors do observe a difference between the genotypes at E12.5 – quantifications would substantiate these conclusions.

5. Figure 3: There are several issues also with Figure 3. First of all, Fig. 3G-L are identical to 2X-CC, i.e. show F-actin expression, but the text discusses E-cadherin which is not shown at all.
Fig. 3I-I' are supposed to report ß-catenin staining ("the ß-catenin expression in evaginating epithelium of WntGOF was reduced and localized to the suprabasal layer (Fig. 3I-I")), but I did not find these data either (and there is no Figure 3I').
Figure S3A-B shows Golgi marker GM130 but the images are so low magnification that it is impossible to evaluate the claim of the authors (apical location of Golgi both in control and in GOF mutants).

6. After analyzing F-actin, the authors move on to analyze the mesenchyme and show a clear ectopic and precocious condensation of the dental mesenchyme (ColVI staining) (Fig. 4A-H). This mesenchymal condensation phenotype is the most interesting finding of the manuscript and it is a pity that the authors do not analyze this phenomenon further. How does epithelial Wnt/ß-catenin signaling regulate mesenchymal condensation? In the Discussion, the authors go very lightly from correlation to causality: "However, when the mesenchymal condensation precedes the epithelial invagination, as seen in WntGOF, it may lead to evagination of the epithelium." This is an overstatement. On the other hand, it is also reported that WntLOF mutants have increased mesenchymal cell density and increased cell density. What do these finding mean?
From ColVIA data, the authors jump back to Figure 2M-P (which should be moved to panel 4), a set of images supposedly reporting pMLC staining (the upper panel M-O has a label F-actin though) and the authors conclude: In the mesenchyme of control molar mesenchyme, there was circumferential expression of phospho-myosin light chain." Unfortunately, I do not see this nor understand what is meant with "circumferential expression". Perhaps arrows would help the reader here. I do see some cells (endothelial cells?) showing very high intensity of pMLC in the mesenchyme of all three genotypes.

If this indeed is pMLC staining, I am curious to know why the suprabasal epithelial cells fo control teeth undergoing tissue canopy (that are high in F-actin in Figure 2G) are negative for pMLC (Fig.

2M, P)? Shouldn't that work as a good internal control? Phospo-specific antibodies can be tricky to work with.

7. In the last set of experiments the authors use MifA to block collagen synthesis.
However, to my understanding MifA has also multiple other functions (e.g. blocks the binding of Sp-family transcription factors to DNA) and is also used as a cancer drug and hence it is difficult to draw any conclusions from this experiment.

8. Figure S3: please, reorganize so that the images are referred to in the text in correct order (now S3C-H is referred to in the text before S3A-B).

9. In the first chapter of the Discussion, the authors speculate that the WntLOF phenotype could be due to SHH and FGF pathways being downregulated. The authors should also consider the possibility that the early signaling center does not form at all (expression of neither Wnt10b or Shh, i.e. the two signaling center markers analyzed, was not detected) and this may explain the early arrest in morphogenesis. Were any other signaling center markers analyzed? Or the developmental arrest simply reflects increased cell death?

Second revision

Author response to reviewers' comments

Reply to referee comments

Editor's note: Please address, to your best ability, the issues raised by Reviewer 2: (i) relationship of "signalling centre" to molar progenitor and (ii) stage of tooth morphogenesis. Most of the specific comments can be addressed by modifying the presentation of data/results and revising the accompanying text.

The first point raised by Reviewer 2 can be addressed by explaining that abundant evidence in the literature shows that highly dynamic *Shh* (signaling center) expression appears as an anterior- to-posterior succession of spots, initially transiently in diastemal epithelium (i.e. anterior to the *Fgf8*-positive region), and then within the *Fgf8* region, where the molar actually forms. Each of these transient signaling centers is associated with at least some thickening, but only the definitive molar contains the FGF8-Cre-labelled cells and can develop to later stages as an isolated tooth slice. With this succession of events in mind, the discrepancies pointed out by Reviewer 2 fall away. We have amended the text to explain this, although briefly as this is not the focus of this work. (We also recognize that some of the cell migration reported in Prochazka et al. (2015) may have been a partial detection of the centripetal movements of canopy contraction detailed in Panousopoulou et al. (2016), but as we cannot be absolutely sure of this and because it is not directly relevant to this manuscript, we have not addressed this fine point specifically.)

Reviewer 2's second point is about the staging of disappearance of the *Shh* expression. We can only report the observations we have made using the staging methods we used (specifically developmental features, such as auditory hillocks, anterior foot plates and tongue (eMouseAtlas), as is fairly standard for the odontogenesis field). Reviewer 2 is right to imply that the strain might have an influence on the precise staging reported, and we now provide additional details of the strain backgrounds used and the staging criteria. Hopefully, this will alleviate any confusion.

Reviewer 1 Advance Summary and Potential Significance to Field:
I already reviewed this manuscript in April 2020 and I was already in favor of publications. Clearly, the authors properly addressed my comments and in particular further discussed the role of Col VI in the mesenchyme. I think this is an important and elegant study on the role of the WNT signaling pathway on the early stages of molar formation. Once again, this manuscript deserves publication.

Reviewer 1 Comments for the Author:

I already reviewed this manuscript in April 2020 and I was already in favor of publications. Clearly, the authors properly addressed my comments and in particular further discussed the role of Col VI in the mesenchyme. I think this is an important and elegant study on the role of the WNT signaling pathway on the early stages of molar formation. Once again, this manuscript deserves publication.

We are grateful for the reviewer's assessment of the manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
This study uses Fgf8-promoter driven loss- and gain-of-function approach to manipulate epithelial ß-catenin to assess its role in early molar development. Their findings show an early arrest of molar tooth development when ß-catenin is deleted, and tissue evagination when ß- catenin is stabilized.

Reviewer 2 Comments for the Author:
In this Manuscript, Kim et al. study the role of ß-catenin, the mediator of the canonical Wnt pathway, in early molar tooth development by using genetic loss- and gain-of-function approach, as well as pharmacological manipulations. Their report confirms previous studies showing the early requirement for epithelial ß- catenin in tooth morphogenesis and suggest that when stabilized, it leads to supernumerary tooth formation, as also previously shown. Additionally, it is shown that early activation leads to epithelial evaginations rather than the typical invagination of the tooth bud – yet ultimately supernumerary teeth form, in line with previous reports. Thus, in this respect the manuscript does not provide much advancement in the field.

Before going to the detailed comments, I have a couple of more general issues that should be clarified. The first one is the authors' view on early molar development. The article depicts the early signaling center as a group of cells separate of the actual molar (e.g. Fig. 1A and Fig. S1A). Presumably this view stems from the authors' previous paper proposing that the molar forming cells are derived from a Fgf8+ 'rosette' (Prochazka et al., 2015) located posteriorly to the signaling center. However, to my understanding there is no evidence showing that the molar would be situated there, only that cells posterior to the signaling center contribute to the growing molar. Do the authors wish to suggest that the signaling center is a solitary group of cells that is not part of an epithelial thickening, and that the only epithelial thickening present at E11.5-E11.75 is what is depicted as "molar" in their schematic pictures? This is very confusing.
Numerous previous studies have shown (based on in situ hybridization on tissue sections) that the early signaling center is part of an epithelial thickening, and the very recent paper by Mogollon et al. (published in Development) provides 3D data confirming that the early signaling center is part of the molar placode. So are the authors proposing that there are two epithelial thickenings at the same time? If yes, this should be shown. And if the authors think that there are two thickenings of the molar at E11.5-E11.75, which one are they studying e.g. in their E11.5 slice tissue explants?
The second issue to be clarified is the staging of mouse embryos. Please, detail how the embryos were staged, because the staging used seems to deviate about half-a-day from what is commonly used in the field, i.e. the embryos appear older than what the labeling suggests. According to the authors, the early molar signaling center genes are no longer expressed at E12.5 (Fig. S2, right hand side of the panel) – the genes analyzed include Wnt10b and Shh. The latter in particular, has been studied by many research groups by the same method than here (whole mount ISH), and all report Shh expression in the molar placode at E12.5, while later on is known to wane (~E13.0) (e.g. Zhang et al., Dev Dyn 1999; Kangas et al., Nature 2004; Fujimori et al., 2010; Nakatomi et al, J Dent Res 2013; Ahtiainen et al., J Cell Biol 2016, Mogollon et al., Development 2021). Likewise, the authors call the E11.5 stage as placode stage while this seems to be the stage when there is only a very thin epithelial thickening, and e.g. the Shh expression domain has not yet split from the initial 'whisker' pattern (continuous between incisor and molar region) to two separate expression incisor and molar signaling centers (see e.g. Dassule and McMahon, Dev Biol 1998). This is an important issue as staging that differs from the conventional one will create misunderstandings. It would also be important to report the strain of the mouse models used in the study.

A third general issue relates to the sample sizes. Although these are clearly stated when cellular level results are reported (such as EdU+ or TUNEL+ cells), I did not find this information when it comes to other phenomenon that were not quantified (which are quite numerous: SEM data, time-lapse data, F-actin and immunostaingings, in situ hybridizations).

In addition to counting embryonic days, we also now point out more clearly in the manuscript that we used developmental features, such as auditory hillocks, anterior foot plates and tongue, to normalize the stages of the embryos within and between litters (eMouseAtlas). Our approach to staging these embryos is standard for the odontogenesis field. Importantly, our *in situ* hybridization data reveal the loss of *Shh* expression in the signaling center as early as E11.75. Thus, it is highly unlikely that the absence of *Shh* in WntLOF at E12.5 is related to embryonic staging.

Lastly, we repeated each experiment at least three times with different embryos. This information has now been added to the materials and method section. We appreciate the reviewer pointing this out.

Specific comments

1. The authors use Fgf8-CreERT as their Cre driver. It is said to be expressed in the "molar region" only, yet the pictures (Fig. S2, left hand side, e.g. Wnt10b, Shh) suggest multiple small ectopic expression foci even anterior to the incisor at E11.75. I guess this must mean that there are Fgf8-expressing cells also elsewhere than posterior to the molar signaling center?

We agree with the reviewer that *Fgf8* is enriched in the molar region but that its expression may not be exclusive by to the molar progenitors. We have modified the text to reflect the possibility raised by the reviewer as below:

- "*Fgf8*-driven WntGOF, which is expressed only in the molar progenitors" was changed to "*Fgf8*-driven WntGOF, which is expressed primarily in the molar progenitors"
- "*Fgf8*-expressing molar epithelium" was changed to "*Fgf8*-enriched molar epithelium"
- "*Fgf8*-driven WntGOF, which is expressed only in the molar progenitors" to *"Fgf8*-driven WntGOF, which is expressed primarily in the molar progenitors"

We have already discussed in the manuscript that Wnt signaling is likely part of a finely-regulated reaction-diffusion network that initiates the tooth development. Therefore, hyperactivation of Wnt pathway may perturb the homeostatic dynamics of the reaction-diffusion system, leading to ectopic expression of odontogenic genes. Another possible explanation is that hyperactivated Wnt signaling promotes *Fgf8*+ cell proliferation and survival, which is one of the well-known roles of Wnt signaling in different organs. This point has been addressed in the revised manuscript.

2. The authors use inducible K14-Cre line to delete epithelial porcupine, a gene essential for Wnt secretion. It is stated that the phenotype is similar to the ß-catenin deletion but to me this looks quite different as an enamel organ forms later on. Revise the text, please. Based on the phenotype the authors conclude: "WntLOF phenotype at early stages confirms that the latter is likely due to a loss of canonical signaling and not primarily loss of ß-catenin-dependent cell adhesion". I agree with the conclusion, but I disagree that it is the porcupine deletion that shows this. For that purpose, the same Fgf8-CreERT model should have been used, and if the same early phenotype was observed, this would be a strong argument.

We used the *Krt14-CreER*^{T2};*Porcn*$^{f/f}$ line to ensure maximal deletion of the WNT-producing signaling cells; while the *Krt14* expression has a later onset of expression, it is broader than the *Fgf8* driver. To further address the issue of whether the WntLOF phenotype is indeed caused by a signaling defect rather than a cell adhesion defect, we assessed expression of γ-catenin, which has been shown to compensate for the loss of ß-catenin in cell-cell adhesion. We found that γ-catenin is present throughout the WntLOF epithelium. We appreciate the reviewer's comment and have revised the text as follows: "Abrogating secretion of Wnt in oral and dental epithelium at E11.5 using *Krt14-CreER*^{T2};*Porcn*$^{fl/f}$ led to a delay in tooth development by E18.5. "

3. Figure S2 shows always only one control for both WntLOF and WntGOF in situ hybridization, but apparently, it can represent littermate control only for one of them. Which one? Were all samples (control, GOF, LOF) for a given gene always in the same ISH experiment? How many samples were analyzed for each stage and gene?

For each WntLOF and WntGOF mutant, we used littermate control for experiments. We did not detect any difference in *in situ* hybridization results between any of the WntLOF and WntGOF controls, and thus, we showed a representative control to avoid redundancy. We have added below the *in situ* hybridization results from WntLOF and WntGOF controls for reference. In addition, the text has been also edited to explain this clearly.

We have removed unpublished data provided for the referees in confidence.

4. Figure 2: There's quite a lot of things that need to be clarified regarding these data, and therefore, in its current state, it is difficult to evaluate the findings. Please, indicate what exactly was quantified – number of TUNEL+ cells (per what? per section, per ?). The quantification suggests that there is maybe 15x more TUNEL+ cells in LOF than in control or GOF -- but this seems odd as it is not obvious from the images shown (2G-I). Overall, it remains unclear how the values were obtained – for example, how many sections from each embryo were quantified for the obtained values? The EdU+ cells should be compared to littermate controls from the same injection, but the images suggest that control embryos were only analyzed either from GOF or LOF litters (but not clear which one). Please, provide proper controls. Furthermore, the text reads: "No significant difference was detected in EdU or TUNEL labeling in WntGOF mutants, in both epithelium and mesenchyme." Yet, Figure 2P shows a significant increase in mesenchymal EdU+ cells. The authors should also describe how they defined the mesenchyme, i.e. what was the region that was used to quantify EdU+ and TUNEL+ cells? Within a certain distance from the epithelial-mesenhcymal forder? A fixed area? I also find many graphs (Fig. 2M-Q) misleading as they Y scale does not start from 0. The mesenchymal cell density graph (2M) lacks information of the value – what do these numbers stand for? Figure 2R-W shows snapshots of live imaging videos. The information content of these videos is low without quantifications. All statements on cell movements (in Results and Discussion) should be omitted without quantifications. ("movement of epithelial cells towards the center of the placode was observed within 9 hours as the width decreased and the depth increased (Fig. 2R-T), consistent with previous work (Panousopoulou and Green, 2016). This cell convergence was not observed in WntLOF.") If this is obvious to the authors, then it should be possible to quantify these movements. Finally, in Fig. 2X-CC and S3C-H, the authors analyze F-actin by phalloidin staining. The text reads: "At E11.5, prior to invagination, there was no F-actin enrichment in the molar placodes of the control and both mutants." However, I find that phalloidin staining is a lot more intense in WntGOF that in other samples. Were these specimens stained at the same time (again the same issue with controls – whose littermate controls are they?). The authors do observe a difference between the genotypes at E12.5 – quantifications would substantiate these conclusions.

The total number of nuclei and the EdU+ nuclei were counted in 7 sequential sections of the molar placode, and the proportion of EdU+ nuclei to the total number of nuclei was used for quantitative analyses. As for the mesenchyme, the region of analysis was defined to include 5-6 layers of mesenchymal cells from the basement membrane surrounding the invaginating epithelium. The EdU and TUNEL counts were comparable between the WntLOF control and WntGOF controls and were thus pooled. This information was also included in the submitted manuscript in the materials and methods section.

Reviewer also mentioned that many graphs are misleading because the Y scale doesn't start at 0. We focused on the range that includes our measurements to help visualize the individual values better. In addition, the statistical analyses were provided to assist the interpretation of the data.

As for the Y-range of Fig. 2Q, which visualizes the number TUNELE+ cell count, includes sub- zero range. This is because most of the values (i.e. the number of TUNEL+ cells) are 0 with one positive outlier. We assume normal distribution of the values for analysis, and thus, the range of standard deviation (not the actual values) inevitably falls below zero.

Mesenchymal cell density was calculated by dividing the number of nuclei in a defined area (in arbitrary unit) automatically calculated by Fiji. The area for counting was defined as explained above. This information is now added to the text in the materials and methods section.
There were technical hurdles that limited us from getting a movie with resolution for cellular

tracking. These hurdles include manually generating a thin and clean tissue slice through the molar placode at E11.5 for imaging and the depth penetration of the spinning disk confocal microscope. Regardless, the overall movement towards the midline of the placode in the movie is consistent with F-actin and immuno-staining. Furthermore, this medial movement of cells is also consistent with the subsequent placode morphology at 9 hours, which became narrower in width and grew deeper into the underlying mesenchyme. Although we cannot provide the quantitative analysis requested, these data add to our 2D analyses on epithelial cell mechanics and are therefore important to include.

5. Figure 3: There are several issues also with Figure 3. First of all, Fig. 3G-L are identical to 2X-CC, i.e. show F-actin expression, but the text discusses E- cadherin which is not shown at all. Fig. 3I-I' are supposed to report ß-catenin staining ("the ß-catenin expression in evaginating epithelium of WntGOF was reduced and localized to the suprabasal layer (Fig. 3I-I")), but I did not find these data either (and there is no Figure 3I') Figure S3A-B shows Golgi marker GM130 but the images are so low magnification that it is impossible to evaluate the claim of the authors (apical location of Golgi both in control and in GOF mutants).

We apologize for the error that occurred while preparing the figures for resubmission. Fig. 3G-I" have been updated with the correct E-cadherin data, and the text has been modified accordingly.

The apical location of GM130 relative to the nucleus serves as a surrogate marker for apicobasal polarity. To aid the visualization, we have increased the image size and annotated Fig. S3G and H to point out GM130 expression and nuclei. A published study by Li et al. shows a GM130 expression upon partial loss of apicobasal polarity, with the localization of GM130 at the basolateral surface in several MCF-10A cells (Li et al., 2014; image below). This expression pattern is significantly different from the GM130 expression in both control and WntLOF epithelium, which had no GM130 staining basal to the nuclei (*i.e.* closer to the basement membrane than the nucleus).

We have removed unpublished data provided for the referees in confidence.

6. After analyzing F-actin, the authors move on to analyze the mesenchyme and show a clear ectopic and precocious condensation of the dental mesenchyme (ColVI staining) (Fig. 4A-H). This mesenchymal condensation phenotype is the most interesting finding of the manuscript and it is a pity that the authors do not analyze this phenomenon further. How does epithelial Wnt/ß-catenin signaling regulate mesenchymal condensation? In the Discussion, the authors go ver lightly from correlation to causality: "However, when the mesenchymal condensation precedes the epithelial invagination, as seen in WntGOF, it may lead to evagination of the epithelium."

This is an overstatement. On the other hand, it is also reported that WntLOF mutants have increased mesenchymal cell density and increased cell density. What do these finding mean? From ColVIA data, the authors jump back to Figure 2M-P (which should be moved to panel 4), a set of images supposedly reporting pMLC staining (the upper panel M-O has a label F-actin though) and the authors conclude: In the mesenchyme of control molar mesenchyme, there was circumferential expression of phospho-myosin light chain.
Unfortunately, I do not see this nor understand what is meant with "circumferential expression". Perhaps arrows would help the reader here. I do see some cells (endothelial cells?) showing very high intensity of pMLC in the mesenchyme of all three genotypes. If this indeed is pMLC staining, I am curious to know why the suprabasal epithelial cells fo control teeth undergoing tissue canopy (that are high in F-actin in Figure 2G) are negative for pMLC (Fig. 2M, P)?
Shouldn't that work as a good internal control? Phospo-specific antibodies can be tricky to work with.

We appreciate this thought-provoking critique, but we have endeavored to be conservative in drawing conclusions about the increase in mesenchymal cell proliferation in WntLOF. One possible explanation is that, while there is increased cell proliferation, WntLOF mesenchyme lacks additional mechanisms to promote mesenchymal condensation (*e.g.* buildup of ECM such as collagen VI). The reviewer also mentioned that it may be an overstatement to claim that the premature mesenchymal condensation in WntGOF that precedes the epithelial invagination may be

involved in evagination. We respectfully disagree, as our suggestion that the premature mesenchymal condensation could contribute to evagination is based on our data on mesenchymal cell count, collagen VI expression and rescue experiments using Mithramycin A, as well as other published studies on mesenchymal condensation contributing to evagination in feather bud and intestinal villi development (Widelitz et al., 2003; Hughes et al., 2018; Shyer et al., 2017). We were also careful not to make overly assertive statements on this point.

We appreciate the reviewer's suggestion to add arrows to highlight the circumferential arrangement of the pMLC-positive cells in the control mesenchyme (now in revised Fig. S3 A and B). This has been clarified in the manuscript as well. We also agree with the reviewer that pMLC staining can be tricky, and we have used mouse intestine as an experimental control to confirm that the antibody detects pMLC (image below). There is also accumulation of pMLC in cells undergoing mitosis within our control and WntLOF images, which serves as a good internal control for the pMLC staining (images below). Although not as strong as F-actin expression, there is epithelial expression of pMLC in control as well. We therefore feel comfortable that our internal and external controls for the pMLC staining render our data on pMLC valid.

We have removed unpublished data provided for the referees in confidence.

We have removed unpublished data provided for the referees in confidence.

7. In the last set of experiments the authors use MifA to block collagen synthesis. However, to my understanding MifA has also multiple other functions (e.g. blocks the binding of Sp-family transcription factors to DNA) and is also used as a cancer drug and hence it is difficult to draw any conclusions from this experiment.

We agree that Mithramycin A (Mit A) can alter other cellular mechanisms, such as the ones pointed out by the reviewer. However, Mit A has been shown to be a potent inhibitor of collagen synthesis and has been used in many published studies for that purpose.

Our results reveal ectopic collagen VI expression in the evaginating mesenchyme of WntGOF, partial rescue of the evaginating phenotype, and a clear reduction of Col VI expression upon Mit A treatment. Based on these findings, we suggested that the premature and dysregulated accumulation of ECM proteins, such as collagen VI, promotes evagination in WntGOF mutants. We also agree with the reviewer that we cannot disregard the possibility of other effects of Mit A on rescuing the evagination phenotype, and this point has been reflected in the revised manuscript.

8. Figure S3: please, reorganize so that the images are referred to in the text in correct order (now S3C-H is referred to in the text before S3A-B).

We appreciate the suggestion and have rearranged Fig. S3 to reflect the order of the figures in the main text of the manuscript.

9. In the first chapter of the Discussion, the authors speculate that the WntLOF phenotype could be due to SHH and FGF pathways being downregulated. The authors should also consider the possibility that the early signaling center does not form at all (expression of neither Wnt10b or Shh, i.e. the two signaling center markers analyzed, was not detected) and this may explain the early arrest in morphogenesis. Were any other signaling center markers analyzed? Or the developmental arrest simply reflects increased cell death?

The reviewer raises an interesting question regarding whether the phenotypes are solely driven by the proliferative defects or whether the signaling defects lead to other morphogenetic defects. We have indeed examined other odontogenic pathways. Both the BMP (Fig. S2S-U) and Eda/Edar pathways (images below), which have been shown to have effects on the formation of the enamel knot among other odontogenic processes, were altered by WntLOF and WntGOF mutations. In addition, we have discussed in our manuscript that SHH and FGF pathways promote cell convergence in the suprabasal cell layer and cell proliferation of embryonic molar, respectively. The loss of convergence as suggested by F-actin staining and live imaging could be mediated by SHH pathway. Taken together, our data indicate that the WntLOF and WntGOF phenotypes are the result of more than just altered proliferation. We agree with the reviewer that there is a theoretic possibility of complete loss of the early signaling center. However, given the space constraints, we were not able to address this question in detail.

We have removed unpublished data provided for the referees in confidence.

Third decision letter

MS ID#: DEVELOP/2021/199685

MS TITLE: Early perturbation of Wnt signaling reveals patterning and invagination-evagination control points in molar tooth development

AUTHORS: Ophir D Klein, Rebecca Kim, Amnon Sharir, Jeremy B.A. Green, Tingsheng Yu Drennon, Jan Prochazka, and Jingjing Li

ARTICLE TYPE: Research Report

I am satisfied with your response and the revision of the paper. The manuscript has been accepted for publication in Development, pending our standard ethics checks.