Pinhead signaling regulates mesoderm heterogeneity via the FGF receptor-dependent pathway

Olga Ossipova, Keiji Itoh, Aurelian Radu, Jerome Ezan and Sergei Y. Sokol
DOI: 10.1242/dev.188094

Editor: Patrick Tam

Review timeline
Original submission: 9 January 2020
Editorial decision: 14 February 2020
First revision received: 9 April 2020
Editorial decision: 12 May 2020
Second revision received: 1 August 2020
Accepted: 4 August 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/188094

MS TITLE: Role of Pinhead signaling in mesoderm heterogeneity

AUTHORS: Olga Ossipova, Keiji Itoh, Aurelian Radu, Jerome Ezan, and Sergei Sokol

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms on the lack of insights into how PNHD acts in the FGF signaling cascade, and if this protein also impacts on or being regulated by other signaling pathways to induce mesoderm differentiation, and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only ONE round of major revision.

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

Reviewer 1

Advance summary and potential significance to field

This manuscript makes a significant and novel contribution to our understanding of fundamental developmental mechanisms, and is therefore in principle suitable for publication in DEVELOPMENT.

Comments for the author

- The title sounds too descriptive and the manuscript would benefit from a more affirmative title, e.g. “Pinhead signaling regulates mesoderm heterogeneity via FGF receptor-dependent pathway”.
- The authors introduce germ layers, but in a very conventional way. Since the authors then describe regulation of only a subsections of mesodermal marker gene expression it might be useful for them to consider introducing and/or discussing the more recent concept of mesendoderm.
- The authors describe how Pinhead functions in an FGF receptor-dependent manner by activating FGF signaling-associated signal transduction mechanisms (i.e. ERK1), leaving it unclear whether Pnhd might function as an alternative to FGF ligands. This seems to be an obvious and relevant question. The authors may consider testing whether experimentally applied Pnhd can rescue FGF ligand knockdown-induced mesoderm development defects (not just FGF receptor knockdown-induced mesoderm development defects or those caused by chemical inhibitors of FGF receptor function); and whether experimentally applied relevant FGF ligands can rescue Pnhd signal knockdown-induced mesoderm development defects. To address a similar scientific question, also the experiments with the animal-vegetal conjugates could be expanded to include FGF signal-induced mesoderm in animal explants (with and without the pnhd knockdown).
- It is not clear what is meant at the bottom of page 12 about some additional inducing signal, are the authors referring to Pnhd or yet another inducer?
- The authors may consider discussing any potential functional overlap of their pnhd-regulated genes with those described as co-regulated by Wnt and FGF signaling (e.g. Nakamura et al., 2016).

The figures are of high standard and the text is well written.

Reviewer 2

Advance summary and potential significance to field

This paper aims to characterize the mode of action and function of the Xenopus protein pinhead (Pnhd). The authors show that it is a secreted protein that activates a subset of mesoderm markers in the presumptive ectoderm. Consistent with this, knockdown of Pnhd using morpholinos reduces expression of a subset of mesodermal markers. The activity of Pnhd requires FGF receptor activity, and Pnhd can induce phosphorylation of ERK. Finally, the authors show that Pnhd can only induce mesoderm in stage 10 animal caps and not in stage 8 animal caps, suggesting that it requires early inducing signals before it can act to induce a subset of mesodermal markers. This is interesting as Pnhd seems to be a novel mesoderm inducer in Xenopus.

Comments for the author

Overall the work is well done and the data provided support the conclusions. I think though that the work is too preliminary at this stage and too many questions are left unanswered as described below.

The authors show the detailed expression pattern of Pnhd over time, and then focus on its role as a mesoderm inducer. It is essential that they determine what regulates Pnhd at the transcriptional level, at least at the early stages.
Their data in Figure 8 suggest that it is activated by early inducing signals.
What are these?

The authors show that both overexpression of Pnhd and its knockdown by morpholinos gives rise to a reduced head phenotype. This is never explained.
There is also nothing to tie its activity as a mesoderm inducer, activating genes like tbxt, vegt, esr5 etc to the reduced head phenotype when knocked down or overexpressed. The authors need to link the Pnhd target genes to the phenotype.

The authors show nicely that the activity of Pnhd depends on the FGF receptor and it can induce Erk phosphorylation. The authors need to demonstrate the mechanism underlying this. Is it a novel FgfR ligand? Does it interact directly with Fgf receptors? Does it activate other signaling pathways downstream of Fgf receptors? Why does Pnhd only activate a particular subset of mesoderm targets.
Are these all Fgf targets?

Other points
1. The authors do not say much about the sequence of Pnhd and whether it is conserved in other species or what other sequence motifs or domains it has other than the cysteines. More information on the gene and protein at the beginning of the Results would be useful.

2. In Fig 3D I think that qPCR should be used so that the authors can really quantitate the transcripts.

3. The elongation of animal caps is reminiscent of the activity of Nodal signals. Can the activity of Pnhd in this assay be inhibited by Nodal receptor inhibitors like SB-431542 or SB-505124?

Reviewer 3

Advance summary and potential significance to field

This is a very good manuscript describing the activities of the pnhd gene, isolated from Xenopus. There are previous reports on the gene from both Xenopus and Ascidians, but this manuscript delves further into the function of the PNHD protein by gain and loss of function experiments. The conclusions are that pnhd is needed for a subset of the mesoderm to form, and that it functions through the FGF pathway, activating characteristic FGF targets, and requiring FGF receptor signaling.
The manuscript offers a somewhat different spin on the function from previous reports, which makes it interesting, but it does not get deeply into the mechanism of Pnhd action. The protein is clearly shown to be secreted, and nicely shown to have effects similar to those of FGF signaling. However, how it works through FGF signaling and whether it differs at all in its activity is not so clear. Perhaps the simplest possibility is that the protein actually does function through the FGF receptor, since its activity is blocked by DN FGFR and SU5402.
The paper also does not make clear what subset of mesoderm the gene is required for, and whether the necessity is any different from that for modest FGF inhibitions. Whether the protein works before or after the FGF receptor might be testable with epistasis experiments using activated components, as well as dominant negatives.
So while the quality of the work is high, and the approaches are good, the manuscript is limited in its insights.
While the scientific quality of the work is high, the impact is perhaps modest.

Comments for the author

This is a very good manuscript describing the activities of the pnhd gene, isolated from Xenopus. There are previous reports on the gene from both Xenopus and Ascidians, but this manuscript delves further into the function of the PNHD protein by gain and loss of function experiments. The conclusions are that pnhd is needed for a subset of the mesoderm to form, and that it functions through the FGF pathway, activating characteristic FGF targets, and requiring FGF receptor signaling.
The manuscript offers a somewhat different spin on the function from previous reports, which makes it interesting, but it does not get deeply into the mechanism of Pnhd action. The protein is clearly shown to be secreted, and nicely shown to have effects similar to those of FGF signaling.

However, how it works through FGF signaling and whether it differs at all in its activity is not so clear. Perhaps the simplest possibility is that the protein actually does function through the FGF receptor, since its activity is blocked by DN FGFR and SU5402.
The paper also does not make clear what subset of mesoderm the gene is required for, and whether the necessity is any different from that for modest FGF inhibitions. Whether the protein works before or after the FGF receptor might be testable with epistasis experiments using activated components, as well as dominant negatives.
So while the quality of the work is high, and the approaches are good, the manuscript is limited in its insights.
While the scientific quality of the work is high, the impact is perhaps modest.

First revision

Author response to reviewers' comments

Responses to Reviewer 1

Advance summary and potential significance to field
This manuscript makes a significant and novel contribution to our understanding of fundamental developmental mechanisms, and is therefore in principle suitable for publication in DEVELOPMENT.

Reviewer 1 Comments for the author
•The title sounds too descriptive and the manuscript would benefit from a more affirmative title, e.g. “Pinhead signaling regulates mesoderm heterogeneity via FGF receptor-dependent pathway”.

We changed the title to the one suggested by the reviewer.

•The authors introduce germ layers, but in a very conventional way. Since the authors then describe regulation of only a subsections of mesodermal marker gene expression it might be useful for them to consider introducing and/or discussing the more recent concept of mesendoderm.

We introduced mesendoderm and added a brief discussion of different mesodermal cell types induced in the early embryo. Like FGF, Pnhd signaling promotes the formation of ventroposterior paraxial mesoderm marked by cdx4, tbxt, wnt8 and msgn1, rather than dorsoanterior (nodal or goosecoid) or ventral (sizzled, bambi or admp2) mesoderm. Notably, the Tbx and Cdx transcription factor families, Msgn1 and Wnt8a have been all implicated in paraxial mesoderm development.

•The authors describe how Pinhead functions in an FGF receptor-dependent manner by activating FGF signaling-associated signal transduction mechanisms (i.e. ERK1), leaving it unclear whether Pnhd might function as an alternative to FGF ligands. This seems to be an obvious and relevant question. The authors may consider testing whether experimentally applied Pnhd can rescue FGF ligand knockdown-induced mesoderm development defects (not just FGF receptor knockdown-induced mesoderm development defects, or those caused by chemical inhibitors of FGF receptor function); and whether experimentally applied relevant FGF ligands can rescue Pnhd signal knockdown-induced mesoderm development defects. To address a similar scientific question, also the experiments with the animal-vegetal conjugates could be expanded to include FGF signal-induced mesoderm in animal explants (with and without the pnhd knockdown).

The referee asked whether the functions of Pnhd and FGF are interchangeable. Since multiple FGF ligands with redundant activities are expressed during gastrulation, it is technically challenging to determine whether Pnhd can function in the marginal zone in the absence of all FGF ligands. Our experiments reveal that Pnhd can act as an alternative to FGF. In fact, among many FGF targets, Pnhd also induced several FGF ligands (FGF3, FGF8 and FGF16, according to our RNA sequencing data). Furthermore, our new result confirms that Pnhd itself is induced by FGF (new Fig. S5), in agreement with previous reports (Branney et al., 2009). We note that, due to this feedback, the

epistatic relationship cannot be readily established and rescue experiments would not be meaningful.

Nevertheless, prompted by the referee, we assessed whether Pnhd is required for mesoderm induction by FGF. The depletion of Pnhd reduces FGF-dependent induction of cdx4 and tbxt by 50-80 % (new Fig. S8), reinforcing our conclusions obtained for animal-vegetal recombinants. This result further supports the positive feedback between Pnhd and FGF.

•It is not clear what is meant at the bottom of page 12 about some additional inducing signal, are the authors referring to Pnhd or yet another inducer?

To clarify our conclusion regarding the synergy of Pnhd and FGF, we revised the text on p. 13, bottom, and added an experimental scheme (Fig.8A). Our key finding is that Pnhd only triggers mesoderm formation in animal caps isolated at stage 10, but not stage 8. We explain that the only difference between the two groups of explants containing Pnhd in Figs. 8B and 8C is the time of their contact with the adjacent mesendoderm". During this time interval, between stage 8 and stage 10, the explanted tissue must have received additional signals from the marginal zone. These signals synergize with Pnhd leading to upregulation of cdx4 and tbxt, as has been previously reported for Wnt8 (Sokol, 1993). We hope that this observation sheds light on the synergy of Pnhd and FGF in mesoderm formation.

•The authors may consider discussing any potential functional overlap of their pnhd-regulated genes with those described as co-regulated by Wnt and FGF signaling (e.g. Nakamura et al., 2016).

Prompted by the reviewer, we compare Pnhd-regulated genes with the common targets of Wnt and FGF pathways in the revised manuscript and discuss the substantial overlap.

The figures are of high standard and the text is well written.

Responses to Reviewer 2

Advance summary and potential significance to field
This paper aims to characterize the mode of action and function of the Xenopus protein pinhead (Pnhd). The authors show that it is a secreted protein that activates a subset of mesoderm markers in the presumptive ectoderm. Consistent with this, knockdown of Pnhd using morpholinos reduces expression of a subset of mesodermal markers. The activity of Pnhd requires FGF receptor activity, and Pnhd can induce phosphorylation of ERK. Finally, the authors show that Pnhd can only induce mesoderm in stage 10 animal caps and not in stage 8 animal caps, suggesting that it requires early inducing signals before it can act to induce a subset of mesodermal markers. This is interesting as Pnhd seems to be a novel mesoderm inducer in Xenopus.

Reviewer 2 Comments for the author
Overall the work is well done and the data provided support the conclusions. I think though that the work is too preliminary at this stage and too many questions are left unanswered as described below.

1. The authors show the detailed expression pattern of Pnhd over time, and then focus on its role as a mesoderm inducer. It is essential that they determine what regulates Pnhd at the transcriptional level, at least at the early stages. Their data in Figure 8 suggest that it is activated by early inducing signals. What are these?

The revised text clarifies that Pnhd is a transcriptional target of FGF and Wnt signals as demonstrated in our new experiments (new Fig. S5) and by previous studies (Branney et al., 2009; Ding et al., 2018; Kjolby and Harland, 2017).

2. The authors show that both overexpression of Pnhd and its knockdown by morpholinos gives rise to a reduced head phenotype. This is never explained. There is also nothing to tie its activity as a mesoderm inducer, activating genes like tbxt, vegt, esr5 etc to the reduced head phenotype when knocked down or overexpressed. The authors need to link the Pnhd target genes to the phenotype.

Prompted by the referee, we revised the text to explain the connection between the activity of Pnhd and the headless phenotype. The synergy of Pnhd with FGF signaling fits nicely with the posteriorizing activity of FGF, resulting in embryos lacking anterior head structures (Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995). By contrast, the headless phenotype of Pnhd morphants is mechanistically very different, being caused by mesoderm deficiency.

3. The authors show nicely that the activity of Pnhd depends on the FGF receptor and it can induce Erk phosphorylation. The authors need to demonstrate the mechanism underlying this. Is it a novel FgfR ligand? Does it interact directly with Fgf receptors? Does it activate other signaling pathways downstream of Fgf receptors?

The referee brought up several interesting questions. In the past, we have done many experiments attempting to address these questions. So far, we were unable to detect a direct interaction of Pnhd with FGF receptors (data not shown). This outcome is not unexpected, considering that Pnhd activity is distinct from that of FGF ligands. Unlike FGF, Pnhd is unable to stimulate Erk1 in early ectoderm even at high doses (see new Fig. 8D). We have attempted to identify Pnhd signaling intermediates by quantitative proteomics and tested the effect of Pnhd on Smad1/5/8 and Smad2, but failed to identify significant effects. Importantly, we found that Pnhd, but not FGF, consistently inhibits Akt phosphorylation in early ectoderm explants (new Fig. 8D). Although this observation is of significant interest, we feel that the analysis of the Akt role in Pnhd signaling is beyond the scope of this study and should be carried out by future experiments.

4. Why does Pnhd only activate a particular subset of mesoderm targets. Are these all Fgf targets?

As discussed in the revised manuscript, Pnhd target genes are largely similar to the published list of FGF targets. We suggest that Pnhd and FGF are involved in the feedback regulation of the same gene-regulatory network (GRN). Such GRNs are characteristic of many developmental processes, e. g. neural crest GRN. Mesoderm is known to be regulated by combinations of different signals including FGF, Wnt and Activin/Nodal and we believe that Pnhd predominantly regulates ventroposterior paraxial mesoderm. The dynamic regulation of Pnhd expression (Fig. 2) indicates that, at later stages, Pnhd must be involved in other developmental processes.

Other points
1. The authors do not say much about the sequence of Pnhd and whether it is conserved in other species or what other sequence motifs or domains it has other than the cysteines. More information on the gene and protein at the beginning of the Results would be useful.

We provide more information about the protein structure, discuss the composition and the degree of conservation of Pnhd in other species. As we discuss, only three CK domains have been detected in Pnhd and the conservation is throughout the structure of the protein. Sequence alignment of several distantly related Pnhd homologues is presented in the revision in new Fig. S1.

2. In Fig 3D I think that qPCR should be used so that the authors can really quantitate the transcripts.

Most of the genes shown in Fig. 3D have been further evaluated by RT-qPCR, as shown in Fig. 4. Moreover, the changes in gene expression has been assessed by multiple independent rounds of RNA sequencing as discussed in the revised text and shown in Tables 1 and 2. We are, therefore, confident about the presented expression changes. Moreover, we added additional RT-qPCR analysis to Fig. S4 to document no change in the expression of the ventral mesodermal marker Admp2.

3. The elongation of animal caps is reminiscent of the activity of Nodal signals. Can the activity of Pnhd in this assay be inhibited by Nodal receptor inhibitors like SB-431542 or SB-505124?

Prompted by the reviewer, we assessed whether SB505124, an Alk5 inhibitor, influences Pnhd ability to stimulate its target genes. We have observed a strong inhibition of tbxt and hoxd1, indicating that Pnhd activates its mesodermal targets synergistically with both FGF receptor and Nodal/activin receptors. These results are now presented in new Fig. S6.

Responses to Reviewer 3

Advance summary and potential significance to field
This is a very good manuscript describing the activities of the pnhd gene, isolated from Xenopus. There are previous reports on the gene from both Xenopus and Ascidians, but this manuscript delves further into the function of the PNHD protein by gain and loss of function experiments. The conclusions are that pnhd is needed for a subset of the mesoderm to form, and that it functions through the FGF pathway, activating characteristic FGF targets, and requiring FGF receptor signaling.

The manuscript offers a somewhat different spin on the function from previous reports, which makes it interesting, but it does not get deeply into the mechanism of Pnhd action. The protein is clearly shown to be secreted, and nicely shown to have effects similar to those of FGF signaling. However, how it works through FGF signaling and whether it differs at all in its activity is not so clear.

1.Perhaps the simplest possibility is that the protein actually does function through the FGF receptor, since its activity is blocked by DN FGFR and SU5402.

Our attempts to physically link Pnhd to the FGF receptor were not successful. This is not surprising, because Pnhd and FGF activities are quite different. Fgf rapidly stimulates Erk1 phosphorylation in blastula animal caps, whereas Pnhd can activate Erk1 only when animal caps are prepared at stage 10 (Fig. 8B). By contrast, Pnhd, but not FGF, consistently inhibits Akt phosphorylation in early ectoderm explants. This is presented in new Fig. 8D, however, we feel that the detailed mechanistic analysis of Pnhd activity should be carried out by future studies. See also our response to point 3 of Reviewer 2.

The paper also does not make clear what subset of mesoderm the gene is required for, and whether the necessity is any different from that for modest FGF inhibitions.

The revised manuscript clarifies that Pnhd predominantly regulates ventroposterior paraxial mesoderm that is characterized by msgn, tbx, cdx and hox genes. Since these genes are also known FGF targets (Branney et al., 2009; Chung et al., 2004), we propose that Pnhd and FGF are involved in the feedback regulation of the same gene-regulatory network (GRN).

Whether the protein works before or after the FGF receptor might be testable with epistasis experiments using activated components, as well as dominant negatives. So while the quality of the work is high, and the approaches are good, the manuscript is limited in its insights. While the scientific quality of the work is high, the impact is perhaps modest.

The reviewer suggested that we should perform additional epistasis studies. Our revised manuscript now includes new experiments demonstrating that Pnhd is induced by both FGF and Wnt proteins (Fig. S5) and that FGF signaling depends on Pnhd (Fig. S8). Combined with the finding that Pnhd can activate Erk1, we propose that FGF and Pnhd function interdependently, in a positive regulatory feedback. We did not carry out further epistasis experiments, because we feel that they would be of limited value. This decision is also influenced by our current inability to conduct wet lab experiments due to the restrictions imposed by our Institution because of Covid-19.

Second decision letter

MS ID#: DEVELOP/2020/188094

MS TITLE: Pinhead signaling regulates mesoderm heterogeneity via FGF receptor-dependent pathway

AUTHORS: Olga Ossipova, Keiji Itoh, Aurelian Radu, Jerome Ezan, and Sergei Sokol

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 BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees have raised the concerns that the study was incomplete and recommend a substantial revision of your manuscript, which may involve further experiments, before we can consider publication. While we will normally permit only one round of major revision, in this case we are prepared to receive another version of the manuscript if you are able to revise the manuscript along the lines suggested to address satisfactorily the reviewers' major concerns.

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.

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

Reviewer 1

Advance summary and potential significance to field

This article studies the role of pinhead in mesoderm patterning in the vertebrate model system Xenopus. It makes a significant and novel contribution to our understanding of fundamental developmental mechanisms and is therefore in principle suitable for publication in DEVELOPMENT. However, some aspects of the manuscript appear too preliminary for publication.

Comments for the author

The authors have addressed in their revised manuscript my concerns in the previous version of the manuscript about
- the title,
- the introduction of of germ layers, and
- the discussion of overlap between pnhd-regulated genes with those regulated by FGF and Wnt signalling.

The authors explain why they could not address the issue of how PNHD functions in an FGF-receptor-dependent manner, which obviously detracts from the appeal of the study; and that they meant yet another inducer, I understand activin/nodal in addition to FGF from the marginal zone, but Fig.8E looks trivial, it doesn't really seem to explain any biology.

I understand that much of the additional material in the revised manuscript is provided in response to reviewers' requests, but it leaves the revised manuscript still with too many loose ends. It remains in many aspects too preliminary. For instance the activin/nodal connection is not further explored and explained, the surprising negative effect on AKT phosphorylation is mentioned but what was the experiment, in the result section, stage 8 is mentioned, on the figure, stage 10 is mentioned with the control, and in the figure legend stage 11 is mentioned for when explants were collected; and this link to AKT phosphorylation was also not further explored and explained, it is not even mentioned in the Discussion, and in a reply to one reviewer the authors admit it is beyond the scope of this study.

I wonder whether the project is just not ready for this format of manuscript.

Reviewer 2

Advance summary and potential significance to field

This paper studies the functional role of the Xenopus protein pinhead (Pnhd). The authors show that it is a secreted protein that activates a subset of mesoderm markers in the presumptive ectoderm. Consistent with this, knockdown of Pnhd using morpholinos reduces expression of a subset of mesodermal markers. The activity of Pnhd requires FGF receptor activity, and Pnhd can induce phosphorylation of ERK. Pnhd does not though appear to act directly as an Fgf ligand. Finally, the authors show that Pnhd can only induce mesoderm in stage 10 animal caps and not in stage 8 animal caps, suggesting that it requires early inducing signals before it can act to induce a subset of mesodermal markers. Thus, Pnhd seems to be a novel mesoderm inducer in Xenopus, which is an important advance for the field.

Comments for the author

The authors have revised their paper in line with my comments and suggestions and as a result have substantially improved it.

I am now convinced that it is suitable for publication in Development, and have no further comments.

Reviewer 3

Advance summary and potential significance to field

DEVELOP/2020/188094 revision

This revision strengthens the case that pnhd is a mediator of positive feedback in FGF signaling, and also clarifies that it is required for paraxial mesoderm formation. In principle I would support publication, but it seems bizarre to publish a paper claiming that pnhd is required for paraxial mesoderm formation, without showing that directly. The evidence is instead from early gene expression, explants and RNA seq. Surely the authors looked at the tadpoles by in situ hybridization or immunostaining? One hesitates to request such revision in the COVID19 era, but this seems to me a central point. I would argue that it is worth delaying publication so that the knock down embryos can be properly characterized to make a compelling case for the authors' contentions, including whether paraxial mesoderm is reduced or absent, whether the lateral plate mesoderm and blood islands might be expanded, as suggested by Kumano and Smith for reduced FGF signaling. And why not intermediate mesoderm for thoroughness? since the gene has been around for at least 16 years, and so I don't see great urgency for publication, and better support for the conclusions would add strength to the manuscript.
Minor comments: In the revised introduction, the sentence "Factors secreted from the organizer…..mesoderm into dorsal (notochord), paraxial (somites), intermediate (kidney and gonads) and lateral/ventral (e. g., blood) types " should include a reference to signals from the marginal zone, reviewed in the references cited, and not least because pnhd and wnt8 are in the margin.
There are three references to pnhd as novel. But pnhd was described in 2004. Similarly the authors assume "key" differences in mesoderm specification in mammals and pnhd carrying animals. But what makes this "key" Could this be one of those empty buzzwords that the definitive journals should avoid?

Comments for the author

DEVELOP/2020/188094 revision

This revision strengthens the case that pnhd is a mediator of positive feedback in FGF signaling, and also clarifies that it is required for paraxial mesoderm formation. In principle I would support publication, but it seems bizarre to publish a paper claiming that pnhd is required for paraxial

mesoderm formation, without showing that directly. The evidence is instead from early gene expression, explants and RNA seq. Surely the authors looked at the tadpoles by in situ hybridization or immunostaining? One hesitates to request such revision in the COVID19 era, but this seems to me a central point. I would argue that it is worth delaying publication so that the knock down embryos can be properly characterized to make a compelling case for the authors' contentions, including whether paraxial mesoderm is reduced or absent, whether the lateral plate mesoderm and blood islands might be expanded, as suggested by Kumano and Smith for reduced FGF signaling. And why not intermediate mesoderm for thoroughness? since the gene has been around for at least 16 years, and so I don't see great urgency for publication, and better support for the conclusions would add strength to the manuscript.
Minor comments: In the revised introduction, the sentence
"Factors secreted from the organizer…..mesoderm into dorsal (notochord), paraxial (somites), intermediate (kidney and gonads) and lateral/ventral (e. g., blood) types " should include a reference to signals from the marginal zone, reviewed in the references cited, and not least because pnhd and wnt8 are in the margin.
There are three references to pnhd as novel. But pnhd was described in 2004. Similarly the authors assume "key" differences in mesoderm specification in mammals and pnhd carrying animals. But what makes this "key" Could this be one of those empty buzzwords that the definitive journals should avoid?

Second revision

Author response to reviewers' comments

We would like to thank all the reviewers for their effort and the constructive comments that helped us improve the manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:
This article studies the role of pinhead in mesoderm patterning in the vertebrate model system Xenopus. It makes a significant and novel contribution to our understanding of fundamental developmental mechanisms and is therefore in principle suitable for publication in DEVELOPMENT. However, some aspects of the manuscript appear too preliminary for publication.

Reviewer 1 Comments for the Author:
The authors have addressed in their revised manuscript my concerns in the previous version of the manuscript about

- the title,
- the introduction of of germ layers, and
- the discussion of overlap between pnhd-regulated genes with those regulated by FGF and Wnt signalling.

The authors explain why they could not address the issue of how PNHD functions in an FGF-receptor-dependent manner, which obviously detracts from the appeal of the study; and that they meant yet another inducer, I understand activin/nodal in addition to FGF from the marginal zone, but Fig.8E looks trivial, it doesn't really seem to explain any biology.

The model in Figure 8 has been modified to include the Nodal and FGF pathways. The legend emphasizes that *Pnhd functions together with the FGF pathway within the newly induced mesodermal layer* rather than outside as proposed for Nodal ligands. Both the text and the figures have been edited throughout the manuscript to clarify the experimental details and place our results in the context of mesoderm formation in response to Pnhd, FGF and Nodal signals. To further help the readers, additional background references have been added.

I understand that much of the additional material in the revised manuscript is provided in response to reviewers' requests, but it leaves the revised manuscript still with too many loose ends. It remains in many aspects too preliminary. For instance the activin/nodal connection is

not further explored and explained, the surprising negative effect on AKT phosphorylation is mentioned but what was the experiment, in the result section, stage 8 is mentioned, on the figure, stage 10 is mentioned with the control, and in the figure legend stage 11 is mentioned for when explants were collected; and this link to AKT phosphorylation was also not further explored and explained, it is not even mentioned in the Discussion, and in a reply to one reviewer the authors admit it is beyond the scope of this study. I wonder whether the project is just not ready for this format of manuscript.

Initially, we wanted to keep the paper brief and considered the directions suggested by the referee beyond the scope, because they were related to our new findings incorporated after the first revision. Nevertheless, we have explored the two issues as recommended and present the following new results:

1. Prompted by the reviewer, we further explored the connection to Activin/Nodal signaling. We now demonstrate that Pnhd potentiates the effect of Activin on Smad2 phosphorylation (**new Fig. 7G**). Revised discussion clarifies that Pnhd- mediated mesoderm formation requires both FGF and Nodal signaling.

2. Prompted by the referee, we corrected the experimental details and added a schematic of our experiments with Akt (**Figs. 7A-D).** Please note that *both the time of explant dissection and the time of explant lysis are critical for the outcome*. We also note that the inhibitory effect of Pnhd on Akt has been reproduced more than ten times at different developmental stages.

3. We further evaluated a potential role of Akt in Pnhd signaling. Neither stimulation of Akt by the constitutively active PI3 kinase (p110CAAX) nor its inhibition by PTEN influenced the ability of Pnhd to stimulate Erk phosphorylation (**new Fig. 7D, Fig.S8**). Whereas PI3K-Akt signaling does not seem to regulate Erk activation by Pnhd, we state that it is likely to be involved in a parallel pathway.

Reviewer 2 Advance Summary and Potential Significance to Field:

This paper studies the functional role of the Xenopus protein pinhead (Pnhd). The authors show that it is a secreted protein that activates a subset of mesoderm markers in the presumptive ectoderm. Consistent with this, knockdown of Pnhd using morpholinos reduces expression of a subset of mesodermal markers. The activity of Pnhd requires FGF receptor activity, and Pnhd can induce phosphorylation of ERK. Pnhd does not though appear to act directly as an Fgf ligand. Finally, the authors show that Pnhd can only induce mesoderm in stage 10 animal caps and not in stage 8 animal caps, suggesting that it requires early inducing signals before it can act to induce a subset of mesodermal markers. Thus, Pnhd seems to be a novel mesoderm inducer in Xenopus, which is an important advance for the field.

Reviewer 2 Comments for the Author:
The authors have revised their paper in line with my comments and suggestions and as a result have substantially improved it.

I am now convinced that it is suitable for publication in Development, and have no further comments.

Reviewer 3 Advance Summary and Potential Significance to Field:
DEVELOP/2020/188094 revision

This revision strengthens the case that pnhd is a mediator of positive feedback in FGF signaling, and also clarifies that it is required for paraxial mesoderm formation. In principle I would support publication, but it seems bizarre to publish a paper claiming that pnhd is required for paraxial mesoderm formation, without showing that directly. The evidence is instead from early gene expression, explants and RNA seq. Surely the authors looked at the tadpoles by in situ hybridization or immunostaining? One hesitates to request such revision in the COVID19 era, but this seems to me a central point. I would argue that it is worth delaying publication so that the

knock down embryos can be properly characterized to make a compelling case for the authors' contentions, including whether paraxial mesoderm is reduced or absent, whether the lateral plate mesoderm and blood islands might be expanded, as suggested by Kumano and Smith for reduced FGF signaling. And why not intermediate mesoderm for thoroughness? since the gene has been around for at least 16 years, and so I don't see great urgency for publication, and better support for the conclusions would add strength to the manuscript.

We have performed WISH at later developmental stages with several mesodermal markers as requested by the referee and present the results in the amended manuscript (new **Fig. S5**). We observe that *myoD* is disorganized in Pnhd-depleted embryos reflecting abnormal somite segmentation (**Fig. S5A, B**). Additionally, *chordin* is expressed in a narrower domain as compared to uninjected controls (**Fig. S5E, F**). By contrast, the blood marker ¤-*globin* has not been significantly changed (**Fig. S5C, D**).
The analysis of *lim1*, an intermediate mesoderm marker, has been variable and inconclusive and we decided not to include it. Since *pnhd* expression in the mesoderm sharply decreases at the end of gastrulation, the early defect of morphants may be compensated by other signaling factors during later development. For this reason, the text has been revised to emphasize the early role of Pnhd in ventroposterior mesoderm formation.

Minor comments: In the revised introduction, the sentence "Factors secreted from the organizer…..mesoderm into dorsal (notochord), paraxial (somites), intermediate (kidney and gonads) and lateral/ventral (e. g., blood) types "should include a reference to signals from the marginal zone, reviewed in the references cited, and not least because pnhd and wnt8 are in the margin.

The relevant sentence has been modified and the relevant references included.

There are three references to pnhd as novel. But pnhd was described in 2004. Similarly the authors assume "key" differences in mesoderm specification in mammals and pnhd carrying animals. But what makes this "key" Could this be one of those empty buzzwords that the definitive journals should avoid?

The references to novelty and the buzzword 'key' have been removed.

Third decision letter

MS ID#: DEVELOP/2020/188094

MS TITLE: Pinhead signaling regulates mesoderm heterogeneity via FGF receptor-dependent pathway

AUTHORS: Olga Ossipova, Keiji Itoh, Aurelian Radu, Jerome Ezan, and Sergei Sokol
ARTICLE TYPE: Research Article

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