The RNA helicase DDX3 induces neural crest by promoting AKT activity

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DOI: 10.1242/dev.184341

Editor: Steve Wilson

Review timeline
Original submission: 29 August 2019
Editorial decision: 29 October 2019
First revision received: 2 July 2020
Editorial decision: 30 July 2020
Second revision received: 29 October 2020
Accepted: 2 December 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/184341

MS TITLE: The RNA helicase DDX3 induces neural crest by promoting AKT activity

AUTHORS: Mark Perfetto, Xiaolu Xu, Natasha Yousaf, Jiejing Li, and Shuo Wei

I have now received two 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 and recommend substantial revisions to your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

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

Reviewer 1

Advance summary and potential significance to field

Summary:
This manuscript by Perfetto et al. examines the role of the RNA helicase DDX3 in neural crest induction in Xenopus tropicalis. The authors found that knockdown of DDX3 resulted in craniofacial abnormalities typically associated with neural crest defects. Through a combination of knockdown

and epistasis experiments, the authors nicely demonstrated a novel and interesting role for DDX3 upstream of Rac1 and Akt in the control of Wnt signaling and neural crest induction. However, there are some important concerns that must be addressed to render the paper acceptable for publication in Development.

Comments for the author

1) A major conclusion of this manuscript is that DDX3 regulates Rac1 to control Akt activation and, subsequently, NC induction. The authors strongly intimate that DDX3 regulates Rac1 post-transcriptionally, at the step of translation initiation. To demonstrate a direct, post-transcriptional effect, as is alluded to at several points in the manuscript, the authors need to demonstrate that the RNA levels of Rac1 have not also decreased with a decrease in protein levels. If this isn't demonstrated, the phrasing should be softened.

2) The authors use immunoprecipitation and Western blot experiments to demonstrate DDX3 knockdown reduces levels of Rac1 protein in Figure 6. First, the blot for Rac1 for the DDX3 knockdown in Fig. 6B isn't clear, and as such, is uninterpretable. Further, conclusions can't be drawn from the Westerns without adequate biological replicates, which should be described, quantified, and presented. Also, I don't agree with the authors' decision to immunoprecipitate for Rac1 in order to demonstrate a reduction in protein levels in Fig. 6C; the loading control isn't useful or applicable in this context, and I don't believe this experiment convincingly demonstrates that DDX3 knockdown reduces Rac1 levels in embryos. Western blots of embryo lysates (without the IP) would be acceptable. Alternatively, a way to satisfy this concern as well as concern (1) above would be to use polysome profiling—a shift of Rac1 transcript from the polysomal actively-translating fractions to monosomal or non-translating fractions would be a very convincing assay with embryo lysates…

3) Given that Akt signaling has known roles in cell survival, and inhibition of Akt is known to induce caspase-mediated apoptosis, cell death assays should be conducted as well, to rule out reductions are specific to DDX3 (etc.) functions and not a general cell death phenotype. This is particularly a concern because it appears that the embryo in Fig. 4C (dnAkt) is blebbing…?? Please address.

General concerns/comments:

1) All Western blots need biological replicates and quantitation.

2) Western blot loading controls in a few places look over-saturated (e.g. Fig. 3A-B), and the DDX3 KD lane with anti-DDX3 in Fig. 3B looks like there was a bubble over the band. Please replace these images with better blots.

3) For Western analyses, please provide more details regarding how protein lysates were collected (what kind of lysis buffer?), how much total protein was loaded per lane, and what type of gels were run (percentage and other pertinent details). The Methods are sparse in this regard.

Reviewer 2

Advance summary and potential significance to field

In this study, Perfetto and colleagues explore the function of the RNA helicase DDX3 in early neural crest development. DDX3 is found mutated in human, causing central nervous system defects linked to intellectual disabilities, as well as various symptoms reminiscent of neurocristopathies. To test if DDX3 is involved in neural crest induction, the authors propose a suite of experiments in X. tropicalis embryos. They confirm that ddx3 is expressed ubiquitously early on, as previously shown (Cruciat et al., Science 2013) which is compatible with a role on neural crest development, among the other tissues potentially affected. DDX3 depletion, using morpholino depletion, is known to block embryo posteriorization and Wnt signaling (Cruciat et al., 2013). As for other posterior neural markers, the authors show here that DDX3 depletion prevents activation of neural border and neural crest markers, especially the ones responding to Wnt signaling. Later on, pharyngeal

cartilages are reduced. Interestingly, and in contrast to previous studies, the depletion phenotype seems rescued by a wtDDX3 form but not by a helicase-dead mutant. The authors then test the effects of DDX3 depletion on β-catenin levels (total and active) and Wnt signaling in vivo and in HEK293 cells, show decreased GSK3b-Ser9 phosphorylation and AKT phosphorylation. Finally, they show that DDX3 activity is likely mediated by Rac1, suggesting that DDX3-Rac1 act upstream of AKT-GSK3b-Wnt signaling in neural crest induction.
As a whole, this study brings novel and interesting data on the role of the DDX3 helicase during neural crest development. It also explores the potential mechanism of its activity with careful experiments and many controls (rescues). There are however several points that can be improved for the study to be fully convincing, as detailed below.

Comments for the author

Major points
1. Due to variability between batches of embryos and variability even between siblings from the same batch, it is unlikely that all phenotypes can be classified in a simple "on/off" [e.g. "normal/reduced"] manner: all scoring should be detailed with at least three categories of phenotype: severe / mild or partial / normal. This is especially important for the rescue experiments, since a mild phenotype is either interpreted as "rescued" [e.g. Fig1B, snai2 or msx1 rescue by wtDDX3] or "not rescued" [e.g. Fig 1C, sox9 not rescued by the AAA mutant]. Similarly, Fig. 3B is unconvincing as the phenotype in DDX3MO is more severe than the one in DDX3MO / AAA: a partial rescue is thus possible. In this case, a dose-response analysis will show if higher doses of AAA mutant help improve rescue levels. The authors need to show groups of embryos and detailed phenotype scoring as supplemental figures to better illustrate the observed phenotype range and strengthen their conclusions.
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3. In the same line of ideas, the morphant phenotype, decreasing Wnt reporter activity in vivo, should be tested for rescue with both wt and AAA forms of DDX3. Similarly, the drug RK33 can be used in vivo, with a careful dose-response assay, to see if Wnt signaling reporter is affected.
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5. In the introduction, the existing knowledge about DDX3 and Wnt signaling should be better detailed, rather than put in the result section.
6. The literature on the role of AKT signaling during neural crest early and later development has grown recently. Introduction and discussion should mention articles such as Bahm et al., 2017 (PMID: 28526750); Ciarlo et al.,2017 (PMID: 28832322); Figueiredo et al., 2017 (PMID: 29038306).
7. In Figure 2G, how does the RK33 inhibitor affect β-catenin stability? Is the stabilized β-catenin resistant to the drug?
8. In Figure 3, exogenous snai1 is used to test post-transcriptional regulation, independently of potential transcriptional regulation. This should be better explained.
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2- As DDX3 depletion was studied before (Crucial et al., 2013), the authors should compare zic1 response to otx2 and sox2 neural phenotypes.
3- Please indicate protein molecular weights on the western blots

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First revision

Author response to reviewers' comments

We thank both reviewers for their highly constructive suggestions. The following is a point-by-point rebuttal, with the reviewers' original comments colored in blue.

Reviewer 1:

This manuscript by Perfetto et al. examines the role of the RNA helicase DDX3 in neural crest induction in Xenopus tropicalis. The authors found that knockdown of DDX3 resulted in craniofacial abnormalities typically associated with neural crest defects. Through a combination of knockdown and epistasis experiments, the authors nicely demonstrated a novel and interesting role for DDX3 upstream of Rac1 and Akt in the control of Wnt signaling and neural crest induction. However, there are some important concerns that must be addressed to render the paper acceptable for publication in Development.

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The reviewer raised an excellent point here. To address this question, we carried out RT-qPCR using two pairs of primers, and detected little reduction in *rac1* mRNA in *X. tropicalis* embryos upon DDX3 KD (Fig. 6E; results are highlighted in pp. 16, 1st paragraph of the current version). Note that these are the same batches of embryos that show ~90% down-regulation in RAC1 protein (Figs. 6C and D). Thus, the mechanisms through which DDX3 regulates RAC1 in *X. tropicalis* embryos seem to be primarily post-transcriptional, consistent with what was observed previously in mammalian cells (see discussion in pp. 15, 2nd paragraph).

2) The authors use immunoprecipitation and Western blot experiments to demonstrate DDX3 knockdown reduces levels of Rac1 protein in Figure 6. First, the blot for Rac1 for the DDX3 knockdown in Fig. 6B isn't clear, and as such, is uninterpretable. Further, conclusions can't be drawn from the Westerns without adequate biological replicates, which should be described, quantified, and presented. Also, I don't agree with the authors' decision to immunoprecipitate for Rac1 in order to demonstrate a reduction in protein levels in Fig. 6C; the loading control isn't useful or applicable in this context, and I don't believe this experiment convincingly demonstrates that DDX3 knockdown reduces Rac1 levels in embryos. Western blots of embryo lysates (without the IP) would be acceptable. Alternatively, a way to satisfy this concern as well as concern (1) above would be to use polysome profiling—a shift of Rac1 transcript from the polysomal actively-translating fractions to monosomal or non-translating fractions would be a very convincing assay with embryo lysates…

We originally decided to perform IP for RAC1 in embryo lysates using two different antibodies, because we were unable to obtain a clean blot with either antibody. As the reviewer suggested, we spent a lot of efforts to optimize the western blot protocol for RAC1, and were finally able to detect a single band at the predicted size without doing the IP (Fig. 6C). Experiments included in the first draft of this manuscript were initiated when we were at West Virginia University, and were completed after we moved to the University of Delaware. As such, biological replicates of western blot for the same proteins were often carried out using different imagers and occasionally with films. Due to these variations, the results cannot be combined for statistical analyses. During the current revision, we repeated several key experiments and obtained statistically meaningful data by including 3 batches of embryo lysates (3 biological replicates) in the same gel and performing detection and quantification using a state-of-the-art LI-COR Odyssey imager (Fig. 6D). We have also replaced the original Fig. 6B with a clearer blot, and quantified the RAC1 levels obtained from HEK293T cells (Figs. 6A and 6B).

3) Given that Akt signaling has known roles in cell survival, and inhibition of Akt is known to induce caspase-mediated apoptosis, cell death assays should be conducted as well, to rule out reductions are specific to DDX3 (etc.) functions and not a general cell death phenotype. This is particularly a concern because it appears that the embryo in Fig. 4C (dnAkt) is blebbing…?? Please address.

The reviewer was correct that AKT is well known to protect cells from apoptosis. We therefore carried out TUNEL staining, but did not detect any apparent increase in apoptosis in stage ~12.5 DDX3 morphants (Fig. S4; results highlighted in pp. 14 of the current version). This is consistent with a previous report that inhibition of AKT activation by KD of PFKFB4 does not induce apoptosis in *Xenopus* embryos until stage ~14 (Pagoraro et al., *Nat. Commun.* 2015). It should also be noted that not all NC/NPB markers are reduced upon DDX3 KD. For example, *zic1* expression is essentially unaltered and is sometimes expanded in DDX3 morphants (Fig. 1B).
Thus, it is unlikely that the reduction of NC is a general cell death phenotype.

General concerns/comments:

1) All Western blots need biological replicates and quantitation.

As explained above in response to the reviewer's Point #2, we did have biological replicates for all western blots. In the current version, we have included quantification for all western blots. In addition, we have also repeated the critical experiments in *X. tropicalis* embryos in a more quantifiable way, and included statistics in the current version of the manuscript (Figs. 3C and 6D).

2) Western blot loading controls in a few places look over-saturated (e.g. Fig. 3A-B), and the DDX3 KD lane with anti-DDX3 in Fig. 3B looks like there was a bubble over the band. Please replace these images with better blots.

As the reviewer suggested, we have replaced the old images with better blots.

3) For Western analyses, please provide more details regarding how protein lysates were collected (what kind of lysis buffer?), how much total protein was loaded per lane, and what type of gels were run (percentage and other pertinent details). The Methods are sparse in this regard.

The requested information has been added to pp. 24, 2nd paragraph of the current version of the manuscript.

Reviewer 2:

In this study, Perfetto and colleagues explore the function of the RNA helicase DDX3 in early neural crest development. DDX3 is found mutated in human, causing central nervous system defects linked to intellectual disabilities, as well as various symptoms reminiscent of neurocristopathies. To test if DDX3 is involved in neural crest induction, the authors propose a suite of experiments in X. tropicalis embryos. They confirm that ddx3 is expressed ubiquitously early on, as previously shown (Cruciat et al., Science 2013) which is compatible with a role on

neural crest development, among the other tissues potentially affected. DDX3 depletion, using morpholino depletion, is known to block embryo posteriorization and Wnt signaling (Cruciat et al., 2013). As for other posterior neural markers, the authors show here that DDX3 depletion prevents activation of neural border and neural crest markers, especially the ones responding to Wnt signaling. Later on, pharyngeal cartilages are reduced. Interestingly, and in contrast to previous studies, the depletion phenotype seems rescued by a wtDDX3 form but not by a helicase-dead mutant. The authors then test the effects of DDX3 depletion on β-catenin levels (total and active) and Wnt signaling in vivo and in HEK293 cells, show decreased GSK3b-Ser9 phosphorylation and AKT phosphorylation. Finally, they show that DDX3 activity is likely mediated by Rac1, suggesting that DDX3-Rac1 act upstream of AKT-GSK3b-Wnt signaling in neural crest induction.
As a whole, this study brings novel and interesting data on the role of the DDX3 helicase during neural crest development. It also explores the potential mechanism of its activity with careful experiments and many controls (rescues). There are however several points that can be improved for the study to be fully convincing, as detailed below.

Major points
1. Due to variability between batches of embryos and variability even between siblings from the same batch, it is unlikely that all phenotypes can be classified in a simple "on/off" [e.g. "normal/reduced"] manner: all scoring should be detailed with at least three categories of phenotype: severe / mild or partial / normal. This is especially important for the rescue experiments, since a mild phenotype is either interpreted as "rescued" [e.g. Fig1B, snai2 or msx1 rescue by wtDDX3] or "not rescued" [e.g. Fig 1C, sox9 not rescued by the AAA mutant]. Similarly, Fig. 3B is unconvincing as the phenotype in DDX3MO is more severe than the one in DDX3MO / AAA: a partial rescue is thus possible. In this case, a dose-response analysis will show if higher doses of AAA mutant help improve rescue levels. The authors need to show groups of embryos and detailed phenotype scoring as supplemental figures to better illustrate the observed phenotype range and strengthen their conclusions.

We agree with the reviewer that more detailed classification of the phenotypes can better illustrate the observed phenotype range. However, we would also like to point out that most of the phenotypes described in this manuscript were characterized by in situ hybridization, in which the signal strength varies greatly depending on gene expression levels, probe quality/concentration, length of development, etc. As a consequence, the difference between “moderate” and “severe” phenotypes is quite arbitrary. For example, an embryo that is classified as “moderate” reduction in one gene may be classified as “severe” for another gene if the expression level for the latter is lower and staining is weaker in general, as little signal may be detected even after prolonged development. Even for the same gene, the inconsistency also exists due to variations in experimental conditions (such as variation in signal strength caused by fresh vs. reused probes). For these reasons, we decided to forgo the three-category classification (normal, moderate and severe), which we used previously (Wei et al., *Dev. Cell* 2010; Wei et al., *Dev. Biol.* 2012), and replace with the more reproducible two-category system (normal and reduced/increased), which is more generally adopted by the *Xenopus* research community (Li et al., *Development* 2018; Li et al., *Sci. Rep.* 2019; for examples of most recent publications on *Xenopus* neural crest induction from other labs, please see Marquez et al., *J. Clin. Invest.* 2020, Scerbo and Monsoro-Burq, *Sci. Adv.* 2020, Pegge et al., *Dev. Biol.* 2020, Schwenty-Lara et al., *Hum. Mol. Genet.* 2020). However, if the reviewer insists, we will be happy to go back and rescore the embryos.

2. A major claim of this study is that the helicase activity of DDX3 is important for the neural crest phenotype and for Wnt signaling in this context. This contrasts with the previous study, and relies on the proposed lack of rescue of the morpholino phenotype by the AAA mutant (lacking helicase function) and on the use of a helicase-specific inhibitor RK33. Unfortunately, the sox9 in situs shown here (Figure 1C) can be improved and better analyzed as mentioned above. Moreover, since neural border defects are proposed as a cause for the phenotype, the rescue with AAA mutant should include analyses of the genes shown in Fig. 1B as well. Additionally, the drug RK33 can probably be used in vivo, to test its effects on neural border and neural crest genes.

We have included high-contrast pictures of *sox9* in situ in the current version of the manuscript (Fig. 1C). We chose to use *gbx2* as another marker for the rescue with the AAA mutant because a) *gbx2* is a direct Wnt target, and b) loss of *gbx2* leads to loss of NPB markers *pax3* and *msx1* (Li

et al., *Development* 2009; note that *zic1* is largely unaltered upon DDX3 KD). Thus, we believe that the *gbx2* in situ can "kill two birds with one stone" – it serves as a readout for both Wnt signaling activity and the NPB markers.

As the reviewer suggested, we incubated *X. tropicalis* embryos in RK-33, but did not observe any NC phenotypes or effect on Wnt signaling even at a concentration twice as high as we used for HEK293T cells. One possible explanation is that RK-33 cannot penetrate the embryos (see below for LY294002). Therefore, these experiments are inconclusive.

3. In the same line of ideas, the morphant phenotype, decreasing Wnt reporter activity in vivo, should be tested for rescue with both wt and AAA forms of DDX3. Similarly, the drug RK33 can be used in vivo, with a careful dose-response assay, to see if Wnt signaling reporter is affected.

We carried out the suggested experiment, and our preliminary data suggest that the AAA mutant did not rescue the reduced Wnt signaling caused by DDX3 KD. However, our building was locked down due to a COVID-19 outbreak on campus in early March, and we were unable to collect statistically meaningful data with stringent controls. We did not observe any effect on Wnt signaling with RK-33, but this may be due to the inability of this drug to penetrate the embryos (see above).

4. It is unclear if HEK293 cells were treated with a Wnt ligand in figure 2F, to obtain high levels of total β-catenin. The discrepancy with the previous results is puzzling since the same cellular model is used and the same mutants: how do the authors compare their results (obtained in whole cell extracts) to the previous results in Cruciat et al. Do they observe the same results if they employ cell nuclear vs cytoplasmic fractionation?

We would like to clarify that we did not treat the HEK293T cells with a Wnt ligand in Fig. 2F of the original version. The levels of total β-catenin appear high probably because we loaded a large amount of protein on the gel and used a high-sensitivity HRP substrate for detection. In fact, in line with previous studies and as pointed out by the reviewer, the endogenous β-catenin levels in HEK293T cells were low, as exogenous β-catenin enhanced Wnt activity in these cells by >100 fold (Figs. 2F and 2G of the current version).

Cruciat et al. attributed the reduced Wnt signaling caused by DDX3 KD to inhibited nuclear localization of β-catenin. However, it is not very clear if the cytosolic β-catenin levels were affected when they knocked down DDX3 in HEK293T cells. In fact, the cytosolic β-catenin bands from cells transfected with control and DDX3 siRNAs appear to be from two separate western blots and cannot be compared with each other directly (Cruciat et al., *Science* 2013, Fig. 1B). Likewise, our data do not rule out an additional effect of DDX3 on β-catenin nuclear localization. Therefore, we do not believe our results contradict with those published in Cruciat et al., *Science* 2013.

5. In the introduction, the existing knowledge about DDX3 and Wnt signaling should be better detailed, rather than put in the result section.

We have moved the background information on DDX3 and Wnt signaling to Introduction (highlighted in pp. 4, 2nd paragraph of the current version).

6. The literature on the role of AKT signaling during neural crest early and later development has grown recently. Introduction and discussion should mention articles such as Bahm et al., 2017 (PMID: 28526750); Ciarlo et al.,2017 (PMID: 28832322); Figueiredo et al., 2017 (PMID: 29038306).

We thank the reviewer for kindly providing the detailed information on these references, and have added them to both Introduction and Discussion (highlighted in pp. 11, 2nd paragraph and pp. 18, 2nd paragraph).

7. In Figure 2G, how does the RK33 inhibitor affect β-catenin stability? Is the stabilized β- catenin resistant to the drug?

These are excellent questions. In response, we have added data showing that the stabilized β-

catenin is indeed resistant to RK-33, although the Wnt activity induced by wild-type protein was drastically reduced by the same drug in the same experiments (Fig. 2F of the current version).

8. In Figure 3, exogenous snai1 is used to test post-transcriptional regulation, independently of potential transcriptional regulation. This should be better explained.

As the reviewer suggested, we have rephrased to emphasize that Snai1 was expressed exogenously to test if it is regulated by DDX3 post-transcriptionally (highlighted in pp. 12 of the current version).

9. The images presented in Fig4A and Fig 5C are difficult to analyze due to high background or poor contrast. Please show better staining/image.

We have replaced the in situ hybridization data in Figs. 4A and 5C with better images in the current version.

10. AKT-independent roles for PI3K are mentioned. In order to test if PI3K and AKT play similar functions during neural crest induction, the treatment with AKT inhibitor should be directly compared to the published treatment with PI3K inhibitor, with similar doses, at relevant stages. The authors can then discuss if AKT and PI3K inhibition display similar roles and which ones, during neural crest induction, as previously debated in Pegoraro et al., 2015; Figueiredo et al., 2017; Geary and Labonne, 2018.

We treated *X. tropicalis* embryos with the PI3K inhibitor LY294002 as described by Pegoraro et al. (*Nat. Commun.* 2015). Surprisingly, no phenotype was observed at 60-80 µM (the dosages used by Pegoraro et al.) and even up to 160 µM. Western blotting results show that AKT activation in treated embryos was not inhibited (although the same dosages inhibited AKT activation in HEK293T cells), suggesting that the drug did not penetrate the *X. tropicalis* embryos. We did not pursue this further because our data suggest that DDX3 activates AKT via RAC1. Therefore, we feel that testing if PI3K and AKT play similar functions during neural crest induction is beyond the scope of the current study.

11. The epistasis experiments with Rac1 are nice and convincing, but limited to the analysis of the late phenotype only. In order to better characterize the proposed model, it would be important to check normal AKT and GSK3b phosphorylation after acRac1 rescue of DDX3MO in vivo.

To check normal AKT and GSK3β phosphorylation after active RAC1 rescue of DDX3 MO in vivo, we carried out whole-embryo injection of DDX3 MO with active *rac1* mRNA (as western blot detection requires whole-embryo manipulation). Unfortunately, whole-embryo injection of active *rac1* mRNA, even at dosages lower than that used in rescue experiments shown in Fig. 6F, caused severe defects at earlier stages, preventing further analyses at late gastrula stages. However, in a separate study (not included in the current manuscript), we found that CRISPR/Cas9-mediated F0 knockout of RAC1 inhibits endogenous Wnt activity on the injected side in the transgenic Wnt reporter embryos (see below). This result provides indirect evidence supporting that RAC1 regulates Wnt signaling downstream of DDX3.

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

Minor points

1- HEK293 cells are not initially isolated from a tumor, but they display many common points with cancer cells (see for ex. PMID: 26026906). Please rephrase to avoid confusion.

We thank the reviewer for this excellent suggestion, and have rephrased accordingly (highlighted in pp. 10, 1st paragraph).

2- As DDX3 depletion was studied before (Crucial et al., 2013), the authors should compare zic1 response to otx2 and sox2 neural phenotypes.

Cruciat et al. (*Science* 2013) reported no change in *sox3* (not *sox2*) expression in *Xenopus* embryos upon DDX3 KD, but there was an expansion of the forebrain marker *otx2*, presumably due to

anteriorization (as Wnt has posteriorizing effects). We would like to point out respectfully that *zic1* expression is insensitive to Wnt signaling, and that the slight expansion of *zic1* caused by DDX3 KD was likely secondary to reduced *pax3* (as explained in pp. 8, lines 9-11, and pp. 9, lines 7-9). Thus, we feel that the expansion of *zic1* is probably unrelated to the expansion of *otx2*.

3- Please indicate protein molecular weights on the western blots

As the reviewer suggested, we have now indicated the approximate molecular weights on the western blots.

Second decision letter

MS ID#: DEVELOP/2019/184341

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AUTHORS: Mark Perfetto, Xiaolu Xu, Congyu Lu, Yu Shi, Natasha Yousaf, Jiejing Li, Yvette Y. Yien, and Shuo Wei

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As you will see, one of the referees is happy with your revisions but the other has some remaining significant concerns. As these relate to controls and experiments to validate the robustness of your conclusions, it is important for you to address the issues raised.

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.

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Comments for the author

The authors have satisfied my concerns by nicely addressing the issues identified in my previous review.
From my perspective, the manuscript is now acceptable for publication.

Reviewer 2

Advance summary and potential significance to field

In the revised version of their manuscript, Perfetto and colleagues have addressed several of my previous comments. There remains, in my opinion, 3 important points to be addressed to establish robust conclusions key to the study.

Comments for the author

In the revised version of their manuscript, Perfetto and colleagues have addressed several of my previous comments. There remains, in my opinion, 3 important points to be addressed to establish robust conclusions key to the study.

- Point 3: there is a previous study reporting helicase-independent activity of DDX3 on Wnt signaling (Cruciat et al., 2013). It is therefore important to validate all the assays confirming that there is also a helicase-dependent one in the neural crest context. As the authors mention preliminary experiments (not shown), it will be probably rather easy to confirm this important point, which needs to be included. This could be achieved rather fast, with a lower number of embryos per condition than in fig.2A (e.g. ~60, 20 per experiment).

- Point 11, the DDx3MO rescue experiment with rac1 insensitive to DDX3 action: If rac1 alone causes important early defects, it is also possible that it creates late non-specific defects even in the targeted injections. The authors thus need to show the cartilage phenotype of rac1 alone injected in 1 blastomere at 8-cell stage in Figure 6F, as an important control.

- linked to Point 11 and Point 1: One picture cannot render the range of phenotypes obtained (cf my previous point 1 about a more precise description of the phenotypes, not addressed). Specifically, the DDX3 MO cartilage phenotypes shown in Figure 6F are much more severe than in Figure 1A. Consequently, the rescued phenotype (DDx3 MO and rac1) is similar to the morphant phenotype in Figure 1. This is puzzling. Linked to the previous point, how do the authors check rac1 activity? This is another example where a better quantification of the phenotypes (e.g. here, measuring the area of the branchial arches cartilages -ratio injected/control side- and plotting the dispersion of the data in controls versus experimental situation) would be very useful to evaluate the range of phenotypes in each series of experiments. If the authors cannot confirm rescue by rac1 on other parameters than cartilage formation (Akt, Gsk3b), this at least should be carefully quantified.

- This study confirms that Akt signaling is essential for neural border and neural crest induction in vivo, during gastrulation. The authors should make it very clear in the discussion (4th paragraph), that this confirms three earlier studies also using in vivo assays (Ciarlo et al., 2017; Figueiredo et al., 2017 and Pegoraro et al., 2015). As discussed, the apparent controversy arose from experiments conducted by gain-of-function study in animal cap assay in one study (Geary and LaBonne 2018), where one difficulty is to compare to the experimental signaling levels to the in vivo levels of signaling. Please discuss more clearly.

Second revision

Author response to reviewers' comments

We thank the reviewer for taking the time to explain the questions in such detail, and agree that addressing these questions would help understand the mechanisms of action for DDX3 in neural crest induction. We have carried out new experiments as suggested, and the results are consistent

with our conclusions. Together with other data presented in the manuscript and studies published by other groups, we hope that the reviewer finds these results satisfactory.

In the revised version of their manuscript, Perfetto and colleagues have addressed several of my previous comments. There remains, in my opinion, 3 important points to be addressed to establish robust conclusions key to the study.

- Point 3: there is a previous study reporting helicase-independent activity of DDX3 on Wnt signaling (Cruciat et al., 2013). It is therefore important to validate all the assays confirming that there is also a helicase-dependent one in the neural crest context. As the authors mention preliminary experiments (not shown), it will be probably rather easy to confirm this important point, which needs to be included. This could be achieved rather fast, with a lower number of embryos per condition than in fig.2A (e.g. ~60, 20 per experiment).

We have included the results of our rescue experiments in the current version of this manuscript. As shown in the new Figure S3, the AAA mutant of human DDX3X failed to restore the reduced GFP reporter expression in the transgenic Wnt reporter line. This result, together with this mutant's inability to rescue the neural crest marker sox9 (Figure 1C) and gbx2, a direct Wnt target required for neural plate border formation (Figure 2B), as well as a previous study showing that human mutations in the highly conserved residues in the ATP-binding domain and helicase C-terminal domain cause reduced Wnt activity and neural crest-related birth defects (Snijders Blok et al., Am J Hum Genet 2015), strongly indicates the existence of a helicase-dependent mechanism for DDX3 in neural crest development.

- Point 11, the DDx3MO rescue experiment with rac1 insensitive to DDX3 action: If rac1 alone causes important early defects, it is also possible that it creates late non-specific defects even in the targeted injections. The authors thus need to show the cartilage phenotype of rac1 alone injected in 1 blastomere at 8-cell stage in Figure 6F, as an important control.

We would like to point out respectfully that injecting one specific blastomere at cleavage stages is commonly used by Xenopus researchers to circumvent the early defects caused by whole-embryo manipulations. This approach is based on the availability of detailed fate maps of individual blastomeres (http://www.xenbase.org/anatomy/static/xenbasefate.jsp), and is similar to the conditional gene manipulations in other animal models. Figure 6F of our manuscript shows that the craniofacial defects caused by DDX3 knockdown were significantly rescued by rac1 mRNA injection. It is unlikely that this rescue effect was a result of earlier defects caused by RAC1, as the latter would be expected to aggravate the craniofacial defects caused by DDX3 knockdown. As the reviewer suggested, we injected rac1 mRNA into one anterodorsal blastomere of 8-cell stage snai2:eGFP embryos (as in Figure 6F). Upon close examination, we did not observe any apparent defects in the craniofacial structures or any other structures (Figure S7 of the current version).

- linked to Point 11 and Point 1: One picture cannot render the range of phenotypes obtained (cf my previous point 1 about a more precise description of the phenotypes, not addressed). Specifically, the DDX3 MO cartilage phenotypes shown in Figure 6F are much more severe than in Figure 1A. Consequently, the rescued phenotype (DDx3 MO and rac1) is similar to the morphant phenotype in Figure 1. This is puzzling. Linked to the previous point, how do the authors check rac1 activity? This is another example where a better quantification of the phenotypes (e.g. here, measuring the area of the branchial arches cartilages -ratio injected/control side- and plotting the dispersion of the data in controls versus experimental situation) would be very useful to evaluate the range of phenotypes in each series of experiments. If the authors cannot confirm rescue by rac1 on other parameters than cartilage formation (Akt, Gsk3b), this at least should be carefully quantified.

We agree with the reviewer that more detailed description of the phenotypes would help clarify the effects of gene manipulations in each series of experiments. As we explained in the previous round of revision, quantification of in situ hybridization data is quite arbitrary. However, we do feel that quantifying the craniofacial phenotypes is less subjective and more reproducible, as it does not require further processing of the embryos. In the current revision, we included updated Figures 1A and 6F, in which the craniofacial phenotypes are further classified as normal, moderate, and severe (see below). As shown in the new Figure 6F, co-injection of rac1 mRNA increased the

percentage of injected tadpoles with normal phenotype and reduced the percentage of tadpoles with severe phenotypes. We chose to include the images of a tadpole with severe phenotype for DDX3 knockdown group and a tadpole with moderate phenotype for the rescue group, as they are the most representative examples of individual injection groups.

- This study confirms that Akt signaling is essential for neural border and neural crest induction in vivo, during gastrulation. The authors should make it very clear in the discussion (4th paragraph), that this confirms three earlier studies also using in vivo assays (Ciarlo et al., 2017; Figueiredo et al., 2017 and Pegoraro et al., 2015). As discussed, the apparent controversy arose from experiments conducted by gain-of-function study in animal cap assay in one study (Geary and LaBonne 2018), where one difficulty is to compare to the experimental signaling levels to the in vivo levels of signaling. Please discuss more clearly.

As the reviewer suggested, we have revised the discussion to clarify this controversy (see additions highlighted in yellow in pp. 19 of the current version).

Third decision letter

MS ID#: DEVELOP/2019/184341

MS TITLE: The RNA helicase DDX3 induces neural crest by promoting AKT activity

AUTHORS: Mark Perfetto, Xiaolu Xu, Congyu Lu, Yu Shi, Natasha Yousaf, Jiejing Li, Yvette Y. Yien, and Shuo Wei
ARTICLE TYPE: Research Article

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

Reviewer 2

Advance summary and potential significance to field

In this revised version, the authors have addressed all my previous comments in a satisfactory manner.

Comments for the author

In this revised version, the authors have addressed all my previous comments in a satisfactory manner. I think it is now ready and important for the readership of this journal.