Concerted morphogenesis of genital ridges and nephric ducts in the mouse captured through whole-embryo imaging

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MS TITLE: Concerted morphogenesis of genital ridges and nephric ducts in the mouse captured through whole embryo imaging

AUTHORS: Corey Bunce, Jennifer McKey, and Blanche Capel

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 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 (please see Editor's note below). 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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

Bunce et al have revisited the development of the mouse urogenital ridge with a focus on the gonad using tissue clearing based on the iDISCO+ method combined with immunofluorescence and light sheet microscopy. This group is familiar with this method since they used it previously to assess the number of the different types of follicles in the adult ovary (Key el al, 2020, Biol Reprod). Here they show the dynamic of gonad development with the changes in the position of the gonad, the changes in the shape of the gonad and the relationship between the mesonephric tubules, the kidneys and the gonad during the early development of the gonads in the embryo.

The first part is descriptive, and the data clarify how the gonads develop in the embryonic context without disrupting its native morphology. In a second part, the authors show that this method is suitable to investigate the morphological changes associated with the lack of expression of a gene involved in the development of the gonad as exemplified with the Cbx2 knockout mutant embryos. This article contains a wealth of data, which may serve as a resource to investigations of urogenital morphogenesis but, which will be also very useful for teaching. This lab has been for decades at the forefront of the developmental biology of the gonads in mice and has the knowledge for such an analysis. By combining their knowledge and the light sheet imaging, they produce new data which are without doubt of great importance for the whole community working on gonad development and more generally developmental biology. Thus, I believe that this article is valuable both scientifically and technically and fits with the interests of the readership of Development. I would like, however, to make a series of recommendations to improve the impact of the study and the clarity of the manuscript.

Comments for the author

Major recommendations:

Some data have been generated using the Sf1-eGFP transgene (Stalling et al, 2002). While this transgene is a good marker for the gonads, this transgene is a poor marker for the early developing adrenal glands. The the Sf1-eGFP transgene is weakly expressed in the adrenals at E11.5 as previously shown by (Pitetti et al, PLoS Genet, 2013, Figure 6Q) while SF1 (native protein) is strongly expressed in the adrenals from E11.5 as previously shown with SF1 immunostainings (Zubair et al., Mol Endocrinol., 2009).
Accordingly, in figures 2C-S3, the adrenals identified using Sf1-eGFP transgene and coloured in magenta appear as a small structure detached from the gonads at E11.5. The size appears to be underestimated and the adrenals are still connected to the gonads at this stage. The authors should use another way to detect the adrenals at E11.5. They should perform SF1 (or other suitable adrenal markers) immunolocalization analysis to reliably identify the whole adrenals. This will clarify the position and the size of the adrenals in Figure 1F and in Figures 2 C and S3 (E11.5). Moreover, this will clearly define the limits of the Sf1-eGFP transgene. These data should be included in this work.

Line 118 Accordingly, without these new data, the conclusion “The adrenal primordium remained in the same relative position where it formed up to E13.5” is too preliminary.

There are questions about SF1 expression as well. It was previously shown that SF1 is not expressed in the cells of the coelomic epithelium at 20-21ts when these cells actively proliferate in the XY gonads (Schmahl et al., Development, 2000). However, it is not clear whether the transgene Sf1-eGFP is expressed in the coelomic epithelium. The authors should provide a comparison of the expression of the Sf1-eGFP transgene and the SF1 native protein in the coelomic epithelium. This might explain some differences between the GATA4 and Sf1-eGFP expression pattern.

It is not clear to me why the gonads are not shown in the 3 D reconstruction analysis of the urogenital system of the Cbx2 mutant embryos despite GATA4 immunostainings have been performed. To show the contribution of this method to analyze a phenotype, the visualization of the major organs of the urogenital system including the gonads seems essential.

Other recommendations Line 39
Wartenburg et al., is quoted in the manuscript and becomes Wartenberg, H. in the references.
This is a reference about testis development in rabbits. Maybe the authors can add a reference about gonad development in mice.

Line 44
The references include the development of mesonephric tubules and gonads in different species and this should be explicitly indicated. We cannot exclude that the morphogenic processes involved in the development of the urogenital ridge vary according to the species.

Line 75
The following references are quoted for the expression profile of PAX8 (Hu et al., 2013; Plachov et al. 1990; Stalling et al., 2002; Viger et al., 1998;). There is an extra semicolon, or a reference has been omitted.
Kulibin and Malolina ((2020), Dev Dyn. Doi: 10.1002/dvdy.242) have also studied the fate of the nephric tubules using PAX8 (another antibody that does not seem to be as good as the one you used). It would be appropriate to mention this study either in introduction or in discussion.

Figure 1F- Figure 2 C;

When the gonad develops, the adreno-gonadal primordium splits to give the adrenal and gonadal primordia. Then the adrenals remain one of the closest neighboring organs of the gonads. In Figure 1F, the organs are false colored easing their identification, but the adrenals are not shown. Given their common origin with the gonad, the adrenal glands should be identified in the tissue context of the developing gonad.

Figure 1D, E; Figure 2B and following Staging of embryos
Most of developmental biologists can relate somites and developmental stages. However, in the field of gonad development, this is the tail somites that are commonly used to determine precisely when the main events of sex determination occur around E11.5. Could the authors add the numbers of tail somites for the data corresponding to E11.5 to ease the comparisons with data published previously.

Figure 2A-B
This is a clever way to show the position of the gonad in the embryo. This highlights differences between XX et XY embryos, but is it meaningful or just due to the variability between different embryos?
What is the precise stage of development of the embryos (in tail somites) and the number of XX and XY embryos and that were used in these experiments? The n on the right-hand side of the figure 2B could be the number of analysed embryos. This should be clarified in the legend of the figure. When there are two embryos, it is not clear what has been represented on the graph (the position in one embryo, an average, other). Could the authors comments whether they have observed a difference in the position of the gonads between XX and XY embryos at a precise stage.

Figure 2C-S3
The adrenals appear smaller in XY embryos in comparison with XX embryos at E11.5. Is there a difference due to the sex or is it due to the limits of the Sf1-eGFP transgene?
If they are sex differences, it would be relevant to show XX and XY data together with some of the views of both sexes in the main figures and the rest in the supplementary figures.

These potential differences depending on the sex of embryos would be highly relevant in a manuscript on gonad development, but do they really exist? The authors should clarify this point.

Line 139
For proliferation of the CE, Schmahl et al (2000) should be quoted.
Schmahl et al have shown that the cells of the coelomic epithelium are SF1- negative cells at 20-21ts. Could this explain part of the difference observed between the GATA4 and Sf1-eGFP stainings? This should be clarified in the text. When the authors address whether the Sf1-eGFP is expressed in the cells of the coelomic epithelium, they should combine GATA4 immunostainings when possible. A combined analysis would show the degree of overlap of these two proteins preventing

further questions.

Figure 3F
The F is missing

In Figure 4G, are these embryos, XX or XY embryos? For all the figures, it would be very informative to know whether the embryos are XX and XY embryos. This should be added in this figure but included on all figures as well when it was forgotten.

Although the position and elaboration of the reproductive ducts was more similar between wild type and Cbx2-/- embryos at E13.5, the mesonephric tubules appear further apart from the kidneys in the Cbx2-/- embryos in comparison to wildtypes. This suggests either a delay/defects in the descent of the testis (if the embryos are XY) or a delay in the development of the ovaries (XX) or a delayed duct development independently of the sex in the mutant embryos. However, this remains speculations. To show the position of these different structures, the authors should perform a 3D reconstruction of the gonads in addition to the ducts and kidneys these mutant embryos. The GATA4 immunostaining performed in Figure S6 could be used to do this reconstruction as presented in the wildtypes in Figure 3A. The whole will show the position of each structure and evaluate their anomalies in XX and XY mutant embryos.

Lines 304-310
A connection exists between morphogenesis and testis differentiation in the mouse gonad. Different groups have shown that Sry and Sox9 and in turn Sertoli cell differentiation begins within the central part of the XY gonads and extend to the poles. When there is a delay of Sry expression, there is sex reversal at the poles of XY gonads. Do you mean that the more pronounced central changes in the gonads are a consequence of this central expression of Sry/Sox9?

Line 361
PtWH: typo between capital and normal letters

Line 386
imaris: a capital letter is missing

In references
Biason-Lauber at al…I did not find this reference in the manuscript

Reviewer 2

Advance summary and potential significance to field

In this manuscript Bunce and co-workers describe the whole-mount analysis of the development of the urogenital ridges in mouse embryos. The authors present beautiful data, which will be very useful for the field. However some of the conclusion are difficult to follow and Cbx2-knockout was probably not the best choice for uncovering a mechanistic relationship between the two tissues.

Comments for the author

1. The numbers used for the analysis in Figure 2 are quite low (n=2 max). How consistent or how variable are these relationships to the somites? And is there a difference between right and left? It would be good to expand on this.

2. In line 115 it is stated “the urogenital complex moved away from the dorsal body wall” – however this is not obvious in Figure 2C, first column. It would help to indicate in the Figure what is meant or re-word the statement. Similarly, the relative movement of the urogenital complexes from each other (line 116) is only obvious between E11.5 and E12.5, but not after this.

3. Line 118: isn’t the movement of the developing kidney posterior to anterior (or caudal to cranial) and not dorsoventral?!

4. It is unclear why the thickness of the gonads at E10.5 and E11.5 is “consistent with an anterior to posterior pattern of development (Figure 3E) as stated in lines 161 to 163. Overall, the gonads do not appear much thicker at the anterior pole compared to the posterior pole. Similarly, the statement in lines 169-171 (“overall, these data confirmed the presence…”) is difficult to see in the data presented.

5. Lines 181-183: Looking at the graph in Figure 3G, it appears that the distance in many cases was bigger at the anterior and then again at the posterior pole, and not just that “the central region was farther apart than the poles”? The data should be described as it is shown.

6. CBX2 is expressed in the mesonephros (see e.g. Figure 1 in Katoh-Fukui et al., Endocrinology 2012), hence it probably is not the best mouse model to suggest a mechanistic relationship between the nephric ducts and the genital ridges.

7. The conclusion that “the genital ridge undergoes considerable AP movement” (line 206) can cause confusion. It is the position relative to somites and/or hindlimbs and does not mean that the genital ridge “moves”. This (and similar statements) should be re-worded (e.g. to “the genital ridge undergoes relative AP movements”, or similar).

8. Throughout the text, the authors should use “gonadal sex determination” instead of just “sex determination” hen talking about the decision of the genital ridge to develop into a testis or ovary.

9. The official gene and protein names should be used throughout the manuscript (e.g. NR5A1 instead of SF1, which officially stands for splicing factor 1).

Reviewer 3

Advance summary and potential significance to field

This work utilizes the techniques of whole-embryo clearing and light sheet microscopic imaging to conduct a detailed characterization of early gonad development in the mouse. Key findings include a better description of the relationship between the developing gonads and surrounding tissue, with the suggestion of a casual link between their morphogenetic programs, as well as a better understanding of the previously described A/P and center/pole differences in gonad development. They also describe defects in gonad morphogenesis in the Cbx2 mutant, where correlation bw the regionalized defects in the gonad and nephrite ducts implies some casual relationship between development of the two tissues.

Comments for the author

As is typical of the Capel lab, the work is beautifully done and carefully presented. These are challenging experiments and the results represent important information for the field. One major question is whether the work can be seen as making enough of a "mechanistic advance" to be deemed appropriate for Development (which emphasizes mechanism). Sometimes a descriptive paper is more important than any other type of work and can become a classic, often-referenced paper. I suspect that work is being targeted for the Resource/Techniques area of the journal, which is most appropriate for the work here. Also, the manuscript is quite "tight" with 4 figures and a short discussion, raising the question of whether it is more appropriate as a Report vs. and Article. Given that the paper presents a great deal of important and difficult-to-obtain data for the field, I would support publication as a "Resource" in either an Article or Report form.

First revision

Author response to reviewers' comments

We would like to thank the reviewers for their helpful and insightful comments, which have allowed us to improve the quality and value of our manuscript.

Summary of the revisions

The majority of revision to the text and figures have been to improve the clarity of the statements and the data. The main addition is a comparison between the expression of the SF1:eGFP reporter and the endogenous NR5A1 protein, which has helped to further contextualize these methods and results in the field of urogenital development.

Responses to specific reviewer comments Reviewer 1:
Bunce et al have revisited the development of the mouse urogenital ridge with a focus on the gonad using tissue clearing based on the iDISCO+ method combined with immunofluorescence and light sheet microscopy. This group is familiar with this method since they used it previously to assess the number of the different types of follicles in the adult ovary (Key el al, 2020, Biol Reprod). Here they show the dynamic of gonad development with the changes in the position of the gonad, the changes in the shape of the gonad and the relationship between the mesonephric tubules, the kidneys and the gonad during the early development of the gonads in the embryo.
The first part is descriptive, and the data clarify how the gonads develop in the embryonic context without disrupting its native morphology. In a second part, the authors show that this method is suitable to investigate the morphological changes associated with the lack of expression of a gene involved in the development of the gonad as exemplified with the Cbx2 knockout mutant embryos. This article contains a wealth of data, which may serve as a resource to investigations of urogenital morphogenesis but, which will be also very useful for teaching.
This lab has been for decades at the forefront of the developmental biology of the gonads in mice and has the knowledge for such an analysis. By combining their knowledge and the light sheet imaging, they produce new data which are without doubt of great importance for the whole community working on gonad development and more generally developmental biology. Thus, I believe that this article is valuable both scientifically and technically and fits with the interests of the readership of Development. I would like, however, to make a series of recommendations to improve the impact of the study and the clarity of the manuscript.

Major recommendations:
Some data have been generated using the Sf1-eGFP transgene (Stalling et al, 2002). While this transgene is a good marker for the gonads, this transgene is a poor marker for the early developing adrenal glands. The the Sf1-eGFP transgene is weakly expressed in the adrenals at E11.5 as previously shown by (Pitetti et al, PLoS Genet, 2013, Figure 6Q) while SF1 (native protein) is strongly expressed in the adrenals from E11.5 as previously shown with SF1 immunostainings (Zubair et al., Mol Endocrinol., 2009).
Accordingly, in figures 2C-S3, the adrenals identified using Sf1-eGFP transgene and coloured in magenta appear as a small structure detached from the gonads at E11.5. The size appears to be underestimated and the adrenals are still connected to the gonads at this stage. The authors should use another way to detect the adrenals at E11.5. They should perform SF1 (or other suitable adrenal markers) immunolocalization analysis to reliably identify the whole adrenals.
This will clarify the position and the size of the adrenals in Figure 1F and in Figures 2 C and S3 (E11.5). Moreover, this will clearly define the limits of the Sf1-eGFP transgene. These data should be included in this work.

Response 1. We thank the reviewer for highlighting this shortcoming of the SF1:eGFP reporter. We performed a comparison between SF1:eGFP and endogenous NR5A1 protein using whole mount confocal imaging, which revealed low SF1:eGFP signal strength in the adrenal at E11.5, in accord with the reviewer's concerns. We've included our comparison in the manuscript as Figure S4 and addressed the issue in the third paragraph to results section 2 (lines 124-137).
While the reporter likely underestimates adrenal size at E11.5, the whole mount staining confirms the accuracy of the position, dorsomedial to the anterior pole of the gonad. We believe it likely that the reason we see SF1:eGFP reporter expression in the adrenals of our E11.5 embryos is that they are

slightly later into urogenital development than the average embryo found at that stage (See Response 9).

Line 118 Accordingly, without these new data, the conclusion "The adrenal primordium remained in the same relative position where it formed up to E13.5" is too preliminary.
There are questions about SF1 expression as well. It was previously shown that SF1 is not expressed in the cells of the coelomic epithelium at 20-21ts when these cells actively proliferate in the XY gonads (Schmahl et al., Development, 2000). However, it is not clear whether the transgene Sf1-eGFP is expressed in the coelomic epithelium. The authors should provide a comparison of the expression of the Sf1-eGFP transgene and the SF1 native protein in the coelomic epithelium. This might explain some differences between the GATA4 and Sf1-eGFP expression pattern.

Response 2. To address this issue, we extended our comparison of the SF1:eGFP reporter and endogenous NR5A1 (Response 1, Figure S4) to include an E10.5 sample as well as planes that capture the coelomic epithelium at E10.5, E11.5, and E12.5. These data show that the reporter can have slightly lower expression in the surface of the gonad compared to the center, and that some of the surface signal is sporadic, but these discrepancies would not impact our construction of isosurfaces, which are not resolved to the single cell level. Also, our ability to pick up the adrenal in the digital sectioning data (Figure 6A, E12.5), in which we see lower signal than in the coelomic epithelium, indicates that our analyses reliably captured the coelomic epithelium.

It is not clear to me why the gonads are not shown in the 3 D reconstruction analysis of the urogenital system of the Cbx2 mutant embryos despite GATA4 immunostainings have been performed. To show the contribution of this method to analyze a phenotype, the visualization of the major organs of the urogenital system including the gonads seems essential.

Response 3. We have included segmentations of the gonads in the presentation of the Cbx2 mutant embryos (Figure 8). See Response 16 for further details.

Other recommendations

Line 39. Wartenburg et al., is quoted in the manuscript and becomes Wartenberg, H. in the references. This is a reference about testis development in rabbits. Maybe the authors can add a reference about gonad development in mice.

Response 4. We have changed this reference to an appropriate mouse reference (Brambell, 1927a)

Line 44. The references include the development of mesonephric tubules and gonads in different species and this should be explicitly indicated. We cannot exclude that the morphogenic processes involved in the development of the urogenital ridge vary according to the species.

Response 5. We have made sure that the text indicates that these studies were carried out in multiple species. We agree with the reviewer that different species may present different morphogenic processes in urogenital ridge development and find this point interesting as the different species where this has been looked at vary in the functional development of the mesonephric tubules. This matter is of particular interest to us, as whole embryo imaging could be useful in capturing these differences, but we believe that a historical account of this line of investigation is outside the scope of this manuscript.

Line 75. The following references are quoted for the expression profile of PAX8 (Hu et al., 2013; Plachov et al. 1990; Stalling et al., 2002; Viger et al., 1998;). There is an extra semicolon, or a reference has been omitted.

***Response 6**. This issue has been corrected.*

Kulibin and Malolina ((2020), Dev Dyn. Doi: 10.1002/dvdy.242) have also studied the fate of the nephric tubules using PAX8 (another antibody that does not seem to be as good as the one you used). It would be appropriate to mention this study either in introduction or in discussion.

Response 7. We thank the reviewer for pointing out this study and have added it to the discussion.

Figure 1F- Figure 2 C; When the gonad develops, the adreno-gonadal primordium splits to give the adrenal and gonadal primordia. Then the adrenals remain one of the closest neighboring organs of the gonads. In Figure 1F, the organs are false colored easing their identification, but the adrenals are not shown. Given their common origin with the gonad, the adrenal glands should be identified in the tissue context of the developing gonad.

Response 8. The single sagittal sections shown in Figure 2C (Previously Figure 1F) do not capture the adrenal, which lies medial to the gonad. The adrenal can be seen in transverse sections at E11.5 and E12.5. We have made sure that the text indicates the position of the adrenal and points to Supplemental Figure 2, where the adrenal is identified in transverse sections. We added higher magnifications of the transverse sections to make this easier to see.

Figure 1D, E; Figure 2B and following. Staging of embryos. Most of developmental biologists can relate somites and developmental stages. However, in the field of gonad development, this is the tail somites that are commonly used to determine precisely when the main events of sex determination occur around E11.5. Could the authors add the numbers of tail somites for the data corresponding to E11.5 to ease the comparisons with data published previously.

Response 9. We agree with the reviewer on the value of tail somites in the gonad development field. To establish this correlation, we compared total somites and tail somites from E10.5 to E12.5 in our main time course. These data can now be found as Supplemental Figure 1C, where we've added an axis for relating our samples and the typical reference correlation as determined in Hacker et al., 1995. As we find that our E10.5 and E11.5 samples have more tail somites than the typical number associated with those stages established by Hacker, this data is valuable to interpreting our results. We mention the significance of this in the text (Results section 1, paragraph 2, lines 80-86).

Figure 2A-B. This is a clever way to show the position of the gonad in the embryo. This highlights differences between XX et XY embryos, but is it meaningful or just due to the variability between different embryos? What is the precise stage of development of the embryos (in tail somites) and the number of XX and XY embryos and that were used in these experiments? The n on the right-hand side of the figure 2B could be the number of analysed embryos. This should be clarified in the legend of the figure. When there are two embryos, it is not clear what has been represented on the graph (the position in one embryo, an average, other). Could the authors comments whether they have observed a difference in the position of the gonads between XX and XY embryos at a precise stage.

Response 10. We have edited the text to clarify how these data were derived (lines 111-115). 'n' refers to the number of embryos averaged in calculating the anterior and posterior points used in construction of the diagram. Though the figure distinguishes between XX and XY samples, we have not identified any distinct sex differences. As the data include few samples for each sex and stage, and the samples at each stage are not matched for somite number, we are hesitant to make any sex specific claims. The major trends seem to be correlated with somite number. This can be seen in the full diagram of all samples in the time course, which has been added as Supplemental Figure 3 (Response 1 to Reviewer 2). The tail somite counts for the samples from E10.5 to E12.5 can be seen in the new Supplemental Figure 1C.

Figure 2C-S3. The adrenals appear smaller in XY embryos in comparison with XX embryos at E11.5. Is there a difference due to the sex or is it due to the limits of the Sf1-eGFP transgene? If they are sex differences, it would be relevant to show XX and XY data together with some of the views of both sexes in the main figures and the rest in the supplementary figures.
These potential differences depending on the sex of embryos would be highly relevant in a manuscript on gonad development, but do they really exist? The authors should clarify this point.

Response 11. The difference in adrenal size between XY and XX samples is real, but we believe it is most likely due to a stage difference. As indicated by tail somites, the XX embryos may be 4 to 8 hours further developed in the window of time when SF1:eGFP is increasing in adrenal expression. We have added this possible explanation to the main text.

Line 139. For proliferation of the CE, Schmahl et al (2000) should be quoted.

Response 12. While Schmahl et al., 2000 looked at proliferation in the coelomic epithelium, their focus was the sex difference and they did not identify any AP patterns. The mention of CE proliferation in this context is about the anterior-to-posterior pattern of proliferation identified by Brambell, 1927.

Schmahl et al have shown that the cells of the coelomic epithelium are SF1-negative cells at 20-21ts. Could this explain part of the difference observed between the GATA4 and Sf1-eGFP stainings? This should be clarified in the text. When the authors address whether the Sf1-eGFP is expressed in the cells of the coelomic epithelium, they should combine GATA4 immunostainings when possible. A combined analysis would show the degree of overlap of these two proteins preventing further questions.

Response 13. We included GATA4 staining in our comparison between SF1:eGFP and NR5A1 protein, which is presented in the new Supplemental Figure 4 (See Response 1 and 2). These data indicate that discrepancies between the reporter and NR5A1 do not account for the difference between SF1:eGFP and GATA4.

Figure 3F. The F is missing

***Response 14**. This issue has been corrected.*

In Figure 4G, are these embryos, XX or XY embryos? For all the figures, it would be very informative to know whether the embryos are XX and XY embryos. This should be added in this figure but included on all figures as well when it was forgotten.

Response 15. We agree with the reviewer that this is important information and have made sure to include it in the figure as well as the figure legend.

Although the position and elaboration of the reproductive ducts was more similar between wild type and Cbx2-/- embryos at E13.5, the mesonephric tubules appear further apart from the kidneys in the Cbx2-/- embryos in comparison to wildtypes. This suggests either a delay/defects in the descent of the testis (if the embryos are XY) or a delay in the development of the ovaries (XX) or a delayed duct development independently of the sex in the mutant embryos. However, this remains speculations. To show the position of these different structures, the authors should perform a 3D reconstruction of the gonads in addition to the ducts and kidneys these mutant embryos. The GATA4 immunostaining performed in Figure S6 could be used to do this reconstruction as presented in the wildtypes in Figure 3A. The whole will show the position of each structure and evaluate their anomalies in XX and XY mutant embryos.

Response 16. As per the reviewer's recommendation, we have added gonad isosurfaces to Figure 8B (previously Figure 4G). These were made by manually drawing around the edge of the GATA4 positive tissue in these samples, as indicated in the Methods. We agree that these structures help in the interpretation of the nephric duct phenotype. However, we do not intend to make conclusions or hypotheses that involve the kidney or sex differences, as these would be outside the scope of this work. To avoid potential confounding issues due to sex reversal, we have chosen to focus on XX embryos for our assessment of the mesonephric phenotype. To avoid confusion over the scope of our conclusions, we have edited this section to clarify our focus on structures of the mesonephros.

Lines 304-310. A connection exists between morphogenesis and testis differentiation in the mouse gonad. Different groups have shown that Sry and Sox9 and in turn Sertoli cell differentiation begins within the central part of the XY gonads and extend to the poles. When there is a delay of Sry expression, there is sex reversal at the poles of XY gonads. Do you mean that the more pronounced central changes in the gonads are a consequence of this central expression of Sry/Sox9?

Response 17. We have edited this section to clarify our conclusions and ideas (lines 346-356). The reported center-to-pole differentiation of Sertoli cells, which has yet to be mechanistically explained, was one of our primary reasons for analyzing the gonad along its AP axis and comparing the regionality between the mesonephros with the gonad. The results of our analyses (Figure 6 and Figure 7) indicate that morphological center-versus-pole differences exist independent of sex at

these early stages of gonad development. Rather than being a consequence of central expression of *Sry/Sox9*, we are interested in whether the morphological patterns are among the causes, but a thorough investigation of this is beyond the scope of this manuscript.

Line 361
PtWH: typo between capital and normal letters

Response 18. This issue has been corrected.

Line 386
imaris: a capital letter is missing

Response 19. This issue has been corrected.

In references
Biason-Lauber at al…I did not find this reference in the manuscript

Response 20. This issue has been corrected.

Reviewer 2 Comments for the Author:
1. *The numbers used for the analysis in Figure 2 are quite low (n=2 max). How consistent or how variable are these relationships to the somites? And is there a difference between right and left? It would be good to expand on this.*

Response 1. Due to the low numbers factored into these results, we are hesitant to make any claims about general variability or left-right differences. To assist the reader in generating hypotheses about these features, we assembled all of the individual alignments for the left and right side in a single graph in a new supplemental figure (Figure S3) and acknowledged these possibilities in the text. In the expanded figure, the samples are arranged by total somites, making it easier to appreciate the general trends over the time course. Though we did not see any left-right differences consistently across stages, given the low number of samples, we could not rule out this possibility. As such, we focused on just a single side (right) when averaging data for presentation in the main figure (Figure 3B).

2. *In line 115 it is stated "the urogenital complex moved away from the dorsal body wall" - however this is not obvious in Figure 2C, first column. It would help to indicate in the Figure what is meant or re-word the statement.*

Response 2a. We have reworded the section (lines 141-149) to more precisely explain the results and added measurements to Figure 4 (previously Figure 2C) and Figure S5 that indicate the measurement we are highlighting and the distance in each sample (denoted in the figures by an asterisk).

Similarly, the relative movement of the urogenital complexes from each other (line 116) is only obvious between E11.5 and E12.5, but not after this.

Response 2b. To support this claim, we have added measurements to Figure 4 (previously Figure 2C) and Figure S5 for the distance between the center of the left and right gonad (denoted in the figures by a double asterisk).

3. *Line 118: isn't the movement of the developing kidney posterior to anterior (or caudal to cranial) and not dorsoventral?!*

Response 3. The kidney moves dorsally and anteriorly. We have removed the unfamiliar word "dorsoanterior" and further adjusted the language in this section to make it easier to interpret.

4. *It is unclear why the thickness of the gonads at E10.5 and E11.5 is "consistent with an anterior to posterior pattern of development (Figure 3E) as stated in lines 161 to 163. Overall, the gonads do not appear much thicker at the anterior pole compared to the posterior pole. Similarly, the*

statement in lines 169-171 ("overall, these data confirmed the presence…") is difficult to see in the data presented.

Response 4. The previously reported anterior-to-posterior thickening of the gonad initiates in the coelomic epithelium when it is a single layer of cells. It is unknown whether this or other processes reported to occur with a similar pattern lead to any morphological AP patterns at later stages of gonad development. As even a slight AP trend at later stages may be a consequence of the initial pattern of development, we believe it is valuable to highlight these trends in the data. We have reworded the section to connect the claims to specific subsets of the samples to better capture our assessment and interpretation of the data.

5. *Lines 181-183: Looking at the graph in Figure 3G, it appears that the distance in many cases was bigger at the anterior and then again at the posterior pole, and not just that "the central region was farther apart than the poles"? The data should be described as it isshown.*

Response 5. Part of the challenge of explaining these data are that in many cases the points corresponding to the ends of the gonads do not have the same meaning as the other points due to the roundedness of the ends of the gonad. In an attempt to reduce confusion, we have removed the points where X=0 from all of the graphs, which we had already indicated as uninformative in the text. It is not so simple to deal with this problem at the posterior pole, so we have adjusted the wording in this section to be more specific about what we are referring to.
Another challenge is that the shortening of the gonad over this time frame means that the words typically used to refer to the parts of the gonad (anterior/posterior poles and center) do not correspond to empirical distances, which is what we have analyzed. We believe that assigning a certain fraction of the gonad a regional term and averaging the points in that region would be useful for making generalizations about gonads of the same length but would be inappropriate for comparing between stages because we do not yet know the mechanism of gonad shortening. We do not know if, for instance, the posterior third of the gonad remains the same group of cells over the time course. This is why we have chosen to display the data in this way, even though it risks confusion and is more challenging to describe. We have reworded the section to be more explicit about the data points under consideration and the way we arrived at our conclusions.

6. *CBX2 is expressed in the mesonephros (see e.g. Figure 1 in Katoh-Fukui et al., Endocrinology 2012), hence it probably is not the best mouse model to suggest a mechanistic relationship between the nephric ducts and the genital ridges.*

Response 6. We have added information about the *Cbx2* expression pattern to the text (lines 303-308). We have also added an explanation of our reasoning for choosing the *Cbx2* mutant for this analysis. For us, the mesonephric expression of *Cbx2* opens the possibility that a mesonephric effect of the mutation underlies aspects of the gonadal phenotype. Without an available method to conditionally knock out *Cbx2* in one tissue or the other, and prior to expending the resources to develop a method, it is valuable to assess whether similar morphological phenotypes arise in both tissues, and whether they occur simultaneously or sequentially.

7. *The conclusion that "the genital ridge undergoes considerable AP movement" (line 206) can cause confusion. It is the position relative to somites and/or hindlimbs and does not mean that the genital ridge "moves". This (and similar statements) should be re-worded (e.g. to "the genital ridge undergoes relative AP movements", or similar).*

Response 7. We have adjusted the wording on this and similar statements to indicate the specific structures that movements are relative to.

8. *Throughout the text, the authors should use "gonadal sex determination" instead of just "sex determination" when talking about the decision of the genital ridge to develop into a testis or ovary.*

Response 8. We agree with the reviewer on the importance of this distinction and have edited the text to make it clear in all cases that we are referring to gonadal sex determination.

9. *The official gene and protein names should be used throughout the manuscript (e.g. NR5A1*

instead of SF1, which officially stands for splicing factor 1).

Response 9. We have edited the text to use the NR5A1 designation when referring to the endogenous gene and protein as well as the official name of the transgene, Tg(*Nr5a1-GFP*). However, we have decide to keep the name of the expressed reporter as SF1:eGFP for several reasons: we find SF1 to be a more commonly recognized term in the field of gonad development, it emphasizes that the reporter is different from NR5A1 (which we now demonstrate in Figure S4), and it allows figure labels to be more informative than simply reading "GFP" without implicating an endogenous gene. We have edited the introduction of the reporter to include the official designation and make it clear that we are using its common name.

Reviewer 3 Advance Summary and Potential Significance to Field: This work utilizes the techniques of whole-embryo clearing and light sheet microscopic imaging to conduct a detailed characterization of early gonad development in the mouse. Key findings include a better description of the relationship between the developing gonads and surrounding tissue, with the suggestion of a casual link between their morphogenetic programs, as well as a better understanding of the previously described A/P and center/pole differences in gonad development. They also describe defects in gonad morphogenesis in the Cbx2 mutant, where correlation bw the regionalized defects in the gonad and nephrite ducts implies some casual relationship between development of the two tissues.

Reviewer 3 Comments for the Author:
As is typical of the Capel lab, the work is beautifully done and carefully presented. These are challenging experiments and the results represent important information for the field. One major question is whether the work can be seen as making enough of a "mechanistic advance" to be deemed appropriate for Development (which emphasizes mechanism). Sometimes a descriptive paper is more important than any other type of work and can become a classic, often- referenced paper. I suspect that work is being targeted for the Resource/Techniques area of the journal, which is most appropriate for the work here. Also, the manuscript is quite "tight" with 4 figures and a short discussion, raising the question of whether it is more appropriate as a Report vs. and Article. Given that the paper presents a great deal of important and difficult-to-obtain data for the field, I would support publication as a "Resource" in either an Article or Report form.

Response 1. We agree with the reviewer on the value of this work as a Resource/Technique article and have adjusted the text to emphasize this function.

Second decision letter

MS ID#: DEVELOP/2020/199208

MS TITLE: Concerted morphogenesis of genital ridges and nephric ducts in the mouse captured through whole embryo imaging

AUTHORS: Corey Bunce, Jennifer McKey, and Blanche Capel
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. (and see Editor's Note).

Editor's Note:
The response to Reviewer 2 and the revision of text are adequate.
re response 7: Please consider replacing "movement" with "displacement" to describe the change in the position of the structure. This change could be made during proof-reading.

Reviewer 1

Advance summary and potential significance to field

As stated in my previous report, this article is very important in developmental biology for the two following reasons : it contains a wealth of data, which may serve as a resource to investigations on urogenital morphogenesis and it will be very useful for teaching in developmental biology. The additional comments and results strengthen the conclusions of the manuscript. I believe it is now suitable for publication in Development.

Comments for the author

The authors have successfully addressed my previous concerns. I have no further criticisms.

Reviewer 3

Advance summary and potential significance to field

see previous summary

Comments for the author

The authors have adequately addressed reviewer comments