nature portfolio

Peer Review File

A single-nucleotide enhancer mutation overrides chromosomal sex to drive XX male development

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Testis development is initiated by Sry, which activates Sox9 expression in mice. Loss of Sox9 leads to XY sex reversal, whereas its ectopic activation in XX gonads induces testis formation. The main enhancer of Sox9 is Enh13. Deletion of Enh13 in XY gonads results in a sex reversal phenotype similar to that observed with Sox9 loss. In this study, the authors demonstrate that small mutations (-3bp/+1bp) in Enh13 leads to robust Sox9 expression in XX gonads, resulting in testis development. These mutations could disrupt the binding of key transcription factors including RUNX1, NR5A1 and GATA4. Whereas the mutations described here lead to an abnormal activation of Sox9 in XX ovaries, they do not support a repressive role of non-mutated Enh13. The results can be interpreted as follows: in XX gonads, Enh13 is able to drive low levels of Sox9. This is supported by the transgenic analysis of non-mutated Enh13 in XX gonads, where Enh13-LacZ was able to drive LacZ expression (Gonen, 2018). This low level of expression is not sufficient to allow testis development. In contrast, the mutation described in this article leads to strong Sox9 expression, sufficient to induce Sertoli cell differentiation. Showing Sox9 expression at earlier stages in XX mutant gonads would support that abnormal expression of Sox9 triggers the sex reversal phenotype. Overall, this discovery is both intriguing and clinically relevant, particularly for understanding 46,XX DSD in human patients.

Several points warrant further clarification.

Comments on the presentation

Nature Communications' guidelines emphasize the importance of accessibility in figure design, particularly regarding color usage. The journal advises avoiding the use of red and green together for contrast, as this combination is not accessible to individuals with color vision deficiencies. Instead, they recommend using high-contrast, color-safe combinations such as green and magenta or other accessible palettes. Additionally, the journal adheres to Web Content Accessibility Guidelines (WCAG 2.1) level AA, ensuring that all readers, regardless of visual abilities, can interpret the figures effectively.

Furthermore, the figures depicting embryonic gonads would be significantly improved by focusing more closely on the gonadal tissue itself and increasing its relative size within the panels.

In the figures, the statistical significance annotations should explicitly indicate which specific samples or groups are being compared to avoid ambiguity.

The presentation of the results would benefit from clearer descriptions to make the work more accessible. Some terms and comparisons come across as subjective; providing more explicit definitions and explanations throughout would help ensure that the significance of the findings is more broadly appreciated. Some examples are given below.

Introduction

First, genetic sex is defined at fertilization by the provision of an X or a Y chromosome to an X-bearing oocyte. Authors could clarify that the X or Y chromosome is provided by the sperm.

Once differentiated, the gonads produce sex hormones necessary for the development of internal and external genitalia,

secondary sexual characteristics, and then control puberty and fertility of the individual. The reference has been lost.

Here we show that XX mice carrying a 3 bp deletion or a 1 bp insertion within the SOX9 binding site (BS) of Enh13 present complete female-to-male sex reversal. The term “complete” may be misleading, since XX mutants exhibit ovotestis development (see Figure 2) and lack germ cells in adulthood.

Adult XX mice appear externally and internally as males, but with small testes devoid of sperm due to the lack of the Y chromosomes. To illustrate the absence of germ cells in the XX testis, Paul Burgoyne's work on this topic could be cited rather than that of others. See Development (1987) 101 (Supplement): 133–142.

Results

Page 4 :Small mutations in the SOX9 BS of Enh13 lead to XX male development in adulthood

XX mice homozygous for the 3 bp deletion were born fully sex reversed and appeared externally as males. The term 'fully sex reversed' may be overly strong, as 'sex reversed' already conveys a significant change. Consider revising the sentence to simply state: XX mice homozygous for the 3 bp deletion were born sex reversed highlighted by male external genitalia.

Adult mice appeared with small testes void of gonocytes, a phenotype known to occur in XX males as they are not able to support spermatogenesis in the absence of Y chromosome genes. I believe the intended word is "devoid" instead of "void". See my comment above for the reference.

In the extended figure 2, there are inconsistencies in the capitalization of annotations (e.g., "Weeks" vs. "weeks"). To improve clarity and maintain uniformity, I recommend standardizing all annotations in the figures.

XX WT and heterozygous gonads exhibited no expression of Sox9 and Sox8. No expression is overstated. It has been shown by other groups than the cells of the developing rete ovarii express Sox9, which are difficult to dissect out.

Figure 1: Are the Mullerian ducts derivatives present in XX mutant mice at 6 weeks ?

Page 5: The conclusion should be The 3 bp deletion alone allows Sox9 expression to rise above the critical level required to trigger testicular differentiation in XX gonads.
There is no evidence for a repressive threshold.

Altogether, these findings underscore the extraordinary sensitivity of the Sox9 regulatory network to subtle enhancer perturbations, capable of breaching the activation threshold required to initiate testicular development in an XX context. This sentence should be revised to focus more directly on the empirical findings.

Embryonic gonads develop as ovotestis upon small mutations in the SOX9 BS of Enh13

The statement "embryos presented variable phenotypes of ovotestis ranging in severity" requires clarification. The term "severity" needs to be defined.

Normally, at E13.5, ovaries are devoid of clear structural characteristics while the testes contain a coelomic vessel along with testis cords. The use of "Normally" seems unnecessary here. Could the authors clarify what is meant by "structural characteristics"? What exactly is being compared between ovaries and testes at E13.5? Is the absence of a coelomic vessel in ovaries being contrasted with the presence of a coelomic vessel and testis cords in the testes?

half a testis-half an ovary, please clarify how this is defined.

Is the ovarian region systematically in the anterior or posterior part of the gonad? What is the orientation of the gonads in Fig 2A-B, Extended Data Figure 1B, Extended Data Figure 4?

Differences in severity appearance were also evident between the right and left gonads of the same embryo. This must be explained.

or the more common view of ovotestis. Unnecessary.

FOXL2 and SOX9 positive cells were mutually exclusive (Fig. 2B) : this is difficult to appreciate in Figure 2B, may be the authors could show a zoom of the gonad showing the exclusive expression of FOXL2 and SOX9.

they are identical to XX WT or XY WT, respectively. Identical? Similar is maybe better.

XX homozygous mutant gonads from the Enh13SOX9*+1bp-/- (Fig. 2D) and Enh13SOX9*-3bp SRY*-12bp-/- (Extended Data Fig. 4G) strains also presented an ovotestis phenotype, with a more testis-like appearance compared to the Enh13SOX9*-3bp-/- strain. I understand how the +1bp sex reversal could be more complete owing to the presence of an additional potential GATA4 BS, could the authors comment on the reason for a stronger phenotype in the Enh13SOX9*-3bp SRY*-12bp-/- gonads compared to the Enh13SOX9*-3bp-/-.

How is the ovotestis phenotype observed at E13.5 resolved into a testis phenotype at 6 weeks? The authors should provide data on an intermediate stage.

Transcriptomic analysis of E12.5 embryonic gonads demonstrates an ovotestis pattern

Pre-granulosa markers as Foxl2, Fst, Bmp2, Wnt4, Runx1, Emx2 and Lhx9 were highly expressed in XX WT and XX heterozygous gonads, but displayed a low-to-intermediate expression in the XX homozygous mutant gonads of the 3 mouse strains (Fig. 3C and Extended Data Fig. 6B). While Foxl2 and Fst are good markers of pre-granulosa cells, the authors should note that Runx1, Bmp2, Rspo1 and Wnt4 mark pre-supporting cells before their differentiation into pre-granulosa cells. Moreover, Lhx9 and Emx2 are expressed in the CE, pre-supporting cells and stromal/interstitial progenitors.

In contrast, XX homozygous gonads from all three mutant strains exhibit a progressive shift from an ovarian transcriptional identity toward a testicular transcriptional identity. The statement should be modulated. The transcriptome at one single developmental stage cannot show a “progressive shift from an ovarian transcriptional identity toward a testicular transcriptional identity” but rather a mixed ovarian and testicular transcriptomic signature at E12.5.

The authors should address the expression of Sox9/SOX9 by qPCR and/or IF at an earlier stage, to determine the initial time when Sox9 is abnormally upregulated in XX Enh13 mutant gonads.

The SOX9 BS mutations do not abolish the ability of SOX9 to bind and activate Enh13

Electro mobility shift assays (EMSA) with in vitro–translated SOX9 on synthetic Enh13 sequences (WT, –3bp, +1bp). A negative test with an irrelevant sequence to Enh13 would reinforce the results.

Fig 7C. Can the authors show the Luciferase assay results when individual TF are transfected? This is highly relevant as they conclude from Figure 4 that cooperative binding of TF is altered in Enh13 mutants.

The SOX9 BS mutations affect the cooperativity of TFs on Enh13 and enhance its activity

JASPAR analysis of ~2300 vertebrate TFs, including all those expressed in the gonad at this stage indicated no major gain or loss of binding sites was found between WT and mutant Enh13 sequences to explain the phenotype (Extended data Figure 9). The authors show in Figure 4 the creation of a de novo potential GATA motif in the +1bp mutant. Can they comment on this?

In this part, the experiments used materials from human, mouse, and monkey. Are all these reagents equivalent? Please clarify in the Materials and Methods section that they originate from different species.

Together, these results indicate that the mutations not only boost enhancer responsiveness but also render it less susceptible to RUNX1/NR5A1/GATA4-mediated repression, thereby facilitating Sox9 activation. The results shown in Fig. 4B and in Extended Data Fig. 10 show boosted enhancer responsiveness to NR5A1/GATA4 of both enhancers in COS7 cells but only of the +1bp enhancer in HEK293T cells. Can the authors comment on this?

To better understand how the mutations might affect cooperative binding of the different transcription factors the authors should provide the results of luciferase assays with individual transfections of NR5A1 and GATA4 and SOX9 as well as the results of luciferase assays with the double transfections of NR5A1/RUNX1, GATA 4/RUNX1 and SOX9/RUNX1. The authors state that the distinct responses of the two mutants to NR5A1 and GATA4 alone suggest that they may act through different molecular mechanisms to alter enhancer function but the corresponding luciferase assays of single transfections of NR5A1 and GATA4 are not shown.

We also show that the -3bp/+1bp mutations sufficiently modify the 3D structure of Enh13, and hence cooperative binding of TFs. I am not certain that the luciferase assays show this. These experiments show that Enh13 mutations affect the transactivation output of the combined transcription factors, whether this occurs by alteration of the 3D structure and of the TF cooperative binding, or rather by alteration of the 3D structure and the transactivation (positive or negative) abilities of the TF/transcriptional machinery complex is unknown.

Overall, we demonstrate that Enh13 is used for both the activation and repression of Sox9 expression in the testis and ovary, respectively. The role of Enh 13 is to activate Sox9 in XY gonads. However, the mutations described (-3 bp or +1 bp) appear to cause ectopic up-regulation of Sox9 in XX gonads, rather than reflecting a repressive function of Enh13 in XX gonads. These mutations in XX gonads represent an abnormal activation, and it may be more accurate to describe this as such rather than as Enh13 acting as a repressor in the ovary.

Discussion

The two mutations described here show that Enh13 has repressive activity in the presence of bipotential factor in the normal ovary, and the subtle alterations in its sequence can overcome this repressive state. In all experiments the transfection of bipotential factors NR5A1 and GATA4 always results in an increase of luciferase activity, both in the context of WT and mutated Enh13. Therefore, I do not agree with the conclusion that Enh13 has repressive activity. Rather Enh13 would have a weak activating activity in the presence of bipotential factors that is further dampened by RUNX1 (RUNX1 being only maintained in XX supporting cells). This activity would not be sufficient to produce the threshold level of SOX9 protein

required for the self-amplification loop in the XX WT context. In the XX Enh13 mutant contexts the presence of an additional GATA site and/or the disruption of the enhancer structure would result in an increase of the transactivation activity of the enhancer even in the presence of RUNX1, allowing for sufficient SOX9 production and initiation of self-amplification loop.

This conclusion based on activation of Sox9 expression above a given threshold is the one that the authors themselves state in the introduction and in the results:

Introduction p3: Our enhancer reporter assays suggest that the mutations we induced do not abolish the SOX9 BS but rather enable the bipotential factors NR5A1, RUNX1, and GATA4 to acquire sufficient activating capacity to induce Sox9 expression above the minimal threshold present in female gonad.

Results p5 : Altogether, these findings underscore the extraordinary sensitivity of the Sox9 regulatory network to subtle enhancer perturbations, capable of breaching the activation threshold required to initiate testicular development in an XX context.

Other comments: Yet, KO of individual profemale factors including WT1 (-KTS) 18, FOXL2 24-26, RUNX1 27 and components of the WNT pathway 19-23 do not lead to XX male phenotype in mice. It is only when two pro-female factors or more are deleted together, that XX male phenotype start to appear at embryonic stages 27-30. I'm not sure that I understand what the authors mean. Could they elaborate?

Reviewer #2

(Remarks to the Author)
This manuscript is an exciting contribution to the sex determination literature showing that female to male sex reversal can be caused by deletions in a critical enhancer of Sox9. Enh13 has previously been shown to be a principal regulator of Sox9 expression in XY gonadal supporting cells. Several of these authors previously showed that mutations in enh13 disrupt male sex determination and testis development. Surprisingly, they now show that addition of 1 base pair (bp) or deletion of 3 bp in this enhancer show no effect on male sex determination or SOX9 expression in XY gonads but lead to elevated Sox9 expression in XX gonads and female to male sex reversal. This manuscript speaks to the amazing plasticity of the sex determination system at the molecular level and provides an interesting example of cell fate switching hinging on minor changes in an enhancer that may alter the relationship of transcription factors known to bind there. The authors create many new mouse strains to explore this phenotype and each is well characterized. They then turn to a molecular analysis of what could be going on at the locus. Using reporter assays in HEK293 cells and EMSA assays, they demonstrate that SOX9 binding in not inhibited by the indels, but cooperative binding of NR5A1, GATA4, RUNX1 and SOX9 may be affected by changes in spacing. Of course, there is always more to learn but I find this a relatively complete story and have only a few recommendations.

In the abstract the authors say, “Mechanistically, these mutations do not eliminate SOX9 binding”. I think a prior sentence to explain that SOX9 binds it’s own promoter would be helpful.

Later in the manuscript the authors say “These findings rule out
increased SOX9 binding or transcriptional activation as the underlying mechanism.” I found this section confusing and felt this conclusion was misleading. After all, the argument is that Sox9 expression is increased by the absence of repression. Have the authors tested whether RUNX1 can repress Sox9 in the presence of low levels of SOX9? Is there a binding competition between these two factors? An assay in HEK cells increasing the dose of SOX9 in the presence of RUNX1 would be ideal.
I would like to see the wild type ATAC landscape around Enh13 in one of the figures. Would ATAC-seq be a helpful approach in mutants to query how binding is changed?
I find the annotation confusing. Could the mutants be represented as alleles using the usual annotation? Sox9-Enh13$^{Sox9*\text{-}3bp/Sox9*\text{-}3bp}$ (denoting a homozygote) ; compared to Sox9-Enh13$^{Sox9+1bp/+}$ (denoting a het).
Please indicate the A/P axis of gonads in Fig.2. Is the ovarian region always anterior?
Fig. 3B – what is the red sample in the XY section of the sample designation line?

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The manuscript by Elisheva Abberbock and colleagues clearly demonstrates that engineered mutations in a key testicular enhancer of the Sox9 gene (named Enhancer13, or Enh13) lead to an absence of Sox9 repression in XX developing

ovaries. This is followed by testis differentiation without SRY and XX sex reversal in adulthood. The authors elegantly and convincingly demonstrate that Enh13, which was previously described as the key regulatory region for Sox9 upregulation in XY pre-supporting cells, also plays a pivotal role in Sox9 repression in XX ovarian pre-supporting cells. This work confirms and provides molecular explanations for XX sex reversal observed in other species, where abnormal SOX9 gene upregulation has been shown to play a primary role in some human cases and in pig intersexuality.
The major conclusions of the manuscript are stated in the title and throughout the Results section. All of the authors' assertions are supported by clear, statistically validated results from morphological observations of the external and internal genital tracts (including gonads), IHC, gonad transcriptomes by bulk RNA-seq from 45 mice with different genotypes, gene expression levels by qPCR, PBM, EMSA, transfection assays caried onto two different cell types (HEK293T and COS7) and bioinformatic analyses. The main result is impressive: the authors clearly demonstrate that adding one nucleotide or suppressing three in the SOX9 binding site of Enh13 changes the sex of the individual. Additionally, they prove that the molecular mechanism involved is different in each XX sex reversal cases. In conclusion, all of the authors' claims and conclusions are justified by their large number of high-quality, rigorous results.
In addition to these general comments, I have two remarks and few minor corrections for the authors.
1 – My first remark concerns the interpretation of the results proposed by the authors and illustrated in Figure 4E, as well as the binding of RUNX1 and SOX9 to their TFBS, which are very close and overlap by one or two bases in the WT allele, and by an even greater amount in the case of the -3 bp allele. Do the authors not consider that the binding of RUNX1 (expected in XX supporting cells) and SOX9 (expected in XY cells) are mutually exclusive on these TFBS due to their proximity and the steric hindrance of both proteins? In other words, why do they not consider that there is competition between the two transcription factors for binding to this region, with RUNX1 binding resulting in repression (or an absence of activation) and SOX9 binding resulting in activation? When the authors tested the binding capacity of both factors individually using PBM or EMSA, they showed that RUNX1 bound equivalently to all three alleles (WT, +1 and -3 bp), but SOX9 had less affinity for the -3 bp allele. Nevertheless, SOX9 is the dominant factor, since sex reversal occurs. One possible explanation is that, in vivo, the chromatin conformation of this region causes competition between the two TFs for binding to this critical region, and the 3 bp deletion prevents RUNX1 binding and thus promotes SOX9 binding, despite its decreased affinity. For the +1 allele and the creation of a new GATA site in this small region, the competition is now between not only RUNX1 and SOX9, but also GATA4. I leave it up to the authors to decide whether or not to take this comment into account in the discussion of the results.
2 – My second remark concerns the deletion of the SRY BS in addition of the 3-bp deleted in the SOX9 BS (first paragraph of page 5). The authors state that “removal of the SRY BS does not affect the female to male sex reversal of XX homozygous mice”. Why do the authors expect a role of this SRY BS in an XX context where there is no SRY? Moreover, the authors state that deleting SRY BS in addition to the 3-bp aggravate the phenotype of sex reversal (last paragraph of page 6). How could the authors explain this observation since there is no SRY?
3 – Page 7, last paragraph: the authors write “Analysis of bipotential genes, Nr5a1, Wt1, Gata4…..revealed no major differences in expression between WT and HM mutants”. By contrast Nr5a1 and Wt1 are found differentially expressed in Fig6B (cluster a & f, respectively) and also on Extended data Fig6A, at least between WT males and females with slight differences for HM mutants (male type for Nr5a1, female type for Wt1).
4 – Page 8, first paragraph. The authors could add Rspo1 in the list of pre-granulosa markers and Aard in those of Sertoli markers, because expression of these two genes are presented on ExtDataFig6 (B & C, respectively).
5 - Page 10, last paragraph, second sentence: “We also show….even in the absence of male-specific factors”. The authors probably think to SRY. It will be better to write “…even in the absence of SRY”.
6 – Figure 2C: The number of studied samples for XX+/+ is n=5 (as indicated in the parenthesis), but there are 4 points for Sox9 and 6 points for Sox8 and Foxl2.
7 – Legend of Figure 2C : “in” is missing between “involved” and “male”.
8 – Figure 4A: the last base of the consensus sequence for the SOX9 binding site is missing on the Figure (should be A>G=T).
9 – Extended Data Fig.1B: “oviduct” term is present on the fourth animal (on 6 weeks gonads) and should be suppressed.
10 – Page 22; Methods; Paragraph “Design and preparation of sgRNAs mRNA”; lane 5 of this paragraph: please write “concentration” instead of “concertation”.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed my questions and have strengthened the manuscript through their revisions and the inclusion of additional results. Overall, this study provides a compelling demonstration of how subtle alterations in regulatory DNA can lead to major developmental effects, using Enh13, a regulatory element of Sox9, as an example. It delivers novel and clinically meaningful insights into the genetic mechanisms underlying disorders of sex development (DSD). While a few minor typographical and editorial corrections can still be addressed, the manuscript is suitable for publication in Nature Communications.
Few comments

The figures currently use red and green together. Nature Communications recommends high-contrast, accessible color palettes (e.g., green and magenta) for clarity and inclusivity. No changes are required if the current colors are essential, but alternatives can be used if possible.

A typo appears in Reference 4.

In contrast, XX homozygous gonads from all three mutant strains exhibit a progressive shift from an ovarian transcriptional identity toward a testicular transcriptional identity.
Although the authors state that the sentence has been amended, the revised version is unchanged from the original and continues to imply a progressive temporal shift.

The authors have now examined Sox9 expression at earlier developmental stages to determine when Sox9 first becomes abnormally upregulated in XX Enh13 mutant gonads. To allow accurate comparison between samples, it would be helpful if the authors could clarify the developmental staging used for these analyses, in particular by indicating the number of tail somites, if available.

Reviewer #2

(Remarks to the Author)
The authors have carefully responded to the reviewers' comments, performed new experiments, and improved many aspects of the manuscript.
I think it would be helpful if the model in Figure 6 showed that Sox9 is initially expressed at low levels in the XX gonad, where SOX9 is cytoplasmic, as the authors explain on line 6 and 26 (Gasco). The question becomes whether it is upregulated or downregulated as gonads develop. This is a subtle but important difference in the way we think about it.
As we know, first SRY and later SOX9 upregulate Sox9. In this manuscript, the authors convince me that RUNX1 is an important repressor of SOX9 (although I am puzzled as to why in its absence, XX Runx1-/- animals do not develop testes – presumably SOX9 levels are not high enough without up-regulation by SRY). I admire the in vitro TF competition assays and model as an explanation for why these 1 or 3 bp mutations cause sex reversal.
This manuscript moves the field in the important direction of understanding the interaction of TFs on chromatin during the process of cell fate determination and sex determination. Although there is a lot more to do, that's for another manuscript.
The authors have made significant progress in this work.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The revised version proposed by the authors is even more convincing. I fully agree with both the new model and the interpretation of the results set out in this version and I strongly recommend the publication of this Ms.
One final detail regarding the sentence on lines 8–11 of page 13: it is not surprising, in my opinion, that a gain of function of Sox9 induces a very robust XX female to male sex-reversal (Vidal et al., 2001). What is striking is that such subtle modifications of a TFBS can induce such a change in the transcriptional expression of the Sox9 gene. In my opinion, XX sex reversal through loss of function of pro-ovarian genes is a completely different story.
And a last typo on line 15 page 10: suppress one "on the".

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In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.
The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
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Response letter to reviewers

We want to sincerely thank all four reviewers for their useful, instructive, and supportive comments. Your suggestions have been extremely useful and helped us solidify the mechanism mediated by the Enh13 mutations, for which we are grateful for.
We have experimentally and textually addressed all the comments. We provide here a point-by-point reply to all the comments raised by each reviewer and indicate the edits that were done in the manuscript and figures. We have added new data and figures and now able to show that pro-female factors, and more so, RUNX1 are able to repress Enh13 activity and hence *Sox9*.

The reviewers' comments are in black, and our replies are in blue.
We also want to apologise for not adding line numbers in the initial submission which made your work harder in trying to direct us. We have now included line numbers to the revised manuscript.

Reviewer #1

Testis development is initiated by Sry, which activates Sox9 expression in mice. Loss of Sox9 leads to XY sex reversal, whereas its ectopic activation in XX gonads induces testis formation. The main enhancer of Sox9 is Enh13. Deletion of Enh13 in XY gonads results in a sex reversal phenotype similar to that observed with Sox9 loss. In this study, the authors demonstrate that small mutations (-3bp/+1bp) in Enh13 leads to robust Sox9 expression in XX gonads, resulting in testis development. These mutations could disrupt the binding of key transcription factors including RUNX1, NR5A1 and GATA4. Whereas the mutations described here lead to an abnormal activation of Sox9 in XX ovaries, they do not support a repressive role of non-mutated Enh13. The results can be interpreted as follows: in XX gonads, Enh13 is able to drive low levels of Sox9. This is supported by the transgenic analysis of non-mutated Enh13 in XX gonads, where Enh13-LacZ was able to drive LacZ expression (Gonen, 2018). This low level of expression is not sufficient to allow testis development. In contrast, the mutation described in this article leads to strong Sox9 expression, sufficient to induce Sertoli cell differentiation. Showing Sox9 expression at earlier stages in XX mutant gonads would support that abnormal expression of Sox9 triggers the sex reversal phenotype. Overall, this discovery is both intriguing and clinically relevant, particularly for understanding 46,XX DSD in human patients.

We are thankful for the nice summary of our paper and will address your concerns and suggestion below.

Several points warrant further clarification.

Comments on the presentation

Nature Communications' guidelines emphasize the importance of accessibility in figure design, particularly regarding color usage. The journal advises avoiding the use of red and green together for contrast, as this combination is not accessible to individuals with color vision deficiencies. Instead, they recommend using high-contrast, color-safe combinations such as green and magenta or other accessible palettes. Additionally, the journal adheres to Web Content Accessibility Guidelines (WCAG 2.1) level AA, ensuring that all readers, regardless of visual abilities, can interpret the figures effectively.

We thank you for this important comment. Further to your suggestion we have now modified the real time PCR graphs to be presented with Green and Magenta. The new graphs are presented in the revised Figure 1E and 2C.

Furthermore, the figures depicting embryonic gonads would be significantly improved by focusing more closely on the gonadal tissue itself and increasing its relative size within the panels.

Thank you for this point, we have tried to increase the magnification in the brightfield images however, the resolution of the binocular at high magnification is not ideal. To get a closer look at the gonadal tissue, we have added X63 confocal images of the immunostaining performed on the gonads in Figures 2B and 2D.

In the figures, the statistical significance annotations should explicitly indicate which specific samples or groups are being compared to avoid ambiguity.

This is correct. We have added to the figure legends which samples were compared to which.

The presentation of the results would benefit from clearer descriptions to make the work more accessible. Some terms and comparisons come across as subjective; providing more explicit definitions and explanations throughout would help ensure that the significance of the findings is more broadly appreciated. Some examples are given below.

Introduction

First, genetic sex is defined at fertilization by the provision of an X or a Y chromosome to an X-bearing oocyte. Authors could clarify that the X or Y chromosome is provided by the sperm.

Page 2 line 20-22
This sentence was amended and now reads as: "First, genetic sex is defined at fertilization by the provision of an X or a Y chromosome by the sperm, to an X-bearing oocyte."

Once differentiated, the gonads produce sex hormones necessary for the development of internal and external genitalia, secondary sexual characteristics, and then control puberty and fertility of the individual. The reference has been lost.

Page 2 line 26
A reference was indeed missing. We have now added a suitable reference (Ref No. 4).

Here we show that XX mice carrying a 3 bp deletion or a 1 bp insertion within the SOX9 binding site (BS) of Enh13 present complete female-to-male sex reversal.
The term “complete” may be misleading, since XX mutants exhibit ovotestis development (see Figure 2) and lack germ cells in adulthood.

Page 3 line 23-24
This is correct. We removed the word complete and now describe this sentence as: "Here we show that XX mice carrying a 3 bp deletion or a 1 bp insertion within the SOX9 binding site (BS) of Enh13 present female-to-male sex reversal."

Adult XX mice appear externally and internally as males, but with small testes devoid of sperm due to the lack of the Y chromosomes. To illustrate the absence of germ cells in the XX testis, Paul Burgoyne's work on this topic could be cited rather than that of others. See Development (1987) 101 (Supplement): 133–142.

Thank you for this suggestion. We have now added this reference (Ref No. 39) here and in the other places that describe the lack of germ cells in these XX sex reversed mice.

Results
XX mice homozygous for the 3 bp deletion were born fully sex reversed and appeared externally as males. The term 'fully sex reversed' may be overly strong, as 'sex reversed' already conveys a significant

change. Consider revising the sentence to simply state: XX mice homozygous for the 3 bp deletion were born sex reversed highlighted by male external genitalia.

We have amended this sentence as suggested and it now reads as: " XX mice homozygous for the 3 bp deletion were born sex reversed highlighted by male external genitalia and small testes (Fig. 1C)."

Adult mice appeared with small testes void of gonocytes, a phenotype known to occur in XX males as they are not able to support spermatogenesis in the absence of Y chromosome genes. I believe the intended word is "devoid" instead of "void". See my comment above for the reference.

We have replaced the 'void' to 'devoid' and added the Burgoyne reference also here (Ref No. 39).

In the extended figure 2, there are inconsistencies in the capitalization of annotations (e.g., "Weeks" vs. "weeks"). To improve clarity and maintain uniformity, I recommend standardizing all annotations in the figures.

Thank you for spotting this. It appeared in additional figures. We have amended all the figures to have 6 weeks without capital.

XX WT and heterozygous gonads exhibited no expression of Sox9 and Sox8. No expression is overstated. It has been shown by other groups than the cells of the developing rete ovarii express Sox9, which are difficult to dissect out.

This is very correct. We have amended the sentence to be: "XX WT and heterozygous gonads exhibited low expression of *Sox9* and *Sox8* while having high *Foxl2* expression (Fig. 1E). XY WT gonads had high expression of *Sox9* and *Sox8* and no detectable *Foxl2* expression."

Figure 1: Are the Mullerian ducts derivatives present in XX mutant mice at 6 weeks?

In all the XX homozygous mice we have dissected from the three mutated lines we could never see any Müllerian duct derivatives present.

The conclusion should be: The 3 bp deletion alone allows Sox9 expression to rise above the critical level required to trigger testicular differentiation in XX gonads.
There is no evidence for a repressive threshold.

You are correct that in the first draft of the paper, there was no strong enough evidence to suggest that the small mutations in Enh13 prevent repression, normally present in ovaries, on *Sox9* expression.
Due to your questions about repression abilities, as well as the suggestions of the other reviewers, we have now added new Luciferase data and figures that prove that RUNX1 has repressive abilities on Enh13 and it may compete with SOX9 on the overlapping site (New Fig. 4D-E). We show that when we add factors able to induce the enhancer and then add RUNX1, or other pro-female factors, this significantly lower enhancer activity (New Fig. 4D-E).
We hence believe that normally, Enh13 is repressed in females >>leading to block in *Sox9* expression, whereas in males it is strongly activated >> leading to high *Sox9* expression. We suggest that the two mutants prevent the ability to repress Enh13 and hence, *Sox9,* and therefore, this triggers auto-regulation, elevated *Sox9,* and testis development (new model in Fig 6).

But since this new data is only presented in Figure 4, we have amended the sentence here to be as you suggested. This appears in Page 5, Lines 20-23: " The 3 bp deletion alone allows *Sox9* expression to rise above the critical level required to trigger testicular differentiation in XX gonads and XY gonads. Moreover, the female-to-male sex reversal is solely due to the 3 bp deletion and does not involve the SRY motif, to which SOX9 can bind."

Altogether, these findings underscore the extraordinary sensitivity of the Sox9 regulatory network to subtle enhancer perturbations, capable of breaching the activation threshold required to initiate testicular development in an XX context. This sentence should be revised to focus more directly on the empirical findings.

We have amended this sentence to now read as: "Altogether, these findings underscore the extraordinary sensitivity of the *Sox9* regulatory network to subtle enhancer perturbations, showing that specific mutations are capable of inducing *Sox9* expression above a critical threshold required to initiate testicular development in an XX context."

The statement "embryos presented variable phenotypes of ovotestis ranging in severity" requires clarification. The term "severity" needs to be defined.

We have removed the term "ranging in severity". The sentence is now: "XX gonads of Enh13$^{SOX9*\text{-}3bp\text{-}/\text{-}}$ embryos presented variable phenotypes of ovotestis."

Normally, at E13.5, ovaries are devoid of clear structural characteristics while the testes contain a coelomic vessel along with testis cords. The use of "Normally" seems unnecessary here. Could the authors clarify what is meant by "structural characteristics"? What exactly is being compared between ovaries and testes at E13.5? Is the absence of a coelomic vessel in ovaries being contrasted with the presence of a coelomic vessel and testis cords in the testes?

We have rephrased these sentences to a more scientific terms. The revised sentences are in: "At E13.5, WT testis are organised into testis cords, containing Sertoli cells and germ cells, along with an interstitial place where Leydig cell reside and present with a coelomic vessel. WT ovaries on the other hand are devoid of clear structural organisation at that stage and germ cells along with somatic cells are spread across the ovary area."

half a testis-half an ovary, please clarify how this is defined.

Thank you, we have revised that sentence to "While some XX homozygous gonads appeared mostly as testes with coelomic vessel and testis cords, others had a coelomic vessel without clear testis cords and some presented with a testicular gonadal portion alongside an ovarian portion".

Is the ovarian region systematically in the anterior or posterior part of the gonad? What is the orientation of the gonads in Fig 2A-B, Extended Data Figure 1B, Extended Data Figure 4?

In some gonads we see the ovarian portion in both the anterior and posterior parts, but when it is only in one portion, it is always the anterior side. We made sure that all BF and fluorescent images the gonads are presented from A-P and added the labelling on the figures.

Differences in severity appearance were also evident between the right and left gonads of the same embryo.

We have amended this sentence accordingly: "Variability in ovotestis phenotypes were also evident between the right and left gonads of the same embryo where in one the anterior portion was ovarian and the posterior was testicular, whereas the other gonad presented an anterior and posterior ovarian portions and a centralised testicular portion (Fig. 2A, first and second mutant gonads are the right and left gonads of the same embryo)."

or the more common view of ovotestis. Unnecessary.

We have amended this sentence, and it now reads as: "XX Enh13$^{SOX9*-3bp-/-}$ gonads presented with ovotestis with an anterior ovarian portion alongside a testicular posterior portion, or an ovotestis where the testicular tissue is centrally located, flanked by ovarian domains at both poles (Fig. 2B)."

FOXL2 and SOX9 positive cells were mutually exclusive (Fig. 2B) : this is difficult appreciate in Figure 2B, may be the authors could show a zoom of the gonad showing the exclusive expression of FOXL2 and SOX9.

We have now added a bigger magnification (X63) of these gonads in Fig. 2B, D, focusing on the border regions between the ovarian and testicular portions to show there is no overlap between SOX9 and FOXL2 expression.

they are identical to XX WT or XY WT, respectively. Identical? Similar is maybe better.

we changed to 'similar'.

XX homozygous mutant gonads from the Enh13SOX9*+1bp-/- (Fig. 2D) and Enh13SOX9*-3bp SRY*-12bp-/- (Extended Data Fig. 4G) strains also presented an ovotestis phenotype, with a more testis-like appearance compared to the Enh13SOX9*-3bp-/- strain. I understand how the +1bp sex reversal could be more complete owing to the presence of an additional potential GATA4 BS, could the authors comment on the reason for a stronger phenotype in the Enh13SOX9*-3bp SRY*-12bp-/- gonads compared to the Enh13SOX9*-3bp-/-.

This is a good point that you raised, also raised by reviewer 4, and we do not have any explanation to that. The 3 bp deletion in the Enh13$^{SOX9*-3bp}$ is completely identical to that in the Enh13$^{SOX9*-3bp\ SRY*-12bp}$ strain. Perhaps by removing the SRY BS we also removed a repressive BS located nearby that slightly elevated *Sox9* or maybe we altered cooperative binding of other factors in that region in a way that further enhanced *Sox9* expression. We cannot know for sure without going into much experimental detail and we feel this is beyond the scope of the current study which is big and elaborative as is.

How is the ovotestis phenotype observed at E13.5 resolved into a testis phenotype at 6 weeks? The authors should provide data on an intermediate stage.

This is an excellent point. To address this, we have experimentally analysed by bright field and immunostaining gonads of E15.5 and P1 mutant and WT embryos/ mice. This now appears in page 7, lines 18-30
We see that at E15.5, the -3bp line still appear as ovotestis with FOXL2 expression in one or two poles. On the contrary, the +1bp line is mostly expressing SOX9 and very few FOXL2 expression is present, if at all.
In P1 gonads, we see the same pattern where in the -3bp line we were still able to observe some FOXL2 positive cells, while in the +1bp line we observe only a small number of FOXL2 positive cells, if at all. This now appears in the new Extend data Fig. 5 and the text.

Pre-granulosa markers as Foxl2, Fst, Bmp2, Wnt4, Runx1, Emx2 and Lhx9 were highly expressed in XX WT and XX heterozygous gonads, but displayed a low-to-intermediate expression in the XX homozygous mutant gonads of the 3 mouse strains (Fig. 3C and Extended Data Fig. 6B).
While Foxl2 and Fst are good markers of pre-granulosa cells, the authors should note that Runx1, Bmp2, Rspo1 and Wnt4 mark pre-supporting cells before their differentiation into pre-granulosa cells. Moreover, Lhx9 and Emx2 are expressed in the CE, pre-supporting cells and stromal/interstitial progenitors.

Thank you for that correct comment. We have modified the sentence to say: "Markers expressed in pre-granulosa cells as *Foxl2*, *Fst*, *Rspo1*, *Bmp2*, *Wnt4*, *Runx1*, *Emx2* and *Lhx9* were highly expressed in XX WT and XX heterozygous gonads, but displayed a low-to-intermediate expression in the XX homozygous mutant gonads of the 3 mouse strains (Fig. 3C and Extended Data Fig. 7B)"

"In contrast, XX homozygous gonads from all three mutant strains exhibit a progressive shift from an ovarian transcriptional identity toward a testicular transcriptional identity".
The statement should be modulated. The transcriptome at one single developmental stage cannot show a “progressive shift from an ovarian transcriptional identity toward a testicular transcriptional identity” but rather a mixed ovarian and testicular transcriptomic signature at E12.5.

This is correct and we have amended the sentence as you suggested. The new sentence reads as: "In contrast, XX homozygous gonads from all three mutant strains exhibit a progressive shift from an ovarian transcriptional identity towards a testicular transcriptional identity."

The authors should address the expression of Sox9/SOX9 by qPCR and/or IF at an earlier stage, to determine the initial time when Sox9 is abnormally upregulated in XX Enh13 mutant gonads.
Since E11.5 is a very dynamic stage where even a few TS can make a dramatic change (describe my Science paper), we decided to perform WM immunostaining at E11.5 (18-21TS) to analyse SOX9 expression.

Thank you for this excellent suggestion. According to your suggestion we performed wholemount immunostaining of E11.5 gonads (18-21 TS) of XX WT, XX Enh13$^{SOX9*-3bp-/-,}$ XX Enh13$^{SOX9*+1bp-/-,}$ and XY. This appears in the new Ext Data Fig. 12. We did co-staining of SF1 to label the gonad region, DAPI that also labels the mesonephros, and SOX9. We can see SOX9 staining in WT XY gonads, and no staining in WT XX gonads. The XX Enh13$^{SOX9*+1bp-/-}$ gonads clearly express SOX9 whereas the XX Enh13$^{SOX9*-3bp-/-}$ do not yet. This is consistent with the stronger testicular appearance in the +1bp mutant compared with the ovotestis appearance in the - 3bp mutants at E13.5.
This figure and accompanying text is now included in the revised manuscript (Page 12, lines 18-26).

Electro mobility shift assays (EMSA) with in vitro–translated SOX9 on synthetic Enh13 sequences (WT, –3bp, +1bp). A negative test with an irrelevant sequence to Enh13 would reinforce the results.

This was added in the revised extended Data Fig. 8B. We have added an EMSA experiment using either the WT SOX9 BS region in Enh13 or the mutated version used in the Ogawa paper (PMID: 35921234) and also in our NAR paper (PMID: 38499491) where the SOX9 BS was replaced by a poly G sequence. Indeed, the mutant probe shows complete elimination of SOX9 binding.

Supp Fig 7C. Can the authors show the Luciferase assay results when individual TF are transfected? This is highly relevant as they conclude from Figure 4 that cooperative binding of TF is altered in Enh13 mutants.

Thank you for this suggestion. We have performed Luciferase experiments with individual TFs and this is now included in the new Extended Data Fig. 8D. Individual TFs are not able to induce enhancer activity, whereas combination of several TFs is inducing enhancer activity.

Page 9 line 16-19
JASPAR analysis of ~2300 vertebrate TFs, including all those expressed in the gonad at this stage indicated no major gain or loss of binding sites was found between WT and mutant Enh13 sequences to explain the phenotype (Extended data Figure 9).
The authors show in Figure 4 the creation of a de novo potential GATA motif in the +1bp mutant. Can they comment on this?

When we did the JAPSAR analysis, we used a very strict analysis where we only kept binding sites with high Position Probability Matrices (PPM) score. The JAPSAR analysis did not identify the creation of the Neo GATA4 site as it fell below the cutoff score we were using. We went to look for the new GATA4 BS only after the GATA4 EMSA indicated the presence of another complex in the 1 bp mutant and this is how we spotted the new site created upon the T insertion.

In this part, the experiments used materials from human, mouse, and monkey. Are all these reagents equivalent? Please clarify in the Materials and Methods section that they originate from different species.

We have added to the methods the following sentence: " Luciferase assays were performed in HEK293T (human cells) or COS7 cells (monkey cells)".

Page 10, lines 4-6
"Together, these results indicate that the mutations not only boost enhancer responsiveness but also render it less susceptible to RUNX1/NR5A1/GATA4-mediated repression, thereby facilitating Sox9 activation".
The results shown in Fig. 4B and in Extended Data Fig. 10 show boosted enhancer responsiveness to NR5A1/GATA4 of both enhancers in COS7 cells but only of the +1bp enhancer in HEK293T cells. Can the authors comment on this?

There is indeed a slight difference in enhancer activation between HEK293 and COS7 cell. When we add GATA4 and NR5A1, we see in HEK cells significant higher activation in the +1bp but not the -3bp, whereas in COS7 cells, both show very strong activation compared to WT. These two cell lines represent an optimal system to explore these questions as they are naturally devoid of these factors and hence represent "a neutral system" to explore the effect of adding the factors in the reconstruction experiments we did. While addition of only GATA4 and NR5A1was not statistically significant in HEK293, addition of RUNX1, GATA4 and NR5A1 together showed a strong statistical significance in both cell lines, suggesting that it is the combination of the 3 factors that is mediating the difference between the WT and mutants.

To better understand how the mutations might affect cooperative binding of the different transcription factors the authors should provide the results of luciferase assays with individual transfections of NR5A1 and GATA4 and SOX9 as well as the results of luciferase assays with the double transfections of NR5A1/RUNX1, GATA4/RUNX1 and SOX9/RUNX1. The authors state that the distinct responses of the two mutants to NR5A1 and GATA4 alone suggest that they may act through different molecular mechanisms to alter enhancer function but the corresponding luciferase assays of single transfections of NR5A1 and GATA4 are not shown.

This is an excellent suggestion, and we are grateful for this. We have taken your advice and now performed Luciferase assays with either individual TFs (NR5A1, GATA4, RUNX1, SOX9) or combination of RUNX1 with each of the TFs- NR5A1, GATA4 and SOX9. We see that neither individual TFs nor combination of two TFs (when one is RUNX1) is able to induce enhancer activity. It is only combination of NR5A1 and GATA4 that is inducing the WT enhancer but more so the mutated versions. Addition of RUNX1 lowers the WT Enh13 but not the two mutants. This further supports our hypothesis that the two mutants prevent RUNX1-mediated repression that should normally happen. This new data appears in the new Extended Data Fig. 11A.

We also show that the -3bp/+1bp mutations sufficiently modify the 3D structure of Enh13, and hence cooperative binding of TFs. I am not certain that the luciferase assays show this. These experiments show that Enh13 mutations affect the transactivation output of the combined transcription factors, whether this occurs by alteration of the 3D structure and of the TF cooperative binding, or rather by alteration of the 3D structure and the transactivation (positive or negative) abilities of the TF/transcriptional machinery complex is unknown.

We indeed do not provide evidence that the mutations have an effect on the 3D structure of Enh13 and we hence modified the sentence to: " We also show that the –3bp/+1bp mutations sufficiently modify the cooperative binding of TFs, to allow significantly higher activation of *Sox9* even in the absence of SRY."

"Overall, we demonstrate that Enh13 is used for both the activation and repression of Sox9 expression in the testis and ovary, respectively". The role of Enh 13 is to activate Sox9 in XY gonads. However, the mutations described (-3 bp or +1 bp) appear to cause ectopic up-regulation of Sox9 in XX gonads, rather than reflecting a repressive function of Enh13 in XX gonads. These mutations in XX gonads represent an abnormal activation, and it may be more accurate to describe this as such rather than as Enh13 acting as a repressor in the ovary.

You are correct that in the first draft of the paper we did not have strong enough evidence for a repressive role of Enh13 in the ovary. Yet, the new data we present in the new Fig. 4D and 4E shows that when we activate the WT Enh13 with either just bipotential factors (NR5A1, GATA4 and WT1) or also with SOX9 (NR5A1, GATA4, WT1 and SOX9), and then we add pro-female factors, this strongly repress enhancer activity. With the bipotential mix it drops from 10 to 5 (50% reduction) and in the mix with SOX9 it goes from 133 fold to 32 fold induction (75% reduction).
We believe that this new added data does allow us to conclude that normally in XY gonads, the enhancer is activated by bipotential factors and SRY, leading to elevated SOX9, that then replaces SRY and further activates its own expression leading to testis development.
Our revised model is now presented in the new Figure 6 and is describes as such: " In XY gonads, SRY, particularly through its functional interaction with NR5A1 [7], activates Enh13 and induces *Sox9* expression, which subsequently reinforces its own expression through auto-activation, leading to testis development (Fig. 6, XY WT). In XX gonads, bipotential factors, including RUNX1, bind Enh13 and mediate repression of *Sox9*. Around E12.0, upon the onset of FOXL2 expression, pro-female factors act cooperatively to fully repress *Sox*9, thereby allowing ovary development (Fig. 6 XX WT). In the −3 bp mutant, the mutation alters the conformation of Enh13 by bringing RUNX1 and GATA4 proteins closer together on chromatin. Under these conditions, NR5A1-, RUNX1- and GATA4-containing complexes acquire sufficient activating capacity to weakly induce *Sox9* expression, reaching the threshold required for its auto-activation, at which point SOX9 replaces RUNX1 on Enh13. This initiates ovotestis development, which is subsequently resolved into a testis (Fig. 6, XX −3bp). In the +1 bp mutant, the mutation creates an additional GATA4 binding site in close proximity to RUNX1. This results in higher levels of SOX9 protein, which more efficiently reinforces *Sox9* autoregulation, leading to a milder ovotestis phenotype that more closely resembles a testis and ultimately resolves into a testis (Fig. 6, XX +1bp). Taken together, both mutations share a common feature: they bring RUNX1 and GATA4 proteins closer together on chromatin: either directly, in the −3 bp mutant, by shortening the distance between their binding motifs, or indirectly, by creating a *de novo* motif that allows GATA4 to bind in very close proximity to RUNX1 on chromatin." This appears in the discussion page 15, lines 1-17.

Discussion

"The two mutations described here show that Enh13 has repressive activity in the presence of bipotential factor in the normal ovary, and the subtle alterations in its sequence can overcome this repressive state". In all experiments the transfection of bipotential factors NR5A1 and GATA4 always results in an increase of luciferase activity, both in the context of WT and mutated Enh13. Therefore, I do not agree with the conclusion that Enh13 has repressive activity. Rather Enh13 would have a weak activating activity in the presence of bipotential factors that is further dampened by RUNX1 (RUNX1 being only maintained in XX supporting cells). This activity would not be sufficient to produce the threshold level of SOX9 protein required for the self-amplification loop in the XX WT context. In the XX Enh13 mutant contexts the presence of an additional GATA site and/or the disruption of the enhancer structure would result in an increase of the transactivation activity of the enhancer even in the presence of RUNX1, allowing for sufficient SOX9 production and initiation of self-amplification loop.

This conclusion based on activation of Sox9 expression above a given threshold is the one that the authors themselves state in the introduction and in the results:

Introduction p3: Our enhancer reporter assays suggest that the mutations we induced do not abolish the

SOX9 BS but rather enable the bipotential factors NR5A1, RUNX1, and GATA4 to acquire sufficient activating capacity to induce Sox9 expression above the minimal threshold present in female gonad.

Results p5 : Altogether, these findings underscore the extraordinary sensitivity of the Sox9 regulatory network to subtle enhancer perturbations, capable of breaching the activation threshold required to initiate testicular development in an XX context.

We agree that the original paper submitted did not have a good evidence of repression apart from RUNX1. Thanks to all the reviewer's suggestions we have now added new Luciferase experiments highlighting RUNX1 repression abilities and pro-female factor repression abilities of the enhancer (Fig 4D-E).
We have amended this sentence in the discussion to: "Our reporter assay experiments suggest that RUNX1, and later on, additional pro-female factors, are able to repress the enhancer, mediating *Sox9* repression in the ovary that is mandatory for proper ovary development. The two mutations described here show that subtle alterations in Enh13 sequence can overcome this repressive state, allowing low-level activation of *Sox9* independently of the SRY protein." This appears in page 13 lines 18-22.

Other comments: Yet, KO of individual pro-female factors including WT1 (-KTS) 18, FOXL2 24-26, RUNX1 27 and components of the WNT pathway 19-23 do not lead to XX male phenotype in mice. It is only when two pro-female factors or more are deleted together, that XX male phenotype start to appear at embryonic stages 27-30.
I'm not sure that I understand what the authors mean. Could they elaborate?

We apologise that the original sentence was not clear. We have amended the sentence to be: "While in the male pathway, there are two strong players: SRY and SOX9, where loss of either would result in XY sex reversal [5], in the female pathway, loss of an individual pro-female factor including WT1 (-KTS) [20], FOXL2 [26, 27, 28], RUNX1 [29] and components of the WNT pathway [21, 22, 23, 24, 25] do not lead to XX sex reversal in mice. It is only when two pro-female factors or more are deleted together, that the XX male phenotype start to appear at embryonic stages [29, 30, 31, 32]. Therefore, it is striking that single nucleotide insertion within a non-coding regulatory element, located more than 500 kb upstream of the *Sox9* gene, can lead to a robust XX female-to-male phenotype, not generated by ablation of any known pro-female gene."

Reviewer #2 (Remarks to the Author):
This manuscript is an exciting contribution to the sex determination literature showing that female to male sex reversal can be caused by deletions in a critical enhancer of Sox9. Enh13 has previously been shown to be a principal regulator of Sox9 expression in XY gonadal supporting cells. Several of these authors previously showed that mutations in enh13 disrupt male sex determination and testis development. Surprisingly, they now show that addition of 1 base pair (bp) or deletion of 3 bp in this enhancer show no effect on male sex determination or SOX9 expression in XY gonads but lead to elevated Sox9 expression in XX gonads and female to male sex reversal. This manuscript speaks to the amazing plasticity of the sex determination system at the molecular level and provides an interesting example of cell fate switching hinging on minor changes in an enhancer that may alter the relationship of transcription factors known to bind there. The authors create many new mouse strains to explore this phenotype and each is well characterized. They then turn to a molecular analysis of what could be going on at the locus. Using reporter assays in HEK293 cells and EMSA assays, they demonstrate that SOX9 binding in not inhibited by the indels, but cooperative binding of NR5A1, GATA4, RUNX1 and SOX9 may be affected by changes in spacing. Of course, there is always more to learn but I find this a relatively complete story and have only a few recommendations.

We thank you for the nice summary of our paper and results.

In the abstract the authors say, “Mechanistically, these mutations do not eliminate SOX9 binding”. I think a prior sentence to explain that SOX9 binds it’s own promoter would be helpful.

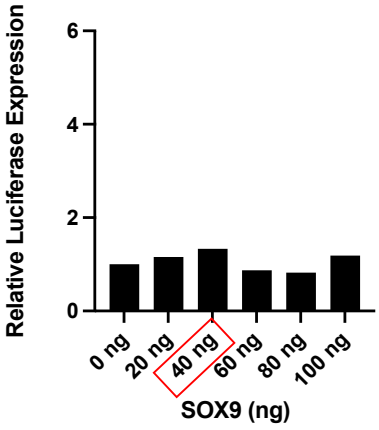
Thank you for that. This is very correct as we did not even mention in the abstract that the mutations are in a SOX9 BS. We have removed this from the abstract.

Later in the manuscript the authors say “These findings rule out increased SOX9 binding or transcriptional activation as the underlying mechanism.” I found this section confusing and felt this conclusion was misleading. After all, the argument is that Sox9 expression is increased by the absence of repression.

This is very correct and we have amended this sentence. This original sentence appeared after we show via PBM and EMSA that the two mutations do not create a more potent SOX9 BS. We amended the sentence accordingly: "These findings rule out that the mutation have created a more potent SOX9 binding site that would lead to higher *Sox9* transcriptional activation as the underlying mechanism."

Have the authors tested whether RUNX1 can repress Sox9 in the presence of low levels of SOX9?
Is there a binding competition between these two factors? An assay in HEK cells increasing the dose of SOX9 in the presence of RUNX1 would be ideal.

You have raised an excellent question, which we addressed experimentally. Following your suggestion, we first tried to increase the dosage of SOX9 in HEK cells and then explore the effect of RUNX1 addition. We were suspecting though that adding SOX9 alone will not induce the enhancer, and this was indeed the case. We tried an increasing array of concentrations of SOX9 alone and could not see any enhancer activation. Hence, it did not make sense to add RUNX1 as we will not be able to see repression. See the graph below.
Normally the concentration we were using of SOX9 were 40 ng but adding even higher concentration did not activate the enhancer.

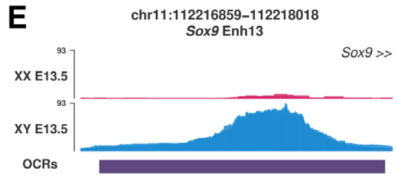


Then, we took a different strategy where we used the WT Enh13 and applied the two combinations that best activate the enhancer: either just bipotential factors (NR5A1, GATA4 and WT1) or the bipotential

factors along with SOX9. We then added RUNX1 into the mix to explore its repressive abilities. We see that addition of RUNX1 dramatically repress Luciferase expression, much more so in the presence of SOX9. This figure now appears as the new Fig 4D-E, and we believe it suggests that RUNX1 normally repress the enhancer by competing with SOX9 on a partially overlapping site. This was added to the text in the result part and discussion.

I would like to see the wild type ATAC landscape around Enh13 in one of the figures. Would ATAC-seq be a helpful approach in mutants to query how binding is changed?

You have raised another excellent suggestion that we were also thinking of. We have recently performed ATAC-seq on sorted Sertoli and pre-granulosa cells from E11.5, E12.5, E13.5 and E15.5 gonads. Enh13 is open and accessible in Sertoli cells from E11.5 whereas it is closed in pre-granulosa cells (PMID: 40712010- Figure 6E, see below).



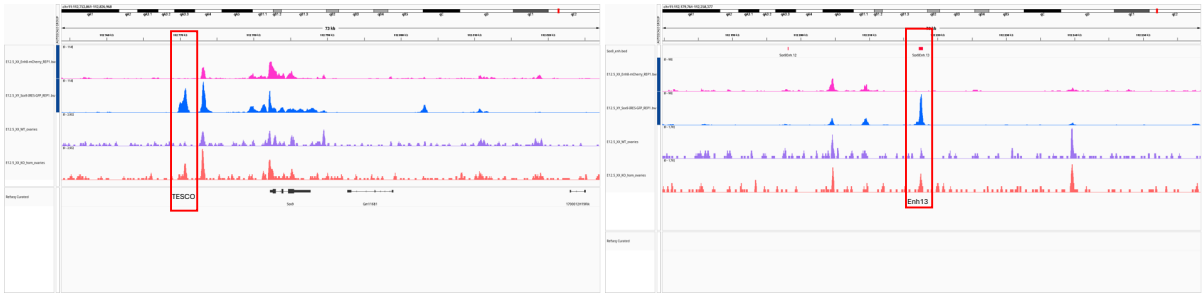
Since in the mutants we have no ability to fluorescently sort Sertoli and pre-granulosa cells, we thought of using whole XY and XX gonads. Normally since WT XX gonads have no signal in Enh13, we were expecting to see accessibility in the XX mutant gonads.

To that aim we performed a preliminary experiment (n=1) and took 100K cells from E12.5 WT XX gonad (separated from the m/s) and 100K cells from E12.5 Enh13 -3bp homozygous gonad.
We did whole gonad ATAC-seq for those two samples and did a low coverage sequencing to get an idea of how it looks.
You can see in the preliminary figure below the results.
The top track is E12.5 sorted pre-granulosa cells from our previous paper (PMID: 40712010).
The second track is E12.5 sorted Sertoli cells from our previous paper (PMID: 40712010).
The third track is E12.5 XX whole gonad
The last track is E12.5 XX Enh13$^{SOX9*\text{-}3bp\text{-}/\text{-}}$ whole gonad

First, looking at the TESCO region, located upstream to the *Sox9* gene you can see it is not accessible in pre-granulosa cells, highly accessible in Sertoli cells, not accessible in XX whole ovary and becoming accessible in the XX homozygous mutant ovaries.
We then looked at the Enh13 region- we see a similar picture of opening of the enhancer in the mutant XX gonads.

These results supported the hypothesis that due to the mutations, there is activation of the enhancer, it becomes accessible, and mediates activation of *Sox9*. However, this is not highly surprising, and it is what we would expect if we saw that *Sox9* is expressed. We would expect that the +1bp mutant will have even more accessibility

Despite these promising preliminary results, we eventually decided to not conduct the full experiment as we would need 3-5 gonads of XX WT, XY WT, XX 3 bp mutant and XX 1 bp mutants. Harvesting the embryos, purchasing the ATAC-seq kit and sequencing all of that turned out to be a substantial amount of money and time that we felt was not proportional for the input it would bring as this is quite an expected outcome. So, we ended up not doing it.
If you were to insist on that, we will do that, but we would hope that these preliminary results will be enough to convince you. Of course, since it is n=1, shallow sequencing and preliminary data, this was not included in the revised manuscript.
We have added a paragraph to the discussion regarding the accessibility of the enhancer. Page 16, lines 1-15.



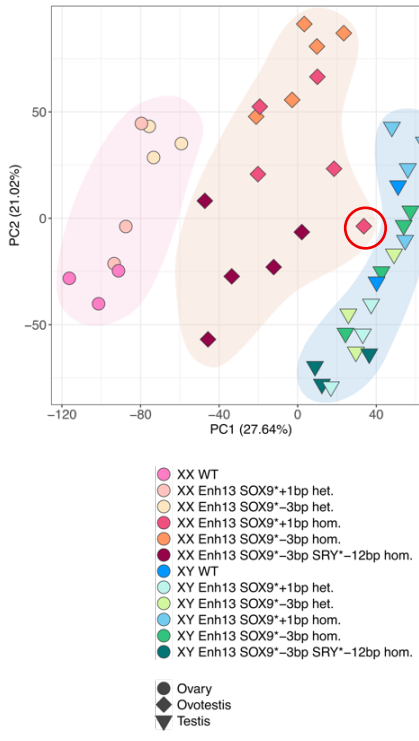
I find the annotation confusing. Could the mutants be represented as alleles using the usual annotation? Sox9-Enh13$^{Sox9*-3bp/Sox9*-3bp}$ (denoting a homozygote) ; compared to Sox9-Enh13$^{Sox9+1bp/+}$ (denoting a het).

Thank you for this comment. There are currently no guidelines in place of how to refer to enhancers. And in addition, we have different mutations within the same enhancer. We considered your option but decided to stay with our original as it would create very long titles that will not fit the boxes labelling the mutants in the figures, so aesthetic wise, it will look less good. In our labelling like "Enh13$^{SOX9*-3bp-/-}$", we mention the enhancer, followed by the specific mutation and then either +/+ for WT, +/- for heterozygous and -/- for homozygous mutant.

Please indicate the A/P axis of gonads in Fig.2. Is the ovarian region always anterior?

This was also raised by reviewer 1. Yes, when there is FOXL2 expression observed at only one pole, the ovarian portion is always at anterior. We have now added A-P labelling to all BF pictures

Fig. 3B – what is the red sample in the XY section of the sample designation line?
The red sample that appears in the XY section is one XX Enh13$^{SOX9*+1bp-/-}$ gonad. This gonad was more masculinised/ testicular than the others and in the PCA plot appeared closer to all the XY gonads. You can see it here labelled with a red circle.
In the heatmap it clusters with the XY samples.



Reviewer #3 (Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank you for your review and welcome you to the reviewing arena.

Reviewer #4 (Remarks to the Author):
The manuscript by Elisheva Abberbock and colleagues clearly demonstrates that engineered mutations in a key testicular enhancer of the Sox9 gene (named Enhancer13, or Enh13) lead to an absence of Sox9 repression in XX developing ovaries. This is followed by testis differentiation without SRY and XX sex reversal in adulthood. The authors elegantly and convincingly demonstrate that Enh13, which was previously described as the key regulatory region for Sox9 upregulation in XY pre-supporting cells, also plays a pivotal role in Sox9 repression in XX ovarian pre-supporting cells. This work confirms and provides molecular explanations for XX sex reversal observed in other species, where abnormal SOX9 gene upregulation has been shown to play a primary role in some human cases and in pig intersexuality.
The major conclusions of the manuscript are stated in the title and throughout the Results section. All of the authors' assertions are supported by clear, statistically validated results from morphological observations of the external and internal genital tracts (including gonads), IHC, gonad transcriptomes by bulk RNA-seq from 45 mice with different genotypes, gene expression levels by qPCR, PBM, EMSA, transfection assays caried onto two different cell types (HEK293T and COS7) and bioinformatic analyses.
The main result is impressive: the authors clearly demonstrate that adding one nucleotide or suppressing three in the SOX9 binding site of Enh13 changes the sex of the individual. Additionally, they prove that the molecular mechanism involved is different in each XX sex reversal cases. In conclusion, all of the authors' claims and conclusions are justified by their large number of high-quality, rigorous results.

We are grateful for your review and the good suggestions.

In addition to these general comments, I have two remarks and few minor corrections for the authors.

1 – My first remark concerns the interpretation of the results proposed by the authors and illustrated in Figure 4E, as well as the binding of RUNX1 and SOX9 to their TFBS, which are very close and overlap by one or two bases in the WT allele, and by an even greater amount in the case of the -3 bp allele.
Do the authors not consider that the binding of RUNX1 (expected in XX supporting cells) and SOX9 (expected in XY cells) are mutually exclusive on these TFBS due to their proximity and the steric hindrance of both proteins? In other words, why do they not consider that there is competition between the two transcription factors for binding to this region, with RUNX1 binding resulting in repression (or an absence of activation) and SOX9 binding resulting in activation? When the authors tested the binding capacity of both factors individually using PBM or EMSA, they showed that RUNX1 bound equivalently to all three alleles (WT, +1 and -3 bp), but SOX9 had less affinity for the -3 bp allele. Nevertheless, SOX9 is the dominant factor, since sex reversal occurs. One possible explanation is that, in vivo, the chromatin conformation of this region causes competition between the two TFs for binding to this critical region, and the 3 bp deletion prevents RUNX1 binding and thus promotes SOX9 binding, despite its decreased affinity. For the +1 allele and the creation of a new GATA site in this small region, the competition is now between not only RUNX1 and SOX9, but also GATA4. I leave it up to the authors to decide whether or not to take this comment into account in the discussion of the results.

We respectfully differ from Reviewer 4's interpretation suggesting that the −3 bp mutant would lead to a competition in favour of SOX9 as a consequence of reduced RUNX1 binding. Several observations argue against this view. First, our EMSA experiments do not reveal any decrease in RUNX1 binding when using the −3 bp probe. Second, at E11.5, SOX9 is not present in the nucleus of XX gonads, as a robust nuclear export mechanism actively excludes SOX9 from the nucleus at this stage.
Instead, we favour an alternative model, which is developed in the revised version of the manuscript. In the −3 bp mutant, the primary change consists of a 3 bp shortening between the RUNX1 and GATA binding motifs. Remarkably, the spacing between these motifs is highly conserved across mammals (Fig. 5A), with only a single base-pair variation observed in rabbit. We propose that this shortening brings RUNX and GATA proteins into closer proximity on chromatin and alters their relative orientation compared with the wild-type configuration, as a consequence of an approximately one-third turn rotation of the DNA double helix. This configuration may facilitate physical interactions between RUNX and GATA proteins, thereby conferring activating potential. Consistent with this idea, RUNX

and GATA proteins are known to interact in hematopoietic cells, and this interaction is evolutionarily conserved up to *Drosophila*.

In the +1 bp mutant, a conceptually similar mechanism is at play: the mutation generates a neo-GATA binding site in very close proximity to the RUNX site, further favouring functional interactions between these factors. Accordingly, the +1 bp mutant displays a stronger phenotype than the −3 bp mutant. Conceptually, a +1 bp mutation that does not create a GATA binding site would be expected to have little or no effect. Importantly, the Enh13 mutants described here cannot be explained solely by changes in transcription factor binding affinity. Rather, our data support a model in which these mutations primarily induce conformational changes of the enhancer itself, thereby reshaping the spatial organization and cooperative interactions of transcription factors bound to Enh13. Looking ahead, our study provides a framework for future conformational analyses of Enh13, calling for three-dimensional modelling and structural biology approaches to determine how subtle sequence changes translate into distinct enhancer architectures and regulatory outcomes.

This model is described in the discussion (pages 14-15) and presented in the new Figure 6.

2 – My second remark concerns the deletion of the SRY BS in addition of the 3-bp deleted in the SOX9 BS (first paragraph of page 5). The authors state that “removal of the SRY BS does not affect the female to male sex reversal of XX homozygous mice”. Why do the authors expect a role of this SRY BS in an XX context where there is no SRY?
Moreover, the authors state that deleting SRY BS in addition to the 3-bp aggravate the phenotype of sex reversal (last paragraph of page 6). How could the authors explain this observation since there is no SRY?

This is a good question. We will first address the first part of it. The SOX9 protein is known to be able to bind (better) to a putative SOX9 BS but it can also bind to other SOX binding sites including the putative SRY BS. Indeed, this was also shown in our recent paper (PMID: 38499491) where we had to delete both the SOX9 and SRY BS of Enh13 to get XY sex reversal. Although females do not have SRY, as they lack the Y chromosome, we expected that the SOX9 protein can mediate its function via either binding to the SOX9 BS or the SRY BS in Enh13, or both. To test this, we generated mice with the same gain-of-function 3 bp deletion in the SOX9 BS but full elimination of the SRY BS. These XX homozygous mice also presented XX male sex reversal, suggesting that the action in this case is not mediated via binding of SOX9 to the SRY BS located 200 bp downstream.
Since this point was not fully clear to you, we have amended the wording in the text. It now reads as such: "Since SOX9 protein can bind to both SRY and SOX9 transcription factor binding sites, we tested whether the elevated *Sox9* expression observed in the XX Enh13$^{SOX9*\text{-}3bp\text{-}/\text{-}}$ is due to SOX9 binding on both the mutated SOX9 BS as well as the SRY BS. To that aim we generated mice carrying the same 3 bp deletion in the SOX9 BS along with a deletion removing the entire SRY BS (Extended Data Fig. 3A, termed Enh13$^{SOX9*\text{-}3bp\ SRY*\text{-}12bp}$)."

We also amended the wording in the same page, slightly below to be as such: "Homozygous XX mice presented as males (Extended Data Fig. 3B-C), demonstrating that removal of the SRY BS, to which SOX9 can bind, does not affect the female-to-male sex reversal phenotype, suggesting that the mutated SOX9 BS is still functional and able to induce *Sox*9 expression"

As to the second part of your question of why do the Enh13$^{SOX9*\text{-}3bp\ SRY*\text{-}12bp}$ embryos present a more testis phenotype compared to the ovotestis of the Enh13$^{SOX9*\text{-}3bp}$, This we do not have a good answer. This was also raised by Reviewer 1.
The 3 bp deletion in the Enh13$^{SOX9*\text{-}3bp}$ is completely identical to that in the Enh13$^{SOX9*\text{-}3bp\ SRY*\text{-}12bp}$ strain. Perhaps by removing the SRY BS we also removed a repressive BS located nearby that elevated *Sox9* or maybe we altered cooperative binding of other factors in that region in a way that further enhanced Sox9 expression. We cannot know for sure without going into much experimental detail and we feel this is beyond the scope of the current study which is big and elaborative as is.

3 –Page 7, last paragraph: the authors write “Analysis of bipotential genes, Nr5a1, Wt1, Gata4…..revealed no major differences in expression between WT and HM mutants”. By contrast Nr5a1 and Wt1 are found differentially expressed in Fig6B (cluster a & f, respectively) and also on Extended data Fig6A, at least between WT males and females with slight differences for HM mutants (male type for Nr5a1, female type for Wt1).

This is correct, we have amended the sentence as such: "Analysis of bipotential genes, *Gata4* and *Zfpm2* (*Fog2*), which are expressed in supporting cell precursors, and later maintained in both Sertoli and pre-granulosa cells, revealed no major differences in expression between WT, heterozygous, and homozygous gonads from the Enh13$^{SOX9*-3bp}$, Enh13$^{SOX9*+1bp}$ and Enh13$^{SOX9*-3bp\ SRY*-12bp}$ mutants (Extended Data Fig. 6A). *Nr5a1* expression in the XX homozygous mutants was more similar to the male expression levels, whereas *Wt1* expression in the homozygous mutants resembles more that of the WT XX gonads."

4 – Page 8, first paragraph. The authors could add Rspo1 in the list of pre-granulosa markers and Aard in those of Sertoli markers, because expression of these two genes are presented on Ext Data Fig6 (B & C, respectively).

Thank you, we have added these genes to the description.

5 - Page 10, last paragraph, second sentence: “We also show….even in the absence of male-specific factors”. The authors probably think to SRY. It will be better to write “…even in the absence of SRY”.

We amended this and replaced male-specific factors with SRY.

6 – Figure 2C: The number of studied samples for XX+/+ is n=5 (as indicated in the parenthesis), but there are 4 points for Sox9 and 6 points for Sox8 and Foxl2.

You are correct, we first had a typo in the legend and there were originally 6 samples of XX WT embryos. All these 6 points appear in the *Sox8* and *Foxl2* graphs but in the *Sox9* graph we now have 5 points and not 6 as one of the points appeared with a value of 10 and was omitted by Prism GraphPad as being an outlier.

7 – Legend of Figure 2C : “in” is missing between “involved” and “male”.

Thank you for spotting this typo. We amended this.

8 – Figure 4A: the last base of the consensus sequence for the SOX9 binding site is missing on the Figure (should be A>G=T).

The SOX9 consensus BS is AACAATGG. The AACAATG are highly conserved, but the last nucleotide is a G/ A/ C in different mammals and hence the software does not place a letter there, but the dashed lines do label all 8 nucleotides of the binding site.

9 – Extended Data Fig.1B: “oviduct” term is present on the fourth animal (on 6 weeks gonads) and should be suppressed.

Many thanks for spotting this typo. We removed this.

10 – Page 22; Methods; Paragraph “Design and preparation of sgRNAs mRNA”; lane 5 of this paragraph: please write “concentration” instead of “concertation”.

Thank you for spotting this typo. We amended this.

Response letter to reviewers Second Round

We are grateful to all four reviewers for their constructive comments and for recommending our manuscript for publication.

The reviewers' comments are in black, and our replies are in blue.

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed my questions and have strengthened the manuscript through their revisions and the inclusion of additional results. Overall, this study provides a compelling demonstration of how subtle alterations in regulatory DNA can lead to major developmental effects, using Enh13, a regulatory element of Sox9, as an example. It delivers novel and clinically meaningful insights into the genetic mechanisms underlying disorders of sex development (DSD). While a few minor typographical and editorial corrections can still be addressed, the manuscript is suitable for publication in Nature Communications.

Many thanks

Few comments

The figures currently use red and green together. Nature Communications recommends high-contrast, accessible color palettes (e.g., green and magenta) for clarity and inclusivity. No changes are required if the current colors are essential, but alternatives can be used if possible.

This is correct, and this comment was raised at the first stage of the revision. We hence amended all real time graphs to be presented with magenta and green. The only thing that stayed red and green are the immunostaining, and for a good scientific reason. When using red and green, if an overlap in protein location exists, it created the very well spot colour of yellow. Green and magenta combination are less clear when overlapping. Since an important scientific question is whether in the ovotestis, it is the same cells that express SOX9 and FOXL2 or different cells, we feel it is important to use green and red to show that we have no yellow cells, meaning although the gonad contain ovarian and testicular portions, these do not overlap at the cellular level.

A typo appears in Reference 4.

Thank you for spotting this. We are unsure of how this happened, but we have now amended the typo.

"In contrast, XX homozygous gonads from all three mutant strains exhibit a progressive shift from an ovarian transcriptional identity toward a testicular transcriptional identity".
Although the authors state that the sentence has been amended, the revised version is unchanged from the original and continues to imply a progressive temporal shift.

We apologise; it was our mistake. This is now amended to: "In contrast, XX homozygous gonads from all three mutant strains exhibit a mixed ovarian and testicular transcriptome signature at E12.5"

The authors have now examined Sox9 expression at earlier developmental stages to determine when Sox9 first becomes abnormally upregulated in XX Enh13 mutant gonads. To allow accurate comparison between samples, it would be helpful if the authors could clarify the developmental staging used for these analyses, in particular by indicating the number of tail somites, if available.

This was now added to Extended Data Figure 12. All gonads were 17-19 tail somites.

Reviewer #2 (Remarks to the Author):

The authors have carefully responded to the reviewers' comments, performed new experiments, and improved many aspects of the manuscript.
I think it would be helpful if the model in Figure 6 showed that Sox9 is initially expressed at low levels in the XX gonad, where SOX9 is cytoplasmic, as the authors explain on line 6 and 26 (Gasco). The question becomes whether it is upregulated or downregulated as gonads develop. This is a subtle but important difference in the way we think about it.
As we know, first SRY and later SOX9 upregulate Sox9.

Thank you for this suggestion. We have now added to the scheme in Figure 6 the presence of low levels of SOX9 in both XY and XX early gonads

In this manuscript, the authors convince me that RUNX1 is an important repressor of SOX9 (although I am puzzled as to why in its absence, XX Runx1-/- animals do not develop testes – presumably SOX9 levels are not high enough without up-regulation by SRY). I admire the in vitro TF competition assays and model as an explanation for why these 1 or 3 bp mutations cause sex reversal.

This is indeed puzzling. We hence delved into the Nicol et al., 2019 paper who first described the role of RUNX1 in the gonads. We also had personal communications with Humphrey Yao and Barbara Nicol about that. Full KO of RUNX1 is embryonic lethal, and hence Nicol et al., used cKO using the *Sf1*-cre line. Data from the lab of Marie-Christine Chaboissier shows that although *Nr5a1* is expressed at E9.0 in the gonads, the *Sf1*-cre line from Keith Parker only starts to work from ~E11.0-E11.5 in the gonads. We believe that this stage is too late, and knocking out RUNX1 at that stage misses the window to prevent *Sox9* repression and lead to XX males. We are hoping that Barbara and Humphrey will use the *Wt1*-cre, which is earlier to drive gonadal expression, in order to do cKO of RUNX1. We anticipate this may lead to XX sex reversal and support our model/ hypothesis.
This will be as part of a follow up work, but we fully agree that this role of RUNX1 is striking and require future investigation.

This manuscript moves the field in the important direction of understanding the interaction of TFs on chromatin during the process of cell fate determination and sex determination. Although there is a lot more to do, that's for another manuscript. The authors have made significant progress in this work.

Thank you

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you

Reviewer #4 (Remarks to the Author):

The revised version proposed by the authors is even more convincing. I fully agree with both the new model and the interpretation of the results set out in this version and I strongly recommend the publication of this Ms.

Thank you

One final detail regarding the sentence on lines 8–11 of page 13: it is not surprising, in my opinion, that a gain of function of Sox9 induces a very robust XX female to male sex-reversal (Vidal et al., 2001). What is striking is that such subtle modifications of a TFBS can induce such a change in the transcriptional expression of the Sox9 gene. In my opinion, XX sex reversal through loss of function of pro-ovarian genes is a completely different story.

We fully agree with you on this statement, but what we are comparing in these sentences is the fact that KO of each pro-female factor that we know of cannot induce XX sex reversal and yet a single insertion in a non-coding genome can do that.

And a last typo on line 15 page 10: suppress one “on the”.

Thank you for that. This was amended on Page 10, line 15. We removed the duplicated "on the"