Identification of regulatory elements required for *Stra8* expression in fetal ovarian germ cells of the mouse

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MS TITLE: Identification of regulatory elements required for Stra8 expression, in fetal ovarian germ cells of the mouse

AUTHORS: Chun-Wei Allen Feng, Cassy Spiller, Kallayanee Chawengsaksophak, Peter Koopman, and Josephine Bowles

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. 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 currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Feng at al., 2020 identify and investigate known and novel upstream regulatory elements involved in Stra8 expression in female germ cells. The authors generate new transgenic reporter lines that enable visualisation of the spatial and temporal expression of Stra8. Using in vitro and in vivo assays, they focus on the interplay between Retinoic Acid Regulatory Elements (RARE) 1 and a newly discovered RARE3, as well as Dmrt1 binding region, all found within a 2.9kb part of the Stra8 promoter. They find that in the mouse fetal ovary Stra8 expression is predominantly driven by RA signalling, with Dmrt1 enhancing this effect. Most impressively, they disrupt RARE1 and RARE3 (and both together) in vivo using CRISPR/Cas9, thus confirming that these sites do regulate Stra8 expression – albeit the effect of individual mutations is mild. Finally, they serendipitously deleted a 173bp region upstream of the TSS, which encompasses RARE1 and a DMRT1 binding site, and this seems sufficient to completely block normal in vivo expression of Stra8.
This study, by a leading lab in the field, makes a significant contribution to our understanding of the control of meiosis initiation, which remains a controversial topic, and so will undoubtedly be of broad interest. The overall approach is sound, and the in vivo work is particular is impressive (and a non-trivial undertaking). I suggest two main areas that could be expanded upon, and then some minor comments beneath. I believe all of these will be straightforwards to address.

Comments for the author

Main issues
1. In vivo characterisation. The in vivo work is overall a strength of the paper. However, a few extra details would be helpful.
a) For the transgenic reporter mice, does the GFP recapitulate the A-P pattern of Stra8 expression at E13.5 and E14.5? Images showing co-staining of GFP/Stra8 and quantification of this would be useful, particularly as the spatio-temporal regulation is specifically mentioned in the Abstract. Also, the overlap in Figure 1F looks good, but can this be quantified?
b) For the RARE1, RARE3, RARE1/3 and especially the Δ173bp mice, can the authors show sequencing date to confirm that only the desired alterations are made? For the Δ173bp mutant it is especially important to show that a knockout allele has not inadvertently been generated.
c) For the homozygous RARE1/3 double mutant mice, is the A-P expression pattern of Stra8 normal? (if not which site is important for this?).
d) Further characterisation of the Δ173bp mice should be shown, including breeding data vs heterozygous controls. Overall does the germ cell phenotype mimic the Stra8 knockout? Does Oct4 protein remain elevated for longer than expected?

2. In vitro assays a) Some basic characterisation of this system is missing (much of which I'm sure the authors have done). Is DMRT1 already expressed in F9? What level is achieved upon transfection? Also, is DMRT1 responsive to retinoic acid in this system (this would impact interpretation of the results). Which retinoic acid receptors are expressed in F9? How much basal RA activity is present in the system? What about BMP activity? Is ZGLP1 expressed?
b) Nagaoka et al., 2020 reported that BMP can also induce Stra8 (in a ZGLP1 dependent manner). It seems this in vitro system would be an ideal opportunity to test this? BMP pathway activators and inhibitors could be added (there may well be BMP activity in the serum used to culture the cells). Is there a synergistic effect of BMP and RA, as suggested in the Nagaoka paper?
c) What is the effect of mutating both DMRT1 and RARE1? This would also serve as a control for the later Δ173bp experiment.
d) For the Δ173bp reporter, can the authors provide evidence that expression is possible from this construct at all? For instance, might high dose RA drive expression? This would add weight to the idea that the mutation does not disrupt something more fundamental than just the RARE1 and DMRT1 binding sites.

e) Have the authors derived pluripotent stem cells from the reporter mice? Do the reporters behave similarly to F9 cells? (please note - this is not a request for these experiments to be performed, if they have not already).

Minor points
1. Line 63 – other explanations are possible – for instance, other TFs or other signalling pathways may play a role, in particular, the BMP pathway. I wonder if Nagaoka et al., 2020 should be referenced and discussed?
2. Line 101 – overall the authors deal elegantly with the (ongoing) controversy regarding RA and meiosis induction in the fetal ovary. Specifically, with regards the 2020 studies by Chassot et al and Vernet et al., I wonder if some discussion of these findings would be helpful (either in the Intro or Discussion), to better explain to the uninitiated why this controversy continues. I am not suggesting the authors need to provide extensive comment, or spend a great deal of time on this. However, as these studies do have relevance to the current work, I think slightly more discussion is required.
3. Line 144. It is not clear to me why the authors included both the 1.4kb and 1.5kb transgenes – as there doesn't appear to be a sequence of interest in the extra 100 or so base pairs?
4. Line 158 -159 'Analysis of tissue sections…'. For added clarity maybe state in the text that this in the 2.9kb mice 5. Fig 2A, 3A/B and 4. It is not clear in all examples which statistical test is used. If multiple groups are being tested, is a Student's t-test appropriate?
6. Line 217, Fig 3A. Is the difference between the first two bars really significant? I wonder if the authors should review the significant comparisons they choose to show in this graph, as it does seem somewhat arbitrary.
7. Figure 4. I wonder if a diagram of the mutations introduced would add clarity?
8. Line 297. I would rather indicate that the line is a valuable tool.
9. Line 353 – 'During the CRISPR/Cas9 process' is an odd turn of phrase, maybe a more full explanation would be clearer.
10. Line 412. Is there an appropriate reference for KH2 cells?
Reference Nagaoka, S. I., Nakaki, F., Miyauchi, H., Nosaka, Y., Ohta, H., Yabuta, Y., et al. (2020). ZGLP1 is a determinant for the oogenic fate in mice. Science (New York, NY), 367(6482). http://doi.org/10.1126/science.aaw4115

Reviewer 2

Advance summary and potential significance to field

The MS DEVELOP/2020/194977 by Feng et al. describes the role of two retinoic acid (RA) response elements (RAREs) for the RA-dependent expression of Stra8 in F9 cells cultured in vitro, in the presence of a supra-physiological dose of RA (50nM). The authors identified a yet unknown RARE located ~1.3kb upstream of the transcription start site (TSS) called RARE3, and confirmed the presence of another one called RARE1, located ~200-bp upstream of the TSS, identified 18 years ago (Giuili et al., 2002, EMBO Rep. 3(8):753) and largely analysed by others since (Tilly et al., 2010, Cell Cycle 9(2):339; Kumar et al., 2011, Nat. Commun. 2:151; Raverdeau et al., 2012, Proc. Natl. Acad. Sci. USA 109(41):16582). The authors showed that each of these RAREs contributes to ~50% of the RA-dependent regulation in F9 cells. When transposed in vivo by CRISPR/Cas9-directed mutagenesis, the RAREs contribute together for ~50% of the total Stra8 expression in the mouse fetal gonad. This finding corroborates the study recently published by Vernet et al. (Sci. Adv. 6:aaz1139), showing that Stra8 expression occurs in germ cells of mutants lacking all RA-receptors (RARs), albeit at a level which is about 50% of the control level. They also confirmed the involvement of DMRT1 in the control of Stra8 expression, a regulation discovered by others several years ago (Matson et al., 2011, Dev. Cell 19(4):612; Krenz et al., 2010, Dev. Biol. 356(1):63). Not surprisingly, they finally showed that ablation of a 173-bp long sequence including both RARE1 and the Dmrt1-binding site located upstream of the TSS, but also numerous binding sites for other factors, totally abolishes Stra8 expression in the mouse fetal gonad.

In the end, this MS describes the contribution of a new RARE to the expression of Stra8. Although interesting, this finding does not add to the questions as to understand "how the gene is so comprehensively silenced in somatic cells at all stages of development and adult life" (line 284) nor "how Stra8 expression is specifically activated in germ cells" (line 288). The authors needlessly fuel a controversy about the role of RA in meiosis initiation (e.g., lines 99-101; lines 344-350; lines 369-

374), even though their present findings perfectly match with the proposal made by others that RA contributes, but is not indispensable, to Stra8 expression (Kumar et al., 2011, Nat. Commun. 2:151; Chassot et al., 2020, Sci. Adv. 6:eaaz1261; Vernet et al., 2020, Sci. Adv. 6:aaz1139).

Comments for the author

Major points:

1) Line 109: using mouse embryonic stem cells induced into epiblast-like cells, which are in turn induced into primordial germ cell-like cells displaying capacity for oogenesis, should be much more representative cells than F9 model system to study Stra8 expression (Miyauchi et al., 2017, EMBO J. 36(21):3100).

2) Line 178: the use of 50nM RA is clearly not suitable to properly "model ovarian germ cells (line 176)" since the physiological concentration of RA is below 25nM (Kumar et al., 2011, Nat. Commun. 2:151). A RA concentration as low as 1nM is actually able to induce Stra8 expression in cultured germ cells (Tedesco et al., 2013, Biol. Reprod. 88(6):145) as well as the expression of a RA-sensitive reporter transgene in the fetal gonad in vivo (Chassot et al., 2020, Sci. Adv. 6:eaaz1261). Characterizing responsiveness of promoter constructs to RA should be performed using RA concentrations as close as possible to the situation in vivo to model regulation of Stra8 in fetal ovarian germ cells in F9 EC cells.

3) Line 189: the reviewer is not convinced that introducing DMRT1 in the reporter system in vitro yields a "synergistic effect". RA alone induces 5-fold (1.4 construct), 4-fold (1.5 construct) or 9-fold (2.9 construct) increase of luciferase activity. DMRT alone induces a ~2-fold increase. The effect of RA +DMRT1 is obviously cumulative rather than synergistic since it induces 10-fold (5x2 for 1.4 construct), 8-fold (4x2 for 1.5construct) and 17-fold (9x2, 2.9 construct). A synergistic effect would have produced much greater increases than those observed.

4) Line 201: binding of RARs to the putative RARE3 sequence should be demonstrated by chromatin immunoprecipitation assays (ChIP), or at least by electrophoretic mobility shift assays (EMSA) if antibodies to RARs are not available to the authors. Otherwise, "RA/RAR/RXR action" on RARE3 is not demonstrated, as stated by the authors (line 329), especially since they seem to consider that RA is capable of producing some effects independently of RARs (see below, points #5 and #6).

5) Line 212: as to the cumulative (but not "synergistic") effect of Dmrt1 on Stra8 expression, the contribution of Dmrt1 alone appears much more marginal in Fig. 3 than in Fig. 2A. Intriguingly, transfecting Dmrt1 had no effect at all on mut-RARE1, mut-RARE3, mut-RARE1+3 constructs (light grey bars, NS). This casts doubts on the involvement of Dmrt1 in Stra8 regulation. The authors should provide evidence that Dmrt1 is expressed or functional in their transfection assays, for instance by analysing the expression of a known Dmrt1 target-gene or by introducing a Dmrt1-reporter construct in their system.

6) Lines 230 and 233: the "dominant instructive role" of RA and the fact that "RA signalling is a dominant activator" of Stra8 are not evident to the reviewer. This concept may be true in vitro, in F9 cells treated with added RA. However, Vernet et al., (2020, Sci. Adv. 6:aaz1139) show that the expression of Stra8 in RAR-null mutants (i.e., in vivo) is delayed, but not switched off: its level at 14.5 dpc reaches the level observed in control ovaries at 13.5 dpc, which indicates a delay but not an impairment. Because all RA-dependent effects necessarily goes through RARs (Kashyap et al., 2013, 126(4):999; Laursen & Gudas, 2018, J. Biol. Chem. 293(30):11891), this means that Stra8 expression can be achieved in vivo in the total absence of a functional RA-signalling pathway. Even though RA enhances Stra8 expression, the notion of "dominant" and "instructive" do not apply in vivo: 50% is not dominant, and something else than RA clearly instructs Stra8 expression.

7) Line 255: the fact that "approximately 40% of Stra8 expression" is retained at 14.5 dpc in mice lacking RARE1 and RARE3 is in agreement with the finding that Stra8 is expressed in RAR-null mutant ovaries. It is not necessary to propose that additional RAREs lie beyond the promoter fragment that was used in the present MS, except if the authors purposely denies the work done by others (Vernet et al., 2020, Sci. Adv. 6:aaz1139). The authors should test the expression of Stra8 at 15.5 dpc and might discovered an expression recovered at a normal level, despite the lack of RAREs

sequences. This would not be surprising since homozygous females harbouring RARE1/3 double mutations are fertile (line 259).

8) Line 263: the finding that deleting 173-bp upstream of the transcription start site (TSS) in mm10 totally abolished Stra8 expression is not surprising on its own, given this region contains binding sites for many other factors such as Setd8 (Niu et al., 2020, J. Cell Mol. Med. 24:4194), Crebbp and Ep300 (Chen et al., 2012, PLoS One 8(6):e66076) or CTCF (Sleutels et al., 2012, Epigenetics Chromatin 5:8). This is also a target region required for acetylation of histone H3 (Chen et al., 2012, PLoS One 8(6):e66076) and nucleosome positioning (Sleutels et al., 2012, Epigenetics Chromatin 5:8).

Minor points:

1) Line 61: in cancer cells Stra8 is expressed as in embryonal carcinoma and embryonic stem cells (e.g., Kuang et al., 2019, Cancer Biomark. 25(2):203-212).

2) Line 146: the choice of integrating the Stra8 promoter construct at the Col1a1 gene is quite surprising given this gene is not expressed in germ cells between 11.5 and 13.5 dpc (Jameson et al., 2012, PLoS Genet. 8(3):e1002575), making the transgene locus possibly closed for proper access to transcription factors.

3) Line 155: figure 1A is not cited in the main text.

4) Line 210: the data show that “mutation of both [RAREs] completely abolishes” the sensitivity of the promoter construct to RA, but not its “expression”, as proposed by the authors.

5) Line 237: given the high error rate generated using the CRISP/Cas9 method, the full sequences of the mutated loci in the mouse lines that were established should be specified as supplementary information.

6) Line 265: the genome reference used by the authors should be mentioned. By the way it is surprising that the TSS of Stra8 gene is at 6:34 870 961 in GRCm38 mm10 in NCBI, while it is at 6:34 870 638 in Ensembl GRCm38.p6. Depending of the genuine TSS, the interpretation of the data presented in this MS might be different since the regulatory regions would be comprised either in the promoter or in the 5’ untranslated region of Stra8 mRNA.

First revision

Author response to reviewers' comments

We have numbered Reviewers queries and our response is in dot-point under each.

Reviewer 1

Major points:

1. For the transgenic reporter mice, does the GFP recapitulate the A-P pattern of Stra8 expression at E13.5 and E14.5? Images showing co-staining of GFP/Stra8 and quantification of this would be useful, particularly as the spatio-temporal regulation is specifically mentioned in the Abstract. Also, the overlap in Figure 1F looks good, but can this be quantified?

- Figure 1B shows our Stra8-GFP transgene displaying an anterior to posterior expression pattern similar to that known for Stra8 expression (Bowles et al, 2006) at both 13.5 and 14.5 dpc.
- We performed new whole-mount immunofluorescence on 13.5 dpc Stra8eGFP ovaries and quantified GFP and STRA8 co-staining as suggested and include this in a new Figure S3. This result is referred to in the main text (lines 175-177) and discussed (lines 325-329).

2. For the RARE1, RARE3, RARE1/3 and especially the Δ173bp mice, can the authors show sequencing date to confirm that only the desired alterations are made? For the Δ173bp mutant it is especially important to show that a knockout allele has not inadvertently been generated.
- We have shown the sequence for the d173bp up to the TSS in Figure S5, C.
- We have added full promoter sequencing data for 2.9Kb promoter, exon 1 (including TSS) and additionally exon 2 (encompassing ATG start site) and flanking intronic region as a new Supplementary Figure (Figure S9 and Data S1), confirming that only the desired alterations were made (new text for new figure: lines 594-599).

3. For the homozygous RARE1/3 double mutant mice, is the A-P expression pattern of Stra8 normal? (if not, which site is important for this?).
- This was a very good suggestion. We have examined the RARE1/3 double mutant mice at 13.5 dpc by whole-mount immunofluorescence and found that STRA8 retains its normal A-P expression pattern. This result has been included as a new Figure S7 and added to the text (lines 276-281) and discussed (lines 380-384).

4. Further characterisation of the Δ173bp mice should be shown, including breeding data vs heterozygous controls. Overall does the germ cell phenotype mimic the Stra8 knockout? Does Oct4 protein remain elevated for longer than expected?
- We have now added breeding data as new Table S4 and also added new Figure S8 (and lines 298-301) that demonstrates retention of OCT4 (POU5F1) protein, corroborating the existing qRT-PCR data in Figures 5 and S6.

5. In vitro assays: Some basic characterisation of this system is missing (much of which I'm sure the authors have done). Is DMRT1 already expressed in F9? What level is achieved upon transfection? Also, is DMRT1 responsive to retinoic acid in this system (this would impact interpretation of the results). Which retinoic acid receptors are expressed in F9? How much basal RA activity is present in the system? What about BMP activity? Is ZGLP1 expressed?
- We have confirmed empirically that Dmrt1 is expressed at low basal levels in F9 cells and that it is not upregulated in response to RA in our system, and included this as new Figure S4. This corroborated microarray data of Laursen et. al. 2012 showing low basal Dmrt1 gene expression and no response to 1uM RA in F9 cells. We have added this information in the Results (lines 199-204).
- We have not determined the levels of DMRT1 achieved upon transfection, and we do not have a suitable antibody, however the data we have presented consists of a minimum of 3x replicates (and 3x technical replicates) performed across multiple days and the induction of Stra8 promoter activity by the transfecting DMRT1 construct (in the presence of RA) appears consistent across all our experiments.
- RAR is expressed in F9 cells with basal levels of RAR and RAR (microarray Laursen et. al 2012). The latter is upregulated upon RA exposure as expected. Discussion of how this RAR expression profile is similar to the profile of fetal pre-meiotic germ cells has been added (lines 341-343). We are not able to assess the basal RA activity, however it is expected to be very low since we are able to elicit a response to RA at with 0.1 nM treatment (data not shown).
- Zglp1 is expressed at very low levels in F9 cells, and is not upregulated by RA (empirical data, new Fig. S4, consistent with microarray data, Laursen 2012). This may indicate absence of BMP activity in the F9 system. Discussion of this difference in Zglp1 expression in the in vitro and in vivo systems, and how it may impact on the discrepancy in Stra8 regulation that we observe between the two, is discussed (lines 403-406).

6. Nagaoka et al., 2020 reported that BMP can also induce Stra8 (in a ZGLP1 dependent manner). It seems this in vitro system would be an ideal opportunity to test this? BMP pathway activators and inhibitors could be added (there may well be BMP activity in the serum used to culture the cells). Is there a synergistic effect of BMP and RA, as suggested in the Nagaoka paper?
- The effect of BMP/ZGLP1 on Stra8 expression is indeed interesting. We are currently addressing this possibility. As our new BMP-related study is extensive, and includes not only in vitro but also in vivo analysis of the role of BMP signalling in mouse fetal ovarian germ cells, we aim to publish it independently of this study. Although we are very happy to acknowledge and discuss this possibility (lines 394-403), we want to keep the focus of this study on the RARE sites we identified and tested in vitro and in vivo. Thanks to the suggestion of this reviewer we have now established that the residual Stra8 expression when RARE1/3 deleted is observed beginning at the anterior end

of the gonad – so the residual expression in the RARE1/3 mutant might be due to distal RAREs not included in our promoter, or due to ZGLP1 together acting with RA, but at a site other than RARE1 or RARE3 (as now discussed in the text). We also include note of another possibility – that RA induces WNT4 expression at the anterior end of the gonad and that this impacts on Stra8 expression, as has been suggested by others (lines 391-393).

7. What is the effect of mutating both DMRT1 and RARE1? This would also serve as a control for the later Δ173bp experiment.
• We agree with the usefulness of this experiment however were not able to generate this additional mutation for this publication (previously we tried and failed). We would require a large amount of time and resources to create this mutation.

8. For the Δ173bp reporter, can the authors provide evidence that expression is possible from this construct at all? For instance, might high dose RA drive expression? This would add weight to the idea that the mutation does not disrupt something more fundamental than just the RARE1 and DMRT1 binding sites.
• We did not see induction of expression using this construct under any of our in vitro culturing conditions (as reported), though we never challenged this with an extremely high RA dose. We do think it possible/likely that other elements in this 173bp region, besides RARE1 and DMRT1 binding sites, are important for transcription (see lines 413-417).

9. Have the authors derived pluripotent stem cells from the reporter mice? Do the reporters behave similarly to F9 cells? (please note - this is not a request for these experiments to be performed, if they have not already).
• A great suggestion, though we have not yet performed this experiment. We will, however, endeavor to do this for future studies.

Minor points
10. Line 63 – other explanations are possible – for instance, other TFs or other signalling pathways may play a role, in particular, the BMP pathway. I wonder if Nagaoka et al., 2020 should be referenced and discussed?
• Thanks, we inadvertently omitted to add the newer manuscript, which should certainly be included. We did mention the work by Miyauchi et. al. 2017 with regards to the possibility of BMP pathway contributing to Stra8 expression. We have now added Nagaoka et. al. 2020 to reference the possibility of Zglp1 as a downstream effector of the BMP pathway and introduced (lines 86-89) and discuss this (lines 398-406).

11. Line 101 – overall the authors deal elegantly with the (ongoing) controversy regarding RA and meiosis induction in the fetal ovary. Specifically, with regards the 2020 studies by Chassot et al and Vernet et al., I wonder if some discussion of these findings would be helpful (either in the Intro or Discussion), to better explain to the uninitiated why this controversy continues. I am not suggesting the authors need to provide extensive comment, or spend a great deal of time on this. However, as these studies do have relevance to the current work, I think slightly more discussion is required.
• We thank the reviewer for understanding that some delicacy is required. We have now added some background regarding the approach taken in the Chassot et al and Vernet et al. 2020 papers (lines 425-434). However, we wish to continue to do so “elegantly” and so have simply used the term ‘knockdown’ to imply that it is possible/likely that complete deletion has not been achieved in these studies. We mention that these studies cannot be accepted to the exclusion of other evidence that indicates that RA is important in the induction of Stra8 expression. We offer our strategy as alternative/complementary to the strategies they used.

12. Line 144. It is not clear to me why the authors included both the 1.4kb and 1.5kb transgenes – as there doesn’t appear to be a sequence of interest in the extra 100 or so base pairs?
• Previous publications such as Kwon, et. al. 2014 tested a 1.5 kb region of the Stra8 promoter. We included this length for comparison to these published findings, for completeness, and to possibly account for any potential discrepancies in results. We have now included this reference/rationale in lines 154-157.

13. Line 158 -159 ‘Analysis of tissue sections…’. For added clarity maybe state in the text that this in the 2.9kb mice

- Done, as suggested (now on line 171).

14. Fig 2A, 3A/B and 4. It is not clear in all examples which statistical test is used. If multiple groups are being tested, is a Student's t-test appropriate?
- We greatly appreciate this suggestion. We have reviewed all of the statistical tests for our results and did identify some misuse of the t-test, where ANOVA would be more appropriate. We have now corrected these errors and the statistical tests used are detailed individually in their respective figure legends.

15. Line 217, Fig 3A. Is the difference between the first two bars really significant? I wonder if the authors should review the significant comparisons they choose to show in this graph, as it does seem somewhat arbitrary.
- Thank you - as we reviewed our statistical tests in response to the previous comment, this was corrected and these slight differences are now no longer statistically significant.

16. Figure 4. I wonder if a diagram of the mutations introduced would add clarity?
- We have added a diagram as suggested (Fig 4).

17. Line 297. I would rather indicate that the line is a valuable tool.
- Agreed and amended (now on line 330).

18. Line 353 – 'During the CRISPR/Cas9 process' is an odd turn of phrase, maybe a more full explanation would be clearer.
- Agreed and we have now given more details of how the d173bp was generated via the NHEJ mechanism in the methods (line 593-594) and results (288-293).

19. Line 412. Is there an appropriate reference for KH2 cells?
- Yes, Beard et. al. 2006 has now been added (lines 159 and 476).

Reviewer 2
Major points:

1. Line 109: using mouse embryonic stem cells induced into epiblast-like cells, which are in turn induced into primordial germ cell-like cells displaying capacity for oogenesis, should be much more representative cells than F9 model system to study Stra8 expression (Miyauchi et al., 2017, EMBO J. 36(21):3100).
- This is a great suggestion, however we did not pursue this method due to its procedural complexity, cellular heterogeneity, low % of resulting PGCLCs and unknown receptiveness to being transfected. To avoid these problems, we used a biologically related, relatively simple and readily transfectable 'workhorse' cell system instead. Importantly, we then followed up our in vitro findings with in vivo mutations, which confirmed the relevance of the F9 system.

2. Line 178: the use of 50nM RA is clearly not suitable to properly "model ovarian germ cells (line 176)" since the physiological concentration of RA is below 25nM (Kumar et al., 2011, Nat. Commun. 2:151). A RA concentration as low as 1nM is actually able to induce Stra8 expression in cultured germ cells (Tedesco et al., 2013, Biol. Reprod. 88(6):145) as well as the expression of a RA-sensitive reporter transgene in the fetal gonad in vivo (Chassot et al., 2020, Sci. Adv. 6:eaaz1261). Characterizing responsiveness of promoter constructs to RA should be performed using RA concentrations as close as possible to the situation in vivo to model regulation of Stra8 in fetal ovarian germ cells in F9 EC cells.
- The complete sentence actually reads 'model ovarian germ cells with respect to Stra8 expression'. Very few cell lines, and only germ cells at particular stages of development, respond to RA by upregulating Stra8. We were testing whether F9 cells respond to modest* doses of RA by upregulating Stra8 (*actually many/most workers use 1 microM RA as standard). We do not claim to have used the precise physiological concentration (as this has not been empirically determined to date). Nonetheless, we consider 50nM reasonable because endogenous ATRA is estimated to be between 7.9 and 122.4nM in various organs of the developing embryo at 10.5 dpc (Horton et. al. 1995). Kumar et al 2011 did not determine the levels of endogenous RA in gonads, but they treated with 'physiological amounts of RA from 25 to 100nM' based on prior work of others. These cell

culture studies were simply used to analyse function of various RARE sites, which we then mutated in vivo, as the method of determining ultimate physiological relevance.

3. Line 189: the reviewer is not convinced that introducing DMRT1 in the reporter system in vitro yields a "synergistic effect". RA alone induces 5-fold (1.4 construct), 4-fold (1.5 construct) or 9-fold (2.9 construct) increase of luciferase activity. DMRT alone induces a ~2-fold increase. The effect of RA +DMRT1 is obviously cumulative rather than synergistic since it induces 10-fold (5x2 for 1.4 construct), 8-fold (4x2 for 1.5construct) and 17-fold (9x2, 2.9 construct). A synergistic effect would have produced much greater increases than those observed.

- A cumulative/additive effect would be: 7-fold (5+2 for 1.4 construct), 6-fold (4+2 for 1.5 construct) and 11-fold (9+2 for 2.9 construct) however these predicted fold changes are all lower than what is actually observed. We think the activation we observed is more accurately described as a "synergistic effect" than as an 'additive' effect.

4. Line 201: binding of RARs to the putative RARE3 sequence should be demonstrated by chromatin immunoprecipitation assays (ChIP), or at least by electrophoretic mobility shift assays (EMSA) if antibodies to RARs are not available to the authors. Otherwise, "RA/RAR/RXR action" on RARE3 is not demonstrated, as stated by the authors (line 329), especially since they seem to consider that RA is capable of producing some effects independently of RARs (see below, points #5 and #6).

- We appreciate the experiments suggested by the reviewer but we do not have access to reliable RAR antibodies for ChIP and also do not have access to purified RAR proteins to show RA/RAR/RXR action by EMSA. We have not suggested that RA is capable of producing effects independently of RARs and have reviewed our manuscript to make sure this point is consistent throughout.

5. Line 212: as to the cumulative (but not "synergistic") effect of Dmrt1 on Stra8 expression, the contribution of Dmrt1 alone appears much more marginal in Fig. 3 than in Fig. 2A. Intriguingly, transfecting Dmrt1 had no effect at all on mut-RARE1, mut-RARE3, mut-RARE1+3 constructs (light grey bars, NS). This casts doubts on the involvement of Dmrt1 in Stra8 regulation. The authors should provide evidence that Dmrt1 is expressed or functional in their transfection assays, for instance by analysing the expression of a known Dmrt1 target-gene or by introducing a Dmrt1-reporter construct in their system.

- Please note that in response to reviewer 1 comments, we have reviewed our statistical calculations and have corrected some errors including in Fig. 2A and Fig 3. For all three constructs, transfection with DMRT1 expression construct does not result in a statistically significant change in Stra8 expression.
- If the effect of DMRT1 is considered synergistic as we have proposed (addressed in point 3 above), it would be consistent to see that DMRT1 alone induces a marginal/non-significant response.
- Please also see Reviewer 1, point 5 above, regarding Dmrt1 expression in our system.

6. Lines 230 and 233: the "dominant instructive role" of RA and the fact that "RA signalling is a dominant activator" of Stra8 are not evident to the reviewer. This concept may be true in vitro, in F9 cells treated with added RA. However, Vernet et al., (2020, Sci. Adv. 6:aaz1139) show that the expression of Stra8 in RAR-null mutants (i.e., in vivo) is delayed, but not switched off: its level at 14.5 dpc reaches the level observed in control ovaries at 13.5 dpc, which indicates a delay but not an impairment. Because all RA-dependent effects necessarily goes through RARs (Kashyap et al., 2013, 126(4):999; Laursen & Gudas, 2018, J. Biol. Chem. 293(30):11891), this means that Stra8 expression can be achieved in vivo in the total absence of a functional RA-signalling pathway. Even though RA enhances Stra8 expression, the notion of "dominant" and "instructive" do not apply in vivo: 50% is not dominant, and something else than RA clearly instructs Stra8 expression.

- The reviewer agrees that in the F9 cell system RA plays a dominant and instructive role in vitro and, since lines 230 and 233 (in the original manuscript) refer to our in vitro experiment, our interpretation remains valid. We have left the first mention of this (now line 248) but have changed the second mention to 'our in vitro F9 cell studies suggest that RA acts directly to activate the 2.9 kb Stra8 promoter, using both RARE1 and RARE3 sites' (now lines 252-254).

7) Line 255: the fact that "approximately 40% of Stra8 expression" is retained at 14.5 dpc in mice lacking RARE1 and RARE3 is in agreement with the finding that Stra8 is expressed in RAR-null mutant ovaries. It is not necessary to propose that additional RAREs lie beyond the promoter

fragment that was used in the present MS, except if the authors purposely denies the work done by others (Vernet et al., 2020, Sci. Adv. 6:aaz1139). The authors should test the expression of Stra8 at 15.5 dpc and might discovered an expression recovered at a normal level, despite the lack of RAREs sequences. This would not be surprising since homozygous females harbouring RARE1/3 double mutations are fertile (line 259).

- We recognise the similarities in Stra8 expression levels between our RARE1/3 mutant and RAR mutant ovaries by Vernet et. al. 2020. However similar expression levels is not enough to assume the same mechanism when our 2 studies were examining different things. We did not remove (or reduce) RA, therefore any RARE other than the two we mutated would be capable of responding to this signal. Therefore, it would be remiss of us to exclude this as a possibility, even in light of Vernet et al 2020.
- At the suggestion of reviewer 1 we have now visualized the residual (40%) STRA8 expression in the RARE1/3 mutant, and we find that begins at the most anterior region of the ovary (Fig. S7). As RA is known to be most prevalent in that region, this interesting finding supports our hypothesis that RAREs may exist further upstream, in vivo. We have also now included other possibilities: that ZGLP1 is driving Stra8 expression, in the presence of RA (lines 398-403) or that RA acts in that region to induce WNT4, which impacts on Stra8 expression, as suggested by others (lines 391-393).
- We are not purposely denying the work of others, we are simply reporting on, and discussing, the results we have found in this study. We have attempted to put our findings into context with respect to all relevant studies. Additional discussion of the work of Vernet et al (2020) and Chassot et al (2020) is now included in the discussion (lines 423-431).
- We were practically unable to collect more data points at 15.5 dpc as suggested, although we did attempt this (colony were numbers low due to COVID and we were unlucky with many non-pregnancies and/or incorrect timepoints). As the Reviewer states, and we agree, Stra8 must reach sufficient levels eventually as these mice are ultimately fertile, as we report here.

8. Line 263: the finding that deleting 173-bp upstream of the transcription start site (TSS) in mm10 totally abolished Stra8 expression is not surprising on its own, given this region contains binding sites for many other factors such as Setd8 (Niu et al., 2020, J. Cell Mol. Med. 24:4194), Crebbp and Ep300 (Chen et al., 2012, PLoS One 8(6):e66076) or CTCF (Sleutels et al., 2012, Epigenetics Chromatin 5:8). This is also a target region required for acetylation of histone H3 (Chen et al., 2012, PLoS One 8(6):e66076) and nucleosome positioning (Sleutels et al., 2012, Epigenetics Chromatin 5:8).

- As with the point above, we do acknowledge that there may be other factors contributing to Stra8 expression and that the positive and mostly negative regulators mentioned by the reviewer are possible candidates. We note that these studies all acknowledge RA as the instructive factor in Stra8 expression despite showing their genes of interest to be involved in regulating Stra8 expression – it is quite likely that other factor(s) act on the deleted 173bp region and contribute to the ablation of Stra8 expression (lines 412-417). We look forward to the collective effort of the scientific community to investigate what this factor may be.

Minor points:

9. Line 61: in cancer cells Stra8 is expressed as in embryonal carcinoma and embryonic stem cells (e.g., Kuang et al., 2019, Cancer Biomark. 25(2):203-212).

- The aberrant expression of Stra8 in cancer is a good point, thanks. We have mentioned this in the introduction (lines 67-68).

10. Line 146: the choice of integrating the Stra8 promoter construct at the Col1a1 gene is quite surprising given this gene is not expressed in germ cells between 11.5 and 13.5 dpc (Jameson et al., 2012, PLoS Genet. 8(3):e1002575), making the transgene locus possibly closed for proper access to transcription factors.

- Indeed we used this approach, of inserting transgenes ~ 500 bp downstream of the Col1a1 3' UTR, as detailed in Beard et al, 2006. The authors of that work chose the locus because 'it has been shown to support high transgene expression even in cell types that do not normally express the type I collagen gene'. In any case, our transgenes clearly are expressed when inserted in this location. We now note in the manuscript Results (line 158) and Discussion (line 331) that we are inserting into an open genomic locus, downstream of Col1a1.
-

11. Line 155: figure 1A is not cited in the main text.

- Fig. 1A is cited on lines 154 and 233 of the main text

12. Line 210: the data show that “mutation of both [RAREs] completely abolishes” the sensitivity of the promoter construct to RA, but not its “expression”, as proposed by the authors.
- Thank you for pointing out this mistake, we have rectified this sentence (now on line 229).

13. Line 237: given the high error rate generated using the CRISP/Cas9 method, the full sequences of the mutated loci in the mouse lines that were established should be specified as supplementary information.
- Agreed, we have now adding sequence data to our supplementary information (Figure S9 and Data S1).

14. Line 265: the genome reference used by the authors should be mentioned. By the way it is surprising that the TSS of Stra8 gene is at 6:34 870 961 in GRCm38 mm10 in NCBI, while it is at 6:34 870 638 in Ensembl GRCm38.p6. Depending of the genuine TSS, the interpretation of the data presented in this MS might be different since the regulatory regions would be comprised either in the promoter or in the 5’ untranslated region of Stra8 mRNA.
- This is a very good suggestion. We have now cited GRCm38.mm10 in our methods (line 601)
- As the co-ordinates of Stra8 TSS varies with each genome assembly version and previous versions may not always be accessible we have also added a definition of the TSS as the beginning of RefSeq. NM_009292.1 in our manuscript (line 602). This definition will remain independent of past and future genome assemblies.

Second decision letter

MS ID#: DEVELOP/2020/194977

MS TITLE: Identification of regulatory elements required for Stra8 expression, in fetal ovarian germ cells of the mouse

AUTHORS: Chun-Wei Allen Feng, Guillaume Burnet, Cassy M Spiller, Fiona Ka Man Cheung, Kallayanee Chawengsaksophak, Peter Koopman, and Josephine Bowles

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 almost opposite responses to your revised version that, I guess, may represent controversy in your field. The reviewer 1 (R1) realized that you made the maxima efforts under this complicated social situation and, thus, expressed the biggest support. In contrast, the R2 may be happy for the technical part of the study, nonetheless, found the interpretation of the study is too much biassed to the people at the another side. I think the experimental setup in this study represents physiological situation and the conclusion should be robust. However, it is also very difficult to exclude all confounding factors that may influence experimental outputs. Could you re-write the text so as to give more emphasis on the controversy in your field? Your revised paper will be re-reviewed by the referees, and acceptance of your manuscript will depend on your addressing satisfactorily her/his 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

The authors have put together a well-argued rebuttal and clarified a number of points. I have a few remaining comments, which amount to textual amendments/suggestions. Nice work, well done.

Remaining issues:
1. Quantitative IF. This is helpful to see. The authors should include the number of cells quantified, and a brief mention of the methodology used would be helpful. This data has revealed a relatively low overlap between the reporter and Stra8. Which I think should be discussed. Presumably this might be technical, variable expression from the targeted locus, or due to the need for other regulatory elements. The absence of ectopic expression is important however, showing the reporter is specific. The interpretation here is particularly important for later statements such as Line 191' includes all regulatory elements' – if all elements are present then why are so many cells negative?

2. Epigenetic regulation:
a) Line 67-72: Epigenetic regulation is pitched as the only possible explanation as to why Stra8 is so tightly regulated. This is of course a reasonable hypothesis, and the authors go on to cite literature regarding epigenetic regulation. However, it seems equally feasible that lack of the right combination of transcription factors, or certain co-factors play a role. Perhaps the authors might reconsider opening with such a strong statement that already seems to presume the regulation is epigenetic.
b) Line 86: 'Hence, as there is no evidence that epigenetic changes are sex-specific'. It is not clear what is meant by this statement – many epigenetic changes are sex-specific.
While many good points are made in this section, I wonder if the overarching narrative could be tidied up somewhat. Finally epigenetic regulation also makes a prominent return in the Discussion (Line 336), which is a bit of a surprise as epigenetic regulation is not a focus of this study.

Minor points and typos:
Line 57. 'Stra8 is expressed exclusively in the germline' perhaps adding 'during normal development', as the authors do mention its expression in tumors later.
Line 64-66: Retinoic acid also induces Stra8 in cultured SSCs (Want et al. 2016, SCR, PMID: 27346680)
Line 102: Typo 'carried in rat'
Line 119: 'To resolve these issues'. The authors have undertaken an interesting investigation here and generated useful tools: a valuable contribution. However, I am not quite sure they have resolved the question of Stra8 regulation and so perhaps this wording should be revised (as they say 'the controversy continues').
Line 300: 'Construct' – is the correct terminology given that the authors are apparently referring to the endogenous locus?
Line 305: The relevance of the fertility of Stra8 heterozygous females is not clear. Has it been shown that Stra8 expression is decreased in these animals?
Finally, is Oct4 a direct target of Stra8? Or does maintained expression reflect a more global failure/delay in differentiation?

Comments for the author

-

Reviewer 2

Advance summary and potential significance to field

identical to the initial review.

Comments for the author

Lines 408-436: While the authors now discuss the possibility of the involvement of BMP in the regulation of Stra8 evoked by reporter # 1, they still do not consider the possibility that the residual expression of Stra8 observed in mutants RARE1 + RARE3 can optionally be independent of retinoic acid. This likely reflects their conviction that retinoic acid necessarily regulates Stra8.

Second revision

Author response to reviewers' comments

Reviewer 1

Major points:

1. Quantitative IF. This is helpful to see. The authors should include the number of cells quantified, and a brief mention of the methodology used would be helpful. This data has revealed a relatively low overlap between the reporter and Stra8. Which I think should be discussed. Presumably this might be technical, variable expression from the targeted locus, or due to the need for other regulatory elements. The absence of ectopic expression is important however, showing the reporter is specific. The interpretation here is particularly important for later statements such as Line 191' includes all regulatory elements' – if all elements are present then why are so many cells negative?

- Total number of cells counted has been added to the figure legend of Figure S3
- Breif description of cell counting methodology was added as suggested (lines 599-602).
- We have integrated the suggested possibilities for the delay in eGFP production when compare to STRA8 to our discussion on this topic (lines 355-363).
- On lines 191-193 we have added a comment that no significant GFP signal was observed outside of the developing ovaries during dissections of 12.5 to 16.5dpc embryos (observational, data not shown).
- The statement now on lines 197-200 has been refined and toned down to correct for the new quantitative data that show more negative cells than originally anticipated.
- The manuscript is impoved by exploration of this point and we thank the reviewer.

2. Epigenetic regulation:

a) Line 67-72: Epigenetic regulation is pitched as the only possible explanation as to why Stra8 is so tightly regulated. This is of course a reasonable hypothesis, and the authors go on to cite literature regarding epigenetic regulation. However, it seems equally feasible that lack of the right combination of transcription factors, or certain co-factors play a role. Perhaps the authors might reconsider opening with such a strong statement that already seems to presume the regulation is epigenetic.

- The possible regulation by absence of critical co-regulator was added as suggested (lines 69-72).
- We feel that it is important to introduce epigenetic regulation of Stra8 so as to be comprehensive, even though they are not the focus of our study (we do not wish readers to think that these are not important considerations). We also wanted to highlight that, to date, such modifications appear to be permissive and not instructive.

b) Line 86: 'Hence, as there is no evidence that epigenetic changes are sex-specific'. It is not clear what is meant by this statement – many epigenetic changes are sex-specific. While many good points are made in this section, I wonder if the overarching narrative could be tidied up somewhat. Finally epigenetic regulation also makes a prominent return in the Discussion (Line 336), which is a bit of a surprise as epigenetic regulation is not a focus of this study.

- Statement was reworked as suggested for better narrative (lines 86-88). We meant that there are no known sex-specific epigenetic changes in this context, but we have removed this wording in any case, to avoid confusion.
- We have retained the mentioning of epigenetic regulation (lines 342-345) to re-enforce that although important, this is not the focus of this study as the reviewer has pointed out. To make the logic more obvious we have added the word 'however' to the next sentence.

Minor points and typos:
Line 57. 'Stra8 is expressed exclusively in the germline' perhaps adding 'during normal development', as the authors do mention its expression in tumors later.
- Great suggestion, done (lines 56-57).

Line 64-66: Retinoic acid also induces Stra8 in cultured SSCs (Want et al. 2016, SCR, PMID: 27346680)
- Wang et al. 2016 and text added as suggested (lines 65-67).

Line 102: Typo 'carried in rat'
- Corrected (line 104)

Line 119: 'To resolve these issues'. The authors have undertaken an interesting investigation here and generated useful tools: a valuable contribution. However, I am not quite sure they have resolved the question of Stra8 regulation and so perhaps this wording should be revised (as they say 'the controversy continues').
- We have revised this sentence (now Lines 121-123)

Line 300: 'Construct' – is the correct terminology given that the authors are apparently referring to the endogenous locus?
- Corrected to 'locus' (line 309)

Line 305: The relevance of the fertility of Stra8 heterozygous females is not clear. Has it been shown that Stra8 expression is decreased in these animals?
- Apologies, this has indeed yet to be shown in a publication so we have removed this statement (line 313). We also removed a similar statement on lines 418-419.

Finally, is Oct4 a direct target of Stra8? Or does maintained expression reflect a more global failure/delay in differentiation?
- Oct4 does not appear to be a direct target of STRA8 as no significant binding is found by ChIP (Kojima et al. 2019 PMID: 30810530) thus its maintained expression is likely to represent a failure/delay in differentiation/meiotic initiation. We have not included this in our MS as we feel that it is beyond the current scope.

Reviewer 2 Comments for the author
Lines 408-436: While the authors now discuss the possibility of the involvement of BMP in the regulation of Stra8 evoked by reporter # 1, they still do not consider the possibility that the residual expression of Stra8 observed in mutants RARE1 + RARE3 can optionally be independent of retinoic acid. This likely reflects their conviction that retinoic acid necessarily regulates Stra8.
- We have amended text throughout the Discussion (lines 421-479) to make it clearer that non-RA factors may also be in play. In particular we have replaced the portion of text that may be considered overly critical of the RA-independent possibilities (originally 450-466) with an new paragraph (lines 448-462) where we discuss how our current work does not necessarily contradict the work by Kumar 2011, Chassot 2020, Vernet 2020 and Bellutti 2019.

Third decision letter

MS ID#: DEVELOP/2020/194977

MS TITLE: Identification of regulatory elements required for Stra8 expression, in fetal ovarian germ cells of the mouse

AUTHORS: Chun-Wei Allen Feng, Guillaume Burnet, Cassy M Spiller, Fiona Ka Man Cheung, Kallayanee Chawengsaksophak, Peter Koopman, and Josephine Bowles

I have now received Reviewer 2's report on the above manuscript, and have reached a decision. The referee's comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referee's comments can be satisfactorily addressed. Please attend to all of the reviewer 27s' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

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Comments for the author

The authors have dealt with all of my comments. I recommend publication. It has been a pleasure to review this manuscript and I hope this has been a productive review process from the authors' side too. Congratulations on a nice piece of work and a job well done.

Reviewer 2

Advance summary and potential significance to field

The MS DEVELOP/2020/194977 by Feng et al. describes the role of two retinoic acid (RA) response elements (RAREs) for the RA-dependent expression of Stra8 in F9 cells cultured in vitro, in the presence of a supra-physiological dose of RA (50nM). The authors identified a yet unknown RARE located ~1.3kb upstream of the transcription start site (TSS) called RARE3, and confirmed the presence of another one called RARE1, located ~200-bp upstream of the TSS, identified 18 years ago and largely analysed by others since. The authors showed that each of these RAREs contributes to ~50% of the RA-dependent regulation in F9 cells. When transposed in vivo by CRISPR/Cas9-directed mutagenesis, the RAREs contribute together for ~50% of the total Stra8 expression in the mouse fetal gonad. This finding corroborates the study recently published, showing that Stra8 expression occurs in germ cells of mutants lacking all RA-receptors (RARs), albeit at a level which is about 50% of the control level.

Comments for the author

Lines 112-115 : The results published in the cited references are not “attempts” fuelling a controversy, but rather facts that the authors cannot brush aside simply because the published results do not fit with their theory. The authors should modify this sentence.

Lines 461-463: The reverse is also true. Although their findings are interesting, they cannot be considered to the exclusion of prior evidence that RAR and RA-synthesizing enzymes do not play a major role in inducing Stra8 expression (Chassot et al., 2020; Vernet et al., 2020). The authors should change their discussion accordingly.

Third revision

Author response to reviewers' comments

Reviewer 2
Lines 112-115 : The results published in the cited references are not “attempts” fuelling a controversy, but rather facts that the authors cannot brush aside simply because the published results do not fit with their theory. The authors should modify this sentence.

- We would like to point out that the data in the referenced published works do show that complete ablation of RARs or RA-producing enzymes was not achieved, and thus we had worded the efforts ‘attempts’ to deplete RARs and RA-producing enzymes. It was not meant to be interpreted as an ‘attempt’ to fuel the controversy as the reviewer’s comment suggested. Nevertheless, we do not intend any offence and we have changed the wording so to remove ‘attempts’. Lines 115-116.

Lines 461-463: The reverse is also true. Although their findings are interesting, they cannot be considered to the exclusion of prior evidence that RAR and RA-synthesizing enzymes do not play a major role in inducing Stra8 expression (Chassot et al., 2020; Vernet et al., 2020). The authors should change their discussion accordingly.

- It appears the reviewer may be referring to the first revision of manuscript where lines 461-463 did read “Although these findings are interesting, they cannot be considered to the exclusion of prior evidence that RA does play a major role in inducing Stra8 expression, as detailed above.”. This sentence was removed in the latest revision.
- We have slightly expanded and modified a related statement on lines 462-464 to make it very clear that we accept that an as yet unidentified factor(s), that functions independently of RARs or RA synthesising enzymes, may be involved.

Fourth decision letter

MS ID#: DEVELOP/2020/194977

MS TITLE: Identification of regulatory elements required for Stra8 expression, in fetal ovarian germ cells of the mouse

AUTHORS: Chun-Wei Allen Feng, Guillaume Burnet, Cassy M Spiller, Fiona Ka Man Cheung, Kallayanee Chawengsaksophak, Peter Koopman, and Josephine Bowles
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

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Comments for the author

As stated previously, I think this paper is ready.
My opinion is that the authors have been very accommodating of alternate hypotheses, and that this work makes an important contribution. The recent conditional knockout papers are of course

interesting too, but there are technical issues which mean they are not fully conclusive - most notably the data on the timing and extent of deletion is not completely clear. I look forwards to reading more papers on this topic in the years to come, and hopefully with more studies we will have an even better understanding and the field will approach consensus.

Reviewer 2

Advance summary and potential significance to field

Idem previous review

Comments for the author

Lines 112-117 in v3 reads : "Although we now know that another RA-synthesising enzyme, encoded by Aldh1a1, is also present and able to produce RA and induce Stra8 in the fetal ovary (Bowles et al., 2016), there have been additional recent attempts to demonstrate that depletion of RA-producing enzymes, or RARs, does not ablate Stra8 expression, and the controversy continues (Bellutti et al., 2019; Chassot et al., 2020; Kumar et al., 2013; Raverdeau et al., 2012; Vernet et al., 2020).

Lines 110-115 in v2, reads "Although we now know that another RA-synthesising enzyme, encoded by Aldh1a1, is also present and able to produce RA and induce Stra8 in the fetal ovary (Bowles et al., 2016), there have been additional recent attempts to demonstrate that depletion of RA-producing enzymes, or RARs, does not ablate Stra8 expression, and the controversy continues (Bellutti et al., 2019; Chassot et al., 2020; Kumar et al., 2013; Raverdeau et al., 2012; Vernet et al., 2020).

The two versions are identical. The wording is therefore not changed, contrary to what is stated by the authors in their rebuttal letter.

Lines 448-463. The modified paragraph now better reflects the facts, as they are published, and the discussion on the possibility of a mechanism other than RA involved in the regulation of Stra8 is appreciated.