

KLB, encoding β -Klotho, is mutated in patients with congenital hypogonadotropic hypogonadism

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

10 February 2017

Thank you for the submission of your manuscript to EMBO Molecular Medicine and importantly, thank you for your patience while we completed the peer-review process. We have now heard back from the two referees whom we asked to evaluate your manuscript.

As you will see from the comments below, both reviewers appreciate the interest of the findings, however both have concerns about the pathogenic role of the heterozygous mutations described and a deeper analysis of the genotypes and putative bigenic role should be provided; referee 2 in addition, would like to see some structural modeling, better discussion and more details to help in defining some mechanism.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the space and time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our guidelines to accelerate the editorial process should your manuscript move forward.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks):

The authors previously identify an FGFR1 p.L342S mutation in a patient with Kallmann syndrome and identify FGF8 as a critical ligand for FGFR1 in GnRH biology during embryonic development. This patient also had metabolic phenotypes with severe insulin resistance. In post-natal biology FGF21 signals through FGFR1C in the presence of beta-Klotho. The authors suspect that defects in FGF21/KLB/FGFR1 signaling pathway may be involved in GnRH deficiency in humans and rodents. A candidate gene approach in 334 patients with congenital hypogonadotropic hypogonadism allowed the identification of 7 heterozygous mutations in KLB, six missense and one inframe deletion. Most patients exhibit metabolic defects. All deletions had a deleterious effect in vitro. Complementation studies in *C. elegans* deleted of the two homologs *klo-1* and *klo-2* showed that the mutants failed or had a decrease ability to rescue the cyst phenotype of the double deleted mutant. *Klb* deficient mice had delayed puberty and subfertility due to defects in GnRH secretion. KLB is expressed in the post-natal hypothalamus. FGF21 stimulates neurite outgrowth in mature GnRH neurons in vitro and induces GnRH secretion in median eminence (ME) explants ex vivo. Peripheral FGF21 reaches GnRH neurons through fenestrated vessels of ME or vascular organ of lamina terminalis in vivo.

This is an interesting work with many different molecular and cellular approaches in vitro and in vivo which demonstrate convincingly the role of KLB in GnRH biology.

However I have questions concerning the pathogenic role of the mutations described in this study:

1) Are these heterozygous mutations sufficient to impair KLB function in vitro and in vivo? Indeed one allele is not mutated in the patients studied. The dominant negative effect of the mutations has not been demonstrated. Increasing amounts of mutated versus wild type KLB expression vectors could highlight such an effect in vitro. Alternatively the authors have not shown the existence of haploinsufficiency. This is critical to establish a direct link between these heterozygous mutations and a pathogenic effect in IHH.

Animal models do not evidence a role for heterozygous mutants: Overexpression in *C. elegans* have not been performed in the context of a normal allele. Complementation in the context of deleted *Klb* *C. elegans* does not reproduce the heterozygous genotypes of the patients. Furthermore a partial rescue of the cyst phenotype is nevertheless observed with four mutants in *klo-2* and *klo1* deleted *C. elegans*, which is in keeping with a partial loss of function of the mutants in this model. The homozygous *Klb* knock mice exhibit only a mild reproductive phenotype. What is the phenotype of heterozygous mice?

If neither dominant negative effect nor haploinsufficiency are found, CGH array should be performed to eliminate a deletion in the second allele not evidenced by Sanger analysis. Why do 83 patients had exome studies and 251 Sanger studies of FGF21 and KLB genes? Which patients had exome studies? Were *Klb* deletions eliminated in those cases?

2) In some cases the mutations are associated with mutations of other genes responsible for IHH and the authors suspect a digenic effect. Why should the same p.F777delF mutation be deleterious when associated with a PROKR2 mutation in one case and alone in a patient with a similar phenotype? In the absence of evidence of dominant negative effect of these heterozygous mutations or of haploinsufficiency the authors should discuss the study of other genes potentially involved. How many such patients had exome studies? What was the result?

Referee #2 (Remarks):

The manuscript by Xu et al reports variants of klotho-beta, the FGF21 co-receptor, as a potentially

new molecular genetic cause of congenital hypogonadotropic hypogonadism. They also characterize the role of klotho beta for reproductive maturation in a knockout mouse, and test the potential roles of FGF21 in hypothalamic GnRH-neurons. The paper is to my opinion interesting and expands knowledge of the roles of FGF21 in signaling and the FGFR1/KLB/FGF21 axis as a cause of CHH.

The authors hypothesized that a specific CHH-associated FGFR1 variant, known to affect FGF8 binding site, also would inhibit binding of beta-klotho (KLB), a necessary coreceptor for FGF21 binding to FGFR1. FGF21 did not show mutations, but in sequencing of 334 patients with CHH, they identified 13 probands with seven different heterozygous rare variants in KLB. Seven probands carried a "hotspot" mutation of F777delF, being enriched to this patient population (frequency 0.2 in CHH vs 0.001 in ethnically matched population). They test these variants in a cell-based reporter system and in *c. elegans* model rescue assay, implicating variable functional defects of the putative mutations, supporting the contributing role of most of them in CHH. These data are quite intriguing.

Comments:

- 1) In their reporter system, the identified KLB variants showed decreased maximal response, with the exception of 309W variant, which is also poorly conserved; *Xenopus* carries the variant W at this site, suggesting it to be a neutral change. Despite some functional findings with this variant (*c. elegans* complementation defective), the lack of conservation and mild functional effects speak against this variant to pathogenic. Similarly, the 574T variant shows little conservation and little functional effects. However, other variants affect highly conserved sites, appear as functionally defective, and therefore as potentially pathogenic. Please revise the text to indicate the likely neutral vs likely pathogenic variants.
2. They find KLB variants to exist in some patients in combination with known CHH/Kallman sdr variants and state: "This finding indicates that some degree of oligogenicity is present in this population." - Just the co-occurrence does not indicate, but at best it may suggest a bigenic contribution. It still remains a possibility that the KLB variants are not required for the phenotype in the case other mutations exist. Are the patients with potential bigenic alleles more severely affected than those that carry single alleles?
3. One subject had KLB F777delF and FGFR1 R78C, which they coexpressed in their reporter assay, showing synergistic effect. In other assays they used the FGFR1 L342 mutant. Do the authors predict that L342 and R78 are close to each other in 3D structure, so that both of them would affect klotho binding? Structural modeling of the mutations to FGFR1 structure would be useful, to illustrate the potential binding site.
4. The second part of their study is the characterization of KLB-KO mice, which are found to have delayed reproductive maturation, nicely fitting to the role of KLB in CHH - but not indicating a link. They find no developmental defects in the organization of GnRH-neurons, or any changes in gonadotropin metabolism, and therefore they conclude that the potential effect of KLB/FGF21 for hypothalamus would be homeostatic. They then go on to study how FGF21 can reach hypothalamic GnRH neurons from the periphery and show that FGF21 stimulates GnRH release from hypothalamus, and that in KLB-KO this does not occur. Whether their identified KLB variants blunt the release, remains open. Any cell culture studies with patient mutations (iPSC derived CHH patient neurons) and their responses to FGF21 would shift the evidence to a completely different level. I do acknowledge that such materials are not easy to get, and therefore this is not a requirement for further experimentation.
5. Figure 5F-G present how fluorescent FGF21 can extravasate specifically in the area of median eminence where BBB is not tight and the GnRH-positive neurons become in contact with periphery. I really tried to see what the authors aim to show in 5F, but the findings were not at all evident from the figures. If they wish to show that FGF21 is first inside the vessels/capillaries and then extravasates, they should at least use a vascular fluorescent marker to point the sites of capillaries vs parenchyma. The labeling in the figure "fluorescent FGF21 vs fluorescent FGF21-GFP" is not clear. GFP is in GnRH neurons, right? Mark this clearly. What is the blue staining?
6. The result that FGF21 stimulates GnRH release would suggest that the liver - the main metabolic organ secreting FGF21 as a response to fasting (in mice, not as clearly in humans) - would regulate reproductive hormone signaling through hypothalamus. This is quite an intriguing possibility,

linking reproductive status to nutrient availability. Is there data suggesting that obese individuals show delayed sexual maturation (lack of fasting periods)? In humans the data suggests the opposite. A short speculation on the relevance of the findings to FGF21 expression and feeding vs. reproduction could be included.

Minor comment:

Terminology: instead of mutants, they should call KLB-changes as variants. This study reports potential variants in CHH, but especially the potential bigenic roles remain to be verified.

1st Revision - authors' response

10 May 2017

We thank you and the Reviewers for the diligent and insightful review of our manuscript. We have responded to each comment and have adapted the manuscript accordingly. The changes are marked in blue in the revised manuscript. We hope that you will find the revised version suitable for publication in *EMBO Molecular Medicine*.

Thank you very much for your kind consideration of our work.

Responses to Reviewers

Referee #1:

The authors previously identify an FGFR1 p.L342S mutation in a patient with Kallmann syndrome and identify FGF8 as a critical ligand for FGFR1 in GnRH biology during embryonic development. This patient also had metabolic phenotypes with severe insulin resistance. In post-natal biology FGF21 signals through FGFR1C in the presence of beta-Klotho. The authors suspect that defects in FGF21/KLB/FGFR1 signaling pathway may be involved in GnRH deficiency in humans and rodents. A candidate gene approach in 334 patients with congenital hypogonadotropic hypogonadism allowed the identification of 7 heterozygous mutations in KLB, six missense and one inframe deletion. Most patients exhibit metabolic defects. All deletions had a deleterious effect in vitro. Complementation studies in C. elegans deleted of the two homologs klo-1 and klo-2 showed that the mutants failed or had a decrease ability to rescue the cyst phenotype of the double deleted mutant. Klb deficient mice had delayed puberty and subfertility due to defects in GnRH secretion. KLB is expressed in the post-natal hypothalamus. FGF21 stimulates neurite outgrowth in mature GnRH neurons in vitro and induces GnRH secretion in median eminence (ME) explants ex vivo. Peripheral FGF21 reaches GnRH neurons through fenestrated vessels of ME or vascular organ of lamina terminalis in vivo.

This is an interesting work with many different molecular and cellular approaches in vitro and in vivo which demonstrate convincingly the role of KLB in GnRH biology.

We thank the Reviewers for the positive review of our study and have responded to each of the points below.

However I have questions concerning the pathogenic role of the mutations described in this study: 1) Are these heterozygous mutations sufficient to impair KLB function in vitro and in vivo? Indeed one allele is not mutated in the patients studied. The dominant negative effect of the mutations has not been demonstrated. Increasing amounts of mutated versus wild type KLB expression vectors could highlight such an effect in vitro. Alternatively the authors have not shown the existence of haploinsufficiency. This is critical to establish a direct link between these heterozygous mutations and a pathogenic effect in IHH. Animal models do not evidence a role for heterozygous mutants: Overexpression in C. elegans has not been performed in the context of a normal allele. Complementation in the context of deleted Klb C. elegans does not reproduce the heterozygous genotypes of the patients. Furthermore a partial rescue of the cyst phenotype is nevertheless observed with four mutants in klo-2 and klo1 deleted C. elegans, which is in keeping with a partial loss of function of the mutants in this model. The homozygous Klb knock mice exhibit only a mild reproductive phenotype. What is the phenotype of heterozygous mice? If neither dominant negative effect nor haploinsufficiency are found, CGH array should be performed to eliminate a deletion in the second allele not evidenced by Sanger analysis. Why do 83 patients had exome studies and 251

Sanger studies of FGF21 and KLB genes? Which patients had exome studies? Were Klb deletions eliminated in those cases?

This is an important comment. A dominant negative mode of action is indeed a feasible mechanism that could underlie the pathogenicity of the heterozygous *KLB* mutations. Through biochemical studies we have previously shown that KLB enhances FGF21-FGFR1c binding and hence promotes FGF21 signaling by simultaneously tethering FGF21 and FGFR1c to itself through two distinct sites (Goetz et al, 2012). If the loss-of-function mutations impaired KLB function without harming the FGF21 or FGFR1c binding sites then such mutant KLB alleles would competitively inhibit formation of KLB-FGFR1c-FGF21 ternary complex containing the wild type KLB allele, i.e. act in a classical dominant negative fashion. To test this possibility, we compared the binding of wild type and mutated KLB alleles with FGFR1c via immunoprecipitation experiments. All the KLB mutants coprecipitated with FGFR1c to the same extent as the wild type KLB, despite the fact they all incurred a loss in the ability to support FGF21 signaling as shown in the initial manuscript (Figure 1C-D). This finding is consistent with a dominant negative effect. We have added the new results in the revised manuscript (Page 8, Paragraph 3, Figure 1E).

As suggested by the Reviewer, we further investigated the reproductive phenotype of *Klb* heterozygous (*Klb*Het) female mice. We found that the *Klb*Het mice exhibit disrupted estrous cycles, blunted LH levels at estrus stage and impaired fertility (assessed by a short-term fertility test) – these phenotypes are similar to those of the *Klb*KO mice. These findings indicate that the phenotype of the *Klb*Het mice is due to haploinsufficiency. We have added the reproductive phenotype of the *Klb*Het mice as well as a discussion point in the revised manuscript (Page 13, Paragraph 2; Page 18, Paragraph 1; Figure EV4).

Thus, we conclude that in patients harboring heretozygous loss-of-function mutations in *KLB*, both mechanisms likely operate in parallel: a dominant-negative effect and a haploinsufficiency effect.

This explanation would be consistent with the partial rescue observed with some mutations in *C. elegans*, where, as the Reviewer correctly points out, no wild-type allele was present. Although the primary aim of the assay was to further test the functionality of *KLB* mutants (loss-of-function *in vitro*), we agree with the Reviewer that the results suggest mechanisms beyond an exclusive dominant-negative effect.

We performed Sanger sequencing in the 1st cohort (n = 251) and exome sequencing in the 2nd cohort (n = 83). Among the 13 *KLB* mutations, 11 were identified by Sanger and 2 by exome (this is now indicated in the revised Table 2). We further performed copy number variation calling in all available exomes; using the XHMM algorithm (Fromer et al, 2012), we did not identify any deletion within or around the *KLB* gene region. Furthermore, we evaluated *KLB* mRNA expression in lymphoblast cells from two available patients from the Sanger cohort (Subject 5 and Subject 7), both of whom harbor the *KLB* p.F777delF mutation. Sequencing the cDNA showed a heterozygous peak at the mutation site, demonstrating that both alleles are expressed at the mRNA level. Based on these findings, we conclude that a deletion of the second allele in *KLB* is unlikely to be a significant contributor in our cohort.

2) In some cases the mutations are associated with mutations of other genes responsible for IHH and the authors suspect a digenic effect. Why should the same p.F777delF mutation be deleterious when associated with a PROKR2 mutation in one case and alone in a patient with a similar phenotype? In the absence of evidence of dominant negative effect of these heterozygous mutations or of haploinsufficiency the authors should discuss the study of other genes potentially involved. How many such patients had exome studies? What was the result?

Indeed, all of the 13 CHH probands harboring *KLB* mutation were screened for other 18 major CHH genes either by Sanger sequencing or by exome sequencing. As outlined in the initial manuscript, 5 out of 13 patients have additional mutations in known CHH genes. Further, the fact that we have 7 patients with the same p.F777delF mutation allowed us to explore genotype-phenotype correlations. In agreement with the Reviewer's comment, we found phenotypic variation in these patients, which suggests that other factors, such as additional unknown genetic modifiers or environmental interactions, may contribute to the pathogenesis of CHH. The fact that genotype-correlations are not optimal is consistent with the generally appreciated fact that there remain 50% of CHH patients for

whom no pathogenic mutation is known (Boehm et al, 2015), suggesting that additional mutations in currently unknown genes remain to be discovered. These points have been added in the Discussion (Page 16, Paragraph 2).

Referee #2:

The manuscript by Xu et al reports variants of klotho-beta, the FGF21 co-receptor, as a potentially new molecular genetic cause of congenital hypogonadotropic hypogonadism. They also characterize the role of klotho beta for reproductive maturation in a knockout mouse, and test the potential roles of FGF21 in hypothalamic GnRH-neurons. The paper is to my opinion interesting and expands knowledge of the roles of FGF21 in signaling and the FGFR1/KLB/FGF21 axis as a cause of CHH.

The authors hypothesized that a specific CHH-associated FGFR1 variant, known to affect FGF8 binding site, also would inhibit binding of beta-klotho (KLB), a necessary coreceptor for FGF21 binding to FGFR1. FGF21 did not show mutations, but in sequencing of 334 patients with CHH, they identified 13 probands with seven different heterozygous rare variants in KLB. Seven probands carried a "hotspot" mutation of F777delF, being enriched to this patient population (frequency 0.2 in CHH vs 0.001 in ethnically matched population). They test these variants in a cell-based reporter system and in c. elegans model rescue assay, implicating variable functional defects of the putative mutations, supporting the contributing role of most of them in CHH. These data are quite intriguing.

We thank the Reviewer for the positive remarks and have responded to each the points below.

Comments:

1) In their reporter system, the identified KLB variants showed decreased maximal response, with the exception of 309W variant, which is also poorly conserved; Xenopus carries the variant W at this site, suggesting it to be a neutral change. Despite some functional findings with this variant (C. elegans complementation defective), the lack of conservation and mild functional effects speak against this variant to pathogenic. Similarly, the 574T variant shows little conservation and little functional effects. However, other variants affect highly conserved sites, appear as functionally defective, and therefore as potentially pathogenic. Please revise the text to indicate the likely neutral vs likely pathogenic variants.

We agree with the Reviewer that R309 residue is not conserved in Xenopus, but actually Xenopus carries N at this site rather than W, thus the difference is likely less dramatic compared to W (R being basic, N being polar and W being nonpolar). We apologize for the low resolution of Figure S1 and have provided figures with higher resolution in our revised manuscript. Further, we have shown that R309W exhibited a 3-fold increase in EC₅₀ compared to WT in the reporter assay (0.52 M vs 0.15 M, $p < 0.001$), consistent with loss-of-function.

We further assessed the KLB variants according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) (Li & Wang, 2017; Richards et al, 2015), which integrate evidence from population data, computational algorithms (including conservation), functional assays and segregation data. All the KLB variants except p.L1011P were classified as pathogenic or likely pathogenic variants, while p.L1011P was classified as 'variant of uncertain significance'. We have added this information in the revised manuscript (Page 10, Paragraph 2; Table 1).

2. They find KLB variants to exist in some patients in combination with known CHH/Kallman sdr variants and state: "This finding indicates that some degree of oligogenicity is present in this population." - Just the co-occurrence does not indicate, but at best it may suggest a bigenic contribution. It still remains a possibility that the KLB variants are not required for the phenotype in the case other mutations exist. Are the patients with potential bigenic alleles more severely affected than those that carry single alleles?

We agree with the Reviewer that the constellation of two CHH gene defects is a suggestion of oligogenicity, but not a direct proof and we have corrected this sentence in the revised manuscript (Page 11, Paragraph 2).

Apart from the observation of additional mutations in 5 out of 13 (38%) CHH patients with KLB mutations, several other lines of evidence also suggest a digenic contribution in those cases: (i) the

other mutations in known CHH gene were all found in heterozygosity, and some are known to be insufficient to cause CHH alone – CHH associated with mutations in *GNRHR* and *PROKR2* is typically inherited in a recessive fashion (Avbelj Stefanija et al, 2012; de Roux et al, 1997); (ii) the cell-based reporter assay testing both KLB F777delF and FGFR1 R78C mutants (found in Subject 9) showed an additive effect on FGF21 signaling, providing *in vitro* evidence supportive of digenicity in this particular case.

Within the families with mutations in two different genes (Pedigree 4 and 5), we observed a more severe phenotype in the probands with digenic mutations compared to their parents with single gene defects. Among probands with *KLB* mutations, nine patients (9/13, 70%) exhibit severe GnRH deficiency. The remaining four patients have partial GnRH deficiency, 3 of whom carry heterozygous *KLB* mutation alone. These observations further suggest that the genetic load may impact the phenotypic severity. We have added this point in the revised manuscript (Page 11, Paragraph 2).

3. One subject had KLB F777delF and FGFR1 R78C, which they coexpressed in their reporter assay, showing synergistic effect. In other assays they used the FGFR1 L342 mutant. Do the authors predict that L342 and R78 are close to each other in 3D structure, so that both of them would affect klotho binding? Structural modeling of the mutations to FGFR1 structure would be useful, to illustrate the potential binding site.

We thank the Reviewer for this useful comment. FGFR1 L342 and R78 residues are not close to each other in the 3D structure. L342 is a key constituent of a hydrophobic groove in the 3rd immunoglobulin domain of FGFR1c that is critical for KLB binding (Goetz et al, 2012). By contrast, R78 lies in the 1st Immunoglobulin domain of FGFR1c which is dispensable for KLB interaction (Goetz et al, 2012). Consistently, the reporter assay studying the co-transfection of FGFR1 R78C and KLB F777delF showed an additive rather than a synergistic effect on FGF21 signaling. We have corrected this in the manuscript (Page 9, Paragraph 1).

4. The second part of their study is the characterization of KLB-KO mice, which are found to have delayed reproductive maturation, nicely fitting to the role of KLB in CHH - but not indicating a link. They find no developmental defects in the organization of GnRH-neurons, or any changes in gonadotropin metabolism, and therefore they conclude that the potential effect of KLB/FGF21 for hypothalamus would be homeostatic. They then go on to study how FGF21 can reach hypothalamic GnRH neurons from the periphery and show that FGF21 stimulates GnRH release from hypothalamus, and that in KLB-KO this does not occur. Whether their identified KLB variants blunt the release, remains open. Any cell culture studies with patient mutations (iPSC derived CHH patient neurons) and their responses to FGF21 would shift the evidence to a completely different level. I do acknowledge that such materials are not easy to get, and therefore this is not a requirement for further experimentation.

We thank the Reviewer for the insightful comment. We agree that our study did not specifically elucidate how the human KLB mutants affect GnRH secretion. Thus we agree with the Reviewer that iPSC-derived CHH patient neurons would be an excellent model to examine this, and we state so in the revised manuscript (Page 19, Paragraph 1).

5. Figure 5F-G present how fluorescent FGF21 can extravasate specifically in the area of median eminence where BBB is not tight and the GnRH-positive neurons become in contact with periphery. I really tried to see what the authors aim to show in 5F, but the findings were not at all evident from the figures. If they wish to show that FGF21 is first inside the vessels/capillaries and then extravasates, they should at least use a vascular fluorescent marker to point the sites of capillaries vs parenchyma. The labeling in the figure "fluorescent FGF21 vs fluorescent FGF21-GFP" is not clear. GFP is in GnRH neurons, right? Mark this clearly. What is the blue staining?

We thank the Reviewer for this comment. To better demonstrate the dynamic diffusion of FGF21 from the fenestrated vessels, we provide a new figure with the straining of laminin as a marker of the blood vessels (Figure EV5) and we clarified the labeling of Figure 5F-G and indicated that the blue staining is the nuclear stain DAPI.

6. The result that FGF21 stimulates GnRH release would suggest that the liver - the main metabolic organ secreting FGF21 as a response to fasting (in mice, not as clearly in humans) - would regulate reproductive hormone signaling through hypothalamus. This is quite an intriguing possibility, linking reproductive status to nutrient availability. Is there data suggesting that obese individuals show delayed sexual maturation (lack of fasting periods)? In humans the data suggests the opposite. A short speculation on the relevance of the findings to FGF21 expression and feeding vs reproduction could be included.

We thank the Reviewer for this comment. Large epidemiologic studies on the timing of puberty have shown an earlier secular trend of pubertal onset in both girls and boys. This advance in pubertal onset is not significant after adjusting for BMI, suggesting that higher BMI contributes to early pubertal onset (Sorensen et al, 2012). However, obesity is also associated with delayed puberty in boys (Crocker et al, 2014) and hypogonadotropic hypogonadism in adult males (Giagulli et al, 1994). In rare cases of congenital obesity caused by mutations in *LEP* and *LEPR*, it is also known that obesity is associated with GnRH deficiency. Thus the relationship between BMI and reproductive fitness is complex in humans. Further, obese individuals appear to have higher circulating FGF21 levels, consistent with a FGF21 resistant state (Fisher et al, 2010; Zhang et al, 2008). We have added this point in the Discussion (Page 19, Paragraph 2).

Minor comment:

Terminology: instead of mutants, they should call KLB-changes as variants. This study reports potential variants in CHH, but especially the potential bigenic roles remain to be verified.

We thank the Reviewer for this suggestion. We have changed the manuscript accordingly. In the revised manuscript, we used 'putative pathogenic variants' to define rare sequencing variants which are predicted to be deleterious. As is commonly practiced, we used the term mutation only after the variants were proven to be loss-of-function. For this reason, we changed the order of the paragraphs on the phenotype-genotype correlation and the functional assays in the Results.

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2nd Editorial Decision

13 June 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

- 1) Please address the minor comments of referee 1. Please provide a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word file) and tune down the conclusion that are not fully supported by experimental data.
- 2) Please indicate in legends exact n= and exact p= values, not a range. Some people found that to keep the figures clear, providing a supplemental table with all exact p-values was preferable. You are welcome to do this if you want to.
- 3) Data deposition: we duly note that you did not obtain explicit consent to deposit the clinical data into a public repository. However I am afraid that you must do so. EGA for example allows access control of datasets should you need it. Please see below:

It is possible to submit information to the EGA while still continuing to manage access via a Data Access Committee (DAC): <https://www.ebi.ac.uk/ega/home>

It's important to stress that the Data Access Committee - which one would need to allow access to the raw data in some way - would remain unchanged. Many studies, each with managed access, do this (see: <https://www.ebi.ac.uk/ega/datasets>).

Be warned that it often takes quite a bit of time for submission. This can be fast tracked but more like 3 or 4 weeks rather than 3 or 4 days. This is because one's data access committee needs to be set up, documentation submitted around it, etc.

TEXT FROM EGA:

Who controls access to this dataset?

For each dataset that requires access control, there is a corresponding Data Access Committee (DAC) who determine access permissions. Data access requests are reviewed by the relevant DAC, not by the EGA.

The text within the study could look like this: "Our datasets were obtained from subjects who have

consented to the use of their individual genetic data for biomedical research, but not for unlimited public data release. Therefore, we submitted it to the European Genome-phenome Archive, through which researchers can apply for access of the raw data."

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Remarks):

In this revised manuscript Cheng Xu et al study the reproductive phenotype of the heterozygous KlbHet mice and now show that they exhibit disrupted estrous cycles, blunted LH levels at estrus stage and impaired fertility. Thus a mechanism of haploinsufficiency is feasible in the patients with heterozygous loss of function mutations of Klb.

However it seems that the number of litters/female mice is more important in homozygous KlbKO mice than in the case of heterozygous KlbHet mice. This is very unlikely. Can the authors check the figures (EV1 and Fig 3) and report complementary comparative studies? What is the reproductibility of such studies?

The authors try also to highlight a dominant negative mechanism by co-immunoprecipitation experiments. The experiments performed showed no difference in co-immunoprecipitation of FGFR1 when WT or mutants Klb were used. However this does not allow any conclusion on a possible dominant negative effect. Only a competitive inhibition of the formation of the Klb-FGFR1C-FGFR1 complex in the presence of increasing concentrations of mutated Klb would allow to highlight an inhibitory effect of the mutated Klb on the WT allele. Thus these conclusions must be deleted from the manuscript.

Referee #2 (Comments on Novelty/Model System):

The manuscript has improved considerably, and meets now high standard.

Referee #2 (Remarks):

The authors have satisfactorily responded to my criticism and I have no further comments. This is an interesting study.

2nd Revision - authors' response

23 June 2017

We thank you for your consideration of our manuscript. We have responded to Reviewers' comments and have adapted the manuscript accordingly. We have also made amendments according to your instructions, the major changes including:

- 1) We double checked the statistical analyses and made the figure legends more precise, with an addition of a supplementary table for all the exact *p* values.
- 2) The clinical and exome dataset deposition in European Genome-phenome Archive is ongoing. We have added the data deposition information in the Methods (Page 22, paragraph 1). We will inform you the accession number as soon as it is available.

Responses to Reviewers
Referee #1 (Remarks):

We thank the Reviewer for his positive comments on our revised manuscript.

In this revised manuscript Cheng Xu et al study the reproductive phenotype of the heterozygous KlbHet mice and now show that they exhibit disrupted estrous cycles, blunted LH levels at estrus stage and impaired fertility. Thus a mechanism of haploinsufficiency is feasible in the patients with heterozygous loss of function mutations of Klb.

However it seems that the number of litters/female mice is more important in homozygous KlbKO mice than in the case of heterozygous KlbHet mice. This is very unlikely. Can the authors check the figures (EVI and Fig 3) and report complementary comparative studies? What is the reproductibility of such studies?

As indicated in the Methods for the fertility assessment in mice, we performed a continuous mating protocol of 7 months in KlbKO mice. Due to the time limitation for the revision, we performed a short-term fertility test in KlbHET mice. The two protocols measure different parameters of fertility: the continuous protocol reports the number of litters per female and litter size, while the short-term protocol evaluates the rate of pregnancy. Therefore, the results cannot be used to compare the degree of a fertility defect between KlbKO and KlbHET. We have modified the Results, the Methods and the legend of Figure 4 (Page 13, paragraph 2; Page 25, paragraph 3; Page 48, paragraph 1) to further clarify the different tests in KlbKO and KlbHET mice.

The authors try also to highlight a dominant negative mechanism by co-immunoprecipitation experiments. The experiments performed showed no difference in co-immunoprecipitation of FGFR1 when WT or mutants Klb were used. However this does not allow any conclusion on a possible dominant negative effect. Only a competitive inhibition of the formation of the Klb-FGFR1c-FGFR1 complex in the presence of increasing concentrations of mutated Klb would allow to highlight an inhibitory effect of the mutated Klb on the WT allele. Thus these conclusions must be deleted from the manuscript.

Although our co-IP result is suggestive of a possible dominant negative effect, we agree with the Reviewer that only a competitive inhibition of receptor complex formation in the presence of increasing concentrations of KLB mutants could confirm the dominant negative effect. The co-IP assay still provides insights into the functional impact of the KLB mutant-FGFR1c complex on signaling transmission. We have thus modified the Results and the Discussion accordingly (Page 8, paragraph 3; Page 18, paragraph 1).

Referee #2 (Comments on Novelty/Model System):

The manuscript has improved considerably, and meets now high standard.

Referee #2 (Remarks):

The authors have satisfactorily responded to my criticism and I have no further comments. This is an interesting study.

We thank the Reviewer for his positive comments on our revised manuscript.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Nelly Pitteloud

Journal Submitted to: EMBO Molecular Medicine

Manuscript Number: EMM-2016-07376

Reporting Checklist for Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Page 20 and 29
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Page 29
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Page 20
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Page 27
4.b. For animal studies, include a statement about blinding even if no blinding was done	Page 27
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical tests are described in the Methods and specific statistic tests are reported in Figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Page 29
Is there an estimate of variation within each group of data?	Yes, variation is reported in each figure as standard error of the mean.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>

<http://1degreebio.org>

<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>

<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>

<http://ClinicalTrials.gov>

<http://www.consort-statement.org>

<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tur>

<http://datadryad.org>

<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>

<http://jij.biochem.sun.ac.za>

http://oba.od.nih.gov/biosecurity/biosecurity_documents.html

<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies specifications are reported in the Methods. Pages 23, 24, 27 and 29.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 22-23 and 28.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Page 25.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 29.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes, page 29.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Page 22.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Page 22.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	The ethical consents from the study participants did not include permission to publish genetic data in public databases.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	The clinical and exome dataset deposition in European Genome-phenome Archive is ongoing.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: Primary Data Wetmore KM, Deuschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA
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