EMBO reports

Manuscript EMBOR-2013-37204

Mammalian iRhoms have distinct physiological functions including an essential role in TACE regulation

Yonka Christova, Colin Adrain, Paul Bambrough, Ashraf Ibrahim and Matthew Freeman

Corresponding author: Matthew Freeman, MRC

Review timeline:

Submission date:	20 February 2013
Editorial Decision:	11 March 2013
Correspondence:	14 March 2013
Correspondence:	15 March 2013
Correspondence:	18 March 2013
Correspondence:	19 March 2013
Revision received:	04 July 2013
Accepted:	23 July 2013

Editor: Barbara Pauly

Transaction Report:

(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 11 March 2013

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that the reviewers appreciate the interest of your findings and support publication of your study in our journal. However, they also point out aspects of your study that would need to be further strengthened before publication. For instance, both referee 1 and 3 state that a more detailed phenotypic characterization of the iRhom1 single and the iRhom1/2 double knock-out mice is needed to give clues about the molecular basis for the observed abnormalities and the identity of additional iRhom substrates. While referee 3 also feels that more effort should be put into the identification of these additional substrates, we would not make this a prerequisite for publication, unless further phenotyping did not provide the hoped-for deeper insights. Of course, if you already have data on additional substrates, for example on additional ADAMs, we would recommend including this information, as all referees feel that any information to this regard would raise the impact of the findings. Referee #2 points out that it should be clarified whether some of the

observed phenotypes might depend on the background of the mice. Finally, all referees also pinpoint several technical and experimental issues that would require strengthening.

Given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only. I would also recommend combining the results and discussion section, as this helps to avoid unnecessary redundancies.

We have also started encouraging authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

This manuscript is an excellent addition to the emerging field of iRhom proteins with appear to have exciting biological and medical functions. The manuscript is a continuation of previous work by the same lab and by Tak Mak's lab. Both previous studies identified iRhom2 as an essential regulator of TACE protease activity in the immune system in vitro and in vivo. The new study generated mice deficient in the homologous protein iRhom1 as well as double deficient mice lacking both iRhom1 and iRhom2. The manuscript contains a detailed characterization of the iRhom1-deficient mice and a partial characterization of the double-deficient mice. The key finding is that both mammalian iRhoms have distinct physiological functions. Another striking and surprising finding is that the double deficient mice show a more severe phenotype than TACE-deficient mice, suggesting that iRhoms have additional client proteins besides TACE. Additionally the authors provide novel

mechanistic insights into the mechanism of iRhom action. In particular the authors demonstrate that iRhom1 is required for TACE maturation and activity outside of the immune system, similar to the known function of iRhom2 in immune cells. This part is in good agreement with a study from the Blobel lab published a few weeks ago in JCI. The manuscript is well written, easy to read, is novel and timely. The conclusions are supported by the data. The study will be of interest to a wide audience as it touches different fields in molecular biology ranging from developmental biology to proteases, immune biology, and protein trafficking.

Some major and minor points should be addressed.

Major points:
1. Given the intensive speculation in the manuscript about additional iRhom client proteins, the phenotype of the double knock-out embryos should be characterized in more detail. The current version of the manuscript mentions that the embryos die at around E9.5-10.5, but does not provide a more detailed description of the phenotype. A better knowledge of the phenotype could be correlated to other known phenotypes and may allow an immediate conclusion about additional iRhom client proteins. For example, do these embryos show skeletal abnormalities similar to Notch or presenilin-deficient mice? At least an initial characterization of the embryos needs to be shown for a better functional and mechanistic understanding of iRhoms. If space is limited in the manuscript, part of the description could be moved to the supplement.
2. Several ADAM proteases were tested and found to be not affected by iRHom deficiency. The choice of ADAMs tested is astonishing as it contains some inactive ADAMs, but lacks some of the proteolytically active ADAMs, such as ADAM12, 21, 28 and 30. In particular ADAM12 has a connection to the heart, such that its impaired maturation and activitiy may be related to the heart phenotype observed in the iRhom-deficient mice. These additional ADAM proteases need to be included into figure 5.

Minor points:
3. Describe in more detail what the 100% level corresponds to in figure 4. What would be the maximal value?
4. Show the whole scale of the x- and y-axes (starting at 0). Otherwise the effect size may be misleading.
5. The Siggs et al paper in Blood from last year needs to be cited. This also demonstrates the requirement of iRHom2 for TACE activity.

Referee #2:

In this manuscript Christova Y. et al characterize the phenotype of mice knock-out for iRhom1. A previous report by the same group showed that iRhom2 is required for the correct maturation of the metalloprotease TACE in macrophages, but not in other cell types. Accordingly, iRhom2 knock-out develop normally and apparently only show an impaired inflammatory response. In the paper reviewed here the authors show that the phenotype of mice knock-out for iRhom1 is far more severe; mice die soon after birth with generalized brain hemorrhage and a variety of somehow subtler defects. Analysis of fibroblasts from mice knock-out for iRhom1, iRhom2 or both iRhoms, shows that both iRhoms participate in the intracellular trafficking of TACE and that both have to be knocked-out to completely prevent the generation of the mature form of the metalloprotease. However the iRhoms do not participate in the maturation of other metalloprotease disintegrins. Since the phentoype of the TACE knock-out mice is not as severe as that of the double iRhom knock-out mice, the authors conclude that iRhoms likely have additional clients.

TACE was identified 15 years ago and yet its regulation is poorly understood. This report builds on the previous work on iRhom2 and greatly expands the observations described in the original Science papers (Adrain et al., 2012; McIlwain et al., 2012). In my opinion the observations presented here will be of great interest for scientists with different backgrounds. The conclusions made by the authors are fully supported by the data presented. I have only some suggestions that, if technically possible, would strengthen this already excellent paper.

The reasoning behind the statement "the fact that the double knockout is worse than a simple combination of the two single mutants implies that there is some redundancy between them" is hard to follow. This conclusion is based on the data corresponding to strain 129S6/SvEvTac, in which the defects induced by iRhom1 knock-down are remarkably milder than in the strain C57BL/6J. Following the same rational, one should conclude that the degree of functional redundancy between iRhoms is different in different strains. This is minor point but, in my opinion, the authors should not highlight the aforementioned conclusion.

Fig. 3A. The total levels of TACE seems to be reduced in the iRhoms knock-out and further reduced in the double knock-out. Is this impression correct? If so, the authors should comment on it.

Immunohistochemisty or indirect inmmunofluoresce to determine the subcellular localization of TACE in double knock-out embryos would greatly support the conclusions drawn from the data presented in Fig. 3. One would expect only intracellular TACE in double knock-outs. These data would also partially address the "formal possibility that mature TACE in only a small proportion of cells would be undetectable".

Referee #3:

Christova et. al. report about the phenotype of iRhom knockout mice with a special focus on the consequences of iRhom1/iRhom2 single or double deficiency on the maturation of the A Disintegrin and Metalloproteinase ADAM17. Based on earlier studies of this and another group it was shown that iRhom2 is a central regulator for the activation of ADAM17 possibly by controling ER exit of the protease. The question of this study was to elucidate the functions of the macrophage specific iRhom2 but also the functions of the more ubiquitously expressed iRhom1. Surprisingly, the suggested major effect of iRhom2 on TACE activity was not reflected by the mild or almost absent phenotype observed in the iRhom2 knockout mice. In contrast, iRhom1 knockout mice have a complex phenotype (not necessarily overlapping with the phenotype of ADAM17-deficient mice) leading to early postnatal death of the animals. Studies in mouse fibroblasts finally revealed an overlapping function of both iRhoms in regulating ADAM17 maturation. The current manuscript closely follows to a recently published paper by Issuree et al. J. Clin. Invest. where in part similar findings were reported.

Despite of some interesting initial observations I strongly feel that this study did not go far enough to characterize the iRhom-deficient mice and to find an explanation for the suggested TACE maturation-independent role of both iRhoms. As the authors correctly point out in their discussion it will be of interest to find other proteins which are regulated by the presence (or in the case of the knockout situation by the absence) of iRhom expression. The phenotype of the iRhom1 knockout mice are only touched at the surface. There are no (molecular or cellular) explanations for the development of brain haemorrhages, the cardiac infarction, the spleen abnormalities, thymus phenotype, the bone abnormalities, and the apparent alterations in the exocrine pancreas. The phenotype of the double knockout mice which were used in the mentioned fibroblast studies was only poorly described in this manuscript. However, such an analysis is needed to better understand the redundancy of both proteins and their regulatory role to regulate ADAM17 trafficking or the ER export of other secretory proteins. The presented experiments in MEF cells are interesting and support earlier studies using siRNA-based approaches showing the redundant role of both iRhoms for regulation of basal and induced ADAM17 (but not other ADAM protease) ER export processes. In summary, unless more insights about the molecular basis of iRhom1 and iRhom2 ADAM17-independent role(s) are presented I cannot support publication. The observed phenotypes in the different tissues, especially in iRhom1-deficient mice should be linked to proteins which are possibly dysregulated due to the absence of this ER traffic-regulator. A more comprehensive phenotyping of the knockout and double knockout mice is required.

Some specific comments and additional major concerns:
- scale bars and statistics are largely missing; higher magnification images as well as electron microscopy studies should complement the phenotype analysis
- the deficiency of iRhoms in the newly generated knockout mice should be demonstrated at the protein level
- to identify the nature of iRhom clients co-precipitation studies using tissue lysates from different

organs (e.g. the affected tissues in the knockouts) followed by identification of the co-precipitated proteins (next to ADAM17) would be of great interest and would significantly increase the impact of this study. An other alternative would be to screen for binding partners using a split-ubiquitin yeast two hybrid assay. A third alternative would be to screen in a for known type 1 or type 2 transmembrane proteins undergoing furin cleavage in post ER compartments. For this type of experiment the available MEF cells would be most suited. May be the double knockout fibroblasts would also offer an excellent tool to analyse the presence of membrane proteins in post-ER membranes as compared to wildtype fibroblasts.
- how do the authors explain the differences between the clinical phenotype and the knockout mice with mutations in iRhom2
- the localization of ADAM17 in the different fibroblast cell lines should be investigated. This would be a nice backup of the immunoblot experiments suggesting ER retention of ADAM17 and the negative findings for the other ADAM members in the double knockout fibroblasts.

Correspondence - authors 14 March 2013

Thanks for handling our ms so quickly and efficiently - it is much appreciated.

I am, however, slightly taken aback by your decision, and I hope you will allow me to discuss the options directly with you now. The basic problem is that the two issues raised by the reviewers are both huge undertakings that are likely to take about a year and if successful would certainly form the heart of other papers. Of course I completely accept your authority to make any editorial decision you think appropriate but I'd like to outline, fairly briefly, a case for rethinking this request.

I don't want this letter to become too long because at this stage I just want to gauge whether there is any scope for revising your opinion. If not, we'll probably just submit elsewhere, since as I think you know from my earlier correspondence with Thomas, on which I think you were copied, that we are in a rush to get this out as soon as possible. So instead of going into great detail, let me just provide the main points of our rebuttal argument. I'll of course be happy to expand on any of it if you think it would be helpful.

1. We were encouraged that Reviewer 1 said that the ms was 'novel and timely'…and of 'interest to a wide audience as it touches different fields in molecular biology ranging from developmental biology to proteases, immune biology, and protein trafficking".

2. Reviewer 2 said "In my opinion the observations presented here will be of great interest for scientists with different backgrounds. The conclusions made by the authors are fully supported by the data presented. I have only some suggestions that, if technically possible, would strengthen this already excellent paper." (my bold)

3. The problem is two-fold. First, it is simply not technically possible to characterise further the phenotypes or to identify new clients in anything less than six months - at a minimum. Second, it is anyway almost certain that the phenotypes are too complex and pleiotropic to lead to any kind of mechanistic conclusions about clients. We are confident about this because we spent two years working on iRhom1 phenotypes before deciding that the only way of making progress was to test a cell biological hypothesis about it's function - and that worked.

4. Reviewer 3 is of course more negative. Probably unsurprisingly, we think that their criticisms and suggestions are unreasonable. We do not think that further phenotyping will reveal the molecular identity of additional iRhom clients. The paper already provides a description of the main aspect of the phenotype and I don't see that the addition of EM data or the use of the split ubiquitin systems in yeast to look for interacting proteins falls within the scope of this work.

5. Of course, we can address the more minor points raised by the reviewers and would be

happy to do so.

6. The reviewers have not challenged any of the main messages that we highlighted: the KOs show that iRhom1 has a much broader role than iRhom1; there is redundancy between the iRhoms but they also have distinct physiological roles; both can promote TACE maturation; iRhoms are new obligate regulators of TACE function; iRhoms must have additional clients (and although these are unidentified, there is solid genetic evidence for this important conclusion); and finally that iRhoms have widespread and profound physiological roles in mammals.

In conclusion, I am hoping that you will reconsider your requirement for the extra work on the grounds that it is simply not feasible, and that with the more minor corrections this paper will be appropriate for EMBO Reports. I of course recognise that you may disagree, in which case I think we will decide to try elsewhere. I do however very much want to publish this paper in EMBO Reports: not only do I think it is good(!), but it is especially timely and addresses questions that have recently become a centre of quite wide attention.

Many thanks for your time and I look forward to hearing from you about the best way forward.

Correspondence - editor 15 March 2013

Many thanks for your email. I apologize if my letter was not clear enough. I do think that a more detailed characterization of the knock-out phenotypes would be useful even if it does not lead to the identification of additional target genes. At least it might provide some clues about the nature of the defects in different organs (and referees 1 and 3 also thought that this would be sufficient and that additional substrates would not need to be identified).

I am sorry for your frustration and I truly hope that we come to an agreement, as I clearly see the potential interest in your paper. Would you at all be willing to invest some time in further characterization of the phenotypes? Or, if you do not want to do this, to test the few candidate substrates that referee 1 suggested?

Again, I want to find a solution that both parties are comfortable with. Please let me know what you think.

Correspondence - authors 18 March 2013

Having now spoken to Yonka and Colin, I'd like to respond to the reviews of our paper in slightly more detail.

First a general explanation, and then specific responses to the three reviews.

The main problem is the complexity of the phenotypes caused by knockout of iRhom1 and the double iRhom1/iRhom2 in mice. We have spent a couple of years analysing the iRhom1 KOs because we initially assumed that their profound abnormalities would lead to mechanistic insight. But the fact that almost all tissues are affected, and the difficulty of distinguishing primary defects from secondary, made us realise that, although valuable as a demonstration of the physiological significance of iRhom1, the phenotype simply could not be used to infer mechanism. At the same time, we had exploited the much narrower phenotype of iRhom2 to discover its cellular function and this allowed us to hypothesise a function for iRhom1, which is proved in this manuscript.

The double knockout dies around 10.5 days of embryogenesis, the time when

organogenesis is starting. This phenotype is worse than the already complicated iRhom1 pathology and even less amenable to deciphering mechanism by phenotyping. There are several hundred genes that cause lethality around this time, many with overlapping phenotypes, so again it will be a slow and not necessarily efficient approach to discovering more mechanistic details; instead we plan to do this mainly by cell biological and biochemical approaches. There is also the practical issue that the person who could best help us in embryonic characterisation is Liz Robertson, who is in my new department in Oxford and who is, I am sure you know, one of the world authorities on embryonic mouse development. I am mid-transition to Oxford and our mice won't be in Oxford until the autumn.

One of the major longterm goals for this project is to identify new clients for iRhoms. This manuscript is an important step in that ambition because it is the first to show that other clients must exist: until now, everything has suggested that TACE is the only mammalian client. But, for exactly the same reasons as above, this is unlikely to be achieved by phenotypic analysis of the mutants. Instead, we are initiating new projects in proteomics and hypothesis-driven cell biology to identify what other proteins iRhoms interact with. Of course, when we have identified strong candidates, the phenotypes of the KOs will be useful to validate their physiological relevance.

I am sure you will understand, therefore, that the frustration I expressed last week was based on the fact that the two issues that arose were precisely the ones that we cannot tackle in the short-term, and which I had discussed with Thomas in some detail at the presub stage. This is not a case of us rebutting the comments out of bloody-mindedness or laziness; it's simply that they cannot be tackled in a reasonable time, and will in fact be the core of future papers.

On a more positive note, both referee 1 and 2 wrote really quite supportively of the manuscript, clearly expressing their opinion of its technical quality, significance and timeliness. Barring the phenotypic analysis, we can address most of their comments quite fast and effectively. Referee 3 is obviously less supportive.

Let me now respond to the specific issues each raises.

Referee 1

1. Obviously we are very pleased with the lines: "The manuscript is well written, easy to read, is novel and timely. The conclusions are supported by the data. The study will be of interest to a wide audience as it touches different fields in molecular biology ranging from developmental biology to proteases, immune biology, and protein trafficking."

2. As discussed above, we simply can't provide more phenotypic detail of the double KO embryos currently. On preliminary inspection, the embryos do not show phenotypes characteristic of well known pathways, but we have initiated a collaboration with Liz Robertson, to start later this year, to analyse them in detail. Nevertheless, for reasons also discussed above, it is very unlikely that this will be the route to further mechanistic understanding of iRhom function.

3. The choice of ADAMs we tested was actually quite justified, and not really 'astonishing'. It's a large family of proteins, many very poorly characterised and it would be rather pointless and quite difficult to do all (several are not easily available). We selected ones where KOs had phenotypes that had any similarity with out KOs. Some of these were catalytically inactive ADAMs: these molecules do have biological functions. Like active ADAMs, they have to be trafficked to the cell surface, hence their inclusion as potential iRhom clients. To be honest, there is an argument that says that anything beyond ADAM10 is not a promising candidate because ADAM10 is very similar to TACE (and is unaffected by iRhoms), whereas all the others are much more distant. However, it is an obvious starting point and that's why we did it. Referee 1 is right that there are a few more which we did not test but would fit our selection logic. This was mainly because they proved hard to find, but I am very willing to try harder and fill in those obvious gaps in the

ADAM table. It almost certainly won't provide any further mechanistic insight, but I'd like to cooperate as much as we can!

4. Minor points are easy to fix.

Referee 2

1. Again, we are gratified with the paragraph: "TACE was identified 15 years ago and yet its regulation is poorly understood. This report builds on the previous work on iRhom2 and greatly expands the observations described in the original Science papers (Adrain et al., 2012; McIlwain et al., 2012). In my opinion the observations presented here will be of great interest for scientists with different backgrounds. The conclusions made by the authors are fully supported by the data presented. I have only some suggestions that, if technically possible, would strengthen this already excellent paper."

2. It is true that the genetics of the redundancy and the background effects are a bit complex. We can describe it better.

3. The referee is right to point this out. It is a robust phenomenon that we do not understand mechanistically but which we suspect is due to a homeostatic feedback mechanism in the ER. We can make this point in the text.

4. It is a nice idea that the cellular localisation of TACE will visibly change in the iRhom KO embryos, but one of the characteristics of TACE is that even in WT cells almost all is in the ER: very little can ever be detected at the cell surface. Part of the explanation for this is that mature TACE has a short half-life, but the ER exit of TACE is also clearly a limiting step. We now believe that this ER retention is due to limiting iRhom levels. For these reasons, this approach is not justified (not to mention that the available antibodies are difficult to use in cell culture and rather unlikely to work in embryonic tissue).

Referee 3

1. This referee is much less supportive in overall tone and it may be difficult to win their support. Nevertheless, I will address their points in detail.

2. My interpretation is that this referee's main criticism is based on somewhat discounting the messages that are already in this paper, and instead wanting us to include what will be substantial future work. But none of the reviewers have challenged any of the current conclusions: the KOs show that iRhom1 has a much broader role than iRhom1; there is redundancy between the iRhoms but they also have distinct physiological roles; both can promote TACE maturation; iRhoms are new obligate regulators of TACE function; iRhoms must have additional clients; and finally that iRhoms have widespread and profound physiological roles in mammals.

3. For reasons I have described above, we think it is unrealistic to believe that further phenotyping of the organ defects in iRhom1 mutants will reveal molecular and cellular mechanistic insight. We provided an overall description and showed examples of some of the tissues affected but it is exactly because it became clear that this was not going to be a mechanistically fruitful route, that we did not spend longer on purely descriptive histology. If it would make a difference, we do have sections of other tissues that we did not show and we could increase the supplemental data by adding some more tissue sections comparing WT and KO. Really not informative or of interest to any but a very few specialist histologists, but we could add it.

4. The mechanistic role of the two iRhoms in TACE trafficking and maturation is addressed in the cellular studies. That iRhom1 and iRhom2 contribute to TACE maturation in some cells is the point of overlap with the Blobel JCI paper; in our ms, however, we look at several cell types, whole embryo extracts, and we use validated genetic KOs rather than siRNA. We note that none of the reviewers describe our paper as being substantially compromised by the Blobel paper.

5. The minor corrections we can of course make.

6. We disagree that the phenotypic analysis would be improved by including EM studies. It is difficult to know where one would start or what useful information would be acquired.

7. The KO mice have been validated at both a genomic and RNA level. We do not agree that a protein level demonstration is also necessary; moreover good enough antibodies are not available.

8. We agree that will be of great interest to identify new iRhom clients by biochemical means and this is indeed one of our next major projects. We do not believe that this kind of proteomic or yeast two hybrid analysis is within the scope of this paper; nor can we realistically test all possible candidates. Not only are these different questions, but they would take a minimum of a year.

9. It is interesting to consider how the human clinical phenotypes correspond to the mouse KOs and this will be the focus of our group and others over the next years. The disease mutations are subtle point mutations and it seems likely that they are not null (our unpublished work). We would be happy to expand on this in the discussion, space permitting.

10. Please see the response to referee 2's point 4.

In summary, Barbara, what I think we can do is, first, to deal with the more minor and specific points raised by the referees; second, we can add a few more ADAMs to the table of almost certainly negative interactions with iRhoms; and third, if it were felt to be helpful, we could add some further histological sections of mutant tissues as supplemental figures. If you will forgive me being a bit blunt, I think that the only one of these that will actually improve the paper or sharpen its message is the first; but the second and third are do-able and I of course understand that you cannot ignore the referees' comments.

Thank you again for your time, and do let me know how you feel about this. I continue to believe that this paper is substantial, will have significant impact, and is especially timely, and for these reasons suitable for EMBO Reports. I do, however, of course understand how the system works, and if you don't feel able to over-rule some of the more substantial requests for further work, I will try elsewhere with minimal hard feelings! If it's easier to talk, I am in Oxford tomorrow and can easily call you.

Correspondence - editor 19 March 2013

Many thanks for your long email and for sharing your thoughts about the referee comments with me. I have discussed the possible options with my colleagues here at EMBO reports again, as well as with referee 1.

While referee 1 still thinks that it would be feasible and informative to describe the double knock out mice in a little more detail, we understand that you are not able to do this right now. Therefore, we would not request this analysis. We also do not insist on testing additional iRhom substrates on a large scale.

However, please test the additional ADAMs as suggested by referee 1. It will also be important to address the other specific comments of the reviewers and I appreciate your willingness to do so. I agree with you that the addition of more tissue sections of the iRhom1 knock-out mice would not add much to the paper, so I think you would not need to do this.

Before submitting the revised version, please also shorten the text as with its currently 40000 characters is exceeds our length restrictions (roughly 29000). It may help to combine the results and discussion section as this avoids redundancies. Please note that Materials&Methods should not be moved to the supplementary section in their entirety.

I hope that you can live with this compromise and look forward to receive the revised version whenever it is ready.

1st Revision - authors' response 04 July 2013

Referee 1

Comment:

Several ADAM proteases were tested and found to be not affected by iRhom deficiency. The choice of ADAMs tested is astonishing as it contains some inactive ADAMs, but lacks some of the proteolytically active ADAMs, such as ADAM12, 21, 28 and 30. In particular ADAM12 has a connection to the heart, such that its impaired maturation and activitiy may be related to the heart phenotype observed in the iRhom-deficient mice. These additional ADAM proteases need to be included into figure 5.

Response

Although we failed to make it explicit, there was in fact logic underlying the choice of ADAMs tested. The ADAMs are a large family of proteins, many of which are poorly characterised; reagents for several are also not easily available. There is in fact an argument that says that anything beyond ADAM10 is not a promising candidate because ADAM10 is much more similar to TACE than any of the others and is unaffected by iRhoms.

We focused on ADAMs for which the knockout phenotype had any similarity with the iRhom KOs. Some of these were indeed catalytically inactive ADAMs: like their active counterparts, these molecules do have biological functions, and have to be trafficked to the cell surface. Hence their inclusion as potential iRhom clients.

Beyond this rather focused starting point, Referee 1 is right that there are a few more ADAMs which we did not test, but would fit our selection logic. We have now cloned and tested five new ADAMs (12, 18, 21, 23, 28). We find that 18, 21 and 28 do not leave the ER in MEFs so cannot be assessed for iRhom involvement. This is not a great surprise since 18 and 21 are testis-specific and not broadly expressed; 28 is also expressed in a very tissue specific manner (in adipose cells). The trafficking of ADAM12 and 23 are unaffected by iRhoms; this data has been added to Figure 5.

Comments

3. Describe in more detail what the 100% level corresponds to in figure 4. What would be the maximal value?

4. Show the whole scale of the x- and y-axes (starting at 0). Otherwise the effect size may be misleading."

Response

Thanks for pointing this out: we have now made this clearer in the Methods and the figure legend. Figure 4 shows the relative mRNA expression levels of iRhom1, iRhom2 and TACE in RNA extracted from a panel of tissues isolated from young adult mice. Equal nanogram amounts of RNA

from each tissue sample were subjected to qPCR. As is standard practice, we established the expression levels of iRhom1, iRhom2 and TACE relative to that of a housekeeping gene (Tbp). After this normalization step, to make comparisons easier, we then took the message that had the highest expression level (the signal for TACE expression in the lung) and fixed this at 100%. Then the mRNA levels in all other samples (all were normalized against Tbp) are expressed as a fraction of this strongest signal (shown on the Y axis). This way of presenting the data allow the most intuitive way of comparing normalised RNA levels. The X axis indicates the tissue source of the individual RNA samples.

Comment

5. The Siggs et al paper in Blood from last year needs to be cited. This also demonstrates the requirement of iRHom2 for TACE activity.

Response

We have now corrected this omission.

Referee 2

Comment

The reasoning behind the statement "the fact that the double knockout is worse than a simple combination of the two single mutants implies that there is some redundancy between them" is hard to follow.

Reponse

Apologies if this was not fully clear. We have modified the text. The point is that the phenotype of double KOs is significantly worse than that of either of the individual single knockouts. If the two iRhoms fulfilled independent roles then the double knockout should simply combine these two individual phenotypes.

Comment

This conclusion is based on the data corresponding to strain 129S6/SvEvTac, in which the defects induced by iRhom1 knock-down are remarkably milder than in the strain C57BL/6J. Following the same rational, one should conclude that the degree of functional redundancy between iRhoms is different in different strains. This is minor point but, in my opinion, the authors should not highlight the aforementioned conclusion.

Response

We agree that the genetics of the redundancy and the background effects are a bit complex. The referee is correct that the degree of functional redundancy may differ in different backgrounds. However, the important conclusion, that iRhoms are functionally redundant, can be concluded from both genetic backgrounds (129S6/SvEvTac and C57BL/6J). In both cases, the double knockouts exhibit embryonic lethality – a phenotype much worse than the single knockouts.

Comment

Fig. 3A. The total levels of TACE seems to be reduced in the iRhoms knock-out and further reduced

in the double knock-out. Is this impression correct? If so, the authors should comment on it.

Response

The referee is right to point this out. It is a robust phenomenon that we do not understand mechanistically but which we suspect is due to a homeostatic feedback mechanism in the ER. We have now made this point in the text.

Referee 3

Comments

Scale bars and statistics are largely missing; higher magnification images as well as electron microscopy studies should complement the phenotype analysis

Response

We have now added scale bars to the images in Figures 1 and 2, and the data in Figures 3C and 5 are now supported by statistics. We have not included higher magnification images because we are not presenting a full histological analysis of the compex phenotype – which would be beyond the scope of this paper (and which we believe would be unlikely to provide any further mechnistic insight). Moreover, the 400X magnification of some of the images is already close to the practical limit of what is useful or standard for histological anaysis.

Comments

How do the authors explain the differences between the clinical phenotype and the knockout mice with mutations in iRhom2

Response

It is interesting to consider how the human clinical phenotypes correspond to the mouse KOs. Unlike the KOs, the human disease mutations are subtle point mutations and we have evidence that they are not null mutations (our unpublished work). Whether they are hypomorphic or neomorphic, whether they affect TACE, and what is the basis of their pathology will be the subject of future work, but is undoubtedly beyond the scope of this work.

2nd Editorial Decision 23 July 2013

Thank you for your patience while your study was reassessed by referee 1. S/he now supports publication of your study and has no further comments. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

I have noticed that information regarding the number of independent experiments performed is missing from the legend of figures 3C and 5C, D and E. We would need you to include this, and also that the bars represent mean +/- standard deviation (I realize the latter information is in the Material and Methods section, but it is more visible here). Please just send us the information by email and we will include it in the relevant figure legends.

We now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to

the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

