THE EMBO JOURNAL

Manuscript EMBO-2016-93801

Transcriptome-based profiling of yolk-sac derived macrophages reveals a role for Irf8 in macrophage maturation

Nora Hagemeyer, Katrin Kierdorf, Kathrin Frenzel, Jia Xue, Marc Ringelhan, Zeinab Abdullah, Isabelle Godin, Peter Wieghofer, Marta Costa Jorao, Thomas Ulas, Gulden Yorgancioglu, Frank Rosenbauer, Percy Knolle, Mathias Heikenwalder, Joachim Schultze, Marco Prinz

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Review timeline:

Submission date:	04 January 2016
Editorial Decision:	15 February 2016
Revision received:	15 April 2016
Editorial Decision:	30 May 2016
Revision received:	13 June 2016
Accepted:	15 June 2016

Editor: Karin Dumstrei

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 15 February 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, both referees find the analysis insightful and support publication here. They raise a number of constructive comments that I anticipate that you should be able to address within a reasonable timeframe. Given the referee comments, I would like to ask you to submit a suitably revised manuscript for our consideration. Let me know if we need to discuss anything further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this study, Hagemeyer, Kierdorf, Frenzel et al analyze the developmental and transcriptional features of mouse macrophage populations in the embryo and in the adult. These populations are identified based on fate mapping experiments using CX3CR1-CreER x Rosa-stop-GFP-stop mice and/or by phenotypic markers. The authors found two distinct subsets of macrophages. One subset (F4/80-high) originates from the Yolk Sac (YS), populates various tissues, has undetectable turnover in microglia and limited turnover in liver and kidney. Another subset (CD11b-high) does not originate from the YS but from a late hematopoietic progenitor, expresses high amounts of CCR2 and undergoes a rapid turnover in kidney and liver.

The origin of macrophages in peripheral tissues has been a recent matter of debate and this paper provides a fresh view on this controversy, demonstrating that a large proportion of peripheral macrophages (F4/80-high) derive from YC progenitors, whereas another population (CD11b-high) derives from peripheral blood monocytes. Remarkably the authors show quite distinct transcriptional signatures of these subsets and identify genes that may be helpful in distinguish them. Moreover, they identify IRF8 as a critical transcription factor that regulates number, surface processes and function of both subsets. Overall, this is an extremely valuable study. Moreover, the gene expression profiles presented here provide a rich resource for scientists in the field.

There are few points that the authors should address

1) The authors provide evidence that CCR2+CD11b+ macrophages derive from Ly6C-hi monocytes. However, also show that Ly6C-hi cells may contribute to kidney F4/80-high cell. This is confusing. Additionally, the authors do not investigate the contribution of Ly6c-lo monocytes to macrophage populations. Do they contribute at all? The issue of Ly6c monocytic subsets and their contribution to macrophage populations should be clarified

2) In the analysis of genes differentially expressed between F4/80-hi and CD11b-hi macrophages, the authors highlight genes indicative of distinct developmental origins. However, they should also include a thorough discussion of differentially expressed genes that drive macrophage functions, such as genes encoding cytokines, antigen capturing molecules, proteolytic enzymes etc,,,

3) I find the section on IRF8 interesting but a bit disconnected with the rest of the study. It seems that IRF8 has no differential effect on the macrophages subsets, rather a general impact on the function of both F4/80 and CD11b subsets

4) Fig 1c. The authors should comment on the heterogeneity of CX3CR1 expression in the A2 population. Is this a pure A2 population or includes both A1 and A2 cells?

Minor points.
1) Introduction is too long, it includes an extensive description of the results that could be deleted
2) The authors should specify in the beginning of the paragraph "Irf8 regulates tissue ...." that they analyze IRF8-/- mice

Referee #2:

In the current manuscript Hagemeier et al. use lineage tracing tools and transcriptomics to analyze distinct tissue resident macrophage populations identified by CD11bhi;F4/80lo and CD11blo;F4/80hi surface marker combinations in several organs (kidney, liver and skin). The authors show that yolk sac (YS) macrophage progenitors can give rise to CD11blo;F4/80hi resident macrophages in the adult. They also demonstrate that embryonic and to a certain degree adult CD11blo;F4/80h macrophages share significant similarity in gene expression with microglia cells and YS progenitors, further supporting a potential developmental relationship. Finally they show that the transcription factor IRF8 is not only important for monocyte derived macrophage development, as previously shown, but also for maturation and gene expression of CD11blo;F4/80hi resident macrophages .

The observations of this study are important because conflicting studies had suggested strict fetal monocyte origin of tissue resident macrophage populations, except for microglia. The current study

makes a significant contribution to the understanding of tissue resident macrophage origin.

A few control experiments and clarifications, however, are required. The authors also need to correct some imprecisions and overstretched claims in their presentation of the data.

1. To validate the CX3CR1-CREER lineage tracing tool for tracking yolk sac (YS) derived macrophages the authors first show that at day 9 of embryonic development, a subpopulation of previously defined A2 YS macrophages expresses CX3CR1, at a time when it is not expressed in the P-Sp region the earliest site of definitive hematopoiesis. It is critical for the conclusions of the paper how long the Cre enzyme is active in the embryo and when CX3CR1 becomes expressed at other sites of hematopoiesis than the YS. The authors show that TAM injection at E7 does not label microglia cells, indicating that TAM is not active anymore at E9 when CX3CR1+ A2 progenitors are present in the YS. However, this still leaves a window of two days. So the author should show that no CX3CR1+ progenitors are present in the sites of definitive hematopoiesis (P-Sp/AGM and FL) up to E11 or they should show that TAM at E8 does not label microglia either and CX3CR1+ cells do not become detectable in the P-SP/AGM region up to E10. Alternatively the authors could test whether CreER induction at E9 with 4-hydroxy-tamoxifen, which has a shorter half life in vivo and thus results in a sharper labeling window than TAM, also results in labeling of CD11blo;F4/80hi resident macrophages, even if at lower efficiency.

2. The results of FigS1C appear very relevant to the main message of the paper and should be included in the main figures. It is important to show that CX3CR1Cre induction at E9 labels E16 F4/80hi macrophages a clearly detectable but significantly reduced contribution to adult F4/80hi macrophages.

3. The statement " Kupffer cells are stable populations which do not undergo significant exchange with blood cells within the first nine months of life." p11 top, is not correct. The CCR2-/- experiments are not sufficient to conclude this and only a small proportion of F4/80hi KC can be labeled with the strategy shown in Fig.2C. This should be discussed in the light of monocyte contribution to this population shown in other studies, for example (Scott et al., Nature Comm., 2016, DOI: 10.1038/ncomms10321 and Bleriot et al., Immunity 2015). The statements on p11 "The results presented above suggested that microglia and F4/80hi MΦ from liver and kidney are derived from CD45+CX3CR1hiF4/80hi A2 progenitors" or on p16 of the discussion "Our results provide therefore the first direct genetic proof that the Kupffer cell population has no turnover from birth until nine months of age" are not entirely true. Only a subpopulation of these cells is shown to be derived from A2 cells.

4. The authors should not only rely on the F4/80;CD11b characterization of resident macrophage populations but also include other markers such as CX3CR1 and MHCII that have been commonly used in other studies, both for contributions in the lineage tracing experiments and for qPCR validation of the presented key genes identified by gene arrays in the F4/80;CD11b defined populations.

5. Only a subpopulation of A2 cells appears to express CX3CR1 at high levels (by FACS and IF) and is therefore likely the origin of the lineage traced resident macrophages. Is there a difference in gene expression between CX3CR1lo and CX3CR1hi A2 cells? This might be relevant for the studied developmental relationships.

6. The authors state in the abstract that IRF8 is vital for the innate immune response but only show altered gene regulation of immune function genes in IRF8-/- resident macrophages. The authors should demonstrate that these changes in gene expression indeed reflect differences in the immune response of IRF8 -/- macrophages, for example by scoring the response to infection mimetics.

7. Several sentences or claims in the title, abstract, highlights and introduction section are imprecise or overstretched and should be corrected.
a. The authors do not really show an effect on macrophage homeostasis in the IRF8 KO. The effects on macrophage numbers are relatively small and it is unclear whether the reduction in number is due to altered differentiation, proliferation or apoptosis. This is not explored in the paper. The effects on cellular morphology and altered expression of immune function genes appear more consistent with a maturation defect of the IRF8 KO macrophages. This should perhaps be the main emphasis.

b. The authors should also be more conservative in the interpretation of the transcriptomics data. They do not really establish a pedigree but show similarities in gene expression that are suggestive of a developmental relationship.
c. The title does not really appear to reflect the key message(s) of the paper. IRF8 was not identified by the transcriptomic analysis, the knockout does not really show an effect on homeostasis (see above). By contrast the more important new observation that IRF 8 has a function YS derived resident macrophages is not mentioned.
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1st Revision - authors' response 15 April 2016

Referee #1:
In this study, Hagemeyer, Kierdorf, Frenzel et al analyze the developmental and transcriptional features of mouse macrophage populations in the embryo and in the adult. These populations are identified based on fate mapping experiments using CX3CR1-CreER x Rosa-stop-GFP-stop mice and/or by phenotypic markers. The authors found two distinct subsets of macrophages. One subset (F4/80-high) originates from the Yolk Sac (YS), populates various tissues, has undetectable turnover in microglia and limited turnover in liver and kidney. Another subset (CD11b-high) does not originate from the YS but from a late hematopoietic progenitor, expresses high amounts of CCR2 and undergoes a rapid turnover in kidney and liver. The origin of macrophages in peripheral tissues has been a recent matter of debate and this paper provides a fresh view on this controversy, demonstrating that a large proportion of peripheral macrophages (F4/80-high) derive from YC progenitors, whereas another population (CD11b-high) derives from peripheral blood monocytes. Remarkably, the authors show quite distinct transcriptional signatures of these subsets and identify genes that may be helpful in distinguish them. Moreover, they identify IRF8 as a critical transcription factor that regulates number, surface processes and function of both subsets. Overall, this is an extremely valuable study. Moreover, the gene expression profiles presented here provide a rich resource for scientists in the field.

We are grateful for this positive statement in general.

There are few points that the authors should address 1) The authors provide evidence that CCR2+CD11b+ macrophages derive from Ly6C-hi monocytes. However, also show that Ly6C-hi cells may contribute to kidney F4/80-high cell. This is confusing. Additionally, the authors do not investigate the contribution of Ly6c-lo monocytes to macrophage populations. Do they contribute at all? The issue of Ly6c monocytic subsets and their contribution to macrophage populations should be clarified.

We would like to refer to Figure 2 E where we included both, mice with a significant reduction of Ly6C^{hi} (Ccr2$^{-/-}$ mice) or Ly6C^{lo} (Nr4a1$^{-/-}$ mice) monocytes. Our data from Nr4a1$^{-/-}$ mice indicate no contribution of Ly6C^{lo} monocytes to the tissue macrophages analyzed. However, in Ccr2$^{-/-}$ mice the number of liver and kidney CD11b^{hi} macrophages as well as F4/80^{hi} kidney macrophages were significantly reduced. This is in line with the data of our turnover experiment presented in Fig. 2B. Here we observed a decline of Cx3Cr1-YFP^{+} F4/80^{hi} kidney macrophages from 4 to 35 weeks post tamoxifen injection in adult Cx3cr1^{CreER}:R26-yfp mice. Taken together our data show that adult F4/80^{hi} kidney macrophages are partially replaced by Ccr2^{+} - dependent progenitors.
Our data are also in line with the findings by Schulz et al, Science, 2012. They showed that Myb-

dependent bone marrow precursors partially contribute to the adult F4/80^{hi} macrophages population in the kidney.
We adapted the corresponding sentence in the revised manuscript to make this point more clear.

2) In the analysis of genes differentially expressed between F4/80-hi and CD11b-hi macrophages, the authors highlight genes indicative of distinct developmental origins.
However, they should also include a thorough discussion of differentially expressed genes that drive macrophage functions, such as genes encoding cytokines, antigen capturing molecules, proteolytic enzymes etc,,,

We performed now further analysis including genes encoding cytokines, antigen capturing molecules, proteolytic enzymes, surface marker, transcription factors and secreted molecules and included the new data in the **new Figure EV3**. The data support our previous finding that F4/80 and CD11b macrophages have a district gene expression pattern that is present upon development (genes marked in red color in the Figure EV3).

3) I find the section on IRF8 interesting but a bit disconnected with the rest of the study. It seems that IRF8 has no differential effect on the macrophages subsets, rather a general impact on the function of both F4/80 and CD11b subsets

This referee is right; IRF8 plays a role in both subsets. However, we found in this study here for the first time that IRF8 plays a detrimental role in maintaining macrophage physiology and function in tissue macrophages. It was postulated before that tissue resident macrophages are not effected at al by the loss of *Irf8* (Hambleton et al 2011) and only for microglia morphological changes were described so far (Minten et al. 2012).

4) Fig 1c. The authors should comment on the heterogeneity of CX3CR1 expression in the A2 population. Is this a pure A2 population or includes both A1 and A2 cells?

As shown before by Bertrand et al., Blood 2005, CX3CR1 is gradually upregulated during maturation on yolk sac progenitors. Therefore, there is no clear cut in CX3CR1 expression between the A1 and A2-progenitor populations.

Minor points.
1) Introduction is too long, it includes an extensive description of the results that could be deleted
We modified the introduction in the revised manuscript.

2) The authors should specify in the beginning of the paragraph "Irf8 regulates tissue ...." that they analyze IRF8-/- mice
We changed this section in the revised manuscript accordingly.

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In the current manuscript Hagemeier et al. use lineage tracing tools and transcriptomics to analyze distinct tissue resident macrophage populations identified by CD11bhi;F4/80lo and CD11blo;F4/80hi surface marker combinations in several organs (kidney, liver and skin). The authors show that yolk sac (YS) macrophage progenitors can give rise to CD11blo;F4/80hi resident macrophages in the adult. They also demonstrate that embryonic and to a certain degree adult CD11blo;F4/80h macrophages share significant similarity in gene expression with microglia cells and YS progenitors, further supporting a potential developmental relationship. Finally they show that the transcription factor IRF8 is not only important for monocyte derived macrophage development, as previously shown, but also for maturation and gene expression of CD11blo;F4/80hi resident macrophages . The observations of this study are important because conflicting studies had suggested strict fetal monocyte origin of tissue resident macrophage populations, except for microglia. The current study makes a significant contribution to the understanding of tissue resident macrophage origin.

We would like to thank the referee for these encouraging comments.

A few control experiments and clarifications, however, are required. The authors also need to correct some imprecisions and overstretched claims in their presentation of the data.

1. To validate the CX3CR1-CREER lineage tracing tool for tracking yolk sac (YS) derived macrophages the authors first show that at day 9 of embryonic development, a subpopulation of previously defined A2 YS macrophages expresses CX3CR1, at a time when it is not expressed in the P-Sp region the earliest site of definitive hematopoiesis. It is critical for the conclusions of the paper how long the Cre enzyme is active in the embryo and when CX3CR1 becomes expressed at other sites of hematopoiesis than the YS. The authors show that TAM injection at E7 does not label microglia cells, indicating that TAM is not active anymore at E9 when CX3CR1+ A2 progenitors are present in the YS. However, this still leaves a window of two days. So the author should show that no CX3CR1+ progenitors are present in the sites of definitive hematopoiesis (P-Sp/AGM and FL) up to E11 or they should show that TAM at E8 does not label microglia either and CX3CR1+ cells do not become detectable in the P-SP/AGM region up to E10. Alternatively the authors could test whether CreER induction at E9 with 4-hydroxy-tamoxifen, which has a shorter half life in vivo and thus results in a sharper labeling window than TAM, also results in labeling of CD11blo;F4/80hi resident macrophages, even if at lower efficiency.

We thank the reviewer for the careful evaluation of our fate mapping tools. We now took the chance to prove the specificity of our experimental setup more in detail.
To evaluate how long the Cre enzyme is active after Tamoxifen application we performed an additional pulse labeling experiment by injecting Tamoxifen at E 8.0 in pregnant females and analyzed the tissue macrophages in the embryos at E 16.0 (**new Fig. EV1 and Fig. 1 in the rebuttal letter**). In the new experimental setup we found a small YFP^+ microglia population (mean 15.5 % vs. 40 % after TAM application at E 9.0). Similarly, the recombination efficiency in $F4/80^{hi}$ macrophages was less in the liver and unchanged in the kidney compared to tamoxifen application at E 9.0 ($F4/80^{hi}$ liver 15,8 % vs. 20,7 %; $F4/80^{hi}$ kidney 15% vs. 15,8%). From this experiment we can conclude that Tamoxifen-induced Cre-activation only lasts approx. for one day. Therefore, the application of Tamoxifen at E 9.0 leads to Cre activation until approx. E10.0.
To analyze if we targeted any HSCs in the fetal liver or AGM at E10.0/E10.5 we next evaluated the fetal liver in E10.5 Cx3Cr1-GFP embryos by immunohistochemistry and included CD31, CD41 and c-kit as marker for HSCs as well as F4/80 as a macrophage marker **(new Expanded Figure 1)**. We detected $CX3CR1^+$ cells which were only double positive for F4/80 but not for CD31, CD41 or cKIT. Moreover, no $CX3CR1^+/CD31^+$ cell were present in the AGM at E10.0. Therefore, we clearly can exclude to target any HSCs in the AGM or fetal liver by our fate mapping approach at this time point.

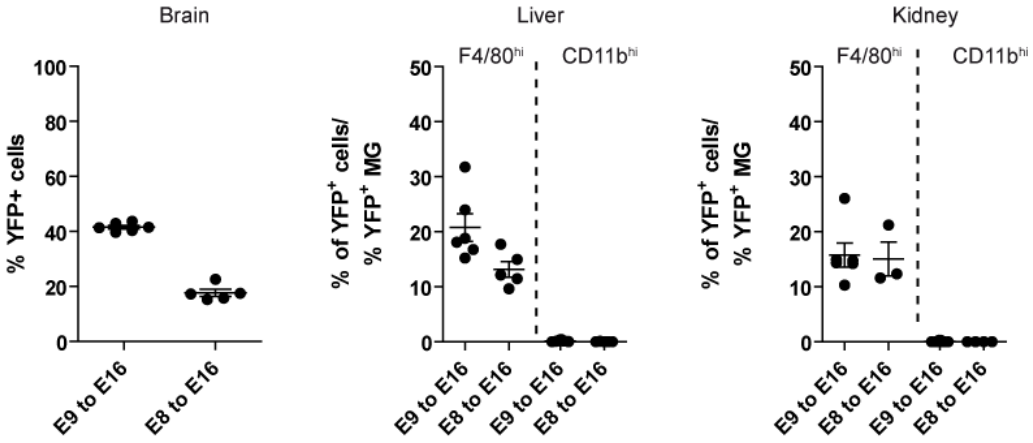

Figure 1: Pulse labeling of yolk sac progenitors at E9 or E8 in $Cx3Cr1Cre^{ER}$:R26-yfp mice

2. The results of FigS1C appear very relevant to the main message of the paper and should be included in the main figures. It is important to show that CX3CR1Cre induction at E9 labels E16 F4/80hi macrophages a clearly detectable but significantly reduced contribution to adult F4/80hi macrophages.

We completely agree with the reviewer and changed the figure 1 accordingly and inserted a **new Fig. 1F**.

3. The statement " Kupffer cells are stable populations which do not undergo significant exchange with blood cells within the first nine months of life." p11 top, is not correct. The CCR2-/- experiments are not sufficient to conclude this and only a small proportion of F4/80hi KC can be labeled with the strategy shown in Fig.2C. This should be discussed in the light of monocyte contribution to this population shown in other studies, for example (Scott et al., Nature Comm., 2016, DOI: 10.1038/ncomms10321 and Bleriot et al., Immunity 2015). The statements on p11 "The results presented above suggested that microglia and F4/80hi MΦ from liver and kidney are derived from CD45+CX3CR1hiF4/80hi A2 progenitors" or on p16 of the discussion "Our results provide therefore the first direct genetic proof that the Kupffer cell population has no turnover from birth until nine months of age" are not entirely true. Only a subpopulation of these cells is shown to be derived from A2 cells.

We re-wrote the corresponding sections in the revised manuscript and included the mentioned studies in the discussion part. However, in these studies the analysis of Kupffer cells was always done under pathological settings and not under homeostatic conditions as we did. We would like to point out that the technical possibilities to analyze the turnover of adult Kupffer cells are currently very limited. It is right that we only targeted a subpopulation of Kupffer cells by our approach. Therefore, the conclusions on that point are only qualitatively and not quantitatively. With the recent finding of *Clec4a* as specific gene for Kupffer cells (Scott et al., Nature Comm., 2016) it might be possible in the future to generate an inducible Clec4^{CreER} line to further analyze the longevity of Kupffer cells.

4. The authors should not only rely on the F4/80;CD11b characterization of resident macrophage populations but also include other markers such as CX3CR1 and MHCII that have been commonly used in other studies, both for contributions in the lineage tracing experiments and for qPCR validation of the presented key genes identified by gene arrays in the F4/80;CD11b defined populations.

As suggested by this referee we now characterized our macrophage populations by analyzing different macrophage marker by flow cytometry (**see Figure2 below in the rebuttal letter**). However, we decided to exclude these markers in our basic gating for the different tissue macrophage populations because e.g. CX3CR1 and MHC II are differentially expressed by tissue macrophages of different organs and during development (e.g. CX3CR1 is not expressed in adult but embryonic and early postnatal F4/80^{hi} liver macrophages). Moreover, MHC II is regulated by Irf8 **(see FigureEV5).** Therefore, CD11b and F4/80 allowed us to reliably define the two macrophage subsets at all stages throughout development in liver and kidney.

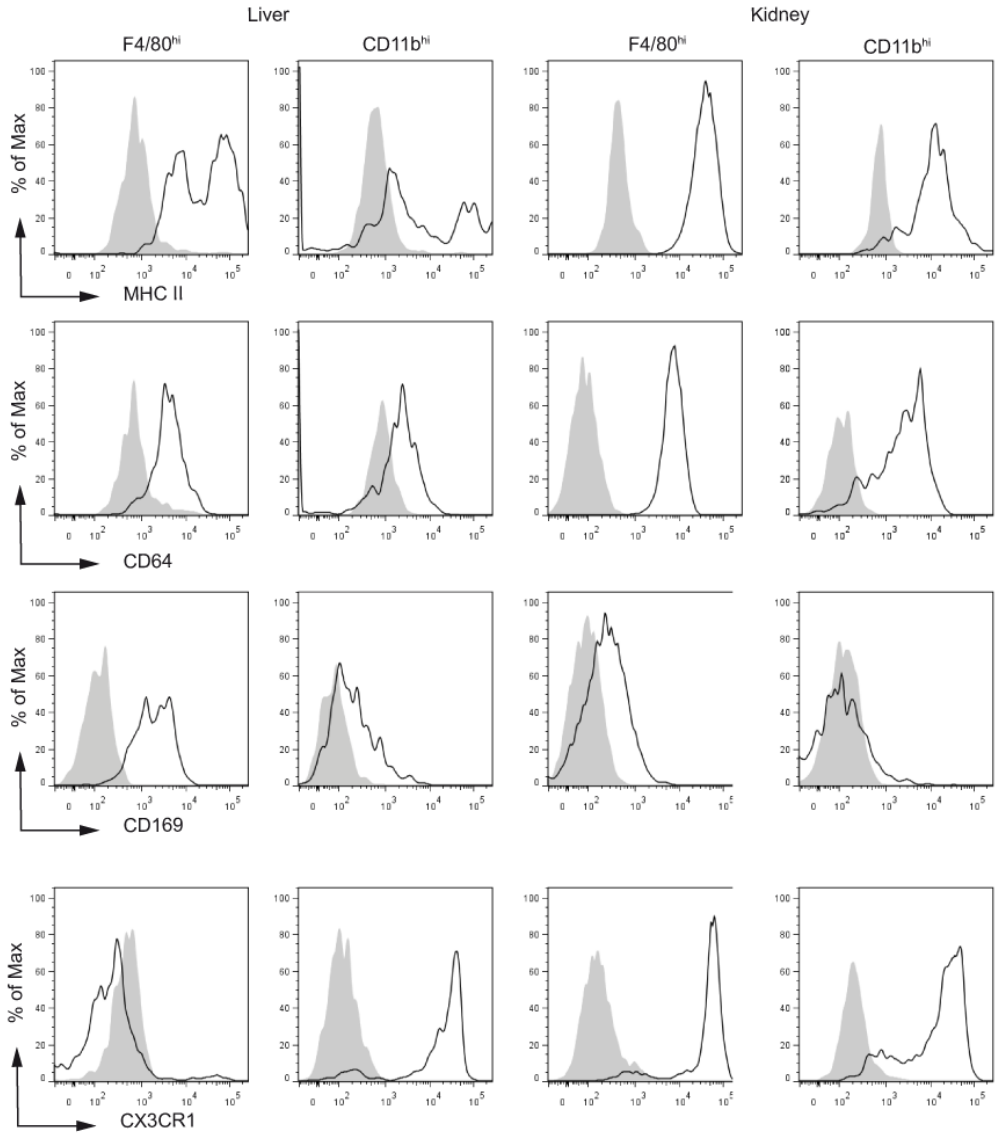

Figure 2: Macrophage marker expression in F4/80^{hi} and CD11b^{hi} liver and kidney macrophage populations.

5. Only a subpopulation of A2 cells appears to express CX3CR1 at high levels (by FACS and IF) and is therefore likely the origin of the lineage traced resident macrophages. Is there a difference in gene expression between CX3CR1lo and CX3CR1hi A2 cells? This might be relevant for the studied developmental relationships.

Please see referee #1, comment 4.

6. The authors state in the abstract that IRF8 is vital for the innate immune response but only show altered gene regulation of immune function genes in IRF8-/- resident macrophages. The authors should demonstrate that these changes in gene expression indeed reflect differences in the immune response of IRF8 -/- macrophages, for example by scoring the response to infection mimetics.

We removed this imprecise statement from the abstract. However, in preliminary experiments we indeed observed a changed immune response in *Irf8*$^{-/-}$ mice. As a model of a non-pathogen-driven inflammatory response we induced acute liver injury by a single injection of carbon tetrachloride (CCl4) in six- to eight-week-old *Irf8*$^{+/+}$ and *Irf8*$^{-/-}$ mice (**see Fig. 3A below in the rebuttal letter**). As expected, the serum alanine aminotransferase (ALT) levels were strongly increased in *Irf8*$^{+/+}$ mice one to three days post injection. In contrast, the ALT levels were significantly lower at the same time points in *Irf8*$^{-/-}$ mice indicating a pathogenetic role of IRF8 during toxic liver damage. In line with these data, immunohistochemical examinations revealed significantly reduced MHC class

II and F4/80 positive areas pointing to reduced macrophage activation in the livers of *Irf8*$^{-/-}$ mice (**Fig. 3B-C in this rebuttal letter**). Additionally, immunostainings for nuclear RelA, as indicator for NF-κB activation, as well as for nuclear STAT1, as indicator for an activated interferon signaling pathway, were significantly reduced in the livers of mice lacking *Irf8* four days post CCl4-treatment (**Fig. 3D,E in this rebuttal letter**). These data suggest a reduced activation and response of liver macrophages as the executive arm of the innate immune response in *Irf8*$^{-/-}$ mice upon CCl4 challenge. However, infiltration of adaptive immune cells such as T- (CD3) and B- (B220) cells was unchanged in *Irf8*$^{-/-}$ compared to *Irf8*$^{+/+}$ mice (Fig. 3F). These data reveal that IRF8 is essential for proper immune response. However, these data are part of a follow up study and were therefore not included in the current manuscript.

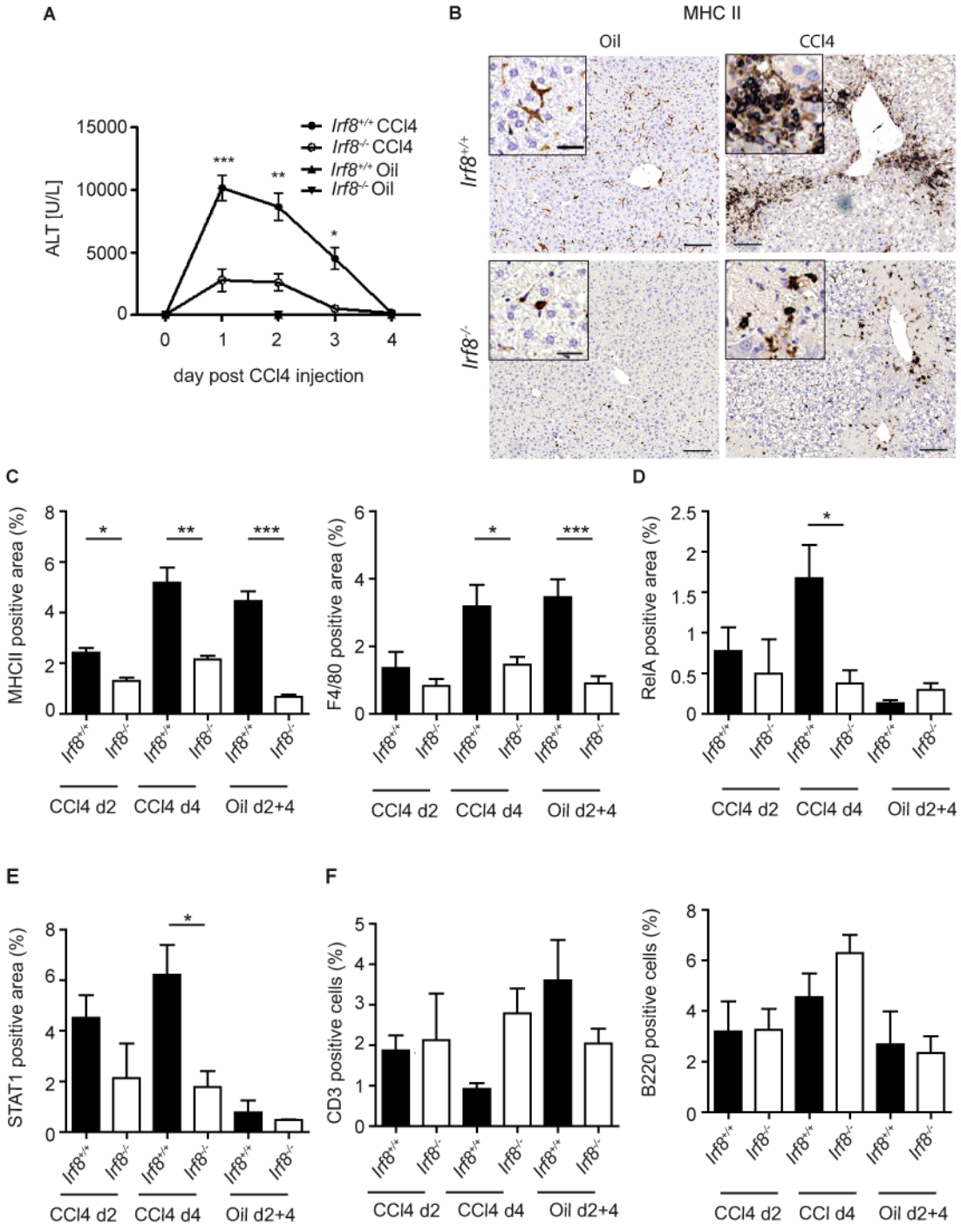

Figure 3: Irf8 deficiency protects from CCl4-induced liver injury

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We agree with the reviewer and adapted this point in the revised manuscript.

b. The authors should also be more conservative in the interpretation of the transcriptomics data. They do not really establish a pedigree but show similarities in gene expression that are suggestive of a developmental relationship.

We considered this point in the revised manuscript.

c. The title does not really appear to reflect the key message(s) of the paper. IRF8 was not identified by the transcriptomic analysis, the knockout does not really show an effect on homeostasis (see above). By contrast, the more important new observation that IRF 8 has a function YS derived resident macrophages is not mentioned.

We adapted the title accordingly.

d. Abstract: the lineage tracing model was not established in this paper but adapted from previous studies.

We rephrased this sentence in the abstract.

e. The authors say in the introduction "that only a small number of transcripts associate with all MΦ " (p5, bottom). This is not correct. Indeed, both Gaultier et al. and Lavin et al. show tissue specific gene expression in macrophages from different tissues but also a majority of shared transcripts. In the current form the sentence is misleading.

This sentence was rephrased in the corresponding section.

f. Introduction, p6 bottom and results p12 bottom: IRF8 does not "heterodimerize" with PU.1. It is an interaction of heterotypic transcription factors. Even the interactions with other IRF proteins do not occur via classical dimerization motifs. The term hetero-dimerization therefore should not be used in this context.

The corresponding sections were changed.

2nd Editorial Decision 30 May 2016

Thanks for sending us the revised manuscript. I have now heard back from the two referees and as you can see below they both appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

There are just a few things to sort out before everything is in order.

- Please take a look at referee #2's remaining comments and respond as you see fit

- The COI statement is missing

- Figure 3 should be uploaded as a single file

- Table EV1 is missing

- I think the arrows in Figure EV1 are a bit difficult to see - would you take a look.

- We need a general summary statement and 3-5 bullet points to place in the synopsis box. I think your highlights are fine - you could use the first one as the summary statement, but take a look.

- Lastly do you have a summary figure that I can place in the synopsis. The size should be 550 wide by [125-400] high (pixels). Let me know if that becomes too much work.

I have provided a link below so that you modify the files.

REFEREE REPORTS

Referee #1:

The authors have thoroughly addressed all my concerns. The paper is significantly improved, advances the field and I recommend publication of the study at this point

Referee #2:

This is an interesting paper and my comments have been adequately addressed now. The paper should be published in EMBO now. Just a few small issues of data presentation remain to be addressed.

1.The title still does not appropriately represent findings reported and should be changed. At the very least "homeostasis should be replaced with "maturation" as done in other parts of the manuscript. The characterization of yolk sac derived macrophages is not mentioned, which would be useful to direct the interested reader.

A suggestion:

"Transcriptome-based profiling of yolk sac derived macrophage development and identification of a role of Irf8 in their maturation"

2. The precise characterization of the lineage tracing tool is important for the reader to judge the manuscript in particular in comparison to other lineage tracing tools recently reported in the literature. This has been done now but figure 1 of the rebuttal letter showing this should also be incorporated into a figure of the manuscript. Extended figure 1 of the manuscript only shows an example of the labelling in microglia cells but is missing the quantification and the labelling data for liver and kidney.

3. "pluripotent" hematopoietic progenitors should be replaced by "multipotent" hematopoietic progenitors (for example p11)

4. remove "homeostasis" from last sentence on p17.

2nd Revision - authors' response 13 June 2016

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The authors have thoroughly addressed all my concerns. The paper is significantly improved, advances the field and I recommend publication of the study at this point

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A suggestion:

"Transcriptome-based profiling of yolk sac derived macrophage development and identification of a role of Irf8 in their maturation"

We thank the reviewer for the suggestion of a modified title and changed the title in the manuscript accordingly.

2. The precise characterization of the lineage tracing tool is important for the reader to judge the manuscript in particular in comparison to other lineage tracing tools recently reported in the literature.
This has been done now but figure 1 of the rebuttal letter showing this should also be incorporated into a figure of the manuscript. Extended figure 1 of the manuscript only shows an example of the labelling in microglia cells but is missing the quantification and the labelling data for liver and kidney.

We now included the data in the main text of the manuscript (please see page 8).

3. "pluripotent" hematopoietic progenitors should be replaced by "multipotent" hematopoietic progenitors (for example p11)

We rephrased the sentence in the manuscript.

4. remove "homeostasis" from last sentence on p17.

We removed the word in the manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Manuscript Number: Prof. Dr. Marco Prinz
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-93801

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 $\chi 2$ 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?	No statistical methods were used to predetermine sample sizes, and exact group numbers were determined by animal availability. However we did ensure that our sample sizes were similar to those generally employed in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Exact group number were determined by animal availability.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples were included.
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.	There was no randomization of mice or samples prior to analysis. However the experimental setup itself let to randomization of groups for the pulse labeling experiments (Figure 1 D-F). Animals were mated to get Cre- and Cre+ mice from the same litter. As pregnant females were treated with tamoxifen all mice were treated in the same way.
For animal studies, include a statement about randomization even if no randomization was used.	See above
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.	To obtain unbiased data, experimental mice of different genotypes per experiment were processed together. The scientist performing the quantifications was blinded for the genotype. After finalization of each experiment, samples were distributed to their genotype. Moreover, Figure 3, 4 and 6: RT-PCR analyses were performed in a blinded way. The investigator did not know the group affiliation of the samples and the samples where ran randomized per PCR-Array plate.
4.b. For animal studies, include a statement about blinding even if no blinding was done	See above
5. For every figure, are statistical tests justified as appropriate?	In the material and method section the statistics are properly justified and in each figure legend the statistical test applied is always mentioned.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data were tested for normality by using the F-test for variance. If normality was given, an unpaired t-test was applied. If the data did not meet the criteria of normality, the Mann–Whitney U-test was applied.
Is there an estimate of variation within each group of data?	The variation of each group is shown with the standard error of mean (S.E.M).
Is the variance similar between the groups that are being statistically compared?	

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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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://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

C- Reagents

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).	For all antibodies used in this study, the company name and clone number is provided in the material and method part.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used.

* 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.	In this study the following mouse lines were used: Cx3cr1GFP/WT, Cx3cr1CreER:R26-yfp, Ccr2-/-, Nr4a1-/-, IRF8-/-, Irf8-PAC-VENUS, Ccr2RFP/WT, C57Bl/6. All mice were group housed and both males and females were used for analysis. Experiments were performed at an age of embryonic day E14, postnatal day 1 as well as 1,2,4,6, or 35 weeks. The age of the mice for each experiment is stated in the figure and figure legend.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were approved by the Federal Ministry for Nature, Environment and Consumers Protection of the state Baden-Württemberg and were performed in accordance to the respective national, federal and institutional regulations.
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.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	The Microarray data are publicly avalaible by the following GEO accession number: GSE73125.
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).	N/A
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).	N/A
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, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis 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	
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.	N/A

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.	N/A