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JOURNAL

One Step CRISPR/Cas9 Method for the Rapid Generation of Human Antibody Heavy Chain Knock-in Mice

Ying-Cing Lin, Simone Pecetta, Jon M. Steichen, Sven Kratochvil, Eleonora Melzi, Johan Arnold, Stephanie K. Dougan, Lin Wu, Kathrin H. Kirsch, Usha Nair, William R. Schief and Facundo D. Batista

Review timeline:

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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 16th Mar 18

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the comments from two referees. I am afraid that the comments are not very encouraging.

As you can see from the comments below, both referees are not convinced that the analysis adds enough insight to be considered for publication in The EMBO Journal. I do see that referee #1 is more supportive than referee #2 but none of them offer strong support for publication here. It is also not clear how the analysis can be extended to make it a stronger candidate for us.

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REFEREE COMMENTS

Referee #1:

This well-written study describes an optimized protocol for the generation of knock-in mice bearing human antibody heavy chain sequences. It relies on CRISPR/Cas9-mediated genome editing in vivo. Outstanding features of the study are: 1. Stringent testing of the efficacy of 10 different guide RNAs in an in vitro system. 2. Probing of the specificity of selected guides also in vitro. 3. An in depth comparison of the homology directed repair efficacy with short and long homology arms. 4. Testing and demonstration of germ line transmission of the altered heavy chain locus.
Development of major B and T cell populations in the gene-edited animals was characterized and contribution of B cells expressing the altered locus Ig was determined by sequence analysis of single

cells. B cell development from the edited GT121 allele was impaired and contribution to mature B cells minimal, hence demonstration of efficient functionality of the engineered allele was not achieved.
The most relevant part of the study is the description of a refined protocol for the rapid generation of animals with an engineered Ig heavy chain locus. While inactivation of genes through "indel" generation due to NHEJ is by now well established and achieved with high efficacy, knock-in of larger sequences or of reporter genes has proven far more complex. Here Lin et al. demonstrate that using two carefully preselected guide RNAs that flank the sequence to be engineered, the efficacy of HDR can be significantly improved by increasing the length of the homology arms provided. Importantly, the sequence to be edited was not located in an open locus like e.g. the R26 locus and the strategy chosen did neither include a selection advantage for the modified sequence, nor was the modification associated with expression of an easily detectable marker. Such strategies were frequently employed in the past, but tend to overestimate the general recombination frequency compared to other loci. While the use of two guide RNAs yielded a modification of the enclosed DNA sequence in about 1 in three cases, the success rate for HDR was only modest. Roughly doubling the length of the homology arms provided a significantly improved HDR while the overall frequency of genome editing remained largely the same.

- specific major concerns essential to be addressed to support the conclusions
The main finding of the study is a technically refined CRISPR/Cas9-based knock-in strategy. Similar approaches were already suggested for and applied in other instances and species (zebrafish). Although it has to be stated that this technology is far from being standard at this time. To fully appreciate the efficacy of the technology in terms of costs, time required and animals used, full disclosure of the numbers of injected embryos would be helpful. The text states that the first line of table 2C corresponds to 400 injected zygotes, assuming similar survival rates the overall generation of ~175 pups would then require the injection of 4,000 - 5,000 zygotes, which is a fair number. As the authors point our correctly, this approach in addition requires elaborate genotyping of the offspring, which due to possible mosaicism has to be done in addition to the founder also for at least the first generation. Here a relevant question is, if the approach can be transferred to ES cells where the seeming disadvantage in terms of speed could potentially be compensated by fast and efficient genotyping upfront in tissue culture and the certainty of the genotype of the generated animals.
An unexpected finding is that in a competitive environment B cells expressing the genome edited human VHJ Ig allele suffer a disadvantage at the preBCR selection checkpoint and appear to be selected against or receptor edited. Failure to pass the check point is very obvious in hemizygous mice that display a extremely low B cell count in peripheral blood. This is completely unexpected, in particular, in light of the fact that the same allele has not caused problems in B cell development in a different study. While it is probably out of the realms of this study to mechanistically explain this finding, it casts some shadow on this approach. The study would be stronger, in particular the claim of the ease with which genome editing can be done rapidly if the technology was applied providing a functional allele, e.g. by knock in of another Ig allele or unrelated sequence.

- minor concerns that should be addressed
Because the immunological message is limited, the study in this form should be suitable as a technical report and should therefore be accessible to non expert immunologist readers. Specific immunological terminology should avoided where possible and all elements in figures should be explained in the legend.
Fig. 1 P corresponding to the murine VHJ558 promotor, HDR
Fig. 2 , T=taq man probes, the position of the primers and the different length of the homology arms should be indicated
Fig. EV2 could be included into the Fig. 2
Fig.3 B the FACS plot for the PGT121+/WT animal appears to correspond the single outlying data point in panel B (70% Hardy population B) and would therefore not be very representative.

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)
None!

Referee #2:

The aim of the manuscript of Lin et al. was to generate human immunoglobulin heavy-chain knock-in mice at a high frequency. The manuscript is a technical report describing an improved method how to efficiently generate knock-in mice by CRISPR/Cas9 mutagenesis in injected zygotes. The main message of the manuscript is that the 'new' CRISPR/Cas9 method facilitates the generation of knock-in mice at an efficiency of 20-50%. The second part of the manuscript deals with the characterization of the human PGT121 Igh kock-in mouse that was generated. This phenotypic analysis revealed that the human PGT121 Igh knock-in allele is counter-selected already at the pre-BCR checkpoint, possibly due to the lack of pairing of the PGT121 heavy-chain with the mouse surrogate light chains. Hence, this analysis did not yield any interesting biological data. Moreover, the 'new' CRISPR/Cas9 method is quite conventional as it is based on the injection of a double-strand plasmid as a repair template together two sgRNAs and Cas9 protein. While this experimental set-up usually yields transgenic mice at a low frequency, Lin et al. report a very high efficiency of 20-50%. The authors attribute this high frequency of homologous recombination to the use of 5-kb long homology arms. However, it is not clear why the extension of the homology arms from 3.9/2.6 kb to 5 kb length has such a dramatic effect. Maybe, the extended arms contain special sequence features that promote this high frequency of homologous recombination. Moreover, the method section is not informative, as the exact concentrations of the different reagents and the injection mode (nuclear versus cytoplasmic) has not been described. The general claim that 5-kb long homology arms facilitate a high recombination frequency has not been validated, as the 'new' method has only been applied to one gene. For such a general claim, multiple genes would have to be targeted to demonstrate that long homology arms consistently promote a high recombination frequency at several loci. Moreover, a recent paper describing the elegant Easi-CRISPR method, which is based on the use of long single-strand DNA as a repair template, has not been cited, although this method leads to knock-in mice at a frequency of 8.5-100% at multiple gene loci (Quadros et al., Genome Biology 18:92). For all the reasons mentioned above, I cannot recommend publication of this manuscript as a resource article in the EMBO Journal.

2nd Editorial Decision 27th Apr 18

Thank you for your email regarding the decision taken on your manuscript.

I have discussed the manuscript and referee comments further with an external advice and I have now received the input back. The advisor does find the described method valuable and supports publication here. S/he also finds the comments raised by referee #1 very constructive. Given this input, we would like to consider a revised manuscript that addresses the concerns raised by referee #1. I agree with referee #1 that the inclusion of a 2nd KI line would be good and strengthen the paper.

It would also be good to publish a detailed protocol for the method used and that this would be helpful for the field.

Let me know if we need to discuss anything further

Revision authors' response 29th May 18

Reviewer #1 (R1):

This well-written study describes an optimized protocol for the generation of knock-in mice bearing human antibody heavy chain sequences. *It relies on CRISPR/Cas9-mediated genome editing in vivo. Outstanding features of the study are: 1. Stringent testing of the efficacy of 10 different guide RNAs in an in vitro system. 2. Probing of the specificity of selected guides also in vitro. 3. An in depth comparison of the homology directed repair efficacy with short and long*

homology arms. 4. Testing and demonstration of germ line transmission of the altered heavy chain locus.
The most relevant part of the study is the description of a refined protocol for the rapid generation of animals with an engineered Ig heavy chain locus. *While inactivation of genes through "indel" generation due to NHEJ is by now well established and achieved with high efficacy, knock-in of larger sequences or of reporter genes has proven far more complex.*

We thank R1 for the encouragement and positive comments on our manuscript.

R1 Specific Comments:

1. ***R1 states:*** *"To fully appreciate the efficacy of the technology in terms of costs, time required and animals used, full disclosure of the numbers of injected embryos would be helpful. The text states that the first line of table 2C corresponds to 400 injected zygotes, assuming similar survival rates the overall generation of ~175 pups would then require the injection of 4,000 - 5,000 zygotes, which is a fair number."*

Response: We thank R1 for raising this point. We apologize for the misunderstanding regarding the number of embryos injected. We would like to clarify that we did not inject 4,000-5,000 zygotes in our experiments. As shown in Table 2C and described on page 8, for our initial CRISPR experiment, we injected 400 zygotes and after that we injected approximately 200 zygotes. The enhanced survival rates after the first two injections could be attributed to an overall improvement of our reagents and techniques. Out of 200 embryos implanted, about 50 pups were born, which is in line with ES transfections in embryos.

2. ***R1 states:*** *"As the authors point our correctly, this approach in addition requires elaborate genotyping of the offspring, which due to possible mosaicism has to be done in addition to the founder also for at least the first generation."*

Response: We apologize to R1 for any confusion created. We would like to point out we followed several different founder lines and observed mosaicism in a minority of lines created (Supplementary Figure EV4). This was revealed by non-Mendelian segregation of the KI gene during animal crossing and not by screening. We follow standard screening procedures which are in line with our routine genotyping assays for each of our KI mice strains, as described in our Methods section on page 19 of the manuscript. The genotyping assays are straightforward, quick and accurate, and allow us to confirm the KI mice genotypes without doing PCR reactions or running agarose gels in-house. We have clarified this point in the text. Please see pages 7 and 19.

3. ***R1 states:*** *" Here a relevant question is, if the approach can be transferred to ES cells where the seeming disadvantage in terms of speed could potentially be compensated by fast and efficient genotyping upfront in tissue culture and the certainty of the genotype of the generated animals."*

Response: We thank R1 for pointing this out and we have addressed this as a potential extension of our approach in the present manuscript. However**,** we have not performed the suggested CRISPR approach into ES cells, therefore we cannot speculate about the efficacy of our method in that system. However, we believe that transferring the CRISPR KI approach into ES cells, will slow down the process of obtaining KI mice. Our goal is to rapidly generate human heavy chain KI mice by CRISPR injection directly into zygotes, overcoming the limitation of the ES cells approach, as explained in pages 14 and 15 of the manuscript.

4. R1 states**:** *An unexpected finding is that in a competitive environment B cells expressing the genome edited human VHJ Ig allele suffer a disadvantage at the preBCR selection checkpoint and appear to be selected against or receptor edited. Failure to pass the check point is very obvious in hemizygous mice that display a extremely low B cell count in peripheral blood. This is completely unexpected, in particular, in light of the fact that the same allele has not caused problems in B cell development in a different study. While it is probably out of the realms of this study to mechanistically explain this finding, it casts some shadow on this approach.*

Response: We thank the R1 for pointing this out. We would like to clarify that the PGT121 germline-reverted heavy chain sequence used in our study is not the same as the as one previously described for PGT121 knock-in mouse immunization (Escolano et al, 2016). Our sequence more likely represents germline-reverted sequences validated by our database and contains changes in the amino acid sequences in the CDR3, which are probably the cause of the differences observed. The origin of our sequence is described on page 6 and in Supplementary Figure EV1. The other difference between our PGT121 IgH KI mouse versus that described in the paper by Escolano et al., is that their KI mouse contains both the PGT121 heavy chain as well as its cognate germline light chain. It is possible that the ability of B cells expressing PGT121 to pass developmental checkpoints in the bone marrow is dependent on the correct pairing of these PGT121 H+L chains.

5. ***R1 states*****:** *The study would be stronger, in particular the claim of the ease with which genome editing can be done rapidly if the technology was applied providing a functional allele, e.g. by knock in of another Ig allele or unrelated sequence.*

Response: We are in agreement with the R1's suggestion to strengthen the study by including a mouse line bearing a knock in of another Ig allele. We have modified the manuscript to include a second IgH KI mouse line expressing BG18-gH, a very potent, recently reported bnAb. This newly created mouse line exhibits normal B cell development and B cells in the periphery, which further validates our approach. We hope that the inclusion of the BG18 KI mouse data will be satisfactory to R1 in supporting our claim that our CRISPR method can be used to rapidly generate KI mice containing functional B cells expressing antibodies of interest for subsequent immunogen validation (Figures 7 and 8; manuscript pages 12-13).

- Minor concerns that should be addressed

Because the immunological message is limited, the study in this form should be suitable as a technical report and should therefore be accessible to non expert immunologist readers. Specific immunological terminology should avoided where possible and all elements in figures should be explained in the legend.
Fig. 1 P corresponding to the murine VHJ558 promotor, HDR
Fig. 2 , T=taq man probes, the position of the primers and the different length of the homology arms should be indicated
Fig. EV2 could be included into the Fig. 2
Fig.3 B the FACS plot for the PGT121+/WT animal appears to correspond the single outlying data point in panel B (70% Hardy population B) and would therefore not be very representative.

Response: We thank the R1 for highlighting these points to make the manuscript readily accessible to non-expert immunologist readers. As suggested, we have explained the terminology in the figure legends, and changed the Figures 2 and 3. We have re-analyzed the early B cell populations in Figure 3B and generated a modified figure.

Referee #2 (R2):

1. ***R2 states:*** *The aim of the manuscript of Lin et al. was to generate human immunoglobulin heavy-chain knock-in mice at a high frequency. The manuscript is a technical report describing an improved method how to efficiently generate knock-in mice by CRISPR/Cas9 mutagenesis in injected zygotes. The main message of the manuscript is that the 'new' CRISPR/Cas9 method facilitates the generation of knock-in mice at an efficiency of 20-50%. The second part of the manuscript deals with the characterization of the human PGT121 Igh kock-in mouse that was generated. This phenotypic analysis revealed that the human PGT121 Igh knock-in allele is counter-selected already at the pre-BCR checkpoint, possibly due to the lack of pairing of the PGT121 heavy chain with the mouse surrogate light chains. Hence, this analysis did not yield any interesting biological data.*

Response: We thank R2 for the detailed remarks on our manuscript. Our area of interest was to investigate the B cell-specific expression of broadly neutralizing antibody (bnAb) germline precursors. To this end, we only focused on CRISPR/Cas9-mediated IgH knock-in in the murine IgH locus. When the rearranged antibody sequence is integrated at the native IgH locus, the knock-

in BCR is not limited in class switch and somatic hypermutation, therefore it can be used as a tool to study the antibody response to a given vaccine in a physiological setting. Until now, most BCR or HIV bnAb knock-in mice targeted at IgH locus were generated by ES cells technology. This approach has the disadvantage of requiring a long time before the murine model can be used in immunological studies. Here we describe the proof of concept of a novel CRISPR injection method to rapidly generated human heavy chain KI mice for vaccine development. Although B cells of PGT121 KI mice couldn't pass the bone marrow checkpoint in B cell development, possibly due to autoreactivity, we have now modified the manuscript to include immunocharacterization data from a second IgH KI mouse line expressing BG18-gH. Please see Figures 7 and 8; manuscript pages 12-13.

2. ***R2 states:*** *Moreover, the 'new' CRISPR/Cas9 method is quite conventional as it is based on the injection of a double-strand plasmid as a repair template together two sgRNAs and Cas9 protein.*

Response: We respectfully disagree with R2 on this point. We believe that our work provides insights into CRISPR-Cas9-mediated targeting of IgH VDJ segments to the murine Ig locus to generate IgH KI mice in a fast and reliable manner. These animal models are going to be extremely important in helping immunogenicity evaluation in mice, which are key in preclinical trials evaluation. We have is a great deal of enthusiasm about our methodology and Ig KI models. We believe that our methodology will have direct implications in human health.

3. ***R2 states:*** *While this experimental set-up usually yields transgenic mice at a low frequency, Lin et al. report a very high efficiency of 20-50%. The authors attribute this high frequency of homologous recombination to the use of 5-kb long homology arms. However, it is not clear why the extension of the homology arms from 3.9/2.6 kb to 5 kb length has such a dramatic effect. Maybe, the extended arms contain special sequence features that promote this high frequency of homologous recombination.*

The general claim that 5-kb long homology arms facilitate a high recombination frequency has not been validated, as the 'new' method has only been applied to one gene. For such a general claim, multiple genes would have to be targeted to demonstrate that long homology arms consistently promote a high recombination frequency at several loci.

Response: We thank the R2 for highlighting a concern regarding the length of the homology arms. While we are unsure about the reason why the extension in arm length leads to such as dramatic improvement in knock-in efficiency, we have performed further two injections with short arm DNA donor which had a similar knock-in rate [1 of 15 pups, (6.7%)], Figure 2C. According to our injection results, we found that the length of arms indeed affected the success of recombination frequency from 6.7% to 20~50% at the murine IgH locus.

4. ***R2 states:*** *Moreover, the method section is not informative, as the exact concentrations of the different reagents and the injection mode (nuclear versus cytoplasmic) has not been described.*

Response: We followed the protocol described in Generating genetically modified mice using CRISPR/Cas9-mediated genome engineering, *Nature Protocols* 2014 Aug;9(8):1956-68. We have described our procedure in detail in the Materials and Methods section of the manuscript.

5. ***R2 states:*** *Moreover, a recent paper describing the elegant Easi-CRISPR method, which is based on the use of long single-strand DNA as a repair template, has not been cited, although this method leads to knock-in mice at a frequency of 8.5-100% at multiple gene loci (Quadros et al., Genome Biology 18:92).*

Response: We thank R2 for bringing this publication to our attention. We have now referred to this paper in our Discussion section.

3rd Editoral Decision 18th Jun 18

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has know been seen by the original referee#1 and the comments are provided below.

As you can see, the referee appreciates the introduced changes and supports publication here. There are just a few minor text changes needed. I am therefore very pleased to let you know that we will accept the manuscript for publication here. Before sending you the formal acceptance letter there are just a few things to sort out - I have provided a revision link below for you to upload the files. Once I get the revised version in I will send you the formal acceptance letter.

REFEREE COMMENTS

Referee #1:

Comments to EMBOJ-2018-99243R1
In their revised version of the manuscript ""One Step CRISPR/Cas9 Method for the Rapid Generation of Human Antibody Heavy Chain Knock-in Mice" the authors have addressed my previous criticisms faithfully. Generation of a second mouse model with a different Ab knock in has significantly strengthened the manuscript and it is certainly a convincing proof for the claim of rapid generation of knock ins by this technology.
Minor points:
- Please introduce the abbreviation broadly neutralizing Antibody (bnAb) also upon its first use in the text body.
- Page 12 5th line form the bottom, I think the reference to Fig. 4G is misplaced here, BG18 data are exclusively shown in Fig. 7.
- Page 16 5th line from the bottom, please check sentence: "Of specific interest is whether the BG18-GL IgH accumulate adequate somatic hypermutation and mature into functional memory B cells are capable of rapid and durable antibody response". Probably "and" should be inserted after B cells?

Revision - authors' response 26th June

Reviewer #1 (R1):

In their revised version of the manuscript ""One Step CRISPR/Cas9 Method for the Rapid Generation of Human Antibody Heavy Chain Knock-in Mice" the authors have addressed my previous criticisms faithfully. Generation of a second mouse model with a different Ab knock in has significantly strengthened the manuscript and it is certainly a convincing proof for the claim of rapid generation of knock ins by this technology.

We thank R1 for the encouragement and positive comments on our manuscript. Our point-by-point response is below:

R1 Specific Comments:

4. **R1 states:** "*Please introduce the abbreviation broadly neutralizing Antibody (bnAb) also upon its first use in the text body.*"

Response: We have introduced the abbreviation broadly neutralizing antibody (bnAb) on page 4.

5. ***R1 states:*** "Page 12 5th line form the bottom, I think the reference to Fig. 4G is misplaced here, BG18 data are exclusively shown in Fig. 7."

Response: We thank R1 for pointing this out. The misplaced figure number has been deleted.

6. ***R1 states:*** *"Page 16 5th line from the bottom, please check sentence: "Of specific interest is whether the BG18-GL IgH accumulate adequate somatic hypermutation and mature into functional memory B cells are capable of rapid and durable antibody response". Probably "and" should be inserted after B cells?"*

Response: This sentence has been corrected to read as follows: Of specific interest is whether the BG18-GL IgH accumulates adequate somatic hypermutations and matures into functional memory B cells capable of rapid and durable antibody response.

Accepted 5th July

Thank you for submitting your revised manuscript to The EMBO Journal. We now have everything in and I am very pleased to accept the manuscript for publication in the EMBO Journal.

The only thing missing is that we need one Appendix file with a Table of Content that has the appendix tables. You can send us this via email and we will upload it for you.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Facundo D. Batista
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99243R1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	Sample sizes were chosen on the basis of published work in which similar phenotypical characterization and similar defects were reported. For the immunocharacterization experiments, we used at least 3 mice to detect an effect size of 5% with power of 90% in specific cells population. Statistical analyses were performed using Prism (GraphPad Software). (Page 21)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes were chosen on the basis of published work in which similar phenotypical characterization and similar defects were reported.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from analysis. Criteria for analyses were pre-established for all types of experiments.
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.	Mice were randomly chosen to perform assays.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly chosen to perform assays.
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.	'Blinding' of investigators to sample identity was not done in this study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	'Blinding' of investigators to sample identity was not done in this study.
5. For every figure, are statistical tests justified as appropriate?	Statistical parameters including the exact value of n with the description of what n represents, the mean, the SEM and the p value are reported in the Figures and the Figure Legends. Statistical analyses were performed using Prism (GraphPad Software) and compared with Student's t-test, p values < 0.05 were considered significant.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of samples was assumed on the basis of published studies with analysis similar to ours.

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-tun

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/

Is there an estimate of variation within each group of data?	All data are provided with standard error of the mean, as specified in the respective figure legend section in the text.
Is the variance similar between the groups that are being statistically compared?	N/A

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).	Reported in Matherials and Methods section (Page 18-21)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* 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.	Reported in Materials and Methods section (Page 18-19). C57BL/6J WT mice (8-10 weeks old) were purchased from The Jackson Laboratory (Stock No: 000664). Mice used in this work were housed at the the Ragon Institute's animal facility.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Reported in Material and Methods section (Page 19). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Harvard University and the Massachusetts General Hospital and conducted in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care (AAALAC).
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.	Our work in in compliance with the ARRIVE guidelines.

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 a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Results from our mAb sequencing data are shown in Supplementary Tables 5-8 accompanying this manuscript.
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. 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

22. 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