EMBO reports

Bardet-Biedl Syndrome ciliopathy is linked to altered hematopoiesis and dysregulated self-tolerance

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DOI: N/A

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Review Timeline:

Transfer from Review Commons:	30th Apr 20
Editorial Decision:	27th May 20
Revision Received:	27th Oct 20
Editorial Decision:	26th Nov 20
Revision Received:	4th Dec 20
Accepted:	9th Dec 20

Editor: Achim Breiling

Review COMMONS

Transaction Report: This manuscript was transferred to EMBO Reports following peer review at Review Commons.

1st Editorial Decision 27th May 2020

Dear Dr. Stepanek,

Thank you for transferring your manuscript from Review Commons to EMBO reports. I went through your manuscript, the referee reports (attached again below), and your revision plan (point-by-point response), and also contacted an expert advisor regarding the study. Both referees acknowledge the potential interest of the findings. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which you are willing to address during a major revision of the manuscript.

We thus would like to invite you to revise your manuscript for EMBO reports with the understanding that all the referee concerns must be addressed in the revised manuscript and in a final detailed point-by-point response, as you indicated in your revision plan. Acceptance of your manuscript will depend on a positive outcome of a second round of review (using the same referees that have assessed the study before). It is our policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Please have your manuscript proofread by a native speaker (see also the comment of referee #1).

Moreover, our expert advisor who went through your paper and the referee comments indicates that it would be great if you could extend the analysis of BBS mutations and the incidence of autoimmune disease, looking if there is any selectivity among BBS mutations. Moreover, the adviser encourages you to add some more thinking about what kind of mechanism could be behind the observations to the discussion, even if these will be rather speculative. S/he points out that the BBS defect could be in the B cell lineage or equally/more likely in the environment of the immune cells, which is no less interesting, but should be discussed.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

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4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please add an author contributions section to the manuscript after the acknowledgements, and a conflict of interest statement (COI).

10) Please add scale bars in the microscopic images (without writing on them). Please define their size only in the respective figure legend.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO reports

Referee #1:

The manuscript by Oksana Tsyklauri and colleagues reports on the increased incidence rate of certain autoimmune diseases in two larger cohorts of Bardet-Biedel-Syndrome (BBS) patients, as well as on disease-correlated alterations in hemograms of patients in one of the cohorts. This information is gathered by systematic review of medical journals. BBS is an autosomal recessive genetic disorder in which (partial) loss-of-function of any of the hitherto 23 disease-associated genes (OMIM series PS209900) leads to defective formation and/or function of cilia. BBS patients present with multi-phenotypes, whereof obesity is amongst the main features.

Pathogenic BBS variants are found in genes encoding for proteins of the BBSome core complex, a protein complex involved in directional intracellular transport of proteins at the centrosome and the primary cilium. Given the profound similarity in organization of directional intracellular transport by the mother centriole/basal body at the primary cilium and the centrosome at the immunological synapse the authors set out to investigate the significance of complete or lineage-specific loss-of-function of a particular BBS protein of the BBSome core complex, BBS4, in immune cells of different mouse models. Finally, the authors compare insights gained from their mouse studies to blood cell analyses of BBS patients and BMI-matched or BMI-randomized control donors. Combined, these studies identified obesity-dependent and obesity-independent effects of Bbs4 loss-of-function on the immune system in mice whereof some are shared cross-species-wise with human BBS patients.

Please find below my comments, which I believe may help strengthening the conclusions of the study.

Major comments: major issues affecting the conclusions.

-Prevalence of autoimmune diseases in BBS patients :
Given the detailed subsequent analysis of BBS4 defective mice it would be very supportive for the experimental rational to analyze if pathogenic mutations in BBS4 (or other BBSome core complex members) are (more) prevalent amongst the autoimmune disease affected BBS patients.

-Mouse models for studying the role of the BBSome in the immune system / Figure 1
The Bbs4GT/GT mouse shows a hypomorphic phenotype leading the authors to speculate about either very low residual expression of full length or truncated BBS4 isoforms, failed to be detected by WB. Did the authors test for Bbs4 mRNA expression/splice variants in cells/tissues of the Bbs4GT/GT mouse? Further, in humans an N-terminal truncated 347aa Bbs4 isoform utilizing an alternative start codon (Met177 in BBS4 consensus full length protein, https://www.nextprot.org/entry/NX_Q96RK4/exons) is evident. The sequence is conserved between human and mice, where it is encoded by exon 8, 3'-prime of the GT-cassette. Unfortunately, the authors do not provide any information on the epitope used for generation of the a-Bbs4 antibody nor present complete MW ranges of Western Blot membranes (are BBS4 truncates detected?), which would help to interpret the observed hypomorphic phenotype. Possibly, this would even open for a future line of research, if eg tissue/cell type specific expression of an N-terminally truncated isoform is evident.

-Alterations in the immune system of Bbs4 deficient mice / Figure 2
Figure 2B: Could the authors please comment on why they chose to follow a Region Of Interest (ROI) gating strategy, rather than a more common subdivision in quadrants for the determination of percent-wise distribution of IgM/IgD-status subtypes?
Figure 2C & Discussion: The increased frequency of CD24highCD43low pre-B-cells in the bone marrow leads the authors to conclude "(p11) ...that Bbs4-deficiency results in a developmental block at the pre-B-cell stage,..." and is discussed as "(p14, 4th paragraph) ...partial developmental arrest..". However, Bbs4KO/KO mice do develop a mature peripheral B-cell system. Hence, it appears equally possible to concluded that the increased frequency of cells at the pre-BCR selection stage might rather reflect a longer time required to pass this developmental stage or an accelerated transition through earlier developmental stages. This finding might indeed be of particular relevance, since failure of negative selection of autoreactive B-cells at the pre-BCR may lead to escape of clones to the periphery and drive the development of autoimmune diseases. If not B-cell intrisic, Bbs4 defects might affect cytokine release or other important properties of non-immune cells in the pre-B-cell niches in the bone marrow. In fact, this might be documentable in terms of IHC/IF of the bone marrow in BBS4-deficient and control mice. The results should be presented more precise and better clarified/discussed by the authors.

Figures 2E/S2B,C: The determined frequency of IgM-IgD+ late mature B-cells is strictly dependent on the gating strategy, and the reported Bbs4 genotype dependent differences are rather small. The contour plots do hardly show any well-defined populations (peaks). It is thus difficult to follow the applied gating strategy, in particular if to distinguish IgD- from IgD+ cells. This point needs to be clarified by the authors to support their strong conclusion of an obesity-independent, Bbs4 dependent B-cell compartment alteration.

-The role of Bbs4 in B-cell homeostasis is not intrinsic / Figure 3
The authors use the Vav-iCre system to specifically target the Bbs4 locus in the hematopoietic lineage. The results of their analysis of the B-cell compartment are summarized in Figure 3, where no significant differences are observed between control and Cre-induced littermates. Example flow-cytometry data should be provided to support the dot-plot panels, eg in form of a supplementary. More importantly, no data on the efficacy and specificity of Bbs4 targeting are shown. These data are required for the solid interpretation of the results by the reader.
Finally, Vav expression is not only restricted to the B-cell compartment and the Vav-Cre system reported as a pan-hematopoietic and pan-endothelial cell targeting system (Georgiades et al, Genes 34(4), 2002). This fact is not discussed, but may/is likely to impinge on the phenotype.

-Bbs4 deficiency does not intrinsically influence T-cell and B-cell antigenic responses
Figure 4A/B: The hypomorphic phenotype of Bbs4GT/GT mice raises the concern of expression of potential partially functional Bbs4 truncate in selected cell types and/or cells of different activation status. Can the authors conclude definitely that there is no Bbs4 or functional BBSome in BCR-engaged B-cells of the Bbs4GT/GT mice? It is difficult to understand the author's argument for not testing the B-cells of the Bbs4KO/KO background. The experiment is performed on isolated B-cells and thus under controlled ex vivo conditions, ie not under exposure to eg obesity specific cytokine profiles or similar. As a suggestion, I would assume that similar experiments could be performed using alternative ways of T-cell independent B-cell stimulation, hence avoiding the requirement for the B1-8 background.

Figures 4C-E, S3: The conditional targeting of the Bbs4 locus in the CD4 T-cell compartment is well documented, and the intricate system used to study the capability of induction type 1 diabetes appears as an elegant way to study Bbs4-dependency in a complex in vivo system. Further, a

shared feature of the autoimmune diseases in BBS patients is the role of CD4 T-helper cells, adding to the relevance of the experiment. The authors state that they hypothesize that the functionality of the immunological synapse is BBS4 (BBSome) dependent, hence testing for a loss of or delayed onset of type 1 diabetes and blood glucose increase. Though I do understand that the authors here set out to test for a T-cell intrinsic function of BBS4, which is in contrast to the germ-line loss-of-function in BBS patients, wouldn't one expect that CD4 T-cell functionality is UNcompromised in BBS patients to drive the observed autoimmune diseases ?!

-BBS-induced obesity affects blood homeostasis
The authors state that the CRP-levels in BBS patients were significantly higher than in BMI randomized an BMI matched controls. However, the authors show in the right panel of figure 5C that these differences are NOT significant, but that the frequency of patients presenting with CRP >5 is significantly different (left panel). The wording at the end of the first paragraph, p13 may be changed to correct for this.

Minor comments: important issues that can confidently be addressed.

-Table 1:
The authors refer in the figure legend to present "the fold change in prevalence" but do show the prevalence and a calculated Odds Ratio and p-value. The calculations are difficult to recapitulate from the numbers presented in the table (if Odds Ratio to be defined by OR = [BBScases / (BBStotal-BBScases)] / [Normalcases / (Normaltotal-Normalcases)] numbers don't match). Could the authors please add a more specific explanation of chosen statistic tools and calculations in the Methods section?
Apparently, some patients suffered from more than one autoimmune disease (CRIBBS cohort). Could the authors specify which diseases co-occurred?
Given the detailed subsequent analysis of BBS4 defective mice it would be very supportive for the experimental rational to analyze if pathogenic mutations in BBS4 (or other BBSome core complex members) are (more) prevalent amongst the autoimmune disease affected BBS patients.

-T-cell compartment analysis / Figure S1
Examples of flow-cytometry data (contour plots) for the quantifications shown panels S1A-C need to be provided.

-Figures 1,5, S1
coding for statistical significance needs to be explained in figure legend

-general comment:
Though being a non-native English speaker I find that the manuscript, particularly the discussion, may benefit from some language editing by the native English speaking co-authors.

Significance:

The manuscript represents an interesting and relevant collection of findings in BBS patients and Bbs4 mouse models, shedding some new light on the potential role of the immune system in the pathobiology of BBS as well as the hypothesized role of the BBSome in non-ciliated blood cells. Hence, the study represents a reference motivating further research into the causes and consequences of alterations of the immune system in ciliopathy patients. Extended analysis of the bone marrow and peripheral lymphoid organs by IHC and IF allowing eg the analysis of cilia status in

non-immune cells as well as cytokine profile analyses to investigate the functional status of these niches/organs would further advance the impact of the study, but are unfortunately beyond what can be suggested for an experimental revision of the manuscript.

There are to the best of my knowledge no other published systematic reviews on hematopoietic system alterations in BBS or related ciliopathies. Extra-ciliary functions of several other ciliary proteins have been and still are an important topic in understanding the pathobiology of these congenic disorders. Selected individual proteins have been studied for their functions in immune cells at greater much detail than in this manuscript (properly discussed by the authors), but were largely restricted to specific cell lines, not embracing the complexity of interplays underlying homeostasis and activation of a proper immune system.

Referee #2:

This is a thorough and well executed study that uses BBS patients and mouse model to dissect the function of BBS4 in maintaining hematopoietic system and self-tolerance. Specifically, by using a combination of genetic/molecular and cell-based assay approaches the authors elegantly show that the deficiency of BBS4 alters the development and homeostasis of B cells. The BBS patients also have a higher incidence of certain autoimmune diseases. The authors further suggested that some of the hematopoietic systems are altered due to the BBS-caused early-onset obesity. There are not many studies to be performed to investigate the connection between ciliopathy and alternation of immune system, this study thus provides solid evidence that ciliopathy could cause abnormal immune responses in patients.

The quality of the data in this manuscript is high and I only have a few comments for the authors to address.

Major comments:

1) To the best of my knowledge, a total of 22 genes (bbs1-bbs22) so far identified clinically, once mutated, cause BBSome. The authors should mention this in the introduction part.

2) BBS is a heterozygous disorder and not all BBS patients develop early-onset obesity. I am wondering whether it is suitable to conclude that BBS, as a whole, causes altered hematopoietic system as reflected by the manuscript title.

3) Could authors discuss a little bit more about the possible role that the ciliated cells, e.g. the leptin receptor expressing neurons controlling energy homeostats in hypothalamus, play in mediating the hematopoietic system?

Minor comments:

1) Page 11, second paragraph, "for an expression of a truncated BBS4..." should be "for the expression of a truncated BBS4...".

2) Through the whole manuscript, "Bbs4-dificient mouse" should be "Bbs4-dificient mouse".

3) Page 11, third paragraph, "decreased percentages of CD44+..." should be "decreased

percentage of CD44+...".

4) Through the whole manuscript, "B-cell development" should be "B cell development".

5) Page 12, first paragraph, "obesity independent" should be "obesity-independent".

6) Page 12, In the subtitle "Bbs4 deficiency does not intrinsically influence T-cell and B-cell antigenic responses", T-cell and B-cell antigenic responses"
should be "antigen-specific B cell and T cell responses".

7) And more.....

Editorial comments

Please have your manuscript proofread by a native speaker (see also the comment of referee #1).

Our manuscript was proofread by a professional editor.

Moreover, our expert advisor who went through your paper and the referee comments indicates that it would be great if you could extend the analysis of BBS mutations and the incidence of autoimmune disease, looking if there is any selectivity among BBS mutations.

We are presenting a detailed analysis of the incidence of autoimmune diseases in patients stratified according to their causative BBS genes (Appendix Tables 3-4) from the London cohort. It shows that autoimmunity was detected in most of these groups. Moreover, the incidence of autoimmunity is slightly lower in patients with mutated BBS1 than in patients with mutated BBS10, which fits with the overall more severe phenotype of the latter group. However, the number of available patients is too low to perform a statistical analysis with a reasonable power. Unfortunately, the information about the causative gene is not available for patients from the CRIBBS NIH Registry. We mentioned this issue in the Results of the revised manuscript.

On this note, we were able to generate another model of BBSome deficiency based on a whole body knock-out of Bbs18 (alias Bbip1). Although the pre-weaning lethality of this strain was severe (Fig. EV3G), we were able to analyze one litter containing 2 KO, 1 WT, and 3 het littermates (all males). In this small group of animals, we observed the major B-cell related features of Bbs4KO, i.e., increased frequency of B-cell precursors in the bone marrow and decreased marginal zone B cells in the spleen (Fig. EV3H-I). This observation indicates that the role of Bbs4 in B-cell development/homeostasis is not unique, but is probably caused by the dysfunction of the BBSome, which could be caused by deficiency in any BBSome subunit or in the chaperonins assisting the BBSome formation.

Moreover, the adviser encourages you to add some more thinking about what kind of mechanism could be behind the observations to the discussion, even if these will be rather speculative. S/he points out that the BBS defect could be in the B cell lineage or equally/more likely in the environment of the immune cells, which is no less interesting, but should be discussed.

We excluded the possibility that the B-cell phenotype of Bbs4KO mice is caused by the BBS4 deficiency in B cells or in the hematopoietic lineage cells in general. The major evidence is that Bbs4 $^{flox/flox}$ Vav1-Cre mice with Bbs4 deleted specifically in the hematopoietic lineage do not have the B-cell phenotype (Fig 4A-D). Moreover, we added a new experiment showing that B cells from WT and Bbs4 KO animals have very similar response to B-cell receptor crosslinking ex vivo (Fig 3C).

We did our best to uncover the mechanisms behind the B-cell phenotype in BBS4-deficient mice. This task was complicated by the current SARS2 pandemic. Not only we had to deal with work restrictions, but the reduction of mice cages and possibly changes in the animal caretaking pipeline resulted in a very low number of Bbs4 KO (and Bbs18 KO) mice generated in the breedings. Despite all these difficulties, we

obtained data that, in our opinion, substantially improved the importance of the manuscript. Please, see the response to the corresponding comment of the Reviewer 1.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The manuscript by Oksana Tsyklauri and colleagues reports on the increased incidence rate of certain autoimmune diseases in two larger cohorts of Bardet-Biedel-Syndrome (BBS) patients, as well as on disease-correlated alterations in hemograms of patients in one of the cohorts. This information is gathered by systematic review of medical journals. BBS is an autosomal recessive genetic disorder in which (partial) loss-of-function of any of the hitherto 23 disease-associated genes (OMIM series PS209900) leads to defective formation and/or function of cilia. BBS patients present with multi-phenotypes, whereof obesity is amongst the main features.

Pathogenic BBS variants are found in genes encoding for proteins of the BBSome core complex, a protein complex involved in directional intracellular transport of proteins at the centrosome and the primary cilium. Given the profound similarity in organization of directional intracellular transport by the mother centriole/basal body at the primary cilium and the centrosome at the immunological synapse the authors set out to investigate the significance of complete or lineage-specific loss-of-function of a particular BBS protein of the BBSome core complex, BBS4, in immune cells of different mouse models. Finally, the authors compare insights gained from their mouse studies to blood cell analyses of BBS patients and BMI-matched or BMI-randomized control donors. Combined, these studies identified obesity-dependent and obesity-independent effects of Bbs4 loss-of-function on the immune system in mice where of some are shared cross-species-wise with human BBS patients.

Please find below my comments, which I believe may help strengthening the conclusions of the study.

We are very thankful for the positive evaluation of our manuscript and for the valuable suggestions how to improve our manuscript. We believe that the experiments carried out during the revision substantially strengthened the manuscript.

Major comments: major issues affecting the conclusions.

-Prevalence of autoimmune diseases in BBS patients :
Given the detailed subsequent analysis of BBS4 defective mice it would be very supportive for the experimental rational to analyze if pathogenic mutations in BBS4 (or other BBSome core complex members) are (more) prevalent amongst the autoimmune disease affected BBS patients.

We are thankful for this comment. We included the analysis of the prevalence of autoimmune diseases in individual groups of patients based on the causative gene (Appendix Tables 3 and 4 of the revised manuscript). We observed that the autoimmunity is not linked to a particular gene, although we cannot exclude some quantitative associations, as the dataset is very limited (we do not know the identity of causative genes for patients in the CRIBBS Registry). The group of patients with the causative mutation in

BBS4 showed the highest prevalence of autoimmunity (50 %) of all groups, but this is based only on 4 patients in total. For this reason, we cannot make any strong conclusions here.

To address the question, whether the phenotype of Bbs4 KO mice is caused by the BBSome dysfunction or whether it is related to a unique role of BBS4, we generated a second mouse model. We made a whole body BBS18 KO (alias BBIP1 KO) mouse using CRISPR/Cas9. Unfortunately, we were experiencing a strong pre-weaning lethality. It is possible that the SARS2 pandemic might have contributed to that via changes in the animal caretaking routine in our animal facility. Luckily, we obtained one cage with six male littermates, from which we had 2 KO, 3 heterozygous, and 1 WT animal. The analysis of this limited number of animals recapitulated major features of BBS4 KO phenotype, i.e., high frequency of B-cell precursors in the bone marrow and low frequency of marginal zone B cells. This strongly indicated that this phenotype is caused by the BBSome impairment and could be expected in all mice deficient in any of eight BBSome subunits or chaperonins required for the BBSome assembly. The results concerning Bbs18 KO mice are summarized in Fig EV3F-I. Moreover, we resolved the low number of BBS4 KO mice for the analysis of Cxcl12 and Il-7 expression by combining BBS4KO and BBS18KO animals (Fig. 4E) – see below.

-Mouse models for studying the role of the BBSome in the immune system / Figure 1
The Bbs4GT/GT mouse shows a hypomorphic phenotype leading the authors to speculate about either very low residual expression of full length or truncated BBS4 isoforms, failed to be detected by WB. Did the authors test for Bbs4 mRNA expression/splice variants in cells/tissues of the Bbs4GT/GT mouse? Further, in humans an N-terminal truncated 347aa Bbs4 isoform utilizing an alternative start codon (Met177 in BBS4 consensus full length protein, https://www.nextprot.org/entry/NX_Q96RK4/exons) is evident. The sequence is conserved between human and mice, where it is encoded by exon 8, 3'-prime of the GT-cassette. Unfortunately, the authors do not provide any information on the epitope used for generation of the a-Bbs4 antibody nor present complete MW ranges of Western Blot membranes (are BBS4 truncates detected?), which would help to interpret the observed hypomorphic phenotype. Possibly, this would even open for a future line of research, if eg tissue/cell type specific expression of an N-terminally truncated isoform is evident.

We are very thankful for this suggestion and we appreciate the effort the reviewer clearly invested into reviewing our manuscript. We agree that understanding the hypomorphic nature of the BBS4 gene trap allele will increase the relevance of our study as a whole. Moreover, it might reveal general mechanisms of how this widely used strategy for making gene knock-outs could be insufficient.

The anti-BBS4 antibody used for WB recognizes a C-terminally localized peptide in exon 15 (the information is added to the Material and Method section). We never detected a truncated BBS4 variant in tissues of BBS4 GT mice (uncropped immunoblots are shown as Source Data for Figure 1), which means that a truncated variant is not expressed at detectable levels. However, it is possible that this alternative truncated form is present at very low levels in tissues of $Bbs4^{GT/GT}$ mice (see below).

We mapped the exon composition of Bbs4 transcript in tissues of WT, $Bbs4^{GT/GT}$, $Bbs4^{KO/KO}$ mice. First, we made a quantitative analysis of the presence of exons 5 to 11 in the brain (Fig. EV1A), kidney, and testes (not shown – all the tissue gave the same results). Whereas Bbs4 mRNA lacked exon 6 in $Bbs4^{KO/KO}$ mice as expected, the transcript contained all exons 5-11 in $Bbs4^{GT/GT}$ mice. This result was very surprising, because it means that RNA polymerase II is able to read-through two polyadenylation sequences serving as terminators of transcription in the GT cassette. The quantification of the expression levels by RT-qPCR

revealed that the expression of Bbs4 transcript is reduced to ~10% of the WT (Fig. EV1B). Interestingly, we noticed that the region between exon 5 and exon 6 is longer in Bbs4 GT than in Bbs4 WT transcript (Fig. EV1A-B). Indeed, sequencing revealed that the transcript contains a relatively short sequence from the GT cassette (in particular it is a part of En2 gene, whose exon is included in the GT cassette to serve as a generic exon) (Fig. 1EV3). This insert introduces a frameshift into Bbs4 ORF which explains why we do not detect BBS4 in tissues from Bbs4$^{GT/GT}$ mice by immunoblotting, despite of the relatively mild 10-fold reduction in the transcript abundance.

In the next step, we addressed two possibilities which might cause the hypomorophic nature of Bbs4 GT allele. First, the Bbs4 GT transcript could be still translated into (partially) functional protein. Second, there could be a low level of properly spliced Bbs4 mRNA in tissues of Bbs4$^{GT/GT}$ mice. Actually, our data supported both these hypotheses. Over-expression of the Bbs4 GT cDNA with a C-terminal FLAG tag resulted in a very low level expression of truncated BBS4 with MW corresponding to the truncated 347aa variant as suggested by this reviewer (Fig. EV1D). Although it is unclear why such a truncated protein would not be expressed in Bbs4$^{KO/KO}$ as well (as they lack only exon 6), this truncated BBS4 protein could be produced in Bbs4$^{GT/GT}$ mice to partially rescue the Bbs4 KO phenotype. However, we also detected a low level of properly spliced Bbs4 mRNA in the brain of Bbs4$^{GT/GT}$ mice (~1% of WT) (Fig. EV1E), which provides an alternative explanation of the hypomorphic phenotype.

Overall, the hypomorphic phenotype of Bbs4 GT allele is caused by the unexpected ability of polymerase II to read-through the GT cassette in all tested tissues (testes, brain, kidney) which is followed by RNA splicing which is (i) in most cases slightly defective, which can generate very low levels of truncated Bbs4, and (ii) in rare cases precise, presumably resulting in a low-level expression of full-length BBS4.

-Alterations in the immune system of Bbs4 deficient mice / Figure 2
Figure 2B: Could the authors please comment on why they chose to follow a Region Of Interest (ROI) gating strategy, rather than a more common subdivision in quadrants for the determination of percent-wise distribution of IgM/IgD-status subtypes?

We reanalyzed all the respective data using the gating strategy as suggested by the Reviewer (Fig. 2B). The data did not change substantially and the conclusions remained unchanged.

Figure 2C & Discussion: The increased frequency of CD24highCD43low pre-B-cells in the bone marrow leads the authors to conclude "(p11) ...that Bbs4-deficiency results in a developmental block at the pre-B-cell stage,..." and is discussed as "(p14, 4th paragraph) ...partial developmental arrest..". However, Bbs4KO/KO mice do develop a mature peripheral B-cell system. Hence, it appears equally possible to concluded that the increased frequency of cells at the pre-BCR selection stage might rather reflect a longer time required to pass this developmental stage or an accelerated transition through earlier developmental stages. This finding might indeed be of particular relevance, since failure of negative selection of autoreactive B-cells at the pre-BCR may lead to escape of clones to the periphery and drive the development of autoimmune diseases. If not B-cell intrisic, Bbs4 defects might affect cytokine release or other important properties of non-immune cells in the pre-B-cell niches in the bone marrow. In fact, this might be documentable in terms of IHC/IF of the bone marrow in BBS4-deficient and control mice. The results should be presented more precise and better clarified/discussed by the authors.

First, we rephrased the corresponding part of the manuscript to avoid the terms such as "developmental block" or "developmental arrest", as they might be misleading.

To uncover the mechanism behind the B-cell lineage phenotype of BBS model mice, we focused on the expression of two key signaling molecules, IL-7 and CXCL12, in the whole bone marrow by RT-qPCR. Whereas the expression of Il-7 was not altered, we found out that the expression of Cxcl12 is reduced in the bone marrow of $Bbs4^{KO/KO}$ and $Bbs18^{KO/KO}$ mice (Fig. 4E). We realized that Cxcl12 expression is lower in mouse embryonic fibroblasts derived from $Bbs4^{KO/KO}$ than in those derived from WT mice (Fig. 4F), suggesting that BBS4 promotes the expression of Cxcl12 in mesenchymal cells. We also observed decreased Cxcl12 expression in two out of four Bbs4KO clones of bone marrow stromal cell line ST2 (Fig. EV5A-B). Because the BBSome down-regulates canonical WTN response (Gerdes et al. Nat Genet, 2007. 39(11): p. 1350-60) and because the canonical WNT signaling down-regulates Cxcl12 (Fig. EV3 and Tamura et al. Int J Biochem Cell Biol, 2011. 43(5): p. 760-7), we conclude that the BBSome deficiency leads to increased canonical WNT signaling which downregulates Cxcl12 expression in bone marrow stromal cells. Interestingly, deficiency of CXCR4, the receptor for CXCL12, in the B cells lineage has a phenotype resembling the Bbs4 and Bbs18 deficiencies (Nie at al. JEM, 2004. 200(9): p. 1145-1156). Moreover, the deficiency of a negative regulator of CXCR4, WBP1L, has the opposite phenotype (Borna et al. Journal of Cellular and Molecular Medicine, 2020. 24(2): p. 1980-1992). We believe that we provide the mechanistic explanation of the B-cell lineage phenotype, which substantially improves the quality of the manuscript. Of course, more in-depth studies are needed to clarify the importance of the BBSome and cilia in general for particular cell types in the bone marrow niche.

Figures 2E/S2B,C: The determined frequency of IgM-IgD+ late mature B-cells is strictly dependent on the gating strategy, and the reported Bbs4 genotype dependent differences are rather small. The contour plots do hardly show any well-defined populations (peaks). It is thus difficult to follow the applied gating strategy, in particular if to distinguish IgD- from IgD+ cells. This point needs to be clarified by the authors to support their strong conclusion of an obesity-independent, Bbs4 dependent B-cell compartment alteration.

We looked at corresponding data carefully once again. We agree that the differences between WT and $Bbs4^{KO/KO}$ mice were rather small. However, we believe that the discrimination between IgD^- and IgD^+ gates is quite obvious (as there are almost no IgM^- IgD^- B cells). Perhaps the reviewer meant the discrimination between IgM^- IgD^+ and IgM^+ IgD^+ subsets, which is indeed somewhat arbitrary. We took advantage of the discrimination between IgM^+ IgD^- and IgM^- IgD^-, which is relatively clear, to set up the gates for the IgM-positive population. Of course, the gates were always set identical to all the samples in the same experiment. To make the manuscript more convincing at this point, we made two changes:

1. We realized that the representative plots in the previous version of the manuscript are actually coming from an experiment, where the gating was the least clear. For this reason, we replaced them with FACS plots from a more representative experiment in the revised manuscript (Fig. 2D). To document that the difference was reproducible and that the swap of the FACS plots is legitimate, we are showing the overview of all individual experiments here (Figure 1, below).

2. We included another type of analysis, which is independent of the respective gating strategy. We first gated on IgD^+ B cells and subsequently quantified the geometric MFI of the IgM signal. We always normalized the data in each experiment to WT signal =1 and observed that the IgM expression in $Bbs4^{KO/KO}$ samples is lower than 1 (Fig. 2F).

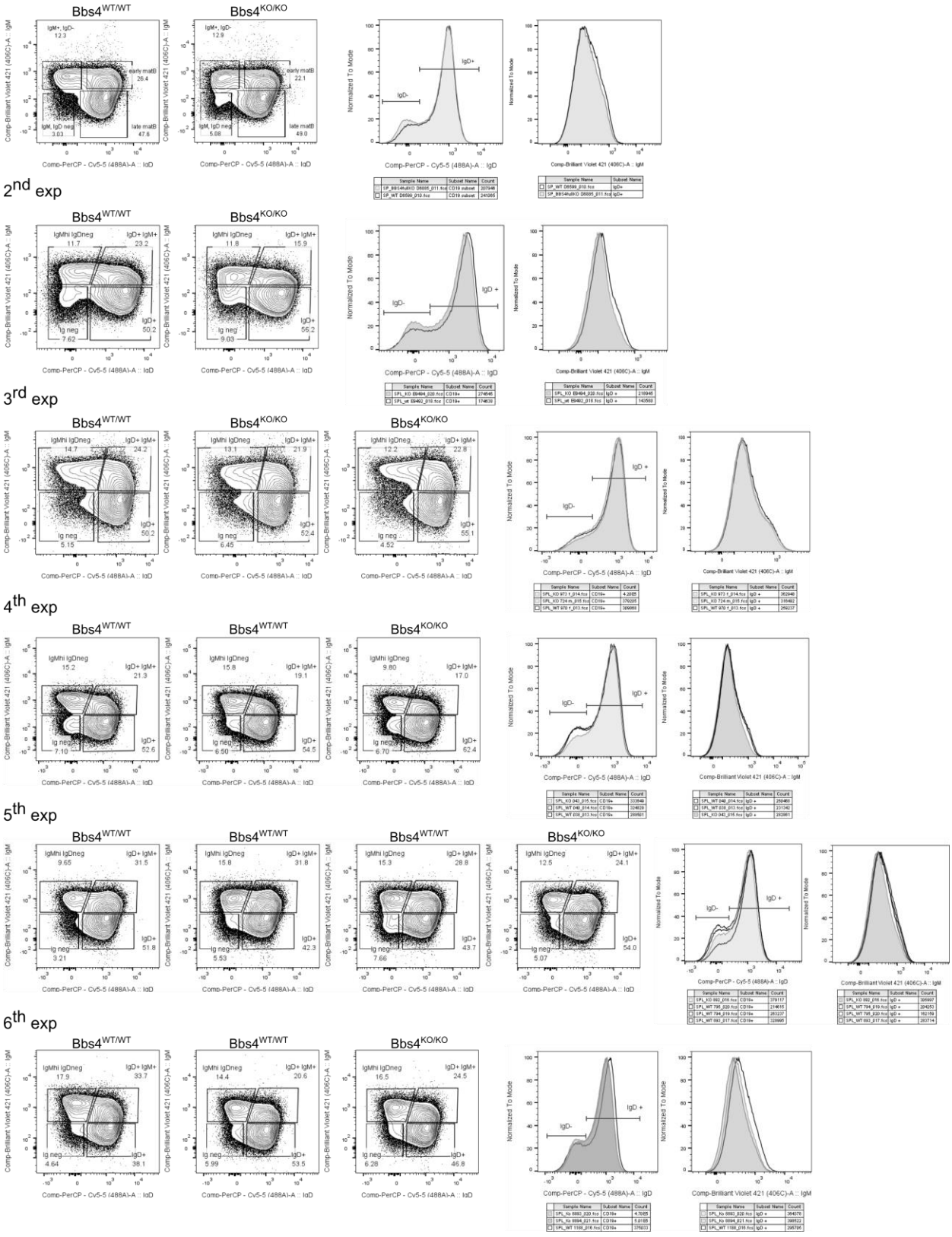

Figure 1. Overview of all replicates of the experiment shown in Figure 2D-E of *the revised manuscript.*

-The role of Bbs4 in B-cell homeostasis is not intrinsic / Figure 3

The authors use the Vav-iCre system to specifically target the Bbs4 locus in the hematopoietic lineage. The results of their analysis of the B-cell compartment are summarized in Figure 3, where no significant differences are observed between control and Cre-induced littermates. Example flow-cytometry data should be provided to support the dot-plot panels, eg in form of a supplementary. More importantly, no data on the efficacy and specificity of Bbs4 targeting are shown. These data are required for the solid interpretation of the results by the reader.

We added the representative FACS plots to the Figure 4 of the revised manuscript as requested by the reviewer.

We agree with the reviewer that the data concerning the efficacy and specificity of the Vav-iCre driven deletion are missing. As we see no difference between the WT and cKO, the data concerning efficacy would be more relevant than data showing the specificity. We are a bit embarrassed to admit that we did not collect the respective samples. After we realized that there is no difference between the strains, we terminated the BBS4FL Vav-iCre mouse strain for ethical reasons (3R principle). Only after this happened, we realized that we do not have samples for the WB analysis. Although we openly admit this unintended omission, we believe that in this particular case, our data could be still interpreted with a relatively high level of confidence. Exon 6 can be deleted in the BBS4 FL allele as shown by the production of the BBS4 KO (driven by Act-Cre) and BBS4 cKO in T cells (driven by CD4-Cre). Moreover, Vav-iCre is one of the best characterized CRE-expressing strains. A recent paper from a neighboring laboratory at the Institute of Molecular Genetics documented the efficacy of the particular Vav-iCre line bred in our facility (Kardosova at al. Haematologica. 2018 Aug;103(8):e331-e335, Supplementary Figure 2 there). We are not aware of a single case of the incompatibility between a tissue specific Cre transgene and a floxed gene allele if both work efficiently in combinations with other "partners". For the sake of the animal welfare and for time reasons, we decided not to re-create this strain.

Finally, Vav expression is not only restricted to the B-cell compartment and the Vav-Cre system reported as a pan-hematopoietic and pan-endothelial cell targeting system (Georgiades et al, Genes 34(4), 2002). This fact is not discussed, but may/is likely to impinge on the phenotype.

We are thankful for this comment and we added the reference to the revised version of the manuscript.

-Bbs4 deficiency does not intrinsically influence T-cell and B-cell antigenic responses
Figure 4A/B: The hypomorphic phenotype of Bbs4GT/GT mice raises the concern of expression of potential partially functional Bbs4 truncate in selected cell types and/or cells of different activation status. Can the authors conclude definitely that there is no Bbs4 or functional BBSome in BCR-engaged B-cells of the Bbs4GT/GT mice? It is difficult to understand the author's argument for not testing the B-cells of the Bbs4KO/KO background. The experiment is performed on isolated B-cells and thus under controlled ex vivo conditions, ie not under exposure to eg obesity specific cytokine profiles or similar. As a suggestion,

I would assume that similar experiments could be performed using alternative ways of T-cell independent B-cell stimulation, hence avoiding the requirement for the B1-8 background.

We are thankful to the reviewer for this comment. Indeed, we did not cross the Bbs4$^{KO/KO}$ strain with B1-8 mice because of the partial pre-weaning lethality of the BBS4$^{KO/KO}$. We performed the suggested assay for the activation of B cells using plate-bound anti-BCR antibody ex vivo (Fig. 3C). This experiment did not reveal any differences between responses of B-cells isolated from WT and BBS4$^{KO/KO}$ mice.

Figures 4C-E, S3: The conditional targeting of the Bbs4 locus in the CD4 T-cell compartment is well documented, and the intricate system used to study the capability of induction type 1 diabetes appears as an elegant way to study Bbs4-dependency in a complex in vivo system. Further, a shared feature of the autoimmune diseases in BBS patients is the role of CD4 T-helper cells, adding to the relevance of the experiment. The authors state that they hypothesize that the functionality of the immunological synapse is BBS4 (BBSome) dependent, hence testing for a loss of or delayed onset of type 1 diabetes and blood glucose increase. Though I do understand that the authors here set out to test for a T-cell intrinsic function of BBS4, which is in contrast to the germ-line loss-of-function in BBS patients, wouldn't one expect that CD4 T-cell functionality is UNcompromised in BBS patients to drive the observed autoimmune diseases ?!

We employed this assay to assess the priming, expansion, migration, and target cell killing of pathological T cells on per cell basis in vivo. The reviewer is correct that the presence of autoimmunity in patients does suggest T-cell defects. However, we also addressed the theoretical possibility that the BBSome might be a negative regulator of T-cell activity, for instance via retrograde transport of the proteins in the immunological synapse. This would be an analogy to the role of the BBSome in removing ciliary cargoes such as GPR161 from the cilia. We clarified this motivation in the revised manuscript.

-BBS-induced obesity affects blood homeostasis

The authors state that the CRP-levels in BBS patients were significantly higher than in BMI randomized an BMI matched controls. However, the authors show in the right panel of figure 5C that these differences are NOT significant, but that the frequency of patients presenting with CRP >5 is significantly different (left panel). The wording at the end of the first paragraph, p13 may be changed to correct for this.

We thank for this remark. We realized that the right panel is confusing.

We wanted to show originally that not only there are more individuals with CRP > 5 among BBS patients than in controls, but also that the average CRP levels in those patients with CRP > 5 is higher among BBS patients. As the reviewer pointed out, this is not statistically significant. Unfortunately, we cannot compare the levels of CRP in all BBS patients and control individuals as the majority of analyzed donors have indicated CRP below a certain level (e.g., lower than 5) and not an exact value. No straightforward (frequentist) statistical approach can be applied for such comparison. For this reason we are showing only the percentage of patients with CRP>5 in the revised manuscript and we adjusted the manuscript accordingly.

Minor comments: important issues that can confidently be addressed.

-Table 1:
The authors refer in the figure legend to present "the fold change in prevalence" but do show the prevalence and a calculated Odds Ratio and p-value. The calculations are difficult to recapitulate from the numbers presented in the table (if Odds Ratio to be defined by OR = [BBScases / (BBStotal-BBScases)] / [Normalcases / (Normaltotal-Normalcases)] numbers don't match). Could the authors please add a more specific explanation of chosen statistic tools and calculations in the Methods section?

We are thankful for this remark. During the preparation of the original manuscript, we changed our minds concerning the presentation of the data as fold-differences or Odds ratios and we did not adjust all parts of the manuscript accordingly (the table actually showed Fold change in prevalence, not Odds ratio). We are sorry for this mistake. In the revised version of the manuscript, we are showing both the Odds ratios and Fold difference in prevalence for the sake of clarity. We described the statistical approach in a greater detail in the Table 1 legend and in Methods and we submitted the R script for the statistical analysis to Zenodo (https://doi.org/10.5281/zenodo.3733230).

Apparently, some patients suffered from more than one autoimmune disease (CRIBBS cohort). Could the authors specify which diseases co-occurred?

We added the analysis of co-occurrence of multiple autoimmune diseases (Appendix Table 1-2) and we changed the Results accordingly. We refer to this phenomenon in the legend to Table 1 as well.

Given the detailed subsequent analysis of BBS4 defective mice it would be very supportive for the experimental rational to analyze if pathogenic mutations in BBS4 (or other BBSome core complex members) are (more) prevalent amongst the autoimmune disease affected BBS patients.

We added the analysis of the prevalence of autoimmune diseases in groups of patients based on the causative genes (Appendix Table 3-4). Indeed, patients with mutated Bbs4 have the highest prevalence of autoimmune diseases. However, we have analyzed only four patients with diagnosed causative mutations in BBS4 (unfortunately, the causative genes are not known for patients from the NIH cohort), from which two suffered from autoimmunity. Concerning the most common mutations, the prevalence of autoimmunity was slightly higher in patients with mutations in BBS10 than in patients with mutations in BBS1, which goes in line with the generally more severe phenotype in the latter group (Niederlova et al. Hum Mutat. 2019 Nov;40(11):2068-2087.). However, the number of patients per group is too low to allow for statistical testing with a reasonable power.

-T-cell compartment analysis / Figure S1
Examples of flow-cytometry data (contour plots) for the quantifications shown panels S1A-C need to be provided.

We added the representative FACS plots to the respective figure (Fig. EV2).

-Figures 1,5, S1
coding for statistical significance needs to be explained in figure legend

We added the legend for the coding of the statistical significance as requested.

-general comment:
Though being a non-native English speaker I find that the manuscript, particularly the discussion, may benefit from some language editing by the native English speaking co-authors.

The manuscript was proofread by a professional editor.

Reviewer #1 (Significance (Required)):

Nature and significance of the advance:

The manuscript represents an interesting and relevant collection of findings in BBS patients and Bbs4 mouse models, shedding some new light on the potential role of the immune system in the pathobiology of BBS as well as the hypothesized role of the BBSome in non-ciliated blood cells. Hence, the study represents a reference motivating further research into the causes and consequences of alterations of the immune system in ciliopathy patients. Extended analysis of the bone marrow and peripheral lymphoid organs by IHC and IF allowing eg the analysis of cilia status in non-immune cells as well as cytokine profile analyses to investigate the functional status of these niches/organs would further advance the impact of the study, but are unfortunately beyond what can be suggested for an experimental revision of the manuscript.

There are to the best of my knowledge no other published systematic reviews on hematopoietic system alterations in BBS or related ciliopathies. Extra-ciliary functions of several other ciliary proteins have been and still are an important topic in understanding the pathobiology of these congenic disorders. Selected individual proteins have been studied for their functions in immune cells at greater much detail than in this manuscript (properly discussed by the authors), but were largely restricted to specific cell lines, not embracing the complexity of interplays underlying homeostasis and activation of a proper immune system.

Audience.

The main audience for this manuscript are medical doctors (primary) and scientist in the field of ciliopathies and immunology.

Reviewer's expertise:

Molecular cell biology of centrosome/primary cilia/ciliopathies/cancer; immunology, mainly B-cell development and B-cell lymphoma/leukemia; no MD background

We are very pleased by the overall positive evaluation of our manuscript by this reviewer. We are very thankful for all the valuable comments that will definitely lead to a substantial improvement of our study.

New experiments added during the revision indicated that bone marrow stromal cells of BBS4$^{KO/KO}$ (and BBS18$^{KO/KO}$) mice have lower expression of CXCL12 (but not IL-7) than WT mice. This can be explained by the role of the BBSome in suppressing canonical WTN signaling, which downregulates Cxcl12 expression. Overall, we believe that these additional experiments extended our original analyses by providing the mechanistic explanation of the extrinsic role of BBSome in the regulation of B-cell development.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

This is a thorough and well executed study that uses BBS patients and mouse model to dissect the function of BBS4 in maintaining hematopoietic system and self-tolerance. Specifically, by using a combination of genetic/molecular and cell-based assay approaches the authors elegantly show that the deficiency of BBS4 alters the development and homeostasis of B cells. The BBS patients also have a higher incidence of certain autoimmune diseases. The authors further suggested that some of the hematopoietic systems are altered due to the BBS-caused early-onset obesity. There are not many studies to be performed to investigate the connection between ciliopathy and alternation of immune system, this study thus provides solid evidence that ciliopathy could cause abnormal immune responses in patients.

The quality of the data in this manuscript is high and I only have a few comments for the authors to address.

We are very thankful for the overall positive assessment of our manuscript and for the effort invested by the reviewer to help us to improve the manuscript.

Major comments:

1) To the best of my knowledge, a total of 22 genes (bbs1-bbs22) so far identified clinically, once mutated, cause BBSome. The authors should mention this in the introduction part.

Thank you for the remark. We checked the literature and there is actually already 24 genes associated with BBS. We adjusted the Introduction accordingly.

2) BBS is a heterozygous disorder and not all BBS patients develop early-onset obesity. I am wondering whether it is suitable to conclude that BBS, as a whole, causes altered hematopoietic system as reflected by the manuscript title.

We thank the author for this comment. Although we did our best, we could not think of a title that would comprehensively characterize the aim of our study together with the conclusions and explain the heterogeneity among the BBS patients at the same time. Unfortunately, titles are usually too short to reflect all aspects of the study, including some major ones. Perhaps the editor or this reviewer might suggest how the title can be improved.

3) Could authors discuss a little bit more about the possible role that the ciliated cells, e.g. the leptin receptor expressing neurons controlling energy homeostats in hypothalamus, play in mediating the hematopoietic system?

We discuss that leptin signaling in neuronal cells can influence in the immune system in the revised version of the manuscript. We cite a study showing how leptin signaling in the CNS regulates immunity

*(Tschop et al. Journal of Neuroscience, 2010. **30**(17): p. 6036-6047).*

Minor comments:

1) Page 11, second paragraph, "for an expression of a truncated BBS4..." should be "for the expression of a truncated BBS4...".
We corrected this error in the revised version of the manuscript.

2) Through the whole manuscript, "Bbs4-dificient mouse" should be "Bbs4-dificient mouse".

We use Bbs4-deficient mouse (which we believe is the correct spelling) throughout the revised manuscript.

3) Page 11, third paragraph, "decreased percentages of CD44+..." should be "decreased percentage of CD44+...".
We corrected this error in the revised version of the manuscript.

4) Through the whole manuscript, "B-cell development" should be "B cell development".

We believe that B-cell is a correct form when used as an adjective (i.e., B-cell development, B-cell receptor etc.). However, our experience is that different journals prefer different forms (i.e., hyphenated or not) in these cases. For this reason we defer this issue to the typesetting editor.

5) Page 12, first paragraph, "obesity independent" should be "obesity-independent".

We corrected this error in the revised version of the manuscript.

6) Page 12, In the subtitle "Bbs4 deficiency does not intrinsically influence T-cell and B-cell antigenic responses", T-cell and B-cell antigenic responses"
should be "antigen-specific B cell and T cell responses".

We partially corrected this in the revised version of the manuscript (we used the term "antigen-specific"). Concerning B-cell vs. B cell and T-cell vs. T cell, please, see response to comment 4.

7) And more.....
Please, see our response to comment 4.

Reviewer #2 (Significance (Required)):

My expertise is about the BBSome-IFT assembly and ciliary transport of the BBSome and its signaling cargoes. This paper significantly advances our understanding of how BBS patients suffer some autoimmune diseases. The manuscript should be of broad interest to the readership, especially those in clinic.

We are very thankful for the positive evaluation of the manuscript and for the valuable comments.

1st Revision - Editorial Decision 26th Nov 2020

Dear Dr. Stepanek,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Referee #1 has a few final suggestions and questions, we ask you to address in a final revised manuscript.

Moreover, I have these editorial requests I also ask you to address in a final revised manuscript:

- I would suggest a slightly different title. Could we add ciliopathy somehow? How about:
Bardet-Biedl Syndrome ciliopathy is linked to altered hematopoiesis and dysregulated self-tolerance

- Please provide the abstract written in present tense.

- Please reduce the number of key words to 5.

- We would like to publish your manuscript (as also indicated by you) as Report. However, for a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Please add a formal "Data Availability section" to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary large datasets have been generated or deposited').

- We have recently changed our reference format. Please make sure the final manuscript file is formatted accordingly:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- In Figure EV1D the same actin loading control is shown for both exposures (30 sec. and 5 min.). Please indicate this in the figure legend. Or show also a 5 min. exposure of actin under the right blot.

- The callout for Appendix Table S6 is missing the 'S'. Please check.

- In the legends you state several times that data from independent experiments are shown. Please indicate in each case if these are biological or technical replicates.

- In general, please make sure that regarding data quantification and statistics, the number "n" for how many independent experiments (biological vs technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is specified in the respective figure legends (ALSO in the Appendix). Please provide statistical testing where applicable. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

- For Fig. EV1E you state than an average of two technical replicates is shown in the bar diagram on the left. Could you show the values separated for each replicate and condition? I think that

would be the most transparent representation.

- Please also make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript text.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

I am pleased to see the revised version of this MS and am thankful for the detailed point-by-point response. The original conclusions are now substantially supported and refined by an extended set of control (re-)analyses and new experiments, which have been performed despite the stressful COVID-19 situation. It is understandable that limited lab and in particular animal facility access as well as ethical considerations impacted on the implementation of a revision plan.

I am happy to recommend the publication of this MS in EMBO Reports.

The improved MS adequately addressed all my comments/concerns, which I am glad to see were indeed as constructive as intended. The MS also gained in clarity and readability, and the provided supporting information (EV) strengthens the data presented in the main part as well as allows the reader to assess the experimental raw data.
In respect to flow cytometry data: As argued in my original report, percentage differences in sub-populations that are determined by flow-cytometry are strongly dependent on the gating strategy (and staining conditions). This factor becomes even more challenging in case of small differences. I would for future experiments advise to use bar-coding techniques for comparative analyses, which enables the staining and analysis of control and treated cells under the exact same conditions. This will ultimately ease the analysis and will increase the confidence in obtained results, particularly if small differences are observed.

The thorough analysis of the Bbs4GT/GT mouse clarified my concerns, and is supported by the proteomic analyses (which, however, to a certain point are limited by the specificity of the Bbs4

antibody). The new experimental data on in vitro BCR stimulation, analysis of IL7/CXCL12-CXCR4 signaling axis in the bone marrow and stroma cells, as well as the introduction of the Bbs18 model collectively feed into the presented model of an altered bone marrow stroma signaling as an underlying cause of the observed defects in B-cell hematopoiesis (and likely self-tolerance). Hence, the study presents novel insights into alterations of the hematopoietic system of BBS-affected individuals at the molecular, cellular and (model) organism level, and thus provides a solid foundation for further investigations into this line of research.

Further work is required to establish a direct mechanistic link between the observed altered B-cell maturation in the bone marrow and the altered self-tolerance that drives auto-immune responses. In that context, looking at the data of co-occurring autoimmune disorders in affected individuals of the two cohorts it is striking to note a high frequency of hypothyroidism/Hashimoto's disease. Can the authors speculate about why this might be the case? And: Do autoimmune diseases occur in aged Bbs4ko/ko or Bbs18ko/ko mice?

Finally, a minor comment on the Cover Art synopsis:
"Normal/Altered B cells" should be changed to "Normal/Altered B cell lymphopoiesis" or "Normal/Altered B cell compartment" since the data does not support a functional defect in B cells per se.

Referee #2:

Accept it as it is.

Dear Dr. Breiling,

We are submitting the final version of the manuscript EMBOR-2020-50785V3. The changes are summarized in italics in this point-by-point response to editorial and reviewers' comments.

Moreover, I have these editorial requests I also ask you to address in a final revised manuscript:

- I would suggest a slightly different title. Could we add ciliopathy somehow? How about:
Bardet-Biedl Syndrome ciliopathy is linked to altered hematopoiesis and dysregulated self-tolerance

We accept the suggested title.

- Please provide the abstract written in present tense.

We provide the abstract in present tense.

- Please reduce the number of key words to 5.

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http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

As discussed via email, we prefer publishing the study as an article.

- Please add a formal "Data Availability section" to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary large datasets have been generated or deposited').

We added the "Data Availability Section".

- We have recently changed our reference format. Please make sure the final manuscript file is formatted accordingly:
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We updated the reference format.

- In Figure EV1D the same actin loading control is shown for both exposures (30 sec. and 5 min.). Please indicate this in the figure legend. Or show also a 5 min. exposure of actin under the right blot.

We indicated that the same exposure was used.

- The callout for Appendix Table S6 is missing the 'S'. Please check.

Corrected.

- In the legends you state several times that data from independent experiments are shown. Please indicate in each case if these are biological or technical replicates.

We specified, whenever unclear. For experiments with mice, we use the following formulation, e.g. "n=7 mice in 3 independent experiments", which is self-explanatory and is the best standard in the field. Of course, each mouse is a biological entity, but indicating this as a biological replicate might be confusing. Independent experiments in this context apparently mean repeating the experiment with a new set of mice on a different day. Indicating not only the number of mice, but also the number of independent experiments contributes to transparency.

- In general, please make sure that regarding data quantification and statistics, the number "n" for how many independent experiments (biological vs technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is specified in the respective figure legends (ALSO in the Appendix). Please provide statistical testing where applicable. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

We checked the manuscript and modified it accordingly.

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We are submitting the two-sentence summary, three bullet point highlights, and the schematic figure.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

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Referee #1:

I am pleased to see the revised version of this MS and am thankful for the detailed point-by-point response. The original conclusions are now substantially supported and refined by an extended set of control (re-)analyses and new experiments, which have been performed despite the stressful COVID-19 situation. It is understandable that limited lab and in particular animal facility access as well as ethical considerations impacted on the implementation of a revision plan.

I am happy to recommend the publication of this MS in EMBO Reports.

The improved MS adequately addressed all my comments/concerns, which I am glad to see were indeed as constructive as intended. The MS also gained in clarity and readability, and the provided supporting information (EV) strengthens the data presented in the main part as well as allows the reader to assess the experimental raw data.
In respect to flow cytometry data: As argued in my original report, percentage differences in sub-populations that are determined by flow-cytometry are strongly dependent on the gating strategy (and staining conditions). This factor becomes even more challenging in case of small differences. I would for future experiments advise to use bar-coding techniques for comparative analyses, which enables the staining and analysis of control and treated cells under the exact same conditions. This will ultimately ease the analysis and will increase the confidence in obtained results, particularly if small differences are

observed.

The thorough analysis of the Bbs4GT/GT mouse clarified my concerns, and is supported by the proteomic analyses (which, however, to a certain point are limited by the specificity of the Bbs4 antibody). The new experimental data on in vitro BCR stimulation, analysis of IL7/CXCL12-CXCR4 signaling axis in the bone marrow and stroma cells, as well as the introduction of the Bbs18 model collectively feed into the presented model of an altered bone marrow stroma signaling as an underlying cause of the observed defects in B-cell hematopoiesis (and likely self-tolerance). Hence, the study presents novel insights into alterations of the hematopoietic system of BBS-affected individuals at the molecular, cellular and (model) organism level, and thus provides a solid foundation for further investigations into this line of research.

Further work is required to establish a direct mechanistic link between the observed altered B-cell maturation in the bone marrow and the altered self-tolerance that drives auto-immune responses. In that context, looking at the data of co-occurring autoimmune disorders in affected individuals of the two cohorts it is striking to note a high frequency of hypothyroidism/Hashimoto's disease. Can the authors speculate about why this might be the case? And: Do autoimmune diseases occur in aged Bbs4ko/ko or Bbs18ko/ko mice?

We are very thankful for the positive evaluation of the manuscript. It is probably that the high level of Hashimoto's disease is linked to obesity. We included this in the discussion. We did not examine BBS mice for signs of autoimmunity very carefully.

Finally, a minor comment on the Cover Art synopsis:
"Normal/Altered B cells" should be changed to "Normal/Altered B cell lymphopoiesis" or "Normal/Altered B cell compartment" since the data does not support a functional defect in B cells per se.

We changed the Cover Art synopsis accordingly.

Referee #2:

Accept it as it is.

We are very thankful for the positive evaluation of the manuscript.

2nd Revision - Editorial Decision 9th Dec 2020

Dr. Ondrej Stepanek
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Dear Dr. Stepanek,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Corresponding Author Name: Stepanek
Journal Submitted to: EMBO reports
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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 χ2 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.

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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?	Minimal sample size was estimated using resource equation approach, as the effect size and the standard deviation required for the power analysis approach were uknown before the start of the study.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The statement is included in the section Material and Methods/Mice.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All the available mutant mice and their (sex-matched, if possible) WT littermates were used for experiments. No samples/mice were excluded from the analysis.
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.	No randomization was performed sfor the analysis of genetically modified since the experimental groups were based solely on the genotype of the mice. For the diabetes experiment, sex-matched littermates were equally distributed into the experimental groups based on their ID number prior to any physical contact between the experimenter and the mice.
For animal studies, include a statement about randomization even if no randomization was used.	The statement is included in the section Material and Methods/Mice.
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.	The experiments were not blinded since no subjective scoring method was used.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The statement is included in the section Material and Methods/Mice.
5. For every figure, are statistical tests justified as appropriate?	We used frequentist statistics for comparing the experimental groups. We choose the most appropriate test for each experiment and indicated it in the Figure Legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used non-parametric tests whenever possible. In the very exceptional cases of RT-qPCR experiments with a very low number of replicates (3-4), the non-parametric tests were not applicable. In these cases, we used one sample t test for normalized data. The assumed normal distribution could not be statistically tested because of the low number of replicates.
Is there an estimate of variation within each group of data?	NA (we have not used parametric tests assuming similar variance between samples in this study). For the vast majority of results, we plotted individual data points in the Figures and we provide the source data. We do not believe that calculating estimates of variantion would bring anything to the presentation to most these particular data, but the readers are welcome to perform they own calculations based on the source data. In some rare cases, we used bar graphs. In such cases, the estimates of variation are visualized as error bars and explained in the Figure Legends.
Is the variance similar between the groups that are being statistically compared?	NA (we have not used parametric tests assuming similar variance between samples in this study)

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).	Antibodies to the following antigens were used for flow cytometry: CD1d Pe-Cy7 (1B1, #123524, Biolegend), CD4 BV650 (RM4-5, #100545, Biolegend), CD8a PE-Cy7 (53-6.7, #1103610, SONY), CD8a FITC (53-6.7, #100706, Biolegend), CD19 PE (6D5, #115508, Biolegend), CD23 APC (b3b4, #1108095, SONY), CD24 FITC (M1/69, #101806, Biolegend), CD43 PE (S7, #553271, BD Pharmingen), CD44 PE (IM7, #103008, Biolegend), B220 Alexa Fluor 700 (RA3-6B2, #103231, Biolegend), B220 FITC (RA3-6B2, #103206, Biolegend), CD69 PE (H1.2F3, #104508, Biolegend), CD69 FITC (H1.2F3, #104506, Biolegend), IgM BV421 (rmm-1, #2632585, SONY), IgD Per-CP-Cy5.5 (11-26c.2a, #2628545, SONY), IgLλ APC (RML-42, #407306, Biolegend), TCRβ APC (H57-597, #109212, Biolegend). Antibodies used for immunoblot analysis: BB54 (rabbit, a kind gift from Prof. Maxence Nachury, UCSF, CA, USA, recognizing LQVGEALVWTKPVKDPKSKH peptide in exon 15 of human BBS4), β-actin (mouse, #4967, Cell Signaling), anti-FLAG (M2, mouse, F1804-200UG, SIGMA), α-mouse-HRP, α-rabbit-HRP (both from Jackson ImmunoResearch). Antibodies used for lymphocyte enrichment: biotinylated α-TCRβ (H57-597, #553169, BD Pharmingen), α-CD19 (6D5, #115503, Biolegend). Antibody used for B-cell activation: polyclonal F(ab')2-Goat anti-Mouse IgM (Mu chain), a kind gift from Dr. Tomas Brdicka (Institute of Molecular Genetics of the Czech Academy of Sciences in Prague, henceforth IMG).

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Details about the cell lines used are reported in the section Material and Methods/Cell culture. The cells were not recently authenticated. Cells were tested for mycoplasma contamination regularly using PCR with the following mixture of primers FWD: CGCCTGAGTAGTACGTTCGC, CGCCTGAGTAGTACGTACGC, TGCCTGAGTAGTACATTCGC, TGCCTGGGTAGTACATTCGC, CGCCTGGGTAGTACATTCGC, CGCCTGAGTAGTATGCTCGC; REV: GCGGTGTGTACAAGACCCGA, GCGGTGTGTACAAAACCCGA, GCGGTGTGTACAAACCCCGA.

* 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.	All mice were 5-25 weeks old and had C57Bl/6J background if not indicated otherwise. Mice were bred in specific-pathogen-free facility (Institute of Molecular Genetics) [77]. Animal protocols were approved by the Czech Academy of Sciences, in accordance with the laws of the Czech Republic. Males and females were used for the experiments. If possible, age- and sex-matched pairs of animals were used in the experimental groups. If possible, littermates were equally divided into the experimental groups. No randomization was performed since the experimental groups were based solely on the genotype of the mice. In case of the RIP.OVA mice used for the autoimmune diabetes model, mice were assigned to experimental groups randomly (defined by their ID numbers) prior to the visual contact between the experimenter and the mice. The experiments were not blinded since no subjective scoring method was used. For animal studies, minimal sample size was estimated using resource equation approach. As the number of mutant mice was limited due to preweaning lethality, all the available mutant mice (and their WT littermates) were used for experiments. For the diabetes experiments, the number of mice was estimated based our previous experience with this method. B1-8 [57], RIP.OVA [78], OT-I Rag2KO/KO [59], Vav-iCre [79, 80], Cd4-Cre [81] strains were described previously. Bbs4+/GT sperm (Bbs4tm1a(EUCOMM)Hmgu) was obtained from KOMP (UC Davis, CA, USA) and used for in vitro fertilization. Bbs4+/+ and Bbs4GT/GT or Bbs4KO/KO littermates were generated by intercrossing heterozygous animals.
8. Continued	Mice with a null-mutation in Bbs18 (alias Bbip1) were generated in a C57BL/6N background using a CRISPR genome-editing system. For this purpose, Cas9 protein and gene-specific single guide (sg) RNAs (Integrated DNA Technologies, Coralville, IA, USA) were used for a zygote electroporation using a protocol described previously [82]. sgRNA sequences with the PAM motif in bold (3' end) were as follows: sgRNA target 1: CTCTTCCCTGAAAATCGGTGAGG sgRNA target 2: GGAATAACCAACTGGTCTTTAGG See Materials and Methods/Mice.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal protocols were approved by the Czech Academy of Sciences, in accordance with the laws of the Czech Republic (see Material and Methods/Mice).
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.	All the relevant aspects of animal studies were reported as recommended.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study protocol was approved by the Great Ormond Street Hospital Research Ethics Committee (Project Molecular Genetics of Human Birth Defects – mapping and gene identification, reference #08/H0713/82) the and by the ethical committee of the Institute of Molecular Genetics of the ASCR (p.8-9).
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.	The statement is included in the section Material and Methods/Analysis of the clinical data of BBS patients.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide 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	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA (we have not generated any 'large data' in this study and we submitted primary data as Source Data) .
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).	NA
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.	This is generally NA for our work. However, we deposited one R script for a slightly complex statistical analysis to Zenodo. https://doi.org/10.5281/zenodo.3733230

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.	NA