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Research article

Tissue-dependent differences in Bardet–Biedl syndrome gene expression

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Background Information. Primary cilia are highly conserved multifunctional cell organelles that extend from the cell membrane. A range of genetic disorders, collectively termed ciliopathies, is attributed to primary cilia dysfunction. The archetypical ciliopathy is the Bardet–Biedl syndrome (BBS), patients of which display virtually all symptoms associated with dysfunctional cilia. The primary cilium acts as a sensory organelle transmitting intra- and extracellular signals thereby transducing various signalling pathways facilitated by the BBS proteins. Growing evidence suggests that cilia proteins also have alternative functions in ciliary independent mechanisms, which might be contributing to disease etiology.

Results. In an attempt to gain more insight into possible differences in organ specific roles, we examined whether relative gene expression for individual *Bbs* genes was constant across different tissues in mouse, in order to distinguish possible differences in organ specific roles. All tested tissues show differentially expressed *Bbs* transcripts with some tissues showing a more similar stoichiometric composition of transcripts than others do. However, loss of *Bbs6* or *Bbs8* affects expression of other *Bbs* transcripts in a tissue-dependent way.

Conclusions and Significance. Our data support the hypothesis that in some organs, BBS proteins not only function in a complex but might also have alternative functions in a ciliary independent context. This significantly alters our understanding of disease pathogenesis and development of possible treatment strategies.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Primary cilia are highly conserved multifunctional cell organelles that extend from the cell membrane. These microtubule-based appendages are vital for development and homeostasis of different organs and tissues and play a role in transduction of intra- and extracellular signals. In contrast to motile cilia, which are found on specialised tissues, primary cilia are a component of virtually all vertebrate cells, and functional defects cause a wide spectrum of clinical phenotypes. A range of genetic disorders, collectively termed ciliopathies, is attributed to primary cilia dysfunction. The archetypical ciliopathy is the Bardet–Biedl syndrome (BBS), patients of which display retinopathy, kidney dysfunction, obesity, polydactyly, behavioural dysfunction and hypogonadism [Forsythe and Beales, 2013].

The primary cilium acts as a sensory organelle transmitting intra- and extracellular signals [Ishikawa and Marshall, 2011] thereby transducing

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Key words: bardet-biedl syndrome, cilia, ciliopathy, gene regulation, mRNA.

Abbreviations: Arl6, ADP-ribosylation factor-like protein 6; ARVO, Association for Research in Vision and Ophthalmology; BBS, Bardet–Biedl syndrome; CCT/TRiC, chaperonin-containing TCP1 complex; cDNA, complementary deoxyribonucleic acid; DNA, deoxyribonucleic acid; DEPC, diethyl pyrocarbonate; GPCR, G protein-coupled receptor; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; IFT, intraflagellar transport; Mkks, McKusick-Kaufman syndrome; mRNAs, messenger ribonucleic acids; OD, optical density; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acids; RNF2, RING finger protein 2; TOR, target of rapamycin; UK, United Kingdom; Usf1, upstream stimulatory factor 1.

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various signalling pathways including Wnt [May-Simera and Kelley, 2012], Hedgehog [Goetz and Anderson, 2010], Notch [Ezratty et al., 2011], PDGF [Schneider et al., 2005], TOR [Yuan et al., 2013] and Hippo [Habbig et al., 2011]. Correct signal transduction is essential for tissue development and homeostasis and the Bardet–Biedl syndrome proteins have been shown to play a crucial role in this. To date, 23 BBS genes (BBS1 - 23) have been reported. Most recently two BBS disease causing loci have been found in other cilia associated genes, namely IFT74/BBS22 and SCAPER/BBS23 [Lindstrand et al., 2016; Schaefer et al., 2019; Wormser et al., 2019]. A subset of these genes encodes proteins that form an octameric complex termed the BBSome, which is crucial for ciliary trafficking [Jin et al., 2010]. The assembly of this complex is facilitated by chaperonin-like BBS proteins [BBS6/MKKS, BBS10 and BBS12; Seo et al., 2010; Zhang et al., 2012]. Although a defect in any BBS gene gives rise to a BBS diagnosis, there is a huge degree of phenotypic variation with no clear genotype to phenotype correlation even within families.

Growing evidence suggests that cilia proteins also have alternative functions in ciliary independent mechanisms. Such extraciliary functions include cell cycle regulation, non-ciliary trafficking, regulation of the DNA damage response, and transcriptional control [Vertii et al., 2015; Hua and Ferland, 2018]. In light of this, it is plausible that the BBSome and BBS chaperonin-like proteins also have alternative functions, possibly in an individual protein dependent manner. In an attempt to gain more insight into this, we examined whether relative gene expression for individual Bbs genes was constant across different tissues, in order to distinguish possible differences in organ specific roles. Furthermore, we wanted to examine the effect of Bbs protein loss on other BBSome or chaperonin-like components.

Results

Differential expression of BBSome transcripts across mouse tissues

To determine tissue specific expression levels of BBSome and chaperonin-like *Bbs* transcripts, we assessed their relative expression in a variety of adult mouse tissues. Quantitative real-time PCR (qRT-PCR) was used to examine the abundance of mRNA transcripts in brain, kidney, lung, spleen, heart, oviduct and retina. Because alternative splicing can produce transcripts with different stabilities, we designed primers to recognise as many of the different *Bbs* transcripts as possible (Table 1). An important consideration is that expression levels of housekeeping genes can vary across tissues, therefore caution must be used when comparing gene expression levels across multiple tissues and normalizing to expression of a single housekeeping gene. We chose *Gapdh* as a housekeeping control gene since the expression of *Gapdh* was more stable across the tissues examined than *Usf1* or other cytoskeletal markers (Supporting Information Figure 1). Furthermore we saw no differences in levels of *Gapdh* expression between either of our mutants and their littermate controls.

Our results show that *Bbs* transcripts are differentially expressed in different tissues when normalised against the housekeeping control *Gapdh* (Figure 1a–h). The expression levels of most BBSome transcripts were highest in the retina with the exception of *Bbs18* (Figure 1h). This correlates with the functional role of the BBSome in trafficking across the connecting cilium in photoreceptors [Datta et al., 2015]. Although the BBSome has a trafficking role in all cilia, the volume of traffic required to build and maintain the photoreceptor outer segment is particularly high and requires continuous turnover of ciliary trafficking proteins. Expression of *Bbs18* was most abundant in the spleen and oviduct.

Expression of BBSome transcripts within a tissue is not stoichiometric

Comparison of BBSome expression levels within a specific tissue revealed variable BBSome composition across different tissues. Expression of BBSome mRNAs were not stoichiometric, rather they were differentially expressed in specific tissues (Figure 2a–g). Interestingly, the expression profiles in brain and kidney were strikingly similar (Figures 2a and 2b). *Bbs1* was the most abundantly expressed transcript in brain and kidney, yet one of the least abundant transcripts in heart and oviduct (Figure 2a, 2b, 2e, and 2f). *Bbs18* was most abundant in the spleen, heart and oviduct, yet contributed the least in the retina (Figure 2g). Interestingly, *Bbs9* was highly abundant in the retina and spleen (Figures 2d and 2g). However, mutations in the *BBS9* gene have been implicated in nonsyndromic craniosynostosis [Barba et al., 2018]. These results

Table 1 | Gene symbol, primer sequences, primer efficiency for each evaluated Primer

Gene symbol	Primer sequencesForward n Reverse	Primer efficiency	Transcript identified
Bbs1	CCCTACTTCAAGTTCAGCCTG TCTGCCTTTTCCCTGATGTC	114.35%	ENSMUST00000053506.7
Bbs2	ACATTGCCCCACCTCTTG TCTTCCCATCACCGTCAAAG	115%	ENSMUST00000034206.5
Bbs3	GATACCCTTCTGAATCACCCAG CCACGGCTTGTCTTTAATGC	107.23%	ENSMUST00000023405.9 ENSMUST00000099646.3 ENSMUST00000118438.1 ENSMUST00000149797.1
Bbs4	GCTCCAGACTTCCCTATTGTG GCATATTCACATAGCCCCTGAG	109.34%	ENSMUST00000026265.7
Bbs5	ACAAAGTCTATTCTGCCAGTCC AAATACGCCACAAAAGCATCC	98.31%	ENSMUST00000074963.8 ENSMUST00000112286.8 ENSMUST00000134659.7
Bbs6	GTGTGCTCTGCAAGATTTGG AAGACGTGCATTGCTGTTTG	97.74%	ENSMUST00000110089.8 ENSMUST00000028730.12
Bbs7	ATGGATCTGACGTTAAGCCG CCTTTTGTGTAGCCCTTTGTCTTGAGGT	112.60%	ENSMUST00000108156.8 ENSMUST00000040148.10 ENSMUST00000108155.7 ENSMUST00000129671.1
Bbs8	GAGGCAGCTGATGTCTGGTACA CATTGGTGGGCCAAGTTTGT	98.25%	ENSMUST00000085109.9 ENSMUST00000079146.12
Bbs9	ACAAATCTCCTGTCAGTCTGC TCGTTGGGATGTTCTGGAAG	96.84%	ENSMUST00000147712.7 ENSMUST00000150395.7 ENSMUST00000039798.15 ENSMUST00000147405.7 ENSMUST00000127296.7
Bbs10	TCCAGCCTCAGTTTTCATCG ACTGAGATGCCTGAAACTGTG	111.39%	ENSMUST00000040454.4 ENSMUST00000219990.1
Bbs12	CGCCGAGCATTGGATGTAG CATGCACACCCACACGT	104.30%	ENSMUST00000057975.7 ENSMUST00000108121.3
Bbs18	CCCTTAAAATCTCTGACGCTGG TGCCTTTTCTGCCATTTCTTG	102.75%	ENSMUST00000135402.3 ENSMUST00000236885.1 ENSMUST00000235348.1 ENSMUST00000236370.1 ENSMUST00000236098.1 ENSMUST00000235688.1 ENSMUST00000237049.1
Gapdh	CGACTTCAACAGCAACTCCCACTCTTCC TGGGTGGTCCAGGGTTTCTTACTCCTT	99.58%	ENSMUST00000118875.7 ENSMUST00000117757.8 ENSMUST00000073605.14 ENSMUST00000183272.1

suggested either tissue-dependent differences in BBSome composition and/or protein half-life, or that some of these transcripts are required for alternative non-BBSome-related functions in different tissues.

Less variable expression of BBS chaperonin-like transcripts across mouse tissues

Next, we determined the gene expression patterns of the three chaperonin-like *Bbs* transcripts (*Bbs6/Mkks, Bbs10* and *Bbs12;* Figure 3). Overall, we saw less variation of these transcripts across different tissues. With exception of lung and heart, which both had slightly higher levels of expression; the level of *Bbs6* was relatively constant (Figure 3a). The expression pattern of *Bbs10* was largely consistent across different tissues (Figure 3b). *Bbs12* expression varied the most, with lower expression in brain and kidney and higher expression in the spleen, oviduct and retina (Figure 3c).

When looking at these expression levels within specific tissues, we observe a similar stoichiometry of expression in brain and kidney (Figure 3d,e), akin

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Figure 1 | Expression of BBSome transcripts

Bar charts showing gene expression of *Bbs1,2,4,5,7,8,9,18* in different tissues relative to *Gapdh*. Relative expression levels of each sample averaged. Error bars show standard error of the mean. $n = 4$ for all genes, *Bbs8* $n = 3$. Statistics were done using the Dunnett's multiple comparison test $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{*}p \leq 0.001$; ns, not significant.

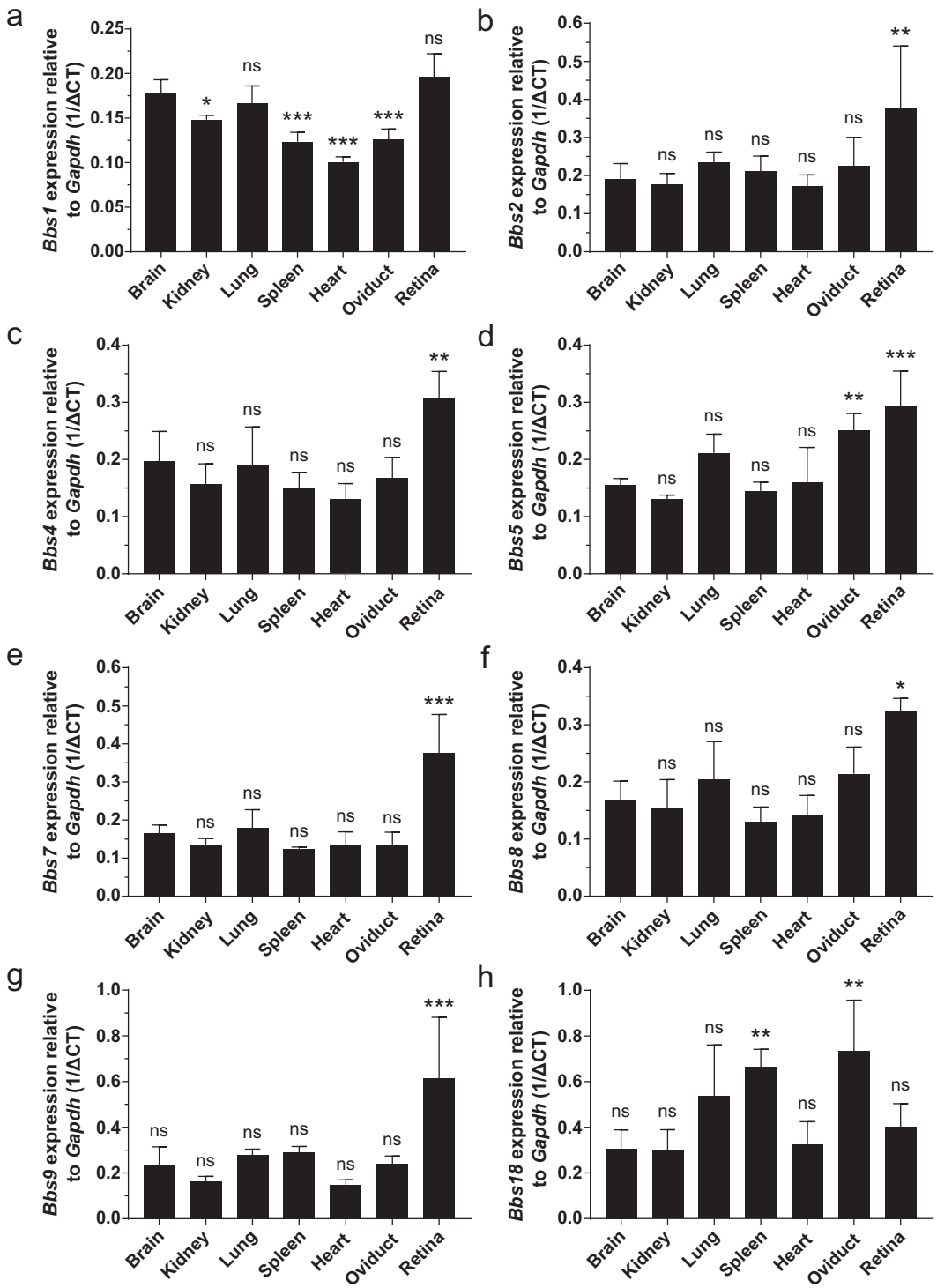
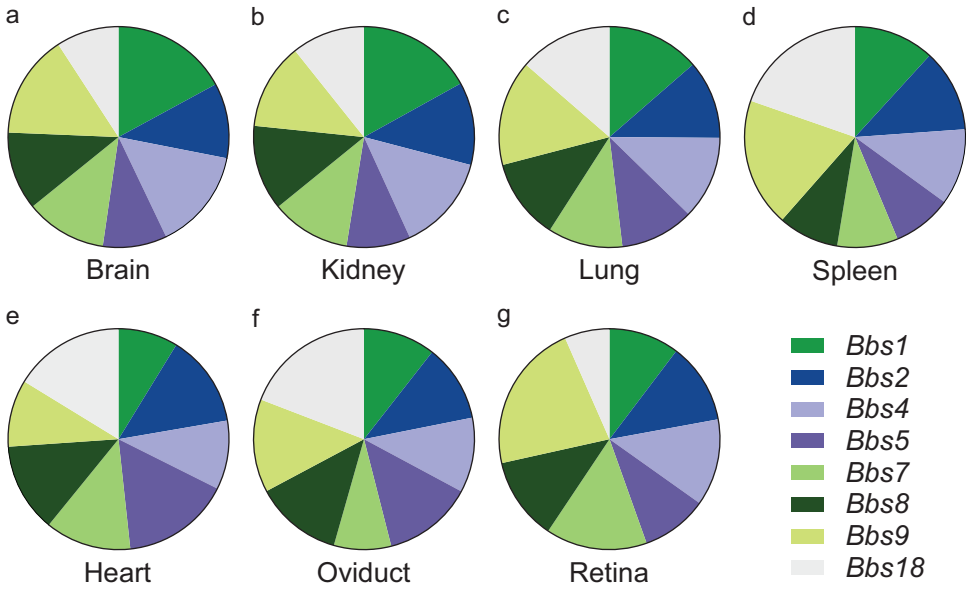


Figure 2 | Relative expression of BBSome transcripts

Pie charts showing relative gene expression of *Bbs1,2,4,5,7,8,9,18* in each tissue. Total expression of all BBSome transcripts is set at 100%. $n = 4$ for all genes, *Bbs8* $n = 3$.



to the trend seen for the BBSome transcripts. The one transcript that stands out is *Bbs12,* which is comparatively more abundant in spleen, oviduct and retina (Figure 3f–j). Overall, relative chaperonin-like *Bbs* expression levels within a specific tissue was less variable across tissues as compared to BBSome transcripts (Figures 3d–j and 2a–g).

Loss of Bbs8 leads to altered expression of other Bbs transcripts

Previous studies from our lab have shown aberrant gene expression of ciliary proteins upon loss of BBS function [Patnaik et al., 2019]; however, very little is known about the transcriptional control of BBSome genes upon loss of one subunit. To assess the expression of BBSome subunits in the absence of *Bbs8/Ttc8*, we utilised a knock out mouse model. We measured mRNA expression levels of the other seven BBSome components as well as the three chaperonin-like genes in tissues harvested from adult $Bbs8^{+/+}$ and $Bbs8^{-/-}$ littermate mice.

We observed significant changes in BBSome mRNA levels in the absence of *Bbs8* suggesting a possible transcriptional control mechanism (Figure 4a–n). mRNA levels were either lower or unchanged in $Bbs8^{-/-}$ mice compared to $Bbs8^{+/+}$ in all tissues analysed (Figure 4a–n). We did not see a significant increase in mRNA expression of other BBSome subunits in an attempt to compensate for the loss. Intriguingly, the expression of *Bbs7* was significantly lower in all knock out tissues tested, while *Bbs9* did not show any significant changes in expression (Figures 4e and 4f). *Bbs18* was only downregulated in heart. Similar to what was observed above, expression patterns in brain and kidney were remarkably similar. In these two tissues, only *Bbs7* was significantly downregulated (Figures 4h and 4i). This could possibly suggest a similar mechanism of transcriptional control in these two organs. Other organs (lung, spleen, heart, oviduct, and retina) have numerous BBSome subunits that are significantly less expressed in $Bbs8^{-/-}$ mice compared to control (Figure 4j–n). Since *Bbs3* (*Arl6*) has extremely close functional links to the BBSome [Fan et al., 2004; Klink et al., 2017], we also analyzed the expression levels of this gene in $Bbs8^{-/-}$ tissues. We found that *Bbs3* had a unique pattern of expression change that did not resemble any of the changes in expression for individual BBSome components (Supporting Information Figure 2a).

We next assessed the mRNA expression levels of chaperonin-like components in $Bbs8^{+/+}$ and $Bbs8^{-/-}$ tissues (Figure 5a–j). Again, we observe tissue- and

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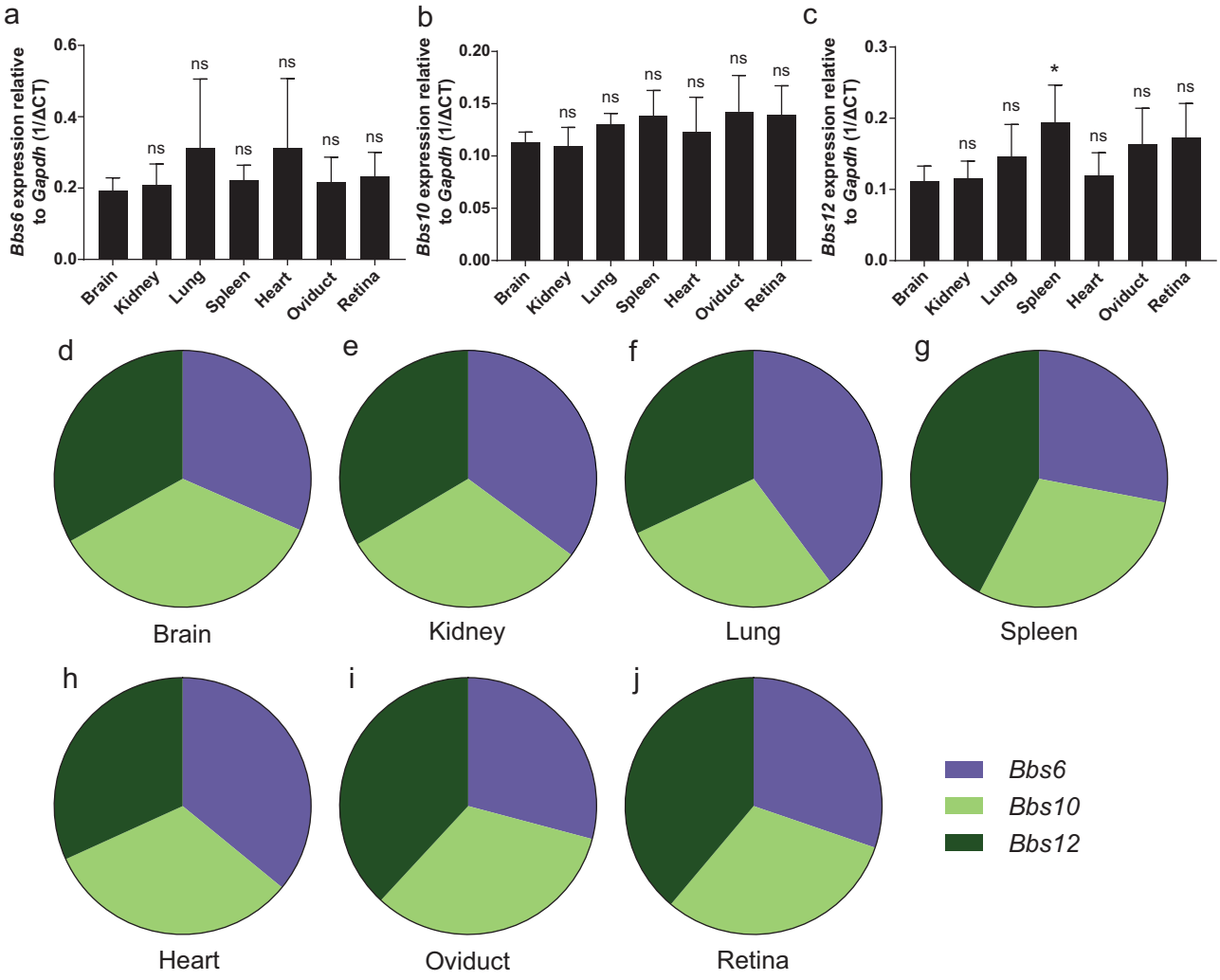
Figure 3 | Expression of BBS chaperonin-like transcripts

a–c) Bar chart showing gene expression of *Bbs6, Bbs10* and *Bbs12* in different tissues relative to *Gapdh*. Relative expression levels of each sample averaged. Error bars show standard error of the mean. Statistics were done using the Dunnett's multiple comparison test *$p \leq 0.05$; ns not significant. **d–j**) Pie charts showing relative gene expression of *Bbs6/Mkks, Bbs10* and *Bbs12* in each tissue. Total expression of all Bbs-chaperonin transcripts is set at 100%. $n = 5$ for *Bbs10* and 12, *Bbs6* $n = 3$.



gene-dependent differences. Loss of *Bbs8* affected the expression levels of all three transcripts, *Bbs6/Mkks*, *Bbs10* and *Bbs12,* variably (Figure 5a–c). In mutant mice *Bbs6* expression was reduced compared to control in brain, oviduct and retina, while *Bbs10* showed lower expression in lung and heart (Figures 5a and 5b). The expression of *Bbs12* was comparable between mutant and control with the exception of oviduct, in which *Bbs12* was increased (Figure 5c). This was the only case in which loss of *Bbs8* lead to an increase in expression of an alternative *Bbs* gene. When focusing on individual tissues, brain and retina had a similar change in expression pattern, as well as lung and heart (Figures 5d, 5j, 5f and 5h). Decreased expression of *Bbs6* is only compensated by increased expression of *Bbs12* in oviduct (and not in brain or retina). This highlights a possible importance of *Bbs6* in the reproductive system. The expression of chaperonin-like genes were unaffected in kidney and spleen (Figures 5e and 5g).

Figure 4 | Expression of BBSome transcripts in *Bbs8*$^{-/-}$ tissues

a–g) Bar chart showing gene expression of *Bbs1,2,4,5,7,8,9,18* in different tissues from *Bbs8*$^{-/-}$ tissues relative to control *Bbs8*$^{+/+}$ (red line). Coloured bars indicate significantly downregulated genes. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$,**$p \leq 0.01$,*$p \leq 0.05$. **h–n**) Graphical representation of different tissue with respective genes downregulated (coloured boxes) in *Bbs8*$^{-/-}$ tissues relative to control. Experiments were performed in triplicates from three individual animals.

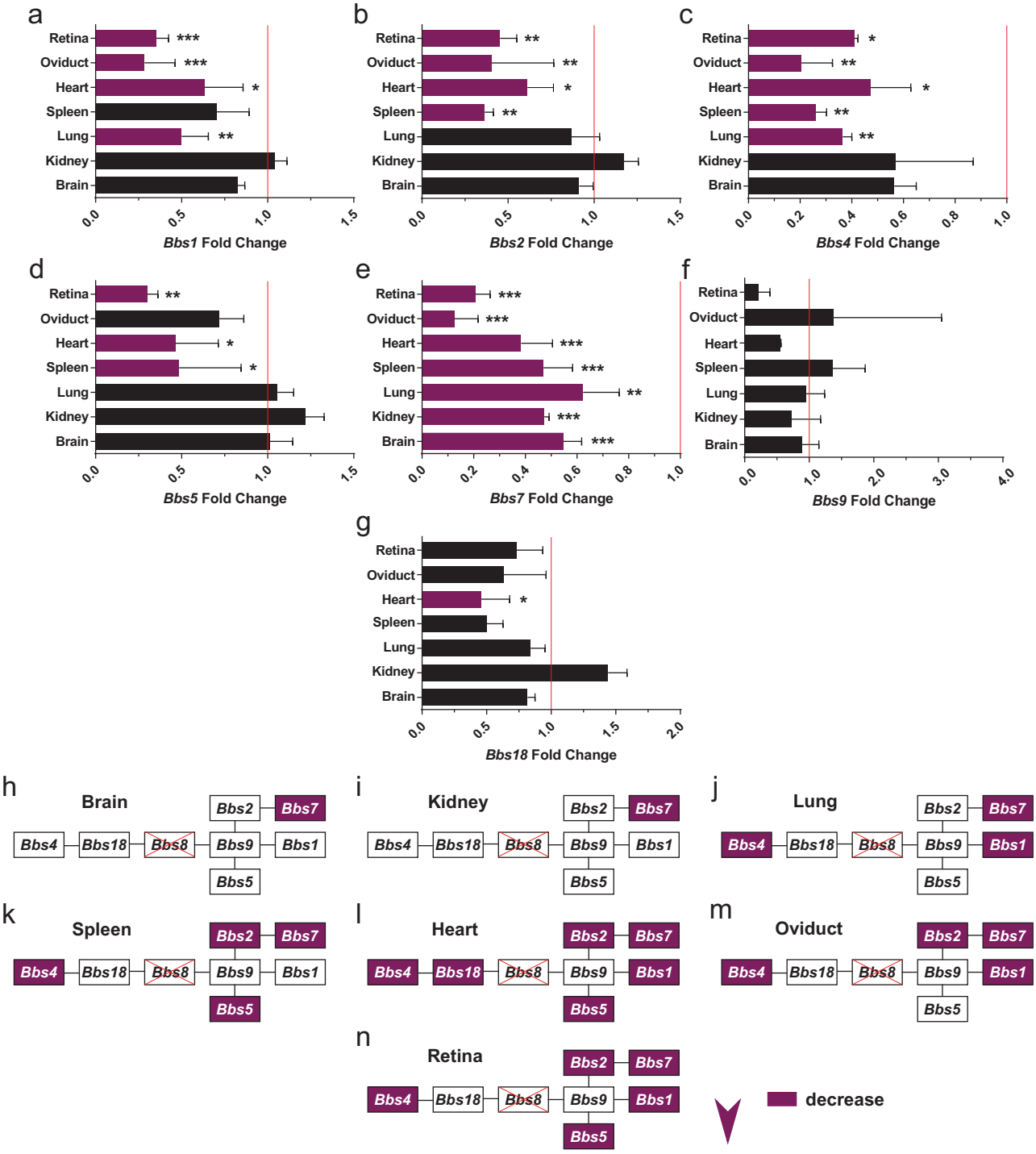


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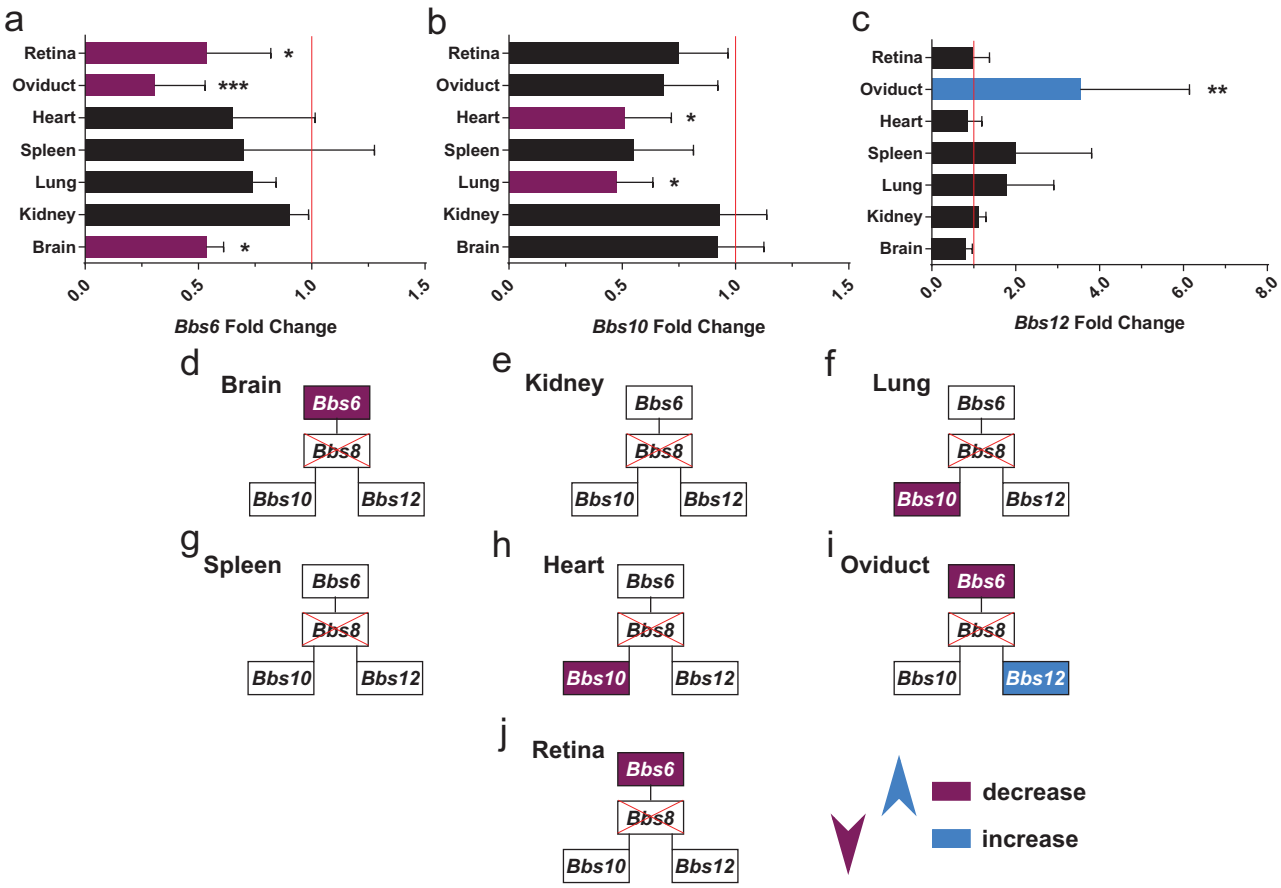
Figure 5 | Expression of BBS chaperonin-like transcripts in *Bbs8*$^{-/-}$ tissues

a–c) Bar chart showing gene expression of *Bbs6, 10 and 12* in different tissues from *Bbs8*$^{-/-}$ tissues relative to control *Bbs8*$^{+/+}$ (red line). Purple coloured bars indicate significantly downregulated genes, blue coloured bar indicates significantly upregulated gene. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. **d–j**) Graphical representation of different tissues with respective genes downregulated or upregulated (coloured boxes) in *Bbs8*$^{-/-}$ tissues relative to control. Purple indicates significantly downregulated genes, blue significantly upregulated genes. Experiments were performed in triplicates from three individual animals.



Effect of Bbs6 loss on other Bbs transcripts

Lastly, we examined the loss of a BBS chaperonin-like component on *Bbs* transcript expression. We started by analysing the expression variability of BBSome components in different tissues in *Bbs6*$^{-/-}$ adult mice compared to *Bbs6*$^{+/+}$ littermate controls. Perhaps unsurprisingly, in contrast to *Bbs8*$^{-/-}$ mutant mice, the expression of BBSome mRNA remained stable in most tissues compared to the control, with the exception of spleen and lung (Figure 6a–o). All BBSome components except for *Bbs2* showed significantly higher expression in spleen (Figure 6l). In *Bbs6*$^{-/-}$ lung *Bbs2, Bbs4* and *Bbs18* expression was increased (Figure 6k). This increased expression (albeit only in spleen and lung) is in contrast to the decreased expression of BBSome transcripts in *Bbs8*$^{-/-}$ mice. As for the *Bbs8*$^{-/-}$ tissue, we also analyzed the expression levels of *Bbs3* in *Bbs6*$^{-/-}$ tissues. Similarly, we found that *Bbs3* also had a unique pattern of expression change upon loss of *Bbs6* (Supporting Information Figure 2b).

Similar to the BBSome transcripts, expression levels of chaperonin-like components were also less variable in *Bbs6*$^{-/-}$ adult mice compared to controls (Figure 7a–i). The only differences were observed in spleen, in which *Bbs10* was significantly increased

Figure 6 | Expression of BBSome transcripts in *Bbs6*$^{-/-}$ tissues

a–h) Bar chart showing gene expression of *Bbs1,2,4,5,7,8,9,18* in different tissues from *Bbs6*$^{-/-}$ tissues relative to control (red line). Coloured bars indicate significantly upregulated genes. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. **i–o**) Graphical representation of different tissues with respective genes upregulated (coloured boxes) in *Bbs6*$^{-/-}$ tissues relative to control. Experiments were performed in triplicates from three individual animals.

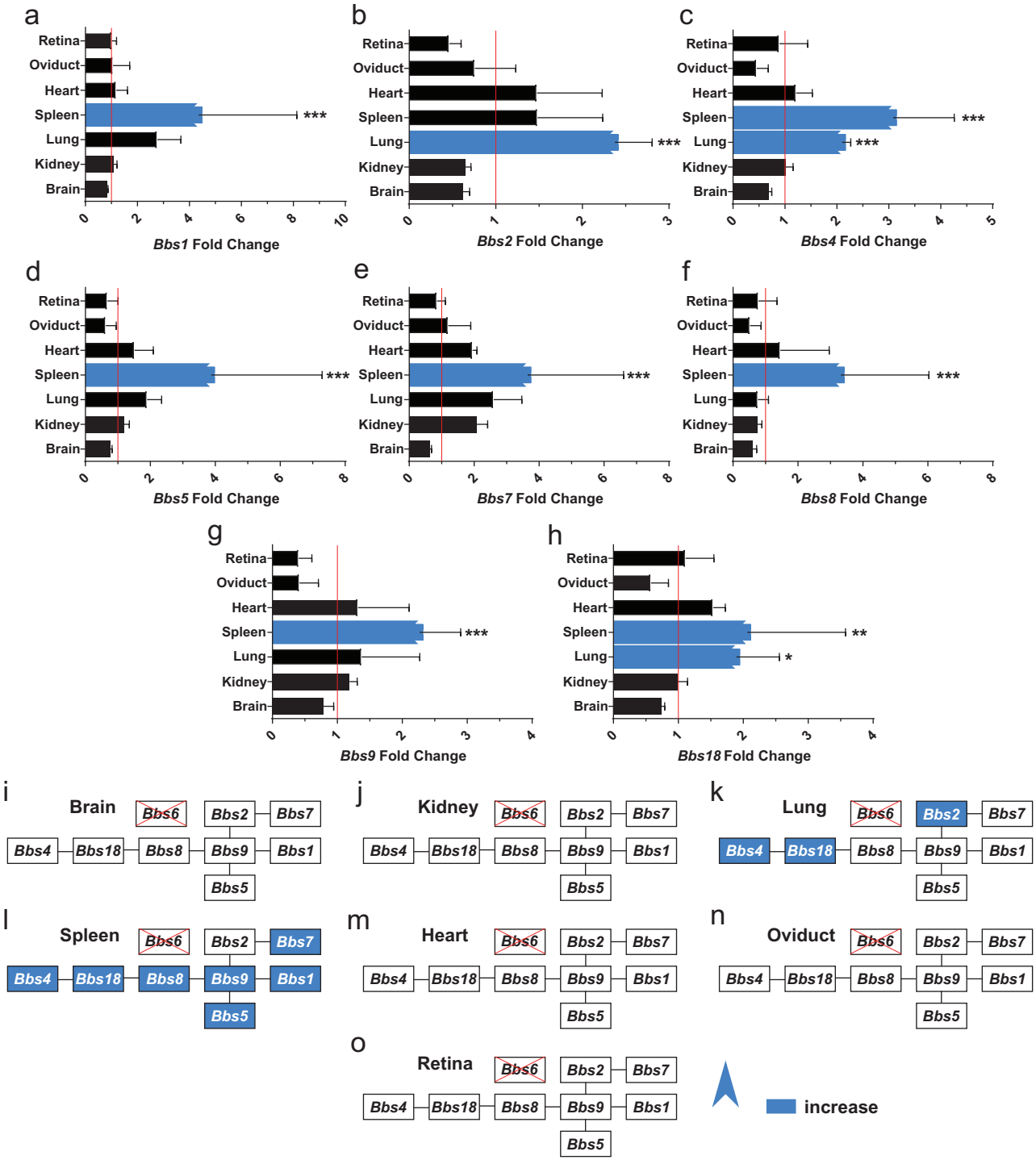


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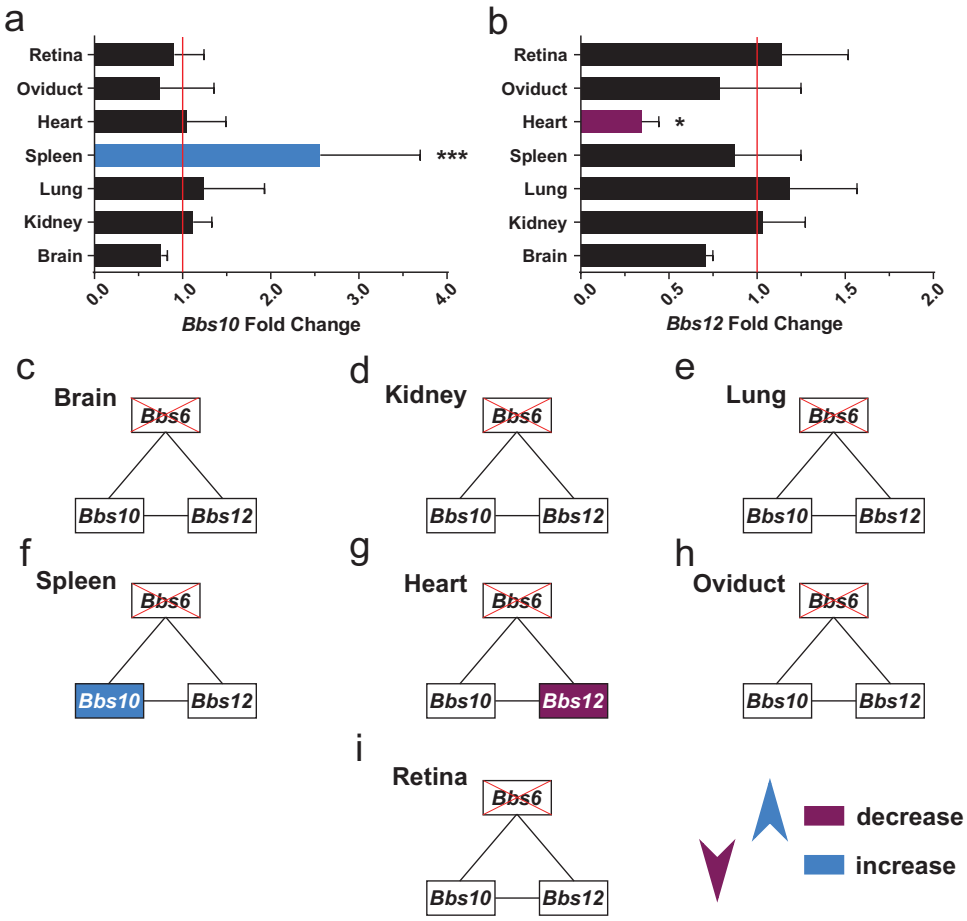
Figure 7 | Expression of BBS chaperonin-like transcripts in *Bbs6*$^{-/-}$ tissues

a and **b**) Bar chart showing gene expression of *Bbs6, 10 and 12* in different tissues from *Bbs6*$^{-/-}$ tissues relative to control (red line). Blue coloured bar indicates significantly upregulated gene, purple coloured bars indicates significantly downregulated gene. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$, **$p \leq 0.01$,*$p \leq 0.05$. c–i) Graphical representation of different tissues with respective genes upregulated or downregulated (coloured boxes) in *Bbs6*$^{-/-}$ tissues relative to control. Blue indicates significantly upregulated genes, purple significantly downregulated genes. Experiments were performed in triplicates from three individual animals.



(Figure 7a and 7f), and in heart in which *Bbs12* was significantly decreased (Figures 7b and 7g). Overall, loss of the chaperonin-like component *Bbs6* did not have a profound effect on transcripts encoding BBSome or chaperonin-like mRNA expression.

Discussion

Mutations in *BBS* genes cause a multitude of phenotypes affecting various organs and tissues. The predominant understanding is that BBS proteins facilitate ciliary membrane trafficking. In this context, the BBSome proteins (BBS1, 2, 4, 5, 7, 8, 9 and 18) form an octomeric protein complex that bind GPCRs and other receptors as a cargo adaptor during IFT [Nachury et al., 2007; Klink et al., 2017; Liu and Lechtreck, 2018]. In particular, BBSome-mediated trafficking is crucial for retrieval of GPCRs back into the cell [Wei et al., 2012; Nager et al., 2017; Ye et al., 2018]. The *BBS* chaperonin-like genes encode proteins (BBS6, 10, 12) that form a complex with the CCT/TRiC family chaperonins which is essential for BBSome assembly. However, the idea that BBS proteins are only functional in a ciliary context

might be an oversimplification. In recent years, there has been increasing interest in highlighting functions of ciliary proteins at extra-ciliary sites and in non-ciliary contexts. Such functions include intracellular trafficking, regulation of the cytoskeleton, mitosis, cell cycle regulation, regulation of the DNA damage response and transcriptional control [Vertii et al., 2015; Hua and Ferland, 2018]. Several studies also suggest that some of the BBS proteins might take on extra-ciliary roles, possibly in a non BBSome/chaperonin dependent manner that could be relevant to the aetiology of the disorder [Novas et al., 2015]. Alternative functions might be dependent on both time point and tissue type.

To gain more insight into BBS functionality, we set out to examine whether relative gene expression for individual *BBS* genes was constant across different tissues. We wanted to test the stoichiometric composition of *Bbs* at the level of mRNA across tissues. We postulated that if the BBSome and Bbs chaperonin-like genes only encode proteins that function in a defined complex, then their relative expression levels should be stoichiometrically conserved between different tissues.

We found that *Bbs* transcripts are differentially expressed in different tissues. Overall, there was a higher degree of variation among BBSome transcripts as opposed to BBS chaperonin-like transcripts. These results suggested possible tissue-dependent differences in BBSome composition and/or protein half-life, or that some of these transcripts might also be required for alternative non-BBSome-related functions in different tissues. A more stoichiometric expression of BBS chaperonin-like transcripts suggests that their individual functions might be more closely coupled. Interestingly, several tissues had similar distribution of expression, which might allude to similar functionality on a molecular level. For example in brain and in kidney, the stoichiometry of both BBSome and BBS chaperonin-like transcripts were more similar compared to other tissues.

When we analysed mRNA expression of other BBSome components upon loss of BBSome component Bbs8, we observed that numerous transcripts were decreased, which might suggest a transcriptional control mechanism. Alternatively loss of Bbs8 might have a stronger impact on tissue health or function and therefore a greater impact on overall gene transcription. Surprisingly, we did not see a significant increase in mRNA expression of other BBSome subunits that would suggest compensation for the loss of *Bbs8*. Interestingly there was little consistency in which transcripts were affected with the exception of *Bbs7,* which was significantly decreased in all tissues examined, and *Bbs9,* which was stable across tissues. Loss of *Bbs8* also affected the expression of individual chaperonin-like genes differently. *Bbs6* and *Bbs10* were decreased in several tissues. In contrast, *Bbs12* was found to be upregulated in oviduct. As in the control tissues, several tissues "responded" in a similar manner, such as brain and kidney in terms of BBSome transcripts and brain and retina in terms of chaperonin-like transcripts, alluding to similar functionalities of these molecules in these tissues.

Loss of BBS chaperonin-like component Bbs6 had little effect on the expression of BBSome transcripts in most tissues, yet surprisingly virtually all BBSome mRNAs in spleen and half in lung were upregulated. This might suggest that Bbs6 has some alternative role in spleen and lung tissue that is not present in any of the other tissues. Loss of *Bbs6* also increased the expression of the other chaperonin-like component, *Bbs10,* but only in spleen. Again, this suggests a possible alternative molecular function of Bbs6, which is possibly more prevalent in spleen. Although spleen defects are not readily reported in BBS patients.

Since alternative splicing can produce transcripts with different stabilities, we designed primers to recognise as many of the different BBS transcripts as possible. Nonetheless, alternative transcripts could result in different mRNA levels, which might contribute to the differences in expression levels described above. Absolute expression values must always be measured in relation to a housekeeping control the selection of which is crucial. We chose *Gapdh*, since it was the best option available to us, but are aware that variation in expression of housekeeping controls across tissues, which can always distort comparisons.

While our approach reveals differences in transcription that presumably affect protein abundance and consequent function, we were unable to show this directly due to technical limitations related to BBS proteins. Antibodies against these proteins are notoriously inconsistent and difficult to use. Numerous Western blots were performed using various tissues but give that certain antibodies failed or either detected bands at incorrect sizes or in knock

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out tissues, the data were unreliable. To overcome issues related to antibody specificity, protein abundance was also quantified via mass spectroscopy but proved imprecise due to the relatively low abundance of Bbs proteins in each sample.

As mentioned above previous studies have already shown that BBS protein functions are not restricted to the primary cilium. Such functions include intracellular trafficking. BBSome components have been found to assist retrograde dynein mediated melanosome transport in zebrafish [Yen et al., 2006] as well as trafficking of various receptor molecules (Notch and Vangl2) to the cell membrane [Leitch et al., 2014; May-Simera et al., 2015]. An active role in regulation of the cytoskeleton has also been shown for BBS4, 6 and 8 via manipulation of actin polymerisation [Hernandez-Hernandez et al., 2013]. Associations with the centrosome, centriolar satellites and the mid body might also underlie BBS4 and 6 facilitation of cell cycle regulation and mitosis [Kim et al., 2004, 2005; Zhang et al., 2014]. More recently, there have been reports showing that several BBS proteins enter the nucleus where they can influence gene expression through interactions with the polycomb group member protein RNF2 [Gascue et al., 2012; Scott et al., 2017].

Although our results suggest BBS proteins might have alternative functions independent of each other in different tissues, it is important to mention the evidence that argues against this hypothesis. Overall there is little evidence of a genotype–phenotype relationship among individuals affected with BBS, with a lack of tissue-specific defects in BBS patients carrying mutations in different BBS genes. The one exception here is the renal phenotype. A recent meta-analysis study in the Czech Republic found that the core BBSome subunits BBS2, 7 and 9 manifest as more critical in the kidney [Niederlova et al., 2019]. Similarly, the risk factor for severe renal disease were found to vary between patients harbouring BBS1, 2, 9, 10 or 12 mutations in a detailed study with 350 BBS patients in the UK [Forsythe et al., 2017]. An additional argument is that most functionally tagged BBSome proteins tend to show the same expression pattern (exclusively ciliary localisation) in cultured cells [Barbelanne et al., 2015; Ye et al., 2018]. Lastly, biochemical analyses have shown that the BBSome proteins consistently build one stable functional complex [Nachury et al., 2007; Klink et al., 2017].

In conclusion, we have seen that *Bbs* transcripts are not stoichiometrically expressed in different tissues and that loss of Bbs function affects expression of other transcripts differently. These data support the hypothesis that in some tissues at least, BBS proteins do not only function in a complex but might also have alternative functions in a ciliary independent context independent of one another.

Materials and methods

Animals

All mouse work was performed as per ethical approval from appropriate governing bodies. Experiments were performed in accordance with guidelines provided by Association for Research in Vision and Ophthalmology (ARVO). Animals were maintained on a cycle of 12 hours of light (200 lux) and 12 hours of darkness. The generation and characterisation of *Bbs6*/*Mkks* and *Bbs8*/*Ttc8* knock out (KO) mice have been previously described. For analysis of wild-type tissues, organs were harvested from C57BL/6 mice aged between 6 and 8 months. For comparison between control and *Bbs* knock out tissues, littermate controls of the same age were used in all experiments.

Biological materials

Mice were euthanised by cervical dislocation. Brain, kidney, lung, spleen, heart, oviduct and retina tissue samples were dissected from adult female mice. Tissues were placed immediately in TRIzol Reagent (Thermo Fisher Scientific) (for RNA) or snap frozen (for Western blotting) and stored at −80°C until further use.

RNA isolation

Total RNA was isolated from tissue samples using TRIzol Reagent (Thermo Fisher Scientific). Tissues were homogenised using a FastPrep® -24 classic (MP Biomedicals) bead-basher at a setting of six (6 m/s) for 20–60 s, periodically placing the samples on ice in between pulses. RNA extraction was performed according to manufacturer's instructions and stored at −80°C. RNA quality and quantity were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) following manufacturer's instructions. Only samples with an $OD_{260/280}$ reading between 1.8 and 2.1 were used for gene qRT-PCR experiments.

Reverse transcription and qRT-PCR

For analysis of target gene mRNA expression, 4 µg of RNA was reverse transcribed into cDNA in a 20 µL reaction volume using the SuperScript™ III first-strand synthesis system (Thermo Fisher Scientific) according to manufacturer's instructions.

Reverse transcription products were diluted in DEPC H_2O. The cDNA was diluted 1:50 or 1:20 while using *Gapdh* or *Bbs* primers respectively. One microliter of the diluted cDNA was used in each qPCR reaction, with a total volume of 10 µl. qRT-PCR amplification was performed using the SYBR® -Green reagent (Life Technologies) on a Step One Plus™ Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific,

Inc.). The thermocycler conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s; and a final extension at 72°C for 1 min. mRNA expression of the *Bbs* genes were calculated using the $2^{-\Delta\Delta Ct}$ method. All primer sequences used for qRT-PCR analysis are listed below (Table 1).

Melt curve analysis was performed to assess the amplification of single specific product (Supporting Information Figure 3). Primer amplification efficiency was determined prior to carrying out qPCR analysis (Table 1). Since alternative splicing can produce transcripts with different stabilities, we designed primers to recognise as many of the different BBS transcripts as possible (Table 1).

'ΔCt' is the difference in expression of a gene of interest (*Bbs* genes) and the reference gene, namely *Gapdh*. '1/ΔCt' termed as 'expression factor' was used to show the relative gene expression across tissues. The expression factor (mean of 1/ΔCt values) was used to make the pie charts. The expression factors of BBSome mRNAs (*Bbs1*, *Bbs2*, *Bbs4*, *Bbs5*, *Bbs7*, *Bbs8*, *Bbs9* and *Bbs18*) in different organs were normalised to either brain or heart. The sum of normalised values of all BBSome components in each organ was set as 100%. The percentage expression of individual Bbs gene was calculated and represented as a pie chart. Similarly, all three chaperonin-like gene expression percentages (*Bbs6*, *Bbs10* and *Bbs12*) are plotted as pie charts.

Statistical Analysis

Statistical differences between multiple groups were assessed using ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 6.0, GraphPad Software, San Diego, CA). Error bars represent the mean ± standard deviation. Results are considered statistically significant if $^{*}p < 0.05$.

Author Contribution

S.R.P. and H.L.M.-S. were responsible for conception and experimental design. S.R.P., A.F., A.-K.V., L.B., S.S. and V.K. carried out experimental work. S.R.P. generated figures. S.R.P. and H.L.M.-S. co-wrote the manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

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