A 5' UTR Mutation Contributes To Down-Regulation Of *Bbs7* In The Berlin Fat Mouse

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Abstract

The Bardet Biedl syndrome 7 (*Bbs7*) gene was identified as the most likely candidate gene causing juvenile obesity in the Berlin Fat Mouse Inbred (BFMI) line. *Bbs7* is significantly lower expressed in brain, adipose tissue, and liver of BFMI mice compared to lean C57BL/6NCrI (B6N) mice. DNA sequence comparison between BFMI and B6N revealed 16 sequence variants in the *Bbs7* promoter region. Here, we tested if these mutations contribute to the observed differential expression of *Bbs7*.

In a cell-based dual luciferase assay we compared the effects of the BFMI and the B6N haplotypes of different regions of the *Bbs7* promotor on the reporter gene expression. A single SNP was identified causing a significant reduction in the reporter gene expression. This SNP (rs29947545) is located in the 5' UTR of *Bbs7* at Chr3:36.613.350. The SNP is not unique to BFMI mice, but occurs also in several other mouse lines, where the BFMI allele is not associated with lower *Bbs7* transcript amounts. As such, a second factor alongside the SNP rs29947545 or interacting with the SNP location is likely required to cause the reduction of *Bbs7* expression which leads to the obese phenotype in BFMI mice.

Introduction

More than one-third of the world's adult population is overweight, and the incidence is further increasing¹. In recent years, in particular, childhood obesity has become a global epidemic, occurring at high rates all over the world². Besides malnutrition, heritability plays a major role in the development of obesity. The contribution of genetic predisposition to the onset of obesity is particularly high in children^{3,4}.

The Berlin Fat Mouse Inbred line (BFMI) carries a natural mutation causing juvenile obesity. Males and females of the BFMI line have a high body fat content already under a standard breeding diet at the young age of 6 to 10 weeks^{5,6}. In addition, BFMI mice show several features of the metabolic syndrome such as impaired lipid metabolism and reduced insulin sensing⁷⁻⁹. Therefore, BFMI is a suitable model for understanding genetic determinants and mechanisms contributing to juvenile obesity.

Previous research identified the *jObes1* locus on chromosome 3 which explains about 40% of the variation of the ratio between total body fat mass to total body lean mass in an F2 crossbred population between obese BFMI and lean C57BL/6NCrI (B6N) mice¹⁰. Subsequent fine mapping of the *jObes1* locus in an advanced intercross line and complementation tests with knockout mouse lines identified the Bardet Biedl syndrome 7 (*Bbs7*) gene as the most likely causal gene for the juvenile obesity phenotype of BFMI mice¹¹.

The Bardet Biedl-Syndrome 7 protein (BBS7) interacts with several other proteins to form the BBSome, an intracellular transport complex that is essential for the biogenesis of ciliary membranes and the transport of proteins to the cell membrane¹². Defects in any of the BBS proteins impair the formation and function of the BBSome¹³. Knock-down studies of BBSome subunits showed the same direction of effects, loss or impairment of the formation of the BBSome^{14,15}. For instance, studies on *Bbs1*^{-/-}, *Bbs2*^{-/-}, and *Bbs4*^{-/-}

knockout mice provided evidence for the significant role of the BBSome for the translocation of G protein-coupled receptors to the cell membrane of primary cilia in neurons¹⁶. This was shown for the leptin receptor¹⁷, the somatostatin receptor 3¹⁸, the dopamine receptor¹⁹, and the melanin-concentrating hormone receptor 1¹⁶. The knockdown of *Bbs7* led to reduced axonal growth and brain abnormalities²⁰. The missing BBS7 protein affected motile cilia structures in the ependymal layer of the brain¹³. Homozygous *Bbs7* knockout mice at 12 weeks age were significantly heavier than control males and females due to increased food intake¹³.

In humans, diverse BBS protein variants have been associated with the Bardet Biedl-Syndrome²¹. Nonetheless, there are cases of Bardet Biedl Syndrome which cannot be explained by protein variants. Therefore, the impairment of gene regulation could be an additional mechanism besides the loss of the protein function which might cause increased bodyweight in patients with Bardet Biedl syndrome.

In our mouse model, we could exclude the BBS7 protein variant of BFMI mice as a causal factor for obesity, since this protein variant occurs in several mouse lines which are not obese (Ensembl release 102)²². In contrast, reduced gene expression of *Bbs7* in different tissues of BFMI mice in comparison to B6N provided a hint that down-regulation, might serve as an additional potential mechanism causing obesity in BFMI mice¹¹. The comparison of the DNA sequences in the promoter region of *Bbs7* (36.613.319–36.614.267 Ensembl release 102) of BFMI and B6N identified 16 DNA sequence variants²².

In the current study, we tested the DNA sequence variants of the promoter region in reporter gene assays in order to identify the cause for the expression differences of the *Bbs7* gene seen between BFMI and B6N. The aim was to further elucidate the molecular mechanism that causes juvenile obesity in BFMI mice.

Results

Effects of variants in the *Bbs7* promoter region on the expression of the luciferase gene

The tested promoter region of 949 base pairs (3:36,613,319 and 3:36,614,267 Mb) comprises 856 base pairs upstream of the *Bbs7* start codon and 159 base pairs of the 5' UTR. The region contains 16 sequence variants between BFMI and B6N; 15 SNPs (12 upstream of the 5' UTR in the promoter region, 3 in the 5' UTR) and an 11 bp deletion upstream the start codon (Fig. 1). In the following, we use the numbers 1 to 16 for the sequence variants to facilitate the understanding of their effects on the reporter gene expression.

First, the effect of BFMI and B6N haplotypes of the whole promoter region with all 16 sequence variants (fragment A, Fig. 1) on the reporter gene expression was tested. The normalized expression of the luciferase gene behind the BFMI promoter was reduced to a fold change of 0.37 compared to the B6N promoter (fragment A, $FC_{(BFMI/B6N)A} = 0.37$, Fig. 1). The magnitude of reduction of the reporter gene

expression with the BFMI promoter is consistent with the reduction of the expression of *Bbs7* in 10-week-old BFMI versus B6N mice in the whole-brain ($FC_{(BFMI/B6N)} = 0.39$)¹¹.

The BFMI promoter variants of the two shorter fragments B and C, containing the sequence variants 8 to 16 and 12 to 16, respectively, showed also a reduction of the expression of the reporter gene. The reduction was in the same range as for the long BFMI fragment A ($FC_{(BFMI/B6N)} B = 0.43$, $FC_{(BFMI/B6N)} C = 0.51$, Fig. 1). Since the longest as well as the shorter BFMI variants of fragments A, B and C cause a reduction of the reporter gene expression, the sequence variants 1 to 11 in the region between 3:36,613,573 and 3:36,614,267 Mb, which occur in fragments A and B only, could be excluded from being causal for the difference in expression. The excluded region also comprises the 11 bp deletion upstream of the 5' UTR of *Bbs7* in BFMI mice (Fig. 1)²². After testing the fragments A, B, and C, the sequence variants 12 to 16 remain important to test for gene expression.

The short promoter fragment D contains the sequence variants 8 to 14, but not 15 and 16. This fragment was designed to test whether the two sequence variants 15 and 16 which are located in the 5' UTR downstream of the 11 bp deletion are necessary for the reduction of expression in BFMI (Fig. 1). The reporter gene assay did not show any difference in the expression between the BFMI and B6N promoter fragments D ($FC_{(BFMI/B6N)} D \approx 1$). Based on this result we dismissed the three sequence variants 12, 13, and 14 between 3:36,613,413 and 3:36,613,573 Mb from being causal and focused on the remaining two sequence variants 15 and 16 which are located in the 5' UTR.

To figure out which of the two remaining sequence variants 15 and 16 are causal for gene expression differences, the four fragments E to H were synthesized which all comprise the sequence between 3:36,613,319 and 3:36,613,413 Mb. Fragment E contains the reference sequence of B6N, fragment F the BFMI allele of sequence variant 15, fragment G the BFMI allele of sequence variant 16, and fragment H the BFMI alleles of sequence variants 15 and 16. Normalized expression levels of the luciferase reporter gene showed consistently a reduction of expression when the BFMI allele C of sequence variant 16 was present in the sequence. This was the case for the mutant fragments G and H compared to the B6N fragment E ($FC_{(G/E)} = 0.35$, $p = 0.001$; $FC_{(H/E)} = 0.37$, $p = 0.001$, Fig. 1). Moreover, no difference was evident in the normalized expression between the fragments F and E ($FC_{(F/E)} = 1.01$, $p = 0.959$, Fig. 1). Therefore, sequence variant 15 was excluded from being causal.

The exclusion of all sequence variants except number 16 led to the conclusion that SNP rs29947545 with the allele C in BFMI is the only candidate that could cause the reduction in expression in the reporter gene assay.

The effects reported above were observed in HEK 293A cells, which originate from human embryonic kidney, which are easy to transfect and which express *Bbs7*. We verified those results in 3T3-L1 cells, which originate from murine preadipocytes. However, it was challenging since the transfection efficiency in the 3T3-L1 cells was very low compared to HEK 293A cells. Since fewer cells were transfected, lower luciferase expression levels were observed. Nonetheless, the normalized luciferase expression of

fragments E, F, G, and H in 3T3-L1 cells showed similar expression levels of luciferase in 3T3-L1 cells when compared to HEK 293A cells with reduced expression under fragments G and H. These observations further validate the findings in HEK cells (Supplementary Fig. 1).

Occurrence of the identified BFMI SNP allele in other mouse inbred strains

To examine the potential function of the identified SNP rs29947545 for obesity we checked in which other inbred mouse strains the BFMI allele (C instead of T) occurs. Surprisingly, 30 out of 43 (69%) commonly used inbred strains for which whole-genome sequence data are available³³ carry the BFMI allele (C) (Supplementary file 3). Frequently, they do not only carry the C allele at rs29947545, but the whole BFMI haplotype of neighboring SNPs. Yet, most of those strains are lean. Therefore, we suggest interaction of the site around the SNP with a second genomic factor.

To further clarify a potential interaction of the SNP rs29947545 with another genomic factor, we investigated the strains AKR (prone to DIO) and SJL (resistant to DIO). These two strains have a *Bbs7* promoter similar to BFMI (table 1) but have never been reported to develop juvenile obesity under a normal diet.

Bbs7 expression in B6N, BFMI, AKR, and SJL

AKR and SJL share the *Bbs7* promoter haplotype with BFMI between 3:36,613,319 and 3:36,614,267 Mb except for variants 3 and 11 where they carry the B6N alleles. The measurement of *Bbs7* transcript amounts in tissue from the brain confirmed the previous finding of lower expression in BFMI compared to B6N¹¹ ($FC_{(BFMI/B6N)} = 0.61$, $p = 0.014$, Fig. 2). However, no significantly reduced *Bbs7* expression was observed for AKR ($p = 0.880$) and SJL ($p = 0.136$) compared to B6N.

On the right side, a bar chart shows the normalized luciferase expression (GLuc / SEAP ratios) of the tested BFMI promoter variants versus B6N as the reference. Significantly reduced expression was evident for BFMI fragments A, B, and C ($FC_{(BFMI/B6N)A} = 0.37$, $FC_{(BFMI/B6N)B} = 0.43$, $FC_{(BFMI/B6N)C} = 0.51$), but not for D. Testing the short synthesized fragments provided evidence for the importance of SNP 16 (rs29947545). The BFMI allele of SNP rs29947545 is required (fragments G and H) for significantly reduced expression ($FC_{(G/E)} = 0.35$, $FC_{(H/E)} = 0.37$, $FC_{(F/E)} = 1.01$). Normalized luciferase expression (GLuc / SEAP ratio) was plotted as mean $\pm$ standard deviation of triplicates.

Discussion

In this study, we examined the regulatory effects of promoter variants of the *Bbs7* gene on its gene expression. The *Bbs7* promoter variants of BFMI and B6N mice were tested using a dual luciferase reporter gene assays performed in HEK 293A cells. The experiments provided clear evidence that SNP rs29947545 caused expression differences in our luciferase assay.

Although our reporter gene assay was performed in HEK 293A cells, we ensured that the results observed in this human cell line are replicated in a mouse cell line (3T3-L1). However, transfection rates and subsequent luciferase expressions were much lower in 3T3-L1 cells. Nonetheless, the BFMI/B6N ratios of luciferase expression observed in 3T3-L1 cells were similar to the reduction in HEK 293A cells. Therefore, we are confident that the BFMI allele C of SNP rs29947545 is causal for the reduction of expression in the transfected cells.

The SNP rs29947545 is located in the 5' UTR of *Bbs7*, a region that is crucial for post-transcriptional regulatory mechanisms^{34–37}. UTRs generally are recognized to have critical roles in post-transcriptional regulation of gene expressions, such as modulation of the transport of mRNAs out of the nucleus³⁸, mRNA stability³⁹, and subcellular localization⁴⁰. DNA variations in the 5' UTR, such as SNP rs29947545, could regulate gene expression through one of the mentioned post-transcriptional pathways.

Another way in which this SNP in the 5' UTR could affect transcription levels is through disruption of an existing transcription factor binding site (TFBS)⁴¹. Whether a SNP potentially disrupts the binding of transcription factors (TF) or leads to a new TFBS can be valuable information. This information can help in interpreting distinct results from different mouse lines sharing the same SNP. Notably, it can help us explain the mechanisms at the molecular level by which the DNA variant directly affects it. For this reason, we used SwissRegulon⁴² and PROMO^{43,44} to predict if SNP rs29947545 is located at currently known TFBSs. Unfortunately, the results from the two mentioned databases were inconclusive. Moreover, there was no currently known TFBS in ENSEMBL²².

The BFMI allele C of SNP rs29947545 occurs in 69% of inbred mouse lines sequenced by the mouse genome project (MGP)³⁸. This SNP might be causal in BFMI's obese phenotype but we can not make the same assumption for the other mice with the same SNP in the MGP. Since most of the other mice that harbor SNP rs29947545 are not obese, we can state that the mutation alone is not sufficient for causing obesity.

With respect to the mechanism causing low *Bbs7* expression in BFMI mice, we identified in a cell-based system, a single SNP in the promoter region that significantly contributes to the regulation of transcription, but, which is not sufficient to reduce the transcript amount in other mouse lines than BFMI. We assume that a second factor interacting with the region of SNP rs29947545 is necessary to affect the expression of *Bbs7*. This secondary factor is most likely only present in the BFMI mouse. The other mouse lines likely have a genetic background that does not express this secondary factor, or that contains a second genomic region that physically interacts with the promoter region where the SNP resides. This hypothesis is supported by the observation that the AKR and SJL lines which share the same SNP allele and almost the entire *Bbs7* haplotype with BFMI, did not show the reduced *Bbs7* expression seen in BFMI mice.

BBS7 protein is one of the key proteins for the formation of the BBSome¹⁵. Our hypothesis is that a lower transcript amount of *Bbs7* leads to a lowered production of the BBS7 protein in BFMI mice. Since the

absence of BBS7 in knock-out mice has been shown to lead to obesity¹³, we hypothesise that the lack of BBS7 in BFMI is sufficient to cause obesity. To confirm this hypothesis, further research is required to evaluate and measure the BBS7 protein in BFMI mice compared to B6N mice.

Conclusion

The results of this study based on the dual luciferase assays imply that the BFMI allele of SNP rs29947545 is the causal variant for the reduction of the expression of *Bbs7* in transfected cells. However, two-thirds of the mouse lines in the Mouse Genome Project share the BFMI SNP allele in their 5' UTR of the *Bbs7* promoter region. Hence, we conclude that a second factor is needed to induce the reduction in *Bbs7* expression in the BFMI mice which causes juvenile obesity. Our results provide fundamental information on the transcriptional regulation of *Bbs7* for future studies, to fully unravel the molecular mechanism leading to obesity.

Material And Methods

Mouse populations

The Berlin Fat Mouse Inbred line BFMI 860 – 12 (BFMI, Humboldt-Universität zu Berlin, Berlin, Germany) and C57BL/6NCrl (B6N, Charles River Laboratories, Sulzfeld, Germany) were used as DNA sources. The BFMI and B6N lines have previously been used for mapping and fine-mapping of the juvenile obesity locus *Jobs1*^{10,11}. For real time gene expression measurements, brain from 6 weeks-old BFMI and B6N mice were collected (4 samples each). For comparison reasons, we also used AKR/J (AKR, Technical University of Munich, Munich, Germany) and SJL/NDIfe (SJL, German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE), Potsdam, Germany) mice (5 samples each). AKR and SJL share a similar promoter haplotype with BFMI mice. Nevertheless, AKR mice are prone to diet-induced-obesity (DIO) while SJL mice are resistant to DIO.

Husbandry conditions

All experimental treatments of animals were authorized by the German Animal Welfare Authorities (approval no. G0174/19). All methods were performed in accordance with the relevant guidelines and regulations of German Animal Welfare laws and also in accordance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. All mice were maintained under conventional conditions and a 12:12 h light: dark cycle at a temperature of $22 \pm 2^\circ\text{C}$. Animals had *ad libitum* access to food (rodent standard diet (V1534-000 ssniff R/M-H; ssniff Spezialdiäten GmbH, Soest, Germany)) and water.

DNA extraction and sequencing

DNA was isolated from BFMI and B6N animals for amplification and subsequent cloning following the DNA extraction protocol for mouse tail clips by Wang and Storm et al²³. The Illumina HiSeq platform (Illumina Inc., San Diego, CA, USA) was used for paired-end sequencing of the BFMI and B6N genomes

isolated from the spleen. Reads were trimmed with Trimmomatic²⁴ after which reads were aligned to the mouse genome (MM10, GRCm38.p6) using the Burrows-Wheeler Aligner (BWA)²⁵. The subsequent SAM files were converted to BAM files, indexed, and sorted utilizing Samtools^{26,27}. Duplicate reads were removed by Picard tools v2.19.0²⁸. Subsequently, indel realignment and base recalibration was done using the GATK v4.1.0.0²⁹, based on GATK best practices³⁰. BCFtools²⁷ was used to call sequence variants.

Primer design

The DNA sequence of the promoter region of *Bbs7* (chr3: 36.613.319–36.614.267, Ensembl release 102) from BFMI and B6N mice was loaded into SnapGene v5.0.7 (<http://www.snapgene.com>, GSL Biotech LLC, Chicago, IL, USA) to define the exact regions to be inserted into the linearized ZX-103 plasmid (GeneCopoeia™, Rockville, MD, USA) (see Fig. 1). Primers were designed to contain overhangs complementary to the sticky ends of the respective restriction site of the linearized plasmid. All primer sequences are available in supplementary table 1 and their purpose of use is mentioned in supplementary table 2.

Generation of a set of promoter fragments of BFMI and B6N

This study investigated 16 sequence variants in a 949 base pair region of the *Bbs7* promoter between 3:36,613,319 and 3:36,614,267 Mb. This region contains 159 base pairs of the 5' UTR of *Bbs7*²². Fragments of the target region for subsequent cloning into the ZX-103 plasmid were generated for BFMI and B6N genotypes either by PCR (fragments A to D) or by synthesis of oligonucleotides (E to H) (Fig. 1). Depending on the length of the PCR products, the BFMI and B6N fragments were haplotypes containing all BFMI or all B6N alleles of up to 16 sequence variants within the examined region. The sequence variants were assigned to numbers 1 to 16 (Fig. 1).

For each PCR reaction, 25 µl of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with 2.5 µl forward and 2.5 µl reverse primers (10 µM), 5 µl DNA (50 ng to 1 µg) and 15 µl nuclease-free water. Thermal cycler conditions were: 95°C for 1 minute for the initial denaturation followed by 40 cycles of amplification with denaturation at 95°C for 30 seconds, annealing at 60°C to 65°C depending on the primer's melting temperature for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. The primer sequences and their melting temperatures are given in supplementary table 1.

DNA sequences were synthesized to obtain short fragments mimicking either the natural B6N sequence or the alternative BFMI sequence. These are the fragments E to G (Fig. 1). Synthesis was carried out in service (Eurofins Genomics Germany GmbH, Ebersberg, Germany).

Cloning

For cloning, we used the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, MA, USA) comprising of NEB 5-alpha Competent *E. coli* together with the plasmid ZX-103. The ZX-103 plasmid was linearized using the restriction enzymes HindIII and EcoRI (New England Biolabs, Ipswich, MA, USA). For restriction digest, we added 1 µg of the plasmid ZX-103 to a 50 µl reaction mixture consisting of 1 µl of HindIII (10 units), 1 µl of EcoRI (10 units), 5 µl CutSmart buffer, and nuclease-free water. The restriction digest reaction was incubated at 37°C for 1 hour. All inserts were designed to carry sticky ends. The assembly protocol from the NEBuilder HiFi DNA Assembly Cloning Kit was carried out to insert the target promoter sequences into the linearized ZX-103 plasmid using the recommended DNA ratio of plasmid to insert by 1:2, with the total amount of fragment in the range of 0.03 to 0.2 pmol. After ligation at 50°C for 15 minutes, the transformation was performed according to the kit's protocol by adding 2 µl of the ligation reaction to 50 µl of NEB 5-alpha competent *E. coli* cells. The tubes containing this mixture were left on ice for 30 minutes before they were incubated in a 42°C water bath for 45 seconds and directly put back on ice for 2–3 minutes. Afterwards, 1 ml lysogeny broth (LB) culture medium was added to each mixture and incubated at 37°C on a Thermo-Shaker with 800 RPM for 1 hour. Subsequently, 100 µl of the culture was spread on LB Agar Kanamycin (50 µg/ml) plates. Kanamycin was used since the ZX-103 plasmid contains a Kanamycin resistance cassette. Plates were incubated at 37°C overnight. The following day single colonies were picked for further use.

Plasmid DNA isolation

Plasmid DNA was isolated from bacteria culture and purified using the GeneJET Plasmid Midiprep or Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). Both kits use silica-based membrane filters with spin columns. The concentration of the purified plasmid DNA was measured on a NanoDrop™ One/One^C device (Thermo Fisher Scientific, Waltham, MA, USA). For the subsequent reporter gene assay, the concentration of recombinant plasmids was adjusted to 500 ng/µl.

Cell culture

For the reporter gene assay, we used HEK 293A cells. They were cultured in high glucose, HEPES supplemented Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA), with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 0.2% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). 10⁴ cells per well of a standard 24 well plate were seeded in 0.5 ml medium. Prior to transfection, cells were incubated at 37°C in a 5% (v/v) CO₂-enriched air for 24 h. The medium was changed after the 24 h incubation before the cells were transfected.

The 3T3-L1 cell line was also used in this study with the same conditions and treatments as the HEK 293A cells. They were seeded in standard 24 well plates with 10⁴ cells per well in 0.5 ml medium. As expected, the transfection efficiency for the 3T3-L1 cell line was low given that it is an embryonic fibroblast cell line. Nonetheless, we performed the dual luciferase experiment on the few transfected cells

using the plasmids containing E to G fragments (supplementary Fig. 1). Data are shown in supplementary file 2.

Transfection

Transfection of HEK 293A cells with 500 ng plasmid DNA was done with the transfection reagent EndoFectin™ Max (GeneCopoeia™, Rockville, MD, USA) according to the manufacturer's protocol. For the dilution gradient of plasmid DNA, Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific, Waltham, MA, USA) was used. 2 µl transfection reagent was used for each well. Transfected cells were incubated for 48 h; the medium was not changed after transfection.

Dual luciferase assay

The dual luciferase reporter system Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia™, Rockville, MA, USA) was used to investigate the effect of the inserted promoter fragments on the activities of Gaussia Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP). The GLuc reporter gene is located downstream of the multiple cloning site of the ZX-103 plasmid where the promoter fragments were inserted. The experimental control gene SEAP is located downstream of a Cytomegalovirus (CMV) promoter about 600 bp downstream of the GLuc gene. Luciferase and SEAP activity was measured 48 h post-transfection using the GL-H and AP buffer, respectively.

Signal normalization was done by calculating the ratio of GLuc/SEAP activity, in order to eliminate the effects of transfection efficiency differences (supplementary file 2). Fold change (FC) was calculated to explain how much the luciferase expression changed between the BFMI and B6N promoter variants. Tecan's Infinite 200 plate reader (Tecan, Männedorf, Switzerland) was used to measure the luciferase activity (reporter gene) and SEAP (experimental control).

RNA extraction and cDNA synthesis

Flash-frozen whole-brains of 6-week-old BFMI, B6N, AKR, and SJL mice that had been stored at -80°C were used for RNA extraction. The brain was disrupted by grinding with a pestle and mortar to a fine powder in the presence of liquid Nitrogen. About 30 mg of the ground tissues were used for total RNA isolation using the NucleoSpin RNA isolation (MACHEREY-NAGEL, Düren, Germany) as described in Hesse et al ³¹. The LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA) was used for cDNA synthesis following the manufacturer's protocol using 1 µg of total RNA as input for each reaction.

Real time PCR

Real time PCR (RT-PCR) was carried out to compare the expression of *Bbs7* in the brain of 6-week-old BFMI, B6N, AKR, and SJL mice (*Bbs7* and *ACTB* primers in supplementary table 1). Takyon™ No ROX SYBR 2X MasterMix blue dTTP (Eurogentec, Seraing, Belgium) was used for RT-PCR. For each sample 2.5 µl Takyon™ MasterMix was used followed by the addition of 0.8 µl primer pair (10 µM), 1 µl of cDNA, and 1.1 µl nuclease-free water resulting in a 5 µl reaction volume. The PCR cycles comprised: 95°C for 3

minutes, followed by 40 cycles consisting of 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 40 seconds. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method³². The fold change of BFMI/B6N was calculated to show the differences in the quantity of *Bbs7* expression between BFMI and B6N.

Declarations

competing interests

The author declares no competing interests.

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Ethical approval

All experimental treatments of animals were approved by the German Animal Welfare Authorities (approval no. G0174/19) and all methods were performed in accordance with the relevant guidelines and regulations. This study is reported in accordance with ARRIVE guidelines.

Author contributions

KM performed the statistical analysis, conducted all of the experiments, drafted the manuscript and figures. DH critically read the manuscript and gave feedback. DA performed the sequence alignment, SNP identification, critical reading of the manuscript, and feedback. GAB designed the experiment, critically read the manuscript, and gave feedback.

Data availability statement

The datasets supporting the conclusions of this article are included within the article and its supplementary files. DNA sequencing data was deposited at the NCBI Sequence Read Archive (SRA) under BioProject ID: PRJNA717237 and is available at <https://www.ncbi.nlm.nih.gov/bioproject/717237>.

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Table

Table 1 is available in the Supplemental Files section.

Figures

Figure 1

On the left side, promoter fragments are shown that were tested for their effect on the expression of luciferase as the reporter gene in the dual luciferase plasmid (ZX-103). The longest fragment A comprises the whole *Bbs7* promoter region of 949 bp between 3:36,613,319 and 3:36,614,267. The 5' UTR is shown with a patterned black and white box. Fragments B, C, and D are shorter cloned promoter fragments. Fragments E through H (3:36,613,319 to 3:36,613,413) were synthesized. E comprises the B6N haplotype; F, G, and H contain BFMI alleles in different combinations. On top, all 16 sequence variants across the promoter fragment are shown with their ID and numbers from 1 to 16. Above every fragment, the B6N alleles are depicted (black), below the BFMI alleles (red).

Figure 2

Normalized *Bbs7* expression (RT-PCR) in the brain of 6-week-old BFMI, B6N, AKR, and SJL males. Beta actin was used as the housekeeper gene. The expression of *Bbs7* was decreased by almost 40% in BFMI *versus* B6N mice only ($FC_{(BFMI/B6N)} = 0.61$). No significant difference was observed when comparing AKR and SJL with B6N.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementaryfile1tablesandfigures.pdf](#)
- [Supplementaryfile2DualLuciferaseanalysisandresults.xlsx](#)
- [supplementaryfile3MGPSNPChr3JObes1.xlsx](#)
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