

Overexpression of the mitochondrial methyltransferase TFB1M in the mouse does not impact mitoribosomal methylation status or hearing

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ABSTRACT

Mitochondrial dysfunction is a well-established cause of sensorineural deafness, but the pathophysiological events are poorly understood. Non-syndromic deafness and predisposition to aminoglycoside-induced deafness can be caused by specific mutations in the 12S rRNA gene of mtDNA and are thus maternally inherited traits. The pathophysiology induced by mtDNA mutations has traditionally been attributed to deficient oxidative phosphorylation, which causes energy crisis with functional impairment of multiple cellular processes. In contrast, it was recently reported that signalling induced by ‘hypermethylation’ of two conserved adenosines of 12S rRNA in the mitoribosome are of key pathophysiological importance in sensorineural deafness. In support for this concept, it was reported that overexpression of the essential mitochondrial methyltransferase TFB1M in the mouse was sufficient to induce mitoribosomal hypermethylation and deafness. At variance with this model, we show here that 12S rRNA is near fully methylated *in vivo* in the mouse and thus cannot be further methylated to any significant extent. Furthermore, bacterial artificial chromosome (BAC) transgenic mice overexpressing TFB1M have no increase of 12S rRNA methylation levels and hear normally. We thus conclude that therapies directed against mitoribosomal methylation are unlikely to be beneficial to patients with sensorineural hearing loss or other types of mitochondrial disease.

INTRODUCTION

Mutations of mtDNA have for more than two decades been recognized as an important cause of human mitochondrial disease (1) and are also implicated in the ageing process (2). The genes in mammalian mtDNA are very densely packed, and this relatively small genome of ~16.5kb encodes 11 mRNAs (translated to 13 proteins), 2 rRNAs and 22 tRNAs (3). Oxidative phosphorylation is dependent upon the expression of mtDNA (3) and the coordinated expression of hundreds of nucleus-encoded proteins and their import into mitochondria (3). The lack of compartmentalization between transcription and translation in mitochondria is reminiscent of prokaryotic systems where there is crosstalk between the mechanisms regulating the various stages of gene expression that involve genome maintenance, transcription, RNA maturation (including processing, stability, polyadenylation and base modifications), ribosomal biogenesis and translational coordination (3).

Sensorineural impairment of hearing is a common symptom in patients with reduced mitochondrial translation, and can be caused by point mutations in the mitochondrial-encoded tRNA or rRNA genes (1). For example, the m.1555A>G mutation in the gene for the small ribosomal subunit 12S rRNA (corresponding to nucleotide 1490 in helix 44 of 16S rRNA, using the standard *Escherichia coli* numbering system) is typically homoplasmic with a prevalence of ~1:500 in European children and adults (4, 5) and predisposes patients to hearing loss after treatment with aminoglycosides (Fig. 1) (6, 7). The m.1555A>G mutation can also cause maternally inherited deafness (a.k.a. non-syndromic deafness) without previous exposure to antibiotics (6, 8, 9). A second mutation at m.1494C>T (nucleotide 1410 in *E. coli*) on the opposite side of helix 44 (Fig. 1) has been reported in other families with maternally inherited non-syndromic deafness (10). The equivalent nucleotides in

bacterial 16S rRNA form part of the decoding sequence where aminoglycoside antibiotics bind to perturb the accuracy of protein synthesis (Fig. 1) (11). Chimeric ribosomes displaying the 12S rRNA decoding sequence within bacterial 16S rRNA become hypersensitive to aminoglycosides when the m.1494C>T and m.1555A>G mutations are introduced (12, 13), supporting the view that the ototoxic side effects of aminoglycosides in mitochondria are directly linked to their disruption of ribosome function (12, 13).

Although the side effects of aminoglycosides are explained by their interaction on the mitoribosome, a number of pathophysiological issues remain unresolved. Given the importance of mitochondrial translation in nearly all types of mammalian cells, it is unclear why most other organs remain unaffected by these drugs. Furthermore, while sensorineural deafness is very often linked with mitochondrial diseases (14), not all carriers of the m.1555A>G mutation develop deafness (9). Possibly, nuclear genes could contribute to the deafness phenotype (8), and such roles have been suggested for the nuclear-encoded mitochondrial transcription factor B1 (TFB1M), the mitochondrial translational optimization protein (MTO1), a GTP binding protein (GTPBP3) (15) and a mitochondrial tRNA-specific modification enzyme 2-thiouridylase (MTU1) (16).

The TFB1M enzyme dimethylates the N6-positions of two adjacent adenosines (A1006 and A1007 in mouse) within the loop of helix 45 at the 3'-end of 12S rRNA (Fig. 1) (17-19). The TFB1M-directed methylations are essential for mitoribosomal biogenesis and assembly of the small subunit is impaired in their absence (17). TFB1M belongs to a family of highly conserved adenosine dimethyltransferases found in bacteria, archaea and eukaryotes (cytosol and mitochondria), and in all cases modify the adenosine nucleotides (A1518 and A1519,

E. coli numbering) at the corresponding positions in helix 45 (Fig. 1) (18-24). Loss of A1518/A1519 methylation by inactivation of the homologous enzyme RsmA (formerly KsgA) in *E. coli* perturbs the binding site of the antibiotic kasugamycin (25) to confer resistance (26, 27). The loss of RsmA methyltransferase function is advantageous for the cell only in the presence of kasugamycin and otherwise has a biological cost, probably resulting from stalling of the ribosomal subunit maturation process (28).

It has been proposed from studies on human cell lines that the two equivalent adenosines in 12S rRNA (Fig. 1) are less than stoichiometrically methylated and that their “hypermethylation” influences the pathogenicity of the m.1555A>G mutation causing impairment of mitochondrial biogenesis, decreased mitochondrial membrane potential and hypersensitivity to stress-induced apoptosis (29). Continuing in this vein, a later study claimed that overexpression of TFB1M in transgenic mice increased methylation of 12S rRNA *in vivo*, which in turn induced stress-signaling, apoptosis of critical cells in the inner ear and, ultimately, deafness (30). These reports are difficult to reconcile with another study showing that 12S rRNA adenosine dimethylation is essential for ribosome biogenesis *in vivo* (17). An independent study further showed near full methylation of 12S rRNA in human cell lines and patients, regardless of whether the m.1555A>G mutation was present or not (31). Weighing the recently proposed concept of 12S rRNA hypermethylation as a target for therapeutic intervention to treat deafness (30) against the conflicting views surrounding this important pathophysiological issue, we chose to revisit the subject and reevaluate the 12S rRNA methylation status and hearing in mice overexpressing the TFB1M methyltransferase.

RESULTS

Generation of transgenic mice overexpressing TFB1M

Mice that ubiquitously overexpressed TFB1M were created using a bacterial artificial chromosome (BAC) transgenic approach (32-34). We routinely use this approach as it is relatively insensitive to positional effects and results in low to moderate ubiquitous overexpression of nucleus-encoded mitochondrially-targeted proteins (32-34). The recombinant *Tfb1m* gene (BAC-TFB1M) and its transcript could be tracked via a silent point mutation that was introduced to abolish an *Xma I* site in exon 3 (Fig. 2A). The BAC transgenic line C7.1 exhibited ubiquitous TFB1M overexpression levels (Fig. 2B-D) similar to those in the previously published mtTFB1 transgenic mouse line (30) (Fig. 3B), and was selected for further studies. To check for unwanted effects caused by genes flanking *Tfb1m* in the BAC clone, we generated transgenic mice harbouring a version of the BAC clone (BAC-KO) in which *Tfb1m* expression was silenced by deletion of exon 3. Both the BAC-KO mice and the BAC transgenic C6.1 line, which only showed a minor increase of TFB1M mRNA expression (Fig. 2B), appeared to be completely healthy. A more marked increase in TFB1M expression was observed in the C7.1 line (Fig. 2B-D), and these transgenic mice were subjected to extensive phenotypic analyses. No changes were detected in their metabolism, cardiovascular function, lung function, eye function, grip strength or rotarod performance; similarly, haematological analyses of peripheral blood, broad spectrum clinical chemistry of plasma samples and pathology studies of various tissues showed no evidence of any change that could be related to the C7.1 genotype.

TFB1M overexpression does not alter the mtDNA copy number or the expression of respiratory chain subunits

The level of mtDNA was not altered in BAC-TFB1M (C7.1) mice (Fig. 3A), consistent with the unaltered amounts of TFAM protein in their heart tissues (Fig. 2C). TFAM is the main protein packaging mtDNA into nucleoids (35, 36) and is thus indicative of mtDNA copy number. In addition, expression of respiratory chain subunits in BAC-TFB1M-mouse heart and liver was seen to be very similar to controls on western blots (Fig. 3C). Tissue samples from the previously published Tg-mtTFB1 mice overexpressing TFB1M (30) showed levels of TFB1M protein similar to that in the BAC-TFB1M (C7.1) mice we generated (Fig. 3B). Again, expression of respiratory chain subunits was unchanged in the Tg-mtTFB1 mice relative to their littermate controls (Fig. 3C).

TFB1M overexpression does not affect 12S rRNA adenosine dimethylation

N6-dimethylation of adenosine in RNAs can be detected by several methods including primer extension analysis (37), radioisotope labelling (38, 39) or mass spectrometry (40). Due to methodological limitations and the restricted availability of pure mitochondrial RNA, primer extension remains the most practical method for analyzing mammalian systems (17, 19, 30). We thus used a primer extension assay with fluorescent, Cy5-labeled primers to quantify 12S rRNA adenosine dimethylation (Fig. 1, primer in green font).

High levels of dimethylated A1006 and/or A1007 (> 97%) were measured in 12S rRNA from liver and heart of BAC-TFB1M (C7.1) and littermate control mice (Fig. 4A and B). Similarly, samples from the previously published Tg-mtTFB1 mouse strain (30) showed, in our hands, near complete (~ 97%) 12S rRNA

dimethylation that did not differ from littermate control mice in liver (Fig. 4C) and heart (Fig. 4D). We proceeded to isolate total RNA from liver and heart also from tissue-specific homozygous TFB1M knockout mice (-/-), heterozygous TFB1M knockout mice (+/-), wild-type mice (+/+), BAC-TFB1M mice and BAC-KO mice. With the exception of the homozygous TFB1M heart knockout, there was no difference in the methylation status of 12S rRNA (Fig. 4C and D). The heart-specific TFB1M knockouts developed a progressive cardiomyopathy and, consistent with a previous study (17), by the age of ten weeks less than 40% of the 12S rRNA in the heart tissue remained dimethylated (Fig. 4D).

TFB1M overexpression does not impair hearing in mice over a range of ages

We attempted to confirm the report that the Tg-mtTFB1 mice have higher auditory brainstem response (ABR) thresholds across the entire range of frequencies at 3–6 months of age (30). We investigated hearing in BAC-TFB1M (C7.1) mice, after establishing that they overexpress TFB1M to the same extent as Tg-mtTFB1 mice (Fig. 4C and D). Male (Fig. 5A) and female (Fig. 5B) BAC-TFB1M and wild-type mice aged four months showed very similar ABR responses. However, a subtle, but statistically significant, elevation in ABR thresholds for click ABR and at 24 kHz was seen in female mice of this age (Fig. 5B). ABR measurements were therefore carried out on slightly older mice at seven and nine months, and revealed no additional difference between BAC-TFB1M and wild-type mice of either sex (Fig. 5A and B). An increase in hearing thresholds at higher frequencies was to be expected, together with a general age-related decline in hearing but, importantly, the TFB1M-genotype had no influence on this phenomenon.

DISCUSSION

We report here that 12S rRNA nucleotides A1006 and/or A1007 are $\geq 97\%$ dimethylated *in vivo* in the mouse; this level of methylation is not increased in transgenic mice overexpressing TFB1M, nor do these mice suffer from impaired hearing. In addition, we have analysed heart and liver samples from another Tg-mtTFB1-mouse strain that also overexpress TFB1M (30) and show that the 12S rRNA adenosines are likewise dimethylated to the same high levels in these tissues. Our findings thus contradict the previous claim that overexpression of TFB1M causes higher levels of methylation of 12S rRNA in transgenic mice *in vivo*, and contradict the idea that there is a causal link between TFB1M overexpression, increased 12S rRNA methylation and stress-signaling-induced deafness (30).

The necessity to resolve these contradictory reports is immediately evident and is directly connected with the huge medical problem that deafness represents, together with the concomitant consumption of research and societal resources. The suggestion that a chain of events initiated by TFB1M-directed “hypermethylation” of 12S rRNA is linked with deafness (30) would require that substoichiometrical methylation is the normal physiological state of 12S rRNA. We show here that this is clearly not the case. Furthermore, targeting TFB1M as a means of therapeutic intervention against deafness need to be re-evaluated.

An explanation for how the contradictory data have arisen is less evident. The data presented here represent the overall dimethylation status of 12S rRNA molecules, the relative ratios of methylated and non-methylated molecules (30) might be heavily influenced by a minor change in the proportion of non-methylated rRNA, a fraction ($< 3\%$) that is too small to feasibly affect the function of the collective pool of mitoribosomes. Dimethylation at the N6-position of adenosines halts the progress

of reverse transcriptase, whereas the enzyme proceeds past N6-monomethylated adenosines under the experimental conditions used here (40). In a recent study of synthetic human mitochondrial rRNA templates, it was demonstrated that dimethylation of the first adenosine encountered in the primer extension reaction (A1584, corresponding to A1007 in mouse) results in a full stop (31). Dimethylation at the second adenosine, A1583 (A1006 in mouse), would therefore be detected only if incomplete methylation at A1584 allowed reverse transcriptase to proceed this far. Studies of rRNA modification in bacteria demonstrated that both adenosines are dimethylated in concert (25, 28, 38), indicating that the primer extension assay would give a reliable estimate of the overall dimethylation status at both adenosines. Dimethylation of these adenosines is one of most conserved rRNA modifications in nature (24, 41), and the importance of the modification for ribosome biogenesis has been demonstrated in bacteria (27, 38), yeast (20), plants (42, 43) and mammals (17-19). Furthermore, adenosine dimethylation is crucial for the last steps in the ribosomal subunit maturation process in bacteria (28) and yeast (20) and occurs first when the small ribosomal subunit has been assembled. Thus, it is to be expected that there will always be a small contingent of unmethylated 12S rRNA molecules still undergoing subunit assembly, and it is unrealistic that dimethylation levels could exceed those observed here.

A small statistically significant difference in hearing was detected in females at four months of age when comparing BAC-TFAM and control mice, however, this could not be substantiated by further measurements in older ages. An age-related shift towards higher thresholds for higher frequencies occurs in older mice (44) and, furthermore, the C57BL/6J and C57BL/6N mouse strains harbour a mutation in the *Cdh23* gene that makes them more prone to age-associated hearing loss (44, 45). We

found higher ABR thresholds in the fully backcrossed C57BL/6N wild-type mice than in the partially backcrossed C57BL/6J wild-type mice (30), consistent with previously reported hearing differences between these two sub-strains of C57/BL6 mice (46). Importantly, however, we found no aggravation of age-related hearing loss in C57BL/6N mice overexpressing TFB1M. Thus, taking into account that the C57BL/6N mice we used are more susceptible to hearing loss than C57BL/6J mice (46), if any age-related increase of the ABR threshold of the severity previously reported (30) were induced by TFB1M overexpression, it would not have gone unnoticed in our present study.

MATERIAL & METHODS

Generation of transgenic animals. A bacterial artificial chromosome clone containing the *Tfb1m* gene (BAC-TFB1M) was isolated, and a silent point mutation was introduced to abolish an XmaI-endonuclease recognition site in exon 3 (Fig. 2A). The BAC-TFB1M construct was injected into pronuclei of one cell-stage mouse embryos; the DNA of founder mice was genotyped using PCR followed by XmaI digestion. Control mice were generated from a construct in which exon 3 of the *Tfb1m* gene had been deleted (BAC-KO). BAC-KO mice cannot express TFB1M, whereas all of the sequences flanking the disrupted *Tfb1m* gene remain unchanged. All BAC transgenic mice were backcrossed to C57BL/6N mice for at least eight generations. The heterozygous *Tfb1m* knockouts (+/-) and the tissue-specific knockout mice (KO) with disruption of *Tfb1m* expression in the heart were generated as previously reported (17). The animal study was performed in strict accordance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Quantitative RT-PCR. RNA was isolated with the TRIzol Reagent (Invitrogen), resuspended in nuclease-free water (Ambion) and 2 µg samples of RNA were reverse transcribed using the High Capacity cDNA kit (Applied Biosystems). Quantitative RT-PCR was performed using a TaqMan probe for TFB1M (Mm00524825_m1).

Quantification of mtDNA. Total DNA was isolated from heart by using the DNeasy Blood and Tissue kit (Qiagen), and 5 ng was used for quantitative PCR in a 7300 Real Time PCR system (Applied Biosystems) with TaqMan probes (Life Technologies) specific for the CoxI (Mm04225243_g1) and beta-actin (Mm00607939_s1).

Western blot analysis. Total proteins were extracted from mouse heart and liver, separated by SDS/PAGE and transferred to PVDF membranes (GE Healthcare). Polyclonal antibodies against mouse TFB1M and TFAM were generated in rabbits (17, 34). OXPHOS subunits (NDUFB8, SDHB, UQCRC2, MTCO1, ATP5A) were assayed with Total OXPHOS Rodent WB Antibody Cocktail from MitoSciences. Monoclonal antibodies were obtained against VDAC (MitoSciences) and tubulin (Calbiochem).

RNA isolation and primer extension. Total RNA was isolated by phenol/chloroform extraction of heart and liver tissues from transgenic mice. Samples of 4 µg RNA were analysed by primer extension as previously described (17, 37) modifying the procedure to use 1 pmol Cy5-labeled primer (5'-Cy5-ATTATTCCAAGCACAC-3') and extending with 100 µM dTTP and 100 µM ddCTP. The primer extension products were precipitated and separated in 13 % polyacrylamide/7 M urea gels. The

fluorescent signal was analysed by scanning of the gels with a Typhoon FLA9500 (GE Healthcare) instrument.

Auditory brain stem response (ABR) analysis. Mutant and control animals of each sex were analysed at the ages of 4, 7 and 9 months at the German Mouse Clinic (47, 48). Mice were anesthetized with ketamine/xylazine, transferred onto a heating blanket in the acoustic chamber and three subcutaneous needle electrodes were placed. Since the stimuli were present as free-field sounds from a loudspeaker, the head of the mouse was placed on the calibrated distance. This distance was determined by the calibration by white noise with the calibration microphone every day prior to starting the measurements. For threshold determination, clicks (0.01 ms duration) or tone pips (6, 12, 18, 24, and 30 kHz of 5 ms duration, 1 ms rise/fall time) were produced by a Tucker Davis Technologies hardware with customized software stimuli (kindly provided by Wellcome Trust Sanger Institute) over a range of intensity levels from 5-85 dB SPL in 5 dB steps. Values above the measurement limit (85db) were replaced by 100. The sound intensity threshold was chosen manually from the first appearance of the characteristic waveform. For quality control, this choice was re-checked routinely by an independent observer.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Nucleotide sequence and predicted structure of the penultimate (helix 44) and ultimate (helix 45) stem-loops at the 3'-end of the mouse mitochondrial 12S rRNA. Variant nucleotides in the highly similar human 12S rRNA sequence are shown (red boxes). The sites of nucleotide dimethylation (helix 45 loop) and nucleotide substitutions implicated in deafness (helix 44) are indicated using the arcane nucleotide numbering systems for the mouse (*Mm*, black) and human (*Hs*, red) structures, together with the standard bacterial rRNA numbers from *E. coli* (*Ec*, blue). The binding site of aminoglycosides within the decoding region of helix 44 overlaps the human nucleotides 1494 and 1555 linked with deafness, and can be seen to be at a structurally distinct location from the dimethylated adenosines (1583/1584 in human; 1006/1007 in mouse). The Cy5-labeled DNA primer (green) was used in primer extension assays to determine the methylation status of these two adenosines in mouse 12S rRNA, and hybridizes to the region shown. The *Mm* and *Hs* helix 44 structures extend a further 48 nucleotides (48n).

Figure 2. Generation of bacterial artificial chromosome transgenic mice overexpressing TFB1M. (A) Organization of the TFB1M gene. An XmaI-endonuclease recognition site in exon3 was abolished by introduction of a silent point mutation to facilitate genotyping of transgenic animals. (B) Relative mRNA expression of TFB1M in two different BAC-TFB1M mouse lines (C6.1 and C7.1, n=3 of each genotype). Error bars represent \pm SD. (C) Western blot analysis of heart extracts of wild type littermates (WT) and BAC-TFB1M (C7.1) mice at 20 weeks of age. The expression levels of TFB1M, TFAM, and VDAC (loading control) were determined. Heart extracts from tissue-specific TFB1M knockout mice (KO) at 10

weeks of age were used as a negative control. **(d)** Relative levels of TFB1M protein expression in littermate control (blue bar) and BAC-TFB1M transgenic (red bar) mice.

Figure 3. Effect of TFB1M overexpression on mitochondria. **(A)** Quantitative RT-PCR analysis of mtDNA (CoxI) to nuclear DNA (beta actin) ratios in hearts from wild type controls (WT) and BAC-TFB1M (C7.1) transgenic mice at 20 weeks of age (n=3 of each genotype). Error bars represent \pm SD. **(B)** Steady-state levels of TFB1M protein in heart (upper panel) and liver (lower panel) from heterozygous *Tfb1m* knockout (+/-), wild-type (+/+) and heterozygous BAC-TFB1M transgenic mice (+/T) and a previously published mouse model overexpressing TFB1M (Tg-mtTFB1) as analysed by immunoblotting of total protein extracts. Heart extracts from tissue-specific TFB1M knockout mice (-/-) at 10 weeks of age were used as a control. Tubulin was used as a loading control. **(C)** Steady-state levels of OXPHOS subunits as determined by immunoblotting of total protein extracts from heart (left) or liver (right) from heterozygous *Tfb1m* knockout (+/-), wild-type (+/+) and heterozygous BAC-TFB1M transgenic mice (+/T) at 20 weeks of age. Heart extracts from tissue-specific TFB1M knockout mice (-/-) at 10 weeks of age was used as a control. The following subunits were analysed: complex I (CI-NDUFB8), complex II (CII-SDHB), complex III (CIII-UQCRC2), complex IV (CIV-MTCOI) and complex V (CV-ATP5A).

Figure 4. Dimethylation of 3'-terminal loop adenosines in mitochondrial 12S rRNA from BAC-TFB1M transgenic mice.

(A) The methylation status of the mitochondrial 12S rRNA was determined by primer extension analysis of total RNA extracts from heart and liver in control (+/+) and BAC-TFB1M transgenic (+/T) mice at 20 weeks of age. (B) Relative dimethylation at the A1006 and A1007 residues of 12S rRNA in control (blue bars) and BAC-TFB1M transgenic (red bars) mice (n=3 of each genotype). Error bars represent mean \pm SD. (C) Primer extension analysis of 12S rRNA adenosine dimethylation in total RNA extracts from liver. The percentage stop at A1007 (the first nucleotide encountered by reverse transcriptase) corresponds to the degree of dimethylation at this adenosine; the percentage stop at A1006 reveals dimethylation here only in molecules where there is read-through from A1007 (and therefore no dimethylation at A1007); stopping at G1005 occurs upon ddCTP incorporation and represents the percentage of rRNA molecules without dimethylation at A1006 or A1007. The percentages below indicate primer extension stops due to adenosine dimethylation at A1007 and A1006, as well as lack of adenosine dimethylation, which is evident as transcripts reading through to G1005. Dimethylation at A1006 and/or A1007 was consistently $\geq 97\%$ in all tissue samples. (D) Primer extension analysis of 12S rRNA adenosine dimethylation in total RNA extracts from heart. Total RNA extracts from heart of homozygous TFB1M tissue-specific knockout mice (-/-) at 10 weeks of age were used as a control. The percentages below indicate primer extension stops due to adenosine dimethylation at A1007 and A1006, as well as lack of adenosine dimethylation which is evident as transcripts reading through to G1005. With the exception of the homozygous TFB1M knockout in heart (-/-), dimethylation at A1006 and/or A1007 was consistently $\geq 97\%$ in all tissue samples.

Figure 5. Normal auditory brain stem responses in BAC-TFB1M transgenic mice.

The auditory brain stem responses (ABR) of control (WT, blue lines) and BAC-TFB1M transgenic (C7.1, red lines) mice are shown. The hearing sensitivity of control and transgenic mice was assessed by measuring ABR after different auditory stimuli. Stimulus intensity thresholds were measured at different frequencies. The medians and quartiles are shown. Male (**A**) and female (**B**) mice were analysed at 4, 7, and 9 months of age. Error bars represent \pm SD.

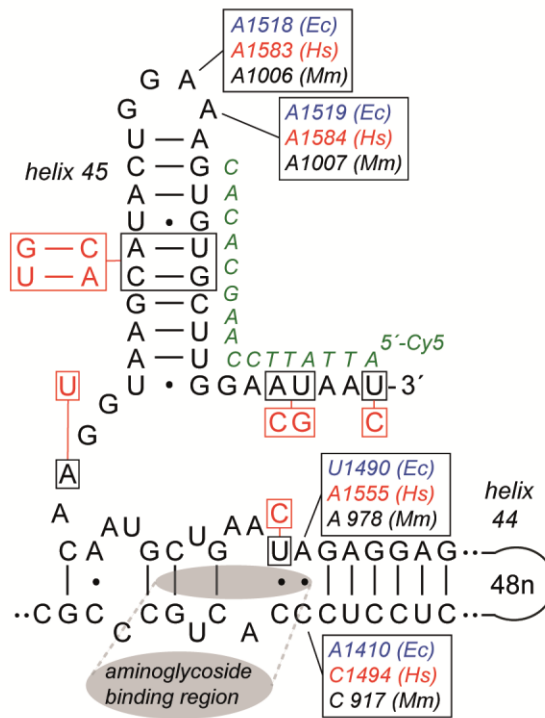


Figure 1

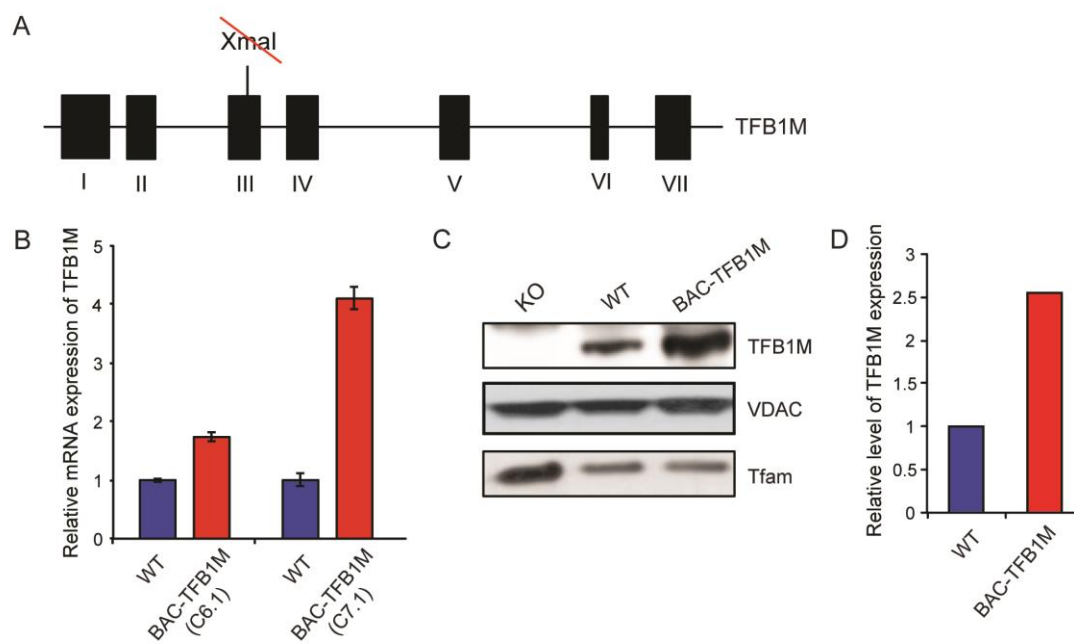


Figure 2

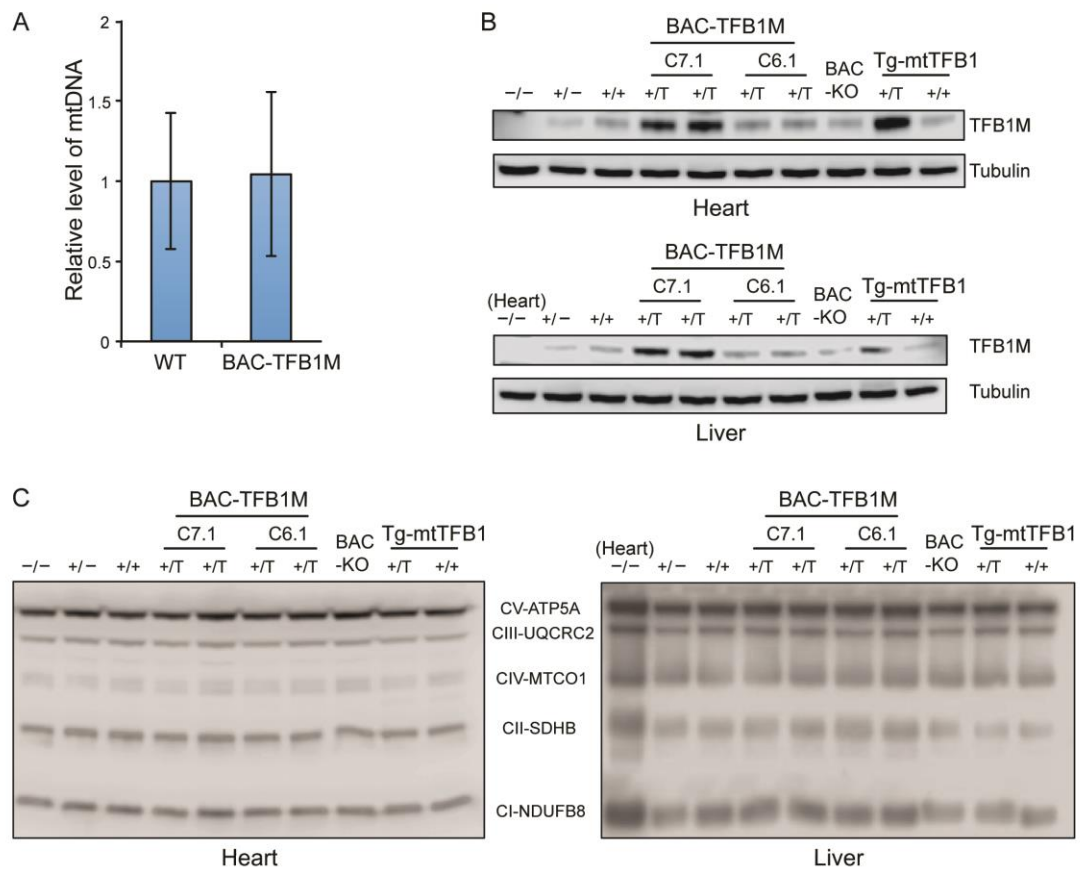


Figure 3

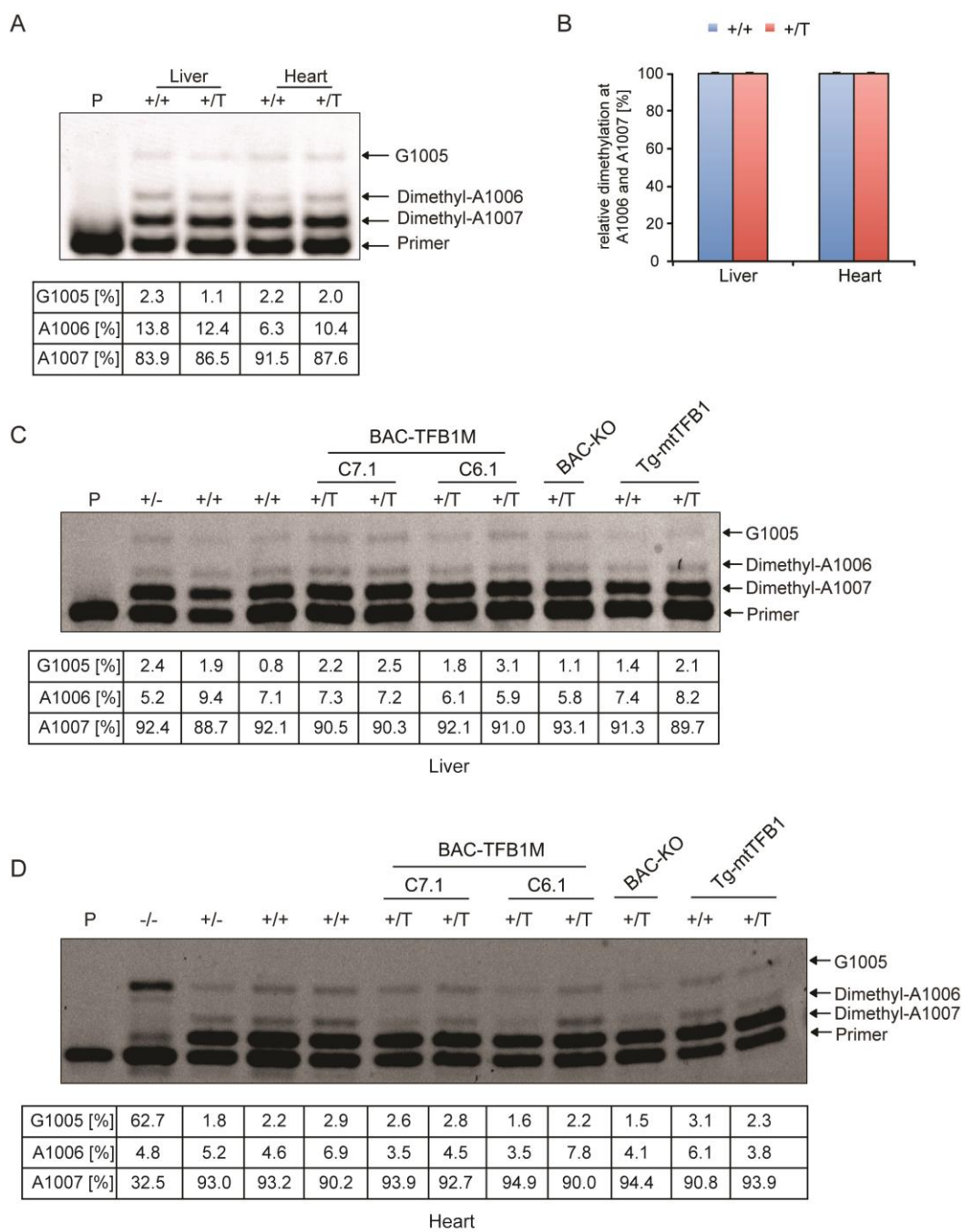


Figure 4

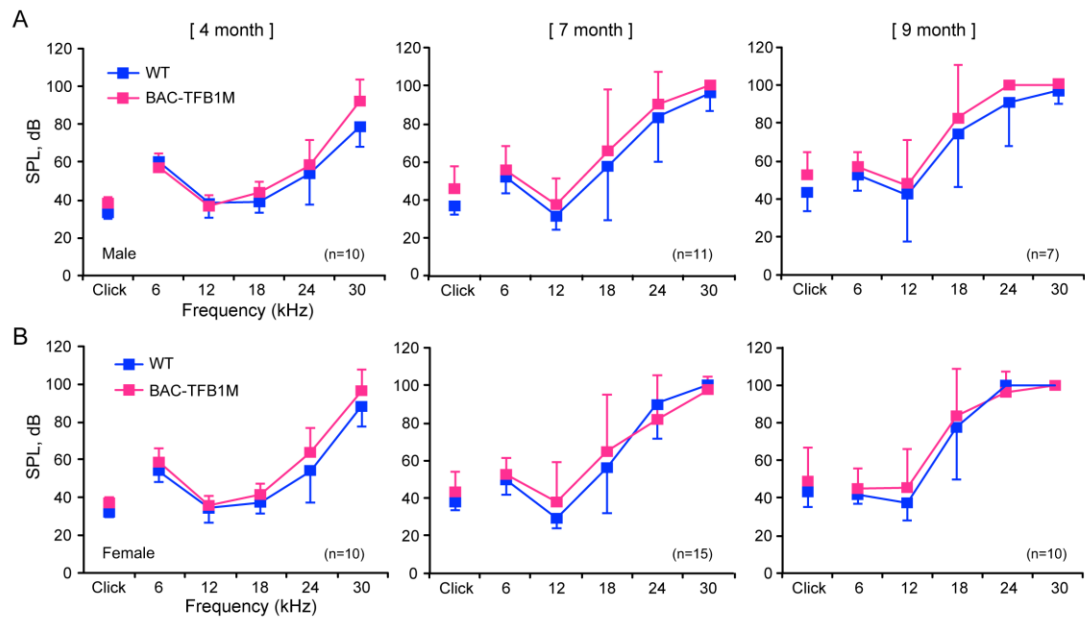


Figure 5