HHS Public Access

Author manuscript

Mamm Genome. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Mamm Genome. 2015 August ; 26(0): 338–347. doi:10.1007/s00335-015-9574-y.

A QTL on Chr 5 modifies hearing loss associated with the fascin-2 variant of DBA/2J mice

Kenneth R. Johnson[1], **Chantal M. Longo-Guess**[1], and **Leona H. Gagnon**[1]

Kenneth R. Johnson: ken.Johnson@jax.org

[1]The Jackson Laboratory, Bar Harbor, ME 04609, USA

Abstract

Inbred mouse strains serve as important models for human presbycusis or age-related hearing loss. We previously mapped a locus (*ahl8*) contributing to the progressive hearing loss of DBA/2J (D2) mice and later showed that a missense variant of the *Fscn2* gene, unique to the D2 inbred strain, was responsible for the *ahl8* effect. Although *ahl8* can explain much of the hearing loss difference between C57BL/6J (B6) and D2 strain mice, other loci also contribute. Here, we present results of our linkage analyses to map quantitative trait loci (QTLs) that modify the severity of hearing loss associated with the D2 strain $Fscn2^{ahl8}$ allele. We searched for modifier loci by analyzing 31 BXD recombinant inbred (RI) lines fixed for the predisposing D2-derived $Fscn2^{ahl8/ahl8}$ genotype and found a statistically significant linkage association of threshold means with a QTL on Chr 5, which we designated *M5ahl8*. The highest association (LOD 4.6) was with markers at the 84–90 Mb position of Chr 5, which could explain about 46 % of the among-RI strain variation in auditory brainstem response (ABR) threshold means. The semidominant nature of the modifying effect of *M5ahl8* on the $Fscn2^{ahl8/ahl8}$ phenotype was demonstrated by analysis of a backcross involving D2 and B6.D2-Chr11D/LusJ strain mice. The Chr 5 map position of *M5ahl8* and the D2 origin of its susceptibility allele correspond to *Tmc1m4*, a previously reported QTL that modifies outer hair cell degeneration in $Tmc1^{Bth}$ mutant mice, suggesting that *M5ahl8* and *Tmc1m4* may represent the same gene affecting maintenance of stereocilia structure and function during aging.

Introduction

Inbred strains of mice vary widely in onset time and severity of age-related hearing loss (AHL), making strain background an important consideration when assessing the effects of a mutation on hearing. Like human presbycusis, the progressive hearing loss exhibited by inbred strains is non-syndromic and due to the effects of multiple genes with hypomorphic and low penetrant alleles that are manifested in a quantitative rather than qualitative manner (Noben-Trauth and Johnson 2009). More than 15 quantitative trait loci (QTLs) have been mapped that contribute to progressive hearing loss in laboratory mouse strains (Drayton and Noben-Trauth 2006; Johnson et al. 2008; Johnson and Zheng 2002; Johnson et al. 2000; Keller et al. 2011; Keller and Noben-Trauth 2012; Latoche et al. 2011; Mashimo et al. 2006; Nagtegaal et al. 2012; Nemoto et al. 2004; Noben-Trauth et al. 2010; Zheng et al. 2009), and

Correspondence to: Kenneth R. Johnson, ken.Johnson@jax.org.

four underlying genes have been identified: *Cdh23* (Noben-Trauth et al. 2003), *Gipc3* (Charizopoulou et al. 2011), *Cs* (Johnson et al. 2012), and *Fscn2* (Shin et al. 2010).

Although the DBA/2J (D2) and C57BL/6J (B6) strains share the same AHL-predisposing *Cdh23* allele (Noben-Trauth et al. 2003), D2 mice exhibit a much earlier onset and more rapid progression of hearing loss than the B6 mice (Zheng et al. 1999). Scanning electron microscopic analysis has shown that hair cell stereocilia abnormalities and degeneration are likely responsible for the rapid hearing loss of D2 mice (Perrin et al. 2013; Shin et al. 2010). A locus contributing to much of the hearing loss difference between these two strains was mapped to distal Chr 11 and designated *ahl8* (Johnson et al. 2008). A missense variant of the *Fscn2* gene, unique to the D2 strain, was later shown to underlie *ahl8*-related hearing loss (Shin et al. 2010). Although the *Fscn2* genotype is a large contributor, it does not account for all of the ABR threshold variation seen in admixtures of D2 and B6 strain mice. Here, we describe our efforts to further dissect the genetic basis of the hearing loss differences between these two strains by analyses of derivative congenic strains, recombinant inbred strains, and a linkage backcross. We identified a QTL on Chr 5 with a statistically significant effect on hearing thresholds, which we designate *M5ahl8* because of its modifying effect on the severity of hearing loss associated with the $Fscn2^{ahl8}$ variant of D2 mice Table 1.

The analysis of modifier genes in mice is a powerful approach for identifying pathways of gene interactions and gaining insight into mechanisms of pathology, which may suggest novel therapeutic interventions in human genetic diseases (Friedman et al. 2000; Nadeau 2001). Although modifier genes are frequent in human disease, allelic heterogeneity and environmental variation obscure their effects in small cohort sizes, and the inability for genetic manipulation limits systematic analysis, increasing the potential value of animal models (Hamilton and Yu 2012). Genetic modifiers can be detected in mice by looking for strain background differences in inheritance or phenotype of a mutation and then mapped by analyses of appropriate linkage crosses, recombinant inbred strains, and congenic lines (Johnson et al. 2006). This approach has been used successfully to identify several strain-specific modifiers of hearing in mice, including modifiers of hearing impairment caused by mutations of *Atp2b2* (Noben-Trauth et al. 1997), *Tub* (Ikeda et al. 1999), *Tmc1* (Noguchi et al. 2006), *Eya1* (Niu et al. 2008), and *Pou1f1* (Fang et al. 2011). Molecular identification of the gene responsible for *M5ahl8* will add to the list of strain-related hearing modifiers in mice and provide insight into *Fscn2*-related hearing loss.

Materials and methods

Mice

The DBA/2J and C57BL/6J inbred strains, the B6.D2-11D/LusJ, B6.D2-$Fscn2^{ahl8}$/4Kjn, and D2.B6-$Fscn2^{+}$/Kjn congenic strains, and the BXD recombinant inbred strains examined in this study originated from The Jackson Laboratory (http://www.jax.org/). The B6.D2-11D/LusJ strain, developed and characterized by other investigators (Davis et al. 2005), is no longer available from The Jackson Laboratory. Experimental mice were housed in the Research Animal Facility of The Jackson Laboratory, and all procedures involving their use were approved by the Institutional Animal Care and Use Committee. The Jackson

Laboratory is accredited by the American Association for the Accreditation of Laboratory Animal Care.

Genetic characterization of B6.D2-*Fscn2*^{ahl8}/4Kjn and D2.B6-*Fscn2*$^{+}$/Kjn congenic strains

We developed the D2.B6-*Fscn2*$^{+}$ and B6.D2-*Fscn2*^{ahl8} reciprocal congenic strains by backcross introgression of B6- and D2-derived segments of Chromosome 11 (containing the *Fscn2* locus) into the genomes of D2 and B6, respectively, as previously described: D2.B6-*Fscn2*$^{+}$ (Shin et al. 2010) and B6.D2-*Fscn2*^{ahl8} (Perrin et al. 2013). To define the extent of the congenic regions in each homozygous congenic strain, multiple markers along the length of Chromosome 11 were genotyped. The approximately 3 Mb B6-derived congenic region of D2.B6-*Fsc-n2*$^{+}$ extends from a point distal to *D11Mit104* (119.3 Mb) to the distal-most end of Chr 11, including *rs3726373* (120.2 Mb), *Fscn2* (120.4 Mb), and *rs29441064* (120.9 Mb). The approximately 5 Mb D2-derived congenic region of B6.D2-*Fscn2*^{ah8} extends from a point distal to *D11Mit203* (116.4 Mb) to the distal-most end of Chr 11, including *D11Mit104* (119.3 Mb), *Fscn2* (120.4 Mb), and *rs29441064* (120.9 Mb).

Hearing assessment by auditory-evoked brainstem response (ABR)

Hearing in mice was assessed by ABR threshold analysis, as previously described (Zheng et al. 1999). Mice were anesthetized with an intraperitoneal injection of tribromoethanol (0.2 ml of 12.5 mg/ml stock per 10 g of body weight), and then placed on a heating pad in a sound-attenuating chamber. Needle electrodes were placed just under the skin, with the active electrode placed between the ears just above the vertex of the skull, the ground electrode between the eyes, and the reference electrode underneath the left ear. High-frequency transducers were placed just inside the ear canal, and computer-generated sound stimuli were presented at defined intervals. Thresholds were determined for 8, 16, and 32 kHz pure-tone stimuli by increasing the sound pressure level (SPL) in 10-dB increments followed by 5-dB increases and decreases to determine the lowest level at which a distinct ABR wave pattern could be recognized. Stimulus presentation and data acquisition were performed using the Smart EP evoked potential system (Intelligent Hearing Systems, Miami, FL).

Genotyping mice

Genomic DNA for genotyping mice was rapidly prepared from tail tips by hot sodium hydroxide and Tris (Hot SHOT) procedure (Truett et al. 2000). Individual DNA samples from linkage mice were genotyped for multiple microsatellite markers and SNPs located on Chr 5. PCR was performed in a Bio-Rad PTC-200 Peltier Thermal Cycler. Amplification consisted of an initial denaturation at 97 °C for 30 s followed by 40 cycles, each consisting of 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 30 s for the first cycle, and then increasing by 1 s for each succeeding cycle (extension). After the 40 cycles, the product was incubated for an additional 10 min at 72 °C (final extension). PCR products were separated on 2.5 % SeaKem gels (Lonza, Rockland, ME) and visualized by ethidium bromide staining.

The region of the *Fscn2* gene that is associated with *ahl8* in the DBA/2J inbred strain (Shin et al. 2010) was amplified by PCR from the genomic DNA of each of the BXD strains. To

genotype *Fscn2*, we used a previously described single nucleotide polymorphism (SNP) genotyping method based on PCR amplification and *Eag*I restriction enzyme digestion (Shin et al. 2010), which alleviated the need for DNA sequencing.

Genetic mapping and statistical analysis

The online WebQTL mapping program (http://www.genenetwork.org/home.html) was used to analyze linkage associations of the mean ABR thresholds of 31 BXD RI strains with hundreds of previously typed SNP and microsatellite markers. Empirical estimates of the genome-wide significance of association scores were computed by performing 2000 permutations (Churchill and Doerge 1994). A significant association is defined as a statistical value that is expected to occur at random 0.05 times or less in a genome-wide scan for linkage (Lander and Kruglyak 1995). Further ABR data analysis of the BXD strains was performed using the JMP 7.0 interactive statistics and graphics software program (www.JMP.com). One-way ANOVA was used to determine the statistical significance (F-ratio probability) of *M5ahl8* genotypes on ABR thresholds and to estimate the percentage of among-strain ABR threshold variation that could be attributed to the *M5ahl8* locus (adjusted R-square statistic).

Individual DNA samples from linkage backcross mice were genotyped for 120 SNP markers distributed throughout the genome. SNP genotyping was outsourced to LGC Genomics (http://www.lgcgroup.com/our-science/genomics-solutions/). ABR thresholds for 8, 16, and 32 kHz stimuli were evaluated as quantitative traits, and the MapManager QTXb20 program of least-squares linear regression (Manly et al. 2001) was used to analyze linkage and localize marker associations with ABR thresholds.

Results

Reciprocal congenic strains

We previously reported on a D2.B6-*Fscn2*$^{+}$ congenic line with a hearing loss profile at 2 months of age that was much less severe than that of the D2 recipient strain and very similar to that of the B6 donor strain, indicating that the B6-derived *Fscn2* genotype has a large effect on ameliorating the hearing loss of mice on an otherwise D2 background (Shin et al. 2010). Here, we report on our analysis of B6.D2-Chr 11D/LusJ (Davis et al. 2005), a B6 congenic strain with D2 donor strain alleles at the distal third of Chr 11, including the *Fscn2*^{ahl8} allele, that contributes to the hearing loss of D2 mice. In contrast to the large hearing loss difference between the D2.B6-*Fscn2*$^{+}$ congenic strain and the D2 parental host strain, the hearing loss profile of the B6.D2- Chr 11D/LusJ congenic strain was similar to that of the B6 parental host strain and much less severe than that of the D2 donor strain (Fig. 1), indicating that the D2-derived *Fscn2* genotype (*ahl8/ahl8*) has little effect on hearing in the context of the B6 genome at the 2-month test age. However, when B6.D2- Chr 11D/LusJ congenic mice were mated with D2 mice, the F1 hybrid progeny exhibited a severe hearing impairment at 2 months of age (Fig. 1). Because the congenic mice and F1 hybrids have the same *Fscn2*$^{ahl8/ahl8}$ genotype, the striking difference in their ABR thresholds indicates the influence of modifier genes, in which heterozygosity for D2 alleles outside of the congenc region worsens hearing loss in the *Fscn2*$^{ahl8/ahl8}$ F1 hybrids.

Because the D2-derived Chr 11 congenic region of B6.D2- Chr 11D/LusJ is so large—about 40 Mb (Davis et al. 2005)—we produced four subcongenic lines to refine the location of *ahl8* (Fig. 2). To accentuate the effect of *ahl8*, mice from each subcongenic line were mated with D2 mice and the resulting F1 hybrids were tested for ABR thresholds at 2 and 4 months of age. F1 hybrid mice derived from Line 1 and Line 4 exhibited threshold elevations of about 20 dB at 2 months of age and about 60 dB at 4 months of age, compared with the normal thresholds of Line 2 and Line 3, indicating that these two lines contained the *ahl8* locus (Fig. 2). Because Line 4 contained the smallest D2-derived segment (~5 Mb), it was chosen for development and distribution as the B6.D2-*Fscn2*^{ahl8}/4Kjn (Stock #009629) congenic strain, hence designated B6.D2-*Fscn2*^{ahl8}. Hearing loss progression in this congenic strain has been previously analyzed (Perrin et al. 2013), but here we present a hearing loss profile of mice from this strain at 3 and 6 months of age that is directly comparable (same test ages, same laboratory) with those of the reciprocal D2.B6-*Fscn2*$^{+}$/Kjn (Stock #12438) congenic strain, here designated D2.B6-*Fscn2*$^{+}$, and the B6 and D2 parental strains (Fig. 3). At 3 months of age, mice of both congenic strains had 8 and 16 kHz ABR thresholds similar to those of B6 mice and 32 kHz thresholds similar to those of D2. At 6 months of age, the 8 kHz thresholds of the D2.B6-*Fscn2*$^{+}$ congenic strain mice were similar to those of the B6 parental strain, whereas the 8 kHz thresholds of the B6.D2-*Fscn2*^{ahl8} congenic strain mice were intermediate to those of the parental strains, the 16 kHz thresholds of both congenic strains were intermediate to those of the parental strains, and all strains had highly elevated 32 kHz thresholds. Clearly, the introgressed regions containing *Fscn2* have an effect on hearing loss in both congenic strains, although the effect is seen at earlier ages in the D2.B6-*Fscn2*$^{+}$ congenic strain than in the B6.D2-*Fscn2*^{ahl8} congenic strain.

Mapping hearing modifiers with BXD recombinant inbred strains

As a first approach to map loci that modify *ahl8*-related hearing loss, we took advantage of the existing BXD recombinant inbred (RI) strains. We obtained mice from the Jackson Laboratory's Genetic Resources Repository: 5–8 retired breeders (7.5–9 months of age) from each of the 31 BXD RI lines that are homozygous for the D2 *Fscn2* allele. Thus, variation among these RI strains cannot be attributed to *Fscn2* variation. Considerable threshold variation was observed among the RI strains (Fig. 4), suggesting that other genetic factors modify the hearing loss effect of the D2 *Fscn2* variant. For the 8 kHz and 16 kHz stimuli, a high proportion of the total ABR threshold variation was explained by among-strain variance (ANOVA adjusted R-square values of 0.68, and 0.55, respectively), indicating a strong genetic component. Because of the old age of the mice when tested, the high frequency 32 kHz ABR thresholds were uniformly high in mice of all RI strains (adjusted R-square = 0.13) and therefore not useful for mapping.

We used the WebQTL mapping program of the GeneNetwork website (http://www.genenetwork.org/home.html) to perform QTL mapping analysis, treating 8 and 16 kHz ABR thresholds as quantitative traits. The set of BXD RI lines has been genotyped for over 750 markers, providing a high marker density for phenotype linkage associations (Williams et al. 2001). A genome-wide scan for linkage associations revealed a single genomic region (on Chr 5) with statistically significant LOD scores (Fig. 5a). The peak association for 8 kHz

threshold associations was with the SNP markers rs13478357 and rs13478361 (LOD 4.6) at the 84 Mb position of Chr 5, with the 1.5-LOD confidence interval extending from 80 to 92 Mb, and the more conservative 3-LOD confidence interval from 74 to 116 Mb (Fig. 5b). Genotypes of markers at the peak association can explain 46 % (adjusted R-square value) of the among-strain threshold variation. Analyses of 16 kHz thresholds gave similar results. We named this QTL *M5ahl8* to indicate that it is on Chr 5 and modifies *ahl8*-related hearing loss. Average 8 and 16 kHz ABR thresholds of RI lines with DBA/2J alleles at the *M5ahl8* locus are statistically significantly higher than those of RI lines with C57BL/6J alleles at this locus: mean elevations of 18 dB for 8 kHz thresholds and 13 dB for 16 kHz thresholds (Fig. 5c).

M5ahl8 effects on hearing confirmed with a linkage backcross

To test the effect of the *M5ahl8* QTL on ABR thresholds in another population of mice segregating B6 and D2 alleles but at a younger age and with different genotypes, we analyzed a backcross involving the B6.D2-Chr 11/Lus congenic strain and D2 mice. We mated congenic strain mice with D2 mice and then backcrossed the F1 hybrids to the congenic strain mice. We use the notation (B6.D2-Chr11D × D2) × B6.D2-Chr11D to represent this back-cross. All loci in N2 progeny from this cross will be either homozygous for B6 alleles (BB) or heterozygous for B6 and D2 alleles (BD) except for loci in the distal Chr 11 congenic region (containing *ahl8*), which is homozygous for D2 alleles (DD). 180 N2 progeny from the (B6.D2-Chr11D × D2) × B6.D2-Chr11D backcross mice (all having *ahl8/ahl8* genotypes) were tested for ABR thresholds at 12 weeks of age. The numbers of backcross mice in each of seven 16 kHz threshold categories show a skewed and slightly bimodal frequency distribution (Fig. 6a), with a large peak centered around normal thresholds (10–20 dB SPL) and a second minor peak centered around 55–60 dB SPL. This type of frequency distribution suggests that the modifier effect on hearing is caused by multiple loci with small effects rather than few loci with large effects.

We undertook a genome-wide screen for linkage of ABR thresholds with 120 SNP markers located throughout the genome. Out of a total of 180 N2 backcross mice produced, we chose 43 mice with the lowest thresholds and 42 mice with the highest thresholds for linkage analysis. SNP rs3023047 (92 Mb position on Chr 5) showed a statistically significant linkage association with 16 kHz ABR thresholds (LOD = 3.3), but not with 8 kHz (LOD = 1.9) or 32 kHz thresholds (LOD < 1.5) thresholds. No other marker loci in the genome screen showed significant linkage with ABR thresholds. Because SNP rs3023047 was within the *M5ahl8* region defined by the BXD RI analysis, we used it as a marker for *M5ahl8* to analyze the effect of this locus on the ABR thresholds of N2 backcross mice. The average 16 kHz threshold of N2 mice with BD genotypes was about 15 dB higher than that of mice with BB genotypes (Fig. 6b). That linkage analysis of RI strain mice and backcross mice both detected a significant linkage association of ABR thresholds with the *M5ahl8* QTL provides strong evidence for the legitimacy this locus in modifying $Fscn2^{ahl8}$-related hearing loss. The backcross results show a modifying effect of the heterozygous BD genotype on hearing loss in $Fscn2^{ahl8/ahl8}$ mice that is not as strong as that of the DD genotype of RI strain mice, suggesting the the D2-derived *M5ahl8* allele acts in a semidominant fashion.

Discussion

Auditory function is a highly complex trait requiring the proper functioning and interactions of hundreds of genes. Hearing loss caused by disruptions of major genes often can be easily detected; however, the degree of hearing loss can be quite variable depending on genetic background. Identification of interacting genes or modifiers can provide much insight into the underlying pathways of auditory pathology, and mice offer advantages for such studies (Hamilton and Yu 2012; Nadeau 2001). Here, we report on our mapping of a QTL (*M5ahl8*) that modifies the progressive hearing loss associated with a D2-specific variant of FSCN2 by analyzing phenotypic variation in admixtures of B6 and D2 strain mice.

The correspondence of mapping results from RI lines and backcross mice provide confirmation that the *M5ahl8* QTL lies within the 74–116 Mb region of Chr 5 and has a significant modifying effect on the hearing loss caused by $Fscn2^{ahl8}$ homozygosity. A previously reported modifier QTL (*Tmc1m4*) that affects outer hair cell degeneration in Beethoven ($Tmc1^{Bth/+}$) mutant mice was mapped to the same region of Chr 5 between *D5Mit394* (54.3 Mb) and *D5Mit161* (126.9 Mb) and, like *M5ahl8*, involves a susceptibility allele derived from the D2 strain (Noguchi et al. 2006). *Tmc1m4* was mapped by analysis of $Tmc1^{Bth/+}$ N2 progeny from a (C3H-$Tmc1^{Bth}$ × D2) F1 hybrid × C3H backcross. Like D2-$Fscn2^{ahl8/ahl8}$ mice (Perrin et al. 2013; Shin et al. 2010), $Tmc1^{Bth/+}$ mice exhibit a progressive outer hair cell degeneration, which is worsened by a single copy of the D2-derived Chr 5 modifier allele. D2-derived alleles of both *M5ahl8* and *Tmc1m4* worsen the degree of outer hair cell degeneration, with more severe degradation in the basal turn, consistent with the tonotopic organization of the cochlea and an initial onset of high-frequency hearing loss. These similarities suggest that *M5ahl8* and *Tmc1m4* may represent the same D2-derived hearing loss modifier gene.

Both FSCN2 and TMC1 are components of the hair cell stereocilia and, therefore, it is reasonable that the same gene could modify the hair cell loss phenotypes of both *Fscn2* and *Tmc1*. Beta actin (ACTB1), another component of hair cell stereocilia, was shown to cooperate with FSCN2 to maintain stereocilia length, and defects of both proteins were shown to cause a more severe hearing loss than single mutants, suggesting a common stereocilia maintenance pathway (Perrin et al. 2013). A genetic factor such as *M5ahl8/Tmc1m4* may be involved in the same pathway and could modify the deleterious effects associated with defects in other pathway components (such as FSCN2 and TMC1). D2 mice also have a deleterious variant of CDH23 (Noben-Trauth et al. 2003) that is epistatic to the effects of the FSCN2 variant (Johnson et al. 2008). CDH23 is a component of the stereocilia tip link, supporting the view that the polygenic nature of the progressive hearing loss of D2 mice is at least partially due to deficiencies in multiple stereocilia components conferring functional integrity. The increased need to repair functionally compromised stereocilia could lead to oxidative stress and hair cell apoptosis.

A recently reported linkage study of (DBA/2J × C57BL/6J) F1 × DBA/2J backcross mice identified three QTL likelihood peaks in the 53–126 Mb region of Chr 5 that are associated with hearing loss susceptibility in DBA/2J mice (Suzuki et al. 2015); one of the peaks overlaps the *M5ahl8* QTL map position and may represent the same D2 gene variant. While

our study focused on mapping loci that modify the hearing loss of *Fscn2*$^{ahl8/ahl8}$ homozygous mice, the results recently reported by Suzuki et al. (2015) suggest that the modifier locus we identified on Chr 5 may also affect the hearing loss of *Fscn2*$^{+/ahl8}$ heterozygotes.

Age-related hearing loss 2 (*ahl2*) is another hearing-related QTL mapping to the *M5ahl8* region on Chr 5 (Johnson and Zheng 2002). The genetic map position of *ahl2* overlaps with the *M5ahl8* QTL, but because *ahl2* was derived from the NOD strain and *M5ahl8* from the D2 strain, they probably are not the same ancestral gene variant (NOD and D2 are not closely related lineages). They could, however, be independently arising deleterious alleles of the same gene or variants of linked genes within the candidate region.

The large differences in ABR thresholds between B6.D2-Chr 11D/LusJ congenic strain mice and (B6.D2-Chr 11D/LusJ × D2) F1 hybrids (Fig. 1) indicated that heterozygous expressions of D2-derived alleles at modifier loci worsen the hearing loss of mice with the *ahl8/ahl8* genotype. We sought to genetically map these modifier loci by analysis of (B6.D2-Chr 11D/LusJ × D2) F1 × B6.D2-Chr 11D/LusJ backcross mice, which were homozygous for D2 alleles in genes at the distal region of Chr 11 (obligate *ahl8/ah8* genotype) but had either B6/B6 (BB) or B6/D2 (BD) genotypes in genes at other regions of the genome. The ABR frequency distribution among progeny from this backcross indicated that the modifier effect of the D2-derived alleles was polygenic in origin, with no single locus having a large effect (Fig. 6). If a single locus was primarily responsible for the modifier effect, then a strongly bimodal threshold frequency distribution would be expected among the backcross mice. Instead we found that the locus with the largest effect (*M5ahl8*) could account for only 16 % (LOD 3.3) of the variation in 16 kHz thresholds among the backcross mice. In contrast, *M5ahl8* could account for 46 % (LOD 4.6) of the variation in 8 and 16 kHz thresholds among the BXD RI strains, possibly because the homozygous DD genotype of the RI strain mice has a greater effect on hearing loss susceptibility than does the heterozygous BD genotype of the backcross mice. An intercross mapping strategy, which could also distinguish semidominant from dominant effects of the alleles, may have shown a stronger effect than the backcross.

Although several genes with mutations that affect hearing are located within the 3-LOD confidence interval of *M5ahl8* (74–116 Mb region of Chr 5), including *Kit, Scarb2, Gfi1, Cplx1, P2rx2,* and *Trpr4*, none contain SNP variants that differ between B6 and D2 and that are predicted to cause missense or splice site mutations. We did, however, find three genes with SNPs fitting these criteria that have been shown by Unigene EST profiles to be expressed in the inner ear: ankyrin repeat domain 17 (*Ankrd17*, 90.3 Mb), SDA1 domain containing 1 (*Sdad1*, 92.3 Mb), and matrix extracellular phosphoglycoprotein (*Mepe*, 104.3 Mb). *Ankrd17* is widely expressed in many tissues including the inner ear. Only a single SNP variant of the *Ankrd17* gene differs between B6 and D2 and is predicted to cause a missense mutation. Five *Sdad1*-encoding ESTs from the inner ear (one from adult and four from otocyst) are listed in Unigene. Four SNP variants in the *Sdad1* gene differ between C57BL/6J and DBA/2J and are predicted to cause missense mutations. Although *Mepe* is thought to be involved in bone mineralization, the *Mepe* gene shows a high and nearly exclusive expression in the inner ear (8 out of 9 Unigene entries), and there are 27 SNP

variants in *Mepe* that differ between B6 and D2 and that are predicted to cause missense mutations. Other genes with undiscovered inner ear expression or with B6-D2 DNA differences that occur outside of coding regions must also be considered as possible candidates for the *M5ahl8* phenotype.

New resources such as the complete genome sequences of multiple inbred mouse strains (http://www.sanger.ac.uk/resources/mouse/genomes/) and new tools such as CRISPR/Cas9 for rapid candidate gene validation (Inui et al. 2014) should help identify the genes underlying modifier QTLs that have been detected by genetic mapping but not molecularly defined. Identification of the gene responsible for the *M5ahl8* QTL will provide insight into the molecular pathways underlying $Fscn2^{ahl8}$-related progressive hearing loss and identify genetic interactions and molecular pathways that contribute to AHL in mice, which could aid in the discovery or validation of human candidate genes that may contribute to presbycusis.

Acknowledgments

We thank Sandra Gray for her skilled mouse colony management and assistance in the development of the congenic strains. We also thank personnel of the Jackson Laboratory's Genetic Resource Science Repository for providing us with retired breeder mice from the BXD RI strains. This research was supported by R01 Grant DC005827 (KRJ) from the National Institutes of Health (NIH) and National Institute on Deafness and Other Communication Disorders (NIDCD). The Jackson Laboratory institutional shared services are supported by NIH National Cancer Institute Support Grant CA34196.

References

Charizopoulou N, Lelli A, Schraders M, Ray K, Hildebrand MS, Ramesh A, Srisailapathy CR, Oostrik J, Admiraal RJ, Neely HR, Latoche JR, Smith RJ, Northup JK, Kremer H, Holt JR, Noben-Trauth K. Gipc3 mutations associated with audiogenic seizures and sensorineural hearing loss in mouse and human. Nat Commun. 2011; 2:201. [PubMed: 21326233]

Churchill GA, Doerge RW. Empirical threshold values for quantitative trait mapping. Genetics. 1994; 138:963–971. [PubMed: 7851788]

Davis RC, Schadt EE, Smith DJ, Hsieh EW, Cervino AC, van Nas A, Rosales M, Doss S, Meng H, Allayee H, Lusis AJ. A genome-wide set of congenic mouse strains derived from DBA/2J on a C57BL/6J background. Genomics. 2005; 86:259–270. [PubMed: 16039824]

Drayton M, Noben-Trauth K. Mapping quantitative trait loci for hearing loss in Black Swiss mice. Hear Res. 2006; 212:128–139. [PubMed: 16426780]

Fang Q, Longo-Guess C, Gagnon LH, Mortensen AH, Dolan DF, Camper SA, Johnson KR. A modifier gene alleviates hypothyroidism-induced hearing impairment in pou1f1dw dwarf mice. Genetics. 2011; 189:665–673. [PubMed: 21840860]

Friedman T, Battey J, Kachar B, Riazuddin S, Noben-Trauth K, Griffith A, Wilcox E. Modifier genes of hereditary hearing loss. Curr Opin Neurobiol. 2000; 10:487–493. [PubMed: 10981618]

Hamilton BA, Yu BD. Modifier genes and the plasticity of genetic networks in mice. PLoS Genet. 2012; 8:e1002644. [PubMed: 22511884]

Ikeda A, Zheng QY, Rosenstiel P, Maddatu T, Zuberi AR, Roopenian DC, North MA, Naggert JK, Johnson KR, Nishina PM. Genetic modification of hearing in tubby mice: evidence for the existence of a major gene (moth1) which protects tubby mice from hearing loss. Hum Mol Genet. 1999; 8:1761–1767. [PubMed: 10441341]

Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, Yamashita S, Asahara H, Fukami M, Takada S. Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. Sci Rep. 2014; 4:5396. [PubMed: 24953798]

Johnson KR, Zheng QY. Ahl2, a second locus affecting age-related hearing loss in mice. Genomics. 2002; 80:461–464. [PubMed: 12408962]

Johnson KR, Zheng QY, Erway LC. A major gene affecting age-related hearing loss is common to at least ten inbred strains of mice. Genomics. 2000; 70:171–180. [PubMed: 11112345]

Johnson KR, Zheng QY, Noben-Trauth K. Strain background effects and genetic modifiers of hearing in mice. Brain Res. 2006; 1091:79–88. [PubMed: 16579977]

Johnson KR, Longo-Guess C, Gagnon LH, Yu H, Zheng QY. A locus on distal chromosome 11 (ahl8) and its interaction with Cdh23(ahl) underlie the early onset, age-related hearing loss of DBA/2J mice. Genomics. 2008; 92:219–225. [PubMed: 18662770]

Johnson KR, Gagnon LH, Longo-Guess C, Kane KL. Association of a citrate synthase missense mutation with age-related hearing loss in A/J mice. Neurobiol Aging. 2012; 33:1720–1729. [PubMed: 21803452]

Keller JM, Noben-Trauth K. Genome-wide linkage analyses identify Hfhl1 and Hfhl3 with frequency-specific effects on the hearing spectrum of NIH Swiss mice. BMC Genet. 2012; 13:32. [PubMed: 22540152]

Keller JM, Neely HR, Latoche JR, Noben-Trauth K. High-frequency sensorineural hearing loss and its underlying genetics (Hfhl1 and Hfhl2) in NIH Swiss mice. J Assoc Res Otolaryngol. 2011; 12:617–631. [PubMed: 21594677]

Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet. 1995; 11:241–247. [PubMed: 7581446]

Latoche JR, Neely HR, Noben-Trauth K. Polygenic inheritance of sensorineural hearing loss (Snhl2, -3, and -4) and organ of Corti patterning defect in the ALR/LtJ mouse strain. Hear Res. 2011; 275:150–159. [PubMed: 21185929]

Manly KF, Cudmore RH Jr, Meer JM. Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome. 2001; 12:930–932. [PubMed: 11707780]

Mashimo T, Erven AE, Spiden SL, Guenet JL, Steel KP. Two quantitative trait loci affecting progressive hearing loss in 101/H mice. Mamm Genome. 2006; 17:841–850. [PubMed: 16897347]

Nadeau JH. Modifier genes in mice and humans. Nat Rev Genet. 2001; 2:165–174. [PubMed: 11256068]

Nagtegaal AP, Spijker S, Crins TT, Borst JG. A novel QTL underlying early-onset, low-frequency hearing loss in BXD recombinant inbred strains. Genes Brain Behav. 2012; 11:911–920. [PubMed: 22989164]

Nemoto M, Morita Y, Mishima Y, Takahashi S, Nomura T, Ushiki T, Shiroishi T, Kikkawa Y, Yonekawa H, Kominami R. Ahl3, a third locus on mouse chromosome 17 affecting age-related hearing loss. Biochem Biophys Res Commun. 2004; 324:1283–1288. [PubMed: 15504353]

Niu H, Li X, Makmura L, Friedman RA. Mapping of genetic modifiers of Eya1 (bor/bor) in CAST/EiJ and BALB/cJ that suppress cochlear aplasia and associated deafness. Mamm Genome. 2008; 19:634–639. [PubMed: 18836772]

Noben-Trauth K, Johnson KR. Inheritance patterns of progressive hearing loss in laboratory strains of mice. Brain Res. 2009; 1277:42–51. [PubMed: 19236853]

Noben-Trauth K, Zheng QY, Johnson KR, Nishina PM. *mdfw*: a deafness susceptibility locus that interacts with deaf waddler (*dfw*). Genomics. 1997; 44:266–272. [PubMed: 9325047]

Noben-Trauth K, Zheng QY, Johnson KR. Association of cadherin 23 with polygenic inheritance and genetic modification of sensorineural hearing loss. Nat Genet. 2003; 35:21–23. [PubMed: 12910270]

Noben-Trauth K, Latoche JR, Neely HR, Bennett B. Phenotype and genetics of progressive sensorineural hearing loss (Snhl1) in the LXS set of recombinant inbred strains of mice. PLoS One. 2010; 5:e11459. [PubMed: 20628639]

Noguchi Y, Kurima K, Makishima T, de Angelis MH, Fuchs H, Frolenkov G, Kitamura K, Griffith AJ. Multiple quantitative trait loci modify cochlear hair cell degeneration in the Beethoven (Tmc1Bth) mouse model of progressive hearing loss DFNA36. Genetics. 2006; 173:2111–2119. [PubMed: 16648588]

Perrin BJ, Strandjord DM, Narayanan P, Henderson DM, Johnson KR, Ervasti JM. beta-Actin and fascin-2 cooperate to maintain stereocilia length. J Neurosci. 2013; 33:8114–8121. [PubMed: 23658152]

Shin JB, Longo-Guess CM, Gagnon LH, Saylor KW, Dumont RA, Spinelli KJ, Pagana JM, Wilmarth PA, David LL, Gillespie PG, Johnson KR. The R109H variant of fascin-2, a developmentally regulated actin crosslinker in hair-cell stereocilia, underlies early-onset hearing loss of DBA/2J mice. J Neurosci. 2010; 30:9683–9694. [PubMed: 20660251]

Suzuki S, Ishikawa M, Ueda T, Ohshiba Y, Miyasaka Y, Okumura K, Yokohama M, Taya C, Matsuoka K, Kikkawa Y. Quantitative trait loci on chromosome 5 for susceptibility to frequency-specific effects on hearing in DBA/2J mice. Exp Anim. 201510.1538/expanim.14-0110

Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques. 2000; 29(52):54. [PubMed: 10907077]

Williams RW, Gu J, Qi S, Lu L. The genetic structure of recombinant inbred mice: high-resolution consensus maps for complex trait analysis. Genome Biol. 2001; 2(11):research0046.1–0046.18. [PubMed: 11737945]

Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear Res. 1999; 130:94–107. [PubMed: 10320101]

Zheng QY, Ding D, Yu H, Salvi RJ, Johnson KR. A locus on distal chromosome 10 (ahl4) affecting age-related hearing loss in A/J mice. Neurobiol Aging. 2009; 30:1693–1705. [PubMed: 18280008]

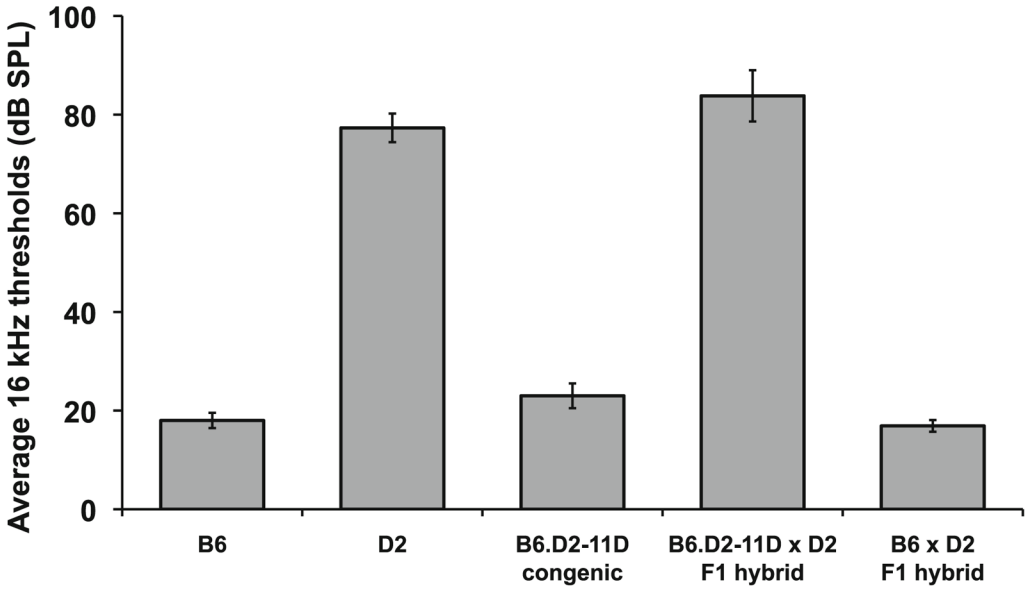

Fig. 1.

ABR thresholds of B6.D2-Chr11D/LusJ congenic strain mice and effects of hybridization with DBA/2J mice. Average 16 kHz thresholds are shown for host strain C57BL/6J (B6) mice (*N* = 20), donor strain DBA/2J (D2) mice (*N* = 11), B6.D2-Chr11D/LusJ (B6.D2-11D) congenic strain mice (*N* = 5), (B6.D2-11D × D2) F1 hybrids (*N* = 4), and (B6 × D2) F1 hybrids (*N* = 10). All inbred strain mice and F1 hybrids were tested at 12 weeks of age. *Bars* represent standard errors of the means

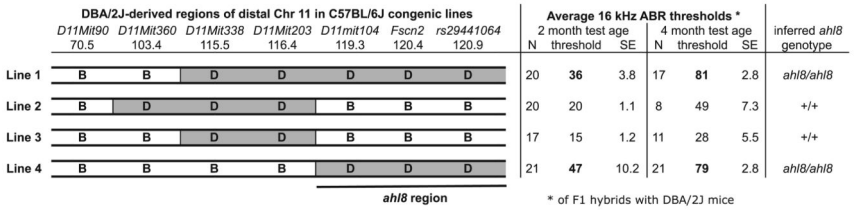

Fig. 2.

B6.D2-Chr11 congenic lines refine the *ahl8* locus. Different segments of distal Chr 11 from DBA/2J (D2) mice were introgressed into the C57BL/6J (B6) genome and made homozygous. B6/B6 (B) or D2/D2 (D) genotypes of microsatellite markers *D11Mit90*, *D11Mit360*, *D11Mit338*, *D11Mit203*, and *D11Mit104*, the *Fscn2* gene, and the *rs29441064* SNP were used to define the D2-derived congenic regions of each line, shaded in gray. The number below each marker locus indicates its Chr 11 Mb position (GRCm38). F1 hybrids between mice of each homozygous line and D2 mice were tested for ABR thresholds at 2 and 4 months of age, and *ahl8* genotypes were inferred from the average threshold values (elevated thresholds indicating hearing loss are shown in boldface). ABR thresholds of line 4 mice were not significantly different ($p > 0.1$) from those of line 1 mice at either 2 months or 4 months of age, but were highly significantly different from those of mice from lines 2 and 3 at both ages ($p < 0.0001$). The minimal region for the *ahl8* locus deduced from these data is shown below the congenic line figures. Line 4 is now designated as strain B6.D2-*Fscn2*^{ahl8}/4Kjn. *N* number of mice tested; *SE* standard error or threshold means

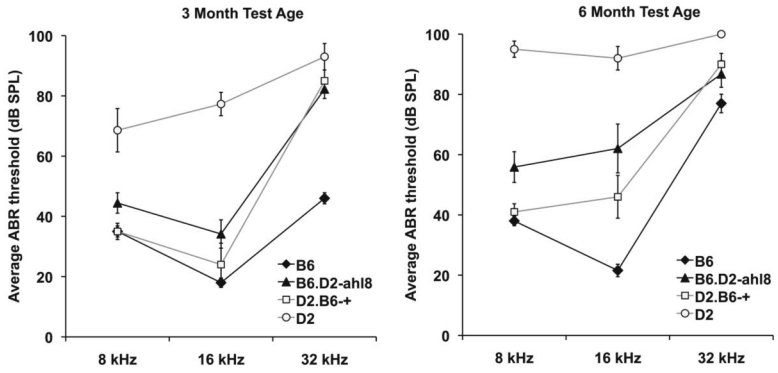

Fig. 3.

ABR thresholds of reciprocal B6.D2- and D2.B6-*Fscn2* congenic strain mice. ABR threshold means for 8 kHz, 16 kHz, and 32 kHz stimuli are shown for mice of the B6.D2-*Fscn2*^{ahl8} (B6.D2-ahl8, $N = 18$) and D2.B6-*Fscn2*$^{+}$ (D2.B6-+, $N = 8$) reciprocal congenic strains compared with those of C57BL/6J (B6, $N = 18$) and DBA/2J (D2, $N = 11$) parental strain mice at 3 and 6 months of age. *Bars* represent standard errors of threshold means. The effects of the *Fscn2* locus on hearing loss are apparent in both congenic strains. The 8- and 16-kHz thresholds of D2.B6- + congenic mice are significantly lower than those of parental D2 mice ($p < 0.01$) at 3 months of age, and the 8- and 16-kHz thresholds of B6.D2-ahl8 congenic mice are significantly higher than those of parental B6 mice ($p < 0.01$) at 6 months of age

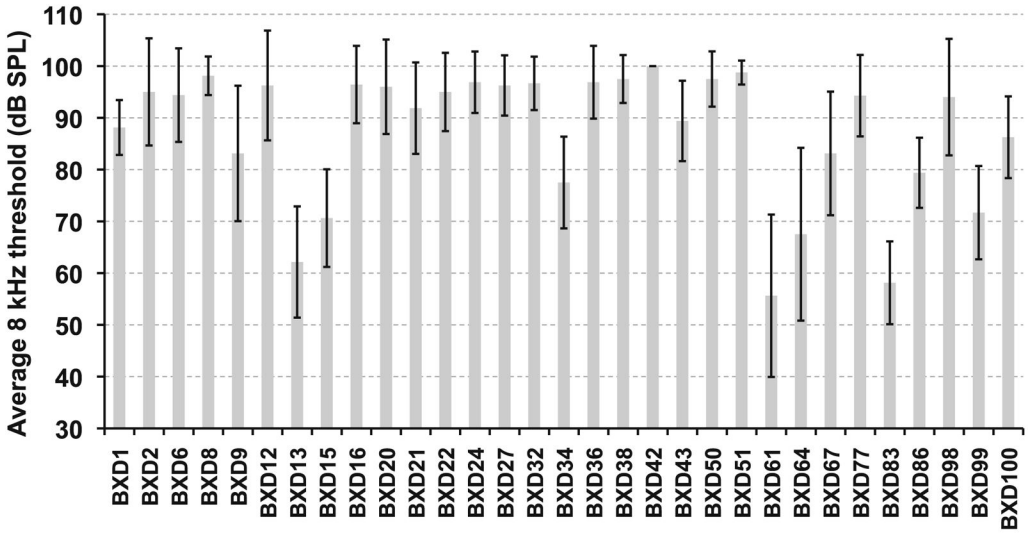

Fig. 4.

ABR threshold variation among BXD recombinant inbred strains with DBA/2J-derived *Fscn2* alleles. 8 kHz threshold means and standard deviations are shown for 6–10 mice from each of 31 BXD RI strains, all homozygous for the DBA/2J-derived allele of *Fscn2*. All mice were tested from 7.5 to 9 months of age. Note that the among-strain threshold variation is greater than the within-strain variation, which indicates that genetic factors have a significant effect on threshold values

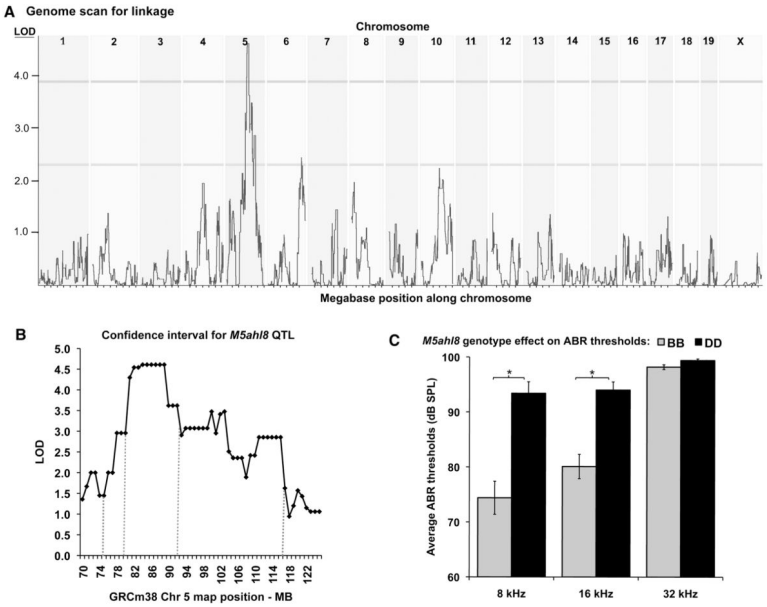

Fig. 5.

BXD RI strain linkage analysis and effects of *M5ahl8* genotype on ABR thresholds. **a** Genome-wide linkage associations of markers with 8 kHz ABR threshold means of the RI strains shown in Fig. 4. The top *horizontal line* indicates statistically significant linkage (LOD = 3.88), and the lower line suggestive linkage (LOD = 2.29). Only markers on Chr 5 showed statistically significant linkage. **b** Detail of Chr 5 marker associations, with innermost *dashed lines* indicating the extent of the 1.5-LOD confidence interval (80–92 Mb) for *M5ahl8* and outer dotted lines the 3.0-LOD confidence interval (74–116 Mb). **c** The effect of *M5ahl8* genotypes on ABR thresholds of RI strain mice. Markers located in the 82–88 Mb region of Chr 5 were used to assign *M5ahl8* genotypes for each RI strain: DD, homozygous for DBA/2J alleles; BB, homozygous for C57BL/6J alleles. The average 8 and 16 kHz thresholds of RI strain mice with DD genotypes at *M5ahl8* were statistically significantly higher ($p < 0.0001$) than those of RI strain mice with BB genotypes

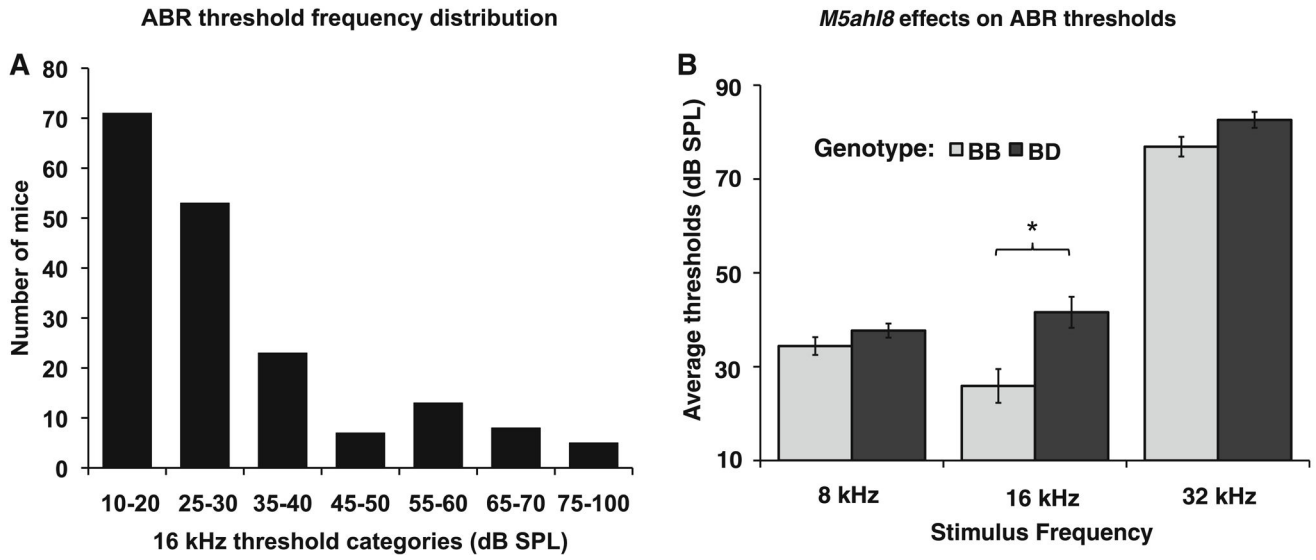

Fig. 6.

Backcross linkage analysis confirms *M5ahl8* effect on ABR thresholds. **a** 180 N2 mice from the (B6.D2-*ahl8* × D2) × B6.D2-*ahl8* backcross were tested for ABR thresholds at 12 weeks of age. The numbers of backcross mice in each of the ascending 16 kHz threshold categories show a skewed and slightly bimodal frequency distribution. **b** 85 of the 180 backcross mice with the lowest and highest 16 kHz ABR thresholds were selected for genome-wide linkage analysis with 120 SNP markers. The highest association detected (LOD = 3.3) was with rs3023047 at the 92.3 Mb position of Chr 5, and this marker was used to assign *M5ahl8* genotypes of the backcross mice: BD, heterozygous for C57BL/6J and DBA/2J alleles; BB, homozygous for C57BL/6J alleles. The 16 kHz threshold mean of backcross mice with BD genotypes was statistically significantly higher than that of mice with BB genotypes ($p < 0.001$)

Table 1

ABR thresholds of BXD RI strain mice

RI strain	Number tested	8 kHz thresholds			16 kHz thresholds			32 kHz thresholds		
		Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
BXD01	3f, 5m	88.1	5.3	1.9	76.9	7.5	2.7	100.0	0.0	0.0
BXD02	2f, 6m	95.0	10.4	3.7	97.5	3.8	1.3	100.0	0.0	0.0
BXD06	2f, 6m	94.4	9.0	3.2	87.5	15.6	5.5	93.8	8.8	3.1
BXD08	8m	98.1	3.7	1.3	98.1	2.6	0.9	99.4	1.8	0.6
BXD09	4f, 4m	83.1	13.1	4.6	94.4	7.3	2.6	100.0	0.0	0.0
BXD12	3f, 5m	96.3	10.6	3.8	97.5	7.1	2.5	98.1	3.7	1.3
BXD13	2f, 5m	62.1	10.7	4.1	74.3	9.3	3.5	99.3	1.9	0.7
BXD15	4f, 4m	70.6	9.4	3.3	80.0	10.7	3.8	100.0	0.0	0.0
BXD16	4f, 3m	96.4	7.5	2.8	96.4	7.5	2.8	100.0	0.0	0.0
BXD20	4m	96.0	9.1	4.6	86.0	16.6	8.3	100.0	0.0	0.0
BXD21	8m	91.9	8.8	3.1	96.3	4.4	1.6	98.8	2.3	0.8
BXD22	1f, 7m	95.0	7.6	2.7	96.9	4.6	1.6	99.4	1.8	0.6
BXD24	5f, 3m	96.9	5.9	2.1	97.5	3.8	1.3	100.0	0.0	0.0
BXD27	3f, 5m	96.3	5.8	2.1	93.8	11.9	4.2	100.0	0.0	0.0
BXD32	6m	96.7	5.2	2.1	97.5	2.7	1.1	100.0	0.0	0.0
BXD34	8m	77.5	8.9	3.1	89.9	13.6	4.8	100.0	0.0	0.0
BXD36	3f, 5m	96.9	7.0	2.5	94.4	5.0	1.8	99.4	1.8	0.6
BXD38	3f, 5m	97.5	4.6	1.6	97.5	3.8	1.3	100.0	0.0	0.0
BXD42	1f, 7m	100.0	0.0	0.0	98.8	2.3	0.8	100.0	0.0	0.0
BXD43	2f, 6m	89.4	7.8	2.8	8.8	8.3	3.0	100.0	0.0	0.0
BXD50	4f, 4m	97.5	5.3	1.9	94.4	5.0	1.8	100.0	0.0	0.0
BXD51	7f, 1m	98.8	2.3	0.8	98.1	2.6	0.9	97.5	4.6	1.6
BXD61	3f, 5m	55.6	15.7	5.5	76.9	4.6	1.6	97.5	4.6	1.6
BXD64	1f, 7m	67.5	16.7	5.9	86.9	10.0	3.5	98.8	3.5	1.3
BXD67	2f, 6m	83.1	11.9	4.2	94.4	5.0	1.8	98.8	3.5	1.3
BXD77	2f, 5m	94.3	7.9	3.0	91.4	9.4	3.6	99.3	1.9	0.7
BXD83	4f, 4m	58.1	8.0	2.8	61.9	13.9	4.9	96.9	3.7	1.3

RI strain	Number tested	8 kHz thresholds			16 kHz thresholds			32 kHz thresholds		
		Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
BXD86	4f, 4m	79.4	6.8	2.4	80.0	8.9	3.1	96.9	5.3	1.9
BXD98	5f, 5m	94.0	11.3	3.6	96.0	6.6	2.1	99.5	1.6	0.5
BXD99	4f, 5m	71.7	9.0	3.0	71.7	4.6	1.5	96.7	2.5	0.8
BXD100	3f, 5m	86.3	7.9	2.8	86.3	13.3	4.7	100.0	0.0	0.0

Within-strain 8 kHz, 16 kHz, and 32 kHz threshold means with associated standard deviations (SD) and standard errors (SE), of mice from 31 BXD strains, tested at 7.5–9 months of age. All 31 strains have the homozygous DBA/2J genotype of *Fscn2*, so genetic variation in thresholds must be due to other loci. No statistically significant sex effects (ANOVA F-ratio probability 0.394) were detected in ABR thresholds at any frequency, so male (m) and female (f) values were combined