Research Article

A Chromosome 17 Locus Engenders Frequency-Specific Non-Progressive Hearing Loss that Contributes to Age-Related Hearing Loss in Mice

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ABSTRACT

The 129S6/SvEvTac (129S6) inbred mouse is known for its resistance to noise-induced hearing loss (NIHL). However, less is understood of its unique age-related hearing loss (AHL) phenotype and its potential relationship with the resistance to NIHL. Here, we studied the physiological characteristics of hearing loss in 129S6 and asked if noise resistance (NR) and AHL are genetically linked to the same chromosomal region. We used auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) to examine hearing sensitivity between 1 and 13 months of age of recombinant-inbred (congenic) mice with an NR phenotype. We identified a region of proximal chromosome (Chr) 17 (*D17Mit143-D17Mit100*) that contributes to a sensory, non-progressive hearing loss (NPHL) affecting exclusively the high-frequencies (>24 kHz) and maps to the *nr1* locus on Chr 17. ABR experiments showed that 129S6 and CBA/CaJ F1 (CBACa) hybrid mice exhibit normal hearing, indicating that the hearing loss in 129S6 mice is inherited recessively. An allelic complementation test between the 129S6 and 101/H (101H) strains did not rescue hearing loss, suggesting genetic allelism between the *nphl* and *phl1* loci of these strains, respectively. The hybrids had a milder hearing loss than either parental strain, which indicate a

possible interaction with other genes in the mouse background or a digenic interaction between different genes that reside in the same genomic region. Our study defines a locus for *nphl* on Chr 17 affecting frequencies greater than 24 kHz.

Keywords: auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), age-related hearing loss (AHL), non-progressive hearing loss (NPHL), 129S6/SvEvTac (129S6), CBA/CaJ (CBACa), 101/H (101H)

INTRODUCTION

In the USA alone, 37 million adults suffer from acquired hearing loss (Pleis and Lethbridge-Cejku 2007), due primarily to a lifetime accumulation of harmful environmental factors and presbycusis, the progressive decline of hearing associated with aging. Inbred mice strains with age-related hearing loss (AHL) are commonly used to understand the genetic basis of presbycusis. The 129S6 inbred mouse is a unique model of presbycusis in that it has early-onset AHL starting by 1 month of age, as well as having a remarkable resistance to noise-induced hearing loss (NIHL) (Yoshida et al. 2000; Rosowski et al. 2003; Ohlemiller and Gagnon 2004; Street et al. 2014). Other known mice with AHL have a predisposition for NIHL, suggesting these traits are associated (Erway et al. 1996; Davis et al. 2001; Vázquez et al. 2004). Most

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of the work in the 129S6 strain has focused on understanding the genetic basis for noise resistance (NR). In this study, we aim to get a better understanding of the genetic and physiological basis of AHL in the 129S6 strain.

Linkage analysis between 129S6 backcrossed to the CBACa mouse identified six NR quantitative trait loci in the mouse genome. The strongest statistical association with NR was found on Chr 17, indicating that genes in these regions are most likely to contribute to the NR phenotype. Chr 17 was linked to two loci, *nr1* on proximal Chr 17 (between markers *D17Mit143-D17Mit100*) and *nr6* on distal Chr 17 (between markers *D17Mit119-D17Mit1*) (Street et al. 2014). Independently, other research has shown that these genomic regions were also associated to other AHL genes (Mashimo et al. 2006; Nemoto et al. 2004).

The C57BL/6J (B6) mouse, which harbors the Cadherin 23 *ahl* locus (*Cdh23^{ahl}*) on Chr 10 (Johnson et al. 1997; Noben-Trauth et al. 2003), also carries the *ahl3* locus on distal Chr 17. The B6 mouse with a transferred distal Chr 17 segment from the MSM inbred mouse has reduced susceptibility to AHL and NIHL (Morita et al. 2007). Hearing loss in the 101H inbred strain is early-onset never exhibiting normal hearing thresholds; its phenotype was termed progressive hearing loss (PHL). PHL is the product of an epistatic interaction between two loci mapped to proximal Chr 17 (*phl1*) and Chr 10 (*phl2*) that result in a profound hearing loss by 1 year of age (Mashimo et al. 2006).

The *phl1* locus on proximal Chr 17 and the *ahl3* locus on distal Chr 17 are in the same regions that were shown on 129S6 mouse to have *nr1* and *nr6* loci, respectively (Street et al. 2014). In light of this evidence, we speculated whether the AHL phenotype in 129S6 mice is genetically linked to the NR loci of Chr 17. We used auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) measurements on previously generated congenic mice to investigate the presence of hearing loss genes on Chr 17 of 129S6. We provide evidence of a locus for *nphl* that maps to the *nr1* locus of the 129S6 mouse and is non-complimentary to the *phl1* locus in the 101H inbred strain.

METHODS

Rearing and Breeding of Experimental Animals

All experimental animals were born and reared in our animal care facility in a quiet environment (<40 dB SPL) with 12-h light/dark cycle and food and water available ad libitum and held to various ages between 1 and 13 months depending on experiments. Animals were male and female inbred mice: CBA/CaJ (CBACa;

Jackson laboratory, ME, USA), 129S6/SvEvTac (129S6; Taconic, NY, USA), and 101/H (101H; MRC Harwell, Oxfordshire, UK); or congenic mice and filial crosses of the inbred strains named above.

CBACa and 129S6 breeders were replaced every three generations with supplier stock to maintain isogeneity in the strains. All 101H mice used were less than five generations removed from supplier's stock. Congenic mice were generated by the speed congenic method (Markel et al. 1997) and maintained by filial crossing. As characterized previously, 97 simple sequence length polymorphism (SSLP) markers with an average distance of 13.7 cM were used to selectively backcross 129S6 to the CBACa recipient strain for seven and five generations to create the CBACa.129S6-(*D17Mit143-D17Mit100*)/Tem and CBACa.129S6-(*D17Mit119-Dmit1*)/Tem congenic strain, respectively (Street et al. 2014). For brevity, regions (*D17Mit143-D17Mit100*) and (*D17Mit119-D17Mit1*) will be referred to as (*prox17*) and (*dist17*), respectively. Eleven SSLP markers with an average distance of 4.5 cM were used on Chr 17 to define in high resolution the regions of interest (Fig. 1).

All physiological experiments were approved by the University of Washington Institutional Animal Care and Use Committee. Physiological tests were conducted in a sound-attenuating chamber. Mice were anesthetized with ketamine (130 mg/kg, i.p.) and xylazine (6.5 mg/kg, i.p.) and their heart rate monitored continuously with an oscilloscope (Tetronix TDS 1002, OR, USA). For ABR, body temperature was maintained at 37 °C throughout the experiment with an isothermal heating pad

FIG. 1. Representation of Chr 17 from congenic strains used in the study. Homozygous regions defining the contribution from 129S6 (blue), CBACa (white), and the unknown interval (hatched). The centromere (not drawn) and the telomere of the Chr are located at the top and bottom portion of the schematic, respectively. The physical location of 11 polymorphic SSPL markers used on Chr 17 to establish congenic mice are given in centimorgans (cM) and megabase pairs (Mbp). Distance of markers along the chromosome was obtained from the Genome Reference Consortium Mouse Build 38 (GRCm38/mm10). Congenic mice CBACa.129S6-(*D17Mit143-D17Mit100*)/Tem and CBACa.129S6-(*D17Mit119-D17Mit1*)/Tem will be referred to as CBACa.129S6-(*prox17*) and CBACa.129S6-(*dist17*), respectively. Scale bar 10 Mbp.

(Deltaphase, Braintree Scientific, MA, USA). For DPOAE, temperature was monitored by a rectal probe and maintained at 37 °C with a heating plate and temperature feedback controller (RET-3, HP-1 M, TCAT-2, Physitemp Instruments, NJ, USA).

Physiological Tests by ABR

We examined auditory sensitivity and neural responses in the mice by free-field ABR. Experiments were conducted with custom software. Stimuli were digitally generated clicks (0.1 ms) or tones (5 ms, 0.5 r/f) presented 13 times per second with alternating polarity and delivered from a transducer 1 ft away from animal's head. Neural response to the stimuli was recorded from two subcutaneous needle electrodes inserted at the vertex and caudal to the left pinna at the mastoid with a ground electrode in the hind leg. Evoked responses were initially filtered (0.1–3 kHz passband) and amplified (1000×; Grass Telefactor P55 A.C. pre-amplifier, RI, USA), followed by a second filtering (<10 kHz passband) and amplification (30 dB; Krohn-Hite, Model 3362 Filter, MA, USA). Responses were digitized (96,500 samples/s) and averaged for 350 or 500 samples at threshold intensities. To determine threshold, stimuli intensity was decreased from 80 dB SPL in 20 dB steps, with smaller 5 dB steps around threshold. We defined hearing threshold as the lowest sound level (± 5 dB) that elicited a reproducible ABR wave within 10 ms of stimuli onset. When responses were absent at the maximum level presented or at 100 dB, a threshold value 5 dB higher was assigned. The ABR wave I component was identified and the peak to peak amplitude computed by off-line analysis of stored waveforms and defined as the suprathreshold response amplitude.

We verified the accuracy of ABR threshold calls with a goodness-of-fit analysis to a linear regression ($X=Y$). A trained audiologist blind to the genotype and testing conditions was instructed to estimate the threshold for a subset (35 %) of all ABR recordings performed. The threshold values from the experimenter and the blind observer were plotted on an XY coordinate and run through a linear regression model ($X=Y$) to determine how similar were the values obtained by the two observers. The results show a high degree of correlation ($R^2=0.9462$, slope of best-fit values 0.9816, results not shown). These results provide evidence that threshold calls from experimenter and blind observer concord and accurately represent the hearing sensitivity of the subjects and not the experimenter bias for the genotype or age of subjects.

Physiological Tests by DPOAE

Sensory response or outer hair cell (OHC) function was measured by DPOAE. Prior to DPOAE measure-

ments, the ear canal and middle ears were evaluated under microscopic observation for otitis media, obstructions, and other abnormalities. Recordings were conducted and analyzed using the EPL PXI hardware/software system (Eaton-Peabody Laboratories, MA, USA) as previously described (Kujawa and Liberman 2006). Primary tones had a frequency ratio of $f_2/f_1=1.2$ and the f_1 level presented 10 dB higher than the f_2 level ($L_1=L_2+10$ dB). Primary tones were incremented together in 5 dB steps to 80 dB. Ear-canal sound pressure was amplified and digitally sampled. DPOAEs corresponding to the cubic difference distortion product ($2f_1-f_2$) frequency and the surrounding noise floor were extracted from each sound level. We determined DPOAE threshold by interpolating from the I/O growth function the intensity of f_2 that produced $2f_1-f_2$ distortion of 0 dB. All distortions above 60 dB were assigned a threshold value of 65 dB because these were beyond the dynamic range of the system at 60 dB.

Data Analysis

All data were analyzed and graphed using PRISM 5 (GraphPad Software, La Jolla, CA). We used within-subjects, two-way analysis of variance (ANOVA) for ABR and DPOAE analyses. A Bonferroni correction for multiple comparisons (t test) was used as a posttest to compare group means by frequency when applicable.

RESULTS

Physiology of Early-Onset, High-Frequency Hearing Loss

Murine models of presbycusis are defined by hearing that deteriorates in an age-dependent manner. In the 129S6 mouse, hearing loss starts in the high-frequency range above 32 kHz (Yoshida et al. 2000). We looked for these two conditions to determine the presence of AHL genes on Chr 17 of 129S6 mice. The ABR waveform represents the evoked auditory response recorded from subcutaneous electrodes as neural signals travel from the cochlea through the brainstem. We measured hearing sensitivity by ABR from inbred (CBACa, 129S6) and congenic [CBACa.129S6-(*prox17*), CBACa.129S6-(*dist17*)] mice at 1 month of age (30 ± 2 days), which is the earliest evidence of a high-frequency hearing loss in 129S6 (Ohlemiller and Gagnon 2004). Data from male and female subjects were pooled as no sex differences were found within any of our four strains (data not shown). Consistent with previous findings, 129S6 mice had an average hearing loss of 18.76 dB between 24 and 40 kHz when compared to age-matched CBACa mice. The

CBACa.129S6-*(prox17)* strain had a similar hearing deficit as the 129S6 mice with an average 19 dB hearing loss between 24 and 40 kHz (Fig. 2). A two-way ANOVA revealed a significant effect of genotype in the hearing sensitivity of 1-month-old mice ($F_{3, 44}=22.03$, $P<0.001$). Frequency and genotype together explain 71.94 % of the variance observed between strains. The CBACa.129S6-*(dist17)* strain had an average threshold difference of 2.7 dB between 24 and 40 kHz compared to the CBACa mouse. The ABR thresholds from the CBACa.129S6-*(dist17)* strain were not statistically different from CBACa mice at any frequency tested.

Measures of Early-Onset OHC Deficit

Statistically, 129S6 and CBACa.129S6-*(prox17)* mice had normal ABR thresholds in the mid-frequency range (8–16 kHz) at 1 month of age. Qualitatively, however, 129S6 mice showed reduced ABR amplitude at these frequencies compared to CBACa (Fig. 3A). In mice, wave I of the ABR represents the auditory nerve afferents firing synchronously (Møller 1994), the amplitude of which is an indirect measure of the strength of the auditory transduction mechanism that precedes the spiral ganglion cells (SGC). We conducted a suprathreshold analysis at 80 dB for frequencies with statistically normal thresholds. Evaluating wave I amplitude and latency may reveal differences in peripheral hearing sensitivity not observed with ABR thresholds (see review; Tremblay and Burkard 2007). Compared to CBACa, the 129S6 mouse had a reduced wave I amplitude ($F_{3, 44}=4.25$, $P<0.01$) of 1.39 μ V at

12 kHz ($t=4.75$, $P<0.001$) and 1.04 μ V at 16 kHz ($t=3.57$, $P<0.01$), but not at 8 kHz ($t=0.1084$, $P>0.05$; Fig. 3B). On average, the 129S6 mouse had longer wave I latencies ($F_{3, 44}=4.74$, $P<0.01$) at 12 kHz ($t=3.618$, $P<0.01$) and 16 kHz ($t=2.494$, $P<0.05$), but not at 8 kHz ($t=2.33$, $P>0.05$) compared to the CBACa mouse (Fig. 3C). Wave I amplitude was measured from the baseline to the peak of the wave because the increased peak latency in 129S6 mice did not allow for the accurate measure of the negative trough generally used for amplitude measures (Markand 1994). No significant suprathreshold differences in amplitude or latency were present at any frequency tested in either congenic strain when compared to the CBACa mouse.

To understand the contribution of OHCs to the differences in ABR suprathreshold responses observed, we conducted DPOAE measurements in a new cohort of mice different from those used in the ABR and suprathreshold analysis. ABR thresholds in this new cohort (data not shown) were not statistically different from those shown in Figure 2. DPOAE measurements and visual assessment of the external auditory canal and middle ear were not conducted in CBACa.129S6-*(dist17)* mice because there was no evidence of a phenotype significantly different from the CBACa controls. In all three strains analyzed, the ear canals, tympanic membranes, and middle ear cavities appeared normal and unobstructed; however, the external auditory meatus of 129S6 mice was qualitatively smaller than either CBACa or CBACa.129S6-*(prox17)*. DPOAE thresholds differ between the three mice strains tested ($F_{2, 54}=26.76$, $P<0.001$). Compared to CBACa mice, the CBACa.129S6-*(prox17)* strain had normal DPOAE threshold between 8 and 16 kHz, but elevated thresholds at 32 kHz (16.43 dB, $t=3.148$, $P<0.05$) and 40 kHz (15.21 dB, $t=3.19$, $P<0.05$). Conversely, 129S6 mice had elevated DPOAE thresholds at all frequencies tested except 8 kHz (Fig. 4A). Genotype and frequency account for approximately 34.89 and 15.22 % of the total variance observed, respectively. The DPOAE data from 129S6 are consistent with OHC-dependent reduced wave I amplitude in the mid-frequency range and elevated ABR thresholds in the high-frequency range. The DPOAE I/O growth function highlights threshold results at 12 kHz (Fig. 4B) and 32 kHz (Fig. 4C) where 129S6 mice showed reduced growth function. The CBACa.129S6-*(prox17)* strain showed reduced growth function only at 32 kHz.

FIG. 2. Early-onset hearing loss in 129S6 mice. Mean ABR thresholds ($\pm$ SD) as a function of frequency in 1-month-old mice shows high-frequency (>24 kHz) hearing loss in 129S6 ($n=14$) and CBACa.129S6-*(prox17)* ($n=10$). CBACa ($n=15$) and CBACa.129S6-*(dist17)* ($n=9$) have normal hearing thresholds at all frequencies tested. Bonferroni post-hoc test compared CBACa to each genotype. *** $P<0.001$.

Ahl Physiology in 129S6 Mice

A presbycusis model requires that hearing deteriorates in an age-dependent manner. Mice with early-onset deafness similar to that seen in 129S6 mice

FIG. 3. ABR supratherreshold reveals amplitude and latency differences. ABR Wave I has reduced amplitude and increased peak latency at frequencies with normal thresholds in 129S6 mice. **A** Mean ABR waveform for all ABR subjects assayed at 80 dB for 12 kHz. (**A, insert**) Wave I amplitude (peak I) was calculated as the difference of the baseline recording 1.0 ms after the onset of the stimuli to the positive most point of wave. **B** Mean ($\pm$ SEM) peak

amplitude for the three frequencies with normal ABR threshold shows 129S6 is statistically different from CBACa at 12 and 16 kHz, but there is no difference between CBACa.129S6-(*prox17*) and CBACa.129S6-(*dist17*). **C** Mean ($\pm$ SEM) peak I latency in 129S6 was increased at 12 and 16 kHz but not at 8 kHz. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

generally have rapidly progressive phenotypes within the first 2 postnatal months (Mashimo et al. 2006; Shin et al. 2010; Latoche et al. 2011). We recorded ABR thresholds in a subset of mice at selective time points to address progression of hearing loss, which has not been previously assessed in a longitudinal study in this strain. In 129S6 mice, hearing sensitivity remained stable within the first 8 postnatal weeks. The first evidence of AHL was recorded at 7 months with a 13–18 dB loss between 12 and 40 kHz (t test, $P < 0.01$) and a 13–26 dB loss between 8 and 40 kHz (t test,

$P < 0.001$) by 13 months ($F_{4, 72} = 83.80$, $P < 0.001$; Fig. 5A). Noise exposure in young mice renders the inner ear significantly more vulnerable to aging pathology and hearing loss (Kujawa and Liberman 2006), especially in mice susceptible to AHL (Erway et al. 1996; Davis et al. 2001; Vázquez et al. 2004; Nemoto et al. 2004). As such, we wanted to examine if repeated ABR testing would have any effect on subsequent hearing threshold results. We compared 129S6 mice that have had a total of five ABR tests by 13 months ($n = 10$) to mice that received a single ABR test at 13 months

FIG. 4. One-month-old 129S6 shows increased DPOAE thresholds. Early-onset OHC dysfunction in the mid-frequency range despite normal ABR thresholds. **A** Mean ($\pm$ SD) DPOAE audiogram based on the f_2 level that elicits a 0-dB response of the $2f_1-f_2$ distortion product. 129S6 ($n = 5$) is significantly different from CBACa ($n = 6$) at frequencies between 12 and 40 kHz. CBACa.129S6-(*prox17*)

($n = 4$) is different from CBACa only at 32 and 40 kHz. Mean ($\pm$ SD) DPOAE I/O growth function of the $2f_1-f_2$ distortion product as a function of the f_2 level at 12 kHz (**B**) and 32 kHz (**C**). Dashed line represents 0-dB criteria for DPOAE threshold. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

($n=18$). We found that repeated ABR testing does not alter significantly hearing thresholds in 129S6 mice ($F_{1, 26}=0.27$, $P=0.6$; Fig. 5B). These results indicate the technique can be used with confidence since repeated ABR measurements do not accelerate the hearing loss trait in the 129S6 strain.

We compared ABR thresholds between 1-month and 13-month mice to assess the amount of AHL in our inbred and congenic mice. All “aged” (average 13 months) mice received no prior ABR measures to maintain comparison consistency with other strains but with the confidence that ABR does not significantly affect ABR thresholds in 129S6 as shown in Figure 5. Aged CBACa mice maintained good audito-

FIG. 5. 129S6 has a slow progressive hearing loss. **A** 129S6 mice ($n=4$) were assayed at various time points, within a short interval at 4, 6, and 8 weeks in the first 2 months and at 7 and 12 months. All hearing thresholds were compared to 4 weeks results. No statistical difference was found by 8 weeks. At 7 months, hearing thresholds were statistically different at all frequencies tested except 8 kHz. By 12 months, all frequencies tested were statistically different from 4-week-old thresholds. Bonferroni post-hoc test, $P<0.01$ where significance is applicable. **B** Multiple hearing assays do not affect progressive hearing loss in 129S6. Mice with five ABR assays by 1 year of age ($n=10$) have mean ($\pm$ SD) threshold indistinguishable from animals with a single ABR assay by the same age ($n=18$).

ry sensitivity compared to 1-month-old mice ($F_{1, 29}=1.04$, $P=0.3136$; Fig. 6A). In contrast, aged 129S6 mice had increased thresholds at all frequencies tested by 1-year of age ($F_{1, 30}=33.61$, $P<0.001$; Fig. 6B). Aged CBACa.129S6-(*prox17*) mice ($F_{1, 26}=2.25$, $P=0.1457$; Fig. 6C) showed no significant AHL affecting the high-frequency range despite having early-onset, high-frequency hearing loss. However, both the CBACa mice ($F_{5, 145}=2.49$, $P<0.05$) and the CBACa.129S6-(*prox17*) strain ($F_{5, 130}=4.77$, $P<0.001$) showed an interaction effect such that age had a different effect on the auditory thresholds at different frequencies. By 13 months, CBACa mice had an average hearing loss of 6.35 dB at 24 kHz ($t=2.98$, $P<0.05$), and CBACa.129S6-(*prox17*) mice had an average hearing loss of 12.89 dB at 16 kHz ($t=3.20$, $P<0.05$). The CBACa.129S6-(*dist17*) strain had significantly better thresholds by 10 months of age ($F_{1, 17}=10.09$, $P<0.01$); at 40 kHz, there was an average of 7.91 dB better hearing threshold ($t=3.150$, $P<0.05$; Fig. 6D). No statistical differences to CBACa were observed when CBACa.129S6-(*dist17*) mice were reanalyzed for hearing loss at 13-months (data not shown). The inbred 101H strain exhibited acute early-onset hearing loss in the mid-to-high frequency range and profound hearing loss at all frequencies by 1 year of age ($F_{1, 19}=236.91$, $P<0.001$; Fig. 6E).

Middle Ear and OHC Function Explains AHL

We used DPOAE measurements from aged mice to determine changes in OHC function by comparing 1-month-old to 13-month-old DPOAE data. The ear canals of aged CBACa mice were normal and unobstructed; middle ears showed no obvious signs of middle ear pathology. DPOAE thresholds of CBACa mice did not vary significantly with age and neither did the DPOAE growth functions ($F_{1, 43}=2.34$, $P=0.1336$; Fig. 7A–C). Consistent with earlier findings, the external auditory meatus of 129S6 mice was qualitatively narrower than in CBACa mice, which prevented visual middle ear assessment in three 129S6 mice. However, there was no indication that these three mice suffered from closure of the external auditory canal. In five out of seven of the 129S6 mice analyzed, the tympanic membrane had an opaque appearance with a white or yellow buildup behind the membrane, which is indicative of possible otitis media with effusion. We did not conduct a DPOAE statistical comparison between 1-month-old and 13-month-old 129S6 mice because 82 % of the aged DPOAE thresholds could not be experimentally determined (Fig. 7D). Either no DPOAE response was observed above the noise floor, consistent with middle ear effusion (Qin et al. 2010), or responses could only be generated with stimuli beyond the dynamic range of

FIG. 6. Comparison between 1-month and 13-month ABR reveals differences in AHL progression. Hearing thresholds ($\pm$ SD) at 1 month represent those previously presented in Figure 2 except for the 101H strain. Aged mice for CBACa (A), 129S6 (B), and CBACa.129S6-(*prox17*) (C) were on average 13 months old ($\pm$ 1 month). Aged CBACa.129S6-(*dist17*) (D) mice were 10 months of age. CBACa and CBACa.129S6-(*prox17*) mice had no effect of age when thresholds were compared to young mice. The asterisks in A and C reflect the interaction effect between age and frequency at 24 kHz in CBACa

and 16 kHz in CBACa.129S6-(*prox17*). Aged 129S6 and CBACa.129S6-(*dist17*) had an effect of age where 129S6 have significantly increased thresholds by 13 months and CBACa.129S6-(*dist17*) have significantly better threshold by 10 months. E Inbred 101H mice have significantly worst hearing thresholds by 13 months. Data for 1-month-old CBACa (black) are graphed in B through E for visual comparison and not for statistical analysis. Bonferroni post-hoc test $*P<0.05$, $***P<0.001$, arrows represent mean threshold above 100 dB.

the system (60 dB SPL). Results from I/O growth function indicate a lack of measurable distortion product in aged 129S6 mice (Fig. 7E,F). Congenic CBACa.129S6-(*prox17*) mice had an age-dependent change in DPOAE threshold ($F_{1,45}=19.94$, $P<0.001$) with higher average threshold only at 16 kHz (15.88 dB, $t=5.030$, $P<0.001$; Fig. 7G-I). All other frequencies tested at 13 months did not show a significant difference to 1 month DPOAE thresholds in CBACa.129S6-(*prox17*) mice.

Hearing Loss in 129S6 Mice Is Autosomal Recessive

AHL-susceptible strains with markedly different onset of hearing loss have a similar *ahl* allele suggesting that hearing loss genes may be common amongst mice strains derived from a common genetic background (Johnson et al. 2000). The locus for *phl1* was mapped to proximal Chr 17 in the 101H strain with profound early-onset hearing loss. The 101H and 129S6 inbred

FIG. 7. Chr 17 does not contribute to progressive OHC dysfunction in the high-frequency range. The *first column* represents the DPOAE threshold as the f_2 level that elicits a 0-dB response of the $2f_1-f_2$ distortion product for CBACa (A), 129S6 (D), and CBACa.129S6-(*prox17*) (G). Dashed line on the first column represents the 60-dB limit of the system's dynamic range. Statistical analysis were conducted comparing 1-month-old mice (closed symbol) previously shown on Figure 4 to aged mice (open symbols; 13 $\pm$ 1 month). Statistical analysis for 129S6 mice was not determined because 82 %

of the values from aged subjects never elicited a response above noise level or were above the 60-dB dynamic range of the system. The *second and third columns* represent the DPOAE I/O growth function of the mean dB ($\pm$ SD) of the $2f_1-f_2$ distortion product as a function of the f_2 level for 12 and 32 kHz, respectively, for CBACa (B, C), 129S6 (E, F), and CBACa.129S6-(*prox17*) (H, I). Dashed line on second and third columns represents 0-dB criterion that determined the DP threshold. Bonferroni post-hoc test *** $P<0.001$.

strains share similar genetic background and a common ancestor (Beck et al. 2000; Witmer et al. 2003). Since the *phl1* locus in 101H mapped to the same Chr 17 region as the hearing loss in the 129S6 strain, we wanted to know if 101H and 129S6 mice share a similar hearing loss gene at proximal Chr 17. We conducted an allelic complementation test to answer this question.

The F1 hybrids between CBACa mice crossed to either 129S6 or 101H mice had normal hearing thresholds, suggesting genes for hearing loss in the 101H and 129S6 strains are autosomal recessive ($F_{2,32}=3.15$, $P>0.05$). These results also indicate that gene

alleles from CBACa mice do not contribute to hearing loss in either the 129S6 or 101H strains (Fig. 8A). We then tested two additional hybrids, 101H crossed to either 129S6 or CBACa.129S6-(*prox17*). Progeny from the former, 129S6101HF1 mice, examined genetic complementation at all alleles shared between 101H and 129S6. Progeny from the latter cross, CBACa.129S6-(*prox17*)101HF1 mice, examined genetic complementation only at the alleles shared between 101H and 129S6 at the *prox17* (*D17Mit143-D17MIT100*) locus (Fig. 8B). The ABR tests revealed a significant difference in the hearing sensitivity of the 101H and 129S6 hybrids compared to CBACa mice

FIG. 8. Hearing loss genes in 129S6 are recessive and non-complementary with 101H genes. **A** Mean ($\pm$ SD) hearing thresholds of 1-month-old hybrid CBACa129S6F1 ($n=7$) and CBACa101HF1 ($n=18$) mice have normal hearing compared to CBACa ($n=10$). **B** Representation of hybrid mice generated to test genetic complementation between 101H (green) and 129S6 (blue) (left), and between 101H mice and proximal Chr 17 of 129S6 in a CBACa (white) background mouse (right). **C** Hybrid 129S6101HF1 ($n=17$) and CBACa.129S6-(*prox17*)101HF1 ($n=16$) have high-frequency hearing loss at 32 and 40 kHz. Data for 1-month-old 129S6 and 101H previously reported in Figure 2 are shown here for visual comparison and not for statistical analysis. Bonferroni post-hoc test. Where significance is applicable, * $P<0.05$, *** $P<0.001$.

controls ($F_{2, 40}=11.65$, $P<0.001$). The 129S6101HF1 mice had significantly elevated thresholds at 8 kHz (6.56dB, $t=3.11$, $P<0.05$), 32 kHz (9.29 dB, $t=3.11$,

$P<0.01$), and 40 kHz (21.88 dB, $t=4.408$, $P<0.01$). The CBACa.129S6-(*prox17*)101HF1 hybrids had significantly higher thresholds at 32 kHz (13.38 dB, $t=13.38$, $P<0.01$) and 40 kHz (27.56 dB, $t=27.56$, $P<0.01$). At all frequencies tested, thresholds between these two hybrid lines were not statistically different suggesting that they share at least one genetic allele in the proximal Chr 17 interval that contributes to hearing loss.

We examined how genetic complementation affected AHL by assessing ABRs in 13-month-old hybrids. Both, 129S6101HF1 ($F_{1, 24}=99.12$, $P<0.001$; Fig. 9A) and CBACa.129S6-(*prox17*)101HF1 ($F_{1, 23}=8.03$, $P<0.01$; Fig. 9B) mice, showed age-dependent hearing loss by 13 months of age. In 129S6101HF1 hybrids, thresholds were significantly higher by an average of 18.55 dB at all frequencies tested except at 8 kHz (Fig. 9A). In CBACa.129S6-(*prox17*)101HF1 hybrids, thresholds were significantly higher to a lesser extent by an average of 10.36 dB only at 32 kHz ($t=4.5$, $P<0.001$) and 40 kHz ($t=5.91$, $P<0.001$; Fig. 9B). These results show that 101H and 129S6 share a gene in the proximal Chr 17 region that engenders high-frequency NPHL.

DISCUSSION

Hearing Loss Linked to Proximal Chr 17

The inbred 129S6 mouse had an average hearing loss of 19 dB between 24 and 40 kHz by 30 days of age consistent with previous results (Yoshida et al. 2000; Rosowski et al. 2003; Ohlemiller and Gagnon 2004). When searching for the presence of AHL genes in NR loci (Street et al. 2014), we found CBACa.129S6-(*prox17*) mice had an average hearing loss similar to the 129S6 strain. Conversely, CBACa.129S6-(*dist17*) did not carry susceptibility for AHL up to 13 months. We focused the remainder of our study analyzing the phenotype present in proximal Chr 17 (*D17Mit143-D17Mit100*) of the 129S6 mouse.

AHL in 129S6 Mice

We found ABR thresholds to be stable up to 2 months of age and unaffected by multiple ABR measurements in 129S6 mice. Our results indicate the reduced wave I amplitude is due to abnormal OHC function which is consistent with the observed reduced DPOAE thresholds and large subject variance shown in this study and previously reported (Martin et al. 2007). Our ABR and DPOAE measures are in agreement indicating an early-onset sensory hearing loss. In the 129S6 strain, DPOAE and ABR suprathresholds are more sensitive measures of hearing deficit and are better predictors of mid-frequency AHL.

FIG. 9. 101H genes do not rescue AHL in 129S6 mice. ABR comparison between 1-month and 13-month hybrid mice shows **A** 129S6101HF1 ($n=9$) hybrid mice at 13 months have progressive hearing loss at all frequencies tested except 8 kHz. **B** CBACa.129S6-(*prox17*)101HF1 hybrid mice have progressive hearing loss at 32 and

40 kHz. Data for 1-month-old CBACa previously reported in Figure 2 are shown here for visual comparison and not for statistical analysis. Bonferroni post-hoc test. Where significance is applicable, *** $P<0.001$.

The consistent appearance of what may be middle ear fluid in 13-month-old 129S6 mice presents a greater challenge for determining the source for AHL. Mice with effusion-like conditions in the middle ear have large increases in ABR thresholds, between 12 and 19 dB (Qin et al. 2010). However, the hearing loss we observed from ABR thresholds in aged 129S6 mice, between 17 and 23 dB, was greater than what is expected from a fluid-filled middle ear alone. Aged 129S6 mice have reduced middle ear transfer functions (Rosowski et al. 2003) and reduced OHC density in the lower turns of the organ of Corti (Ohlemiller and Gagnon 2004), which suggest a combinatory effect of conductive and sensory hearing loss that is responsible for the AHL in the 129S6 mouse.

High-Frequency *Nphl* Locus at Proximal Chr 17

One of our findings is the minimal and frequency-specific hearing loss in CBACa.129S6-(*prox17*) mice. The CBACa.129S6-(*prox17*) strain had early-onset, high-frequency hearing loss similar to 129S6 mice. However, ABR suprathresholds and DPOAE thresholds were similar to CBACa mice in the mid-frequencies with no considerable AHL by 13 months. On average, all frequencies tested preserved 1-month-old ABR thresholds except at 16 kHz, which is the only frequency exhibiting marginal decreased in hearing sensitivity.

The recipient strain of the congenic mice, CBACa, is known for having good hearing throughout its life (Zheng et al. 1999); however, it is not resistant to age-related changes in hearing sensitivity (Sergeyenko et al. 2013). In our hands, the CBACa mouse develops an average of 5 dB hearing loss centered between 16

and 24 kHz by 13 months of age. Similarly, the CBACa.129S6-(*prox17*) congenic strain develops an average of 10 dB hearing loss centered between 16 and 24 kHz, which is within the expected values for the CBACa mouse based on our ABR threshold criteria of ± 5 dB. Conversely, the 129S6 strain develops between 16 and 22 dB of hearing loss uniformly spread between 8 and 40 kHz. The above data suggest that genes on proximal Chr 17 by themselves produce an autosomal recessive, early-onset high-frequency hearing loss and not the overall AHL we observed in the 129S6 strain. Henceforth, we define a high-frequency *nphl* locus by markers *D17Mit143-D17Mit100*. Models of AHL commonly have numerous genes that contribute to their AHL phenotype (Erway et al. 1993; Keithley et al. 2004; Latoche et al. 2011; Keller and Noben-Trauth 2012; Kane et al. 2012). The extensive broad-frequency hearing loss in aged 129S6 mice which is not present in CBACa.129S6-(*prox17*) suggests that numerous genes in the 129S6 background contribute to the 129S6 AHL phenotype. We propose the *nphl* locus harbors at least one of the genes that affects AHL in the 129S6 mouse. Our data also suggests that the *nphl* locus causes OHC dysfunction at 16 kHz by 13 months of age, which was not detected by DPOAE or ABR suprathresholds at 1 month of age in our congenic mice. Alternatively, the *nphl* locus may exacerbate the existing hearing susceptibility we observed between 16 and 24 kHz in the CBACa background at 13 months.

Genetic Non-complementation in 129S6 Mice

PHL in the 101H inbred strain is the product of an epistatic interaction between genes mapped to Chr 17

(*phl1*) and Chr10 (*phl2*) (Mashimo et al. 2006). We studied the interaction between *nphl* and *phl1* in a genetic complementation study. A cross between 129S6 and 101H mice (129S6101HF1) did not rescue the high-frequency hearing loss, which suggests that the *phl1* and *nphl* loci are allelic. This is plausible because 129S6 and 101H share similar genetic and ancestral backgrounds (Beck et al. 2000; Witmer et al. 2003). The 129S6101HF1 and CBACa.129S6-(*prox17*)101HF1 hybrid mice have identical phenotypes at 1 month of age suggesting the 129S6 mouse does not carry the *phl2* locus that gives the 101H mouse its profound deafness. The phenotypes for both these hybrid mice are not as severe as in either parental strain suggesting the *nphl* locus exerts varying levels of hearing loss severity depending on other interacting genes in the background of the affected mouse. An alternative hypothesis is that there are two genes for hearing loss within proximal Chr 17. A double heterozygous mouse for each gene could produce non-complementation if a digenic interaction exists between genes that rely on each other structurally or they are part of a similar molecular pathway. This digenic inheritance has been shown in double heterozygous *Cdh23* and protocadherin 15 (*Pcdh15*) mutant mice (Zheng et al. 2005) and in patients with double heterozygous Connexin 26 (*GJB2*) and Connexin 31 (*GJB3*) mutations (Liu et al. 2009). In both cases, the double heterozygous subjects exhibit deafness, whereas individuals heterozygous for a single mutation lack a deafness phenotype.

By 13 months of age, the 129S6101HF1 hybrid had extensive AHL, between 15 and 28 dB similar to that seen in aged 129S6 mice between 12 and 40 kHz. By the same age, the CBACa.129S6-(*prox17*)101HF1 hybrid had a marginal AHL, between 9 and 12 dB at 32 and 40 kHz, characteristic of the homozygous CBACa.129S6-(*prox17*) trait. Both hybrid strains analyzed have 50 % of their genome from 101H with different AHL phenotypes by 13 months suggesting heterozygous 101H alleles alone are not sufficient to cause the extensive AHL. The interaction between the 101H and 129S6 strains at a locus outside of *Prox17* could induce the extensive AHL observed in 129S6101HF1. Alternatively, 129S6 genes outside of Chr 17 may cause autosomal dominant AHL in the 129S6101HF1 hybrid. These results together confirm that 101H and 129S6 share a gene in the proximal Chr 17 region that engenders frequency-specific NPHL.

Candidate Genes

Over 300 protein-coding genes are present in the genomic region defining the *nphl* locus. We considered the function of these genes and propose a list of

candidate genes for the *nphl* phenotype, which have been found to cause auditory or vestibular traits. The NADPH oxidase 3 (*Nox3*) and NADPH oxidase organizer 1 (*Noxo1*) genes are critical for reactive oxygen species (ROS) production necessary for otoconia synthesis (Paffenholz et al. 2004; Kiss et al. 2006). While important in the vestibular system, ROS have toxic effects in the cochlea and may exacerbate hearing loss (Henderson et al. 2006). The Claudin 6 (*Cldn6*) and Claudin 9 (*Cldn9*) genes play a critical role in the maintenance of the endocochlear potential and structural integrity between OHC and Deiter cells (Kitajiri et al. 2004a, b; Nunes et al. 2006). A point mutation in *Cldn9* disrupts the ionic composition of the microenvironment at Nuel's space causing hearing loss in mice (Nakano et al. 2009). The Axin gene, an inhibitor of the Wnt signaling pathway, is mutated in the *Fused* and *Kink* mice showing various degrees of deafness and circling behavior (Dunn and Caspari 1945; Zeng et al. 1997).

The candidate genes above or a non-identified gene in this region may be implicated in any of the hearing phenotypes attributed to the proximal Chr 17 region. A 41-Mb region of Chr 10 has five loci involved in hearing loss, from which two distinct genes have been identified (Noben-Trauth et al. 2003, 2010; Mashimo et al. 2006; Charizopoulou et al. 2011; Latoche et al. 2011). It is possible that the 30 Mb proximal Chr 17 region has separate genes that contribute to high-frequency NPHL and 16 kHz AHL in 129S6, as well as to PHL in 101H mice.

Similar to 129S6, the NIH Swiss mouse has three loci specific for high-frequency hearing loss (*hfh1-1,-2,-3*), however with a mild AHL at all frequencies tested (Keller et al. 2011; Keller and Noben-Trauth 2012). The stable high-frequency OHC function and non-progressive nature of the phenotype in CBACa.129S6-(*prox17*) suggest that the *nphl* locus may not be deleterious to OHCs, which often die as a secondary effect of abnormal function and result in AHL. NR and NPHL could be allelic if the *nphl* locus has a frequency-specific effect to reduce sensitivity to high-frequency stimuli that prevents acoustic injury from the overstimulation of OHCs.

Human Non-progressive Deafness

To our knowledge, there are two loci for non-progressive, high-frequency, sensorineural hearing loss in humans, with post-lingual (Zanchetta et al. 2000) and pre-lingual (DFNA24; Häfner et al. 2000) onset. Mutations in the *TECTA* gene cause prelingual non-progressive, mid-frequency hearing impairment (DFNA 8/12; Govaerts et al. 1998; Kirschhofer et al. 1998). All reported cases are autosomal dominant. The number of affected individuals with non-

progressive hearing loss may be misrepresented in the general population. Patients with this type of condition may be unaware of the problem throughout their lives, and it could be misdiagnosed in the clinic.

Many of the inbred 129 substrains have an AHL phenotype (Zheng et al. 1999). In 129S6, the AHL is not attributed to the *Cdh23^{ahl}* allele as in other strains of the same background (Noben-Trauth et al. 2003). However, the common genetic background between 129 substrains suggests they may share the *nphl* locus found in the 129S6 mouse. Efforts to use quantitative trait loci to locate AHL genes in 129 substrains should consider the large impact the *nphl* locus has on the onset of hearing loss, which does not account for the entire AHL phenotype in these strains. Analysis of the AHL phenotype at multiple frequencies and ages may provide a better assessment of the location of the AHL genes affecting 129 substrains. Our current study provides additional understanding of the genetic characteristics and hearing physiology associated with 129S6 and the possible interaction between the *nphl* locus and other hearing genes. These findings provide strong support to exclude the use of 129S6 and 129 substrains in the use of embryonic stem cells for targeting of hearing-related genes in recombinant mice.

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Conflict of Interest

The authors would like to declare no commercial or other conflict of interests.

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