The Genetic Identification and Physiological Characterization of a Novel Locus for Non-Progressive Hearing Loss on Mouse Chromosome 17

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Abstract

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Presbycusis, the progressive loss of hearing that occurs with aging, is a widespread condition with serious economical and social ramifications. Mice with age-related hearing loss (AHL) are commonly used as models of presbycusis because of their physiological and genetic homology to the human auditory system. The inbred mouse, 129S6/SvEvTac (129S6), often used to study its resistance to noise-induced hearing loss (NIHL) also has early-onset progressive hearing loss. However, little is known about the AHL trait of 129S6 or the identity of the underlying genes. This dissertation will describe studies conducted in 129S6 to understand the physiological mechanisms associated with AHL, its genetic manifestation and inheritance, and the physical location of the causal genes.

Hearing sensitivity was examined in subjects using non-invasive auditory-evoked potentials and otoacoustic emissions. My research will show that hearing loss in 129S6 is early-onset, slow progressive, and a combination between sensory and conductive hearing loss. The research will show that hearing loss in the 129S6 strain is autosomal recessive with possible contribution by multiple genes. Selective breeding and the creation of recombinant-inbred mice

were used to define a novel 3.7 megabase (Mb) locus for non-progressive hearing loss (*nphl*) on mouse proximal Chromosome (Chr) 17 that contributes exclusively to high-frequency (>24 kHz). The *nphl* locus is linked genetically to the same region on Chr 17 exhibiting resistance to NIHL and a progressive hearing loss (PHL) locus.

Collectively, the work in this dissertation more accurately defines the physiological and genetic characteristics of hearing loss in the 129S6 mouse. The understanding of this phenotype and the effects in mice will provide insight to future therapeutic advantages and preventative measures for homologous human conditions.

DEDICATION

To the women in my life who give me support and encouragement in everything I do,

mi madre Mildred Milene, mi esposa Stephanie, e hija Soleil Leone.

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LIST OF ABBREVIATIONS

101H	101/H inbred mouse strain
129S6	129S6/SvEvTac inbred mouse strain
ABR	auditory brainstem response
AHL	age-related hearing loss
ANOVA	analysis of variance
B6	C57Bl/6J inbred mouse strain
CBACa	CBA/CaJ inbred mouse strain
Cdh23	Cadherin 23 gene
CS	Citrate Synthase gene
Chr	chromosome
Cldn6	Claudin 6 gene
Cldn9	Claudin 9 gene
dB	decibel
DBA2	DBA/2J inbred mouse strain
DC	Deiter cells
DFNA	nonsyndromic deafness, autosomal dominant
DFNB	nonsyndromic deafness, autosomal recessive
dist17	Chromosome 17 region between genetic markers D17Mit119 and D17Mit1
DPOAE	distortion product otoacoustic emissions
EP	endocochlear potential
FSCN2	Fascin 2 protein

Gipc3	GAIP-interacting protein C terminus 3 gene
GJB2	Gap junction beta-2 protein (connexin 26)
GJB3	Gap junction beta-3 protein (connexin 31)
GRHL2	Grainyhead-like 2 gene
GRM7	Metabotropic glutamate receptor 7 gene
hscy	hurry-scurry mouse
IHC	inner hair cells
Jams1	juvenille audiogenic monogenic seizure 1 locus
kHz	kilohertz
Lhfpl5	Lipoma HMGIC fusion partner-like 5 gene
Mb	Megabase
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NIHL	noise induced hearing loss
Nox3	NADPH oxidase 3 gene
Noxo1	NADPH oxidase organizer 1 gene
NPHL	non-progressive hearing loss
NR	noise resistant
OHC	outer hair cell
Pcdh15	Protocadherin 15 gene
PHL	progressive hearing loss
prox17	Chromosome 17 region between genetic markers D17Mit143 and D17Mit100
QTL	quantitative trait locus analysis
ROS	reactive oxygen species

SGC	spiral ganglion cells
Slc26a4	Solute carrier anion transporter family 26, member 4 gene
Slc26a5	Solute carrier anion transporter family 26, member 5 gene
Slc26a8	Solute carrier anion transporter family 26, member 8 gene
SINE	short interspersed nucleotide element
SNHL	sensorineural hearing loss
SNP	single-nucleotide polymorphism
Sod2	Superoxide dismutase 2 gene
SPL	sound pressure level
Scnn1	Epithelial sodium channel gene
SSLP	simple sequence length polymorphism
TECTA	Tectorin alpha gene
TMHS	Tetraspan membrane protein of hair cell stereocilia
Tmprss3	Transmembrane protease serine 3 gene

EPIGRAPH

"I said in Dorian Gray that the great sins of the world take place in the brain: but it is in the brain that everything takes place. We know now that we do not see with the eyes or hear with the ears. They are really channels for transmission, adequate or inadequate, of sense impressions. It is in the brain that the poppy is red, that the apple is odorous, that the skylark sings."

Oscar Wilde, De Profundis 1897

CHAPTER 1:

Introduction

The auditory system is designed to sense pressure changes, or vibrations, of the local physical medium, which in humans are perceived as sounds. It is arguably the most important sensory system for the survival of many animal species used for: navigation, foraging, predator avoidance, and social communication to attract mates and defend territories. In humans, the auditory system does not play an essential role for our survival, but it remains a central part of our daily social and working lives. Congenital or prelingual deafness may have dramatic effects on language acquisition and educational progress in children. Acquired hearing loss is linked to various physical and mental health complications in the adult population. At all stages of life, deafness may lead to social adversities and economic hardships creating a current public health concern.

This dissertation will present research conducted in mice to improve our understanding of the physiological characteristics and genetic basis of age-related hearing loss (AHL). This introductory chapter will cover the various forms of hearing loss, placing an emphasis on presbycusis and the mice models of AHL. The anatomy and physiology of the mammalian auditory system will be reviewed as it relates to the areas important for the understanding of the pathologies related to presbycusis. These topics will provide a substantial overview to understand the research conducted, the results obtained, and the conclusions drawn in this dissertation. The findings in this dissertation may help identify genes responsible for the underlying causes of presbycusis so that these may help elucidate preventative and therapeutic interventions.

1.1 Epidemiology of hearing impairment

Hearing loss is the most common sensory deficit, affecting over 5% of the world's population. There are numerous causes for hearing loss, but they are all classified as either congenital or acquired. The incidence of each type can vary by region and community but acquired hearing loss remains the most prevalent form of deafness in industrialized nations. Understanding the epidemiology of hearing loss helps assess the need for research to address our understanding of the normal and diseased state of the auditory system.

1.1.2 Congenital hearing loss

Congenital hearing loss is defined by hearing impairment that is present at birth or soon after birth due to either genetic or environmental factors. The prevalence of each can vary by community or region. For example, congenital hearing loss due to environmental factors is most common in developing nations that have a higher incidence of infections and diseases that go untreated in expecting mothers and newborns. In regions were consanguinity is practiced, there is a larger number of recessively inherited congenital hearing impairment (Zakzouk et al., 1993; Zakzouk, 2002; Bener et al., 2005). Overall, the incidence of congenital hearing loss is 1/1000 live births, 50% of which are due to genetic mutations (Bitner-Glindzicz, 2002). Genes responsible for hearing loss may be syndromic, where hearing loss is one aspect of various physical or mental abnormalities present in a patient. In contrast, nonsyndromic forms of hearing loss affect only the auditory system. Over 135 non-overlapping loci related to nonsyndromic hearing loss have been identified; 55 genes for nonsyndromic deafness, autosomal dominant (DNFA) and 30 genes for nonsyndromic deafness, autosomal recessive (DFNB) have been mapped or cloned in the human population (Friedman and Griffith, 2003;

HereditaryHearingLoss.com). The identification of genes that affect the auditory system offer insight into the development, function, and maintenance of the auditory system and provide a platform by which we can investigate therapeutic interventions for hearing loss.

1.1.2 Acquired hearing loss

The majority of all hearing impairment develops over a lifetime due mostly to presbycusis, the progressive decline of hearing associated with aging, and the accumulation of noxious environmental factors. In the USA alone, 37 million adults suffer from acquired hearing loss (Pleis and Lethbridge-Cejku, 2007). The increased life expectancy of the general population means a greater percentage of individuals will suffer from presbycusis in the future. The incidence of NIHL is on the rise amongst children and young adults exposed daily to damaging loud sounds during leisure activities (Niskar 2001; Chung 2005; Shargorodsky 2010). Hearing loss has been linked to higher rates of depression (Huang et al., 2010), higher incidence of falls (Viljanen et al., 2009a; Viljanen et al., 2009b), and decreased cognitive acuity (Lin et al., 2013) amongst other health problems. The high prevalence of acquired deafness makes hearing loss a widespread public health concern with negative economic ramifications on our society. Thus, it is imperative to conduct research aimed to elucidate the source of acquired deafness. In this dissertation, I address presbycusis and its possible sources.

1.2 The mammalian auditory system

1.2.1 Peripheral auditory transduction

To understand how presbycusis occurs it is important to understand auditory transduction in the peripheral auditory system and how each part contributes to signal amplification. The peripheral auditory system is divided in three sections: the outer, the middle, and the inner ear, each one responsible for an important part of sound amplification and signal transduction.

The outer ear is composed of the pinna, the concha, and the ear canal. One of its primary functions is to collect sound from the environment and funnel it to the tympanic membrane producing a signal amplification of about 15-20 dB (SPL; sound pressure level) in humans. The middle ear is made up of the tympanic membrane and the ossicular chain, comprised of the three smallest bones in the human body: the incus, the malleus, and the stapes. Sound vibrations hit the tympanic membrane, which causes motion of the ossicular chain that drives the footplate of the stapes into the oval window, an opening into the fluid filled cochlea. The middle ear transfers the energy of air vibrations into displacement of fluid inside the cochlea, and amplifying the stimuli's energy about 30 dB in humans (Figure 1.1A).

The inner ear is separated into two functional parts, the vestibular system responsible for the sense of balance and orientation in space, and the cochlea dedicated for hearing transduction. The cochlea is the most intricate part of the peripheral auditory system and we discuss here the most important parts necessary for sound transduction. The cochlea is a coiled tube divided in lengthways into three compartments: the scala vestibuli, the scala media, and the scala tympani. Both the scala vestibuli and the scala tympani are filled with perilymph, fluid with an ionic concentration similar to the extracellular milieu (high in sodium and low in potassium).
The scala media is filled with endolymph, the ionic composition of which is similar to that found

in the intracellular space (high in potassium and low in sodium). Reissner's membrane separates the scala vestibuli from the scala media and the basilar membrane separates the scala media from the scala tympani. The basilar membrane has varying physical properties throughout its length. It is narrow and taut at the base of the cochlea and becomes wide and loose at the apex. When the footplate of the stapes displaces the perilymph fluid at the scala vestibuli, the basilar membrane resonates at the base of the cochlea from high frequency fluid displacement, while the apex responds best to low frequency fluid displacement, which correlate to high and low frequency sounds, respectively. The basilar membrane functions as a Fourier transform by taking complex sounds and separating them to simpler individual frequency components along the length of the cochlea (Figure 1.1B).

Within the scala media lies the stria vascularis and the organ of Corti. The stria vascularis is a group of cells on the lateral wall of the cochlea responsible for maintaining the ionic concentration of the endolymph that produces the endocochlear potential (EP) necessary for sound transduction. The organ of Corti sits on top of the basilar membrane and is responsible for transduction of sound from a mechanical to an electrical form. Its major cell types are three rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs) that span the length of the cochlea. Stereocilia bundles, connected to each other via tiplinks, project from the apical end of hair cells bathed in the endolymph. Upward motion of the basilar membrane deflects stereocilia opening mechanotransducer channels attached to the tiplinks. Gating of potassium and calcium ions from the endolymph into the OHC cause a change in the cell's electrical potential that produces OHC motility responsible for the frequency specific 40-50 dB amplification of auditory stimuli. The IHCs are the primary sensory transducer of the auditory system. Gating of potassium and calcium ions from the endolymph into the IHC cause a change in the cell's

electrical potential and the release of neurotransmitters at the basolateral end. IHC are innervated by spiral ganglion cells (SGC), which make up the auditory nerve (AN) and send information centrally to the brain (see review; Pickles, 2008).

1.2.2 The physiological basis of AHL

Early work on presbycusis examining audiograms and postmortem temporal bones classified three distinct types of presbycusis: (i) metabolic, (ii) sensory, and (iii) neural presbycusis. Each type highlights anatomical changes directly affecting one of the cochlea's active processes that independently or in combination lead to AHL (Figure 1.2) (Schuknecht, 1964; Schuknecht, 1993; Schuknecht and Gacek, 1993).

Metabolic presbycusis affects mainly the stria vascularis and other cell types responsible for the maintenance of the endocochlear potential (EP). The EP is the positive voltage of the endolymph that bathes the stereocilia of the IHCs and OHCs necessary for hair cell depolarization. The profile of this type of hearing loss is comprised of a 10 to 40 dB hearing loss at low frequencies (1.5 kHz) coupled with a greater loss in the higher frequencies.

Sensory presbycusis primarily affects the OHCs and the support cells of the organ of Corti. OHCs depolarize when the basilar membrane moves towards the tectorial membrane deflecting the stereocilia. OHC are most susceptible to the mechanical motion of the basilar membrane and the metabolic demands placed on the cell from continuous activity.

Neural presbycusis is a common finding accompanying other forms of presbycusis. It affects mainly the IHCs, the SGC and the associated nerve fibers that transduce mechanical vibrations into neural impulses (see review; Schmiedt, 2010).

1.2.3 Studying the genetic basis of AHL

A longitudinal study of families over several generations revealed presbycusis has a strong genetic component with up to 55% and 42% heritability of sensory presbycusis and metabolic presbycusis, respectively (Gates et al., 1999). Yet, we know little of the genes that contribute to presbycusis. Genome-wide linkage and association studies take advantage of large families and heritability patterns to locate regions of interest in the genome that exhibit a statistical association with hearing loss. Only two genes, the metabotropic glutamate receptor 7 (*GRM7*; Friedman et al., 2009; Newman et al., 2012) and grainyhead like 2 gene (*GRHL2*; Van Laer et al., 2008) have a significant association with presbycusis. Seventy additional genes known for their involvement in hearing loss were assayed showing no statistical association with presbycusis (Van Laer et al., 2008). For these two studies, over 3,000 samples pooled from multiple European medical centers with genomic and audiogram data were analyzed making it an arduous and expensive undertaking with few positive results. Genome-wide linkage studies have provided some indications of genes involved in presbycusis; however, they have been fruitless in providing strong evidence. Inheritance and association studies are difficult to conduct when considering the vast heterogeneity of the human genome and the late age of trait onset after a lifetime accumulation of confounding non-genetic factors such as acoustic trauma, pathology, and ototoxicity that contribute to acquired hearing loss.

1.3 Murine models of hearing loss

To circumvent the inherent problems with human research, inbred mice are commonly used to understand the genetic basis of human presbycusis. The systematic breeding of the mouse, *Mus musculus*, for more than 20 generations has created isogenic strains that maintain

stereotyped auditory phenotypes such as AHL. By using these strains in experiments under controlled environmental conditions, we can study the physiological and genetic basis of AHL. The auditory system of the mouse is similar in both structure and physiology to humans. Thus, by understanding the physiological and genetic basis of mice AHL we may elucidate similar mechanisms in humans. Furthermore, we can use the same physiological techniques as in humans to measure hearing loss.

1.3.1 Tools to study hearing loss in mice

Auditory brainstem response (ABR) is a standard non-invasive measure of hearing sensitivity that can be conducted under anesthesia in mice; thus, behavioral response from mice is not necessary. ABR is an electroencephalographic response of the auditory system produced at the auditory nerve and brainstem nuclei in the first 10 ms following a brief acoustic stimulation. The brain's electrical response is time-locked to the stimulus, amplified, and averaged over many presentations. The stereotyped auditory-evoked response is composed of five waves representing the summed action potential of the electrical signal traveling through the brainstem nuclei (Willott, 2006; Markand, 1994). By decreasing the sound stimulus intensity we can determine the lowest sound level at which a reproducible electrophysiological response is obtained. This intensity corresponds to the hearing "threshold" and it correlates to the hearing sensitivity of the animal. Mice with hearing loss require a louder sound stimulus to produce an ABR response, which correspond to a higher threshold.

Distortion product otoacoustic emission (DPOAE) is an independent assessment of OHC function, which allows us to measure sensory presbycusis in animals. Otoacoustic emissions are sounds found in the ear canal as a result of the physiological activity in the cochlea (Kemp,

2002). These sounds are a by-product of normal OHC function and require an intact middle ear for the sounds to travel out of the cochlea and into the ear canal. When two tones with a predetermined frequency ratio are presented simultaneously into the ear canal, the signal amplification by OHCs produces an audible acoustic signal. A microphone in the ear canal picks up the sounds produced by the OHCs. We can measure the loudness of the sound, which is a direct measure of OHC function. The lack of these sounds generally corresponds to aberrant function or loss of OHCs (see review; Manley and Fay, 2007).

1.3.2 Mice as models of AHL

Models for each of the three types of presbycusis have been studied. The most common models are those for sensorineural hearing loss (SNHL) that describe a combinatory effect of neural and sensory presbycusis. Nineteen loci and four corresponding genes for AHL have been mapped in mice (Table 1.1). The most studied model, C57BL/6J (B6), suffers from SNHL that develops in the high frequencies (>32 kHz) and spreads to mid-frequencies (12-28 kHz) by one year of age (Mikaelian, 1979). The locus for this trait in B6 was denoted as *ahl* and was mapped to Chromosome (Chr) 10. *Ahl* is attributed to a single-nucleotide polymorphism (SNP) in Cadherin 23 (*Cdh23*) presumably causing reduced stereocilia stability and increased AHL susceptibility (Johnson et al., 1997; Noben-Trauth et al., 2003a). The $Cdh23^{ahl}$ allele is the most common form of AHL in more than ten different strains of mice (Johnson et al., 2000). In humans, mutations in *CDH23* are associated with Usher Syndrome (Bork et al., 2001); however, no association to presbycusis has yet been found (Hwang et al., 2012). The *ahl5* locus in the Black Swiss mouse was also mapped to Chr 10; caused by missense mutation in the PDZ domain of the GAIP-interacting protein C terminus 3 (*Gipc3*) gene (Drayton and Noben-Trauth, 2006;

Charizopoulou et al., 2011). Mutations in *GIPC3* is also responsible for nonsyndromic hearing loss in humans (DFNB 15/72/95/; Ain et al., 2007; Chen et al., 1997; Rehman et al., 2011).

AHL traits can result in complex genetic interactions where polygenic inheritance can lead to early-onset severe hearing loss. The DBA/2J (DBA2) mouse has a rapid progressive hearing loss and is autosomal recessive for three hearing loss genes; *Cdh23^{ahl}, ahl8, and ahl9* (Erway et al., 1993; Johnson et al., 2000; Nagtegaal et al., 2012). The *ahl8* locus on distal Chr 11 was identified as an R109H variant on the actin-crosslink protein Fascin 2 (FSCN2), which interacts with the CDH23 protein (Johnson et al., 2008; Shin et al., 2010). Similarly, the A/J strain with the Cdh23^{ahl} allele develops hearing loss by 4 weeks of age and has a second locus *ahl4* on Chr 10 (Zheng et al., 2009). A SNP in the Citrate Synthase (*Cs*) gene causes an amino acid change in the highly conserved protein, suggesting a functionally important implication to hearing (Johnson et al., 2012).

In the 101/H (101H) strain, hearing loss in the high-frequency range (>32 kHz) is evident by 38 days postnatally and profound by one-year of age. In this strain the loci were named progressive hearing loss (PHL). The causal genes were mapped to Chr 17 (*phl1*) and Chr 10 (*phl2*) which produce an epistatic interaction giving 101H its profound hearing loss phenotype (Mashimo et al., 2006b). Hearing loss in the ALR/LtJ mouse is early-onset and profound with three sensorineural hearing loss (SNHL) loci (*snhl2, -3, -4*) responsible for 90% of the phenotypic variations; each locus having strong individual effect and combined have additive or cumulative effects (Latoche et al., 2011).

Environmental factors may also influence AHL genes and the traits they exhibit. Mice with *Cdh23^{ahl}* or the *ahl3* locus have increased NIHL susceptibility (Erway et al., 1996; Vázquez et al., 2004; Morita et al., 2007). Black Swiss mice with *Gipc3^{ahl5}* have juvenile

audiogenic monogenic seizure (*jams1*) when exposed to high intensity noise (Misawa et al., 2002; Charizopoulou et al., 2011). Hearing loss may affect selectively the cochlea in certain frequencies. The NIH Swiss mouse has three loci for high-frequency hearing loss (*hfhl-1,-2,-3*) but also carries a mild progressive AHL at all frequencies (Keller and Noben-Trauth, 2012; Keller et al., 2011). In all reported cases of SNHL the trait is complex and causes age-related changes in hearing sensitivity resulting in AHL.

1.3.3 Hearing loss in 129S6/SvEvTac

The 129S6/SvEvTac (129S6) is a unique model of auditory traits with a remarkable resistance to NIHL (Yoshida et al., 2000a; Rosowski et al., 2003). Noise resistance (NR) in 129S6 is recessive and complex mapping to 6 chromosomal regions (Street et al., 2014). Despite its NR, 129S6 has early-onset AHL, which starts affecting high-frequencies (>24 kHz) by 4 weeks postnatally (Ohlemiller and Gagnon, 2004). Together these findings suggested that 129S6 mouse is the first demonstration of an AHL model that does not produce NIHL susceptibility (Yoshida et al., 2000b).

A large-scale screen for hearing function in multiple inbred strains, identified various 129 substrains with AHL not attributed to $Cdh23^{ahl}$ (Zheng et al., 1999; Noben-Trauth et al., 2003b). The common genetic background between 129 substrains suggests they may share a common hearing loss mutation that has not been previously mapped. Given the above evidence we were motivated to use the 129S6 strain to get a detailed characterization of the mechanisms of hearing loss and the possible genes. This research may help identify genes, which could be used for preventative and therapeutic interventions in presbycusis.

In Chapter 2, you will read about the studies conducted to understand the physiology and genetics of hearing loss in 129S6. The research will show that AHL in 129S6 mice is complex and potentially a result of multiple interacting genes. I also provide evidence of a novel *nphl* locus that maps to the same region as the *nr1* locus on Chr 17 and is non-complimentary to the *phl1* locus in the 101H inbred strain. In Chapter 3, you will read about the use of genetic tools and selective breeding techniques to identify in high resolution the genetic locus of *nphl*. In Chapter 4, I provide a summary of the findings and speculate on the possible candidate genes and mechanisms of action for the phenotypes on proximal Chr 17 as well as possible future studies.

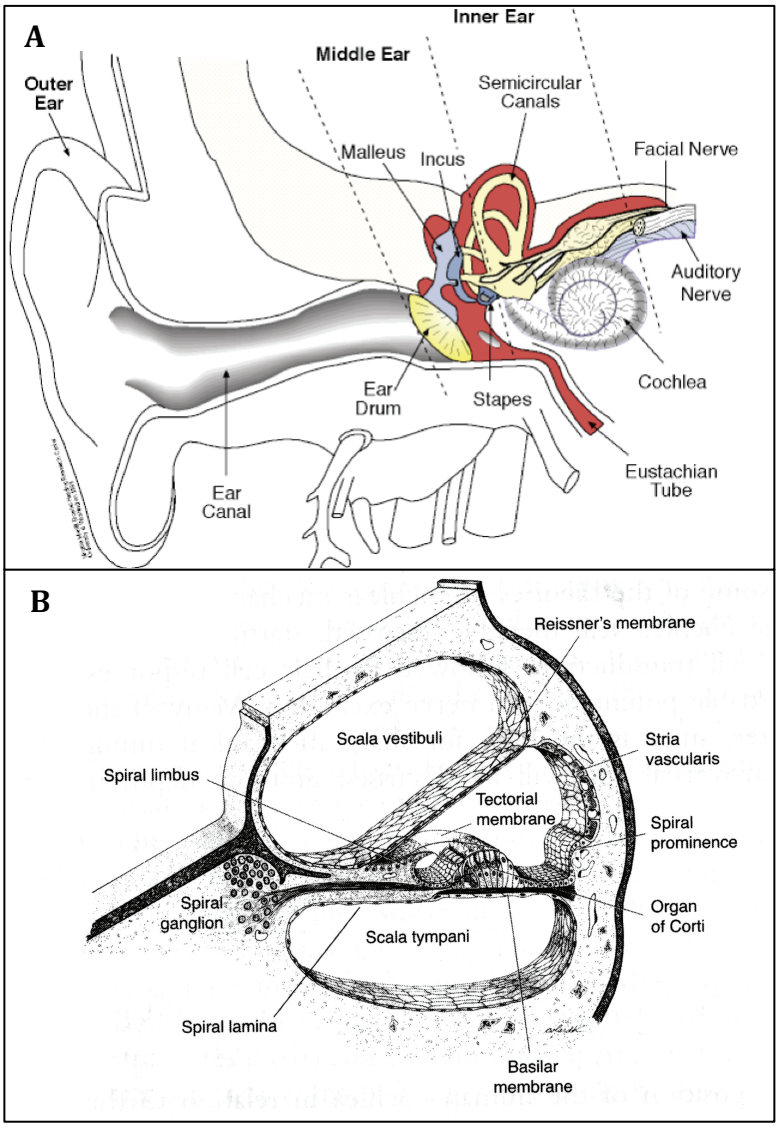
A
Inner Ear
Middle Ear
Outer Ear
Semicircular Canals
Malleus
Incus
Facial Nerve
Auditory Nerve
Cochlea
Ear Drum
Stapes
Eustachian Tube
Ear Canal
B
Reissner's membrane
Scala vestibuli
Stria vascularis
Spiral limbus
Tectorial membrane
Spiral prominence
Spiral ganglion
Organ of Corti
Scala tympani
Spiral lamina
Basilar membrane

Figure 1.1 The human auditory system and cochlea.

(A) Schematic of a coronal section of the human's auditory system emphasizing the outer, middle and inner ear. Sound is funneled to the external auditory meatus reverberating against the tympanic membrane (ear drum). The ossicular chain (Incus, Malleus, Stapes) in the middle ear transfers sound energy into the fluid filled cochlea in the inner ear. The inner ear is comprised of the vestibular and auditory sensory organs. **(B)** A cross section of the cochlea cavity with the scala vestibuli, scala media, and scala tympani. The stria vascularis at the lateral wall is responsible for maintaining the endocochlear potential (EP). The organ of Corti seating on top of the basilar membrane is responsible for sound transduction by converting the mechanical motion of the basilar membrane into an electrical signal that travels centrally to the brain through the spiral ganglion cells (images adapted from Pickles, 2008).

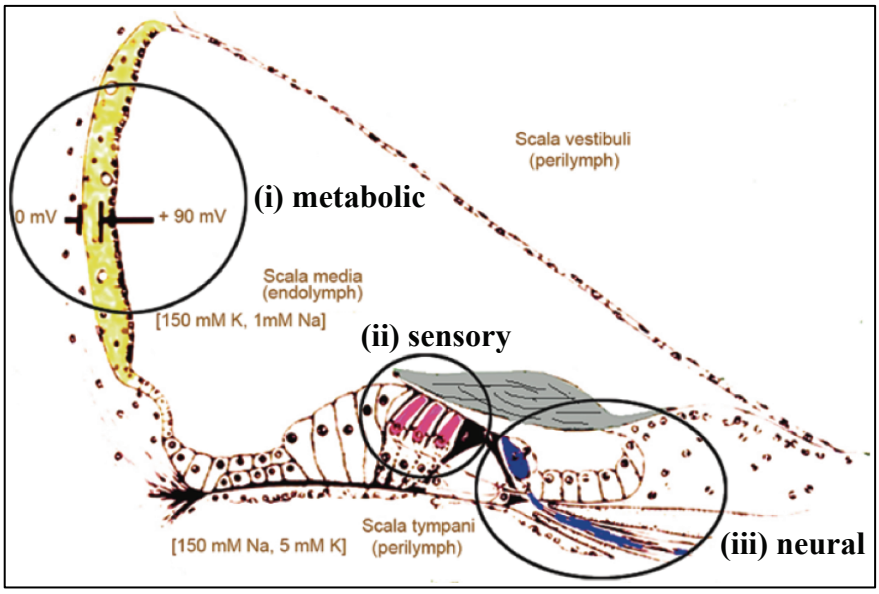

Figure 1.2 A cross section of a single turn of the cochlea.

The three systems responsible for the active processes underlying basic cochlear function and the source of the three types of presbycusis are highlighted and circled. Metabolic presbycusis affects the stria vascularis on the lateral wall, which produces the endocochlear potential (EP) of the scala media. Sensory presbycusis affects the outer hair cells (OHCs) and support cells involved in the frequency specific amplification of the cochlea's mechanical vibration. Neural presbycusis affects the inner hair cells (IHCs), the spiral ganglion cells (SGC), and associated afferent nerve fibers responsible for the transduction process where mechanical vibrations are transduced to neural impulses that are sent to the brain and are perceived as sounds. (Image adapted from Schmiedt, 2010)

Locus (gene)	Chr	Location	Affected strain	References
ahl (*Cdh23*)	10	60 Mb	Many	1, 2, 3
ahl2	5	65-100 Mb	NOD/LtJ	4
ahl3	17	65-69 Mb	C57BL/6J	5, 6
ahl4 (*Cs*)	10	120-130 Mb	A/J	7, 8
ahl5 (*Gipc3*)	10	81Mb	Black Swiss	9, 10
ahl6	18	64-76 Mb	Black Swiss	10
ahl8 (*Fscn2*)	11	120 Mb	DBA/2J	11, 12
ahl9	18	73-77 Mb	DBA/2J	13
hfhl1	7	20-38 cM	NIH Swiss	14, 15
hfhl2	8	20-32 cM	NIH Swiss	14
hfhl3	9	30-50 cM	NIH Swiss	15
phl1	17	8-29 Mb	101/H	16
phl2	10	48-70 Mb	101/H	16
snhl1	10	54-60 Mb	ISS	17
snhl2	1	133-172 Mb	ALR/LtJ	18
snhl3	6	121-143 Mb	ALR/LtJ	18
snhl4	10	49-90 Mb	ALR/LtJ	18
mtdna (mt-Tr)	mitochondria		A/J	19

Table 1.1 List of loci and genes identified to cause sensorineural hearing loss in mice strains.

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CHAPTER 2:

Characterization of AHL in 129S6 Mouse and

The Identification of a Novel Locus for Non-Progressive Hearing Loss

Parts of this chapter are in preparation for a manuscript publication

With the following title and authors:

Short title: Non-progressive Hearing loss

A Novel Locus for Non-Progressive Hearing Loss Maps to Proximal Chromosome 17 of the 129S6/SvEvTac Mouse.

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2.1 Summary

Hearing loss is the most common sensory deficit, affecting over 5% of the world's population. In the USA alone, 37 million Americans suffer from acquired hearing loss (Pleis and Lethbridge-Cejku, 2007), due primarily to a lifetime accumulation of noxious environmental factors as well as presbycusis, the progressive decline of hearing associated with aging. Studies aimed to elucidate the genetic basis of progressive hearing loss and its effects on the cochlea are difficult to conduct due to the vast heterogeneity of the human genome and the lifetime accumulation of environmental insult that contribute independently to hearing loss among individuals.

To circumvent these confounds, inbred mice strains with AHL are commonly used to understand the genetic basis of presbycusis. The 129S6/SvEvTac (129S6) inbred mouse is a unique model of presbycusis in that it has early-onset AHL starting by one month of age, as well as having a remarkable resistance to NIHL (Yoshida et al., 2000a; Rosowski et al., 2003; Ohlemiller and Gagnon, 2004; Street et al., 2014). All previous mice with AHL had a predisposition for NIHL, suggesting these traits were associated (Erway et al., 1996; Davis et al., 2001; Nemoto et al., 2004a; Vázquez et al., 2004). The 129S6 mouse is the first model of AHL that is not vulnerable to NIHL (Yoshida et al., 2000a). Most of the work in the 129S6 strain has focused on understanding the genetic basis for 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 CBA/CaJ (CBACa) mouse identified six NR quantitative trait loci (QTL) in the mouse genome. The strongest statistical association with NR was consistently 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., 2006a; Morita et al., 2007).

The C57BL/5J (B6) mouse, which harbors *ahl* locus on Chr 10 attributed to a SNP in Cadherin 23 (*Cdh23*) (Johnson et al., 1997; Noben-Trauth et al., 2003a), also carries a second AHL allele, *ahl3*, on distal Chr 17. Congenic B6 mice have reduced susceptibility to AHL and NIHL when carrying distal Chr 17 from the MSM inbred mouse derived from *Mus musculus molossinus* that shows resistance to AHL (Nemoto et al., 2004b; Morita et al., 2007).

In the 101H inbred mouse strain, hearing loss is early-onset, never showing an indication of good hearing and becoming profoundly deaf by 1 year of age. Its phenotype was named progressive hearing loss (PHL) and two loci were mapped to proximal Chr 17 (*phl1*) and Chr 10 (*phl2*). These genes have an epistatic interaction that results in a profound hearing loss phenotype (Mashimo et al., 2006b). To date, the identity of the PHL genes is unknown as well as how many additional strains of mice may share the same hearing loss-causing genes.

The *phl1* locus on proximal Chr 17 and the *ahl3* locus on distal Chr 17 are in the same regions of 129S6 mouse that were shown 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 previously generated congenic mice to investigate the presence of hearing loss genes on Chr 17 of 129S6. We used non-invasive ABR and DPOAE measurements to get a detailed characterization of the hearing loss mechanisms in this strain and the congenic mice. Consistent with previous studies, 129S6 has early-onset AHL. Our research shows AHL in 129S6 mice is complex and multigenic. We also provide evidence of

a novel *nphl* locus that maps to the *nr1* locus of 129S6 and is non-complimentary to the *phl1* locus in the 101H inbred strain.

2.2 Methods

2.2.1 Rearing and breeding of experimental animals

All experimental animals were born and reared in our animal care facility in a quiet environment (<40 dB) with 12-h light/dark cycle, food and water available *ad libitum*, 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 recombinant inbred (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. Ninety-seven 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 7 and 5 generations to create the CBACa.129S6-(*D17Mit143-D17Mit100*)/Tem and CBACa.129S6-(*D17Mit119-DMit1*)/Tem congenic mice, 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 (Figure 2.1).

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.) xylazine (6.5 mg/kg, i.p.) and their heart rate monitored continuously with an oscilloscope (Tetronix TDS 1002, OR, USA). Body temperature was maintained at 37^{o}C throughout the experiment with an isothermal heating pad when conducting ABR (Deltaphase, Braintree Scientific, MA, USA) or with a heating plate monitored by a rectal probe and temperature feedback controller when conducting DPOAE (HP-1M, RET-3, TCAT-2, Physitemp Instruments, NJ, USA).

2.2.2 Physiological tests by ABR

We examined auditory sensitivity and neural responses in the mice by free-field ABR. 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 they were delivered from a transducer one foot away from animal's head. To determine threshold, stimuli intensity was decreased from 80 dB in 20 dB steps, with smaller 5 dB steps around threshold. Neural response to the stimuli was recorded from two subcutaneous needle electrodes inserted at the vertex and ventrolateral to the left pinna with a ground electrode in the hind leg. Evoked responses were initially filtered (0.1-3 kHz passband) and amplified (1,000x; 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 (96500 samples/sec) and averaged for 350 or 500 samples at threshold intensities. We defined hearing threshold as the lowest sound level ($\pm$5 dB) that elicited a reproducible ABR wave within 10 ms of stimuli onset. Experiments were conducted with custom software and waveforms were saved for later analysis. When responses

were absent at the maximum level presented or above 100 dB, a threshold value 5 dB higher was assigned.

2.2.3 Physiological tests by DPOAE

Sensory response or outer hair cell function of the auditory system was measured by DPOAE. Prior to DPOAE measurements, 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 f1 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. DPOAE's 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/0 growth function the intensity of f_2 that produced $2f_1$-f_2 distortion of 0 dB. All responses above 60 dB were assigned a threshold value of 65 dB because non-biological distortions were observed for intensities greater than 60 dB.

2.2.4 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 post-test to compare group means by frequency when applicable.

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 the values were. 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.

2.3 Results

2.3.1 Physiology of early-onset, high-frequency hearing loss

Murine models of presbycusis are defined by two general criteria; (i) hearing loss that begins in the high-frequency range, usually above 32 kHz, and (ii) hearing that deteriorates in an age-dependent manner. 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 one month of age (30 days $\pm$ 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 strain (data not shown). Consistent with previous findings, 129S6 mice had an average hearing loss of 18.76 dB SPL between 24-40 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 24-40 kHz (Figure 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 24-40 kHz compared the CBACa mouse. The ABR thresholds from the CBACa.129S6-(*dist17*) strain were not statistically different from CBACa mice at any frequency tested. Threshold estimates from the experimenter and the blind observer show a high degree of correlation (R^2=0.9423, slope of best-fit values 0.9956; Figure 2.2B). These results are evidence that visual threshold calls are an accurate representation of hearing sensitivity observed via ABR and do not represent experimenter's bias.

2.3.2 Measures of early-onset outer hair cell deficit

Statistically, 129S6 and CBACa.129S6-(*prox17*) mice had normal ABR thresholds in the mid-frequency range (8-16 kHz). Qualitatively, however, 129S6 mice showed reduced ABR amplitude at these frequencies compared to CBACa (Figure 2.3A). In mice, Wave I of the ABR represents the auditory nerve afferents firing synchronously (Moller, 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 of frequencies with statistically normal thresholds by evaluating Wave I amplitude (peak I) and latency because these may reveal differences in peripheral hearing sensitivity not observed via ABR threshold (see review; Tremblay and Burkard, 2007). The 129S6 mouse had reduced Wave I amplitude ($F_{3, 44}$=4.19, P<0.05) of 1.39μV at 12 kHz (t=4.55, P<0.001) and 1.04μV at 16 kHz (t=3.418, P<0.01) compared to the CBACa mouse (Figure 3B). On average 129S6 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 (Figure 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 in either congenic strain when compared to the CBACa mouse.

To understand the contribution of the outer hair cells (OHCs) to the differences in ABR suprathreshold 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 were not statistically different from those shown in Figure 2.2 (data not shown). 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 significant phenotype difference from the CBACa controls. In all three strains, 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, the CBACa.129S6-*(prox17*) strain had normal DPOAE threshold between 8-16 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 (Figure 4A). Genotype and frequency account for approximately 34.89% and 15.22% of the total variance observed, respectively. The DPOAE data 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 (Figure 4B) and 32 kHz (Figure 4C) where 129S6 mice showed reduced growth function. The CBACa.129S6-(*prox17*) strain showed reduced growth function only at 32 kHz.

2.3.3 Ahl physiology in 129S6 mice

The second criterion for a presbycusis model requires that hearing deteriorate in an age-dependent manner. Mice with early-onset deafness similar to that seen in 129S6 mice generally have rapidly progressive phenotypes within two post-natal months (Mashimo et al., 2006a; 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 progressive hearing loss was recorded at 7 months with a 13-18 dB loss between 12-40 kHz (t-test, $P<0.01$) and a 13-26 dB loss between 8-40 kHz by 13 months ($F_{4, 72} = 83.80$, $P <0.001$; Figure 2.5A). 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; Nemoto et al., 2004a; Vázquez 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 5 ABR tests by 13 months (n= 10) to mice that received a single ABR test at 13 months (n=18). We found that repeated ABR testing does not alter significantly hearing thresholds in 129S6 mice ($F_{1, 26}=0.27$, $P=0.6$; Figure 2.5B). 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 1-month to 13-month ABR thresholds to assess the amount of AHL in the inbred and congenic mice under investigation. All ”aged” (average 13 months) mice used 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 2.5. Consistent with previous reports, CBACa mice maintain good auditory sensitivity past one year of age (Zheng et al., 1999). No age effect was found in the thresholds of 13-month-old compared to 1-month-old CBACa mice ($F_{1, 29}$=1.04, P=0.3136; Figure 2.6A). The 129S6 strain had age-dependent hearing loss by one-year of age ($F_{1, 30}$=33.61, P<0.001; Figure 2.6B). Conversely, CBACa.129S6-(*prox17*) mice demonstrated no age-dependent hearing loss despite having early-onset, high-frequency hearing loss ($F_{1, 26}$=2.25, P=0.1457; Figure 2.6C). There is an interaction effect between frequency and age for CBACa ($F_{5, 145}$=2.49, P<0.05) and CBACa.129S6-(*prox17*) ($F_{5, 130}$= 4.77, P<0.001) mice, 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 at 16 kHz (t=3.20, P<0.05). CBACa.129S6-(*dist17*) mice had significantly better thresholds ($F_{1, 17}$=10.09, P<0.01) by 10 months of age. On average, their thresholds are reduced by 7.91 dB at 40 kHz (t=3.150, P<0.05; Figure 2.6D). No statistical differences to CBACa were observed when mice were reanalyzed for hearing loss at 13-months (data not shown). The 101H inbred strain exhibits acute early-onset hearing loss in the mid-to-high frequency range and profound hearing loss at all frequencies by one year of age ($F_{1, 19}$=236.91, P< 0.001; Figure 2.6E). Threshold estimates from the experimenter and the blind observer show a high degree of correlation (R^2=0.9497, slope of best-fit values 0.9691; Figure 2.6F). These

results are evidence that visual threshold calls are an accurate representation of hearing sensitivity observed via ABR and do not represent experimenter's bias.

2.3.4 Middle ear and OHC function explains AHL

We used DPOAE measurements from a subset of 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; Figure 2.7A, B, 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 mice subjects. However, there was no indication that these three mice suffered from closure of the external auditory canal. In 5 out of 7 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 (Figure 2.7D). 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 the system (60 dB). Results from I/O growth function indicate a lack of measurable distortion product in aged 129S6 mice (Figure 2.7E, 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; Figure

2.7G, H, I). All other frequencies tested at 13 months did not show a significant difference to 1 month DPOAE thresholds in CBACa.129S6-(*prox17*) mice.

2.3.5 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 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 ($F_{2, 32}$= 3.15, P>0.05), suggesting genes for hearing loss in the 101H and 129S6 strains are autosomal recessive. These results also indicate that gene alleles from CBACa mice do not contribute to hearing loss in either strain (Figure 2.8A). 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 (Figure 2.8B). The ABR tests revealed a significant effect of genotype ($F_{2, 40}$= 11.65, P<0.001) on the hearing sensitivity of the 101H and 129S6 hybrids. Compared to CBACa mice controls, 129S6101HF1 mice had significantly elevated thresholds

(between 4-21 dB) at 8 (t=3.11,P<0.05), 32 (t=3.11, P<0.01), and 40 kHz (t=4.408, P<0.01). In the CBACa.129S6-(*prox17*)101HF1 hybrids had significantly higher thresholds (between 13-27 dB) at 32 and 40 kHz (t=13.38, 27.56 respectively, P<0.01). Thresholds between these hybrids were not statistically different from each other at all frequencies.

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) and CBACa.129S6-(*prox17*)101HF1 ($F_{1,23}$=8.03, P<0.01) hybrid mice, showed age-dependent hearing loss by 13 months of age. However, in 129S6101HF1 thresholds were significantly higher by an average of 18.55 dB at all frequencies tested except at 8 kHz (Figure 2.9A). 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; Figure 9B). These results confirm that 101H and 129S6 share a gene in the proximal Chr 17 region that engenders NPHL.

2.4 Discussion

2.4.1 AHL linked to nr1 locus in 129S6 mice

Consistent with previous results, the inbred 129S6 mouse has an average hearing loss of 18.76 dB between 24-40 kHz by 30 days of age. (Yoshida et al., 2000a; Rosowski et al., 2003; Ohlemiller and Gagnon, 2004). When analyzing for the presence of AHL genes in NR loci, we find CBACa.129S6-(*prox17*) mice have an average hearing loss of 19 dB by 30 days similar to the parental 129S6 strain. Conversely, CBACa.129S6-(*dist17*) does not carry susceptibility for AHL at 30 days, 10 months, or 13 months. We focused the remainder of our study on analyzing the phenotype present on 129S6 and proximal Chr 17 (D17Mit143-D17Mit100).

2.4.2 AHL in 129S6 mice

We found that ABR thresholds in 129S6 mice are stable past two months of age and multiple ABR measurements did not accelerate hearing loss (Figure 2.5). As early as 4 weeks postnatally, we observed a decreased ABR Wave I amplitude in 129S6 mice (Figure 2.3A). Wave I of the ABR waveform corresponds to the synchronous firing of spiral ganglion cells (SGCs). A reduced Wave I amplitude may indicate problems in the cochlea's active processes: (i) reduced endocochlear potential (EP) (Hellstrom and Schmiedt, 1990; Schmiedt et al., 2002), (ii) the loss of SGC, inner hair cells (IHC) and their synapses (Kujawa and Liberman, 2009; Sergeyenko et al., 2013), and (iii) the loss of outer hair cells (OHC), or a combination of these factors. Ohlemiller and Ganon (2004) previously showed that 129S6 mice maintain normal EP levels and SGC numbers despite their AHL phenotype. Thus, the decrease in Wave I amplitude could be due to abnormal OHC function which we measured via DPOAE. We observed reduced DPOAE thresholds and a large subject variance in the DPOAE measurements of 129S6 mice (Figure 2.4) as shown previously (Martin et al., 2007). Our ABR and DPOAE measures are in agreement with an early-onset sensory hearing loss, which is categorized by OHC dysfunction with normal EP levels and SGC and IHC numbers. The reduced DPOAE threshold and suprathreshold ABR measures are the earliest indication of hearing deficit and predict what later will become reduced ABR thresholds in the mid-frequency range indicating AHL in 129S6 mice.

The consistent appearance of what may be middle ear fluid in our "aged" (>13 months) 129S6 mice, presents a greater challenge for determining the source for AHL. Mice with effusion-like conditions in the middle ear similar to those observed in 129S6 mice have elevated ABR thresholds by an average of 12-19 dB (Qin et al., 2010). However, the hearing loss we observed from ABR thresholds in aged 129S6 mice 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 suggests these additional factors contribute to the levels of hearing loss we see in 129S6 mice. These data together suggest a combinatory effect of conductive and sensory hearing loss that is responsible for the progressive phenotype in 129S6 mice. A careful analysis of sensory and conductive hearing loss in the same subjects is warranted to tease apart their individual contributions to the AHL phenotype.

2.4.3 nphl locus at proximal Chr 17

One of our most interesting findings is the absence of AHL in CBACa.129S6-(*prox17*) mice. Similar to 129S6 mice, the CBACa.129S6–(*prox17*) strain had early-onset, high-frequency hearing loss. However, at 1 month it had ABR suprathresholds and DPOAE thresholds similar to CBACa in the mid-frequencies (8-16 kHz), and no significant age related hearing loss by 13 months. The presence of normal mid-frequency thresholds at 1 month correlates with good hearing sensitivity at later ages except at 16 kHz, which is the only frequency exhibiting AHL as evidenced by DPOAE threshold.

The recipient strain of our congenic mice, CBACa, is known for having good hearing throughout its life; however, it is not resistant to age-related changes in hearing sensitivity (Sergeyenko et al., 2013). In our hands, CBACa develops as much as 6 dB of hearing loss by 13 months while the 129S6 strain develops an average hearing loss of 60 dB by13 months. The average hearing loss in the CBACa.129S6-(*prox17*) strain is 5 dB which is within what we expect for the CBACa background. The above data demonstrate that genes on proximal Chr 17 contribute exclusively to early-onset, high-frequency hearing loss, but not the AHL we observed

in the 129S6 strain. Henceforth, we define a locus for non-progressive hearing loss (*nphl*) by markers D17Mit143-D17Mit100. The extensive hearing loss in aged 129S6 mice which is not present in CBACa.129S6-(*prox17*) suggests that multiple genes in 129S6 mice cause AHL, a common occurrence amongst other AHL models (Erway et al., 1993; Keithley et al., 2004; Kane et al., 2012; Latoche et al., 2011; Keller and Noben-Trauth, 2012).

2.4.4 Genetic Non-complementation in 129S6 mice

The PHL phenotype described in the 101H inbred strain is early-onset by 38 days and profound by one year, a product of the epistatic interaction between genes mapped to Chr 17 (*phl1*) and Chr10 (*phl2*) (Mashimo et al., 2006a). We studied the interaction between *nphl* and *phl1* in a genetic complementation study since both genes mapped to the same proximal region of Chr17 and they are inherited recessively (Figure 2.8A). A cross between 129S6 and 101H mice (129S6101HF1) did not rescue the high-frequency hearing loss observed in the 129S6 mouse, which suggests that *phl1* and *nphl* are allelic. This is plausible because 129S6 and 101H share similar genetic and ancestral backgrounds (Beck et al., 2000; Witmer et al., 2003). The hybrids 129S6101HF1 and CBACa.129S6-(*prox17*)101HF1 mice have identical phenotypes suggesting that 129S6 does not carry the *phl2* locus at Chr 10 that gives the 101H mouse its profound deafness from the epistatic interaction between *phl1* and *phl2* (Mashimo et al., 2006a). It is worth noting that the phenotype of the 129S6101HF1 and CBACa.129S6-(*prox17*)101HF1 hybrids are not as severe as the phenotype in either parental strain. These findings suggest that the *nphl* gene may interact differently with genes in the 129S6 and 101H background giving each mouse a unique hearing loss phenotype. The CBACa.129S6-(*prox17*)101HF1 cross may represent the effects of the *nphl* gene in isolation of other interacting hearing loss genes. An

alternative hypothesis is that there are two genes for hearing loss within proximal Chr 17. In this alternative hypothesis, a double heterozygous mouse for each gene could produce non-complementation in a digenic interaction if the genes 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.

The study of AHL in 129S6101HF1 and CBACa.129S6-(*prox17*) hybrids reveal further differences in the phenotypes. The 129S6101HF1 hybrid has AHL between 15-28 dB similar to that seen in aged 129S6 mice between 12-40 kHz. The CBACa.129S6-(*prox17*) hybrid has a marginal AHL between 9-12 dB at 32 and 40 kHz, characteristic of the results seen in homozygous CBACa.129S6-(*prox17*). The above results further support the findings that genes at proximal Chr 17 do not contribute to the progressive hearing deterioration in 129S6, thus a different locus outside of this region in 129S6 is responsible for AHL. Both hybrid strains have 50% of their genome from 101H and different AHL phenotypes, suggesting alleles in the 101H background alone are not sufficient to cause AHL. The interaction between 101H and 129S6 at an alternative locus outside of *Prox17* could induce AHL. Alternatively, 129S6 genes outside of Chr 17 may cause autosomal dominant AHL in the 129S6101HF1 hybrid. Since the CBA129S6F1 hybrid had normal hearing at 1 month of age, we did not age these mice to study a possible AHL phenotype.

2.4.5 Candidate genes

In the Chr 17 region proximal to marker *D17Mit175*, we propose the following candidate genes for the *nphl* locus, 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 the production of reactive oxygen species (ROS) necessary for otoconia synthesis. Mutant mice exhibit impaired vestibular response from the lack of otoconia in the utricle and saccule (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).

Three claudin genes are found within the proximal region of Chr 17. These proteins play a critical role in the maintenance of the EP by creating the ion selective barrier of the endolymph, essential for the proper function of the sensory hair cells. The Claudin 6 (*Cldn6*) and Claudin 9 (*Cldn9*) genes create tight-adherens junction complexes for ionic selectivity and structural integrity between OHC and Deiter cells (Nunes et al., 2006a). 129S6 mice have normal EP levels throughout their lives (Ohlemiller and Gagnon, 2004) which suggests these may not play a role; however, a point mutation in *Cldn9* disrupts the ionic composition of the microenvironment at Nuel's space causing OHC death despite having normal EP levels (Nakano et al., 2009). Claudin 20 has not yet been studied in the auditory system, but it may also play a role in the proper function of the peripheral auditory system.

Within this region, two genes causing non-syndromic hearing loss in humans are found. One, The Lipoma HMGIC Fusion partner-like 5 (*Lhfpl5*) gene is responsible for autosomal recessive hearing loss in humans (DNFB67; Shabbir et al., 2006), and deafness in the hurry-scurry (hscy) mouse (Longo-Guess et al., 2005). This gene codes for the Tetraspan Membrane

Protein of Hair Cell Stereocilia (TMHS), a component of the mechanotransduction machinery of hair cells (Xiong et al., 2012). Two, the Transmembrane Protease Serine 3 (*Tmprss3*) gene. Mutations in *Tmprss3* causes hearing loss in humans (DFNB8/10; Ben-Yosef et al., 2001) and is thought to activate the epithelial sodium channel (*Scnn1*) which is co-expressed with *Tmprss3* in the cochlea and presumably plays a role in EP function (Guipponi et al., 2002).

The *Axin1* 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). Lastly, the anion exchange transporter (*Slc26a8)* is a candidate gene although no link to hearing or vestibular function has been reported. *Slc26a8* is part of the same solute carrier family as Pendrin (*SLC26A4*) that causes pendred syndrome associated with profound sensorineural auditory impairment (Luxon et al., 2003), and Prestin (*Slc26a5*) the motor protein of OHC required for electromotility and the cochlear amplifier (Zheng et al., 2000; Liberman et al., 2002).

In addition to the non-progressive hearing loss (*nphl*) described here, there are two additional overlapping loci within proximal Chr 17, *nr1* in 129S6 mice and *phl1* in 101H mice {Street 2014; Mashimo 2006). Any of the candidate genes above or a non-identified gene in this region may be implicated in any of the three phenotypes attributed to the proximal Chr17 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., 2003b; Mashimo et al., 2006a; Noben-Trauth et al., 2010; Latoche et al., 2011; Charizopoulou et al., 2011). It is also likely that the 30Mb proximal region of Chr 17 with similar gene density has separate genes for NR and NPHL in 129S6 as well as a different PHL gene in the 101H strain.

The NIH Swiss mouse has three loci for high-frequency hearing loss (*hfhl-1,-2,-3*) with similar frequency selective hearing loss but with mild progressive threshold shift at all frequencies tested (Keller and Noben-Trauth, 2012; Keller et al., 2011).The non-progressive nature of the hearing loss at Chr 17 and the stable OHC function past one year of age suggest that the *nphl* gene may not be deleterious to the OHCs, which often die as a secondary effect of abnormal function and result in AHL. Gene products can have graded expression along the organ of Corti. The *nphl* gene might have a frequency-specific effect to reduce sensitivity to high-frequency stimuli. This in turn could prevent acoustic injury from the over stimulation of OHCs. In this hypothesis, NR and NPHL could be allelic.

2.4.6 Human non-progressive deafness loci

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 in contrast with our gene at *nphl* that is autosomal recessive. The number of affected individuals with non-progressive hearing loss may be misrepresented in the general population as patients with this type of condition may be unaware of the problem throughout their lives and it could be misdiagnosed as a speech problem in infancy or hearing loss due to noisy environments in adults. There is a clear relevance to the human population as we can better identify genes and treatments that affect frequency-selective hearing loss.

As for the relevance in mouse physiology and genetics, the common genetic background between 129 substrains suggests they may share a common AHL hearing loss allele not previously mapped (Zheng et al., 1999; Noben-Trauth et al., 2003b). Our current study provides additional understanding of the genetics and physiology of the hearing traits associated with the 129 background whose embryonic stem cells are commonly used for genetic targeting of recombinant mice, and hearing loss trait currently preclude their use in targeting of hearing-related genes.

marker	cM	Mbp	CBACa.129S6-(*prox17*)	CBACa.129S6-(*dist17*)
D17Mit143	4.9	8.4	129S6	CBACa
D17Mit113	8.1	12.0	129S6	CBACa
D17Mit100	13.1	26.2	129S6	CBACa
D17Mit175	17.3	31.8	CBACa	CBACa
D17Mit51	19.7	43.5	CBACa	CBACa
D17Mit67	24.9	49.3	CBACa	CBACa
D17Mit180	26.7	51.4	CBACa	CBACa
D17Mit109	30.2	58.1	CBACa	CBACa
D17Mit119	38.2	66.9	CBACa	129S6
D17Mit93	45.2	73.8	CBACa	129S6
D17Mit1.1	56.7	88.2	CBACa	129S6

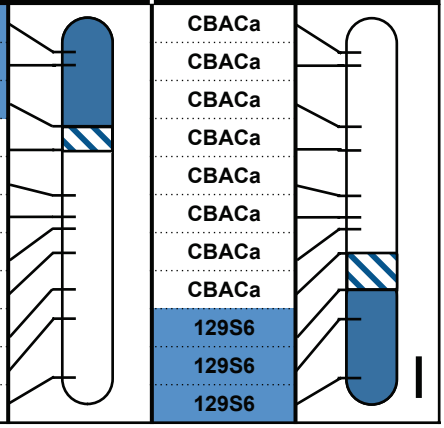

Figure 2.1 Representation of Chr 17 in congenic strains.

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 10Mbp.

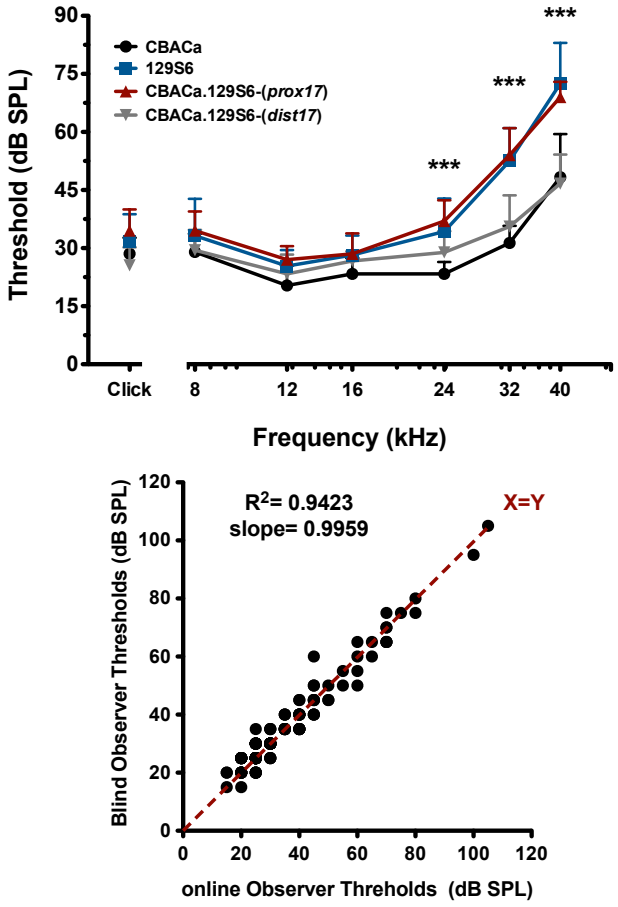

Figure 2.2 Early-onset hearing loss in 129S6 mice.

(A) Mean ABR thresholds (±SD) as a function of frequency in one-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 with each genotype. ***P<0.001. **(B)** Blind observer verification of visual estimates of ABR thresholds. Each point represents the threshold value for a representative ABR response. The dashed line represents the unity line (X=Y). R^2 and slope of best-fit values are indicated. Blind observer threshold estimates (y-axis) for a subset (45%) of recordings in (A) correlate with estimates made by the author (x-axis).

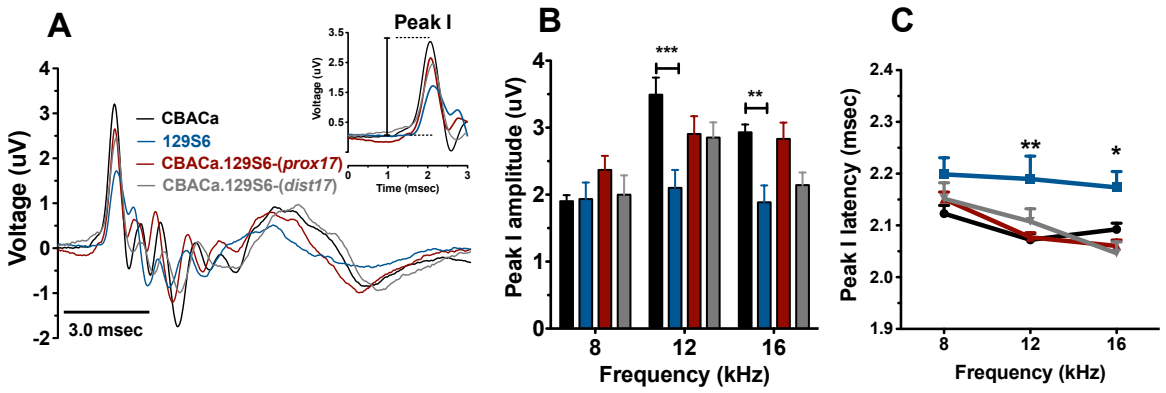

Figure 2.3 ABR suprathreshold reveal amplitude and latency differences.

Reduced wave I amplitude and increased peak I latencies at frequencies with normal ABR thresholds in 129S6 mice. (**A**) Mean ABR waveform for all ABR subjects assayed at 80dB for 12 kHz. (**A insert**) Wave I amplitude (peak I) was calculated as the difference of the baseline recording 1.0 msec after the onset of the stimuli to the peak of wave I. (**B**) Mean (± SEM) peak amplitude for the three frequencies with normal ABR threshold show 129S6 is statistically different from CBACa at 12 kHz and 16 kHz, but there is no difference between CBACa.129S6-(*prox17*) or CBACa.129S6-(*dist17*). (**C**) Mean (± SEM) Peak I latency in 129S6 was increased at 12 kHz and 16 kHz but not at 8 kHz. **P*<0.05, ***P*<0.01, *** *P*<0.001.

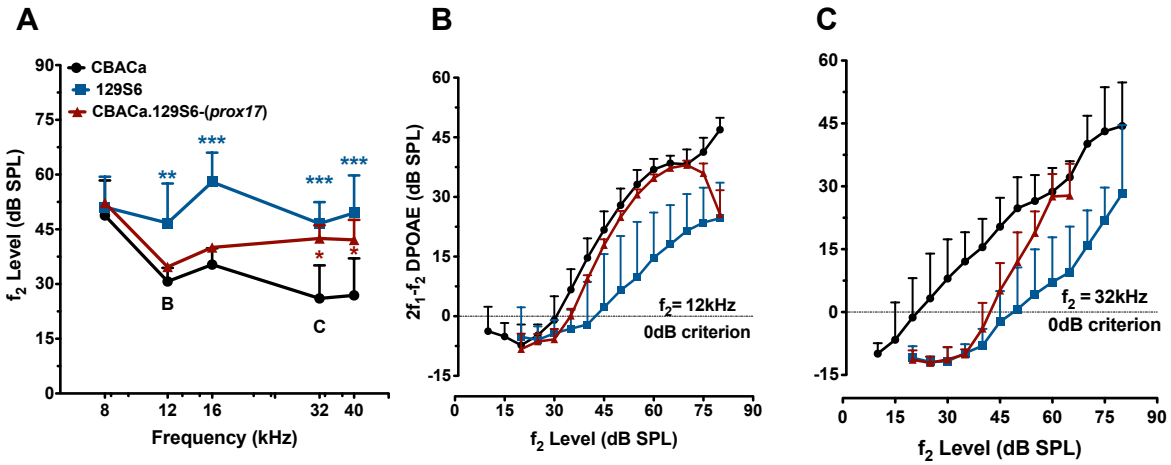

Figure 2.4 One-month old 129S6 show increased DPOAE thresholds.

Early-onset OHC dysfunction in the mid-frequency range despite normal ABR thresholds. (**A**) Mean (±SD) DPOAE audiogram based on the f2 level that elicits a 0 dB response of the 2f1-f2 distortion product. 129S6 (n=5) is significantly different from CBACa (n=6) at frequencies between 12-40 kHz. CBACa.129S6-(*prox17*) (n=4) is different from CBACa only at 32 kHz and 40 kHz. Mean (±SD) DPOAE I/O growth function of the 2f1-f2 distortion product as a function of the f2 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.

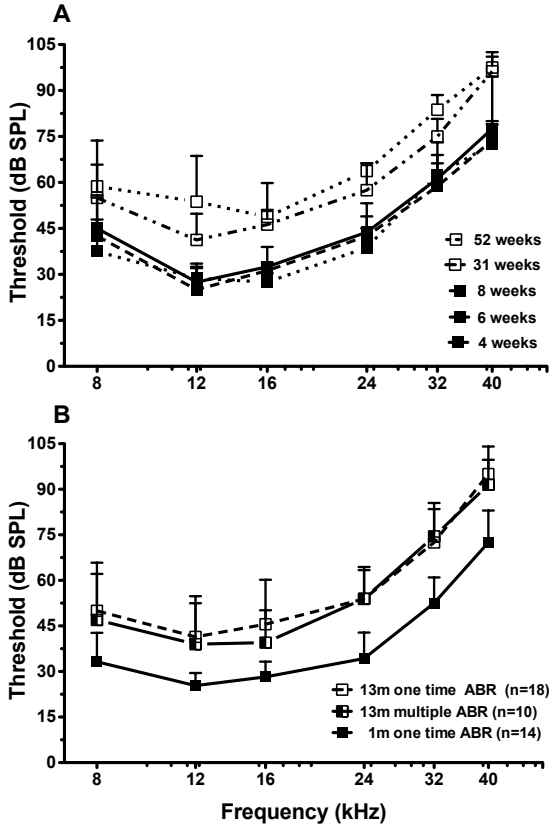

Figure 2.5 129S6 has a slow progressive hearing loss.

(**A**) 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 5 ABR assays by one-year of age (n=10) have mean ($\pm$SD) threshold indistinguishable from animals with a single ABR assay by the same age (n=18).

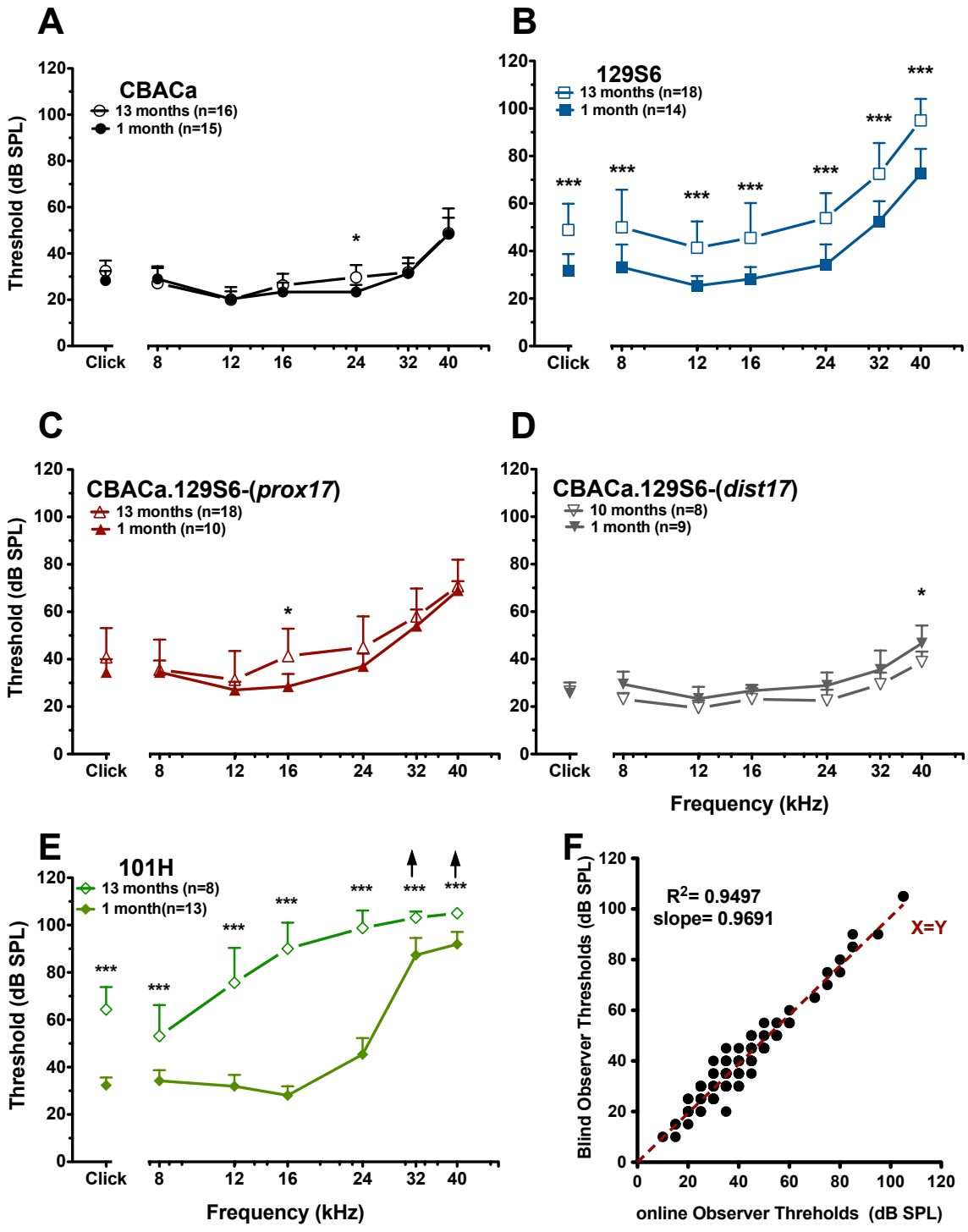
A
CBACa
13 months (n=16)
1 month (n=15)
Threshold (dB SPL)
Click
8
12
16
24
32
40
B
129S6
13 months (n=18)
1 month (n=14)
C
CBACa.129S6-(prox17)
13 months (n=18)
1 month (n=10)
D
CBACa.129S6-(dist17)
10 months (n=8)
1 month (n=9)
Frequency (kHz)
E
101H
13 months (n=8)
1 month(n=13)
Frequency (kHz)
F
R2= 0.9497
slope= 0.9691
X=Y
Blind Observer Thresholds (dB SPL)
online Observer Threholds (dB SPL)

Figure 2.6 Genes on proximal Chr 17 do not contribute to AHL despite early-onset phenotype.

Hearing thresholds (±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 (± 1 monrh). 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. There was an interaction effect such that age affects frequencies differently at 24 kHz in CBACa and 16 kHz in CBACa.129S6-(*prox17*). 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. Bonferroni post-hoc test $*P<0.05$,$***P<0.001$, arrows represent means threshold above 100 dB. (**F**) Blind observer verification of visual estimates of ABR thresholds. Each point represents the threshold value for a representative ABR response. The dashed line represents the unity line (X=Y). R^2 and slope of best-fit values are indicated. Blind observer threshold estimates (*y*-axis) for a subset (53%) of the recordings from aged mice from panels (A-E) correlate with estimates made by the author (*x*-axis).

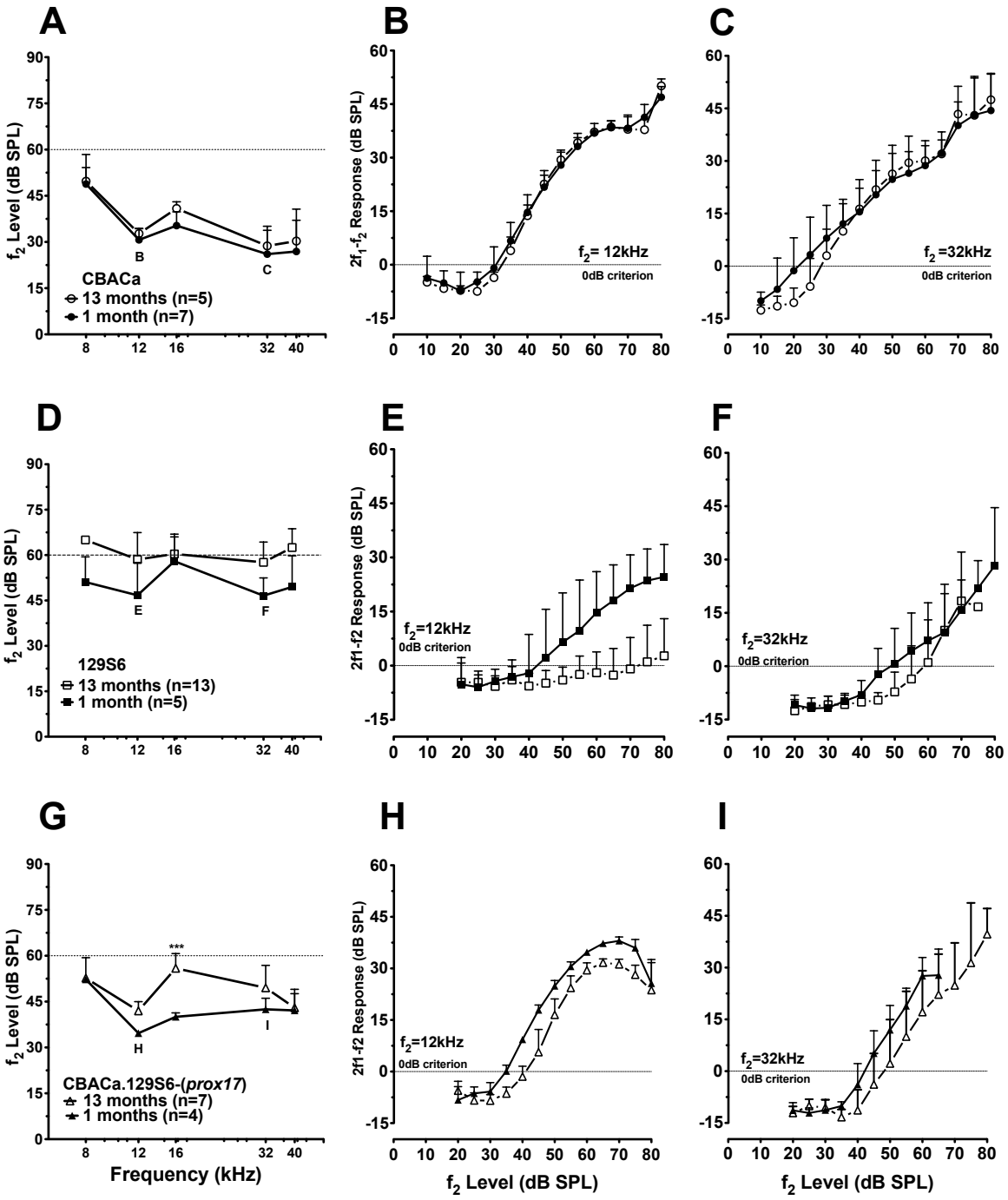
A
B
C
D
E
F
G
H
I
f2 Level (dB SPL)
2f1-f2 Response (dB SPL)
CBACa
13 months (n=5)
1 month (n=7)
f2= 12kHz
f2 =32kHz
0dB criterion
129S6
13 months (n=13)
1 month (n=5)
f2=12kHz
f2=32kHz
CBACa.129S6-(prox17)
13 months (n=7)
1 months (n=4)
***
Frequency (kHz)
f2 Level (dB SPL)

Figure 2.7 Chr 17 does not have progressive OHC dysfunction.

First column represents the DPOAE threshold as the f2 level that elicits a 0 dB response of the 2f1-f2 distortion product for CBACa (**A**), 129S6 (**D**), and CBACa.129S6-(*prox17*) (**G**). Dashed line on first colum 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 months ±1 month). Statistical analysis for 129S6 could not be accurately 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 (± SD) of the 2f1-f2 distortion product as a function of the f2 level for 12 kHz 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 ***$P<0.001$.

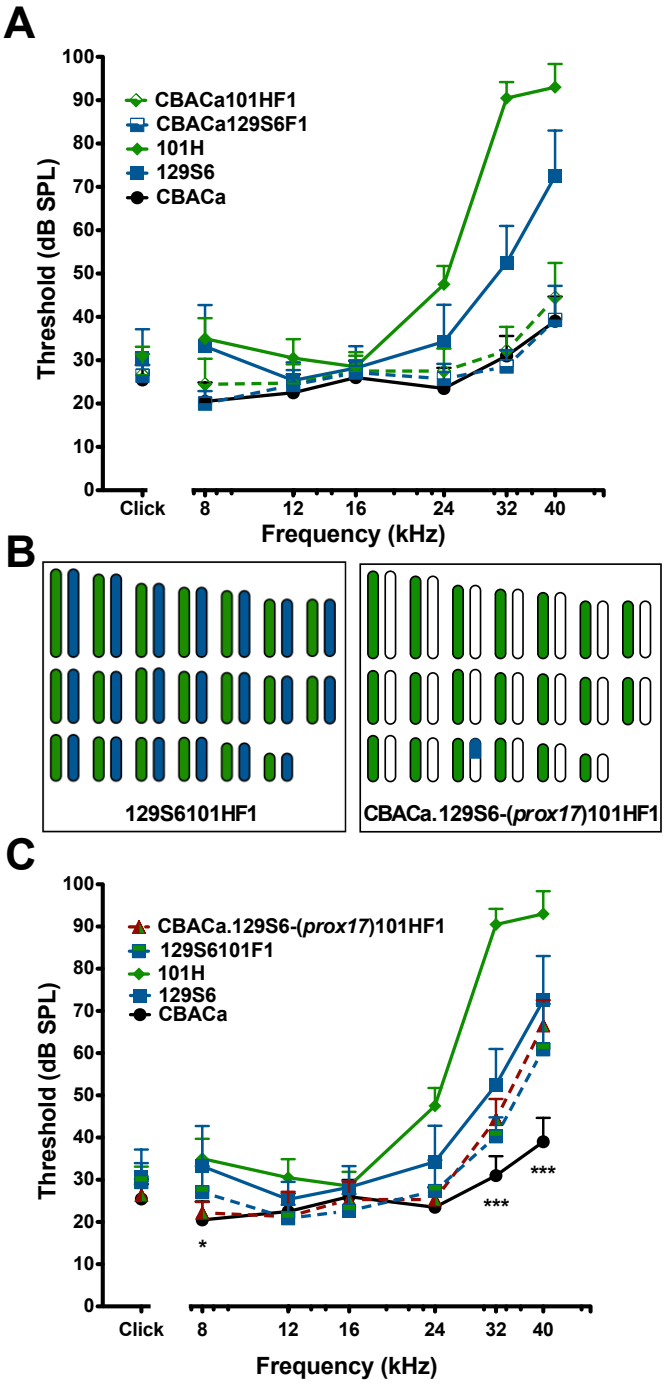
A
Threshold (dB SPL)
CBACa101HF1
CBACa129S6F1
101H
129S6
CBACa
Click
8
12
16
24
32
40
Frequency (kHz)
B
129S6101HF1
CBACa.129S6-(prox17)101HF1
C
Threshold (dB SPL)
CBACa.129S6-(prox17)101HF1
129S6101F1
101H
129S6
CBACa
*
***
***
Click
8
12
16
24
32
40
Frequency (kHz)

Figure 2.8 Hearing loss genes in 129S6 are recessive and non-complementary with 101H genes.

(**A**) Mean (±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 were previously reported in Figure 2 and 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.

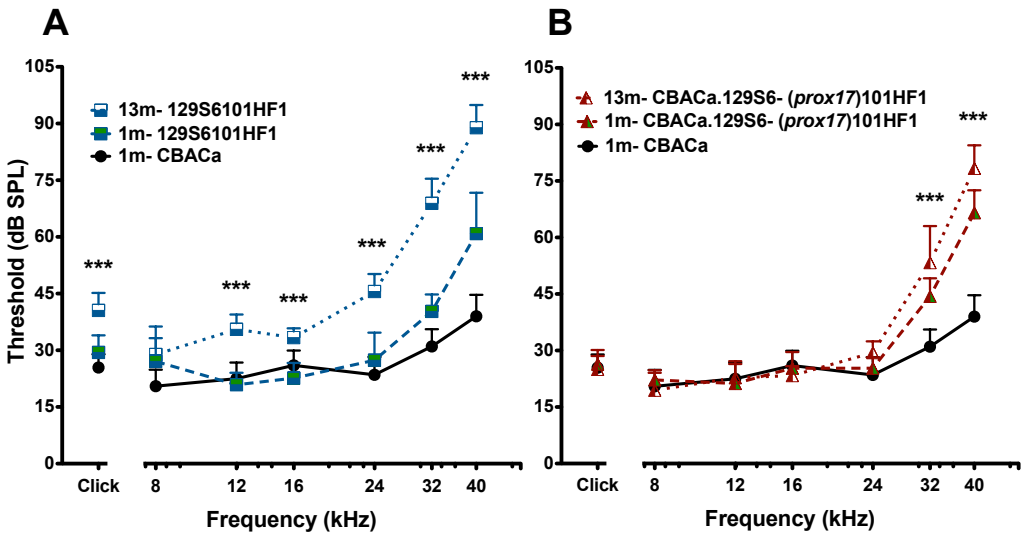

Figure 2.9 101H does not rescue AHL in 129S6.

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 have progressive hearing loss at 32 and 40 kHz. Bonferroni post-hoc test. Where significance is applicable, ***p<0.001.

CHAPTER 3:

Fine Mapping the Locus for Non-Progressive Hearing Loss on Mouse Chr 17

3.1 Summary

Numerous inbred mice strains maintained by brother-sister mating for 20+ generations have developed stereotyped auditory phenotypes. Scientific studies of these mice and their auditory phenotypes have contributed to our understanding of the development and function of the human auditory system. Identifying the genes responsible for these traits in mice may elucidate genetic and molecular processes in the human auditory system.

One such example is the 129S6 inbred mouse that has two auditory phenotypes; early-onset high-frequency (>24 kHz) hearing loss and resistance to NIHL (Yoshida et al., 2000a; Rosowski et al., 2003; Ohlemiller and Gagnon, 2004; Street et al., 2014). The genes responsible for these two phenotypes in the 129S6 mouse are unknown. Research in the Tempel laboratory indicate the proximal Chr 17 region between genetic markers D17Mit143 and D17Mit100 is associated with the *nr1* locus contributing to noise resistance and the *nphl* locus contributing to non-progressive hearing loss. Additionally, Mashimo et al. (2006) mapped a third locus for progressive hearing loss, *phl1*, to the same chromosomal region in the 101H strain.

The proximal 30 Mb of Chr 17 covers 422 protein-coding genes. Our *in silico* analysis of this region identified 9 candidate genes related to hearing or vestibular phenotypes (*Nox3, Cldn6, Cldn9, Cldn20 Noxo1, Axin1, Lhfpl5, Slc26a8, and Tmprss3*). The candidate genes above or a novel gene may be implicated in any of the hearing phenotypes attributed to the proximal Chr17 region. Our goal in this study was to determine with greater resolution the region that defines the *nphl* locus in the 129S6 mouse. The CBACa.129S6-(*prox17*) congenic mouse has the segment between the centromere and SSLP marker D17Mit100 derived from 129S6 mouse in a CBACa background mouse. This congenic mouse created in the Tempel laboratory provides an excellent tool to identify the genes contributing to *nphl*. We used SSLP and SNP markers to create seven

new congenic mice with shorter homozygous segments of Chr 17 derived from 129S6. We tested the new lines for early-onset, high-frequency hearing loss. Based on the mapping analysis, we defined the locus of *nphl* to a 3.7 Mb region on proximal Chr 17.

3.2 Methods

3.2.1 Genotyping protocol

DNA for genotyping was collected from 20-day-old mice tail samples digested overnight in proteinase K and prepared using the Qiagen Puregene Mouse Tail Kit (cat. 158267, Qiagen, Valencia, CA) according to the manufacturer's specification.

Table 3.1 shows all SSLP and SNP markers used for genotyping and determining the breakpoints of new congenic lines that define the contribution of either CBACa or 129S6 mouse background. TaqMan® Probed-Based Genotyping Assays from Applied Biosystems (CA, USA) were used with the following 5 SNPs: rs13482862 (cat. M_22947608_10), rs13482886 (cat. M_23770054_10), rs13479574 (cat. M_23496636_10), rs13482921 (cat. M_23287169_10), and rs13482938 (cat. M_22518445_10). Assays were conducted using the SsoAdvanced Universal Probes supermix (BioRad CA, USA Cat. 172-5280) according to manufacturer's specification in a BioRad iQ5 Real-Time PCR System (BioRad CA, USA).

Defining breakpoints of new congenic lines were conducted using standard Sanger sequencing techniques using SSLP D17Mit213, D17Mit29, D17Mit61 and SNPs rs238316053 and rs13482926. Results were aligned with Codon Code Aligner (v.3.5, Centerville MA, USA) using the BL6 reference sequence from the Genome Reference Consortium Mouse Build 38 and compared for number of dinucleotide repeats and SNP variants.

3.2.2 Generating new congenic mice

Figure 3.1 illustrates the breeding paradigm conducted to create six new congenic mice with shorter homozygous segments derived from the 129S6 mouse in the proximal 28 Mb of Chr17. The CBACa.129S6- (*prox17*) mouse and the CBACa mouse were used as parental strains and crossed to produce heterozygous CBACa and 129S6 mice on Chr 17 between the centromere and marker D17Mit29 (N1). A heterozygous mouse from the N1 generation was backcrossed to CBACa to produce the second backcross generation (N2). One out of seven or 14% of gametes from the heterozygous N1 mouse will have a single homologous recombination event in the proximal 28 Mb segment of Chr 17 based on the 14 cM genetic distance of the region. In the N2 generation, we selected offspring with homozygous recessive CBACa alleles at any of the screening SNP markers, which indicated a breakpoint in the desired chromosomal segment. At the N3 generation, we selected for female and male offspring that maintained the new breakpoints within the proximal 28 Mb Chr 17 segment. The F4 generation was a filial cross between the two heterozygous mice with the new breakpoint selected from the N3 generation. One quarter of the offspring from F4 were homozygous recessive for the shorter segment of Chr 17 derived from 129S6 mouse and became the new established congenic line.

3.2.3 Physiological tests by ABR

The new congenic mouse lines were tested for early-onset, high-frequency hearing loss at 30 days ($\pm$2 days) of age using open-field ABR. ABR stimuli were digitally generated clicks (0.1 ms) or tones (5 ms, 0.5 r/f) presented 13 times per second with alternating polarity and they were delivered from a transducer one foot away from animal's head. To determine threshold, stimuli intensity was decreased from 80 dB SPL in 20 dB steps, with smaller 5-dB steps around threshold. We defined hearing threshold as the lowest sound level ($\pm$5 dB SPL) that elicited a

reproducible ABR wave within 10 ms of stimuli onset. ABR recording parameters are identical to those previously used on Chapter 2.

2.2.4 Data Analysis

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

3.3 Results

3.3.1 Defining ambiguous region in the CBACa129S6-(prox17) congenic strain

The development and catalogue of SSLP markers throughout the mouse genome has been instrumental for mapping the ancestral haplotype of mice strains (Witmer et al., 2003). We identified polymorphic markers between 129S6 and CBACa to better determine the region in the CBACa.129S6-(*prox17*) congenic strain that is derived from the 129S6 mouse that carries the *nphl* locus.

The D17Mit100 genetic marker at 26.1 Mb of Chr 17 defines the distal most segment of the *nphl* locus with a known 129S6 genotype. The D17Mit175 marker at 31.8 Mb defines the proximal most region outside the *nphl* locus with a known CBACa genotype. The 5.7 Mb segment between SSLP marker D17Mit100 and D17Mit175 is considered the ambiguous interval because the breakpoint that defines the contribution from either the CBACa or the 129S6 mouse is unknown (Figure 3.2A). There are 103 protein-coding genes and 4 out of the 9 candidate genes proposed in the *nphl* locus are within the ambiguous interval. We wanted to define with greater resolution the segment between markers D17Mit100 and D17Mit175 that defines the

contribution from the 129S6 mouse. We identified 19 SSLP markers in the ambiguous interval; 3 out of 5 markers screened were polymorphic between 129S6 and CBACa mice. In the CBACa.129S6-(*prox17*) congenic mouse, marker D17Mit29 at 28.7 Mb defined the distal most segment derived from 129S6 and D17Mit61 at 30.6 Mb defined the proximal most segment derived from the CBACa mouse (Figure 3.2B).

3.3.2 High-resolution mapping of nphl with new congenic mice

In the mouse genome, polymorphic SNPs between the CBACa and the 129S6 mouse can be used as markers for genetic mapping and haplotype analysis of congenic strains. We identified five SNP markers polymorphic between 129S6 and CBACa that covered the 20Mb distance between SSLP markers D17Mit143 and D17Mit29. We used these SNP markers in a selective breeding paradigm to establish six new congenic mice lines with shorter homozygous segments of proximal Chr 17 derived from the 129S6 background (see methods; Figure 3.1). We derived 5 new congenic mice from 44 generation N2 offspring, which is within the expected probability (1/7) of observing a single homologous recombination event in the 28Mb (14 cM) region of Chr 17.

Figure 3.3A displays the physical map of the proximal Chr 17 segment that defines the contribution of 129S6 in the CBACa.129S6-(*prox17*) mouse as well as the schematic for six new congenic lines (A through F) created from a selective breeding paradigm backcrossing the CBACa strain with the CBACa.129S6-(*prox17*). Congenic line A mice between the centromere to SNP rs13482926 were homozygous 129S6 mouse. Congenic lines B and C mice between the centromere to SNP rs13482886 were homozygous 129S6 mouse. Congenic line D mice between SNPs rs13482862 and SNP rs13482938 were homozygous 129S6 mouse. Congenic line E mice had SSLP D17Mit213 to SNP rs13482938 derived from the 129S6 mouse. Line F mice were

generated from line E mice with genomic segment between SSLP D17Mit213 and SNP rs13479574 derived from the 129S6 mouse.

We conducted open-field ABR assay at 30 days (±2 days) to determine presence of early-onset, high-frequency hearing loss in the new congenic mice. Both male and female mice were used to study hearing loss because no sex difference is found in the hearing threshold of mice with the *nphl* locus (see Chapter 2). A two-way ANOVA revealed a significant effect of genotype in the hearing threshold of 1-month-old mice ($F_{6, 56} = 28.34$, $P<0.0001$; Figure 3.3B). Frequency and genotype together explain 57.94% of the variance observed between the strains. A post-hoc test revealed that congenic strains A, D, and E had an average of 17.74 dB hearing loss between 24-40 kHz compared to CBACa. At 8 kHz the same congenic strains had an average hearing loss of 10.81 dB. Conversely, congenic lines B, C, and F had ABR thresholds that were indistinguishable from CBACa with an average threshold difference of 0.37 dB between 24-40kHz and 2.05 dB at 8kHz.

To confirm the candidate region of the *nphl* locus, we generated an additional mouse crossing congenic line A and E, both positive for high-frequency hearing loss. Since the NPHL phenotype is inherited recessively, the new F1 hybrid between congenic line A and E would be homozygous recessive at the allele that contributes to the NPHL phenotype (Figure 3.4A). The F1 hybrid was significantly different from CBACa ($F_{1, 16}= 42.57$, $P<0.0001$, Figure 3.4B) with an average hearing loss of 19.87 dB between 24-40 kHz and 15.38 dB at 8 kHz.

3.3.4 Defining phl1 locus in 101H by non-complementation

The *phl1* locus in the 101H strain was mapped by QTL to Chr 17 with a peak at SSLP marker D17Mit113 at 11.9 Mb. We conducted complementation study between 101H crossed to either

congenic line C or congenic line E and tested for early-onset, high-frequency hearing loss (Figure 3.5A). There was an effect of genotype on the hearing thresholds of the hybrids compared to CBACa ($F_{2, 31}$= 4.83, P=0.0149, Figure 3.5B). Compared to the CBACa mouse, the hybrid mouse between congenic E and 101H had significantly greater ABR threshold by an average of 19.61 dB between 32 and 40 kHz (T=4.85 and T= 9.66 respectively; P< 0.001). Conversely, the congenic C and 101H hybrid mouse had ABR thresholds indistinguishable from CBACa at all frequencies tested.

3.4 Discussion

3.4.1 Defining the ambiguous interval of CBACa.129S6-(prox17)

Creating a congenic mouse can leave ambiguous regions that are carried over from the transferred mouse background. Using known polymorphic markers between 129S6 and CBACa, we defined the ambiguous interval in CBACa.129S6-(prox17) to a 1.9 Mb region between SSLP markers D17Mit29 and D17Mit61. Since the *nphl* locus is carried in the 129S6 background, genes that have a CBACa haplotype between markers D17Mit100 and D17Mit175 would not be considered candidates. We excluded *Tmprss3* and *Slc26a8* as candidate genes because their haplotype is that of the CBACa mouse. We excluded an additional 17 protein-coding genes that have a CBACa haplotype. We confirmed 403 genes that have 129S6 strain haplotype between marker D17Mit29 and the centromere.

3.4.2 Defining the nphl locus to a 3.7 Mb locus on Chr 17

In this study we define a 3.7 Mb locus for *nphl* on mouse Chr 17 through genetic mapping using different sets of congenic mice. The congenic line E showing high-frequency hearing loss, had an 11.9 Mb segment derived from the 129S6 mouse between SSLP D17Mit213 at 16.6 Mb and SNP rs13482938 at 28.5 Mb. The congenic line F mouse with normal hearing, had a shorter

5 Mb segment derived from the 129S6 mouse between SSLP D17MIT213 and SNP rs13479574 at 21.6 Mb (Figure 3.2). These data together puts the proximal limit of the *nphl* locus at 21.6 Mb with SNP marker rs13479574 defined by congenic E mouse, and the distal limit at 25.3Mb with SNP marker rs13482926 defined by congenic A mouse.

3.4.3 Non-complementation with 101H

We previously showed that *nphl* and *phl1* loci are non-complementary which may indicate the traits are allelic, the same gene contributes to the phenotypes of both the *nphl* and the *phl1* loci. The original complementation test covered an area of 28 Mb which made room for the possibility of two hearing-loss genes, in which double heterozygous mutations did not rescue hearing loss. Our recent complementation experiment shows that the gene for *phl1* in 101H is within the chromosome region defined by congenic line E between SSLP D17Mit213 and SNP rs13482938. The above results provide stronger evidence for the *phl1* and the *nphl* loci to be products of the same gene allele. Our working hypothesis is that the phenotype can vary in hearing loss severity depending on the background of the mouse strain. In the 129S6 background producing a mild AHL phenotype and in the 101H background a profound hearing loss (see Chapter 2, Figure 6). In the CBCACa background however, the locus produces a non-progressive, high-frequency hearing loss trait.

3.4.4 Candidate genes in the nphl locus

In the 3.7 Mb locus of *nphl* there are 88 protein-coding genes. From these genes we have several that may be a good candidate for the *nphl* locus. The *Cldn6 and Cldn9* genes play a critical role in the maintenance of the ionic selectivity of the EP and structural integrity between

OHC and Deiter cells (Nunes et al., 2006a). A mutation in *Cldn9* causes hearing loss in mice (Nakano et al., 2009). The *Axin*1 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 *Noxo1* gene is critical for ROS production necessary for otoconia synthesis (Kiss et al., 2006).

We sequenced the coding exons of all 4 candidate genes looking for any mutations in the DNA sequence that would lead to changes in amino acid sequence that may explain the NPHL phenotype observed in the 129S6 mouse. We sequenced the DNA protein-coding-exons for *Cldn6*, *Cldn9* and *Axin1* which wer identical between CBACa and 129S6 mice suggesting these genes are not implicated in mutations that change amino acid sequence that may lead to the NPHL trait. This does not exclude the possibility that other genetic mutations outside the coding exons may play a role. Mutations in the introns may affect splice variance, mutations in the promoter may affect transcriptional regulation, and mutations in the untranslated sequence may affect mRNA trafficking. These possibilities would have to be studied further by sequencing the entire genomic DNA of the candidate genes.

The *Noxo1* gene is necessary for otoconia synthesis, but its role in the cochlea its unknown. The *Noxo1* protein-coding sequence in the 129S6 mouse has six synonymous single-nucleotide substitutions. Because the amino acid sequence is unchanged, these six mutations are unlikely to cause a phenotype based on differences in protein sequence. There is a single base nonsynonymous substitution in the last coding exon that changes amino acid at position 340 from a glycine to an alanine (G340A). This SNP (rs108666100) is referred to as a common SNP mapped to this location in the reference genome assembly with a frequency of at least 1% (Sherry et al., 2001). This indicates that the substitution is not rare and it is common amongst

other mice strains, thus not likely to contribute to the unique NPHL phenotype found on Chr 17 of the 129S6 mouse.

It is possible that a novel hearing gene or a unidentified gene is responsible for the NPHL phenotype. There are 88 protein-coding genes in the region making Sanger Sequencing an extraneous and time-consuming undertaking. The identification of the causal gene for the *nphl* locus may require next generation sequencing tools to identify more candidate genes and possible mutations.

Genotyping Markers			
Marker ID	Distance	Nearest gene	polymorphism
Chr 17	Mb (cM)		CB / S6
D17Mit143	8.4 (4.9)	T	122 / 120 repeats
rs13482862	8.8 (5.0)	Pde10a	A / G
rs13482886	14.4 (8.9)	Smoc2	T / C
rs238316053	15.7 (8.9	Chd1	G / C
D17Mit213	16.6 (9.7)	Rgmb/Zfp960	127 / 113 repeats
rs13479574	21.5(11.4)	Zfp53	A / T
rs33547845	23.7 (12.0)	Cldn6	A / C
D17Mit100	26.1(13.0)	Axin1	135 / 133 repeats
rs13482921	24.0 (12.2)	Prss30	G / A
rs13482926	25.3 (12.5)	Cacna1h	T / C
rs13482938	28.5 (14.8)	Srpk1/Lhfpl5	C / T
D17Mit29	28.6 (14.8)	Slc26a8	143 / 149 repeats
D17Mit61	30.6 (15.7)	Dnah8	158 / 162 repeats
D17Mit175	31.8 (17.2)	Sik1	124 / 104 repeats

Table 3.1 List of all SSLP and SNP markers used for defining congenic lines.

Location along Chr 17 is given in megabases (Mb) and centimorgans (cM) along with the closest known protein-coding gene for each marker. The polymorphism observed by Sanger sequencing is given as either the number of dinucleotide repeats for SSLP markers or the nucleotide variant for SNP markers.

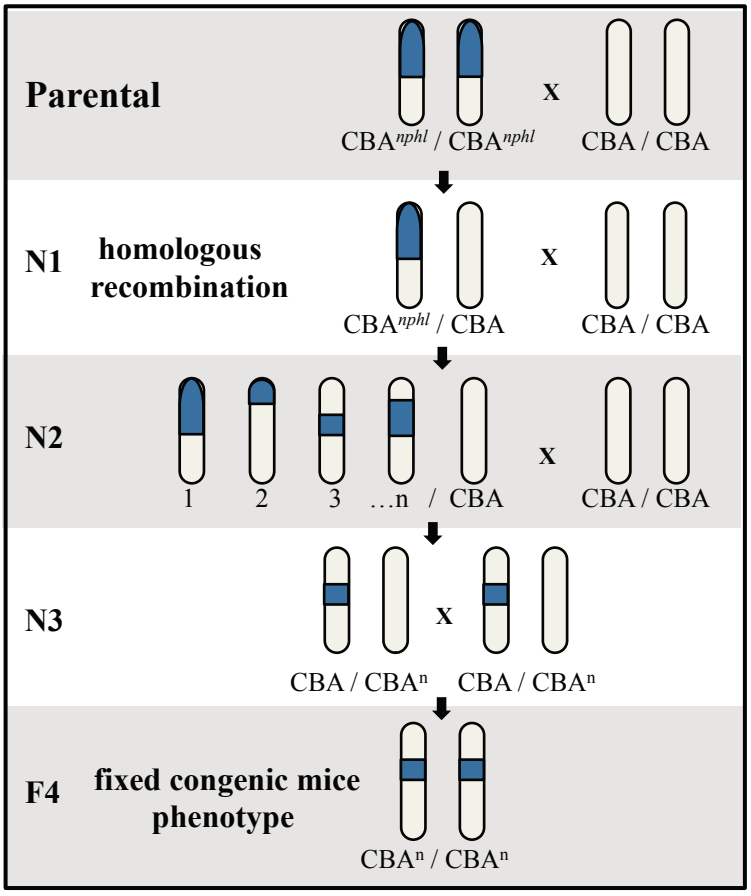

Figure 3.1 Breeding paradigm for creating congenic strains.

Six novel congenic lines were produced with different homozygous contribution from the 129S6 (blue) mouse within the proximal 28 Mb region of Chr 17. The CBACa.129S6-(*prox17*) (CBA^{nphl}) mouse was backcrossed to the CBACa (CBA; cream) mouse. Gametes from the hybrid CBA^{nphl}/CBA mice underwent homologous recombination within the *Prox17* segment with a finite probability. These homologous recombination events were identified in the N2 generation and backcrossed to CBA. In the N3 generation female and male mice that maintained the newly selected breakpoints were chosen as breeding pairs to fix the congenic lines in a filial crossing (F4). The centromere (not drawn) and the telomere of the Chr are located at the top and bottom portion of the chromosome, respectively.

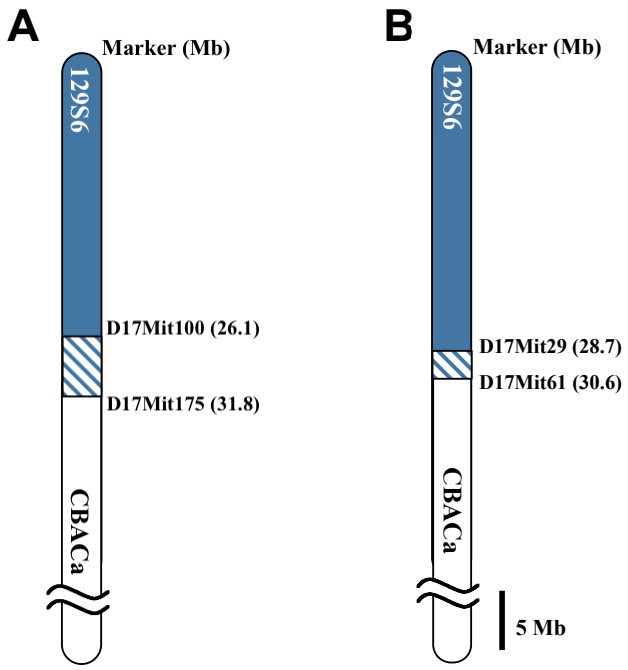

Figure 3.2 Narrowing the ambiguous interval in the CBACa.129S6-(*prox17*) strain. Schematic of Chr 17 from the CBACa.129S6-(*prox17*) mouse displaying the contribution from the 129S6 mouse (blue), the CBACa mouse (white), and the ambiguous interval (hatched). (**A**) The ambiguous segment was originally defined as a 5.7 Mb interval between D17Mit100 and D17Mit175. (**B**) The ambiguous interval was redefined as a 1.9 Mb segment between markers D17Mit29 and D17Mit61. The centromere (not drawn) and the telomere of the Chr are located at the top and bottom portion of the chromosome, respectively.

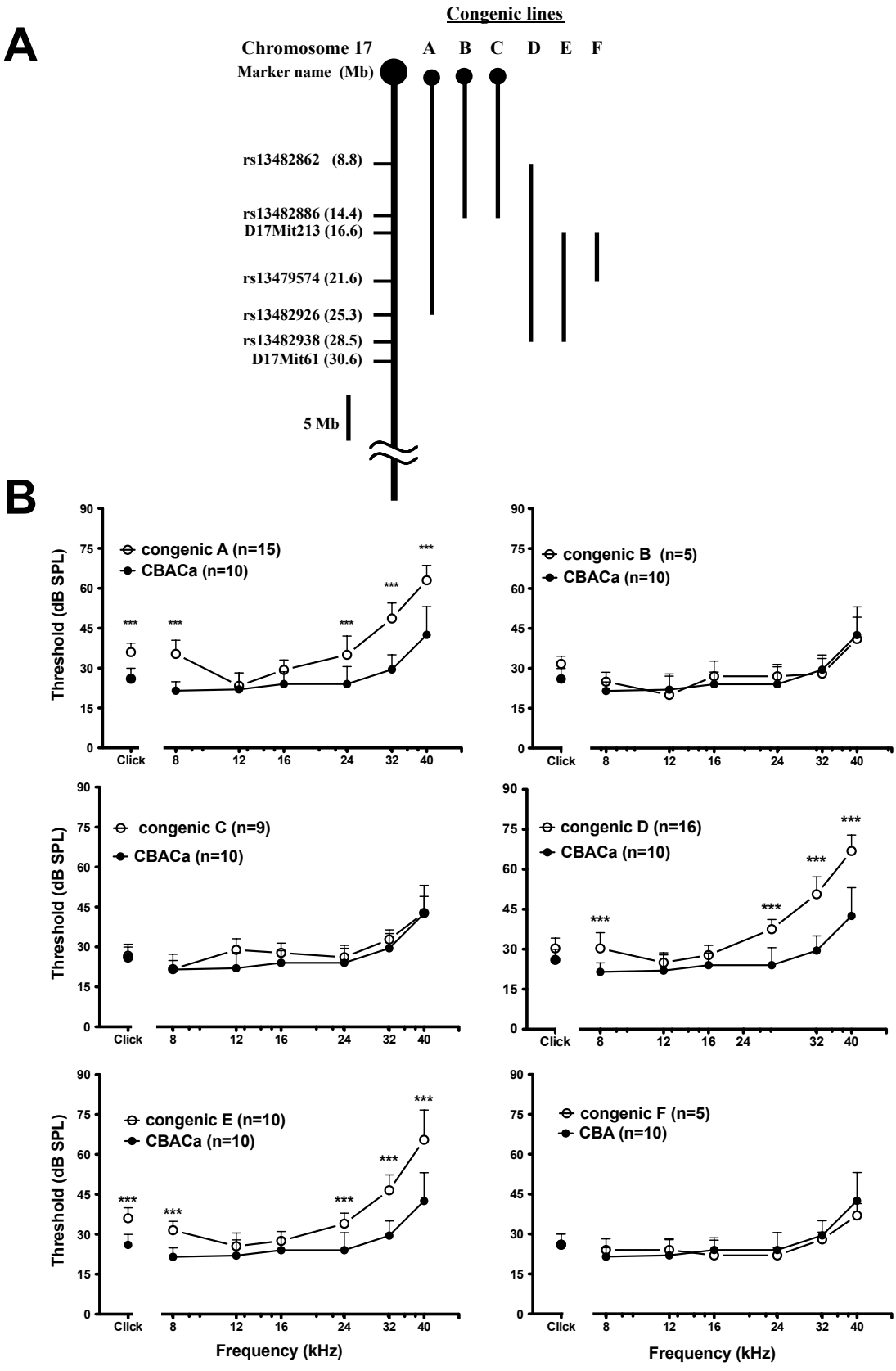
A
Congenic lines
Chromosome 17
Marker name (Mb)
A
B
C
D
E
F
rs13482862 (8.8)
rs13482886 (14.4)
D17Mit213 (16.6)
rs13479574 (21.6)
rs13482926 (25.3)
rs13482938 (28.5)
D17Mit61 (30.6)
5 Mb
B
congenic A (n=15)
CBACa (n=10)
congenic B (n=5)
CBACa (n=10)
congenic C (n=9)
CBACa (n=10)
congenic D (n=16)
CBACa (n=10)
congenic E (n=10)
CBACa (n=10)
congenic F (n=5)
CBA (n=10)
Threshold (dB SPL)
Frequency (kHz)
Click
8
12
16
24
32
40
***

Figure 3.3 Congenic mice with NPHL phenotype.

(**A**) Genetic constitution of congenic mouse lines A through F in a CBACa background. SNP markers on the left show genetic positions (in Mb) on the Chromosome 17. Vertical bars on the right side represent a chromosomal region carrying the 129S6-derived regions. The circle on top of the vertical bars represent the location of the chromosomal centromere. (**B**) ABR thresholds for each congenic line compared to control CBACa mice (n=10) at 30 days (± 2 days). Two-way ANOVA revealed an effect of genotype on the hearing thresholds. Bonferroni post-hoc test was conducted to compare CBACa to congenic lines at each auditory frequency tested. Error bars ± SD ***P<0.001.

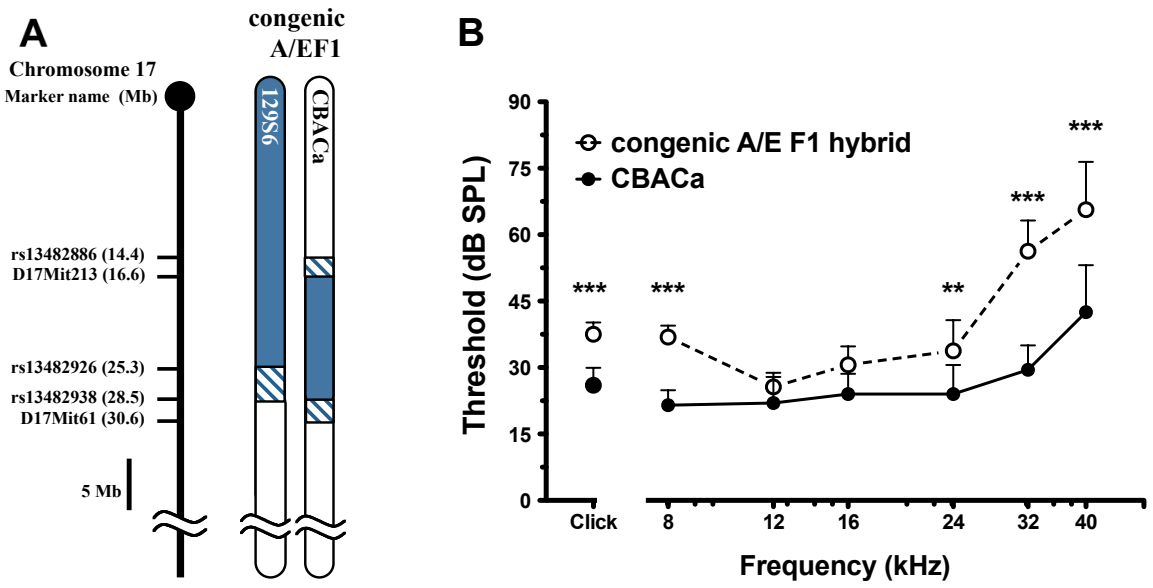

Figure 3.4 The NPHL phenotype in heterozygous congenic mice.

(**A**) The vertical bar represents genetic distance along Chr 17 given by SNP markers in megabases (Mb). Chr 17 schematic with contribution from 129S6 (blue) or CBACa (white) mice and the ambiguous interval (hatched) for the hybrid strain tested. (**B**) There is an effect of genotype on the thresholds of the congenic A/E F1 hybrid (n=8) with 8 kHz and 24-40 kHz significantly different from CBACa (n=10) thresholds. Error bars ± SD, ***P*<0.01, ****P*<0.001.

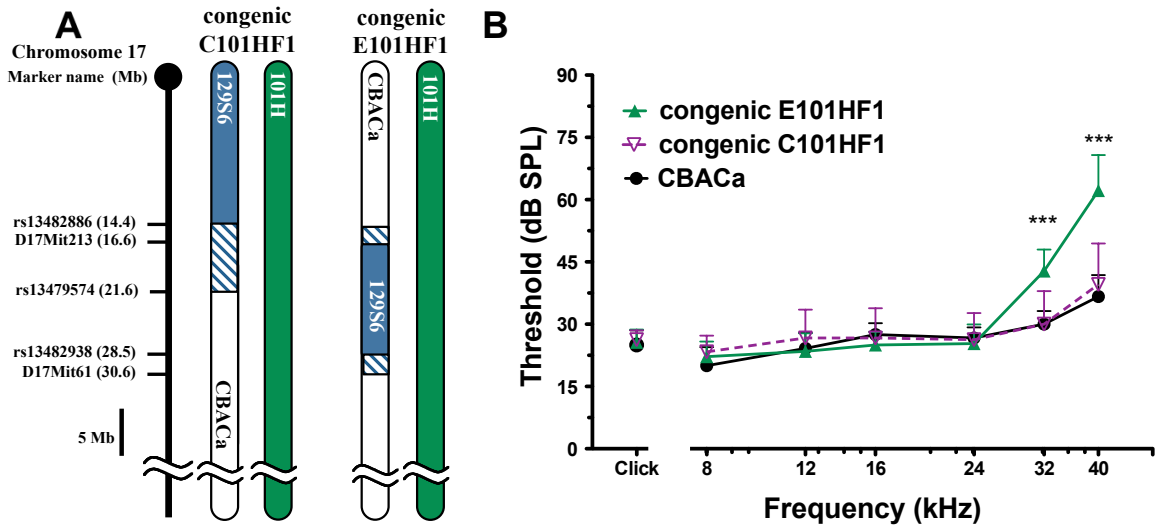

Figure 3.5 The 101H mouse does not rescue hearing loss in congenic mice.

(**A**) The vertical bar represents the genetic distance along Chr 17 given by SNP markers in megabases (Mb). Chr 17 schematic with contribution from 129S6 (blue) or CBACa (white) mice with ambiguous interval (hatched) is shown for each hybrid strain tested. (**B**) Only the congenic E101H hybrid (n=16) has thresholds significantly different from CBACa (n=6) at 32 and 40 kHz. The congenic C101H hybrid (n=12) has normal hearing thresholds compared to CBACa. Error bars ± SD, ***P<0.001.

CHAPTER 4:

Conclusions

The motivation for the work conducted in this dissertation comes from my interest to better understand the genetic basis of presbycusis or AHL. Acquired hearing loss is the most common sensory deficit worldwide and its prevalence its on the rise. Health statistic collected by the United States (Pleis and Lethbridge-Cejku, 2007), The European Union (Roth et al., 2011), the World Health Organization, and other organizations give a stark reminder of the pervasiveness of the problem in our society (see review; Cruickshanks et al., 2009). Acquired deafness can be caused by genetics or environmental factors. The accumulation of noxious environmental factors such as ototoxic drugs, diseases, and noise exposure from leisure and occupational activities can be avoided by practicing good preventative measures. However, our genetic predisposition for AHL is unavoidable. People with a genetic predisposition to AHL may develop hearing loss despite taking preventative hearing loss measures. The aim of this dissertation was to conduct research to better understand the genetic factors that contribute to AHL.

With the recent advances in mouse genetics and bioinformatics, scientists have mapped 19 loci responsible for AHL in mice, but only 5 genes have been identified (see Table 1.1 on Chapter 1). The slow identification of genes that cause AHL in light of the numerous genetic loci mapped is a consequence of the long path from genome mapping to gene identification that can take up to 10 years (Drinkwater et al., 2012). In this dissertation, I take advantage of work previously conducted to fine map an *nr* locus in order to map a novel *nphl* locus on proximal Chr 17.

4.1 Findings

4.1.1 Ahl in the 129S6 mouse.

My research indicates that hearing loss in the 129S6 mouse starts at frequencies above 24 kHz by one month of age (Figure 2.2). These findings are consistent with work previously conducted (Ohlemiller and Gagnon, 2004). What is unique about my findings is the presence of mid- and low-frequency hearing loss (<24 kHz) by 13 months of age in the 129S6 mouse, which was not detected in the work by Ohlemiller and colleagues.

Some of the hearing loss observed in the low and mid frequency range may be in part due to possible effusion of the middle ear observed in 13-month-old 129S6 mice. Middle ear pathology is commonly found in older 129S6 (Rosowski et al., 2003) suggesting 129S6 mice have a genetic predisposition for middle ear infection. Effusion of the middle ear can cause reduced hearing sensitivity (Qin et al., 2010); however, the AHL phenotype we see in the 129S6 mouse is greater than that seen by effusion conditions alone suggesting a primary role of sensory AHL. In future aging experiments, we need to pay close attention to the middle ear condition of aged mice which can go unnoticed when conducting free field ABR. By making a comparison of hearing loss between age-matched mice with and without middle ear pathology, we could get insight into the individual contribution of conductive and sensory hearing loss to the 129S6 AHL phenotype.

Additionally, my research shows one-month-old 129S6 mice have reduced DPOAE thresholds at frequencies below 24 kHz despite showing normal ABR thresholds. These results support previous findings that otoacoustic emissions can detect subtle OHC dysfunctions before they become clinically apparent by other hearing assays such as ABRs (Martin et al., 1990; Liu and Newton, 1997). Future physiological work on the hearing traits of 129S6 mice should

consider relying on DPOAE measures as a more sensitive test to detect normal and abnormal cochlear function.

4.1.2 Nphl in the 129S6 mouse.

My research also indicates that proximal Chr 17 has a locus of *nphl*. Through genotyping and selective recombinant breeding, I successfully mapped the locus for *nphl* to a 3.7 Mb region on proximal Chr 17 between SNP markers rs13479574 and rs13482926 in a region with 88 protein-coding genes. The *nphl* locus in the CBACa background is a non-progressive, high-frequency hearing loss, which is a hearing trait different from that seen in the inbred 129S6 mouse. These phenotype differences in the 129S6 and CBACa background suggest that the *nphl* gene may interact with other genes in the 129S6 mouse background producing the observed 129S6 AHL trait.

To understand how the AHL physiology relates to anatomical changes in the cochlea, Ohlemiller and colleagues conducted an anatomical analysis concurrent with their physiological studies of AHL in the 129S6 mouse. By 15 months, the 129S6 strain had reduced OHC density at the base of the cochlea but normal density of SGC compared to controls. These results indicate sensory AHL due to OHC death with normal IHCs and SGC density (Ohlemiller and Gagnon, 2004). Future experiments characterizing the *nphl* locus in the CBACa background, would need to address the relationship between the physiological and the anatomical changes happening in the cochlea between 1 and 13 months of age.

4.2 *Nphl* Candidate genes

In the 3.7 Mb region of the *nphl* locus, we identified four genes with prior implications to hearing or vestibular phenotypes. The *Axin1* and *Noxo1* genes were excluded because their genetic sequences did not reveal differences between the 129S6 and CBACa mice that would support a possible involvement in the NPHL trait.

The *Cldn6* and *Cldn9* genes at 23.6 Mb represent the best candidate genes for the NPHL phenotype. The family of claudin genes are tight junction proteins important for creating the paracellelular barrier that controls the flow of ions in the intercellular space. In the cochlea, claudins play an important role to separate the three compartments of the cochlea: the scala vestibuli, the scala media, and the scala tympani. Claudin proteins are expressed throughout the cochlea at the organ of Corti, the marginal and basal cells of the stria vascularis, Reissner's membrane, and spiral limbus (Kitajiri et al., 2004b; Kitajiri et al., 2004a). At the organ of Corti, *Cldn6*, *Cldn9*, and *Cldn14* provide two essential roles. They connect OHC and DC providing a dynamic structure to support the mechanical stress of the cells in response to the motion of the basilar membrane, and they provide the ionic selectivity that separates the endolymph and the perilymph fluid at the reticular lamina (Nunes et al., 2006b). Mutations in *CLDN14* causes autosomal recessive deafness (DFNB 29; Wilcox et al., 2001), and mutations in *Cldn9* causes deafness in mice (Nakano et al., 2009) supporting the important role of claudins at the junction of the reticular lamina.

The protein-coding DNA sequence for both *Cldn6* and *Cldn9* are identical between the 129S6 and the CBACa mice suggesting the protein sequence does not play a role in the NPHL phenotype. However, mutations in these genes outside of the coding sequence may affect other parts of gene expression and trafficking. We sequenced the proximal promoter of both genes,

1000 bases upstream of the start of translation site looking for possible proximal promoter changes. We identified a few candidate SNPs that change two possible transcription factor binding sites in *Cldn6* which may disrupt up or down regulation of gene expression during development or in the mature auditory system. In *Cldn9* we identified an insertion of a short interspersed nucleotide element (SINE) which represent remnants of reverse-transcribed RNA molecules which may play a role in transcriptional regulation by causing methylation of active sites or from internal promoter sequences (Takai and Jones, 2002; Nishikiori et al., 2008; Bumaschny et al., 2007). Our laboratory is currently studying the *Cldn6* and *Cldn9* regulation as a possible mechanism for hearing loss and noise resistance attributed to the Proximal Chr 17 region.

4.3 Implications

The work in this dissertation represents the first characterization of a novel locus that affects selectively non-progressive hearing loss in the high frequency range of mice early in life. More importantly, this gene may have different effects depending on the background of the mouse that it is in. In the 101H mouse it may contribute to profound hearing loss, while in 129S6 mouse contributes to a slow progressive AHL phenotype, and a non-progressive hearing loss in the CBACa background. This gene may be implicated in the hearing phenotype of other inbred mice with known loci or genes for hearing loss for which the identified gene or loci alone cannot explain the entire hearing loss phenotype observed. This is a common observation in AHL research when causative genes are taken out of the mouse background and the phenotype is not replicated (Kane et al., 2012; Keithley et al., 2004).

In the human population, an *nphl* phenotype as seen in the CBACa background may go undetected because the high-frequency it affects may be beyond the hearing frequencies used for human communication and often not tested in the clinics. Thus, an affected individual may lead a normal life unaware or untroubled by the high-frequency hearing loss deficiency. However, the AHL trait might be manifested when the *nphl* loci is inherited to an individual with other genetic susceptibilities in their genomic background. In such an instance, it would be difficult to track the genetic inheritance of an AHL gene because the manifestation of the gene changes each generation depending on its interaction with other genes. Within this problem lie the difficulties of conducting AHL research in the human population. It is my expectation that this body of work may elucidate scientific advances towards understanding the genetic basis of AHL.

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VITAE

Braulio Peguero was born and raised in the Dominican Republic until the age of 10 when he migrated to Bridgeport, Connecticut with his mother and older brother where he graduated from Central Magnet High School. He attended Bowdoin College in Brunswick, Maine graduating with a BA, *Cum laud*e in Neuroscience. He received his PhD. From the University of Washington in Neurobiology and Behavior.

EDUCATION

University of Washington. Seattle, WA (2007 - 2014)

Graduate Program in Neurobiology and Behavior

Dissertation: "The Genetic Identification and Physiological Characterization of a Novel Locus for Non-Progressive Hearing Loss on Mouse Chromosome 17"

Advisor: Bruce L Tempel, PhD

Bowdoin College. Brunswick, ME (2001 - 2005)

B.A. cum laude in Neuroscience

Thesis: "The Effects of the Novel Peptide Val[1]-SIFamide on the Central Pattern Generators in the Stomatogastric Ganglion of the American Lobster, *Homarus americanus."*

Advisor: Patsy S. Dickinson, PhD

PROFESSIONAL EXPERIENCE

Program Advisor (2006 - 2007)

Office of Student Activities, Bowdoin College

Supervisor: Allen Delong, PhD

Position description: Planned and executed student programming. Launched and implemented a campus-wide leadership development initiatives. Advised 100 student organizations that developed and executed community events with a collective budget of $600,000. Mentored student class officers, the Campus Activities Board, and the Bowdoin student government.

Research Assistant (2005 - 2006)

Department of Biology, Bowdoin College

Advisor: Patsy S. Dickinson, PhD

Project description: Studied the effects of neuromodulators on the central pattern generators of crustaceans. Worked with a team of scientists to identify and characterize novel neuromodulators with chemical, molecular, and physiological techniques. Supervised undergraduate research projects. Presented findings at scientific conferences in written and oral format.

INROADS Research Intern (summers 2002, 2003)

Monsanto Biotech Company. Mystic, CT

Position description: Conducted and optimized genetic and molecular biology research on agricultural products. Programmed and used robotics for high-throughput genetic testing. As an INROADS scholar, attended bi-weekly workshops and professional development seminars on leadership, teamwork dynamic, and workplace professionalism.

FUNDING AWARDS

- Minority Student Travel Award from the Association for Research in Otolaryngology 2013
- Research Training in Speech & Hearing Sciences (T32DC000033) 2012 - 2013
- Institutional Training Grant for Neurobiology (T32GM007108) 2009 - 2012
- Training Grant in Otolaryngology (T32DC000018) 2008 - 2009
- INBRE Program of the National Center for Research Resources (P20RR016463) 2005 - 2006

MEMBERSHIP TO NATIONAL ASSOCIATIONS

- (SFN) Society for Neuroscience 2004 - present
- (ARO) Association for Research in Otolaryngology 2012 - present
- (SACNAS) Society for the Advancement of Chicanos/Latinos & Native Americans in Science 2010 - present

PROFESSIONAL & COMMUNITY SERVICE

President, (2011 - 2012)
University of Washington SACNAS Chapter

Managed special projects to support diversity and leadership in science: engaging campus, state, and nationwide entities. Led the chapter with the execution of the 2012 SACNAS National Conference in Seattle. Supervised committees and projects responsible for educational outreach and student/faculty engagement and mentorship. Received the 2012 Graduate Chapter of the Year Award for advancing the SACNAS mission to promote diversity and leadership of underserved communities in science.

Graduate Advisor, (2010 - 2012)
Louis Stokes Alliance for Minority Participation (LSAMP)

Mentored undergraduate students in STEM majors to achieve academic excellence. Planned and implemented the first annual LSAMP retreat for 50+ students from five regional universities in Idaho, Oregon, and Washington State. Developed workshops to engage student participation in science and collaborative teamwork. Participated as panelist in regional LSAMP Conferences.

Student Representative, (2008 - 2010)
Neurobiology Seminar Committee

Helped select seminar speakers for the Graduate Program in Neurobiology & Behavior. Facilitated communication between student and faculty interests. Organized and implemented seminars and guest visits.

PUBLICATIONS

Referee Publications

1- Dickinson PS, Stemmler EA, Cashman CH, Brennan HR, Dennison B, Huber KE, **Peguero B**, Rabacal W, Goiney CC, Smith CM, Towle DW, Christie AE (2008) "SIFamide peptides in clawed lobsters and freshwater crayfish (Crustacea, Decapoda, Astacidea): A combined molecular, mass spectrometric and electrophysiological investigation." *General and Comparative Endocrinology* 156: 347–360

2- Stemmler EA, **Peguero B**, Bruns EA, Dickinson PS, and Christie AE (2007) "Identification, physiological actions, and distribution of TPSGFLGMRamide: a novel tachykinin-related peptide from the midgut and stomatogastric nervous system of *Cancer* crabs." *Journal of Neurochemistry* 101: 1351–1366

3- Christie AE, Stemmler EA, **Peguero B**, Messinger DI, Provencher HL, Scheerlinck P, Hsu YA, Guiney ME, de la Iglesia HO, and Dickinson PS (2006) "Identification, distribution, and physiological actions of VYRKPPFNGSIFamide (Val^1-SIFamide) in the stomatogastric nervous system of the American lobster *Homarus americanus*." *Journal of Comparative Neurology* 496(3): 406-421.

Thesis

4- **Peguero B** (2005) "The effects of the novel peptide Val^1-SIFamide on the central pattern generators in the stomatogastric ganglion of the American lobster, *Homarus americanus*." Brunswick, ME: Bowdoin College.

Published Abstracts

5- **Peguero B**, Robinson L, Tempel BL. (2013) "New locus for Age-Related Hearing Loss (AHL) on Chromosome 17 in the 129S6/SvEvTac mouse." Program no. 352.09. Society for Neuroscience 2013. San Diego, CA: Abstracts. Online.

6- **Peguero B**, Robinson L, Tempel BL. (2013) "Genes on Chromosome 17 Contribute to Age-Related Hearing Loss in 129S6/SvEvTac" Assoc. Res. Otolaryngol. Abs.:314

7- Tempel BL, Street V, **Peguero B**, Galitsky T, Carter G, Liberman CM, Kujawa SJ. (2010) "Genes Contributing to Noise Resistance on Chromosome 17 in 129S6 Mice." Assoc. Res. Otolaryngol. Abs.: 691

8- **Peguero B**, Bruns EA, Rabacal W, Messenger DI, Christie AE, Dickinson PS, Stemmler EA (2006) "Identification of a novel tachykinin-related peptide from the midgut and stomatogastric nervous system of Cancer crabs." Program no. 129.3. Society for Neuroscience 2006. Atlanta, GA: Abstracts. Online

9- Goiney CC, Brennan HR, Cashman C, **Peguero B**, Rabacal W, Messinger DI, Easton CR, Hsu YA, Smith CM, Towle DW, Stemmler EA, Dickinson PS, Christie AE (2006) "VYRKPPFNGSIFamide is a genus (Homarus)-specific isoforms within the SIFamide peptide family." Program No. 129.9. Society for Neuroscience 2006. Atlanta, GA: Abstracts.Online

10- **Peguero B**, Scheerlinck P, Stemmler EA, Provencher HL, Guiney ME, Messinger DI, Soon PY, Hsu YA, de la Iglesia HO, Coito M, Christie ME, Dickinson PS (2005) "The peptide Val^1-SIFamide activates multiple patterns in the lobster nervous system." Program No. 30.5, Society for Neuroscience 2005. Washington, DC: Abstracts.Online

11- Dickinson PS, Messinger DI, Schmidt JJ, Hsu YA, Rabacal W, Ho TN, Pott M, **Peguero B**, Stemmler EA, Li L, Christie AE (2005) "SIFamides in Stomatogastric Nervous System and Neuroendocrine Organs of Decapod Crustaceans." Program No. 30.4, Society for Neuroscience 2005. Washington, DC: Abstracts. Online

12- Johnson C, Dunphy T, Scheerlinck P, **Peguero B**, Dickinson PS (2004) "The neuropeptide proctolin alters the interactions between two pattern generators in the lobster, *Homarus americanus*." Program No. 657.21, Society for Neuroscience 2004. San Diego, CA: Abstracts. Online

PRESENTATIONS

Unpublished Abstracts

1- Iwata AJ, **Peguero B**, Li J, Tempel BL (2013) "Analysis of claudin gene expression as a causal basis of differential noise resistance in 129S6/SvEvTac and CBA/CaJ mice" Moleuclar Biology of Hearing and deafness. Palo Alto, CA

2- **Peguero B**, Tempel B (2012) "Genes on Chromosome 17 of Mice Contribute to Age-Related Hearing Loss" 2012 SACNAS National Conference. Seattle, WA

3- **Peguero B**, Shilling D, Liberman CM, Kujawa SJ, Tempel BL (2009) "Molecular and Genetic Analysis of Noise Resistant Candidate Genes in 129S6" Molecular Biology of Hearing and Deafness. Boston, MA

4- **Peguero B**, Pott M, Rabacal W, Dickinson PS (2006) "SIFamide activation in the stomatogastric nervous system of decapod crustaceans." East Coast Nerve Net, MBL. Woods Hole, MA

5- Christie AE, **Peguero B**, Brennan HR, Rabacal W, Donahue R, Ho TN, Rus S, Kotecha V, Goiney CC, Towle D, Stemmler EA, Dickinson PS (2006) "Characterization of a cDNA encoding Val^1-SIFamide on the American Lobster, *Homarus americanus*." East Coast Nerve Net. MBL, Woods Hole, MA

6- **Peguero B**, Stemmler EA, Christie AE, Dickinson PS (2006) **"**Identification and physiological activity of a new tachykinin-related peptide in *Cancer irroratus*." Maine Biological and Medical Sciences Symposium, Mount Desert Island Biological Laboratory. Salisbury Cove, ME

7- **Peguero B**, Scheerlinck P, Dickinson PS (2005) "Val^{1}-SIFamide activates the pyloric pattern generator in the American lobster, *Homarus americanus*." Annual East Coast Nerve Net, MBL. Woods Hole, MA

Research Talks

8- **Peguero B**, Robinson L, Tempel BL (2013) "Genes on Chromosome 17 contribute to age-related hearing loss in129S6/SvEvTac" Assoc. Res. Otolaryngol. Abs.:314

9- **Peguero B**, Tempel BL (2012) "Genes on Chromosome 17 of mice contribute to age-related hearing loss" 2012 SACNAS National Conference. Seattle, WA

10- **Peguero B** (2011) "Characterizing and identifying a novel hearing loss gene in 129S6" Neurobiology and Behavior Graduate Program Retreat. University of Washington. Seattle, WA

11- **Peguero B**, Stemmler EA, Christie AE, Dickinson PS (2006) **"**Identification and physiological activity of a new tachykinin-related peptide in *Cancer irroratus*." Maine Biological and Medical Sciences Symposium, Mount Desert Island Biological Laboratory. Salisbury Cove, ME

Science Outreach Lectures

12- The Seattle Public Library, Healthy Aging Program "From Hearing to Deafness, Learn how aging and environmental factors affect hearing" (Nov. 10, 2012) West Seattle Public Library Branch.

Course Lectures

13- "The Auditory System" (Oct 18, 2010) systems Neurobiology,NBio401. University of Washington

14- "The Olfactory System" (Dec 6, 2010) Systems Neurobiology, Nbio401. University of Washington

15- "Language and Cortex" (Dec 10, 2010) Systems Neurobiology, Nbio401. University of Washington