



Published in final edited form as:

Mamm Genome. 2019 February ; 30(1-2): 5–22. doi:10.1007/s00335-019-09792-6.

Genetic variation in thyroid folliculogenesis influences susceptibility to hypothyroidism-induced hearing impairment

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Abstract

Maternal and fetal sources of thyroid hormone are important for the development of many organ systems. Thyroid hormone deficiency causes variable intellectual disability and hearing impairment in mouse and man, but the basis for this variation is not clear. To explore this variation we studied two thyroid hormone-deficient mouse mutants with mutations in pituitary-specific transcription factors, POU1F1 and PROP1, that render them unable to produce thyroid stimulating hormone. DW/J-*Pou1f1*^{dw/dw} mice have profound deafness and both neurosensory and conductive hearing impairment, while DF/B-*Prop1*^{df/df} mice have modest elevations in hearing thresholds consistent with developmental delay, eventually achieving normal hearing ability. The thyroid glands of *Pou1f1* mutants are more severely affected than those of *Prop1*^{df/df} mice, and they produce less thyroglobulin during the neonatal period critical for establishing hearing. We previously crossed DW/J-*Pou1f1*^{dw/+} and Cast/Ei mice and mapped a major locus on Chromosome 2 that protects against hypothyroidism-induced hearing impairment in *Pou1f1*^{dw/dw} mice: **M**odifier of **dw** hearing (*Mdwh*). Here we refine that map with additional animals and genotypes, and we conduct novel mapping with a DW/J-*Pou1f1*^{dw/+} and 129/P2 cross that reveals 129/P2 mice also have a protective *Mdwh* locus. Using DNA sequencing of DW/J and DF/B strains, we determined that the genes important for thyroid gland function within *Mdwh* vary in amino acid sequence between strains that are susceptible or resistant to hypothyroidism-induced hearing impairment. These results suggest that the variable effects of congenital hypothyroidism on the development of hearing ability are attributable to genetic variation in postnatal thyroid gland folliculogenesis and function.

Keywords

genetic modifier; *Mdwh*; POU1F1; PROP1; thyroglobulin; thyroid hormone

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Disclosure Statement: The authors have nothing to disclose.

Introduction

Congenital hearing impairment affects nearly 1 in every 1000 live births and is the most common birth defect in developed nations (White 2004). Hearing impairment affects speech and language development, the education of children, and career opportunities later in life. Hypothyroidism during pregnancy and in newborns can cause hearing loss in humans and in rodents, if not promptly identified and treated (Brucker-Davis et al. 1996; Glinoyer and Delange 2000; Knipper et al. 2000; Vanderschueren-Lodeweyckx et al. 1983). Individual susceptibility to hypothyroidism-induced hearing impairment is highly variable, and the genetic factors that influence susceptibility are not known (Karolyi et al. 2007; Napióntek et al. 2004). Genetic background significantly affects phenotype in many mouse models of human diseases, including hearing impairment (Hamilton and Yu 2012; Ikeda et al. 2002; Johnson et al. 2006; Kousi and Katsanis 2015; Yan and Liu 2010). The mouse offers a powerful system for identifying complex genetic interactions in hearing development and disease because of the availability of numerous strains of mice with different genetic backgrounds (Niu et al. 2008). It is clear that genetic factors affect the severity of hearing impairment in humans, but it is much more difficult to identify genetic interaction in humans even when large pedigrees are available (Kallman et al. 2008). Due to the conservation of genes among mammals, the identity of candidate modifier genes in humans can be predicted from studies in model organisms such as mice. In order to realize the potential of the genome project to predict disease risk and severity for humans, we need to know more about gene-gene interactions that ultimately dictate the individual's phenotype.

We studied two genetic models of severe hypothyroidism and the effects of genetic background on susceptibility to hearing deficits (Karolyi et al. 2007). DF/B-*Prop1*^{df/df} (p.S83P) and DW/J-*Pou1f1*^{dw/dw} (p.W251C) mice have loss-of-function mutations in two pituitary-specific transcription factors that result in arrested pituitary cell specification early in development and the inability to produce TSH, growth hormone, and prolactin, causing developmental delay, severe hypothyroidism, and growth insufficiency (Li et al. 1990; Sornson et al. 1996). *PROP1* and *POU1F1* regulate two sequential steps in pituitary differentiation, and one of the primary roles of *Prop1* is to activate expression of *Pou1f1*. The pituitary hormone phenotypes of the two mutant mice are indistinguishable. Surprisingly, the severity of the hearing impairment in the two mutants is strikingly different despite their identical effects on pituitary hormone production. DF/B-*Prop1*^{df/df} mutants have a mild hearing deficit of ~15 dB elevation in thresholds at 6 wk and normal hearing by 12 wk (Fang et al. 2012; Karolyi et al. 2007). In contrast, DW/J-*Pou1f1*^{dw/dw} mutants are profoundly deaf at all ages tested (~65 dB elevation) (Fang et al. 2012; Karolyi et al. 2007). When these genetic backgrounds are mixed, the hearing impairment of the two mutants is indistinguishable (~30 dB threshold elevation). These genetic background effects are not maternal effects, but affect the fetus directly, because transfer of DF/B-*Prop1*^{df/df} and DW/J-*Pou1f1*^{dw/dw} fertilized eggs to surrogate mothers did not alter the severity of the hearing impairment (Fang et al. 2012). The hearing impairment of both mutants is rescued by thyroid hormone supplementation initiated during pregnancy and continued during postnatal development, proving that thyroid hormone deficiency is linked to the hearing problems.

Thyroid hormone (thyroxine or 3,5,3',5'-tetraiodothyronine) is necessary for development of hearing and many other body functions including fetal brain development, growth, metabolism, and regulation of body temperature (Brent 2012; van der Spek et al. 2017). Thyroid hormone is synthesized in the follicular cells of the thyroid gland from the protein precursor thyroglobulin (TG). Thyroglobulin is released into the lumen of the follicle, the colloid, where iodine is added to the tyrosine residues of the protein and subsequently oxidized by the action of thyroid peroxidase (TPO). The follicular cell takes up the modified thyroglobulin by endocytosis, and proteolyzes it to produce thyroxine (T4) and triiodothyronine (T3), which are released into the bloodstream in response to stimulation by pituitary thyroid stimulating hormone (TSH). TSH binds the TSH receptor on thyroid follicular cells and stimulates the endocytosis of the colloid, processing of thyroglobulin, and release of T4 into the bloodstream. In addition to its role in regulating thyroid gland function, pituitary TSH is important for stimulating thyroid gland growth and folliculogenesis at 18–20 wks of gestation in human fetuses and in mice after birth (Postiglione et al. 2002). Cells throughout the body take up T4 by active transport, and it is processed into the more biologically active T3 (triiodothyronine) by type 2 deiodinase (DIO2) (Bernal et al. 2015). Both T3 and T4 are inactivated by the deiodinase DIO3. T3 binds thyroid hormone receptors (THRA and THRB) and alters gene expression.

Pleiotropic effects of hypothyroidism on normal auditory development have been demonstrated in several rodent models (Christ et al. 2004; Knipper et al. 2000; Li et al. 1999; Richter et al. 2010; Sprenkle et al. 2001). Thyroid hormone also affects auditory pathways in the brain [reviewed in (Mustapha et al. 2009; Ng et al. 2013)]. The inner ear defects range from immature cochlear morphology, tectorial membrane abnormalities, inner and outer hair cell defects, reduced expression and function of potassium channels, hair cell loss, and impaired oto-acoustic emissions, and they have been thoroughly examined in *Prop1* and *Pou1f1* mutants (Fang et al. 2012; Mustapha et al. 2009; Sundaresan et al. 2016). Middle ear defects can include ossicular malformations and defects in middle ear cavitation, but these have not been explored in either *Prop1* or *Pou1f1* mutants. Many of these abnormalities can be corrected by thyroxine treatment during hearing maturation, although the period of sensitivity to hormone is transient (Knipper et al. 2000; Uziel 1986; Uziel et al. 1983). If hypothyroidism is not corrected promptly, permanent intellectual disability and deafness can result. In contrast, later TH replacement can correct growth insufficiency. In the auditory system, thyroid hormone controls the differentiation of the cochlea during the critical postnatal period between birth and the onset of hearing at 2 wks in rodents [reviewed in (Forrest et al. 2002; Ng et al. 2013)]. In humans, this window occurs largely during fetal development (Moore and Linthicum 2007).

Both genetic and environmental factors can cause hypothyroidism and hearing impairment. For example, environmental iodine deficiency can result in combined maternal and fetal hypothyroidism during pregnancy (Berbel et al. 2007). Genetic defects in thyroid hormone synthesis and action lead to primary hypothyroidism and varying levels of deafness in humans and mice. The variability results from several factors: the specific gene that is mutated, the timing of thyroid hormone deficiency during development, the effects of modifying genes, and the environment. Three relevant examples are illustrative of this. First, siblings with Pendred syndrome who are homozygous for the common *SLC26A4* p.T416P

allele vary in the severity and progression of deafness (Napiontek et al. 2004). Second, humans with mutations in *THRB* that reduce T3 binding affinity and disrupt interactions with transcriptional cofactors have variably penetrant hearing impairment. Notably, only 21% of patients with *THRB* loss of function have hearing loss (Brucker-Davis et al. 1996). Third, *Thrb*^{-/-}; *Thra*^{-/-} mice lack all known thyroid hormone receptors and are deaf due to numerous defects in cochlear physiology and morphology (Rusch et al. 2001). Initial studies of these mutants were carried out on mixed genetic backgrounds. Generation of congenic lines of these mutants on the C57BL/6J strain background reduced individual variability in hearing impairment. Thus, the incidence and severity of deafness due to hypothyroidism can be influenced by both genes and environment.

We previously mapped the effects of genetic background on hypothyroidism induced hearing impairment (Fang et al. 2011). We crossed *Mus musculus musculus* DW/J-*Pou1f1*^{dw/+} with wild type mice of the distantly related subspecies *Mus musculus castaneus* (CAST/EiJ). Forty-one F₂ mice with extreme differences in auditory brain stem responses were genotyped with 90 microsatellite markers. We identified a modifier locus on mouse Chromosome 2 that protects against hearing impairment, which we designated as *Mdwh* (**M**odifier of **dw** hearing). This 20 Mb region of Chr 2 contained one known modifier of hearing, the gene encoding the microtubule-associated protein 1a (MTAP1A). However, additional genetic crosses with the AKR strain demonstrated that protective alleles of *Mtap1a* were neither necessary nor sufficient for normal hearing in *Pou1f1*^{dw/dw} mice. We expect that future identification of the protective modifier gene(s) in mice could enhance our understanding of the mechanisms that underlie susceptibility to hypothyroidism-induced hearing impairment in humans. We report additional genetic and phenotypic analysis and the identification of candidate genes in the critical region of Chr 2 that may protect against hypothyroidism-induced deafness by influencing thyroid gland function.

Materials and Methods

Mice and genotyping

All mice were housed in a 12-h light, 12-h dark cycle in ventilated cages with unlimited access to tap water and Purina 5020 chow. All procedures were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines of the Care and Use of Experimental Animals and approved by our Institutional Animal Care and Use Committee. Ames dwarf mice (*Prop1*^{df/df}) were originally obtained from Dr. A. Bartke (Southern Illinois University, Carbondale, IL) from a non-inbred stock (DF/B). Snell dwarf mice (*Pou1f1*^{dw/dw}) were originally obtained from The Jackson Laboratory from an inbred stock (DW/J) (Bar Harbor, ME). Separate colonies of DF/B-*Prop1*^{df/+} and DW/J-*Pou1f1*^{dw/+} mice have been maintained at the University of Michigan through heterozygous matings. CAST/EiJ inbred mice were from The Jackson Laboratory, and 129P21OlaHsd inbred mice were from Harlan Sprague Dawley Inc. Crosses were conducted to produce (DW/J-*Pou1f1*^{dw} × CAST/EiJ) F₂ hybrids and (DW/J-*Pou1f1*^{dw} × 129P21OlaHsd) F₂ hybrids. The *Prop1*^{df} p.S83P and *Pou1f1*^{dw} p.W251C mutations were genotyped as described (Douglas et al. 2001; Gage et al. 1996). Both male and female mice were used.

Tissue Preparation and Histology

For middle ear histology, mice were euthanized at 3 weeks and 4 months of age. The middle ear was dissected and fixed in 4% paraformaldehyde for 2 hours. The middle ears were washed 3 times for 5' each in 1X Phosphate Buffered Saline (PBS) and then placed in 10% ethylenediaminetetraacetic acid (EDTA) overnight at 4° C. The next day they were washed again 3 times for 5' each in 1X PBS. Thyroid glands were dissected from postnatal day 9 (P9) mice and fixed in 4% paraformaldehyde for 1 hour and then washed 3 times for 5' each in 1X PBS. Both middle ear and thyroid glands were dehydrated to 70% ethanol, and embedded in a Tissue Tek VIP Paraffin tissue processing machine. Sections at 6 µm thickness from at least 3 different animals of each age and genotype were analyzed by hematoxylin and eosin (H&E) staining and 4',6-diamidino-2-phenylindole(DAPI) staining. H&E staining was performed as described previously (Mortensen et al. 2015). The H&E images were captured using a Leica DMRB fluorescent microscope.

For bullae histology, 3-month old mice were euthanized, heads were removed and skinned manually, and the remaining skull tissues were removed by dermestid beetles (Geister et al. 2015). Bullae images were captured with a Leica dissecting microscope.

Thyroid follicle measurement

H&E stained thyroid sections were used for quantifying the number of follicles with colloid per area of thyroid tissue. Three sections were analyzed from the middle of the gland that were 5 sections apart (n=3 for each genotype). The area of the thyroid was measured using the Image J software (National Center for Biotechnology Information), and the number of follicles with colloid was counted. The range of follicle numbers counted was as follows: DF/B-*Prop1*^{+/+} controls (97 to 189 follicles), DF/B-*Prop1*^{df/df} mutants (49 to 110), DW/J-*Pou1f1*^{+/+} controls (60 to 116), and DW/J-*Pou1f1*^{dw/dw} mutants (30 to 54). The average number of follicles with detectable colloid per thyroid area was calculated. Significance was determined using one-way ANOVA.

Real Time PCR

Thyroid glands were dissected from P9 mice and stored in RNALater at -20°C. Thyroids were collected from DF/B-*Prop1*^{+/+}, DF/B-*Prop1*^{df/df}, DW/J-*Pou1f1*^{+/+}, and DW/J-*Pou1f1*^{dw/dw} mice (n=8 for each genotype). RNA was isolated and DNase I treated using the protocols provided in the RNAqueous Micro kit (Life Technologies). The amount and quality of RNA was determined with the NanoDrop 2000 Spectrophotometer (Thermo Scientific). The Superscript II reverse transcription system from Invitrogen (Life Technologies) was used to generate cDNA. Specifically, RNA and oligo dT primers were denatured at 70°C for 10 minutes and then placed on ice. 100 mM DTT, 10 mM dNTPs, 5X first strand buffer and 1 U Superscript II were added to the RNA primer mix. Samples were incubated at 42°C for 50 minutes, then heat-inactivated for 10 minutes at 70°C. Samples without reverse transcriptase were included as negative controls. Quantitative PCR (qPCR) was carried out using Taqman gene expression assays for *Tg* (Mm01200340_m1), *Dio1* (Mm00839358_m1), *Tpo* (Mm00456355_m1), and *Gapdh* (4308313). The qPCR reaction was set up in triplicate with 5 ng cDNA per reaction using Taqman Universal PCR Master Mix and default run parameters on the ABI 7500 Real-Time PCR instrument. Results were

expressed as average CT normalized to *Gapdh*. Triplicates containing the Master Mix and water with the Taqman primer sets were used as negative controls. All reagents and instruments were obtained from Life Technologies. Significance was determined using an ordinary one-way ANOVA.

Quantitative PCR (qPCR) was carried out on liver cDNA using Taqman gene expression assays for *Thrsp* (Mm01273967_m1) and *Gapdh*. The qPCR reaction was carried out in triplicate with 80 ng of cDNA per reaction, using same reagents, run parameters, and machine as previously described (Vesper et al. 2006) (Livak and Schmittgen 2001). Among T3 injected mice, the fold change in expression of DF/B-*Prop1*^{+/+} controls was compared to DF/B-*Prop1*^{df/df} mice, and DW/J-*Pou1f1*^{+/+} values were compared to DW/J-*Pou1f1*^{dw/dw}, and the calculations were performed as described previously (Brinkmeier et al. 2009).

Assessment of response to exogenous thyroid hormone

Two-month old DF/B-*Prop1*^{+/+} (n=5), DF/B-*Prop1*^{df/df} (n=5), DW/J-*Pou1f1*^{+/+} (n=5), and DW/J-*Pou1f1*^{dw/dw} (n=4) mice were injected with 4 µg of T3 (3,3',5-Triiodo-L-thyronine sodium salt, Sigma, catalog # T6397) per g body weight on two consecutive days. On the third day, livers from the injected mice and non-injected control mice of the same genotypes were collected and stored in RNALater at -20°C. RNA was isolated using the RNAqueous 4PCR RNA isolation kit from Ambion (AM1914).

Total T4 Analysis

Eight P9 and 9 adult (~2 mo) mice of each of the following genotypes, DF/B-*Prop1*^{+/+}, DF/B-*Prop1*^{df/df}, DW/J-*Pou1f1*^{+/+}, and DW/J-*Pou1f1*^{dw/dw}, were euthanized with CO₂ and blood was collected by cardiac puncture. After clotting at room temperature for 30 minutes, the blood was centrifuged at 8000 x g for 10 min. 25 ul of serum was analyzed using the Total Thyroxine (T4) Enzyme Immunoassay Test Kit (07BC-1007, MP Biomedicals, Ohio, USA).

Auditory brainstem response

The auditory brain stem response test was used to assess hearing thresholds of the mutant mice (Fang et al. 2011; Karolyi et al. 2007). A total of 183 *Pou1f1*^{dw/dw} F2 mice from a (DW/J-*Pou1f1*^{dw/+} × CAST/EiJ)F1 intercross and 128 *Pou1f1*^{dw/dw} F2 mice from a (DW/J-*Pou1f1*^{dw/+} × 129P2/OlaHsd)F1 intercross were tested. For the (DW/J-*Pou1f1*^{dw/+} × Cast/Ei)F1 intercross, the frequencies of tone bursts presented to the mice were click and 8, 16, 32 kHz at the Jackson laboratory and 10 and 20 kHz at the University of Michigan. Both 10 and 20 kHz frequencies of tone bursts were presented to the (DW/J-*Pou1f1*^{dw/+} × 129P2/OlaHsd)F1 intercross mice.

Genome Scan

Genotyping services were provided by the Sequencing Core at the University of Michigan using a commercially available Medium Density Mouse Linkage Panel (Illumina, San Diego, CA) to genotype 1,449 SNPs on 183 (DW/J-*Pou1f1*^{dw} × CAST/EiJ) *Pou1f1*^{dw/dw} F₂ mice and 128 (DW/J-*Pou1f1*^{dw} × 129P2/OlaHsd) *Pou1f1*^{dw/dw} F₂ mice. The panel carries approximately 3 SNPs per 5 Mb interval across the genome. ABR thresholds for 10 and 20

kHz stimuli were evaluated as quantitative traits, and QTL linkage analysis was performed using PLINK v1.07, which is a free, open-source whole genome association analysis toolset (Purcell et al. 2007). 1359 of the SNPs are located on Chr 1–19, and these were considered in the analysis. Individual samples that failed to type at more than 10% of the loci were excluded from the analysis (N=1 for 129P2 F₂ and N=8 for CAST/Ei F₂). Loci that failed to type on >5% of the samples were excluded from the analysis (N=66 for 129P2 and N=133 for CAST/Ei). Loci that were not informative between DW/J and 129P2 (N=629) or between DW/J and CAST/Ei (N=876) were also excluded. Thus, 664 and 876 informative SNPs were utilized in the analysis of the 129P2 and CAST/Ei crosses respectively. The threshold P value was calculated using the Bonferroni method ($p=0.05/\text{number of informative markers}$). The critical interval was defined by subtracting 1.5 from the LOD score for the peak SNP.

DNA sequencing

Candidate genes for *Mdwh* were selected based on relevance to thyroid biology and reported DNA sequence variation between the susceptible C57BL/6J strain and resistant 129P2 strain (Table S1). To verify the sequences, primers were designed to amplify exons of candidate genes from the DW/J and DF/B strains, and amplification products were subjected to Sanger sequencing (Table S2).

Results

DW/J-*Pou1f1*^{dw/dw} mutants have poor middle ear development

Patients with resistance to thyroid hormone have both conductive and sensorineural deafness (Brucker-Davis et al. 1996). The development of the cochlea had been extensively compared in the hypothyroid DF/B-*Prop1*^{df/df} and DW/J-*Pou1f1*^{dw/dw} mutants, and the results demonstrated that the DW/J-*Pou1f1*^{dw/dw} mutants had essentially every defective feature found in other hypothyroid mice, while the DF/B-*Prop1*^{df/df} mice had none of them despite the lack of TSH and detectable thyroid hormone (Fang et al. 2012; Mustapha et al. 2009; Sundaresan et al. 2016). This led us to hypothesize that the protective locus in DF/B-*Prop1*^{df/df} mice has a global impact on most thyroid hormone-dependent processes, with the exception of body growth. To test whether this predicted protection is true for conductive hearing impairment, we examined the development of the middle ear. The critical role of thyroid hormone in development of the middle ear has been demonstrated by elegant studies in three other mutant mice: the congenitally hypothyroid *Tshr*^{-/-} mutants, TR-deficient *Thra*^{-/-}; *Thrb*^{-/-} double mutants, and dominant-negative TR α 1 transgenics (Cordas et al. 2012). Thyroid hormone is necessary for cavitation of the middle ear space by clearing mesenchymal tissue to create an operational air-filled cavity, which is necessary for sound conduction and normal development of hearing (Jaskoll and Maderson 1978; Richter et al. 2010). It is also necessary for proper development of the ossicles. In mice, mesenchyme begins clearing at P6 and normally clears from the area surrounding the middle ear ossicles by P14, allowing free movement of the ossicles.

We observed complete mesenchymal clearing in 3 wk *Prop1*^{+/+} and *Pou1f1*^{+/+} mice using H&E staining of sections (Fig. 1A and 1C), but both the *Prop1*^{df/df} and *Pou1f1*^{dw/dw} (Fig. 1B and 1D) mutants abnormally retain mesenchymal cells at this age, consistent with

developmental delay due to hypothyroidism and elevated hearing thresholds in both mutants at this age (Karolyi et al. 2007). DAPI staining on adjacent sections confirms that the retained material in the mutants is nucleated cellular debris and that material in the space of normal middle ears is acellular (Supplemental Fig. 1). By 4 months, the mesenchyme is essentially cleared in the *Prop1^{df/df}* (Fig. 1F) and appears similar to wild type DF/B and DW/J (Fig. 1E, G). The *Pou1f1^{dw/dw}* mutants, however, continue to retain mesenchyme in the middle ear cavity (Fig. 1H), which could interfere with conductive hearing properties. DAPI staining confirmed these conclusions as well (Supplemental Fig. 1).

The middle ear ossicles are encased within the auditory bulla, a structure that forms from the fusion of multiple intramembranous ossifications (Hanken and Thorogood 1993). The formation of the auditory bulla in conjunction with cavitation results in an enclosed air space, through which sound vibrations are carried. The bulla is also the seat of the tympanic membrane. Therefore, development of the bulla is critical for hearing. The patterning of the auditory bulla is complete in mice by P9, and it continues to increase in size until P21 (Huangfu and Saunders 1983). At 3 months of age, some defects are evident in the auditory bullae of both the *Prop1^{df/df}* (Fig. 1J) and the *Pou1f1^{dw/dw}* (Fig. 1L) mutants, compared with wild type littermates (Fig. 1I, 1K). The *Pou1f1^{dw/dw}* mutant auditory bulla is much more severely affected than the *Prop1^{df/df}* mutant, including lack of the tympanic ring, which likely contributes to conductive hearing impairment in *Pou1f1^{dw/dw}* mutant.

Thyroid hormone and its cellular actions in *Prop1^{df/df}* and *Pou1^{dw/dw}* mutants

Given the diversity of features that are more severely affected in susceptible hypothyroid mutant strains relative to resistant ones, we considered the possibility that there might be a global difference in thyroid hormone responsiveness between strains affecting regulatory or coding sequences for thyroid hormone transporters, receptors, co-factors or other aspects of the thyroid hormone-mediated regulation of gene expression. If this were the case, then the DW/J strain might respond more poorly to exogenous thyroid hormone administration than the DF/B strain. To test this we examined expression of *Spot 14*, also called *Thrsp* (thyroid hormone responsive protein), a gene for which expression is highly induced by thyroid hormone in the liver (Jump et al. 1984; Seelig et al. 1981). We injected 2-month old *Prop1^{df/df}* and *Pou1f1^{dw/dw}* mutant mice and their wild type littermates with maximal stimulating doses of triiodothyronine (T3) on 2 consecutive days and collected livers 24 hr after the last injection. Non-injected mice served as controls. We determined *Thrsp* and *Gapdh* expression levels using qRT-PCR. As expected, *Thrsp* expression was substantially lower in the non-injected *Prop1^{df/df}* and *Pou1f1^{dw/dw}* mutants compared to their non-injected, normal littermates (38-fold and 33-fold, respectively), consistent with the hypothyroid status of the mutants (data not shown). The *Prop1^{+/+}* and *Pou1f1^{+/+}* wild type mice responded to T3 injection with 2 to 2.6 fold increase in *Thrsp* expression compared with the non-injected controls, respectively. T3 injection caused both mutants to increase liver *Thrsp* expression levels markedly over those of non-injected mutants. A 23-fold increase was observed in *Prop1^{df/df}* mice and 27-fold in *Pou1f1^{dw/dw}* mice (Fig. 2A). Using ANOVA we determined that the level of induction of *Thrsp* by T3 injection is not significantly different between the mutants. Thus, the strain backgrounds of *Prop1^{df/df}* and

Pou1f1^{dw/dw} mutants permit equivalent, robust responses of liver gene expression to exogenous thyroid hormone.

The critical period for thyroid hormone action in the development of rodent hearing is within the first 2 weeks after birth [reviewed in (Ng et al. 2013)]. Accordingly, we measured the serum levels of T4 in *Prop1*^{df/df}, *Pou1f1*^{dw/dw}, and wild type littermates at P9 to determine whether there were differences in circulating thyroid hormone during this critical developmental stage. The T4 levels in the *Pou1f1*^{+/+} wild type mice were 2.2 fold lower than the *Prop1*^{+/+} wild types (4.2±1.5 vs. 9.4±2.3) (Fig. 2B), confirming significant strain differences in circulating thyroid hormone levels. The T4 levels in both the *Prop1*^{df/df} mutants (1.42±0.27) and *Pou1f1*^{dw/dw} mutants (1.01±0.58) were substantially lower than their normal littermates. These values were essentially at the lower limit of detection by the assay. The DF/B and DW/J strain differences in serum T4 levels persisted in adult mice, and the T4 levels in both mutants were near the limit of detection (Fig. 2C).

Thyroid gland folliculogenesis is poorer in *Pou1f1*^{dw/dw} than *Prop1*^{df/df} neonates

To determine whether the lower serum levels of T4 in the normal neonates of the DW/J strain relative to the DF/B strain arose from differences in thyroid gland folliculogenesis or function, we characterized the thyroid follicles in hypothyroid mutants and wild type mice on these two genetic backgrounds using H&E staining of sections from P9 animals. Normal DF/B mice appeared to have more large follicles than normal DW/J mice, and both mutants have smaller and less well-developed follicles than their normal littermates (Fig. 3A). The young *Pou1f1*^{dw/dw} mutants appeared to have much less well developed follicles and more fatty infiltrate relative to *Prop1*^{df/df} mutants. We quantified the differences in the number of follicles with colloid per area of thyroid tissue using ImageJ software and a one-way ANOVA (Fig. 3B). The *Pou1f1*^{dw/dw} mutants had significantly fewer follicles with developed colloid per thyroid area (11.8 ± 2.2) compared to *Prop1*^{df/df} mutants (28.0 ± 6.3), or wild type mice of either strain (34.9 ± 2.5 for DF/B and 25.5 ± 2.3 for DW/J). It is noteworthy that these values in *Prop1*^{df/df} mutants are in the same range as the DW/J wild type mice. There was no significant difference between the values of any of the other genotypes.

To investigate whether the under-developed thyroid follicles of *Pou1f1*^{dw/dw} mutants were associated with reduced expression of genes indicative of thyroid gland function (Amendola et al. 2005), we used qRT-PCR to measure expression of thyroglobulin (*Tg*), iodothyronine deiodinase type 1 (*Dio1*), and thyroperoxidase (*Tpo*) in RNA from thyroid glands of *Prop1*^{df/df}, *Pou1f1*^{dw/dw}, and wild type littermates at P9. All three genes were expressed at significantly lower levels in the mutants compared to wild type mice, which is consistent with expectations for severely hypothyroid animals (Fig. 3C and Supplemental Fig. 2). *Tg* expression was 4.3 fold lower in the *Pou1f1*^{dw/dw} mutants compared to *Prop1*^{df/df} mutants. This finding is compelling since thyroglobulin is the most abundant protein in follicular cells and is the critical precursor in thyroid hormone biogenesis (Di Jeso and Arvan 2016; Salvatore and Edelhoch 1973). Therefore, reduced *Tg* expression is consistent with the histological findings of reduced thyroid folliculogenesis and function in *Pou1f1*^{dw/dw} mutants compared to *Prop1*^{df/df} mutants during the period critical for developing hearing.

We found no significant difference in the expression of *Dio1* and *Tpo* between the *Prop1^{df/df}* and *Pou1f1^{dw/dw}* mutant mice (Supplemental Fig. 2).

A region of mouse Chr 2 from CAST/Ei and 129P2 strains protects *Pou1f1^{dw/dw}* mutants against hypothyroidism-induced hearing impairment

Our previous genetic mapping studies placed the *Mdwh* critical region between ~118 and 138 Mb on Chr 2 and suggested that the DW/J background is more similar to C57 substrains than other inbred strains (Fang et al. 2011). This analysis used 90 microsatellite markers to analyze forty-one F₂ mutant progeny with the most extreme differences in auditory brain stem responses to 16 kHz sounds, and a follow-up screen was done with 196 F₂ mutant progeny and eight microsatellite markers on Chr 2. The lead marker in that study was *D2Mit304* located at 128 Mb. In an effort to narrow the critical region, we conducted a higher resolution genome wide association study (GWAS) with 196 mutant F₂ progeny from the (DW/J-*Pou1f1^{dw/+}* × CAST/EiJ) F₁ intercross using a mouse medium density linkage panel with 876 informative SNPs (Fig. 4A). The association between hearing threshold and SNP genotypes was evaluated with the PLINK whole genome data analysis software. Loci with LOD scores above 4.24 are considered statistically significant using the Bonferroni method ($p=0.05/\text{number of informative markers}$). The lead SNP for *Mdwh* on Chr 2 was rs6228179 at bp position 123 Mb (GRC m38) with a LOD of 9.13. The 1.5 LOD confidence interval was between 114 and 137 Mb. An additional locus on Chr 5 reached significance, rs3705458 with LOD=4.41, suggesting additional genetic complexity in protecting against hypothyroidism-induced hearing impairment.

Recombination rates differ significantly between inbred strains. SNP panels facilitate genetic mapping between closely related strains which may have less suppression of recombination than more distantly related strains (Dumont and Payseur 2011). Therefore, we carried out a second cross to narrow the critical interval using the good hearing strain 129P2/OlaHsd, which is more closely related to DW/J than CAST/EiJ, 23.3% SNP variation vs. 43.6% (Table 1). We conducted genetic mapping in 128 mutant F₂ progeny from a (DW/J-*Pou1f1^{dw/+}* × 129P2/OlaHsd) F₁ intercross. These mice exhibited a broadly distributed range of hearing thresholds (Fig. 4B). Approximately 15% of the progeny had excellent hearing, with ABR thresholds of 35 dB or less at 20kHz. The nearly continuous distribution of hearing thresholds for these mutant progeny contrasts with the bimodal distribution observed in (DW/J × CAST/Ei)F₂ mutant mice.

We conducted a GWAS with the mouse medium-density linkage panel described above. 664 SNPs were informative (Fig. 4C). Loci with LOD scores above 4.12 are considered significant according to the Bonferroni method. The GWAS data from 20 kHz ABR thresholds identified the same major modifier locus on Chr 2 with the same lead SNP rs6228179 as the CAST/EiJ cross, and a LOD of 9.09. The 1.5 LOD confidence intervals were positioned at 112–137 Mb. A peak on Chr 14 achieved significance, rs61543475, LOD 4.87, suggesting that additional loci contribute to the protected phenotype in this strain, and that the additional modifiers are different than for CAST/Ei. Similar results were obtained in a GWAS analysis of the 10 kHz ABR thresholds (data not shown).

These studies demonstrate that the major modifier on Chr 2 is conserved across several strains. However, higher resolution mapping did not substantially narrow the critical interval, even with the more closely related strain. This result suggested that there could be more than one gene within the non-recombinant interval that contributes to the quantitative trait.

Analysis of candidate genes in the Chr 2 critical region

The phenotypic comparison of susceptible and protected strains suggested that thyroid folliculogenesis and/or function could be the process that underlies their difference in auditory development among hypothyroid strains. This is consistent with the independent mapping of a modifier of hypothyroidism, hypothyroid related chr 2 or *Htrc2*, to a region that overlaps with *Mdwh* (Fig. 5) (Amendola et al. 2005; Amendola et al. 2010). We identified seven candidate genes within the *Mdwh* critical region that are relevant to thyroid biology and contain potentially damaging variation between the C57BL/6J (which is closely related to DW/J) and 129P2 strains (Table 2) (Keane et al. 2011). Two to six missense variants were detected between susceptible (C57BL/6J) and resistant (129P2) strains for each of these genes. These include the closely linked dual oxidase genes *Duox1* and *Duox2*, and the dual oxidase maturation factor *Duoxa2* that map within a 68 kb interval. Another candidate gene located 3.1 Mb proximal, *Dnajc17*, encodes a type III heat-shock protein-40 involved in splicing, which has been suggested as a candidate for *Htrc2* (Pascarella et al. 2018). This gene is hypothesized to be the modifier of thyroid gland development and function in *Nkx2.1*, *Pax8* double heterozygotes and affects *Tg* transcription in cell culture (Amendola et al. 2010). Less compelling candidates include three genes involved in thyroid cancer: the spindle checkpoint gene *Bub1b*, the notch ligand *Dll4*, and the P53 binding protein *Trp53bp1*.

We used Sanger sequencing to determine the predicted missense variation and stop gain in each of these genes in the DW/J and DF/B strains relative to the previously reported values for C57/BL6J and 129/P2 (Tables 3 and S1). In all cases the DW/J strain had the same variant as the C57BL/6J strain, and in most cases the 129P2 and DF/B alleles were the same as each other and matched the predominate allele amongst inbred strains that have been sequenced (Table 3). We used phyloP, GERP (genomic evolutionary rate profiling), and SIFT (sorting intolerant from tolerant) to evaluate the conservation of each of the changes between DW/J and 129/P2 (Cooper et al. 2005; Kumar et al. 2009; Pollard et al. 2010). In PhyloP analysis, sites predicted to be conserved have positive scores, and those predicted to be fast-evolving have negative scores. The absolute values of the scores represent -log p-values under a null hypothesis of neutral evolution. Each of these genes has some variants with strongly positive phyloP scores except *Dll4* and *Duox1*. GERP scores that are positive indicate that a deficit of substitutions relative to neutrality and suggest evolutionary constraint. Each of the candidate genes has variants with positive scores greater than 1 except *Trp53bp1*. SIFT scores that are below 0.05 are considered likely damaging. *Bub1b*, *Trp53bp1*, and *Duoxa1* have variants that are likely damaging according to SIFT. Thus, all candidate genes have variants predicted to be deleterious by one of the three prediction programs. These programs suggest variants that are most likely to be affected by the variation in DW/J relative to 129P2 and DF/B, but functional studies are required as follow up.

Discussion

Genetic disorders are frequently classified as either rare, “simple Mendelian” diseases or common, complex diseases. This classification over-simplifies the frequent observation that related individuals with the same Mendelian mutation often have different clinical presentations that range from relatively high functioning to very disabled. This variability in clinical features is attributable to both environmental and genetic factors. An excellent example are the genetic modifiers that have been found to influence the presentation of the autosomal recessive mutation in *CFTR* that causes cystic fibrosis in Caucasian populations (Corvol et al. 2015; Furlan et al. 2016; Sofia et al. 2016; Strug et al. 2016). Mouse models provide the means to detect interacting pathways with strong modifying effects on phenotype, and they have been used to identify over 280 modifier loci (Hamilton and Yu 2012). The mouse has been particularly informative in identifying genetic modifiers of hearing impairment, including age related hearing loss (Johnson et al. 2006). Two spectacular examples bear mention. First, the microtubule associated protein *Mtap1a* is a modifier of the hearing impairment in *Tubby* mice, but it does not affect the obesity or retinal phenotypes, and the effect of the amino acid alterations is only evident in the context of the *Tubby* mutant allele (Ikeda et al. 2002). Second, cadherin 23 (*Cdh23*) is a modifier of the hearing of deaf waddler mutants, which have a defective calcium transporting membrane protein ATP2B2, and hearing loss allele of the adhesion G-protein coupled receptor V1 gene, *Adgv1^{firings}* (Johnson et al. 2005; Noben-Trauth et al. 2003; Noben-Trauth et al. 1997). The sensitive variant *Cdh23^{753A}* also contributes to age-related hearing loss. These mouse studies accurately predicted similar genetic interactions in humans, in which alleles of the *ATP2B2* gene influence hearing impairment caused by mutations in *CDH23* (DFNB12) and *MYO6* (DFNB37) (Schultz et al. 2005). We previously reported mapping a modifier of hypothyroidism-induced hearing impairment in *Pou1f1^{dw}* mice, *Mdwh*, to Chr 2, and we demonstrated that it acts intrinsically in the fetus (Fang et al. 2012; Fang et al. 2011). Here we demonstrate that poor thyroid gland folliculogenesis during the critical period for developing hearing is associated with susceptibility to hypothyroidism-induced hearing impairment (Table 4), and we present four strong candidate genes: *Dnajc17* and the closely linked *Duox1*, *Duox2* and *Duoxa2* genes.

We present the first analysis of middle ear development in *Prop1^{df/df}* and *Pou1f1^{dw/dw}*. We observed persistent mesenchyme in both *Prop1^{df/df}* and *Pou1f1^{dw/dw}* mutants at 3 wk of age, consistent with elevated hearing thresholds at that time (Karolyi et al. 2007), and the mesenchyme persisted through 4 mo of age in *Pou1f1^{dw/dw}* mutants, consistent with earlier reports of *Pou1f1^{dw/dw}* mutants (Marovitz et al. 1968). In contrast, the mesenchyme was completely cleared by 4 mo in *Prop1^{df/df}* mutants, consistent with their acquisition of normal hearing by that time (Fang et al. 2012). Persistent mesenchyme is thought to predispose to middle ear infections, which can influence learning and speech. Genetic and infectious disease models of otitis media have been studied in mice (Hardisty et al. 2003; Rye et al. 2011). We did not observe any difference in otitis media in hypothyroid mice that are susceptible or resistant to hearing impairment. The persistent developmental delay in bone maturation in *Pou1f1^{dw/dw}* mutants also extended to the auditory bullae, in which the tympanic ring failed to develop. We conclude that conductive hearing impairment

contributes to the developmental delay in hearing acquisition of *Prop1*^{df/df} mutants and to the persistent, profound deafness in *Pou1f1*^{dw/dw} mutants. These findings are consistent with observations in thyroid stimulating hormone receptor mutant mice (*Tshr1*^{-/-}) and thyroid hormone receptor deficient mice that are doubly mutant for both receptors (*Thra1*^{-/-}, *Thrabr*^{-/-}). Both have defects in inner ear and middle ear development including persistent retention of mesenchyme surrounding the ossicles, enlarged ossicles, and delayed ossification (Cordas et al. 2012).

We hypothesized that the susceptibility to hypothyroidism-induced hearing loss could result from a systemic difference in thyroid hormone production, cellular transport, bioactivation or degradation, or in coactivators and corepressors that facilitate thyroid hormone receptor mediated changes in transcription (Ng et al. 2013). This idea is supported by the pleiotropic effects of thyroid hormone deficiency on inner and middle ear development in susceptible strains and the relatively normal development of these structures in hypothyroid, resistant strains [(Fang et al. 2012; Fang et al. 2011; Mustapha et al. 2009), and this report]. However, we found no difference in the ability of susceptible and resistant strains to respond to exogenous thyroid hormone by rapid and robust activation of thyroid hormone responsive protein (THRSP or Spot14) expression in the liver. The liver utilizes a variety of thyroid hormone transporters that are also expressed in cochlear development (MCT8, MCT10, LAT1) (Engels et al. 2015). This suggests that thyroid hormone receptors, auxiliary nuclear factors, and thyroid transporters are unlikely candidates for *Mdwh*.

The thyroid gland begins to develop follicles even in the absence of pituitary thyroid stimulating hormone signaling, and the effect of TSH deficiency on thyroid gland development becomes evident in early neonatal life in mice (Beamer et al. 1981; Postiglione et al. 2002). The circulating levels of thyroid hormone in adult mice varies widely among inbred strains (Center for Genome Dynamics). We detected striking differences (~2 fold) between the circulating levels of T4 in normal mice from DF/B and DW/J strains near the normal time for onset of hearing (P9). We were unable to detect significant levels of circulating thyroid hormone in either *Prop1*^{df/df} or *Pou1f1*^{dw/dw} mice at this time or in adult mice. However, the thyroid glands of *Pou1f1*^{dw/dw} mice clearly had a paucity of functional follicles, as evidenced by the lack of colloid, and thyroglobulin transcription was reduced ~10 fold relative to normal littermates. The number of follicles with colloid per thyroid tissue area was not significantly different in *Prop1*^{df/df} mutants from either wild type strain. Taken together these data strongly support the idea that the DW/J strain poorly supports thyroid gland development in the absence of pituitary stimulation, while the DF/B strain supports significant thyroid gland function (Fig. 6).

We mapped *Mdwh* in two inbred strains to the same location on Chr 2. There are several candidate genes for differential regulation of thyroid gland function within the *Mdwh* critical region. Among the most compelling of these are the genes involved in thyroid hormone biogenesis: *Duox1*, *Duox2*, *Duoxa2*, and *Dnajc17*, each of which have likely deleterious amino acid substitutions in the susceptible DW/J strain relative to the resistant 129P2 and DF/B strains. The enzymes encoded by *Duox1* and *Duox2* are critical for generating the hydrogen peroxide required by thyroid peroxidase for incorporation of iodine into thyroglobulin, a crucial step in thyroid hormone production. Defects in DUOX2 gene

expression cause congenital hypothyroidism and hearing impairment in mouse and man, with a high level of phenotypic variability (Fugazzola et al. 2011; Johnson et al. 2007; Zheng et al. 2017). Mutations in dual oxidase maturation factor, DUOX2, also cause severe hypothyroidism in mouse and man (Grasberger et al. 2012; Vigone et al. 2005). The phenotypic consequences are likely to be influenced by genetic background and environmental factors (Weber et al. 2013). This maturation factor is a transmembrane protein in the endoplasmic reticulum that is essential for the deposition of DUOX2 at the plasma membrane (Grasberger and Refetoff 2006). It is intriguing that comprehensive screening for genes mutated in congenital hypothyroidism identified numerous cases of digenic inheritance involving *DUOX2*, *TG*, and *TPO*, providing proof of principle that genetic interaction in this pathway could be pathogenic (Nicholas et al. 2016). Other chromosomal regions contribute to the resistance to hypothyroidism induced hearing impairment, and more than one gene in the *Mdwh* region may contribute to poor thyroid gland development and deafness of *Pou1f1^{dw/dw}* mice on the DW/J strain background. Additive effects of multiple genes within a quantitative trait locus have been observed in many genetic studies (Buchner and Nadeau 2015).

We suspect that the *Mdwh* candidate genes also modify other loci that cause congenital hypothyroidism (Fig. 5). For example, a locus designated *Htrc2* (hypothyroidism-related chromosome 2), that confers resistance to congenital hypothyroidism caused by double heterozygosity for the transcription factors *Nxk2.1* and *Pax8*, was mapped to a narrow interval between 112–121 Mb, near rs13476722, using only 52 doubly heterozygous animals (Amendola et al. 2010). The C57BL6/J (B6) genetic background is susceptible, whereas the 129/SvPasCrl (Sv) strain is resistant, and the effect is polygenic, involving a second locus on Chr 5. The authors considered *Dnaja17* to be the most likely candidate gene for the *Htrc2* locus on Chr 2 because susceptible mice have reduced levels of thyroglobulin during embryogenesis, and the B6 allele of *Dnaja17* suppresses NKX2.1-mediated transcription of thyroglobulin more robustly than the 129 allele. *Duox1*, *Duox2* and *Duoxa2* are outside their critical region. In a second example, a locus for resistance to congenital hypothyroidism, *Lrch*, was mapped to Chr 2 between 104.0–154.3 Mb, with *D2Mit255* as the lead SNP (Hosoda et al. 2012). This interval overlaps the *Mdwh* critical region. A 129-derived strain [129(+Ter)/SvJcI] has a resistant allele, and the DW and B6 strains have susceptible alleles that influence both body growth and the development of the thyroid gland in *Tpst2^{ert}* mutants. These mutants have an inactivating mutation (p.H266Q) in the tyrosylprotein sulfotransferase 2 gene, which catalyzes the sulfation of the thyroid stimulating hormone receptor which is required for TSH signaling (Sasaki et al. 2007). The nature of this modifier is not known, but the authors considered numerous candidate genes on Chr 2 including *Duox2* and *Duoxa2* (candidates for *Mdwh*, Table 2) and *Kcnip3*, a Kv channel interacting protein. *Dnaja17* could also be a candidate for *Lrch*, as it is for *Htrc2* and *Mdwh*. However, we suspect that multiple candidates in the *Mdwh* interval contribute to the resistance to hearing impairment in mice with hypopituitarism because we were unable to substantially narrow the critical interval even with large numbers of mutants or closely related strains.

Congenital hypothyroidism is a common birth defect, affecting ~1 in 3500 children (for reviews, see (Abu-Khudir et al. 2017; Ng et al. 2013; Stoppa-Vaucher et al. 2011)). It can be

caused by several mechanisms, including thyroid gland dysgenesis, where the thyroid gland is missing, ectopic or severely underdeveloped, and dysmorphogenesis, which involves failure to produce adequate amounts of thyroid hormone. These problems have varied incidence by ethnicity. Early detection and treatment of newborns with hormone replacement therapy within the first two weeks of life is effective, and this occurs in most countries, avoiding permanent effects on intelligence and hearing (Bellman et al. 1996; Lazarus et al. 2012; Rovet and Daneman 2003; Rovet and Ehrlich 2000). Women with hypothyroidism need increased thyroid hormone replacement to meet the increased demands of pregnancy. Failure to manage this properly can have lasting effects on the child's hearing, which has been observed in the case of *POU1F1* p.R271W mutation (Cuevas et al. 2005; Haddow et al. 1999; Lavado-Autric et al. 2003; Pine-Twaddell et al. 2013). Our study has identified candidate genes and pathways that could contribute to human variation in susceptibility to hypothyroidism-induced hearing impairment and neurological deficits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the March of Dimes for funding (grant N018020 to SAC), the NIH (T32GM00754 and T32HG000040 to AZD), Stephen H. Hinshaw for access to the dermestid beetle colony at the University of Michigan Ruthven Museum, Dr. Robert H. Lyons, Jr. and members of the University of Michigan DNA Sequencing Core Facility for sequencing and genotyping, members of the Kresge Hearing Research Institute including Jennifer Benson, Lisa Kabala, and David F. Dolan for auditory brainstem response testing, and Jun Z. Li, David T. Burke, Ronald J. Koenig, and Miriam H. Meisler for helpful discussions.

Funding: March of Dime Foundation (N018020) Sally A. Camper

National Institutes of Health (T32GM007544) Alexandre Z. Daly

National Institutes of Health (T32HG000040) Alexandre Z. Daly

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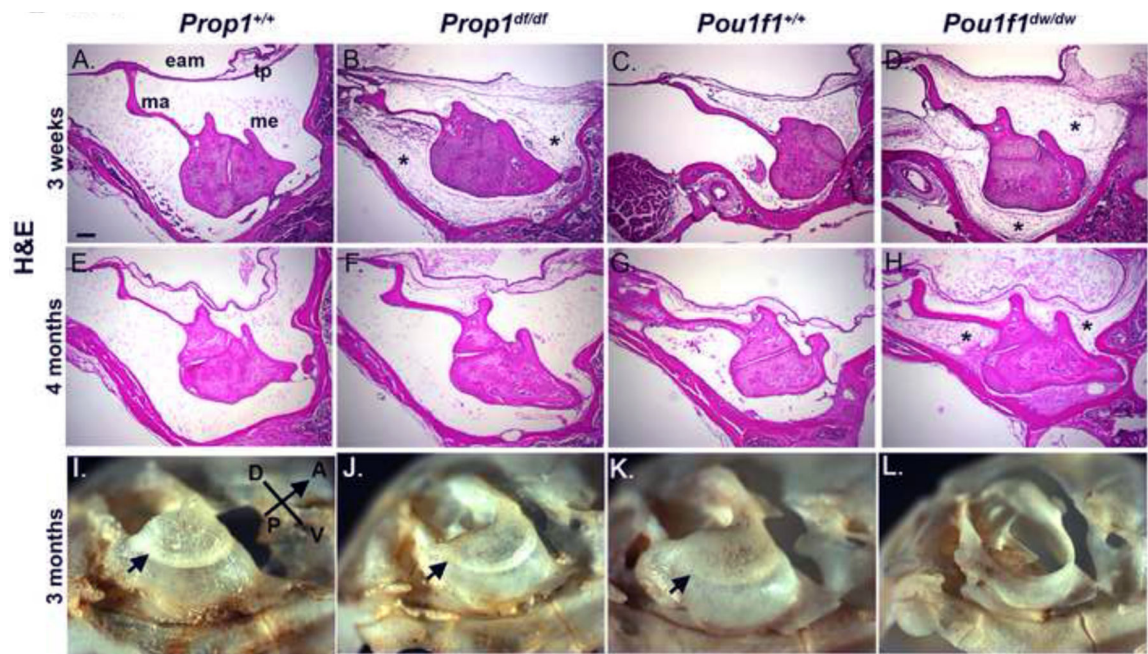


Figure 1. Conductive hearing loss contributes to hearing impairment in *Pou1f1* mutant mice. Sections of the middle ear were stained with hematoxylin and eosin (H&E) and photographed in brightfield or dark field, respectively, at 100x. The scale bar is 200 microns. Samples were taken from *Prop1*^{+/+} (A, E), *Prop1*^{df/df} (B, F), *Pou1f1*^{+/+} (C, G), and *Pou1f1*^{dw/dw} (D, H) mice at 3 wks (A-D) and 4 mo (E-H). The external auditory meatus (eam), tympanic membrane (TP), middle ear cavity (mec) and malleus (ma) structures are indicated. Retained mesenchyme is indicated with an asterisk. Bullae collected from 3 mo old *Prop1*^{+/+} (I), *Prop1*^{df/df} (J), *Pou1f1*^{+/+} (K), and *Pou1f1*^{dw/dw} (L) mice were exposed to dermestid beetles to visualize the bone structure. The sulcus tympanicus, or tympanic ring, is indicated with an arrowhead.

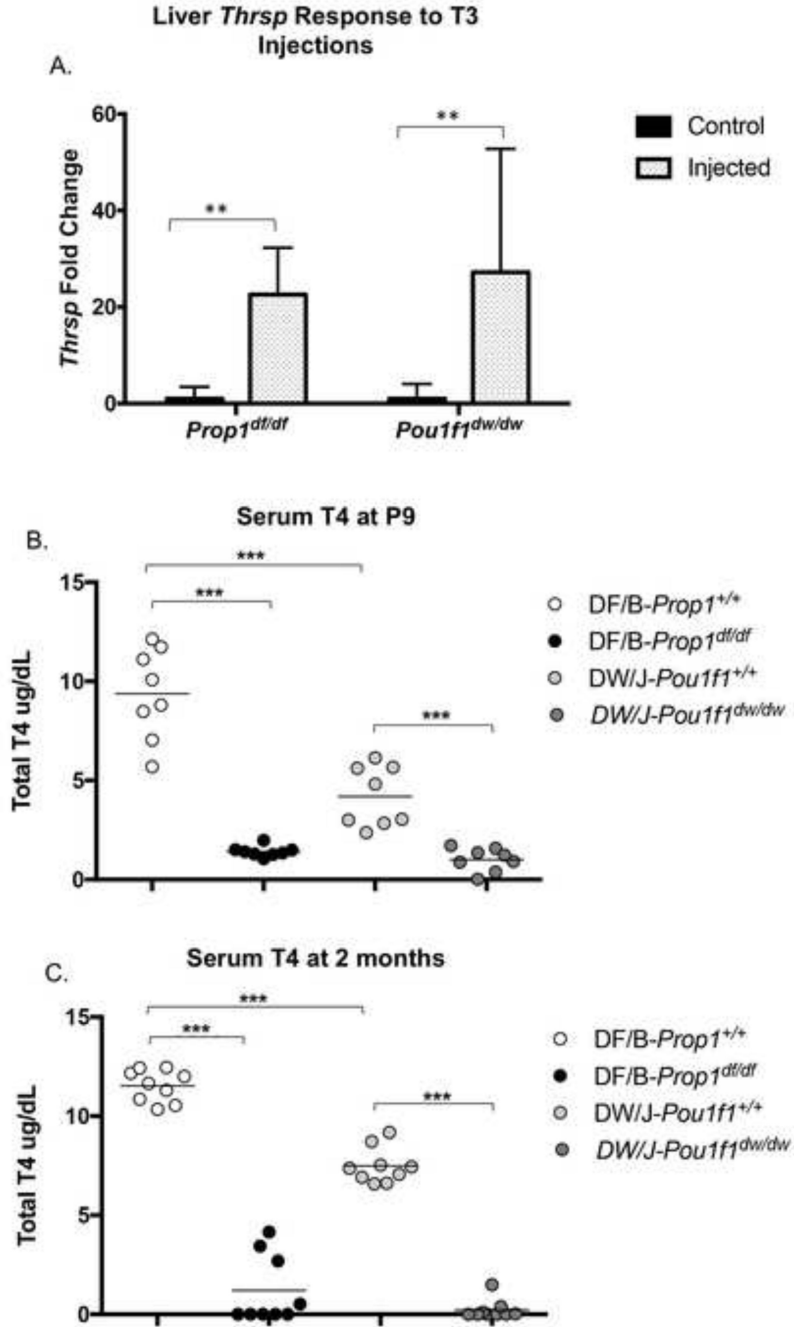


Figure 2. Hypothyroid *Prop1* and *Pou1f1* mutants mount strong responses to exogenous thyroid hormone, and their strain backgrounds have different circulating levels of thyroid hormone.

A. RT-PCR was used to measure expression levels of the thyroid hormone responsive gene *Thrsp* in the livers of *Prop1*^{df/df} and *Pou1f1*^{dw/dw} control and thyroid hormone injected mice relative to *Gapdh*. ** indicates $p < 0.01$. B. Serum T4 levels were measured in 9 day old *Prop1*^{+/+}, *Prop1*^{df/df}, *Prop1*^{+/+}, and *Pou1f1*^{dw/dw} mice. *** indicates $p < 0.001$. C. Serum T4 levels were measured in adult, ~2 mo old animals of the same four genotypes.

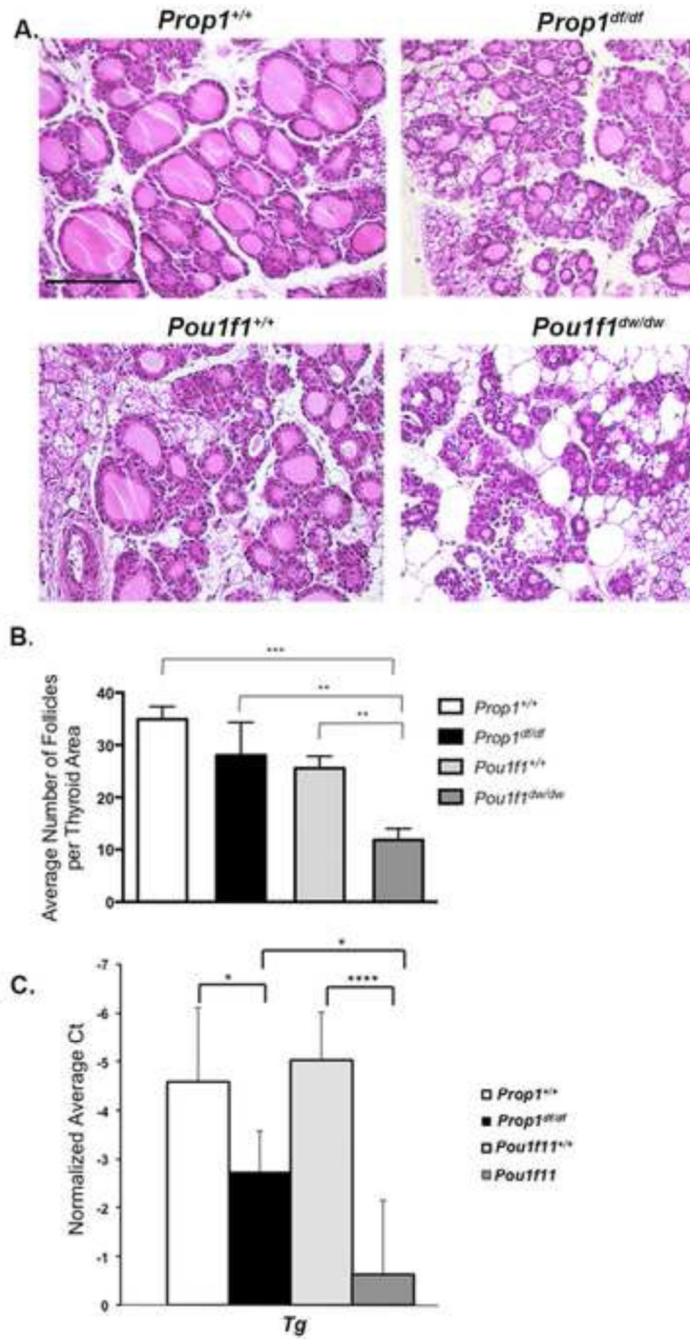


Figure 3. Thyroid development and function is strongly influenced by strain background and pituitary dysfunction.

A. Thyroid glands of P9 *Prop1*^{+/+}, *Prop1*^{df/df}, *Pou1f1*^{+/+}, and *Pou1f1*^{dw/dw} mice were sectioned and stained with hematoxylin and eosin and photographed at 200x. The scale bar is 400 microns. B. The average number of thyroid follicles per area were quantified. ** indicates p<0.01 and *** indicates p<0.001. C. Thyroid gland RNA was prepared from P9 *Prop1*^{+/+}, *Prop1*^{df/df}, *Pou1f1*^{+/+}, and *Pou1f1*^{dw/dw} mice and thyroglobulin (*Tg*) transcripts were measured by RT-PCR and normalized to *Gapdh*. * indicates p<0.05 and **** indicates p<0.0001.

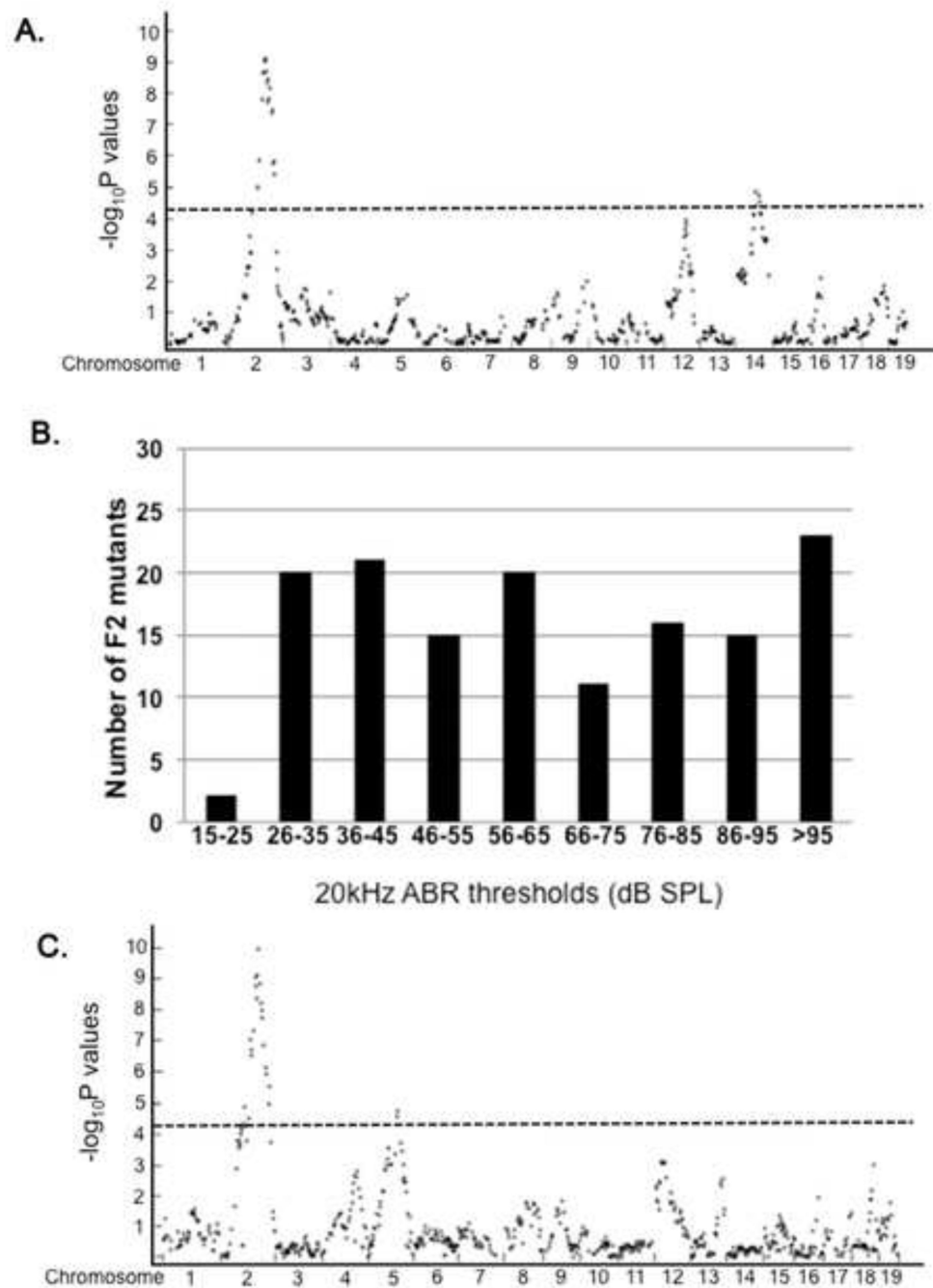


Figure 4. Genetic mapping of hearing as a quantitative trait reveals the same region of Chr 2 in CAST/Ei and 129P2 strains modifies *dw* hearing.

A. A GWAS was carried out to assess the association of hearing ability with SNP typing of mutants from an $F_1(\text{CAST/Ei wild type} \times \text{DW/J-}Pou1f1^{dw/+}) \times F_1$ cross. Each dot represents the $-\log_{10}P$ value for an individual, informative SNP on each chromosome. The dotted line indicates the baseline for significant association. B. The hearing of $Pou1f1^{dw/dw}$ mutants from an $F_1(129P2 \text{ wild type} \times \text{DW/J-}Pou1f1^{dw/+}) \times F_1$ cross was assessed by auditory brain stem response (ABR) to 20kHz tone and the threshold sound pressure level (SPL) recorded in dB. The number of mutants with thresholds in the indicated ranges were recorded. C. A

similar GWAS was carried out to assess the association of hearing ability with SNP typing of mutants from an $F_1(129P2 \text{ wild type} \times DW/J\text{-}Pou1f1^{dw/+}) \times F_1$ cross. Each dot represents the $-\log_{10}P$ value for an individual, informative SNP on each chromosome. The dotted line indicates the baseline for significant association.

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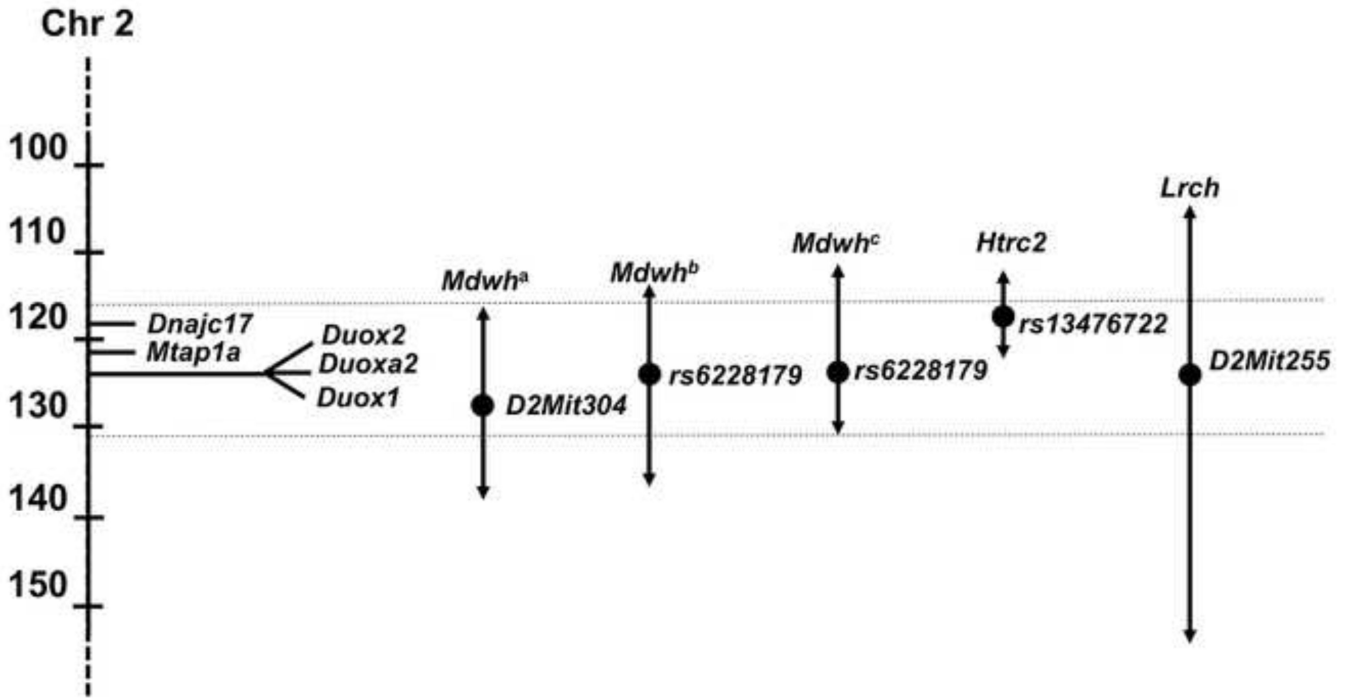


Figure 5. Multiple modifiers of thyroid gland folliculogenesis map on Chr 2.

The 1.5 LOD interval for *Mdwh* was originally mapped with microsatellite markers in mutant progeny of a (DW/J-*Pou1f1*^{dw/+} × CAST/Ei)F1 × F1 intercross (*Mdwh*¹) with the lead marker *D2Mit304* (1128331784). A GWAS analysis of this cross (*Mdwh*²) and a similar intercross with 129P2 (*Mdwh*³) refined the location and identified the same lead SNP in each case, rs6228179 (123293026). The lead candidate genes for *Mdwh* are *DNAJc17* and the cluster containing *Duox2*, *Duoxa2*, and *Duox1*. The *Htrc2* locus was mapped to rs12476722, with *Dnajc17* as the lead candidate, and *Lrch* to *D2Mit255*, completely overlapping *Mdwh*.

Genetic modification of hypothyroidism-induced hearing impairment

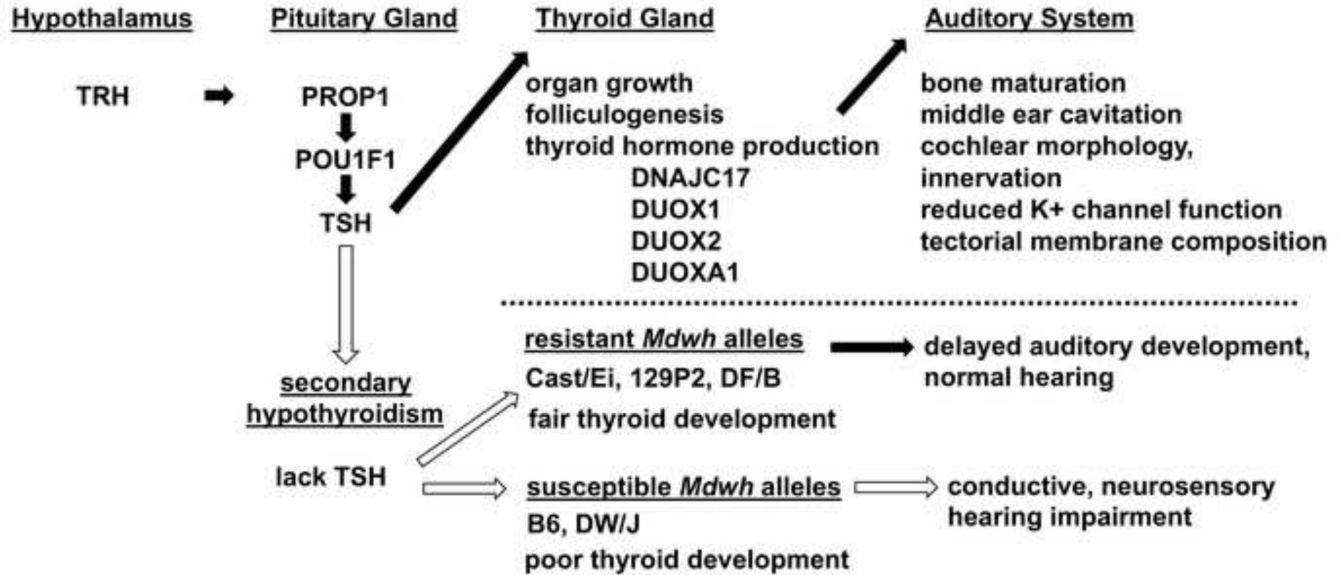


Figure 6. Genetic modification of hypothyroidism-induced hearing impairment.

Hypothalamic production of TRH stimulates the pituitary gland to produce TSH. The transcription factor PROP1 is necessary to activate expression of the transcription factor POU1F1, and both are necessary for development of the cells that produce TSH. Pituitary TSH stimulates thyroid gland growth, folliculogenesis and thyroid hormone production in the postnatal period. DUOX1, DUOX2, and DUOXA1 are necessary for thyroid hormone production from the thyroglobulin precursor protein. DNAJC17 is expressed early in thyroid bud development and is a candidate gene for regulation of thyroglobulin expression. In rodents the critical period for thyroid hormone stimulation of brain and auditory system development is the first two weeks after birth. *Prop1* and *Pou1f1* mutations cause severe TSH deficiency. We propose that the variable effects of TSH deficiency on hearing are caused by genetic modifiers that influence thyroid gland function in the early neonatal period. *Dnajc17*, *Duox1*, *Duox2*, and *Duoxa1* are candidate genes for the resistant *Mdw* alleles that allow for better thyroid gland development and function in the absence of pituitary stimulation.

Table 1.

SNP variation between DW/J and common inbred mouse strains

Strains	# variable SNPs	% variation
DW/J vs. C57BL/6J	47	0.68
DW/J vs. AKR/J	1514	21.97
DW/J vs. BALB/cJ	1521	22.08
DW/J vs. 129P2	1605	23.30
DW/J vs. DBA/2J	1824	26.47
DW/J vs. CBA/J	1986	28.82
DW/J vs. C3H/HeJ	2232	32.39
DW/J vs. CAST/Ei	3004	43.60

Table 2.

Genes in *Mdwh* region with genetic variation and relevance to thyroid biology

Mouse Gene Symbol	Gene Name	Function	Variants 129P2 vs. DW/J and B6	Location	Disease	Human Gene Symbol
<i>Bub1b</i>	Spindle assembly checkpoint	Overexpressed in thyroid carcinoma	6 missense	118,598,211-118,641,592	colorectal cancer, variegated aneuploidy syndrome 1	<i>BUB1B</i>
<i>DNAJC17</i>	DNAJ(Hsp40) homolog, subfamily C, member 17	Expressed in thyroid bud	6 Missense	119,172,500-119,208,795	homozygous lethal in mice	<i>DNAJB1</i>
<i>Dll4</i>	Delta-like 4	Notch pathway, plays a role in thyroid carcinogenesis and angiogenesis	2 missense	119,325,784-119,335,666	heterozygous partial lethal, homozygous embryonic lethal in mice	<i>DLL4</i>
<i>Trp53bp1</i>	Transformation related protein 53 binding protein 1	Nuclear Trp53BP1 foci in thyroid cancer	3 missense	121,267,312-121,271,407	growth retardation, immunodeficiency, thymic lymphoma in mice	<i>TP53BP1</i>
<i>Duox2</i>	Dual oxidase 2	A component of the thyroid H ₂ O ₂ generator critical for thyroid hormone synthesis	5 missense	122,280,437-122,298,165	congenital hypothyroidism, low T4, high TSH, hearing impaired	<i>DUOX2</i>
<i>Duoxa1</i>	Dual oxidase maturation factor 1	Required for targeting of functional DUOX enzymes to the cell surface	3 missense	122,303,549-122,313,730	severe hypothyroidism with growth retardation	<i>DUOXA1</i>
<i>Duox1</i>	Dual oxidase 1	Main source of hydrogen peroxide in rat thyroid cell line PCC13	3 missense	122,315,672-122,347,972	unknown	<i>DUOX1</i>

¹Highly conserved p.Phe273Tyr allele is implicated in altered DNAJC17 activity.

Table 3.

Genetic Variation in candidate gene for *Mdwh* and evaluation of function.

Gene	Position	dbSNP	Amino Acid B6>129 Mouse	Amino Acid Human	PhyloP	GERP	SIFT	DW and B6	I29P2	DF/B	B6/129 allele count ¹
<i>Bub1b</i>	118612729	rs3144766	Gly240Ser	Gly	1.51 ²	2.36	0.46	G	A	A	0/16
	118615032	rs3149979	Thr278Pro	Ala	-0.049	0.959	0.97	A	C	C	0/26
	118615135	rs3149980	Ala312Val	Leu	0.094	1.82	0.35	C	T	T	0/16
	118630960	rs27423851	Glu664Asp	Asp	1.55	-6.63	-	G	C	C	0/26
	118631761	rs27423850	Asp705Asn	Asn	0.50	3.61	0.7	G	A	A	0/26
	118631824	rs252392177	Leu726Ile	Leu	1.29	1.15	0.01	T	A	A	0/14
<i>Dnaje17</i>	119172660	rs8279388	Phe273Tyr ³	Tyr	1.51	1.92	1.0	A	T	T	0/26
	119179372	rs49263590	Gly258Ser	Ser	-0.17	2.53	1.0	C	T	T	0/26
	119180535	rs27453586	Glu184Asp	Glu	0.25	3.04	0.24	C	A	A	0/26
	119180966	rs51167341	Arg161Lys	Lys	1.24	2.9	1.0	C	T	T	0/26
	119180967	rs46745469	Arg161Gly	Lys	1.24	5.33	0.17	T	C	C	0/26
	119183622	rs27453572	Gln152Arg	Gln	1.55	3.27	0.7	T	C	C	0/26
<i>Dila</i>	119332155	rs27423750	Gln419Leu	Leu	0.13	3.58	0.61	A	T	T	0/26
	119332778	rs27423747	Gly627Ser	Thy	-0.054	1.72	1.0	G	A	A	0/26
<i>Trp53bp1</i>	121216105	rs27454672	Lys1071Asn	His	-0.10	0.866	0.47-0.63	T	G	G	0/27
	121243989	rs27439904	Ala427Thr	Thr	0.0088	-3.11	0.55-0.68	C	T	T	0/26
	121244066	rs29674073	Val401Ala	Glu	1.32	-10.4	0.02-0.07	A	G	G	0/28
<i>Duox2</i>	122280988	rs217232017	Cys1410Arg	His	1.66	-4.91	0.61	A	G	G	0/26
	122291506	rs27453362	His627Arg	His	1.56	0.412	0.46	T	C	C	0/27
	122292682	rs32841329	Asp495Val	Asp	0.28	4.34	-	A	T	A ⁴	0/11
	122294092	rs27453345	Asn378Ser	Asn	1.27	5.36	0.36	T	C	C	0/26
122296719	rs219434625	His159Pro/Arg	Pro	1.56	2.14	0.2/1.0	A	C/G	C	0/12	
<i>Duoxal</i>	122303818	rs47922057	Ile318Thr	Ile	1.41	5.43	0.0	A	G	G	0/26

Gene	Position	dbSNP	Amino Acid B6>129 Mouse	Amino Acid Human	PhyloP	GERP	SIFT	DW and B6	129P2	DF/B	B6/129 allele count ¹
	122303917	rs27453307	Gln285Leu	Gln	1.75	4.17	0.68-0.71	T	A	A	0/26
	122305560	rs29816247	Leu112Phe	n.a.	-2.84	4.18	0.0-0.3	G	A	A	0/26
<i>Duox1</i>	122319515	rs236906605	His129Arg	Pro	-0.30	0.712	0.47	A	G	<u>A</u>	0/11
	122319587	rs229793548	Arg153His/Pro	Pro	-0.13	2.59	0.12	G	A/C	C	0/3
	122326535	rs27453258	Ala561Glu	Lys	-0.48	-7.12	1	C	A	<u>C</u>	0/11

¹# mouse strains with alleles matching B6/# matching 129P2.

² Grey highlighting indicates variants more likely to be damaging.

³ This highly conserved amino acid alters DNAJC17 activity.

⁴ Underlining reveals DF/B alleles that are the same as B6.

Summary of evidence for variation in thyroid gland folliculogenesis and function affecting hypothyroidism-induced hearing impairment

Table 4.

Phenotype	Strain and genotype			
	DF/B +/+	DF/B df/df	DW/J +/+	DW/J dw/dw
Strain sensitivity	resistant	resistant	sensitive	sensitive
Pituitary hormones	normal	TSH, GH, PRL deficient	normal	TSH, GH, PRL deficient
Response to T3	2 fold	23 fold	2.6 fold	27 fold
ABR ¹	~28	~40	~38	>100
Adult inner ear	normal	normal	normal	Multiple abnormalities
Adult middle ear	normal	normal	normal	Multiple abnormalities
Thyroid follicles/area	35 ± 3	28 ± 6	26 ± 2	12 ± 2
Thyroglobulin mRNA ²	-4.6 ± 1.5	-2.7 ± 0.9	-5.0 ± 1.0	-0.63 ± 1.5
Thyroid hormone	9.4 ± 2.3	1.4 ± 0.27	4.2 ± 1.5	1.0 ± 0.58

¹ Auditory brain stem response thresholds in dB SPL to 10 kHz in 6 wk old mice

² Normalized average Ct value from qRT-PCR