

A Modifier Gene Alleviates Hypothyroidism-Induced Hearing Impairment in *Pou1f1^{dw}* Dwarf Mice

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ABSTRACT Thyroid hormone has pleiotropic effects on cochlear development, and genomic variation influences the severity of associated hearing deficits. DW/J-*Pou1f1^{dw/dw}* mutant mice lack pituitary thyrotropin, which causes severe thyroid hormone deficiency and profound hearing impairment. To assess the genetic complexity of protective effects on hypothyroidism-induced hearing impairment, an F₁ intercross was generated between DW/J-*Pou1f1^{dw/+}* carriers and an inbred strain with excellent hearing derived from *Mus castaneus*, CAST/EiJ. Approximately 24% of the (DW/J × CAST/EiJ) *Pou1f1^{dw/dw}* F₂ progeny had normal hearing. A genome scan revealed a locus on chromosome 2, named modifier of *dw* hearing, or *Mdwh*, that rescues hearing despite persistent hypothyroidism. This chromosomal region contains the modifier of tubby hearing 1 (*Moth1*) locus that encodes a protective allele of the microtubule-associated protein MTAP1A. DW/J-*Pou1f1^{dw/+}* carriers were crossed with the AKR strain, which also carries a protective allele of *Mtap1a*, and we found that AKR is not protective for hearing in the (DW/J × AKR) *Pou1f1^{dw/dw}* F₂ progeny. Thus, protective alleles of *Mtap1a* are not sufficient to rescue DW/J-*Pou1f1^{dw/dw}* hearing. We expect that identification of protective modifiers will enhance our understanding of the mechanisms of hypothyroidism-induced hearing impairment.

THYROID hormone (TH) is an important regulator of many processes in mammalian development, including body growth (Cabello and Wrutniak 1989) and central nervous system maturation (Bernal 2005). Auditory function is particularly sensitive to the effects of TH, which is required for the complex development and physiology of the cochlea (Deol 1973; Uziel 1986; Sohmer and Freeman 1996). Mutations in several different genes can prevent or interfere with the TH-signaling pathway that is required for normal auditory development and function. In addition to gene mutations that cause hypothyroidism, mutations of genes encoding thyroid hormone receptors (Forrest *et al.* 1996; Rusch *et al.* 2001) and iodothyronine deiodinases (Ng *et al.* 2004, 2009) can diminish the developing cochlea's response to TH and exhibit similar auditory phenotypes. Cases of congenital hypothyroidism (CH) are classified as primary if caused by thyroid gland dysfunction and as secondary if caused by pituitary gland

abnormalities. Mouse models of primary hypothyroidism include the hypothyroid mutation (*hyt*) of the *Tshr* gene, which has residual activity and mild-to-negligible hearing deficit (O'Malley *et al.* 1995; Sprenkle *et al.* 2001; Karolyi *et al.* 2007), and the thyroid dysmorphogenesis mutation (*thyd*) of the *Duox2* gene, which causes severe-to-profound deafness in mice (Johnson *et al.* 2007). *Pax8* knockout mice are athyroid, exhibit ear abnormalities, and lack an auditory brainstem response (ABR) to sound. The utility of this model is limited because the mice survive only to postnatal day 21 (P21), and it is difficult to distinguish between the effects of *Pax8* expression in the thyroid gland and the otic placode (Christ *et al.* 2004).

Hearing impairment has been examined in three mouse models of secondary hypothyroidism: the Snell dwarf (*dw*) mutation of the *Pou1f1* gene (formerly *Pit1*), the Ames dwarf (*df*) mutation of the *Prop1* gene, and a targeted knockout mutation of the *Cga* gene, which encodes an essential subunit of thyrotropin (Karolyi *et al.* 2007). Both *Pou1f1* and *Prop1* encode transcription factors in the same pathway of pituitary gland development. Mice with null mutations in *Prop1*, *Pou1f1*, or *Cga* lack pituitary thyrotropin and have no measurable thyroid hormone in the serum. In spite of these similarities, the

Copyright © 2011 by the Genetics Society of America
doi: 10.1534/genetics.111.130633

Manuscript received May 13, 2011; accepted for publication July 18, 2011

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hearing defects in DW/J-*Pou1f1*^{dw/dw} and *Cga* mutant mice are profound, whereas DF/B-*Prop1*^{df/df} mutants have only a mild hearing impairment. The *Pou1f1*^{dw} and *Prop1*^{df} mutations are on different strain backgrounds, and analysis of a small cross between DW/J-*Pou1f1*^{dw/+} and DF/B-*Prop1*^{df/+} mice showed that genetic background is likely responsible for the different hearing phenotypes (Karolyi *et al.* 2007).

Advances in understanding how TH signaling affects the development and function of the cochlea will help to illuminate the molecular mechanisms that underlie development of normal auditory functions. We chose the *Pou1f1*^{dw} mutant mice because of the severity of their hearing impairment, their responsiveness to TH replacement, and their well-characterized cochlear pathologies (Mustapha *et al.* 2009). Detailed morphological, physiological, and gene expression analyses of *Pou1f1*^{dw} mutants during the course of cochlear development revealed tectorial membrane abnormalities, loss of outer hair cells (OHCs) preceded by their compromised function [as evidenced by an absence of distortion product otoacoustic emissions (DPOAE) and cochlear microphonics], reduced endocochlear potential, and reduced expression of potassium channel proteins (KCNJ10 in the stria vascularis and KCNQ4 in OHCs), all of which are likely contributors to the severe hearing impairment of these mutant mice (Mustapha *et al.* 2009). Similar features, including lack of DPOAE, reduced endocochlear potential, and tectorial membrane abnormalities, are characteristic of the *Cga* knockout mice (Karolyi *et al.* 2007).

Although insights have been gained from studies of existing hypothyroid mouse models, our understanding of the molecular mechanisms underlying the hearing impairment associated with hypothyroidism is still incomplete. The observed influence of genetic background on the severity of hearing impairment in hypothyroid mice offers an opportunity to identify modifier genes and pathways that could enhance our understanding of these mechanisms. Here we describe results from a large linkage cross that was designed to map loci that modify the hearing of hypothyroid DW/J-*Pou1f1* dwarf mice. Heterozygous DW/J-*Pou1f1*^{dw} mice were intercrossed with the wild-derived, inbred strain CAST/EiJ, which have good hearing. F₂ mutant progeny (*dw/dw*) from this intercross ranged from normal hearing to completely deaf, despite the fact that they were all profoundly hypothyroid and equally growth-impaired. By analysis of this cross, we identified a locus on chromosome (Chr) 2 that had a highly significant linkage association (LOD = 10) with the ABR threshold variation exhibited by the mutant intercross mice. This quantitative trait locus (QTL) was given the symbol *Mdwh* (modifier of *dw* hearing). The *Mdwh* interval contains *Moth1*, the modifier of tubby hearing, which varies among strains by the length of an alanine-proline amino acid repeat in microtubule-associated protein 1a (*Mtap1a*) (Ikeda *et al.* 2002). DW/J strain has a susceptible allele of *Mtap1a*, while CAST/EiJ and AKR strains have protective alleles. F₂ *dw/dw* mutants born from an intercross of (AKR × DW/J) F₁ mice are all deaf, suggesting that *Mdwh* is

not *Moth1*, but represents a novel locus that protects against hearing impairment in hypothyroid *Pou1f1*^{dw} mutants.

Materials and Methods

Mice

Mice of the C3H/HeJ-*Pou1f1*^{dw-J/J}, DW/J-*Pou1f1*^{dw}, and CAST/EiJ inbred strains and their derivative F₁ and F₂ hybrids were housed in the Research Animal Facility of the Jackson laboratory. In parallel, stocks of DW/J-*Pou1f1*^{dw}, CAST/EiJ, and AKR were purchased from the Jackson laboratory and bred at the University of Michigan to produce (DW/J-*Pou1f1*^{dw} × CAST/EiJ) F₂ hybrids and (DW/J-*Pou1f1*^{dw} × AKR) F₂ hybrids. All procedures were approved by the Institutional Animal Care and Use Committees of each institution. Both the Jackson laboratory and the University of Michigan are accredited by the American Association for the Accreditation of Laboratory Animal Care and are registered with the U.S. Department of Agriculture as research facilities. Genotyping processes for *dw* and *dw-J* alleles were previously described (Eicher and Beamer 1980; Karolyi *et al.* 2007).

Assessment of hearing by ABR threshold analysis

The ABR test was used to assess hearing thresholds of the mice. C3H/HeJ-*Pou1f1*^{dw-J/J}, DW/J-*Pou1f1*^{dw}, and their derivative F₂ hybrids and 196 (DW/J-*Pou1f1*^{dw} × CAST/EiJ) F₂ hybrids were tested at the Jackson laboratory. At the University of Michigan 68 (DW/J-*Pou1f1*^{dw} × CAST/EiJ) F₂ hybrids and 62 (DW/J-*Pou1f1*^{dw} × AKR) F₂ hybrids were tested.

Similar ABR procedures were used at the Jackson laboratory and at the University of Michigan (Zheng *et al.* 1999; Karolyi *et al.* 2007). All animals were tested binaurally. 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.

Linkage intercross mapping

Individual DNA samples from linkage intercross mice were genotyped by PCR amplification with primer pairs designed to amplify specific microsatellite markers purchased from Integrated DNA Technologies (Coralville, IA). PCR reactions and PCR product visualization methods were as previously described (Gagnon *et al.* 2006). ABR thresholds for click, 8, 16, and 32 kHz stimuli were evaluated as quantitative traits, and QTL linkage analysis was performed using the computer program Map Manager QTX (version b20), which uses a fast regression method to detect and localize quantitative trait loci within intervals defined by genetic markers and can perform pairwise locus analysis to search for QTL interactive effects. This program reports the resulting regression coefficients and a likelihood-ratio statistic that is based on natural logarithms but can be converted to conventional LOD scores by dividing by 4.61.

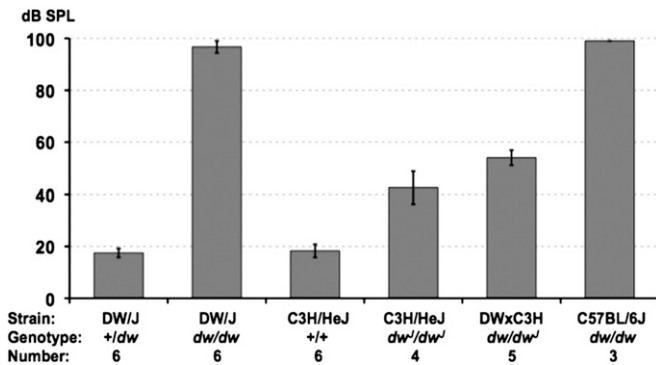


Figure 1 Genetic background modifies severity of hearing impairment in *Pou1f1*^{dw/dw} mice. Average 16-kHz ABR thresholds (with standard error bars) are shown for mice with different *Pou1f1* genotypes on different strain backgrounds. Note that *dwarf* mutant mice on the DW/J and C57BL/6J strain backgrounds have a much more severe hearing impairment than mutant mice on the C3H/HeJ background (dB SPL = decibel sound pressure level).

Candidate gene analysis

PCR for comparative DNA sequence analysis was performed according to the same conditions as described above for linkage mapping. DNA sequences of the PCR primers used to amplify the polymorphic repeat sequence GCTCCA of *Mtap1a* were 5'-TCTGGGACCTCACTCCTCTG (forward) and 5'-GTTTCTCCTGGGCCATTAGC (reverse). PCR products from genomic DNA were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). DNA sequencing was performed using the same primers as for DNA amplification, then run on an Applied Biosystems 3700 DNA Sequencer with an optimized Big Dye Terminator Cycle Sequencing method.

Results

We reported strain background effects on hearing in hypothyroid mice that were produced from an intercross of DF/B-*Prop1*^{df} and DW/J-*Pou1f1*^{dw} strains (Károlyi *et al.* 2007). There were insufficient F₂ progeny, however, to assess the heterogeneity of effects among them (six or fewer for each genotype group). The unique nature of each stock (DW/J and DF/B) and the fact that DF/B are not inbred compelled us to extend this analysis of modifier effects by comparing ABR thresholds of mice with different *Pou1f1* mutant genotypes on multiple genetic backgrounds (Figure 1). As previously reported, *Pou1f1*^{dw/dw} mice on the DW/J strain background are nearly deaf at 4 weeks of age, whereas *Pou1f1*^{dw/+} heterozygotes have normal hearing thresholds. On the C3H/HeJ strain background, mice homozygous for a different null mutation of *Pou1f1*, the *dw*^j allele, show a hearing impairment greater than that of the parental C3H/HeJ strain mice but not nearly as severe as that of *dw* mutants on the DW/J strain background. Both the *dw* and *dw*^j alleles are recessive, complete loss-of-function alleles that result from a missense mutation in the homeo-

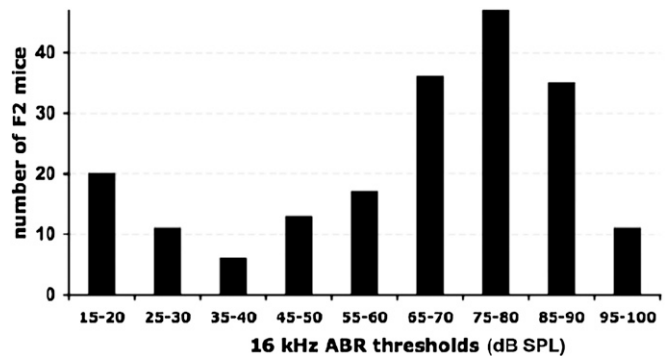


Figure 2 Degree of hearing impairment varies among F₂ *dw/dw* progeny of the (DW-*Pou1f1*^{dw/+} × CAST) intercross. A frequency distribution is shown for 196 *dw/dw* F₂ mice sorted according to their 16-kHz ABR thresholds. All mice were tested at 4–8 weeks of age. Note the bimodal shape of the distribution with peaks at 15–20 and 75–80 dB SPL.

domain that abolishes DNA binding and an intragenic rearrangement, respectively (Camper *et al.* 1990; Li *et al.* 1990). Compound heterozygote (DW/J × C3H/HeJ) F₁ hybrids (*dw/dw*^j) exhibit a hearing impairment that is intermediate between that of DW/J-*dw/dw* and C3H/HeJ-*dw*^j/*dw*^j mutants. Mutant *dw/dw* mice of the B6.DW-*Pou1f1*^{dw}/J congenic strain exhibit severe hearing impairment like that of *dw/dw* mutants on the DW/J strain background. The congenic strain was constructed by transferring the *dw* mutation from the DW/J strain onto an otherwise C57BL/6J strain background by multiple rounds of backcrossing and selection. All of these results support the idea that the genetic background affects the degree of hearing loss of hypothyroid *Pou1f1* mutants.

To investigate the genetic complexity of the strain-related hearing loss variation of *Pou1f1*^{dw} mutant mice and to map the putative modifier genes, we produced a linkage intercross between (DW/J-*Pou1f1*^{dw} × CAST/EiJ) F₁ hybrids. We chose the CAST/EiJ strain because mice of this strain retain normal hearing ability at advanced ages and because of the increased likelihood of genetic marker polymorphisms with the DW/J strain (Zheng *et al.* 1999). *Pou1f1* mutant (*dw/dw*) F₂ progeny from this intercross represented ~25% of the total, as expected, and these mutants were tested for hearing and analyzed for genetic linkage. ABR thresholds for click, 8-, 16-, and 32-kHz stimuli were measured at 1–2 months of age (average 40 days) for each of 196 F₂ mutant mice. The *dw/dw* F₂ mice exhibited a wide range of ABR thresholds that were distributed in a skewed bimodal fashion, suggesting a major influence of one or a few genes (Figure 2). A bell-shaped distribution with a single mode would have suggested that many genes with small effects contribute to the hearing differences among the mutant mice. A substantial portion—16% (*N* = 31)—of the mutant F₂ mice exhibited normal hearing thresholds (<35 dB SPL at 16 kHz). The body weights of the mutant *dw/dw* F₂ mice were about threefold smaller than normal mice, consistent with profound hypothyroidism and growth deficiency. There was no significant correlation between body weight and

Table 1 Body size of dwarf intercross mice is not affected by strain background

ABR threshold (dB SPL)	Count	Average weight (g)	Variance
15–25	23	7.8	0.6
30–50	27	8.0	1.0
55–60	17	7.8	0.6
65–70	36	7.8	0.8
75–80	47	7.6	0.4
85–90	35	7.7	0.6
95–100	11	7.5	0.3

ANOVA					
Source of variation	SS	d.f.	MS	F-value	P-value
Between	3.2	6	0.53		
Within	119	189	0.63	0.8	0.54
Total	122.2	195			

ANOVA confirms that the similar body weights of *dw/dw* F₂ mice from the (DW-*Pou1f1*^{dw} × CAST/EiJ) intercross do not correspond with their highly variable ABR thresholds.

ABR thresholds (Table 1). This suggests that the rescue of hearing does not occur concomitantly with a growth rescue.

Secretory otitis media can affect the hearing functions in both humans and mice, mostly at low frequencies (Hardisty *et al.* 2003; Bluestone and Klein 2007). A study of hearing loss in children with sporadic CH showed no increased susceptibility for middle ear effusion (Vanderschueren-Lodeweyckx *et al.* 1983). In mice of the DW/J background, we observed mild otitis media with a similar frequency in *Pou1f1*^{dw/dw} mutants (8/11) and in wild types (5/11) ($P > 0.05$; our unpublished data). All mutant *Pou1f1*^{dw/dw} mice on the DW strain background exhibit profound hearing impairment, even those without any sign of otitis media. The presence or absence of otitis media, therefore, cannot explain the variable hearing loss observed in *Pou1f1*^{dw/dw} mice of the (DW/J-*Pou1f1*^{dw} × CAST/EiJ) F₂ intercross.

As a first approach for mapping modifier loci for hearing, we performed a genome-wide scan for linkage by limiting our analysis to F₂ mice with the most extreme ABR thresholds, 30 with the lowest (15–30 dB SPL), and 11 with the highest (95–100 dB SPL) thresholds among 192 *dw/dw* F₂ mutants generated at the Jackson laboratory. Selecting mice with extreme phenotypes maximizes information for more efficient linkage scanning without sacrificing detection capability (Darvasi 1997). We typed 90 microsatellite markers spaced at 15-cM intervals on all 20 mouse chromosomes, which covered >90% of the genome. We used the Map Manager QTX computer program (Manly *et al.* 2001) to analyze QTL associated with ABR threshold variation. We evaluated intercross linkage using a free model (2 d.f.) without prior assumptions of dominant, recessive, or additive effects. The LOD scores for associations of 16-kHz thresholds with the 90 markers are shown in Figure 3. With this model, a LOD score of 4.3 is considered a significant intercross linkage value, corresponding to a genome-wide type I error of 0.05 (Lander and Kruglyak 1995). The highest and only statistically significant linkage associations were found with

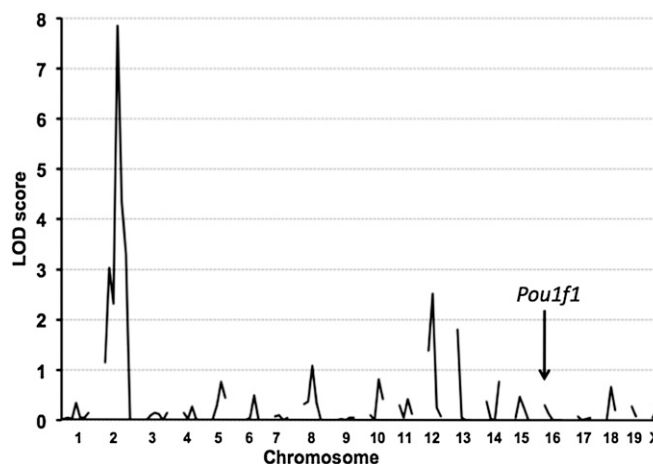


Figure 3 Genome-wide linkage analysis of (DW-*Pou1f1*^{dw/+} × CAST) F₂ mice identifies a hearing modifier locus on Chr 2. The 41 *dw/dw* F₂ mice with the most extreme ABR thresholds were analyzed, and LOD scores are shown for the linkage associations of these threshold values with each of 90 chromosomal markers. Only markers on Chr 2 showed statistically significant linkage. The *Pou1f1* gene is located on Chr 16.

markers on Chr 2, and additional Chr 2 markers were then analyzed (Figure 4A). The maximum LOD score was 7.9 for the linkage associations of *D2Mit134* and *D2Mit304* with 16-kHz ABR thresholds (LOD scores were 7.0, 6.7, and 7.4 for click, 8-, and 32-kHz thresholds, respectively). Given that a LOD score of 4.3 is considered significant, the Chr 2 linkage with a LOD score of 7.9 validated designation of this new QTL as “modifier of *dw* hearing” (*Mdwh*).

To confirm the significance of this linkage and to clarify the additive and dominance components of the phenotypic variance, we extended the linkage studies by genotyping the remaining 155 *dw/dw* F₂ mice from the intercross for Chr 2 markers surrounding the *Mdwh* locus. Identical most likely map positions for the *Mdwh* QTL were determined from all 196 *Pou1f1*^{dw/dw} F₂ mice and the 41 *dw/dw* mice with the most extreme hearing phenotypes (Figure 4). Both analyses gave peak associations with *D2Mit304* (which was nonrecombinant with *D2Mit134* in the 41 extreme mice). The 1.5-LOD support interval, which provides 95% confidence of coverage (Dupuis and Siegmund 1999), for both analyses is the 118- to 138-Mb region of Chr 2 (National Center for Biotechnology Information Build m37). Because of the increased sample size, the LOD scores for linkage associations were higher when all 196 mice were analyzed, with a peak score of 10.1 for marker *D2Mit304* (Figure 4B).

The effects of *Mdwh* on the hearing thresholds of dwarf intercross mice are highly significant and vary little in response to the different auditory stimuli (LOD 9.0–10.1; Table 2). The LOD scores shown in Figure 4 and Table 2 are for an additive inheritance model. These values were essentially the same as those calculated for a free model (no inheritance assumptions) and larger than those of recessive and dominant models, indicating that the effects of *Mdwh* are primarily additive.

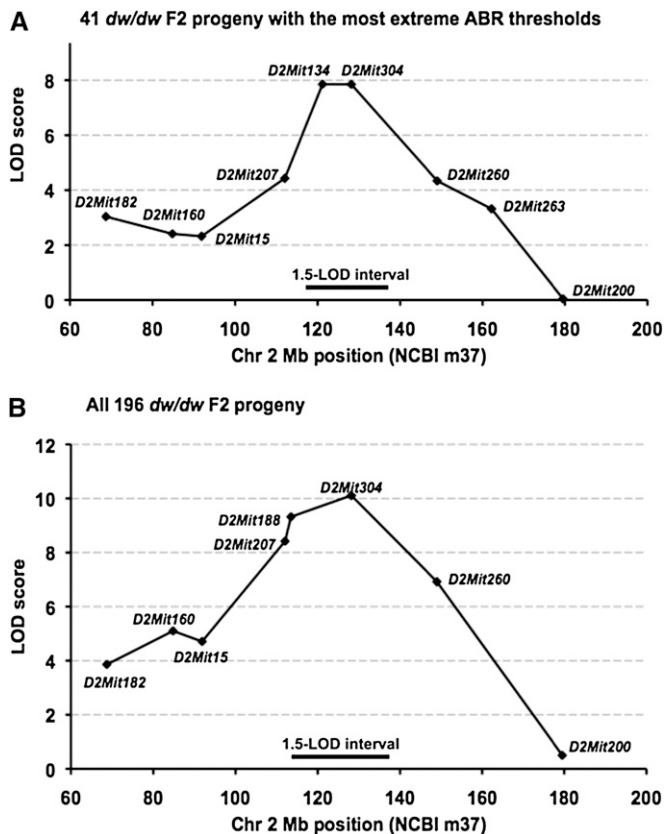


Figure 4 Additional mice and markers refined the map position of *Mdwh* on Chr 2. (A) Map position as determined from the 41 *dw/dw* F₂ mice with the most extreme thresholds. The peak LOD scores (7.9) are for *D2Mit134* at the 121-Mb position and *D2Mit304* at the 128-Mb position. (B) Map position as determined from all 196 *dw/dw* F₂ mice. The peak LOD score (10.1) is for *D2Mit304* at the 128-Mb position. The 1.5-LOD support intervals for both analyses show that *Mdwh* is most likely located within the 118- to 138-Mb region of Chr 2.

The genetic interval containing *Mdwh* was estimated to span ~20 Mb between the 118- and 138-Mb positions of Chr 2 (Figure 4). An interesting candidate gene within this interval is *Mtap1a*, the gene encoding microtubule-associated protein 1 A. *Mtap1a*, located at 121.1 Mb on Chr 2, is particularly intriguing because it modifies the hearing of mice with the tubby (*tub*) mutation in a strain-specific manner (Ikeda *et al.* 2002). The wild-type *Mtap1a* alleles from strains AKR/J, CAST/EiJ, and 129P2/OlaHsd protect tubby mice from hearing deficits, whereas a sequence variant in the C57BL/6J strain confers susceptibility to hearing impairment. We examined the *Mtap1* gene in the DW/J strain and found that it is the same allelic form as that of the C57BL/6J strain (Figure 5), consistent with the possibility that this allele confers hearing loss susceptibility to *Pou1f1^{dw/dw}* mutants as it does for *tub/tub* mutant mice. Both DW/J-*Pou1f1^{dw/J}* and C57BL/6J strains contain five repeats of a 6-bp sequence in the large exon 5 of *Mtap1a*, whereas all other strains that we examined (AKR/J, CAST/EiJ, 129P2/OlaHsd, C3H/HeJ, BALB/cByJ, and DF/B-*Prop1^{df}*/J) contain 15 repeats of this sequence.

Table 2 *Mdwh* locus has a large effect on ABR thresholds of intercross mice

Genotype	ABR thresholds			
	Click	8 kHz	16 kHz	32 kHz
DD, N = 50	97.9 (8.1)	95.9 (8.2)	82.1 (12.6)	97.9 (7.5)
DC, N = 96	84.8 (16.7)	81.6 (16.9)	64.3 (20.8)	87.1 (15.6)
CC, N = 44	72.8 (23.7)	72.4 (21.5)	51.6 (27.1)	75.2 (22.4)
LOD score	10.1	9.5	9.9	9
% variation	22	20	21	20

Average ABR thresholds of *dw/dw* F₂ mice from the (DW-*Pou1f1^{dw}* × CAST/EiJ) intercross are shown sorted by their *D2Mit304* (*Mdwh*) genotypes. LOD scores of linkage associations are shown for an additive inheritance model with 1 d.f., and estimates are given for the percentage of total ABR variation that can be explained by the *Mdwh* locus. Genotype designations: DD, homozygous for DW allele; DC, heterozygous for DW and CAST alleles; CC, homozygous for CAST allele. Mice were tested at 4–8 weeks of age. Standard deviations are in parentheses.

To assess the likelihood that the *Moth1* allele of *Mtap1a* is identical to *Mdwh*, we created F₁ intercrosses between DW/J-*Pou1f1^{dw/+}* and each of the two strains CAST/EiJ and AKR/J at the University of Michigan and assessed the segregation of non-DW/J *Mtap1a* alleles with the ability of F₂ *Pou1f1^{dw/dw}* mutants to hear. Sixty-eight *Pou1f1^{dw/dw}* (DW/J-*Pou1f1^{dw/+}* × CAST/EiJ) F₂ mice were generated at the University of Michigan. Sixteen of them (23.5%) had ABR thresholds <35 dB SPL, which we designated as normal hearing. In both cohorts of (DW/J-*Pou1f1^{dw/+}* × CAST/EiJ) F₂ *dw/dw* mice generated at the Jackson laboratory and the University of Michigan, there is an enrichment of CAST/EiJ *Mtap1a* alleles among the hearing F₂ mutants, but homozygosity for CAST/EiJ alleles is not sufficient for normal hearing ability (Figure 6A). Two F₂ *Pou1f1^{dw/dw}* mice homozygous for DW/J *Mtap1a* alleles also exhibited normal hearing ability (one is 20 dB SPL and one is ~37 dB SPL) (Figure 6C), suggesting that CAST/EiJ *Mtap1a* alleles are not necessary for hearing either. ABR thresholds of mice heterozygous for *Mtap1a* alleles (DC in Table 2) are distributed through the range represented by the homozygous alleles (DD or CC), indicating the contribution of additional genes (Figure 6B).

Sixty-two F₂ *Pou1f1^{dw/dw}* mice were collected from an intercross between (DW/J-*Pou1f1^{dw/+}* × AKR/J) F₁ mice, and none of the progeny had normal hearing ability (Figure 7). We confirmed that 9 of 62 F₂ *Pou1f1^{dw/dw}* (DW/J-*Pou1f1^{dw/+}* × AKR/J) mice were homozygous for alleles of *Mtap1a* that protect against deafness of *Tubby* mutants, despite the lack of protection against *dw* hearing impairment. Thus, the AKR/J genetic background cannot protect against hypothyroidism-induced hearing loss in *Pou1f1^{dw/dw}* mutants even though AKR/J mice have the same *Mtap1a* allele as CAST/EiJ.

The Mouse Diversity Genotyping Array (Yang *et al.* 2009) was used to characterize the genetic background of the DW/J-*Pou1f1^{dw}* strain and compare it to 72 inbred strains (SNP data from Mouse Diversity Array for the DW/J-*Pou1f1^{dw}* strain can be found on the Mouse Phenome Database website at <http://phenome.jax.org>; MPD#: TJL4). In the *Mdwh*

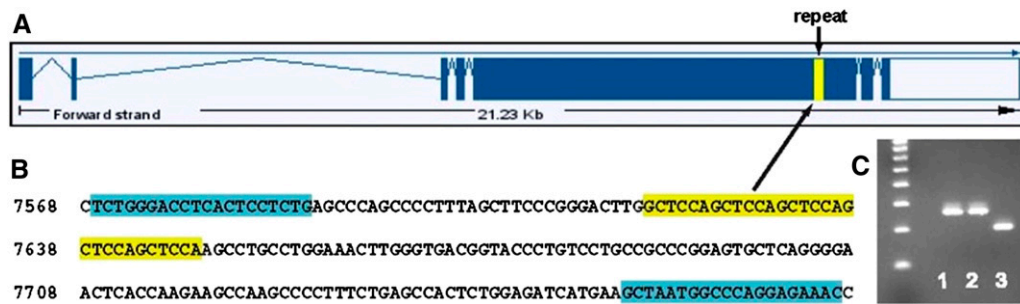


Figure 5 *Mtap1a* is a candidate gene for *Mdwh*. (A) Genetic structure of *Mtap1a* showing the position of a polymorphic repeat sequence (vertical yellow bar) in the large exon 5. (B) Primers (highlighted in blue) were designed to amplify the GCTCCA repeat sequence (highlighted in yellow; from C57BL/6J DNA), which encodes an alanine-proline repeat. (C) Sequencing of

the 268-bp PCR amplification products from CAST/EiJ (lane 1) and C3H/HeJ (lane 2) showed that these strains have 15 repeats of the 6-bp sequence, whereas the 208-bp PCR product from the DW strain (lane 3) showed only 5 repeats.

critical region (Chr 2: 118–138 Mb), the DW/*J-Pou1f1^{dw}* strain is more similar to C57 substrains than to any of the other inbred strains used in the Mouse Diversity Genotyping Array. There are only 34 mismatches of 4508 SNPs analyzed between the DW/*J-Pou1f1^{dw}* and C57BL/6J strains. Almost all of these apparent mismatches occur in cases where the allele of one or the other strain was uncertain. There is only one SNP that is a true mismatch: C/T. The strain distribution pattern (SDP) is the pattern of allelic similarities and differences among strains at a locus (Yalcin *et al.* 2004). The most consistent SDP between the susceptible (DW/*J-Pou1f1^{dw}*, C57BL/6J, AKR) and protective (Cast/EiJ, C3H/HeJ) strains for hypothyroidism-induced deafness is the 124- to 131-Mb region of Chr 2, suggesting that *Mdwh* is in this interval (Table 3).

Discussion

TH deficiency can have major effects on neuronal development, body growth, and cochlear development resulting in cretinism, dwarfism, and deafness if not treated promptly. It is intriguing that the consequences of severe thyroid hormone deficiency can be highly variable in both humans and mice. Mutations in the pituitary transcription factor *Pou1f1* result in severe hypothyroidism, dwarfism, and profound deafness on the DW/J genetic background, but the hearing deficit is milder when crossed to the normal-hearing, but genetically undefined, DF/B strain. Because the critical period for TH replacement is late gestation and early neonatal life, we assessed the contribution of the maternal environment to the differential hearing impairments between DW/J and DF/B strains. Our results prove that this particular genetic background effect is intrinsic to the fetus, rather than an influence of maternal TH during gestation or lactation (Q. Fang, unpublished data).

In this study, we examine the effects of the CAST/EiJ genetic background on the hearing of *Pou1f1* mutants and show that strain-specific alleles of the *Mdwh* locus on Chr 2 have a major influence on the hearing of hypothyroid *Pou1f1* dwarf mice (*dw/dw*). This locus showed a highly significant association (LOD score of 10) with hearing ability and could explain ~20% of the ABR threshold variation of dwarf F₂

mice from a DW/*J-Pou1f1^{dw}* × CAST/EiJ intercross. Given the pleiotropic effects of TH on the inner ear development, it is very surprising that the *Mdwh* appears simple and genetically tractable.

The *Mdwh* modifier locus does not appear to affect the basal serum level of TH in *Pou1f1* mutant mice, which lack thyroid stimulating hormone (TSH). If the modifier had a general effect on TH levels, then the dwarf mice with normal hearing would be expected to be larger than the deaf dwarfs. We have detected such generalized effects in other models with partial restoration of TSH production (Cushman *et al.* 2001). Because the weights of hearing and deaf dwarfs are indistinguishable and only ABR thresholds are variable (Table 1), it is likely that *Mdwh* is modifying processes that affect cochlear development or function even in the absence of systemic TH stimulation. Although we cannot rule out a local effect on cochlear TH production or transport, there are no known TH transporters or deiodinases in the critical region (Mouse Genome Informatics: <http://www.informatics.jax.org>).

The *Mdwh* gene may modify the degree of hearing impairment due to hypothyroidism by altering the basal expression levels of critical gene(s) without enhancing TH production. This model is drawn from a similar case of genetic background effects in which the survival of *Prop1* mutant newborn mice correlated with higher basal levels of surfactant B gene expression in the lung at birth compared to mutants that exhibited lethality (Nasonkin *et al.* 2004). The transcription factor *Nkx2.1* is necessary for surfactant B expression in the lung, and *Nkx2.1* transcription is upregulated by TH. The mutant mice that were cyanotic and died had lower basal levels of *Nkx2.1* expression than those that were viable, suggesting that genetic background affected the basal level of *Nkx2.1* expression in the absence of TH. In the case of *Mdwh*, the DW/J hearing loss susceptibility allele may have a less active basal state (in the absence of TH) than the CAST/EiJ protective allele. If cochlear development and function were sensitive either directly or indirectly to slight variations in the level of *Mdwh* gene activity, then the lower basal levels in hypothyroid F₂ mice with the DW/J allele could explain their more severe hearing impairment, whereas the higher basal level of *Mdwh* gene activity associated with the CAST/EiJ allele would protect from severe

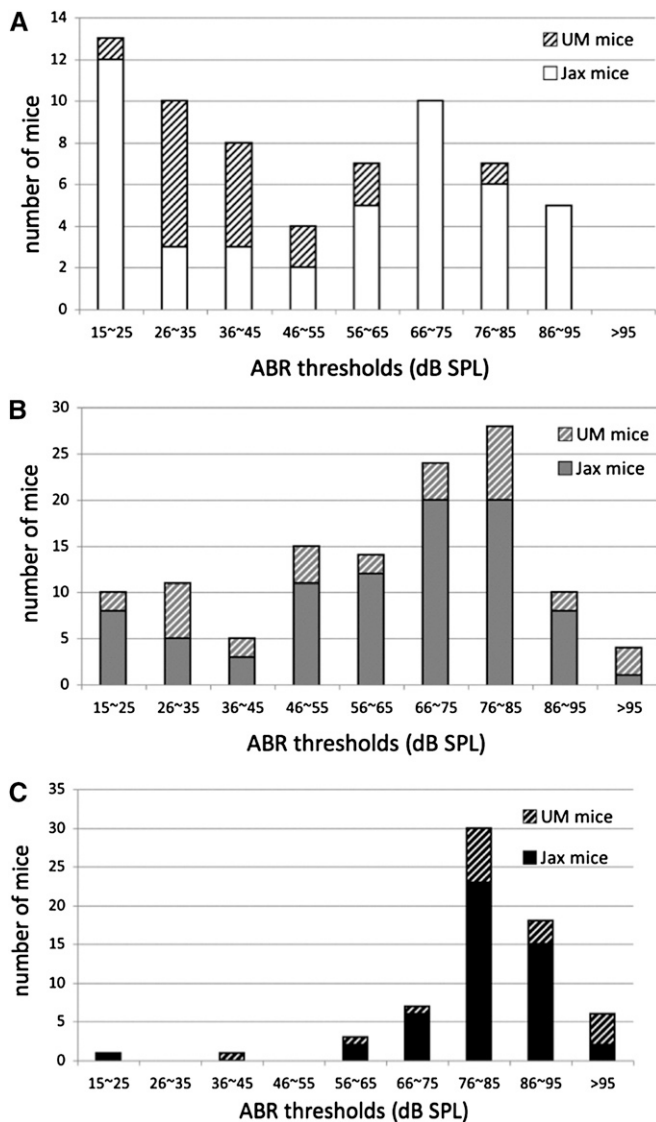


Figure 6 The *Mtap1a* allele correlates with, but is neither necessary nor sufficient for, protection of hearing in *Pou1f1^{dw}* mice. Two cohorts of *dw/dw* F_2 (DW/J-*Pou1f1^{dw/+}* × CAST/EiJ) mice, with a total number of 251, were tested for their hearing sensitivity and genotyped for sequence variants of GCTCCA repeats in the *Mtap1a* gene. Mice were grouped by their *Mtap1a* genotypes, and we plotted the number of mice with ABR thresholds in each 20-kHz interval. Within each bar, the solid or open portion represents the mice generated at the Jackson laboratory (Jax mice); the hatched portion represents the mice generated at the University of Michigan (UM mice). (A) The numbers of mice homozygous for CAST/EiJ *Mtap1a* alleles at each ABR threshold interval. (B) The numbers of mice heterozygous for CAST/EiJ and DW/J *Mtap1a* alleles at each ABR threshold interval. (C) The numbers of mice homozygous for DW/J *Mtap1a* alleles at each ABR threshold interval.

hearing loss and could explain the additive nature of *Mdwh* allele effects on ABR thresholds.

We considered the *Mtap1a* gene as a particularly intriguing candidate gene for *Mdwh*. It maps within the 20-Mb candidate interval on Chr 2 and is the gene responsible for the strain-specific modification of hearing in tubby (*tub*) mutant mice (Ikeda *et al.* 2002). The underlying mechanism

proposed for this modification is an altered protein–protein interaction between MTAP1A (a microtubule-associated protein) and a protein localized to the post-synaptic density in hair cells, PSD95, a protein critical for the cyto-architecture at the synapse. Another hearing-related QTL on Chr 2, which could be equivalent to *Mtap1a* or *Mdwh*, modifies the degree of hearing loss in Beethoven mutant mice, which carry a mutation in *Tmc1* (Noguchi *et al.* 2006). Intriguingly, the phenotype of these mice is outer hair cell loss and reduced DPOAE, the same as that observed in *Pou1f1^{dw}* mutants (Mustapha *et al.* 2009).

Evidence implicating *Mtap1a* as the gene underlying the modifier effects of the *Mdwh* locus includes the following. First, the DW/J strain has the same *Mtap1a* allele as C57BL/6J (Figure 5), which was shown to confer susceptibility to hearing loss in *tub* mutant mice (Ikeda *et al.* 2002). Both DW/J and C57BL/6J strain backgrounds confer an increased susceptibility to hearing impairment in *Pou1f1^{dw}* mutant mice (Figure 1), and both have the same *Mtap1a* allele. Second, the expression of *Mtap1a* is altered by TH in the cerebellum of hypothyroid rats (Benjamin *et al.* 1988) and in the sensory motor cortex of hypothyroid (*hyt*) mutant mice (Biesiada *et al.* 1996). Third, MTAP1A interacts directly with the calcium-activated potassium channel BKCa (Park *et al.* 2004), and defects in *Kcnma1*, which encodes one of the BKCa subunits, cause progressive hearing loss and absence of KCNQ4 expression (Ruttiger *et al.* 2004). Fourth, KCNQ4 is regulated by the thyroid hormone receptors TR α 1 and TR β in outer hair cells (Winter *et al.* 2006), and its expression is reduced in *Pou1f1^{dw}* mutant outer hair cells (Mustapha *et al.* 2009). Finally, recent studies demonstrate that TH is essential for morphological and functional maturation of inner hair cell (IHC) ribbon synapses (Brandt *et al.* 2007; Sendin *et al.* 2007). These studies suggest that pre-synaptic dysfunction of IHCs, which could be proven by demonstrating reduced exocytosis efficiency, is a mechanism in congenital hypothyroid deafness, and MTAP1A has been proposed to be involved in synaptic function (Ikeda *et al.* 2002). Although these lines of evidence seem compelling when taken together, our study on segregation of CAST/EiJ and AKR/J *Mtap1a* alleles in F_2 *Pou1f1^{dw}* mutants showed that CAST/EiJ and AKR/J *Mtap1a* alleles are neither sufficient nor necessary for normal hearing abilities in those mice. We observed the same result in F_2 mutants from an intercross between the DW/J strain and the 129P2/OlaHsd strain (129P2), which contains the same *Mtap1a* allele as CAST/EiJ and AKR/J (data not shown). We conclude that *Mdwh* is not *Mtap1a*, that *Mtap1a* is not the sole component of *Mdwh*, or that additional modifiers obscure the effects of *Mtap1* on hearing.

Given the pleiotropic effects that the thyroid hormone has on cochlear development (Mustapha *et al.* 2009), *Mdwh* could consist of multiple genes, possibly including *Mtap1a*, whose combined actions protect against hypothyroidism-induced hearing impairment in *Pou1f1^{dw}* mutants. The information gleaned by genotyping the DW/J-*Pou1f1^{dw}* genome

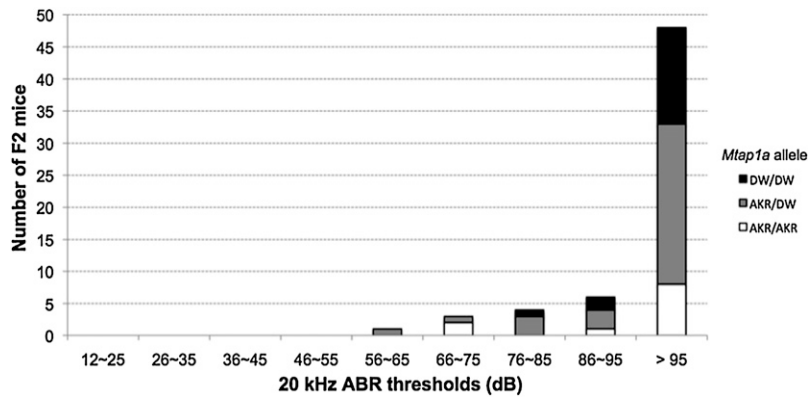


Figure 7 AKR genetic background is not protective against hypothyroidism for the hearing in DW/J-*Pou1f1*^{dw/dw} mice. Sixty-two *dw/dw* F₂ (DW/J-*Pou1f1*^{dw/+} × AKR) mice were tested for their hearing sensitivity and genotyped for sequence variants of GCTCCA repeats in the *Mtap1a* gene. None of the mice showed ABR thresholds <55 dB SPL.

for >570,000 SNPs in the Mouse Diversity Genotyping Array will facilitate the search for candidate genes within the *Mdwh* locus as well as assess candidate regions of additional modifier QTL on other chromosomes. Future directions to identify the protective genes could include refining the critical interval by generating more animals and genotyping more markers on Chr 2 and by creating a congenic mouse line with CAST/EiJ alleles of *Mdwh* and DW/J alleles at other loci to determine if the CAST/EiJ *Mdwh* locus is sufficient to protect against hearing loss. Another strategy would be to directly sequence the candidate genes within the *Mdwh* locus from the susceptible DW/J and/or C57BL/6J strains and compare them with the C3H/HeJ, CAST/EiJ, and other inbred strain sequences generated by the Mouse Genomes Project (<http://www.sanger.ac.uk/resources/mouse/genomes/>). This strategy was successful in identifying two

Table 3 Comparing SNPs of DW-*Pou1f1*^{dw}, C57B6/J, and AKR strains with SNPs of C3H and CAST/EiJ in the Chr 2 candidate region for the *Mdwh* QTL (118–138 Mb)

Mb region	No. of SNPs with appropriate SDP
118.0–118.9	6 (179)
119.0–119.9	1 (185)
120.0–120.9	2 (184)
121.0–121.9	4 (166)
122.0–122.9	1 (142)
123.0–123.9	0 (189)
124.0–124.9	14 (220)
125.0–125.9	46 (209)
126.0–126.9	58 (187)
127.0–127.9	74 (243)
128.0–128.9	46 (301)
129.0–129.9	9 (333)
130.0–130.9	34 (219)
131.0–131.9	2 (235)
132.0–132.9	6 (211)
133.0–133.9	3 (190)
134.0–134.9	0 (222)
135.0–135.9	0 (196)
136.0–136.9	0 (148)
137.0–138.0	0 (175)

Numbers in italics indicate the region with a consistent SDP. Numbers in parentheses are the numbers of SNPs that have allele information in each of the five mouse strains.

other modifier loci (Buchner *et al.* 2003; Amendola *et al.* 2010). There are >1000 genes and >50,000 SNPs between C57BL/6J and CAST/EiJ within the *Mdwh* critical region, but no obvious nonsense SNPs are evident. Thus, genetic studies to narrow the interval are the next logical step for identification of the *Mdwh* modifier gene(s).

In conclusion, identifying the gene(s) that modify the expression of mutant auditory phenotypes related to congenital hypothyroidism will be important for determining the molecular mechanisms that underlie the influence of TH on cochlear development and function. In addition, it is likely that a mechanistic understanding of modifier gene action will provide a conceptual framework for understanding the multi-factorial basis for hearing loss.

Acknowledgments

We thank the following individuals for their important contributions to this work: Mirna Mustapha and Thomas Glaser for discussion and suggestions, Michelle Fleming and Emily Maclary for SNP analysis, Jennifer Benson for auditory brainstem response tests at the University of Michigan, and Sandra Gray for mouse husbandry at the Jackson laboratory. Funding for this study came from March of Dimes grant 6-FY08-262 (S.A.C.), National Institutes of Health/National Institute on Deafness and Other Communication Disorders grants DC04301 (K.R.J.) and P30 DC05188 (D.F.D.), a University of Michigan Barbour Fellowship (Q.F.), and a Rackham Graduate School Summer Grant (Q.F.).

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Communicating editor: F. F. Pardo Manuel de Villena