

Methylthioadenosine Phosphorylase (*MTAP*) in Hearing: Gene Disruption by Chromosomal Rearrangement in a Hearing Impaired Individual and Model Organism Analysis

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Genes with a role in the auditory system have been mapped by genetic linkage analysis of families with heritable deafness and then cloned through positional candidate gene approaches. Another positional method for gene discovery is to ascertain deaf individuals with balanced chromosomal translocations and identify disrupted or dysregulated genes at the site(s) of rearrangement. We report herein the use of fluorescence in situ hybridization (FISH) to map the breakpoint regions on each derivative chromosome of a de novo apparently balanced translocation, t(8;9)(q12.1;p21.3)dn, in a deaf individual. Chromosomal breakpoints were assigned initially by GTG-banding of metaphase chromosomes and then BAC probes chosen to map precisely the breakpoints by FISH experiments. To facilitate cloning of the breakpoint sequences, further refinement of the breakpoints was performed by FISH experiments using PCR products and

by Southern blot analysis. The chromosome 9 breakpoint disrupts methylthioadenosine phosphorylase (*MTAP*); no known or predicted genes are present at the chromosome 8 breakpoint. Disruption of *MTAP* is hypothesized to lead to deafness due to the role of *MTAP* in metabolizing an inhibitor of polyamine synthesis. *Drosophila* deficient for the *MTAP* ortholog, *CG4802*, were created and their hearing assessed; no hearing loss phenotype was observed. A knockout mouse model for *MTAP* deficiency was also created and no significant hearing loss was detected in heterozygotes for *Mtap*. Homozygous *Mtap*-deficient mice were embryonic lethal. © 2007 Wiley-Liss, Inc.

Key words: deafness; chromosomal translocation; FISH; *Drosophila* model; mouse model

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INTRODUCTION

Hearing loss is the most common sensory deficit in humans, affecting approximately 1 in 1,000 newborns [Morton 1991]. Almost half of these cases are attributed to environmental factors, while the other half are believed to have a genetic etiology [Gorlin et al., 1995]. To date, more than 120 loci have been identified and more than 65 genes have been cloned (<http://webhost.ua.ac.be/hhh>, accessed December

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2006). Genes known to be involved in the hearing process belong to a great variety of protein families including gap junctions, ion channels, and collagens. New genes continue to be discovered at an impressive rate, but multiple loci remain for which the gene is not yet known and many processes of the inner ear are not yet elucidated. To add to gene discovery efforts in this field, we have chosen to study an individual who has an apparently balanced de novo chromosomal translocation and is deaf. We have designated this individual as DGAP090 as part of the Developmental Genome Anatomy Project (DGAP, www.dgap.harvard.edu). It was our a priori expectation that, because the translocation is apparently balanced, disruption or dysregulation of gene(s) at the breakpoints could be pathogenetic in the observed phenotype. By identifying these gene(s), we sought to uncover new gene(s) involved in hearing.

The chromosomal location of several genes in the auditory process has been predicted based on their association with chromosomal abnormalities (reviewed in Giersch and Morton [2001]). A de novo chromosomal inversion, *inv(2)(q35q37.3)*, in a sporadic case of Waardenburg syndrome type 1 (WS1), an autosomal dominant disorder characterized by dystopia canthorum, pigmentary abnormalities of the skin, hair and ocular fundus and sensorineural deafness, was invaluable in focusing genetic linkage analyses in this region of chromosome 2 [Ishikiriyama et al., 1989]; assignment of WS1 to 2q37 [Foy et al., 1990] and identification of mutations in *PAX3* [Tassabehji et al., 1992] followed shortly thereafter. Identification of the role of *POU3F4* in X-linked progressive mixed deafness with perilymphatic gusher was aided by recognition of cytogenetically visible deletions in Xq13-q21.1 [Brunner et al., 1988; Wallis et al., 1988]. Several Smith–Magenis syndrome patients who have sensorineural hearing loss (SHL) and deletion of one allele of *MYO15* have been found to have a point mutation in the remaining allele of *MYO15* [Liburd et al., 2001]. A variety of other chromosomal deletions associated with deafness including the 1p36 deletion syndrome, Wolf–Hirschhorn syndrome, and cri-du-chat syndrome are likely to include genes important in hearing.

DGAP090, has a de novo translocation between chromosomes 8 and 9 in addition to a maternally inherited rearrangement, designated 46,XY,t(8;9)(q12.1;p21.3)dn,t(9;11)(q33;q13)mat, and various congenital anomalies including SHL, Mondini defect, mild subcutaneous atrophy, avascular necrosis of the left femoral head, a telangiectatic skin condition with recurrent ulceration, and juvenile rheumatoid arthritis. Using fluorescence in situ hybridization (FISH) and Southern blot analysis, we identified disruption of the sequence encoding methylthioadenosine phosphorylase (*MTAP*) between exons

5 and 6 at 9p21.3. *MTAP* then became an attractive positional candidate for investigation; it plays a key role in the methionine salvage pathway and in the production of endogenous cellular adenine, through processing of its substrate, methylthioadenosine (MTA), to adenine and methylthioribose-1-phosphate. Methylthioribose-1-phosphate is then further metabolized to yield methionine. In addition, MTA is a by-product as well as a potent feedback inhibitor of polyamine synthesis. During the production of the polyamines, spermidine and spermine, decarboxylated S-adenosylmethionine is converted to MTA. MTA inhibits spermidine synthase and spermine synthase. In *MTAP*-deficient cells, the production of polyamines is likely disturbed by impaired metabolism of MTA. Also of note, mice in which the gene for spermine synthase has been deleted, and are therefore deficient in spermine, are deaf [Barkway et al., 1988, 1989; Lorenz et al., 1998]. To study the role of *MTAP* in hearing loss in DGAP090, we developed both *Drosophila* and mouse knockout models.

MATERIALS AND METHODS

Bacterial Artificial Chromosomes (BACs)

BACs for translocation breakpoint mapping were selected using the University of California Santa Cruz Genome Browser and Database (<http://genome.ucsc.edu>) and the NCBI Human Genome Browser and Database (<http://www.ncbi.nlm.nih.gov>). BACs from the RP11 library were acquired from CHORI (Children's Hospital Oakland Research Institute, Oakland, CA) and those from the CTD libraries were obtained from Invitrogen (Carlsbad, CA). DNA was isolated following a standard protocol consisting of alkaline lysis, neutralization, and ethanol precipitation (Qiagen, Valencia, CA).

Fluorescence In Situ Hybridization (FISH)

Slides with metaphase chromosome preparations from DGAP090 EBV-transformed lymphocytes were prepared using standard hypotonic lysis and fixation. The Nick Translation Reagent Kit from Vysis (Downers Grove, IL) was then used to label fluorescently the BAC DNA with either SpectrumGreen or SpectrumOrange conjugated dUTP. *CotI* DNA was added to suppress repetitive sequences and probes were ethanol precipitated and resuspended in 70% Hybri-sol (Vysis). Metaphases were dehydrated in a series of ethanol washes and denatured in 2X SSC at 72°C for 2 min. Probes were then denatured at 37°C for 10 min and applied to slides for hybridization to metaphase chromosomes. Slides were incubated overnight at 37°C using a HYBrite apparatus (Vysis) and then washed in 2X SSC in 50% formamide at 37°C for 20 min and 2X SSC at 37°C for 20 min. 4',

6'-diamidino-2-phenylindole hydrochloride (DAPI) was used as counterstain. Hybridization results were assessed using a Zeiss Axioskop 2 epifluorescence microscope (Thornwood, NY) and images acquired using an Applied Imaging CytoVision cytogenetics workstation (Santa Clara, CA). A minimum of 10 metaphases were scored for each probe hybridization.

Southern Blot Analysis

Sequences of the presumed breakpoint regions were analyzed using Repeat Masker (<http://ftp.genome.washington.edu/cgi-bin/RepeatMasker>) to identify regions in which unique probes could be generated. PCR products ranging in length from 200 to 1500 bp were produced from these nonrepetitive sequences. These products were then labeled with the Megaprime DNA Labeling Kit (Amersham Biosciences, Piscataway, NJ). Fifteen micrograms of both patient and control genomic DNA were digested with restriction endonucleases overnight. Digested DNA was then electrophoresed in 0.7% agarose gels, transferred overnight by capillary action to Gene-Screen Hybridization Transfer Membrane (Perkin-Elmer, Boston, MA), and cross-linked to the membrane using ultraviolet light. Blots were then prehybridized for at least 1 hr in UltraHybe (Ambion, Austin, TX) at 42°C. Probes were denatured at 95°C for 10 min, quenched on ice and added to the hybridization solution. Hybridization was performed overnight at 42°C. Blots were then washed twice at room temperature in 2X SSC in 0.1% SDS for 20 min and twice at 55°C in 0.2X SSC in 0.1% SDS for 30 min and exposed to Kodak XAR-5 film with intensifying screens at -80°C.

Breakpoint Cloning

Translocation breakpoints were cloned using modifications of the protocol as described using the Advantage-2 PCR kit for the long-range PCR steps [Siebert et al., 1995]. All primer and adaptor oligos were purchased from Invitrogen. PCR products were then TOPO TA-cloned into pCRII-TOPO (Invitrogen) and sequenced.

RT-PCR

RNA was isolated from human fetal cochlear tissue and converted to cDNA using reverse transcriptase and oligo dT (Invitrogen). This cDNA was then used as a template for PCR using external and internal primers for the coding sequence of *MTAP*.

Drosophila Melanogaster *MTAP* Knockout Model

A P element insertion [P(SUP^{or}-P)KG03863 (Berkeley *Drosophila* Genome Project, www.fruitfly.org)]

located directly downstream of the gene encoding *Drosophila* *MTAP* (CG4802) was used to create imprecise excision lines that removed CG4802 coding sequence. Excision of CG4802 was verified in three independent lines using PCR amplification of the genomic region using the following primers: excision upper 5'-TATAATTAGAAGGGCTTTGT-TAG-3', excision small lower 5'-ATCGTAGAAGG-TCTGAAGG-3', excision large lower 5'-GCCAATAA-TACCAATGTGTG-3'.

Electrophysiology of *Drosophila Melanogaster*

Auditory recordings of mutant and control flies (N = 10 per genotype) were performed as described [Eberl et al., 2000] 0–2 days after eclosion. Computer-generated pulse song and sine song stimuli were delivered under near-field sound conditions. Sound-evoked potentials (SEPs) were recorded from the antennal nerve using electrolytically sharpened tungsten electrodes. The differential signal was amplified 1,000-fold (DAM-50 differential amplifier (World Precision Instruments, Sarasota, FL)), digitized (InstruNet 100B data acquisition module (GW Instruments, Somerville, MA)), and sampled at 13.3 kHz using Superscope II software v3.0 (GW Instruments).

Mouse *Mtap* Knockout Model

All mouse work underwent review and received approval by the Institutional Animal Care and Use Committee at Harvard Medical School. An Ola/129 ES cell line, RRK081, in which an exon-trapping vector was inserted into the second intron of *Mtap* was created by BayGenomics (<http://baygenomics.ucsf.edu>). Chimeric mice produced from injection of these ES cells into pseudopregnant C57/B6 females were bred to wild-type Ola/129 mice. DNA prepared from tails from the albino offspring of these crosses was then analyzed by PCR for detection of the β -geo insert present in all BayGenomics ES cells. Primers used for β -geo amplification were 5'-AATCGCCTTG-CAGCACATCC-3' and 5'-CGCAACTCGCCGCACATC-3', with an annealing temperature of 60°C and an extension time of 1 min. The insertion site of the exon-trapping vector was determined by PCR using an upper primer from *Mtap* exon 2, 5'-ATCCC-GAAATTTTAGAAGGAAG-3', and a vector lower primer, 5'-AAGGCGATTAAGTTGGGTAAC-3', with an annealing temperature of 55°C and an extension time of 3 min. The product of this reaction was then sequenced. Genotyping was performed using a mixture of three primers: a wild-type upper primer from *Mtap* intron 2, 5'-CCCTTCCCTCAAGTTCTG-CTTC-3'; a wild-type lower primer from within *Mtap* intron 2, 5'-CACAAGGACGCTCGCTCAG-3'; and the vector lower primer described above. The reaction was performed with an annealing temperature of

60°C and an extension time of 1 min. The PCR product using the *Mtap* genotyping upper primer and the wild-type lower primer produced a band of 737 bp. The *Mtap* genotyping upper primer and the vector primer yielded a band of 1,297 bp. The *Mtap*-deficient mice have been deposited in the MMRRRC repository (www.mmrrc.org) (Strain CBaca.129P2-*Mtap*^{Gt(RRK081)Byg}, MMRRRC ID# 15981).

Auditory brainstem response. Auditory brain stem responses (ABRs) were measured in heterozygotes and their wild-type littermates in both ears. For the measurement, mice were anesthetized with xylazine (20 mg/kg ip) and ketamine (100 mg/kg ip). Electrodes were inserted at the vertex and pinna, with a ground electrode positioned near the tail. ABR potentials were evoked with 5-msec tone pips (0.5-msec rise-fall with a \cos^2 onset envelope, delivered at 35/sec). The response was amplified (10,000 \times), filtered (100 Hz–3 kHz), and averaged with an A-D board in a LabVIEW-driven data acquisition system. Sound level was increased in 5 dB steps from 10 dB below threshold to ≥ 80 dB SPL. At each sound level, 1,024 responses were averaged (with stimulus polarity alternated) using an “artifact–reject,” whereby response waveforms were discarded when peak-to-peak amplitude exceeded 15 μ V. On visual inspection of stacked waveforms, “threshold” was defined as the lowest SPL at which any wave could be detected, usually corresponding to the level step just below that at which the peak–peak response amplitude rose significantly above the noise floor (approximately 0.25 μ V). For amplitude versus level functions, the wave I peak was identified by visual inspection at each sound level and the peak-to-peak amplitude computed.

Distortion product otoacoustic emission responses. Animals were anesthetized as for ABR testing. Distortion product otoacoustic emissions (DPOAEs) at $2f_1-f_2$ were recorded with a custom acoustic assembly consisting of two 0.25 inch condenser microphones to generate primary tones (f_1 and f_2 with $f_2/f_1 = 1.2$ and f_2 level at 10 dB $< f_1$ level) and Knowles miniature microphone (EK3103) to record ear canal sound pressure. Stimuli were generated digitally (AO-6, National Instruments, Austin, TX). Ear canal sound pressure was amplified and digitally sampled at 20 msec (A-2000, National Instruments). Fast Fourier transforms were computed and averaged over five consecutive waveform traces, and $2f_1-f_2$ DPOAE amplitude and surrounding noise floor were extracted, a procedure requiring approximately 4 sec of data acquisition and processing time.

RESULTS

Patient Description

All human samples were obtained following review and approval by the Partners HealthCare

System Human Research Committee. DGAP090 is the product of an uncomplicated diamniotic, dichorionic, like-sex, twin pregnancy delivered by Cesarean for transverse lie. He weighed 4,000 g and was 48 cm long. At 6 months of age he was first seen in genetics clinic for anisochoria (which persists) and a peculiar telangiectatic skin condition that produced recurring ulcerations (Fig. 1). These started with mostly painless, dark necrotic ulcers, which eventually healed leaving cutaneous atrophy. Biopsies of the lesions showed no evidence of vasculitis. An evaluation for thrombophilia and collagen vascular disease was negative. The frequency of recurrent ulceration decreased after empiric treatment with low dose aspirin. Asymmetric leg movement while attempting to crawl at 10 months of age eventually led to discovery of avascular necrosis of the left femoral head, which was treated conservatively but healed very slowly. At 6 years of age he had a 2 cm leg length inequality.

Delayed speech at 17 months of age led to detection of a sensorineural hearing loss. On brainstem-evoked response testing, neural transmission could not be assessed due to the absence of wave I bilaterally. Responses to click stimuli were obtained down to 70 dBnHL on the left and 75 dBnHL on the



FIG. 1. The telangiectatic skin condition of DGAP090 on his hand (A) and foot (B) at 2 years of age. The patient presented with recurring ulcerations that started with mostly painless, dark necrotic ulcers, and eventually healed leaving cutaneous atrophy.

right. Subsequent pure tone thresholds indicated a moderate bilateral SHL at 250–500 Hz, sloping to a severe SHL at 1,000–2,000 Hz with a profound SHL at 4,000–8,000 Hz. Speech detection thresholds were in the moderate hearing loss range bilaterally. Immittance testing revealed normal middle ear volume, pressure and compliance bilaterally. Transient evoked otoacoustic emissions were absent bilaterally, suggesting abnormal cochlear function as estimated by the motility of the outer hair cells. Bilateral Mondini defects were noted on the computerized tomograms of the temporal bone. Contiguous 1 mm thick axial and direct coronal scans through the petrous portion of the temporal bone documented normal external auditory canals and normal ossicles. Cochleae were small bilaterally and demonstrated incomplete septation. Vestibules were mildly prominent and the vestibular aqueducts were not enlarged. The internal auditory canals were normal and symmetrical in appearance. Mastoid aeration was normal. DGAP090 had a good response to amplification and began to speak. Over time the hearing loss progressed such that by 5 years of age he had no usable hearing in the high frequencies although he was aided well at 500 Hz. He had a cochlear implant at age 6 years. *GJB2* mutation analysis via DHPLC was negative; the DNA sample had a normal elution profile at the three evaluation temperatures so no direct sequencing was performed. Chromosome analysis was performed after the Mondini malformations were discovered given his multiple, unexplained other clinical findings. Following identification of two translocations in DGAP090, t(8;9)(q12.1;p21.3), and t(9;11)(q33;q13), family studies revealed the t(9;11) to be maternally inherited and also to be present in his co-twin.

At 4 years of age, he developed a swollen right knee. He was diagnosed with pauciarticular juvenile rheumatoid arthritis although no serological tests of inflammation were positive. He responded to steroid injections into the joint space. No systemic symptoms developed and his knee complaints resolved at 10 years of age.

Growth has never been impaired, with his height consistently at the 90th centile and both head circumference and weight at the 75th centile. Although he started kindergarten about 6 months behind his peers, he tested at 7 years of age into the gifted and talented program, in which he has done well academically.

Determination of Genes Disrupted by the Translocation

Sequential FISH experiments were performed until a BAC was identified that hybridized to the normal chromosome and both derivative chromosomes. This BAC was designated as containing the sequence of the breakpoint region. In this manner, the break-

point on chromosome 8 was narrowed to the BAC sequence of RP11-696J24 and that on chromosome 9 to RP11-70L8 (Fig. 2A). Subsequent use of overlapping BACs further refined the breakpoint region. The region on chromosome 9 was narrowed to 1,246 bp when aberrant bands were found in a Southern blot experiment using small PCR probes from the locus (Fig. 2B). The breakpoint was then cloned and sequenced. The sequence of each derivative chromosome is shown in Fig. 2C. The translocation in DGAP090 involves a 7 bp deletion, a 12 bp insertion, and a 19 bp duplication and disrupts *MTAP* between exons 5 and 6. On chromosome 8, the breakpoint is in a gene poor region approximately 200 kb downstream of carbonic anhydrase VIII.

Expression Analysis of *MTAP*

RT-PCR experiments showed that *MTAP* is expressed in human fetal cochlear tissue (data not shown). Northern blot analysis was performed, but an interpretable signal was not observed in any of the tissues tested. Immunohistochemistry was also performed on sections from human fetal inner ear, but no specific signal was observed. Western blots detected with this antibody also did not produce a convincing signal. Thus, our conclusion is that *MTAP* is expressed ubiquitously, but at levels too low for reliable detection by these Northern, Western, and immunohistochemistry analyses.

To assess the expression profile of the *MTAP* *Drosophila* ortholog, *CG4802*, whole mount in situ hybridization was performed on *Drosophila* embryos using the coding sequence of *CG4802* as a probe. Expression of *CG4802* at stages 13 and 14 (Fig. 3) is detected in the fat body and visceral mesoderm.

Drosophila Melanogaster Model for *MTAP* Deficiency

A fly strain was obtained from the Berkeley *Drosophila* Genome Project in which a P element was inserted just 3' of the stop codon for *CG4802*, the fly ortholog of *MTAP*. Because it was possible that such an insertion could interfere with *CG4802* expression, RT-PCR was employed to assess *CG4802* expression. Expression was not affected based on production of a robust PCR product using coding sequence primers for amplification (data not shown). This strain was then crossed with a transposase-expressing strain to yield several excision lines in which the P-element had been removed with the expectation that, in a percentage of cases, imprecise excision would delete some of the surrounding genomic sequence resulting in a fly knockout model for *MTAP*. Three lines were produced in which the complete absence of the

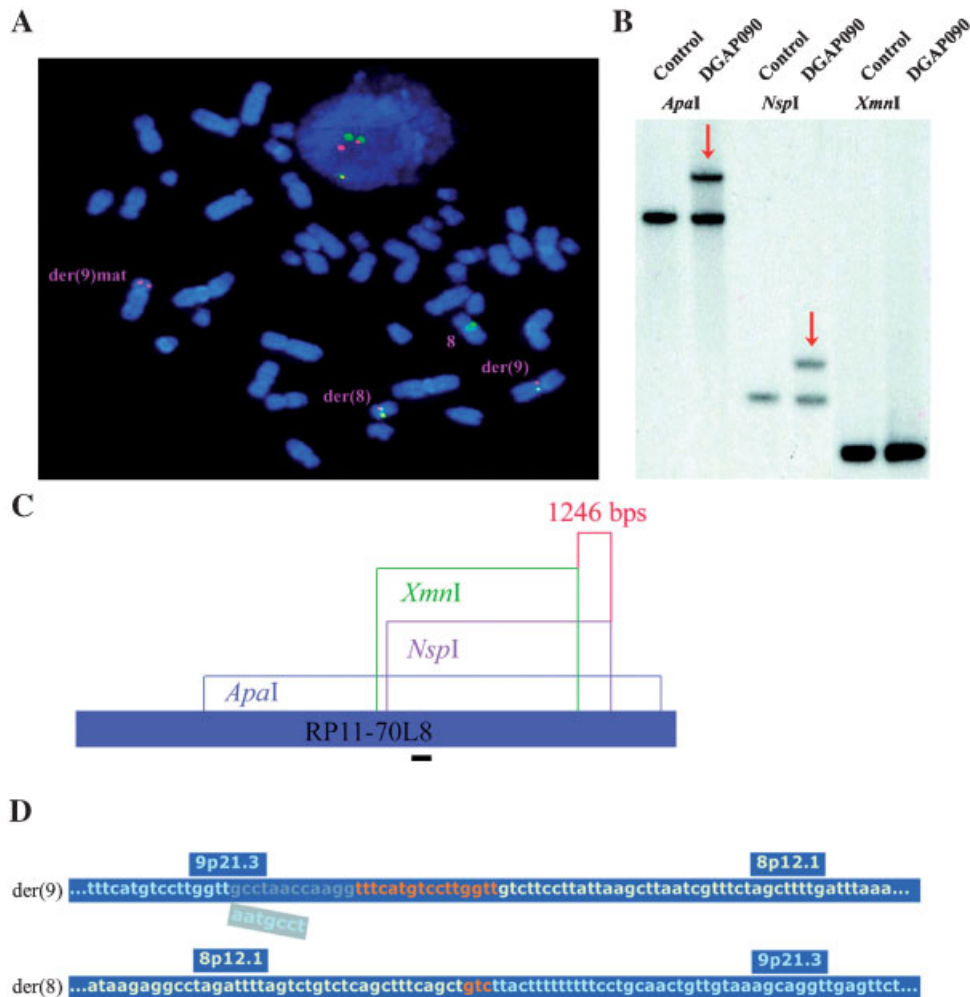


FIG. 2. **A:** FISH was performed with BAC RP11-70L8, mapping to chromosome 9, directly labeled with SpectrumOrange, and with BAC RP11-696J24, mapping to chromosome 8, directly labeled with SpectrumGreen. RP11-70L8 hybridizes to the der(9)mat chromosome and to both the der(9) and der(8) chromosomes, showing that the breakpoint on chromosome 9 is contained within genomic sequence present in RP11-70L8. RP11-696J24 hybridizes to the normal chromosome 8 and to both der(8) and der(9) chromosomes, demonstrating that the breakpoint on chromosome 8 is contained within the genomic sequence present in RP11-696J24. **B:** Southern blot analysis narrowing the chromosome 9 breakpoint region to 1,246 bp. A 579 bp PCR fragment amplified from RP11-70L8 was used to probe DGAP090 genomic DNA and normal male genomic DNA. Aberrant bands (red arrows) are seen in the DGAP090 DNA digested with both *ApaI* and *NspI*. No rearrangement was detected after *XmnI* digestion as expected by a restriction map of the genomic segment. **C:** Restriction map for *ApaI*, *NspI*, and *XmnI* used in defining the chromosome 9 breakpoint within a region of 1,246 bp. The PCR fragment used as the probe in the Southern blot is shown with a black rectangle. **D:** DGAP090 involves collectively a 7 bp deletion (shown in blue letters in the gray box), 12 bp insertion (gray letters), and 19 bp duplication (orange letters). Sequence of the junction fragments cloned from the der(9) shows a 7 bp deletion, 12 bp insertion, and 16 bp duplication, while the sequence of the der(8) shows a 3 bp duplication.

genomic sequence of *CG4802* was verified by PCR. Two of these excisions were homozygous lethal and, presumably, had lost a large segment of surrounding sequence. The third excision was viable when homozygous for the excision. All three strains were tested for auditory responses. No significant difference was seen between the responses of homozygotes, heterozygotes, and wild-type controls.

Mouse *Mtap* Knockout Model

A mouse knockout of *Mtap* was created using an Ola/129 ES cell line, RRK081, in which an exon-trapping vector was inserted into the second intron of

Mtap. The insertion site of the exon-trapping vector was verified by PCR using an upper primer from *Mtap* exon 2 and a vector lower primer. Homozygous deletion of *Mtap* was lethal by embryonic day 8.5. Five and a half week-old mice underwent ABR and DPOAE testing to assess cochlear function. At this age, the cochlea is fully developed, but early-onset progressive hearing loss present in many inbred strains has not begun to develop. Mice were bred onto a pure background of Ola/129 to minimize variability. In the Ola background, the small differences seen in ABR and DPOAE thresholds between *Mtap* heterozygotes and wild-types (Fig. 4A,B) were not significant ($P = 0.256$).

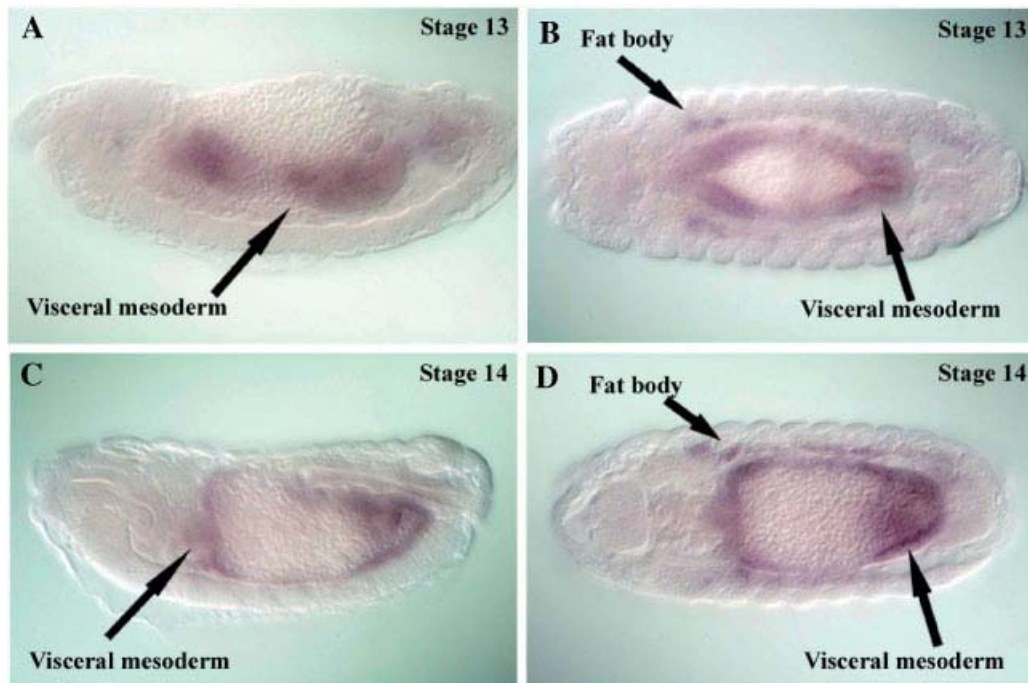


Fig. 3. Embryonic expression of *CG4802*, the fly ortholog of *MTAP*, detected by whole mount in situ hybridization. Lateral (A,C) and ventral (B,D) views of stages 13 (A,B) and 14 (C,D) displaying expression in the visceral mesoderm and the fat body.

Because hearing loss in mice can be strain-dependent, the *Mtap* heterozygote males were out-crossed to wild-type CBA/CaJ females for four generations. Mice were then tested at 5½ weeks and again at 6 months; no significant hearing loss was observed at either time point in this genetic background ($P=0.499$, $P=0.065$) (Fig. 4C,D).

The histopathology of the Ola/129 heterozygotes was then carefully examined. At 6 weeks, no significant pathology was observed. Ola/129 heterozygous mice from 4 to 7 months of age showed various lesions including polyarteritis nodosa, dilated bronchus, and unusual vacuoles in the liver. Because the findings were not consistent throughout the mice tested, the clinical significance is unknown.

DISCUSSION

Metaphase chromosomes and DNA from patients with balanced chromosomal rearrangements can serve as useful biological tools in identifying new genes in developmental processes and is the rationale behind DGAP (www.dgap.harvard.edu). Using cytogenetic and molecular techniques (Fig. 2), *MTAP* was detected to be disrupted in a deaf boy with a de novo translocation between chromosomes 8 and 9, t(8;9)(q12.1;p21.3)dn. *MTAP* is a critical enzyme in the recycling of methionine and adenine. It metabolizes MTA, produced during polyamine synthesis, into adenine and the methionine precur-

sor, methylthioribose-1-phosphate [Backlund et al., 1982]. Additionally, MTA is a potent feedback inhibitor of the polyamine synthesis reaction of which it is a by-product [Pajula and Raina, 1979; Raina et al., 1982]. In the absence of *MTAP*, polyamine levels are presumed to be haploinsufficient and a lower level of polyamines has been observed in cancer cells where *MTAP* is deleted through large genomic rearrangements encompassing *CDKN2A* and *CDKN2B* [Yamanaka et al., 1987]. Disruption of these essential pathways in a homozygous *Mtap* knockout mouse results in an embryonic lethal phenotype.

Based on molecular analysis of the derivative chromosomes in DGAP090, it is reasonable to hypothesize that the phenotype observed may be due to decreased levels of *MTAP*. No evidence for a dominant fusion transcript was found. It would then follow that an analogous model for the patient's phenotype would be *Mtap* knockout heterozygous mice, although differences between mouse models and human phenotypes are well known. It is possible that the altered stoichiometry of polyamine synthesis in DGAP090 impairs hearing. The role of the polyamines, spermidine and spermine, in the auditory system is suggested by the fact that *gyro* mice deficient for spermine synthase are deaf [Lorenz et al., 1998]. The *Phex* gene is also deleted in these mice and *Phex* knockout mice have been shown to demonstrate hearing loss in a background-dependent manner [Lorenz-Depiereux et al., 2004].

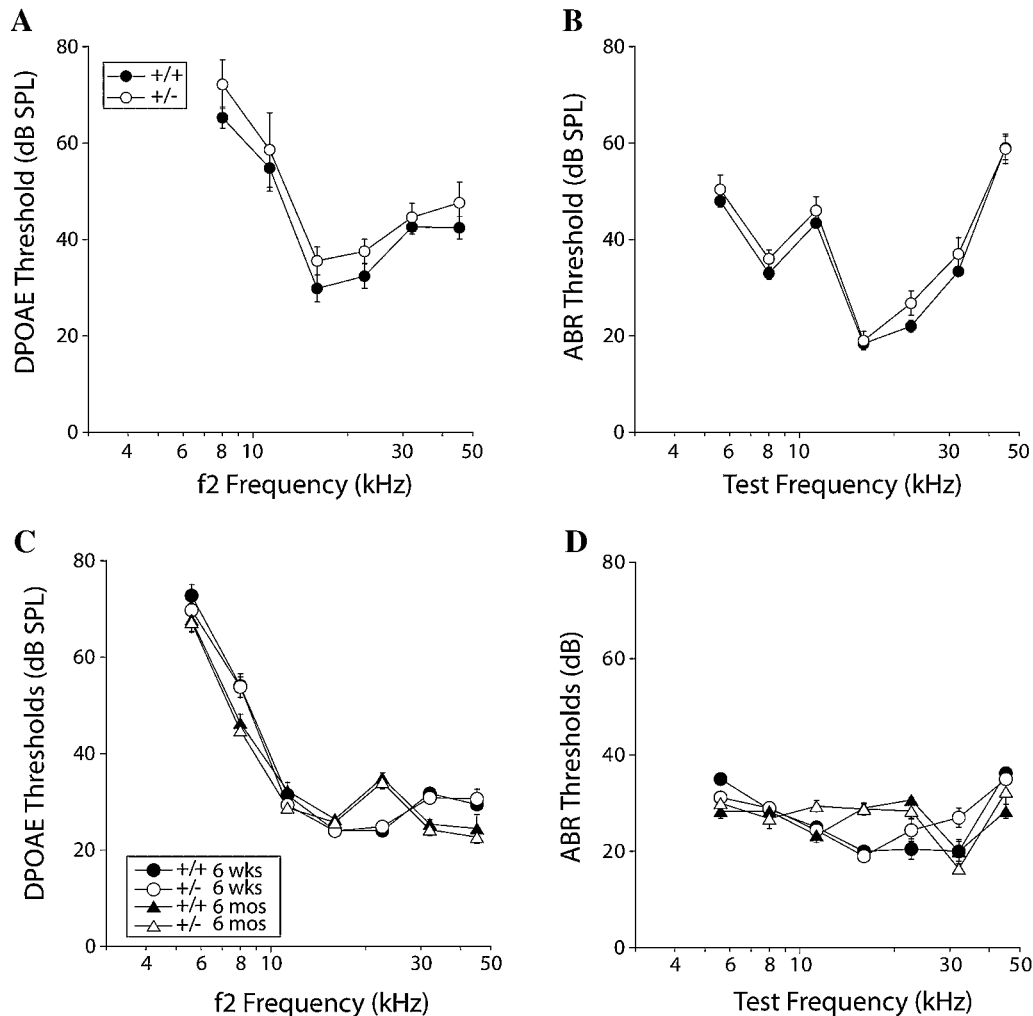


FIG. 4. **A:** Distortion product otoacoustic emissions (DPOAE) thresholds for *Mtap* heterozygous mice in a homogeneous genetic background, Ola/129, tested at 5½ weeks. Data are group mean values (\pm SEM); heterozygous ($n = 5$) and wild-type ($n = 5$). Threshold is defined as the primary level required to produce a DPOAE of 0 dB SPL. **B:** Auditory brainstem response (ABR) thresholds for *Mtap* heterozygous mice in a homogeneous genetic background, Ola/129, tested at 5½ weeks. Data are group mean values (\pm SEM) for the same animals shown in panel A. **C:** Distortion product otoacoustic emissions (DPOAE) thresholds for *Mtap* heterozygous mice in a homogeneous genetic background, CBA/CaJ, tested at 5½ weeks and 6 months. Data are group mean values (\pm SEM); heterozygous ($n = 5$) and wild-type ($n = 4$). Threshold is defined as the primary level required to produce a DPOAE of 0 dB SPL. **D:** Auditory brainstem response (ABR) thresholds for *Mtap* heterozygous mice in a homogeneous genetic background, CBA/CaJ, tested at 5½ weeks and 6 months. Data are group mean values (\pm SEM) for the same animals shown in panel C.

But, the potential importance of spermine synthase is suggested by work done with transgenic *gyro* mice in which the spermine synthase gene has been reintroduced [Wang et al., 2004b]. Though hearing tests of these mice are unpublished, the published work describes how these mice no longer circle, suggesting that the lack of spermine synthase contributes to the hearing loss phenotype of the mice. Further, human patients treated for cancer with α -difluoromethylornithine, DFMO, an inhibitor of polyamine biosynthesis, experience hearing loss that is reversible when administration of the drug is terminated [Abeloff et al., 1984; Sjoerdsma et al., 1984]. Also, another gene involved in the polyamine pathway, spermidine/spermine N^1 -acetyltransferase, *SAT*, is detected to be highly expressed in human fetal cochlea [Robertson et al., 1994].

Polyamines are involved in regulating inwardly rectifying potassium (K_{ir}) channels [Fakler et al., 1994; Ficker et al., 1994; Lopatin et al., 1994]. This is intriguing because the auditory system is critically dependent upon the potential of +80 mV created by a high potassium concentration in the endolymph of the inner ear. It has long been thought that this endolymphatic potential is generated by machinery found in the stria vascularis [Tasaki and Spyropoulos, 1959]. Hibino et al. [1997] identified an ATP-dependent inwardly rectifying potassium channel, $Kir4.1$, localizing to the stria vascularis and absent in deaf W^V/W^V mice. Further evidence supporting the importance of these channels in hearing is provided by patch clamp experiments [Takeuchi and Ando, 1998] in which the main type of current found in intermediate cells from the stria vascularis was a K_{ir}

current. Finally, it was observed that the endocochlear potential is completely absent in a knockout mouse model of *KCNJ10* [Marcus et al., 2002].

The *CG4802* knockout flies do not demonstrate any hearing loss. There are similarities between the hearing process of flies and that of humans and several genes have been identified for which mutations in the human gene and in the *Drosophila* ortholog both cause hearing loss [Weil et al., 1995; Kohlhase et al., 1998, 2002; Dong et al., 2003; Todi et al., 2005]. In flies, sound waves cause movement of the arista on the 3rd segment (a3) of the antenna which leads to a twisting of a3 in relation to the 2nd segment (a2) [Boekhoff-Falk, 2005]. This torque stretches the scolopidia within the Johnston's organ of a2 which causes the opening of ion channels in the scolopodial neuron dendrites and depolarization. Though it has been speculated that the fluid surrounding the scolopodial neuron dendrites is potassium-rich, this has not yet been directly demonstrated [Eberl, 1999]. It is also not known if any inward rectifying potassium channels play a role in the hearing process of *Drosophila*. At this time, three inward rectifying channels have been identified in flies [Doring et al., 2002; MacLean et al., 2002], but there is no evidence that they are expressed in Johnston's organ. In contrast, microarray data have shown that all three channels are highly expressed in the renal tubule, or the Malpighian tubule [Wang et al., 2004a]. Additional *in situ* hybridization and quantitative RT-PCR confirmed this expression [Doring et al., 2002; Evans et al., 2005] and treatment with inward rectifying potassium channel blockers inhibited fluid secretion in the tubule [Evans et al., 2005]. Further studies of the renal function of *CG4802* knockouts may be warranted.

The hearing loss in DGAP090 is not observed in the *Mtap* knockout heterozygous mice. This may reflect differences often seen between the severity of a phenotype in humans and the phenotype of the corresponding mouse model. Such apparent phenotypic differences could be the result of the genetic backgrounds of the organisms (e.g., other "deafness" quantitative trait loci or modifier genes). Alternatively, the phenotypic discrepancy may be due to a progressive loss that will become more significant in older mice. Of note, it is difficult to predict the natural history of the hearing disorder in DGAP090 as a cochlear implant was performed at 6 years of age.

It is also possible that disruption of *MTAP* is not the cause of the hearing loss in DGAP090. The t(8;9) may affect the expression of other genes in the region of the breakpoints through position effects and such observations are well known and have been reported previously (e.g., notably *FOXL2* dysregulation [Beysen et al., 2005; Crisponi et al., 2001]). Alternatively, the translocation may be unrelated to the hearing loss and another gene mutation etiologic. These possibilities are some of the caveats involved

in using balanced translocations as sign-posts for disease-causing genes. Nonetheless, the actual disruption of *MTAP* in this case strongly suggests its involvement in the individual's phenotype. Further investigation of the role of *MTAP* in the hearing process by testing for *MTAP* mutations in individuals with hearing loss could be valuable.

In sum, study of a *de novo* chromosome rearrangement in a deaf child revealed disruption of *MTAP*, which is hypothesized to play a role in the hearing process based on its fundamental role in polyamine synthesis. A knockout mouse model for *Mtap* resulted in an embryonic lethal phenotype, substantiating its critical role in mammalian development.

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