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Deletion of an enhancer near *DLX5* and *DLX6* in a family with hearing loss, craniofacial defects, and an inv(7)(q21.3q35)

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Abstract

Precisely regulated temporal and spatial patterns of gene expression are essential for proper human development. *Cis*-acting regulatory elements, some located at large distances from their corresponding genes, play a critical role in transcriptional control of key developmental genes and disruption of these regulatory elements can lead to disease. We report a three generation family with five affected members, all of whom have hearing loss, craniofacial defects, and a paracentric inversion of the long arm of chromosome 7, inv(7)(q21.3q35). High resolution mapping of the inversion showed that the 7q21.3 breakpoint is located 65 and 80 kb centromeric of *DLX6* and *DLX5*, respectively. Further analysis revealed a 5115 bp deletion at the 7q21.3 breakpoint. While the breakpoint does not disrupt either *DLX5* or *DLX6*, the syndrome present in the family is similar to that observed in *Dlx5* knockout mice and includes a subset of the features observed in individuals with *DLX5* and *DLX6* deletions, implicating dysregulation of *DLX5* and *DLX6* in the family's phenotype. Bioinformatic analysis indicates that the 5115 bp deletion at the 7q21.3 breakpoint could contain regulatory elements necessary for *DLX5* and *DLX6* expression. Using a transgenic mouse reporter assay, we show that the deleted sequence can drive expression in the ear and developing bones of E12.5 embryos. Consequently, the observed familial syndrome is likely caused by dysregulation of *DLX5* and/or *DLX6* in specific tissues due to deletion of an enhancer and possibly separation from other regulatory elements by the chromosomal inversion.

Keywords

DLX5; *DLX6*; enhancer; inversion; 7q21.3

INTRODUCTION

The profoundly complex processes underlying human development are predicated upon precisely regulated temporal and spatial patterns and proper levels of gene expression. Important developmental genes are subject to strict regulation and often reside in a complex and extended genomic regulatory landscape (Kleinjan and van Heyningen 2005). Most of these

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genes, for example *SHH* and *SOX9*, have compact transcriptional units, but are surrounded by large non-coding regions, sometimes hundreds of kilobases long, that must remain intact for appropriate expression. These extended domains likely contain multiple regulatory elements necessary for expression of their corresponding genes in specific tissues. Most developmental genes with associated large regulatory domains have been identified through chromosomal rearrangements. For example, translocations up to 265 kb 5' of *SHH* cause the same phenotype, holoprosencephaly type 3, as do mutations within the *SHH* coding sequence (Belloni et al. 1996; Roessler et al. 1996; Roessler et al. 1997).

Recent whole genome sequencing of many species has now made identification of these long range regulatory elements possible. Because these regulatory elements have important biological function, their sequence is expected to be evolutionarily conserved. Non-genic regions with a high degree of conservation between distant species, such as human and zebrafish, are attractive candidate sequences for regulatory elements. Large scale investigation of these non-genic evolutionarily conserved regions using transgenic mouse reporter assays has revealed that many of these sequences can act as enhancers (Pennacchio et al. 2006). Additionally, specific investigations of the developmental regulatory regions identified by chromosomal rearrangements have uncovered multiple enhancers driving the expression of *SHH*, *PAX6*, and *SOX9* (Bagheri-Fam et al. 2006; Jeong et al. 2006; Kleinjan et al. 2006). In one study, the breakpoint of a translocation causing preaxial polydactyly was mapped to a region approximately 1 Mb 5' of *SHH* and a *SHH* enhancer driving expression in the limbs was subsequently identified in this region (Lettice et al. 2002; Lettice et al. 2003). Point mutations within this enhancer have been found in several families with preaxial polydactyly (Lettice et al. 2003; Gurnett et al. 2007), unequivocally demonstrating that disruption of the action of long range enhancers can cause developmental malformations.

The homeobox transcription factors *DLX5* and *DLX6*, which are important for craniofacial, inner ear, and limb development, also seem to be regulated by *cis*-acting long range enhancers. Chromosomal rearrangements up to 450 kb away from *DLX5* and *DLX6* cause phenotypes similar to complete deletion of these genes (Scherer et al. 1994, Chromosome 7 Annotation Project, <http://www.chr7.org>). In this study, we have mapped and sequenced the breakpoints of an inversion, inv(7)(q21.3q35), in a family with hearing loss and craniofacial defects. We have shown that the 7q35 breakpoint disrupts *CNTNAP2* and the 7q21.3 breakpoint is located 65 and 80 kb from *DLX6* and *DLX5*, respectively. Investigation of these genes implicates dysregulation of *DLX5* and/or *DLX6* in the pathogenesis of the family's phenotype. Additionally, deletion of a 5115 bp sequence at the 7q21.3 breakpoint seems to contribute to the phenotype as it may contain an enhancer essential for expression of *DLX5* and *DLX6* in the ear and in developing bones.

MATERIALS AND METHODS

Patient Ascertainment

The DGAP115 family was ascertained as part of the Developmental Genome Anatomy Project (DGAP). The human study protocol for this project has been reviewed and approved by the Partners Health Care System Human Research Committee.

Fluorescence *in situ* hybridization (FISH) analysis

Peripheral blood samples were collected from the five affected family members and the unaffected grandmother of the proband. Lymphoblastoid cell lines were generated from each blood sample at the Massachusetts General Hospital Cell Transformation Core using standard protocols. Based on the reported karyotypes, BAC clones spanning the breakpoint regions were selected for FISH mapping using the University of California Santa Cruz (UCSC) Genome

Browser (<http://genome.ucsc.edu>). BACs from the RP11 library were obtained from Children's Hospital Oakland Research Institute (Oakland, CA) and BACs from the CTD library were acquired from Invitrogen (Carlsbad, CA). Metaphase chromosome spreads were prepared from the lymphoblastoid cells using standard cytogenetic protocols and FISH performed as previously described (Ney et al. 1993). BACs were directly labeled with either Spectrum Orange or Spectrum Green conjugated dUTP using a nick translation kit (Vysis, Downers Grove, IL) and differentially labeled pairs were hybridized overnight to metaphase chromosome preparations. After washing, chromosomes were counterstained with DAPI and analyzed with a Zeiss Axioskop (Thornwood, NY) microscope and Applied Imaging CytoVision software (Santa Clara, CA). At least 10 metaphases were scored per BAC probe.

Southern Blot Analysis

The 27 kb of overlapping sequence from BACs CTD-2366L13 and RP11-737n20 was analyzed using RestrictionMapper (<http://www.restrictionmapper.org/>) to select restriction enzymes digesting in staggered patterns across the sequence and using RepeatMasker (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>) to identify regions in which unique probes could be generated. DNA was isolated from lymphoblastoid cell lines using the PureGene system (Gentra, Minneapolis, MN). 7.5 ug of DGAP115 and control genomic DNA were digested overnight. Digested DNA was electrophoresed in a 0.7% agarose gel and transferred overnight to Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ) via capillary action. DNA was cross-linked to the membrane with ultraviolet light. Probes of 300–600 bp were created by PCR, radioactively labeled with ^{32}P -dCTP by random priming using the Megaprime DNA Labeling Kit (Amersham Biosciences), and purified using Microspin G-25 columns (Amersham Biosciences). Following prehybridization for at least an hour in UltraHybe (Ambion, Austin, TX) at 42°C, blots were hybridized overnight at 42°C with labeled probes that had previously been denatured at 95°C for 10 minutes. Blots were washed several times, exposed to Kodak Biomax XAR film in cassettes with intensifying screens at –80°C, and developed with a Kodak X-OMAT 2000A processor. Bands detected in patient but not in control DNA indicate localization of the breakpoint within these restriction fragments.

Breakpoint Cloning

The 7q21.3 breakpoint was cloned using the suppression PCR strategy described in (Siebert et al. 1995). The 7q21.3 specific primer sequences were 5'-ATTCTGGAAACGCAGGTGGCTCTGT-3' and 5'-CGCCCTTACCCCTCTTAGAC-3' (nested). DGAP115 and control reactions were electrophoresed in a 1% agarose gel and the band detected only in the DGAP115 reaction was extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The extracted band was cloned into pCRII-TOPO using the Invitrogen TOPO-TA cloning kit and sequenced at the Dana-Farber/Harvard Cancer Center (DFHCC) DNA Resource Core. Based on the sequence of the 7q21.3 breakpoint, PCR primers were designed to amplify the 7q35 breakpoint. The 7q35 PCR product was TOPO-TA cloned and sequenced at the DFHCC DNA Resource Core. Breakpoints were confirmed in all affected family members by PCR and sequencing.

Array Comparative Genomic Hybridization (aCGH)

aCGH was performed by the DFHCC Cytogenetics Core facility using Spectral Genomics 2600 BAC arrays (Houston, TX) with ~1 Mb resolution.

Mouse Whole Mount *In Situ* Hybridization

For mouse whole mount *in situ* hybridization experiments, a 640 bp fragment from the 3'UTR of *Cntnap2* was amplified by PCR from mouse embryonic cDNA and was TOPO-TA cloned into pCRII-TOPO. The vector was linearized with either *Bam*HI or *Eco*RV and both antisense

and sense riboprobes with digoxigenin conjugated UTP were generated by *in vitro* transcription using Sp6 and T7 RNA polymerases (MAXIScript kit, Ambion). Riboprobes were purified with P-30 Tris RNase-free Micro Bio-Spin Columns (Bio-Rad, Hercules, CA). E9.5, E10.5, and E11.5 ICR embryos (Taconic, Germantown, NY) were collected in cold PBS and fixed overnight in 4% paraformaldehyde at 4°C. Embryos were dehydrated in a series of methanol/PBST washes at room temperature and stored in 100% methanol at -20°C until ready for processing. Embryos were rehydrated, bleached in 6% hydrogen peroxide in PBST, and treated with proteinase K for 10 minutes (E9.5), 15 minutes (E10.5), or 20 minutes (E11.5). After postfixing in 4% paraformaldehyde/0.2% glutaraldehyde in PBST, embryos were equilibrated in 50% hybridization solution (50% formamide, 5XSSC at pH 4.5, 1% SDS, 50 ug/ml yeast tRNA, and 50 ug/ml heparin) in PBST and then prehybridized in 100% hybridization solution at 65°C for one hour. Riboprobe was added to the embryos and they were incubated overnight at 65°C. Embryos were washed, blocked with 10% heat inactivated sheep serum and 1% blocking reagent (Roche, Indianapolis, IN) in MABT, and then incubated with anti-digoxigenin-AP antibody (Roche) at a concentration of 1:5000 in 1% heat inactivated sheep serum and 1% blocking reagent overnight at 4°C. Embryos were then washed thoroughly, soaked in NTMT, and developed in BM Purple AP Substrate (Roche) until the reaction was judged complete (about 2 hours). Finally, the embryos were washed, postfixed in 4% paraformaldehyde/0.1% glutaraldehyde for 20 minutes, and washed twice more.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from lymphoblastoid cell lines using Trizol (Invitrogen). 1 ug of RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was amplified with the following primers: 5'-CTCGTGGGATTGACACAA-3' and 5'-GGTCATAGATTTCAGGCACCA-3' for *DLX5* and 5'-CGAACTGGCAGCTTCCTTAG-3' and 5'-AATGCAGGAGTCCAAAATGC-3' for *DLX6*.

Analysis of Deleted Sequence

The 5115 bp deleted sequence from 7q21.3 was submitted to GenomeVista (<http://pipeline.lbl.gov/cgi-bin/GenomeVista>; Bray et al. 2003; Couronne et al. 2003) to assess conservation of the sequence between human, mouse, rat, and chicken genomes. The 5115 bp deleted sequence was also compared to the mouse and rat genomes using both BLASTN and cross-species megaBLAST to identify highly similar regions between the species (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>; Altschul et al. 1990). Additionally, the sequence was submitted to the Genomatix MatInspector program (<http://www.genomatix.de/>; Quandt et al. 1995) to search for known transcription factor binding sites.

Transgenic Embryo Assay

The entire 5115 bp sequence plus the conserved regions at each end of the sequence (chr7:96,401,902-96,407,985 on the March 2006 assembly of the human genome) and a minimal beta globin promoter (5'-AGCTTCCCGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGCA-3') were cloned between the *XhoI* and *NcoI* sites of MCS_pNASS β , which contains a LacZ cassette (gift of Amy Donner, Brigham and Women's Hospital). 20 ug of the construct was digested overnight with *NarI* and *SphI*. Digested DNA was electrophoresed in a 1% agarose gel, the 10 kb band was extracted using the QIAquick Gel Extraction kit (Qiagen), and the extracted band was eluted in 10 mM Tris/0.25 mM EDTA pH 8.0. Following elution, this linearized construct was diluted, injected into the pronucleus of FVB fertilized eggs, and transferred into pseudopregnant mothers by the Brigham and Women's Hospital Transgenic Core facility.

Embryos were collected at E12.5 and their yolk sacs were saved for genotyping. The embryos were washed in PBS and fixed for one hour in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA, and 0.02% NP-40 in PBS. Then they were washed three times in PBS/0.02% NP-40 and stained at 37°C with 5 mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.01% NaDeoxycholate, 0.02% NP-40, and 1mg/ml X-gal in PBS until the reaction was complete. Finally, embryos were washed three times in PBS and postfixed in 4% paraformaldehyde overnight at 4°C. Images of embryos were taken with a dissecting microscope using a digital camera. Ears were dissected and whole mounted images were taken using a digital camera mounted onto a Nikon E800 microscope.

DNA was isolated from the yolk sacs using the DNAeasy Blood and Tissue Kit (Qiagen). PCR was performed on the resulting DNA using a primer in the LacZ gene and a primer in the 5115 bp deleted sequence to identify the transgenic embryos.

RESULTS

We have ascertained a family (designated the DGAP115 family) consisting of five affected members in which hearing loss and craniofacial anomalies segregate with a paracentric inversion of the long arm of chromosome 7. The family consists of five affected members spanning three generations, the proband (designated DGAP115), the proband's mother (DGAP116), and the proband's three children (DGAP117, DGAP118, and DGAP119). All five affected family members have hearing loss, micrognathia, and abnormal pinnae (small and posteriorly rotated with overfolded superior helices) (Figure 1). Some of these individuals also have additional anomalies, including cleft palate, mild developmental delay, early puberty, kyphosis, and abnormal dentition, as detailed in Table 1. X-rays taken for unrelated purposes have also shown femoral head necrosis in one family member. An MRI of the brain and auditory structures of DGAP115 revealed abnormalities of the inner ear. The right inner ear showed incomplete segmentation of the cochlea with an underdeveloped basal turn and a poorly formed modiolus, while the left ear showed hypoplasia of the cochlea with absent segmentation, a poorly formed modiolus, and cochlear aperture stenosis. Vestibular structures and the vestibulocochlear nerve appeared normal on both sides. Structural abnormalities of the middle ear are also likely; however, a follow-up temporal bone CT scan to evaluate the morphology of the ossicles was not performed. The reported karyotype for all affected individuals, based on GTG-banded analysis of metaphase chromosomes, was 46,XX,inv(7)(q22.1q35) or 46,XY,inv(7)(q22.1q35). The proband's maternal grandmother is phenotypically normal and does not have the inv(7). Her maternal grandfather also had a normal phenotype but was not available for further evaluation.

Breakpoint Mapping

To investigate the molecular basis of this family's clinical features, we further mapped the breakpoints of the inversion and examined potential candidate genes in the breakpoint regions. We began by performing FISH on metaphase chromosomes from DGAP115 lymphoblastoid cells. Based on the reported karyotype, inv(7)(q22.1q35), BACs localized to 7q21-q31 and 7q34-q36 on the UCSC website were selected and sequential FISH experiments performed to narrow the breakpoint regions. BAC clones CTD-2350n7 and RP11-879e11 from 7q21.3 hybridized to 7q21.3 on the normal chromosome 7 and to both 7q21.3 and 7q35 on the inv(7) indicating that the breakpoint occurs within the 100 kb region where these BACs overlap (Figure 2A and Figure 3). The ends of a BAC centromeric to the 7q21.3 breakpoint, CTD-2366L13, and a BAC telomeric to this breakpoint, RP11-737n20, slightly overlap but neither shows a split hybridization signal, suggesting that the breakpoint lies within the 27 kb of overlapping sequence (Figure 3). BACs RP11-5p2 and RP11-643a21 from 7q35 hybridized to 7q35 on the normal chromosome 7 and to both 7q21.3 and 7q35 on the inv(7) narrowing

the 7q35 breakpoint to 140 kb (Figure 2B). Based on our FISH studies, which provide a higher resolution analysis than a traditional GTG-banded karyotype, the breakpoints of the familial inversion were revised to inv(7)(q21.3q35).

To delineate the location of the 7q21.3 breakpoint further, Southern blots were performed on control and DGAP115 DNA using probes spanning the 27 kb FISH-defined breakpoint region. A band was detected in the *AfIII*, *KpnI*, and *StuI* DGAP115 digests that was not present in the control digests indicating that the breakpoint occurs within the 3255 bp where these restriction fragments overlap (Figure 2C and Figure 3). No aberrant bands were detected in the *EcoRI* digests, further narrowing the breakpoint to 331 bp (Figure 3). The 7q21.3 breakpoint was then amplified using suppression PCR, cloned, and sequenced. Using this sequence as a guide for primer design, the 7q35 breakpoint was amplified directly by PCR, cloned, and sequenced. Sequencing of both breakpoints revealed a 5115 bp deletion (chr7:96,402,577-96,407,691 on the March 2006 assembly of the human genome) of 7q21.3 sequence, a 2 bp deletion of 7q35 sequence, and a 15 bp duplication of 7q35 sequence on the inv(7) (Figure 3). Sequencing also confirmed that all affected family members have the same exact breakpoints. To ensure that there were no deletions or duplications elsewhere in the genome, DNA from DGAP115 was examined by aCGH. No clinically significant copy number gains or losses were detected.

Identification and Investigation of Genes near the Breakpoints

The 7q35 breakpoint disrupts *CNTNAP2* between exons nine and ten (Figure 4). To determine if *CNTNAP2* is a strong candidate gene for the family's phenotype, its expression pattern during early development was investigated using whole mount *in situ* hybridization on mouse embryos. At E9.5, E10.5, and E11.5, *Cntnap2* expression was seen primarily in the brain (Figure 5). *Cntnap2* expression was absent from most tissues relevant to the family's phenotype, such as the branchial arches and the otic placode/vesicle, suggesting that malformation of these tissues is unrelated to disruption of this gene. Additionally, *Cntnap2* knockout mice have been reported to have "no apparent phenotype" (Poliak et al. 2003) and specific examination of these null mice revealed no craniofacial or ear abnormalities. Recent reports have implicated *CNTNAP2* as the cause of various neurologic disorders, including autism, epilepsy, and schizophrenia (Strauss et al. 2006; Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Friedman et al. 2008); however, disruption of *CNTNAP2* does not appear to be the primary cause of the DGAP115 family's clinical features.

There are no known genes, mRNAs, or ESTs at the 7q21.3 inversion breakpoint. The closest genes to the breakpoint are *DLX6* (65.5 kb telomeric), *DLX5* (80 kb telomeric), and *SHFM1* (225 kb centromeric) (Figure 6). *Dlx5* knockout mice have malformations of the middle and inner ear, abnormal pinnae, and micrognathia (Acampora et al. 1999; Depew et al. 1999; Merlo et al. 2002b), consistent with the phenotype seen in the DGAP115 family. Additionally, individuals with deletions of both *DLX5* and *DLX6* or with chromosomal rearrangements centromeric to *DLX5* and *DLX6* have severe phenotypes which generally include hearing loss, abnormal pinnae, micrognathia, cleft palate, and developmental delay (Chromosome 7 Annotation Project, <http://www.chr7.org>). To assess whether the inversion affects expression of *DLX5* or *DLX6*, reverse transcriptase (RT) -PCR was performed for the DGAP115 family and control lymphoblastoid cell lines using primer sets for both genes. Because both *DLX5* and *DLX6* are expressed at very low levels in lymphoblastoid cell lines, they were not consistently amplified even when the PCR was performed for an increased number of cycles. Previous reports have also noted difficulty in assessing *DLX5* and *DLX6* expression in lymphoblastoid cell lines (Horike et al. 2005; Schule et al. 2007; Miyano et al. 2008) and profiles for both *DLX5* and *DLX6* in the UniGene database (<http://www.ncbi.nlm.nih.gov>) show no expression in blood.

Contribution of 5115 bp Deleted Sequence to Family's Phenotype

To investigate further a role for the 7q21.3 breakpoint in the family's phenotype, the 5115 bp deleted sequence was analyzed. Comparing the deleted sequence with the syntenic regions of the mouse genome and the rat genome using GenomeVista (<http://pipeline.lbl.gov/cgi-bin/GenomeVista>) revealed three regions (1-3MC) with over 75% conservation in both mouse and rat and an additional five regions (1-5C) with over 70% conservation in either mouse or rat when using 100 bp sliding windows (Figure 6). Comparing the deleted sequence to the mouse and rat genomes via BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) revealed two regions with greater than 85% identity. These regions correspond with regions 1MC and 2MC identified by GenomeVista. BLAST also detected a 50 bp region in both mouse and rat with high basepair identity, 91% for rat and 83% for mouse, which corresponds to GenomeVista region 1C. MatInspector (<http://www.genomatix.de/>), which searches for known transcription factor binding sites, identified four *DLX1* and seven *MEF2* consensus binding sites within the 5115 bp deleted sequence. Both *DLX1* and *MEF2C* are known to regulate *DLX5* and *DLX6* expression (Anderson et al. 1997; Zerucha et al. 2000; Verzi et al. 2007).

Because the 5115 bp deleted sequence contains several conserved regions and binding sites for transcription factors that regulate *DLX5* and *DLX6*, this sequence was tested for *in vivo* enhancer activity. The 5115 bp deleted region and a minimal beta globin promoter were cloned into the MCS-pNASS β vector upstream of the LacZ cassette. This construct was then used to generate transgenic embryos to determine if the deleted region could drive expression of the LacZ reporter in developing tissues relevant to the family's phenotype. Eight of the 72 F0 embryos harvested at E12.5 had the transgenic construct integrated into the genome. Two of these eight embryos (25%) showed LacZ expression. Interestingly, the LacZ expression appears in a subset of the tissues where *DLX5* and *DLX6* are expressed at E12.5 (Acampora et al. 1999; Robledo et al. 2002), including the developing bones of the vertebrae and ribs and the inner ear (Figure 7). Further dissection to localize the inner ear expression more specifically revealed intense staining of the endolymphatic sac and the tip of the cochlear duct and minor staining in the posterior semicircular canal (Figures 7D and 7E). The presence of LacZ expression in specific tissues suggests that the 5115 bp deleted region contains an enhancer which potentially regulates expression of genes in the vicinity of the 7q21.3 breakpoint, such as *DLX5* and *DLX6*.

DISCUSSION

Congenital anomalies in individuals with chromosomal rearrangements are often caused by disruption of genes at the rearrangement breakpoints. However, chromosomal rearrangements have also been useful for detecting genes that are subject to the action of long range regulatory elements. Several genes have been identified for which rearrangement breakpoints at a distance of up to 1 Mb away from the gene can cause the same phenotype as a direct disruption of that gene. These genes include *PAX6*, *SOX9*, *POU3F4*, *SHH*, *SIX*, *MAF*, *FOXC1*, *FOXC2*, *FOXL2*, and *TWIST* (Kleinjan and van Heyningen 2005). Expression of these genes is precisely regulated during development by long range regulatory elements and disruption of this regulation can cause severe phenotypes. Mapping the breakpoints of a chromosomal rearrangement in the family reported herein revealed that *CNTNAP2* is directly disrupted at 7q35, but is unlikely to be responsible for the observed heritable phenotype. The 7q21.3 breakpoint does not disrupt any genes, but is located less than 100 kb from *DLX5* and *DLX6* which are compelling candidate genes for the family's disorder.

Exclusion of *CNTNAP2* as a Candidate Gene for the Family's Phenotype

The 7q35 breakpoint disrupts *CNTNAP2* between exons nine and ten effectively separating the first nine exons from the last 15 exons on the inv(7). *CNTNAP2* is a member of the neurexin superfamily of transmembrane proteins that mediate cell-cell interactions in the nervous system. *CNTNAP2* co-localizes with the voltage gated potassium channels, KCNA1, KCNA2, and KCNB2, at the juxtaparanodal region of myelinated axons (Poliak et al. 1999). It is necessary for proper positioning of these channels in the juxtaparanodal region and possibly for formation and maintenance of distinct domains along the axon (Poliak et al. 2003). Expression of *Cntnap2* in developing mice is seen predominantly in the brain. To assess the potential role of this gene in neurologic disease, *Cntnap2* null mice were generated, but reported to have no noticeable phenotype (Poliak et al. 2003). No craniofacial, skeletal, or ear malformations were observed in the null mice.

Recent reports of individuals with other mutations of *CNTNAP2* also support exclusion of *CNTNAP2* as a candidate gene for hearing loss, craniofacial, and skeletal abnormalities. Chromosomal rearrangements disrupting *CNTNAP2* have been reported in individuals with various neurologic conditions, including an insertion in a family with Gilles de la Tourette syndrome and obsessive compulsive disorder (Verkerk et al. 2003), an inversion in a child with autism (Bakkaloglu et al. 2008), and deletions in one individual with autism (Rossi et al. 2008), one individual with epilepsy (Friedman et al. 2008), and two individuals with both epilepsy and schizophrenia (Friedman et al. 2008). Additionally, a recessive mutation in *CNTNAP2* causes cortical dysplasia-focal epilepsy syndrome (Strauss et al. 2006) and other *CNTNAP2* variants increase risk for autism (Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008). None of the individuals with these other mutations have been reported to have hearing loss, craniofacial defects, or skeletal abnormalities. Furthermore, a completely healthy individual with a translocation disrupting *CNTNAP2* has also been reported (Bellosso et al. 2007). Besides mild developmental delay, no neurological conditions have been noted for any of the DGAP115 family members and the family has been described as "very laid back." Based on the phenotype of the knockout mouse and the other patients with *CNTNAP2* mutations, disruption of *CNTNAP2* does not appear to contribute to the syndrome present in the DGAP115 family.

DLX5 and *DLX6* as Candidate Genes for the Family's Phenotype

DLX5 and *DLX6* are transcription factors containing a homeobox domain related to the *Drosophila* *Distal-less* homeobox domain and are important developmental genes involved in patterning of craniofacial structures, the inner ear, the limbs, and the brain (Merlo et al. 2000). They also play a major role in chondrocyte and osteoblast differentiation. In E9.5 mouse embryos, *Dlx5* and *Dlx6* are expressed in the branchial arches and otic placode (Acampora et al. 1999). By E11.5, expression is seen throughout the derivatives of the first branchial arch, in the vestibular portion of the inner ear, in the diencephalon and telencephalon of the brain, and in the apical ectodermal ridge (Acampora et al. 1999). *Dlx5* and *Dlx6* are also expressed in all skeletal elements from initial cartilage formation until ossification (Simeone et al. 1994). *Dlx5* knockout mice are perinatal lethal and can be easily distinguished from their wild-type and heterozygous littermates due to their decreased nasal capsular breadth, micrognathia, and abnormal pinnae (Depew et al. 1999). *Dlx5* knockouts are also characterized by cleft palate, ectopic bones in the middle ear, a shortened cochlear duct, absent semicircular canals, a reduced endolymphatic duct, misshapen teeth, and cranial bone defects (Acampora et al. 1999; Depew et al. 1999; Merlo et al. 2002b). *Dlx5* and *Dlx6* double knockout mice have more severe craniofacial, ear, and bone defects, including ectrodactyly (Merlo et al. 2002a; Robledo et al. 2002). Although neither *DLX5* nor *DLX6* is disrupted by the breakpoint, the DGAP115 family members and the *Dlx5* knockout mice have many similar features.

Furthermore, multiple individuals with deletions of *DLX5* and *DLX6* have been reported. These individuals have severe phenotypes, including ectrodactyly, sensorineural hearing loss, conductive hearing loss due to fixation of ossicles, abnormal pinnae with overfolded helices, cleft palate, micrognathia, developmental delay, and abnormal teeth (Scherer et al. 1994; Haberlandt et al. 2001; Fukushima et al. 2003; Wieland et al. 2004). Affected members of the DGAP115 family also have sensorineural hearing loss, conductive hearing loss, abnormal pinnae with overfolded helices, cleft palate, micrognathia, developmental delay, and abnormal teeth. However, the clinical features of the DGAP115 family are less severe than those seen in the *DLX5* and *DLX6* deletion patients, as they have a milder hearing loss and they do not have ectrodactyly. Interestingly, several individuals with translocations centromeric to *DLX5* and *DLX6* have also been reported with similar phenotypes to the deletion patients (Chromosome 7 Annotation Project, <http://www.chr7.org>) suggesting the existence of long range *cis*-acting regulatory elements centromeric to *DLX5* and *DLX6* that are essential for proper *DLX5* and *DLX6* expression. It is possible that the family's inversion leads to loss of function of an enhancer element which regulates patterning of the ear and craniofacial regions, but is not necessary for other functions of *DLX5* and *DLX6*, such as development of the fingers and toes. Given that the DGAP115 family has clinical features similar to, but milder than, those caused by loss of the *DLX5* and *DLX6* genes, we propose that the *inv*(7) disturbs the function of an enhancer which regulates *DLX5* and/or *DLX6* expression in specific tissues during development.

Identification of an Enhancer near *DLX5* and *DLX6* Driving Expression in Developing Bones and the Ear

Since disruption of the function of an enhancer could be the pathogenic mechanism causing the phenotype of the DGAP115 family, we decided to test for the presence of long range enhancers in the vicinity of the 7q21.3 breakpoint using an *in vivo* reporter assay. Dysregulation of *DLX5* and *DLX6* could result from deletion of an enhancer within the 5115 bp deleted sequence at the breakpoint or from separation of the genes from enhancers on the opposite side of the breakpoint. Bioinformatic analysis of the 5115 bp deleted sequence for conserved regions and relevant transcription factor binding sites indicated that it could potentially harbor regulatory elements. Testing the deleted sequence in transgenic embryos showed that it could drive expression of a LacZ reporter gene in developing skeletal structures and the inner ear, which are areas of *DLX5* and *DLX6* expression and tissues affected in the DGAP115 family members. While these results support the idea that the deleted region contains an enhancer regulating *DLX5* and/or *DLX6* expression, we also expected to see LacZ expression in the developing mandible and middle ear tissues. Embryos were assayed at E12.5 because *DLX5* and *DLX6* are at their widest range of expression at this time. While no expression was seen in the mandible at this time point, the reporter may have been expressed in this area at an earlier time point as the mandible begins developing around E9.5. A MEF2C-dependent enhancer in the promoter region of *DLX6* drives reporter gene expression in the mandible and other craniofacial regions at E9.5 (Verzi et al. 2007); however, in embryos assayed at E11.5 the same sequence drives expression in the midbrain, but not in any craniofacial areas (Vista Enhancer Browser, <http://enhancer.lbl.gov/>), demonstrating the precise temporal specificity of enhancers. Likewise, the middle ear primordium is present at E12.5, but development of the middle ear bones is not detectable until at least E14.5; therefore, the reporter gene may be active in the middle ear later in development. Alternatively, there could be additional enhancers located in the 7q21.3 region centromeric to the breakpoint regulating *DLX5* and *DLX6* expression in the mandible, middle ear, and other craniofacial areas. While two embryos showed similar X-gal staining patterns, the other six transgenic embryos did not show any X-gal staining. There are several possible explanations for this; for example, the construct may have inserted into regions of heterochromatin in the non-expressing embryos or the enhancer in the deleted sequence must act in concert with other elements to drive transcription

effectively. The latter option seems likely as additional conserved areas were also present on both sides of the deleted sequence at 7q21.3. Furthermore, the deleted region is quite large, so it is possible that an enhancer element located at a distance from the minimal promoter will not be able to act on the promoter efficiently when it is outside of its normal genomic context, particularly if elements that promote chromatin looping are not present. The results of the reporter assay indicate that the 5115 bp deleted sequence contains an enhancer that could regulate *DLX5* and *DLX6* expression in the vertebrae, the ribs, the inner ear, and possibly other tissues. Deletion of this sequence from the inv(7) could potentially cause some, if not all, of the clinical features seen in the DGAP115 family.

While the tissues where this potential enhancer is active correlate with the tissues where *DLX5* and *DLX6* are expressed, further work will need to be done to establish conclusively that this enhancer plays a role in the regulation of *DLX5* and/or *DLX6*. For example, transgenic studies using large BAC-based constructs with *DLX5* and *DLX6* in their normal genomic context and the enhancer region either present or absent could be used to show that the enhancer actually directs the expression of the *DLX5* and/or *DLX6* genes. Additional studies would also be needed to determine the exact sequence of the enhancer and the transcription factors that bind the enhancer. The 5115 bp deleted region could be split into smaller segments and those smaller constructs could be used in either transgenic experiments or *in vitro* reporter assays to localize more precisely the enhancer element. Once the enhancer is precisely identified, electrophoretic mobility shift assays (EMSA) could be performed to discover which transcription factors bind to the enhancer, which may provide further insight into the molecular pathways involved in otic, craniofacial, and skeletal development.

In summary, high resolution mapping and sequencing of the breakpoints of a familial inversion associated with hearing loss and craniofacial defects revealed a 5115 bp deletion at one of the breakpoints. This breakpoint occurs less than 100 kb from the *DLX5* and *DLX6* genes, which are regulated in a strict temporal and spatial pattern during development presumably due to the action of long range *cis*-acting regulatory elements. Disrupting the action of these regulatory elements may cause severe abnormalities, including hearing loss, craniofacial defects, and limb defects, like those observed from deletion of these genes. Similarly, dysregulation of *DLX5* and/or *DLX6* due to deletion of an enhancer at the 7q21.3 breakpoint and possibly separation from other regulatory elements is the likely cause of the syndrome observed in the DGAP115 family.

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Figure 1. Side views of the DGAP115 family members (DGAP115-119) and a family pedigree with affected individuals denoted. Note the micrognathia and abnormal pinnae with overfolded superior helices present in all family members (color figure online)

Figure 2. Mapping of the DGAP115 family inversion breakpoints using FISH and Southern blots. A) FISH on metaphase chromosomes showing BAC RP11-879e11 (green), which maps to 7q21.3 and spans the 7q21.3 breakpoint. B) FISH on metaphase chromosomes showing BAC RP11-643a21 (red), which maps to 7q35 and spans the 7q35 breakpoint. C) Southern blot with red arrows marking aberrant bands seen in DGAP115 genomic DNA when digested with *KpnI*, *StuI*, and *AflIII*, indicating that the breakpoint lies within these restriction fragments (color figure online)

Figure 3.

Strategy used to clone and sequence the DGAP115 family inversion breakpoints. FISH mapping with overlapping BACs narrowed the 7q21.3 breakpoint region to 27 kb. BACs with split hybridization signals are colored red. Southern blots with probes spanning the 27 kb FISH-defined region further narrowed the breakpoint to 331 bp. Restriction fragments producing aberrant bands are colored red. The breakpoint was then amplified by suppression PCR and sequenced. Sequencing traces spanning the 7q21.3 and 7q35 breakpoints are shown. 5115 bp were deleted from 7q21.3, 2 bp were deleted from 7q35, and 15 bp of 7q35 sequence were duplicated at the inversion breakpoints (color figure online)

Figure 4.

Map of the 7q35 breakpoint from the UCSC genome browser. The breakpoint is marked by the red line. The 7q35 breakpoint disrupts *CNTNAP2* between exons nine and ten (color figure online)

Figure 5. Whole mount *in situ* hybridization on mouse embryos using a *Cntnap2* riboprobe at E9.5 (A,B), E10.5 (C,D), and E11.5 (E,F). *Cntnap2* expression is seen primarily in the brain at all three time points (color figure online)

Figure 6.

Map of the 7q21.3 breakpoint region from the UCSC genome browser. The 7q21.3 breakpoint is 65 kb and 80 kb from *DLX6* and *DLX5*, respectively. 5115 bp of 7q21.3 sequence is deleted at the breakpoint. This deleted sequence contains several regions that are conserved between the human, mouse, and rat genomes as shown in the GenomeVista plot in the lower half of the figure. The three most conserved regions (1-3MC) with greater than 75% conservation in both mouse and rat are labeled in red. Five additional conserved regions (1-5C) with greater than 70% conservation in either mouse or rat are labeled in black (color figure online)

Figure 7.

X-gal staining of E12.5 transgenic embryo shows LacZ expression in developing bones and the ear. A) View of transgenic embryo showing surface staining. B) Cleared view of transgenic embryo showing staining of internal structures. C) Dissection of the inner ear region shows LacZ expression in the endolymphatic duct (ED), around the tip of the cochlear duct (CD), and in the posterior canal. D) Magnified view of the cochlear duct staining. 100 µm error bars are shown in the top right corner of images C and D (color figure online)

Table 1

Phenotype of affected family members

DGAP #	Gender	Age at Examination	Phenotype
115	female	35	Mild high frequency sensorineural hearing loss in right ear, moderate conductive hearing loss in left ear, abnormal pinnae, micrognathia
116	female	58	Bilateral severe conductive hearing loss and high frequency sensorineural hearing loss, abnormal pinnae, micrognathia
117	male	12	Bilateral mild conductive hearing loss, abnormal pinnae, severe micrognathia, failure to thrive, feeding problems, mild developmental delay, early puberty, bilateral small 5 th toenail
118	female	15	Mild sensorineural hearing loss in right ear, moderate to severe conductive hearing loss in left ear, abnormal pinnae, micrognathia, mild developmental delay, mild kyphosis, right accessory lateral incisor, menarche at age 10
119	male	3	Mild sensorineural hearing loss in right ear, moderate mixed hearing loss in left ear, abnormal pinnae, micrognathia, cleft palate, femoral head necrosis, right accessory lateral incisor, bilateral small 5 th toenail