A Human Homeotic Transformation Resulting from Mutations in \( \text{PLCB4} \) and \( \text{GNAI3} \) Causes Auriculocondylar Syndrome

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Auriculocondylar syndrome (ACS) is a rare, autosomal-dominant craniofacial malformation syndrome characterized by variable micrognathia, temporomandibular joint ankylosis, cleft palate, and a characteristic “question-mark” ear malformation. Careful phenotypic characterization of severely affected probands in our cohort suggested the presence of a mandibular patterning defect resulting in a maxillary phenotype (i.e., homeotic transformation). We used exome sequencing of five probands and identified two novel (exclusive to the patient and/or family studied) missense mutations in \( \text{PLCB4} \) and a shared mutation in \( \text{GNAI3} \) in two unrelated probands. In confirmatory studies, three additional novel \( \text{PLCB4} \) mutations were found in multigenerational ACS pedigrees. All mutations were confirmed by Sanger sequencing, were not present in more than 10,000 control chromosomes, and resulted in amino-acid substitutions located in highly conserved protein domains. Additionally, protein-structure modeling demonstrated that all ACS substitutions disrupt the catalytic sites of \( \text{PLCB4} \) and \( \text{GNAI3} \). We suggest that \( \text{PLCB4} \) and \( \text{GNAI3} \) are core signaling molecules of the endothelin-1-distal-less homeobox 5 and 6 (EDN1-DLX5/DLX6) pathway. Functional studies demonstrated a significant reduction in downstream DLX5 and DLX6 expression in ACS cases in assays using cultured osteoblasts from probands and controls. These results support the role of the previously implicated EDN1-DLX5/DLX6 pathway in regulating mandibular specification in other species, which, when disrupted, results in a maxillary phenotype. This work defines the molecular basis of ACS as a homeotic transformation (mandible to maxilla) in humans.

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manuscript. In each case, mandibular ankylosis was progressive, of variable severity, and characterized by inconsistent fusion to the medial and lateral pterygoid plates. All cases demonstrated a similar phenotype, consisting of a lateral mandibular bony prominence with or without TMJ ankylosis, and had features consistent with classic ACS. The anatomic features of these cases led us to hypothesize that the malformations observed in individuals with ACS were due to a homeotic transformation, with the mandible assuming a maxillary phenotype (Figures 2E–2G).

Other reports of mandibular patterning mutations in mice and zebrafish have implicated the endothelin-1 (EDN1) receptor A (EDNRA) and its downstream targets, distal-less homeobox 5 and 6 (DLX5 and DLX6).13–16 Sanger sequencing of endothelin-1 pathway candidates EDNRA, DLX5, and DLX6 in our probands was negative. We performed exome sequencing on all five probands and parents (excluding A001 [no parental DNA available] and S012-P [inadequate sample]) to identify gene-based coding variants present in individuals with ACS. Genomic libraries were prepared and underwent exome capture with the use of a 28 Mb target derived from the consensus coding sequence (CCDS) database (version 20080902)17 or the ~32 Mb target from Roche Nimblegen SeqCap EZ (version 1.0). Exome-enriched libraries were sequenced on Illumina GAIIx or HiSeq 2000 platforms with paired-end 50 or 76 base reads. Each individual generated ~75 million unique reads mapping to the exome target and nearby flanking regions. Over 93% of the exome positions had a coverage depth > 8×, and the average overall coverage depth was 103×. Data from each individual were processed from real-time base calls and aligned to a human reference (hg19) through the use of the Burrows-Wheeler Aligner.18 Data was processed with the use of the Genome Analysis Toolkit (GATK) reference version 1.0.2905, and variant detection and genotyping were performed with the use of the Unified Genotyper (GATK) tool. We flagged variant sites by using the filtration walker (GATK) to mark sites that were of lower quality and more likely to be false positives. Each individual generated an
average of ~22,000 total variants, with a final-pass filter set of ~18,900 variants annotated for variant function (i.e., missense, synonymous, splicing, insertion or deletion [in/del], etc.) (Genome Variation Server).

Given the consistency among the phenotypic features, we first analyzed exome data from all five probands to identify any genes with novel variants shared among them. Probond variant data (i.e., missense, in/dels, and splice acceptor and donor sites) were initially filtered against a smaller database of ~1,200 control exomes drawn from a subset of individuals from the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) to identify novel variants, those passing GATK filtering, and those evident at a robust coverage level (>20x). Second, we performed discrete filtering to identify any gene in which all probands shared novel missense, insertion/deletion, nonsense, or splice variants consistent with either an autosomal-dominant or -recessive model. Under these models and criteria, no genes were detected in common between all probands.

We expanded our search for de novo mutations in probands (S001, S004, and S008), using data from parental individuals along with stringent parameters for GATK pass filters and a sequence depth of >20x in both parents and probands. This identified six putative genes (ASPSCR1, C2orf16, HERC1, PAIP1, PLCB4, and TCHP) within these three individuals. The PLCB4 (MIM 600810) variant c.1868A>G (NM_000933.3; p.Tyr623Cys) had the highest conservation Genomic Evolutionary Rate Profiling (GERP)\textsuperscript{20,21} score (6.17) and was considered the top candidate on the basis of the core signaling role of its ortholog plcb3 in mandibular patterning of zebrafish.\textsuperscript{22,23} Sanger sequencing of parents S002-P and S003-P (kindred B, individuals I-1 and I-2 in Figure S1) and the proband (S001) confirmed it as a de novo mutation (Table 1). On the basis of this finding, we searched the remaining proband exome data for additional PLCB4 variants and identified, in siblings S004 and S005, a second missense mutation at c.986A>C, which changed the amino acid from asparagine to serine (p.Asn329Ser; Table 1).

kindred, the mutation was transmitted from the mildly affected father (S006-P [kindred A, individual I-1 in Figure S1]), a transmission which supports the variable expressivity of ACS.

The discovery of putative coding-sequence, disease-causing PLCB4 mutations in isolated ACS kindreds led us to use Sanger sequencing to screen the gene coding regions containing the conserved catalytic site (exons 11–26) in two additional multigeneration ACS pedigrees\textsuperscript{5} (family 1 [M001] and family 2 [M002] in Figure S2). We identified two additional novel PLCB4 missense mutations at c.1862G>A (M001) and c.1948A>C (M002) (with resultant protein changes, p.Asn650His and p.Arg621His, respectively) that segregated in affected individuals (Table 1 and Figure S2). We have also identified a family with an ACS proband (family 3 [M003], individual IV-1 in Figure S2; with micrognathia, cleft palate, glossoptosis, and a constriction between the helix and the lobe of the left ear) and his “unaffected” father (individual III-1) who presented with asymmetric ear lobes. The family history is significant for four paternal relatives with ear malformations with or without micrognathia (Table 1; family 3 in Figure S2). Sanger sequencing confirmed a PLCB4 missense mutation at c.1862G>A (p.Arg621Cys) in the proband and his unaffected father, the same amino-acid position (621) as noted in family M001 but a different amino-acid change. The phenotypic variability of ACS in these families was confirmed by the variable expressivity and apparent incomplete penetrance associated with the father (S006-P) of a proband with a PLCB4 mutation.\textsuperscript{5} None of the PLCB4 mutations we discovered had been identified previously in a large set of control exomes containing 10,758 chromosomes (Table 1). Each of the mutations led to substitutions at highly conserved nucleotide positions (those with a GERP score > 5.8 make up the top 0.006% of coding nucleotides\textsuperscript{24}) and evolutionary invariant amino-acid positions (Figure 3).

Discrete filtering of exome data from the PLCB4-mutation-negative probands (S008, S011, and A001) revealed a Sanger-confirmed single-missense mutation in an inhibitory...
Figure 3. Conservation and Substitution Modeling of PLCB4 and GNAI3

(A) PLCB4 sequence alignment at the highly conserved catalytic domains. Alignments were performed between human, Drosophila, and fungus protein sequences with the use of ClustalW2. Conservation among vertebrates, invertebrates, and fungi suggests evolutionary importance for these residues. Identified substitutions are depicted in green boxes; residues conserved in vertebrates, invertebrates, and fungus are depicted in yellow; conservative substitutions are in orange; and divergent residues are in white. Protein sequences used for alignment of PLCB4 include Homo sapiens (NP_877949.2), Drosophila (no receptor potential A, isoform D, NP_001014720.1), and Aspergillus clavatus (Gpa2p, NP_010937.1).

(B) Sequence alignment (similar to panel A) of the GNAI3 catalytic region with the p.Gly40Arg ACS substitution. Protein sequences used for alignment of GNAI3 include Human (NP_006487.1), G protein (GNAI3; MIM 139370) in exon 1 (c.118G>C [NM_006496.2; p.Gly40Arg]) shared by individuals S008 and S011 (Table 1). In both kindreds, the p.Gly40Arg variant was inherited; in one case (S011) it was inherited from an affected mother (S012-P), and in the other (S008), from an unaffected father (S009-P), documenting incomplete penetrance. Using the exome data, we confirmed that these individuals did not share a haplotype region encompassing this mutation, and all other measures of global genomic relatedness, determined by analyzing all exome variants or specific high-frequency variants, were consistent with unrelatedness. These data suggest the possibility of a recurrent mutation at this position. The p.Gly40Arg substitution also was not observed in 10,758 control chromosomes, and it occurred at a highly conserved amino-acid residue (and the Grantham score [125] suggested a moderately radical amino-acid replacement 24) within the guanosine diphosphate (GDP)-binding catalytic domain of this G protein (Table 1; Figure 3). Furthermore, GNAI3 maps in the vicinity of the region on chromosome 1 previously linked with the ACS phenotype.6 Given this, we considered the GNAI3 p.Gly40Arg change to be an ACS candidate because of the known role of phospholipase C (PLC) in G protein-coupled receptor signaling, a core component of the endothelin-DLX pathway14 (reviewed in Clauthia et al.25).

To further evaluate the significance of variants in PLCB4 (p.Asn329Ser, p.Arg621His, p.Arg621Cys, p.Tyr623Cys, p.Asn650His) and GNAI3 (p.Gly40Arg), we performed detailed structural protein modeling of specific substitutions found in ACS probands. All missense variants observed in cases, controls, and accessible databases were mapped to PLCB4 and GNAI3 protein structures and visualized with UCSF Chimera.26 None of the variants found in ACS probands. All missense variants observed in cases, controls, and accessible databases were mapped to PLCB4 and GNAI3 protein structures and visualized with UCSF Chimera.26 None of the variants are recognized by RapGapII and would lead to inhibition of the MAPK pathway activation through Rap (Figure 4).
functions. Minimal to positive influence on stability suggesting further study and led us to examine why this large arginine substitution was tolerated in the conserved core of the G-alpha protein. A detailed assessment revealed a cavity large enough for an organic cationic metabolite in each of the three crystal structures (Figures 3E and 3F, semi-transparent green surface) and large enough to hold and electrostatically balance the guanidinium group. Our protein modeling suggested the presence of hydrogen bonds between the Arg40 side-chain nitrogens and (1) the P loop Gly42 backbone carbonyl, (2) the Asp229 side chain, and (3) the Ser246 side chain (Figure 3F, pink lines). Modeling the p.Gly40Arg variant suggests stabilization of GNAI3 in the active conformation (switch III). Examination of potential interactions with the downstream signaling partner RapGAP II through analysis of the common structural fold between Rap and GNAI3 revealed close recognition of the constitutively activated switch III. Thus, modeling the GNAI3 p.Gly40Arg substitution suggests a gain of function, with stabilization of the active conformation recognized by RapGAP II triggering inhibition of the mitogen-activated protein kinase (MAPK) pathway activation through Rap.

As a proxy for assessing the effects of the substitution on GNAI3 activity, we chose to assay the expression of two known downstream targets of the G protein-coupled endothenin receptor pathway, DLX5 and DLX6, which play a key role in mandibular patterning. The importance of PLC and G protein signaling in this pathway led us to hypothesize that a potential dominant-negative effect of PLCB4 mutations and a gain-of-function GNAI3 mutation would lead to downregulation of DLX5 and DLX6. We measured gene expression levels for DLX5 and DLX6 in available cultured mandibular osteoblasts from our two GNAI3 probands (p.Gly40Arg), one PLCB4 proband (p.Try623Cys), and one unresolved ACS proband (A001). Using quantitative, real-time RT-PCR of steady-state mRNA, we identified a 6-fold reduction of DLX5 (p < 0.005; probands versus controls) and an 8-fold reduction of DLX6 expression (p < 0.001) in mandibular osteoblasts from selective probands relative to 17 randomly selected calvarial osteoblasts from controls. Preliminary RNA sequencing experiments showed similar and relatively strong levels of expression of PLCB4 and GNAI3 in control calvarial osteoblasts (data not shown), and at levels 2–4 fold greater than DLX5 and DLX6. These data support a final common pathway leading to DLX5/6 regulation through suppression of the endothelin pathway as a unifying cause. These data also provide a basis for examining other candidate genes with similar functional effects in our single unresolvable case (A001), negative for mutations in PLCB4 and GNAI3. To do this we looked at our initial exome results for A001 to identify singleton exon sequence variants in candidate genes from the PLC gene family (PLCD1, PLCD1, PLCH1), PLC pathway candidate genes (PL4KA, DOCK1, DOCK6, MIOX, HRASLS, RAC1), or known endothelin-DLX5/6 pathway genes (PBMER, IGF2BP2, WNT7A). Although sequence variants were detected in five candidate genes (from the list above), upon the application of information from our large control set, only DOCK1 and DOCK6 sequence variants were not present in controls (Table S1). As members of the DOCK-C subfamily of the DOCK family of guanine nucleotide exchange factors, both genes have a suggestive function as an activator of small G proteins. Although we are hesitant to draw conclusions from a single proband without parental data, it is intriguing to have identified an exceptionally rare mutation in a G protein activator in a disorder known to be caused by disruption of signaling through a G protein-dependent signaling pathway. Further functional studies will be required to test the putative role of these DOCK-related genes in the endothelin-DLX5/DLX6 pathway and mandibular patterning.

Our primary observation of a de novo mutation in PLCB4 led us to identify other mutations in a second kindred and in three confirmatory pedigrees. Further analysis of PLCB4-negative probands identified a second (GNAI3) mutation in two probands and a DOCK6 sequence variant in another proband, indicating a role for endothelin-1 pathway genes in this heterogeneous condition. In each of the families with transmission of the PLCB4 or GNAI3 mutations, we observed incomplete penetrance and/or variable expressivity of the ACS phenotype. Although variable expression is common among dominant craniofacial disorders, the nature of phenotypic variability in ACS suggests that common craniofacial disorders such as micrognathia, Pierre Robin sequence (MIM 261800), and microtia may also be caused by mutations in this pathway.

The interpretation of our data was supported by knowledge of classic developmental model systems. In zebrafish, mutations in edn1 result in a reduction in lower jaw size and fusion to dorsal structures with loss of the intervening joints. Mutations of schmerle (she), the zebrafish ortholog of PLCB3 (and ortholog of PLCB4), result in a near phenocopy of edn1 mutations, with reduction of the ventral facial skeleton and fusion to dorsal skeletal elements. Two she alleles have been identified in the highly conserved catalytic domains of plcb3, the same...
domains in which we have defined substitutions in PLCB4. she is required for edn1-dependent dx5a, dx6a, and dx3a expression. Furthermore, when plcb3^{-/-} neural crest cells were transplanted into wild-type embryos, they contributed to dorsal, but not ventral or intermediate, jaw structures, suggesting that plcb3 may be necessary for the determination of ventral branchial arch fates and functions downstream of edn1. In another animal model, knockdown of PLCB3 in Xenopus results in reduction of cartilage in the first pharyngeal arch and loss of the jaw joint, in a proportion of treated embryos. The lack of a craniofacial phenotype in the Plcb4 knockout mouse suggests that the catalytic domain substitutions identified in our cases and in zebrafish schmerle may result in dominant negative effects. A similar mechanism has been suggested for a splice variant affecting the catalytic domain of PLCD4, which has a dominant negative effect on the function of other wild-type PLC enzymes. Although studies to confirm this hypothesis are necessary, these data would suggest that the loss of PLC catalytic function, in the absence of effects on protein stability, may confer dominant negative effects.

The observed conversion from a mandibular to a maxillary phenotype, as we have described for ACS, represents a homeotic transformation and is supported by several mouse knockout models (Ednra^{15,16,26} and Edn1^{27} annotated in Figure 4). Mice with targeted mutations of endothelin-converting enzyme 1 (Ece-1), Edn1, Ednra, and G protein alpha subunits (G_{q2}, G_{z13}) each exhibit a reduction in ventral jaw size and fusion to dorsal skeletal components. Similarly, mice lacking expression of Dlx5 and Dlx6 demonstrate transformation of the lower jaw into an upper jaw phenotype.^{13,15,16,25} This homeotic transformation was the first described in any gene other than the classic Hox genes. Furthermore, selective reduction of another member of the endothelin-Dlx5/Dlx6 pathway, Hand2, in the cranial neural crest results in duplication of palatine bones in the mouse mandible and aglossia. These findings support a role for Edn1-Dlx5/Dlx6 as a prototypical signaling pathway (Figure 4) in cranial neural crest cell proliferation in the first branchial arch that is necessary for mandibular specification. As described above, this pathway is further supported by our quantitative real-time PCR analysis in osteoblasts from four ACS cases, which demonstrates a significant reduction in the downstream expression of DLX5 and DLX6 (Figure S3).

We report the identification of extremely rare, highly conserved mutations (both de novo and inherited) in two endothelin pathway signaling enzymes, PLCB4 and GNAI3, as the molecular causes of ACS and further define the role of the endothelin-distal-less pathway in the specification and patterning of the lower jaw of vertebrates. The ACS phenotype is consistent with the transformation of the mandible to a maxillary identity and constitutes a molecularly defined homeotic transformation in humans. The phenotypic variability of ACS suggests that mutations in this pathway, especially those affecting core signaling molecules such as PLCB4 and GNAI3, should be considered as potential candidates for other ear and jaw malformations. The identification of phenotypic variability and incomplete penetrance underscores the utility of these results in genetic counseling of families affected by ACS.

**Supplemental Data**

Supplemental Data include three figures and one table and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources
The URLs for data presented herein are as follows:
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
UCSC Genome Browser, http://genome.ucsc.edu/
UCSF Chimera, http://www.cgl.ucsf.edu/chimera/

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