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A Human Homeotic Transformation Resulting from Mutations in PLCB4 and GNAI3 Causes Auriculocondylar Syndrome

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Abstract:	Auriculocondylar syndrome is an autosomal dominant craniofacial malformation syndrome characterized by variable mandible undergrowth, immobility of the temporomandibular joint, cleft palate, and a characteristic "question mark" ear. We used exome sequencing of three probands and identified three novel missense mutations in core signaling molecules PLCB4 and GNAI3. Two additional PLCB4 mutations were found in multigenerational ACS pedigrees. Protein modeling demonstrates that all ACS mutations disrupt the catalytic sites of these proteins. These results confirm the role of the endothelin-1 (EDN1)/DLX5/6 pathway in regulating mandibular specification. ACS is the first molecularly defined homeotic transformation (mandible to

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A human homeotic transformation resulting from mutations in *PLCB4* and *GNAI3* causes auriculocondylar syndrome

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SUMMARY

Auriculocondylar syndrome is an autosomal dominant craniofacial malformation syndrome characterized by variable mandible undergrowth, immobility of the temporomandibular joint, cleft palate, and a characteristic "question mark" ear. We used exome sequencing of three probands and identified three novel missense mutations in core signaling molecules *PLCB4* and *GNAI3*. Two additional *PLCB4* mutations were found in multigenerational ACS pedigrees. Protein modeling demonstrates that all ACS mutations disrupt the catalytic sites of these proteins. These results confirm the role of the endothelin-1 (EDN1)/DLX5/6 pathway in regulating mandibular specification. ACS is the first molecularly defined homeotic transformation (mandible to maxilla) in humans.

Auriculocondylar syndrome (ACS; *OMIM* 602483; also known as 'question mark ear syndrome' or 'dysgnathia complex') is an autosomal dominant craniofacial malformation syndrome characterized by highly variable mandibular anomalies, including a small and malformed lower jaw often with fusion of the temporomandibular joint, cleft palate, and a distinctive ear malformation consisting of separation of the lobule from the external ear giving the appearance of a question mark. Other frequently described features include prominent cheeks, cupped and posteriorly rotated ears, preauricular tags, and a small mouth. ACS was first described in a mother and her two affected children just over thirty years ago[1], and to date nine well characterized families with multiple affected individuals [2-8] as well as several simplex cases[4,9-11] have been reported.

CASE REPORTS

Our index case (S011) and her affected mother (S012P; see Fig. 1 in Supplementary Appendix) had severe mandibular abnormalities[3]. Specifically, S011 had an unusual congenital mandibular ankylosis with lateral fusion of the mandible to the temporozygomatic suture and medial fusion to the skull base (medial and lateral pterygoids) (Fig. 1A and 1B). Mandibular immobility required that intraoral exam was performed under anesthesia and demonstrated severe reduction in tongue size, elongate soft tissue masses attached at the

posterior floor of the mouth and soft tissue shelves protruding from the medial alveolus of the lower jaw, giving the appearance of a "mandibular palate" (Fig. 1C and 1D).

We previously identified two additional kindreds with dysgnathia complex (ACS)[3], including two simplex case child-parent trios (S001, S008) and a family with two affected siblings (S004 [proband] and S005) whose father had mild mandibular hypoplasia (Fig. 1 in Supplementary Appendix). In each case mandibular fusion was progressive, of variable severity, and characterized by inconsistent fusion to the medial and lateral pterygoids. All cases demonstrated a similar phenotype with a lateral mandibular boney prominence with or without ankylosis (Fig. 1A and 1B and Fig. 2C and 2D) and had features consistent with classic ACS. Screening of these patients for endothelin 1 pathway candidates EDN1, DLX5 and DLX6 were negative (data not shown). Furthermore, the anatomic features of these cases led us to hypothesize that the malformations observed in ACS patients were due to a homeotic transformation with the mandible assuming a maxillary phenotype (Fig. 1G). For additional confirmation of the molecular basis of ACS, we also identified two large, multi-generational pedigrees with classic features of ACS[5] (Fig. 2 in Supplementary Appendix).

METHODS

Clinical Information

This study was approved by Seattle Children's Hospital Institutional Review Board. Written consent was obtained for all participants. Photographic consent was obtained for each participant with identifiable images used in publication. The study is HIPAA compliant.

Exome Sequencing.

Genomic DNA (3.5 micrograms) was extracted from osteoblast cell lines or blood and underwent capture to enrich for the exome (ie. exon coding regions) using a 28 Mb[12] or ~32 Mb target. Massively parallel second-generation sequencing was performed on exome libraries (for full methods see also the Supplementary Appendix) in all probands and parents to identify variants associated with ACS.

Mutation Identification.

Proband and parental DNA variant data (i.e. missense, insertion/deletions and splice acceptor and donor site) were filtered against a database of ~1200 control exomes to identify novel (ie. no occurrences), high quality variants. These genes (and variants) were considered as ACS candidates (additional methodological details are available in the Supplementary Appendix).

Protein modeling of missense mutations on protein structure and function.

All missense mutations observed in cases, controls, and accessible databases were mapped to *PLCB4* and *GNAI3* protein structures and assessed for structural changes, ligand binding and molecular bond profiles (details of analysis are found in the Supplementary Appendix).

RESULTS

Due to the consistency of the phenotypic features, we performed exome sequencing of all four probands to identify genes with novel variants shared among them (see details in the Supplementary Appendix). Discrete filtering for a gene in which all probands shared novel missense, insertion/deletions, nonsense, or splice variants consistent with either an autosomal dominant or recessive model of inheritance failed. We then analyzed the proband and parental variant data and identified a novel *de novo* mutation (*PLCB4* variant at genome position chr20:9389733) leading to a tyrosine to cysteine changes at amino acid position 623 (p.Tyr623Cys) in a proband (S001; Fig. 2 and Table 1 in the Supplementary Appendix), that was subsequently confirmed by Sanger sequencing. Based on this finding, we searched the proband exome data for additional *PLCB4* variants and identified a second missense mutation at genome position chr20:9364980 in sibling samples S004 and S005 changing the amino acid at position 329 to serine from asparagine (p.Asn329Ser; Table 1 in Supplementary Appendix) that was transmitted from their mildly affected father (S006-P; Fig. 1 in the Supplementary Appendix); the latter confirming the variable expressivity of ACS. *PLCB4* was the only gene that shared different novel mutations between multiple probands.

The discovery of disease-causing mutations in *PLCB4* 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 multi-generation ACS pedigrees[5] (Fig. 2 in the Supplementary Appendix). This led to identification of two additional novel *PLCB4* mutations (p.Arg621His [M001]; p.Asn650His [M002]) that segregated in affected individuals (Table 1 and Fig. 2 in the Supplementary Appendix). None of the *PLCB4* mutations we discovered had been identified previously in a large set of control exomes containing more than 4600 chromosomes, (Table 1 in the Supplementary Appendix and methods). Each of the mutations led to substitutions at highly conserved nucleotide positions (GERP score >5) in evolutionary invariant amino acid positions (Fig. 3 in the Supplementary Appendix) localized within the *PLCB4* catalytic, substrate binding domain (Fig. 2G and 2H) and were presumed to inhibit function.

Discrete filtering of exome data from the probands (S008 and S011) without *PLCB4* mutations revealed a single mutation in an inhibitory G protein (*GNAI3*) [p.Gly040Arg] shared by two unrelated probands S008 and S011 (Fig. 1 and Table 1 in the Supplementary Appendix). This p.Gly040Arg mutation was not observed in 4686 control chromosomes and occurred at a highly conserved amino acid residue within the GDP-binding catalytic domain of this G protein (Fig. 3 in the Supplementary Appendix). Protein modeling of this mutation suggests a gain of function with stabilization of the active conformation recognized by RapGapII, triggering inhibition of the MAPK pathway activation through Rap (Fig. 2H and in Fig. 3). Furthermore, *GNAI3* maps to the region on chromosome 1 previously linked with the ACS phenotype[6]. All mutations identified in *PLCB4* and *GNAI3* were confirmed by Sanger sequencing.

DISCUSSION

The observed mandibular to maxillary phenotype, as we have described for ACS (Fig. 1), represents a homeotic transformation and is supported by several vertebrate models. Mice with targeted mutations of endothelin converting enzyme 1 (*Ece-1*), endothelin-1 (*Edn1*), endothelin receptor a (*Ednra*), and G protein alpha subunits ($G\alpha_q$, $G\alpha_{11}$) each exhibit a reduction in ventral jaw size and fusion to dorsal skeletal components [13-15]. Similarly, mice lacking expression of *Dlx5* and *Dlx6* demonstrate transformation of the lower jaw into an upper jaw phenotype[15-18]. This homeotic transformation was the first described in any gene other than the classic Hox genes[18]. Furthermore, selective reduction of another member of the endothelin-Dlx5/6 pathway

(Hand2) in the cranial neural crest results in duplication of palatine bones in the mouse mandible and reduction in tongue size [19]. These findings suggest a role for Edn1-Dlx5/Dlx6 as a prototypical signaling pathway (Fig. 3) in cranial neural crest cell colonization of the first branchial arch that is necessary for mandibular specification. This is further supported by qRT-PCR analysis in calvarial osteoblast cells from an ACS case [S001] demonstrating a reduction in the downstream expression of *DLX5/6*. (Fig. 4 in the Supplementary Appendix).

In zebrafish, mutations in *Edn1* result in a reduction in lower jaw size and fusion to dorsal structures with loss of the intervening joints[17,20]. Mutations of schmerle (*she*), the zebrafish ortholog of *plcb3* (and human *PLCB4)*, result in a near phenocopy of *edn1* mutations with reduction of the ventral facial skeleton and fusion to dorsal skeletal elements[14]

. Two *she* alleles have been identified in the highly conserved catalytic domains of *plcb3* in the same regions in which we have defined mutations in the human ortholog *PLCB4*. The *she* gene is required for *edn1* dependent *dlx5a*, *dlx6a*, and *dlx3a* expression. Furthermore, transplantation of *plcb3*$^{-/-}$ neural crest cells into wild type embryos populated dorsal, but not ventral, jaw structures suggesting that *plcb3* is critical for the determination of ventral branchial arch fates and functions downstream of *edn1*.

In conclusion, we have identified novel mutations in two endothelin pathway signaling enzymes, *PLCB4* and *GNAI3,* as the molecular causes of ACS and further define the role of the endothelin-distalless pathway in the specification and patterning of the lower jaw of vertebrates (Fig. 3). The ACS phenotype is consistent with the mandible assuming a maxillary identity and to our knowledge represents the first example of a molecularly defined homeotic transformation in humans. The phenotypic variability of ACS suggests that additional novel mutations in endothelin-distalless pathway, especially those defining 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.

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AUTHOR CONTRIBUTIONS

MLC, AVH, GEG, CMC, JMJ recruited and characterized patients and performed initial genetic characterizations. SSP, SBE, BDS, carried out experimental methods, MJR, JDS, MLC, AVH performed gene filtering screening and analysis; JAH analyzed protein structure and function of mutations, MJB, DAN provided

critical review, oversight and resources. TCC contributed input on comparative craniofacial anatomy and candidate gene analysis. MJR and MLC wrote the paper.

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Figure Legends

Figure 1. *PLCB4* and *GNAI3* mutations cause a mandibular to maxillary homeotic transformation.

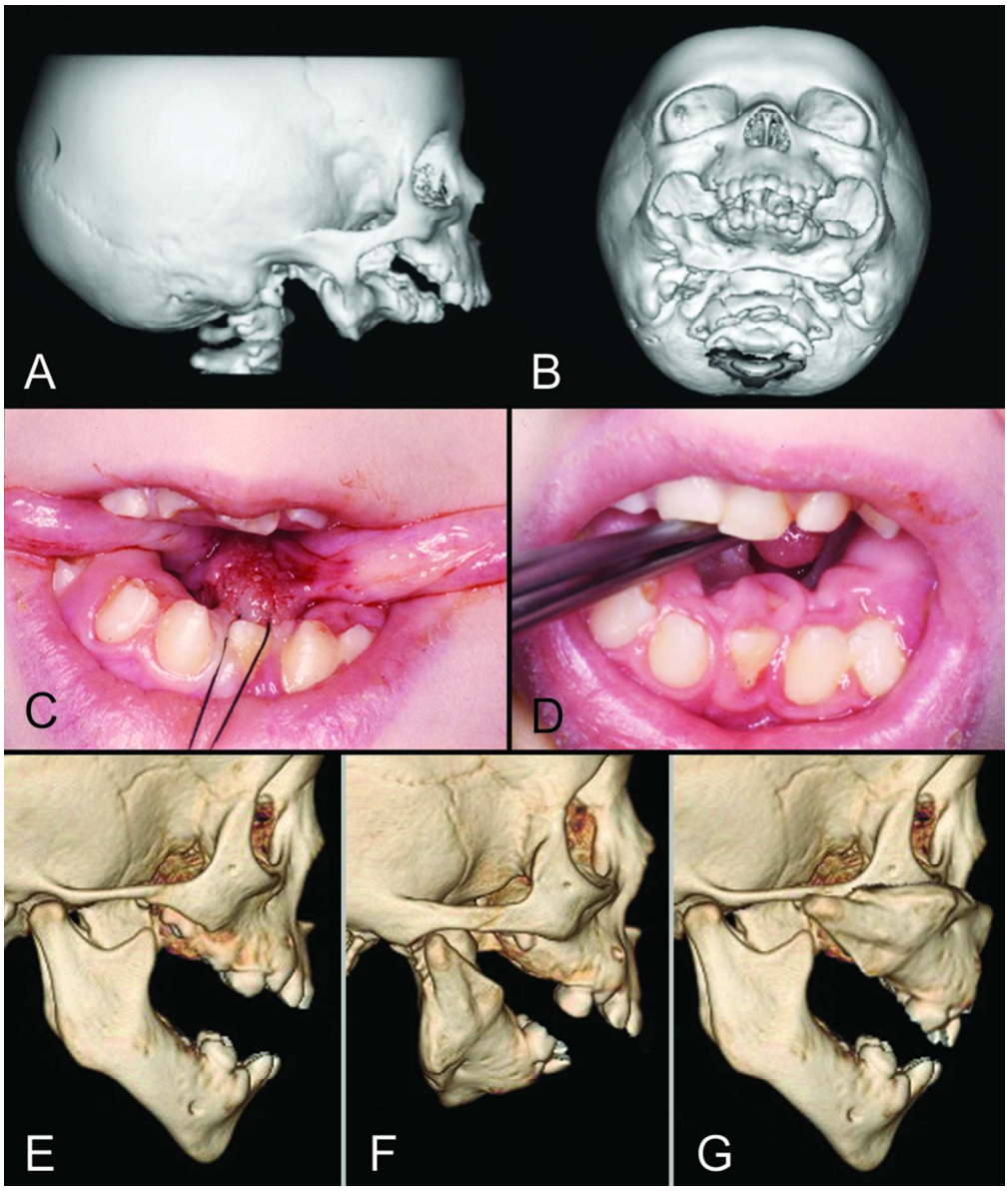
Panel A is a 3D CT scan image of case S011 (*GNAI3* p.Gly040Arg) who demonstrates severe mandibular hypoplasia with fusion of the lateral mandibular process to the temporozygomatic suture. Panel B shows the dorsoventral symmetry of case S011. Panel C depicts the reduction in tongue size and lateral soft tissue projections at the tongue base seen intraoperatively. Panel D demonstrates that removal of the aberrant soft tissue revealed medial "shelves" reminiscent of a palate. Panel E is a control CT image of an age and sex-matched patient showing normal mandible anatomy. Panel F is a CT image of a patient S001 with a *PLCB4* p.Tyr623Cys mutation. Panel G is a composite CT image generated with photo editing software in which the mandible from Panel F was copied, inverted, and placed over the control maxilla in Panel E. The resultant image in Panel G depicts the remarkable alignment of the ACS mandible with the control maxilla, zygomatic arch and midfacial structures, demonstrating transformation to a maxillary identity (a homeotic transformation).

Figure 2. ACS phenotypes and protein modeling of mutations.

Panel A shows a facial photos of proband S001 (*PLCB4* p.Tyr623Cys). Panel B demonstrates a facial photo of proband S008 (*GNAI3* p.Gly040Arg) with severe micrognathia, mandibular dysplasia and the classic "question mark" ear. Panel C and D are CT images of proband S001 and S008, respectively. Panel E shows all four *PLCB4* missense mutations (red) that contribute directly to catabolic release of inositol triphosphate (gray). Panel E' shows a magnification of the active site of PLCB4 and demonstrates that variant residues disrupt catalytic function without destabilizing protein folding, presumed inhibiting MAPK signaling without affecting other protein interactions. Panel F shows the *GNAI3* p.Gly40Arg mutant (red) residue. Panel F' is a

magnification showing that the side chain extends across an empty cavity (transparent green) to form novel hydrogen bonds (pink lines) with asparagine-229 and serine-246, stabilizing the active conformation of switch III (black) recognized by RapGapII and leading to inhibition of the MAPK pathway activation through Rap. Other missense variants observed in controls for these proteins are shown as white spheres.

Figure 3. Homeotic transformation of jaws associated with mutations in the endothelin-DLX5/6 pathway. Several reports [14-19,21] support the premise that mutations in the endothelin pathway cause homeotic transformation of the lower (ventral) jaw to a maxillary (dorsal) identity. Mice with mutations in endothelin 1 (*edn1*), the endothelin receptor A (*Ednra*), the G protein α_q subunit, Dlx5/Dlx6, and most recently *Hand2*[19], each result in a small ventral jaw with dorsal jaw features. Mutations of in zebrafish *plcb3* lead to the same homeotic transformation. In this figure, cartoons of fish, mice and humans indicate gene family members in this pathway which, when mutated, result in a homeotic transformation of the dorsal and ventral jaws. Figure adapted from ref. [22].



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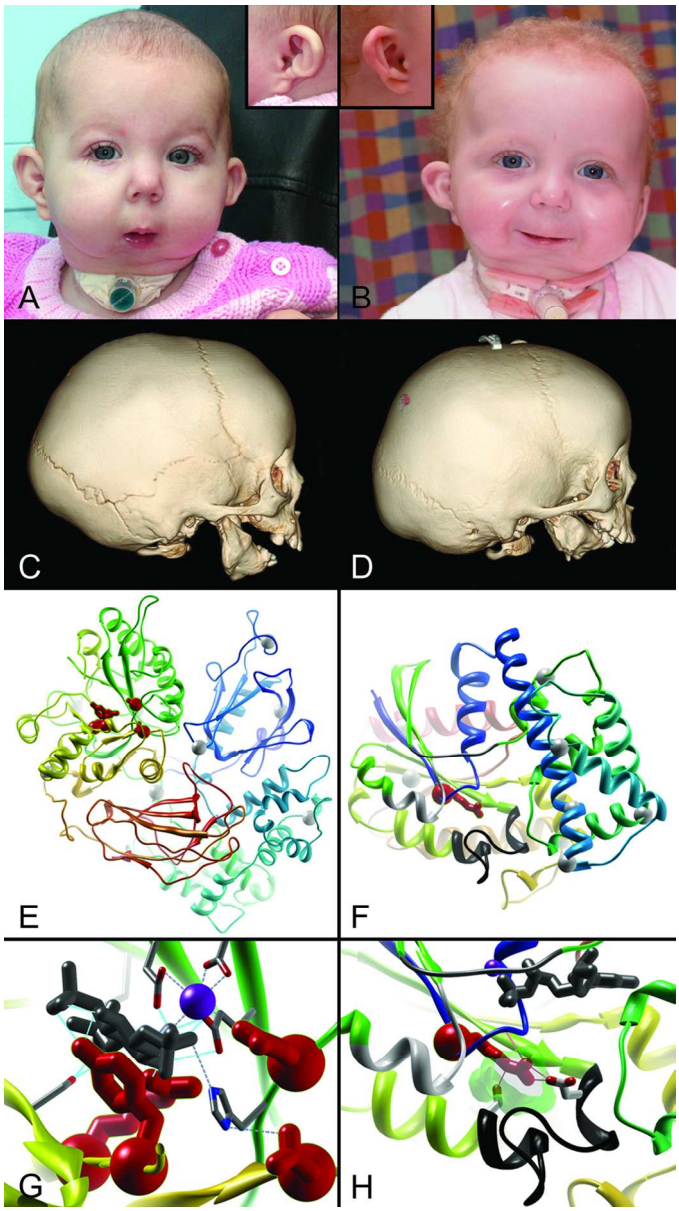

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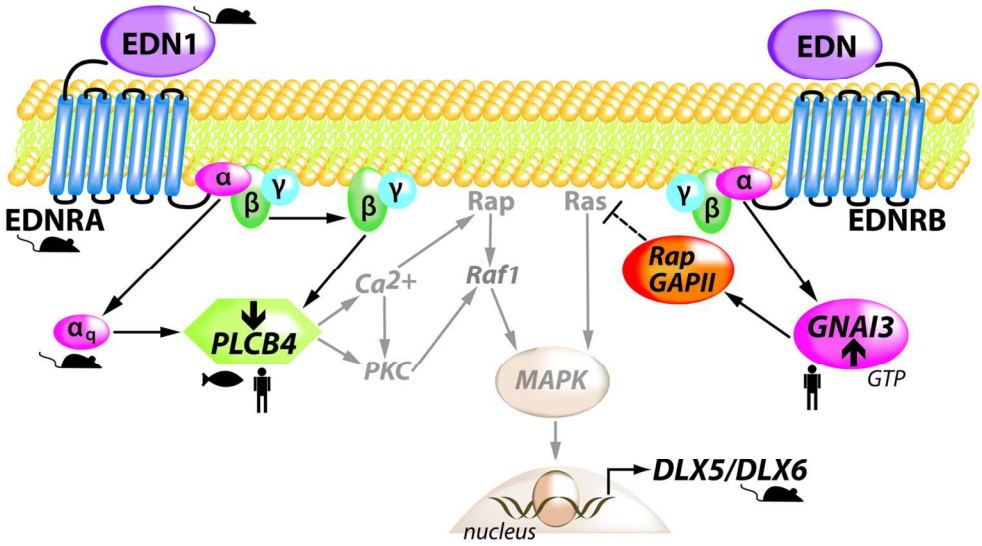

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