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Dominant-negative $G\alpha$ subunits are a mechanism of dysregulated heterotrimeric G protein signaling in human disease

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Abstract

Auriculo-Condylar Syndrome (ACS), a rare condition that impairs craniofacial development, is caused by mutations in a G protein-coupled receptor (GPCR) signaling pathway. In mice, disruption of signaling by the endothelin type A receptor (ET_AR), which is mediated by the G protein subunit $G\alpha_{q/11}$ and subsequently phospholipase C (PLC), impairs neural crest cell differentiation that is required for normal craniofacial development. Some ACS patients have mutations in *GNAI3*, which encodes $G\alpha_{i3}$, but it is unknown whether this G protein has a role within the ET_AR pathway. Here, we used a *Xenopus* model of vertebrate development, in vitro biochemistry, and biosensors of G protein activity in mammalian cells to systematically characterize the phenotype and function of all known ACS-associated $G\alpha_{i3}$ mutants. We found that ACS-associated mutations in *GNAI3* produce dominant-negative $G\alpha_{i3}$ mutant proteins that couple to ET_AR but cannot bind and hydrolyze guanosine triphosphate, resulting in the prevention of endothelin-mediated activation of $G\alpha_{q/11}$ and PLC. Thus, ACS is caused by functionally dominant-negative mutations in a heterotrimeric G protein subunit.

INTRODUCTION

Heterotrimeric G proteins are gate-keepers of signal transduction that play critical roles in physiology. They cycle between inactive [guanosine diphosphate (GDP)-bound] and active [guanosine triphosphate (GTP)-bound] states to control the flow of information from extracellular cues to intracellular effectors (1–4). Resting heterotrimeric G proteins are composed of a GDP-bound $G\alpha$ subunit in complex with $G\beta\gamma$. G protein activation is predominantly carried out by G protein-coupled receptors (GPCRs), which promote the exchange of GDP for GTP on $G\alpha$ (1, 3). This leads to the dissociation of the heterotrimer

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into G α -GTP and free G $\beta\gamma$, both of which act on downstream effector molecules. G α subunits are classified into 4 major families, G α_s , G $\alpha_{i/o}$, G $\alpha_{q/11}$ and G $\alpha_{12/13}$, which are characterized by their ability to modulate different effector molecules and second messengers. For example, G α_i subunits dampen the production of cyclic adenosine monophosphate (cAMP) by inhibiting adenylyl cyclase, whereas G α_q subunits increase intracellular Ca²⁺ by activating phospholipase C (PLC). The evidence that the dysregulation of heterotrimeric G protein signaling causes various diseases is mounting rapidly, but the underlying molecular mechanisms in many of these diseases are elusive. The majority of G protein-associated mutations have been identified in genes encoding G α subunits (5, 6) and are broadly classified as loss-of-function or gain-of-function mutations. Loss-of-function mutations in various G α proteins are associated with the congenital diseases Albright's Hereditary Osteodystrophy (G α_s), Nougaret night blindness (G α_r) and two types of dystonia (G α_{olf}) (5, 7–10). On the other hand, gain-of-function mutations are somatic and exert a dominant effect by rendering the G α subunit constitutively active. The most frequent alteration associated with gain-of-function mutations is GTPase-deficiency, as seen in aberrant G α_s , G α_q and G α_{11} in up to 80% of the patients with some types of cancer (6).

Auriculo-Condylar Syndrome (ACS) (11–13) is a rare condition that impairs craniofacial development. Evidence from genetic studies in humans and animal models indicates that ACS is caused by disruption of an endothelin type A receptor (ET_AR) → G α → PLC pathway that induces the expression of genes encoding the distalless homeobox (DLX) transcription factors DLX5 and DLX6 required for specification and patterning of neural crest cells during craniofacial development (14). In humans, this hypothesis is supported by the identification of mutations in the genes encoding for the natural ET_AR ligand endothelin-1 (15) and the G $\alpha_{q/11}$ effector PLC β 4 (11, 12). This is in agreement with evidence in mice showing that knockout animals lacking G α_q and its close homolog G α_{11} display craniofacial defects that resemble ACS (16, 17). However, this model contrasts with the genetic evidence in humans; no G α_q or G α_{11} mutations have been found in ACS patients to date. Instead, some ACS patients have mutations in another G α subunit, G α_{i3} . Whether G α_{i3} functions between ET_AR and PLC β 4 and the mechanism of action by which G α_{i3} mutations affect this pathway are not yet known.

ACS is classified as type I, II or III based on the presence of mutations in *GNAI3* (encoding G α_{i3}), *PLCB4* (encoding PLC β 4) or *EDN1* (encoding endothelin-1), respectively. Five autosomal dominant mutations in G α_{i3} have been found in type I ACS (11–13). All five mutations affect conserved amino acid positions that cluster within the nucleotide binding pocket (Fig. 1 and fig. S1). It has been speculated that ACS-associated G α_{i3} mutants may behave as dominant-negatives (meaning it interferes with the function of the normal gene product) or as constitutively active G proteins (11, 13, 14). However, none of these hypotheses or the possibility of acting as loss-of-function mutants leading to haploinsufficiency have been formally tested. Here we investigated the molecular basis of type I ACS by systematically characterizing the functional consequences of the five G α_{i3} mutations found in ACS patients using *in vivo* and *in vitro* approaches.

RESULTS

ACS mutations increase the frequency of developmental defects induced by the ectopic expression of *Gai3* in *Xenopus laevis* embryos

As a first approach to investigate the functional consequences of ACS mutations in $G\alpha_{i3}$, we performed experiments with *Xenopus laevis* embryos as a development-relevant model. For this, we took advantage of previous observations (18) showing that ectopic expression of $G\alpha_{i1}$ induces developmental defects in *Xenopus laevis*. During normal gastrulation, embryo cell movements cause the appearance of a new hollow cavity, the archenteron, while simultaneously inducing the progressive removal of another, the blastocoel. In $G\alpha_{i1}$ -injected embryos, this process is disrupted: the archenteron does not inflate and the blastocoel is not removed (18). The abnormal distribution of the internal cavities alters the flotation of the embryo and causes an inversion of its normal gravitational orientation (Fig. 2A). This phenotype can be easily scored at the neurula stage because the neural tube faces downward instead of upward. Although it is not known what specific signaling pathways are disrupted by the ectopic expression of $G\alpha_i$, this system provides a suitable platform to test whether G protein mutants induce a gain or loss of function in the context of embryonic development. First, we validated that ectopic expression of $G\alpha_{i3}$ caused the same defects as those previously observed for $G\alpha_{i1}$ (Fig. 2A). We reasoned that if the ACS-associated mutations induce loss of function in $G\alpha_{i3}$, their expression will not lead to a $G\alpha_{i3}$ overexpression phenotype. We found that this is not the case because the ACS-associated $G\alpha_{i3}$ mutants exerted the opposite effect; expression of any of the five mutants (G40R, G45V, S47R, T48N and N269Y) increased the frequency of gravitational inversion in embryos compared to those expressing wild-type $G\alpha_{i3}$ ($G\alpha_{i3}$ WT); this was observed at two different injected doses of mRNA (Fig. 2, B and C). Embryo bisections revealed that the gravitational inversion in ACS-associated $G\alpha_{i3}$ mutants (of which S47R is shown as a representative in Fig. 2B) was accompanied by defective archenteron inflation and incomplete blastocoel removal, indicating that the underlying cause of the phenotype is the same as in $G\alpha_{i3}$ WT-injected embryos. We ruled out that different abundance of the $G\alpha_{i3}$ mutant proteins compared to that of $G\alpha_{i3}$ WT caused the difference in phenotype frequency by confirming their equal quantities in immunoblots of embryo lysates (Fig. 2C). Together, these results demonstrate that ACS mutations increase the frequency of developmental defects caused by ectopic expression of $G\alpha_{i3}$, indicating that the mutations provide $G\alpha_{i3}$ with dominant properties rather than inducing a loss of function.

ACS-associated $G\alpha_{i3}$ mutants display defective GTP binding

To further characterize the functional consequences of the ACS mutations in $G\alpha_{i3}$, we investigated their biochemical properties *in vitro*. We obtained very poor or no yields of soluble proteins when we attempted to purify $G\alpha_{i3}$ G40R, G45V, T48N or N269Y from *E. coli*, suggesting that the mutations compromise G protein stability. Only $G\alpha_{i3}$ S47R was purified in sufficient quantities for biochemical studies. The ability of $G\alpha_{i3}$ S47R to bind nucleotides was investigated by using a well-established assay that monitors G protein activation upon nucleotide binding by measuring its susceptibility to limited trypsinolysis. Briefly, inactive, GDP-bound G α is readily digested by trypsin whereas active G α , generated by the binding of GTP mimetics GTP γ S or GDP-AlF $_4^-$, adopts a conformation that is

resistant to trypsin digestion outside of a short N-terminal sequence which is cleaved off by trypsin (19). We found that $G\alpha_{i3}$ S47R protection from trypsinolysis after incubation with $GTP\gamma S$ or $GDP\text{-}AlF_4^-$ was dramatically reduced compared to that of $G\alpha_{i3}$ WT (Fig. 3A), indicating that the mutant cannot bind nucleotides and/or it cannot change conformation efficiently upon GTP binding. Next we investigated whether the S47R mutation affects the steady-state GTPase activity of $G\alpha_{i3}$ and found that it was virtually abolished (Fig. 3B). The cause of this defect was failure to bind GTP, as determined by two independent approaches that measure binding of the non-hydrolysable analogue $GTP\gamma S$ (Fig. 3, C and D). To elucidate whether the impaired activation of $G\alpha_{i3}$ S47R was due to a defect specific to GTP binding or to nucleotide binding in general, we analyzed its nucleotide content by high-performance liquid chromatography (HPLC). $G\alpha_{i3}$ proteins were exchanged into nucleotide-free buffer and rapidly denatured to release any bound nucleotide. The HPLC analysis revealed identical chromatograms for $G\alpha_{i3}$ WT and $G\alpha_{i3}$ S47R corresponding to GDP peaks of identical intensity (Fig. 3E). Together, these results demonstrate that $G\alpha_{i3}$ S47R retains the capability to bind GDP but fails to bind GTP, rendering the G protein unable to switch into an active conformation.

Next, we investigated whether the GTP-binding defect of $G\alpha_{i3}$ S47R is a common feature among all the ACS mutants. Because $G\alpha_{i3}$ G40R, G45V, T48N and N269Y mutants could not be purified from bacteria, we measured susceptibility to limited proteolysis after expression in mammalian cells. In initial experiments, we found that all the ACS mutant plasmids expressed lower protein quantities than $G\alpha_{i3}$ WT in human embryonic kidney 293T (HEK293T) cells. To overcome this limitation and be able to make comparisons, we equalized the amount of protein expression by transfecting larger quantities of the corresponding ACS mutant plasmids (2–4 times more than that of the WT). We found that protection from trypsinolysis after $GTP\gamma S$ incubation was reduced for all five ACS-associated $G\alpha_{i3}$ mutants (G40R, G45V, S47R, T48N, N269Y) compared to that for $G\alpha_{i3}$ WT (Fig. 3F). These findings indicate that defective GTP binding and G protein activation is a common feature of ACS-associated $G\alpha_{i3}$ mutants.

ACS-associated $G\alpha_{i3}$ mutants cannot be activated by a GPCR

Our *in vitro* data indicates that ACS mutations abolish the spontaneous exchange of GDP for GTP on $G\alpha_{i3}$. Therefore, we investigated whether they also affect GPCR-catalyzed $G\alpha_{i3}$ activation. GPCRs are guanine nucleotide exchange factors (GEFs) that accelerate the exchange of GDP for GTP, which in turn dissociates $G\alpha\beta\gamma$ trimers into $G\alpha\text{-GTP}$ and $G\beta\gamma$. Because association of $G\alpha$ with $G\beta\gamma$ is an obligatory requirement for GPCR-catalyzed activation, we first examined whether ACS-associated $G\alpha_{i3}$ mutants bind to $G\beta\gamma$ in cells. For this, we used a cell-based bioluminescence resonance energy transfer (BRET) system that monitors the association of free Venus- $G\beta\gamma$ (BRET acceptor) with the C-terminal fragment of its effector, G protein-coupled receptor kinase 3 (GRK3), fused to a modified luciferase (masGRK3ct-Nluc, BRET donor) (20, 21). In the absence of $G\alpha$, free $G\beta\gamma$ associates robustly with the GRK3 probe leading to high BRET signals, whereas expression of $G\alpha$ subunits favors the formation of $G\alpha\text{:}G\beta\gamma$ complexes and diminishes the association of $G\beta\gamma$ with GRK3, thereby quenching the BRET signals (fig. S2A). As expected, we found that ectopic expression of $G\alpha_{i3}$ WT in HEK293T cells decreased the $G\beta\gamma\text{:}GRK3$ BRET signals

compared to cells not transfected with $G\alpha_{i3}$ (fig. S2B). When we transfected cells with plasmids expressing ACS-associated $G\alpha_{i3}$ mutants G40R, G45V, S47R, T48N or N269Y, at the appropriate quantities to obtain protein amounts similar to $G\alpha_{i3}$ WT (fig. S2B, lower panel), we found that the BRET signal was also significantly reduced compared to cells expressing $G\beta\gamma$ alone (fig. S2B, upper panel). However, we also observed that expression of some of the mutants (G40R, G45V, T48N and N269Y) did not quench the BRET signal as efficiently as did expression of $G\alpha_{i3}$ WT (fig. S2B, upper panel), which is indicative of reduced $G\beta\gamma$ binding. These results indicate that, although some of the ACS-associated $G\alpha_{i3}$ mutants display mild to moderate $G\beta\gamma$ binding defects, all of them are capable of forming significant amounts of $G\alpha\beta\gamma$ complexes that serve as GPCR substrates.

We used the same BRET-based experimental system described above to monitor the kinetics of G protein activation upon stimulation of a prototypical G_i -coupled GPCR, the adenosine 1 receptor (A_1R) (Fig. 4A, fig. S2C). In cells expressing $G\alpha_{i3}$ WT, stimulation of A_1R with adenosine resulted in a rapid increase of the BRET signal (Fig 4B, fig. S2C), which is indicative of dissociation of $G\beta\gamma$ from $G\alpha_{i3}$ upon G protein activation. This activation was reversed upon GPCR inhibition, because addition of the A_1R antagonist DPCPX returned BRET signals to basal values (fig. S2C). When analogous experiments were performed with cells expressing any of the five ACS-associated $G\alpha_{i3}$ mutants (G40R, G45V, S47R, T48N or N269Y) in protein amounts equivalent to $G\alpha_{i3}$ WT (as in fig. S2B), the BRET increase upon adenosine stimulation was essentially absent (Fig. 4B). Because under these conditions some ACS-associated $G\alpha_{i3}$ mutants displayed increased basal BRET values that are indicative of impaired association with $G\beta\gamma$ (fig. S2B), we performed additional experiments to rule out the possibility that the observed defect in GPCR-mediated activation was because of the impaired formation of $G\alpha\beta\gamma$ heterotrimers. For this, we adjusted the transfected amounts of $G\alpha_{i3}$ WT and mutant plasmids to obtain similar basal BRET values for all of them, which reflect equivalent $G\alpha\beta\gamma$ heterotrimer formation. We found that under these conditions the BRET increase upon adenosine stimulation was absent for all the $G\alpha_{i3}$ mutants. Because it is also possible that impaired activation of $G\alpha_{i3}$ mutants by GPCRs is because of G protein mislocalization, we performed an additional control by investigating the subcellular localization of the $G\alpha_{i3}$ mutants when expressed in the presence of $G\beta\gamma$. We found that the subcellular distribution of each of the five ACS-associated $G\alpha_{i3}$ mutants was similar to that of $G\alpha_{i3}$ WT (fig. S3), including localization at the plasma membrane as well as endomembranes, as previously reported (22, 23), indicating that defective activation by GPCRs is not due to G protein mislocalization. Together, these results demonstrate that trimeric G proteins bearing $G\alpha_{i3}$ subunits with ACS-associated mutations cannot be activated by GPCRs.

ACS-associated $G\alpha_{i3}$ mutants have increased binding to GEFs

The results presented so far indicate that ACS-associated $G\alpha_{i3}$ mutants exert a dominant effect in *Xenopus* bioassays, cannot bind GTP efficiently, and fail to be activated by the GEF activity of ligand-bound GPCRs. These results suggest that these ACS-associated $G\alpha_{i3}$ mutants may work as dominant-negative proteins by forming non-productive complexes with GEFs, because the dissociation that occurs upon GTP binding to the WT protein (24–27) would not occur for the mutants. If this is the case, we reasoned that ACS-associated

$G\alpha_{i3}$ mutants would associate with GEFs better than the WT protein. We initially explored this idea with the non-receptor GEF Ric-8A. Much like GPCR GEFs, Ric-8A binds with moderate affinity to GDP-bound G proteins, forms a high affinity complex with the nucleotide-free G protein, and dissociates once the G protein binds GTP (Fig. 5A) (25–27). To validate that Ric-8A can be used as a tool to probe for different $G\alpha_i$ conformations in yeast two-hybrid experiments, we used mutants that mimic different states of $G\alpha_{i3}$ along the activation pathway (Fig. 5A). As expected, binding of Ric-8A to $G\alpha_{i3}$ Q204L, a mutant constitutively bound to GTP (19), was diminished compared to WT, which is predominantly bound to GDP. Moreover, binding was enhanced between Ric-8A and $G\alpha_{i3}$ N269D (Fig. 5B, C), a synthetic “GEF-trapping” mutant that mimics the nucleotide-free intermediate state and forms a non-dissociable complex with GPCRs (28). We found that, similar to the $G\alpha_{i3}$ N269D mutant, all five ACS-associated $G\alpha_{i3}$ mutants showed increased binding to Ric-8A compared to $G\alpha_{i3}$ WT, suggesting that they assemble into more stable G protein-GEF complexes. Next, we performed co-immunoprecipitation experiments to directly test whether ACS-associated $G\alpha_{i3}$ mutants bind with higher affinity to ET_A R, the GPCR that governs the signaling pathway dysregulated in ACS (14). We found that all 5 ACS-associated $G\alpha_{i3}$ mutants displayed increased binding (3- to 5-fold) to myc-tagged ET_A R compared to $G\alpha_{i3}$ WT (Fig. 5D). Collectively, these results indicate that ACS-associated $G\alpha_{i3}$ mutants bind to GEFs with high affinity.

ACS-associated $G\alpha_{i3}$ mutants exert a dominant-negative action on the ET-1/ ET_A R/ $G\alpha_q$ pathway

ACS is caused by disruption of the ET-1/ ET_A R signaling axis (14). To further explore the idea that ACS-associated $G\alpha_{i3}$ mutants work as dominant-negative proteins and that this underlies the molecular basis of the disease, we investigated their impact on endothelin-dependent signaling. As a first step, we investigated the coupling efficiency of ET_A R to G_q and G_{i3} proteins using the BRET assay described above (Fig. S4A). We found that both $G\alpha_q$ and $G\alpha_{i3}$ associate with $G\beta\gamma$ with similar efficiency (Fig. S4B), as determined by quenching of the BRET signal compared to cells expressing free $G\beta\gamma$ alone. However, G_q but not G_i was efficiently activated by ET-1-stimulated ET_A R (Fig. 6A, B). In $G\alpha_q$ -expressing cells, ET-1 induced a robust and dose-dependent increase of BRET signals whereas in $G\alpha_{i3}$ expressing cells ET-1 induced only a modest increase at the highest concentrations tested (Fig. 6A, B). These results indicate that, using an identical readout (free $G\beta\gamma$ -mediated BRET), ET_A R couples preferentially to G_q but shows some promiscuity toward G_i proteins. Because ACS-associated $G\alpha_{i3}$ mutants show increased binding to GEFs but cannot be activated, we reasoned that they could work as dominant-negative proteins by precluding G_q coupling to ET_A R (Fig. 6C). To investigate this, we co-expressed $G\alpha_q$ with sub-stoichiometric amounts of $G\alpha_{i3}$ WT or ACS-associated mutants (8:1 ratio) and monitored $G\alpha_q$ activation upon stimulation with 0.3 μ M ET-1. Under these conditions, the BRET changes reflect G_q activation because G_{i3} activation by 0.3 μ M ET-1 was negligible (Fig. 6A). Moreover, the amount of ectopically expressed $G\alpha_{i3}$ proteins was low [the total amount of $G\alpha_{i3}$ in transfected cells is indistinguishable from untransfected controls (Fig. 6D)], and therefore the vast majority of $G\alpha\beta\gamma$ complexes that serve as GPCR substrates are expected to contain $G\alpha_q$ and not $G\alpha_{i3}$. We found that expression of any of the five ACS-associated $G\alpha_{i3}$ mutants decreased G_q activation upon ET-1 stimulation ~65 to 85% (Fig. 6D). On the other

hand, expression of $G\alpha_{i3}$ WT did not change G_q activation, which ruled out that the effect of the mutants was because of sequestration of available $G\beta\gamma$ to form the $G\alpha_q$ - $G\beta\gamma$ complexes. We performed analogous experiments to test the effect of substoichiometric amounts of ACS-associated $G\alpha_{i3}$ mutants on the modest activation of $G\alpha_{i3}$ WT by ET-1/ ET_{AR} (Fig. 6D). We found that cells co-expressing $G\alpha_{i3}$ WT and ACS-associated $G\alpha_{i3}$ mutants had similar BRET response compared to cells expressing $G\alpha_{i3}$ WT alone (Fig. 6D), which suggests that the inhibitory effect of ACS-associated $G\alpha_{i3}$ mutants is specific toward ET-1/ ET_{AR} / $G\alpha_q$ but not ET-1/ ET_{AR} / $G\alpha_{i3}$ signaling. From these experiments we conclude that ACS-associated $G\alpha_{i3}$ mutants exert a dominant-negative effect on the ET-1/ ET_{AR} / $G\alpha_q$ pathway by precluding the coupling of the GPCR to G_q .

DISCUSSION

The main finding of this work is the identification of a new mechanism by which mutations in trimeric G proteins cause human disease. Our data indicate that ACS-associated $G\alpha_{i3}$ mutants are dominant-negative proteins that disrupt a G protein-dependent signaling pathway required for proper craniofacial development. This pathway consists of the activation of ET_{AR} by endothelin-1, which in mice leads to activation of $G\alpha_{q/11}$ -dependent signaling and subsequent transcriptional regulation of neural crest cell development (14). Although ET_{AR} displays some promiscuity in terms of G protein selectivity, our work here and that by others previously (29) have shown that it preferentially activates $G\alpha_q$ over $G\alpha_i$. We propose that the dominant-negative action of ACS-associated $G\alpha_{i3}$ mutants is due to their unproductive coupling to ET_{AR} , which in turn precludes $G\alpha_q$ activation (Fig. 7). The unproductive coupling of ACS-associated $G\alpha_{i3}$ mutants to ET_{AR} is due to their inability to bind GTP (Fig. 3) and their increased binding to GEFs (Fig. 5), which lead to irreversible association with $G\beta\gamma$ and favor sustained association with ET_{AR} even after ligand stimulation. In this scenario, the availability of ET_{AR} for $G\alpha_q$ activation is diminished, and endothelin-dependent signaling important for craniofacial development is impaired.

Previous genetic studies in humans and animal models indicate that disruption of a ET-1/ ET_{AR} / $G\alpha$ /PLC β 4 pathway that controls craniofacial development leads to ACS (14). Two items support that $G\alpha$ subunits of the $G\alpha_{q/11}$ family fulfill the role of mediators in this pathway. One is that PLC β 4, contrary to the rest of PLC β isoforms, is activated exclusively by G_q -related $G\alpha$ subunits and not by $G\beta\gamma$ subunits, which can originate from heterotrimers containing any type of $G\alpha$ (30, 31). The other one is that $G\alpha_q$ / $G\alpha_{11}$ double knockout mice display craniofacial developmental defects analogous to those found in ACS (16, 17). However, results from mouse models should be interpreted with caution, because there is precedent that the signaling mechanism that controls craniofacial development in mice might have differences with humans. For example, knockout mice lacking PLC β 4 do not display a craniofacial phenotype (32), whereas there is at least one case reported of autosomal recessive ACS caused by mutations in PLC β 4 (33). Moreover, no mutations in $G\alpha_q$ and/or $G\alpha_{11}$ have been found in ACS patients. Conversely, the presence of mutations in $G\alpha_{i3}$ indicates that this G protein plays a role in the signaling pathway disrupted in ACS, but the mechanism involved remained unclear until now. Our results support that ACS-associated $G\alpha_{i3}$ mutants disrupt signaling “horizontally”, by blocking GPCR-mediated activation of another G protein, $G\alpha_q$. Although it is possible that ET_{AR} - $G\alpha_{i3}$ signaling participates in

craniofacial development, our results indicate that ACS-associated $G\alpha_{i3}$ mutants do not exert their dominant-negative effect under conditions in which ET_{AR} - $G\alpha_q$ signaling is efficiently inhibited (Fig. 6D).

The lack of dominant-negative inhibition on $G\alpha_{i3}$ is somewhat puzzling because the unproductive coupling of ACS-associated $G\alpha_{i3}$ mutants with ET_{AR} would be expected to interfere with the binding of any G protein to this GPCR. However, the dominance of ACS-associated $G\alpha_{i3}$ mutants could be different depending on the nature of the G_q - ET_{AR} and G_i - ET_{AR} interactions. It is possible that the dominant effect is dampened for $G\alpha_{i3}$ if G_i proteins have higher affinity for ET_{AR} than G_q and/ or if G_i proteins but not G_q “precouple” to ET_{AR} before agonist stimulation. Although further investigation is required to clarify the specificity of the dominant-negative function of ACS-associated $G\alpha_{i3}$ mutants, our results suggest that the primary mechanism by which they cause ACS is “horizontal” interference with $G\alpha_q$ -mediated signaling.

Another question that remains open is why ACS patients bearing mutations in $G\alpha_{i3}$ do not display more pleiotropic phenotypes than the observed effect restricted to neural crest cell differentiation during development. It is possible that the dominant-negative function of ACS-associated $G\alpha_{i3}$ mutants is dampened in other situations due to differences in overall $G\alpha_{i3}$ expression. Because ACS-associated $G\alpha_{i3}$ mutant proteins are intrinsically unstable, the dominant-negative function would become apparent only when sufficient amounts accumulate, for example, by increased gene transcription and/or posttranslational stabilization. Another possibility is that the dominant function is dampened in the presence of higher amounts of other $G\alpha_i$ subunits and/or when multiple GPCRs are present and activated simultaneously. Similarly, if the ACS phenotype is specifically associated with the disruption of $G\alpha_q$ -dependent signaling, the relative amounts of $G\alpha_q$ and $G\alpha_{i3}$ are bound to be critical for the development of ACS. In summary, obtaining additional information on the expression patterns of $G\alpha_{i3}$, $G\alpha_q$ and other signaling components in developing humans will be important to interpret the mechanistic results presented here.

In the past, the characterization of synthetic $G\alpha$ mutants with dominant-negative function has been instrumental in understanding the molecular basis of G protein regulation (28, 34–37). The present work is the first description of naturally occurring $G\alpha$ mutants with dominant-negative function, which demonstrates that this type of mutants are important not only as research tools but also as an underlying cause of human disease. ACS-associated $G\alpha_{i3}$ mutants have similarities and differences with some of the previously described synthetic dominant-negatives. For example, both an N→D mutant in $G\alpha$ G-4 box originally identified in yeast, and $G\alpha_t$ S43N (located in the G-1 box/ P-loop) bind to $G\beta\gamma$ and have increased affinity for GEFs (28, 36, 38), much like $G\alpha_{i3}$ in ACS. These mutants adopt a conformation that mimics an intermediate in the G protein activation cycle that traps GPCRs in an unproductive complex (28, 36). Interestingly, the positions mutated in $G\alpha$ N→D and $G\alpha_t$ S43N, correspond to N269 and S47 in $G\alpha_{i3}$, two of the positions mutated in ACS. However, they are mutated to different residues, meaning N269→Y and S47→R, which may account for some of their different properties. The main difference is that $G\alpha$ N→D and $G\alpha_t$ S43N are capable of binding GTP spontaneously (28, 36) whereas $G\alpha_{i3}$ N269Y and S47R (as well as the rest of ACS mutants) do not. This unique feature of ACS-associated $G\alpha_{i3}$

mutants is bound to contribute to their dominant-negative effect by precluding $G\alpha_{i3}$ from adopting an active conformation and blocking the progression of the G protein cycle.

Another feature shared with other dominant-negative proteins like $G\alpha_{N\rightarrow D}$ is decreased stability (28, 38), which is most readily explained by their mimicry of an unstable nucleotide-free intermediary in the activation cycle (25, 39). All or 4 out of 5 ACS-associated $G\alpha_{i3}$ mutants expressed poorly in mammalian cells and bacteria, respectively. On the other hand, the amount of ACS-associated $G\alpha_{i3}$ mutant proteins ectopically expressed in *Xenopus* or yeast was similar to $G\alpha_{i3}$ WT. This discrepancy is best explained by technical differences among experimental systems. G proteins were expressed at lower temperatures in *Xenopus* and yeast (16°C and 30°C, respectively) compared to mammalian cells (37°C). In addition, the mutants were co-expressed with Ric-8A in yeast, which is a folding chaperone for $G\alpha_i$ (25). Regardless, these results suggest that ACS-associated $G\alpha_{i3}$ mutants are potent dominant-negative proteins because they can efficiently disrupt signaling even when present at quantities lower than endogenous $G\alpha_{i3}$ in mammalian cells.

As for other diseases caused by inherited G protein mutations like dystonias, Nougaret night blindness or Albright's hereditary osteodystrophy, the genetic pattern of type I ACS inheritance is dominant. However, our results indicate that the molecular basis for type I ACS dominant inheritance is different from other diseases caused by G protein mutants. In type I ACS, a single mutant allele is sufficient to cause the disease because of the dominant-negative action of $G\alpha_{i3}$ variants, whereas in other diseases a single G protein mutant allele causes haploinsufficiency. For example, primary torsion dystonia, craniocervical dystonia or Albright's Hereditary Osteodystrophy (AHO) can be caused by non-sense or missense mutations that lead to no protein or severe protein structural defects (5, 9, 10). Nougaret night blindness is caused by a missense mutation in $G\alpha_t$, but it has been unequivocally established that the disease arises from a loss of function not accompanied by any dominant-negative function (7, 8). Conversely, our results rule out haploinsufficiency as the cause of type I ACS because $G\alpha_{i3}$ mutants provoke an increase rather than a decrease of the penetrance of $G\alpha_{i3}$ -dependent phenotypes in embryo development assays (Fig. 2). Based on this, we conclude that type I ACS is the first human disease caused by G protein mutants that arises from a dominant-negative function instead of haploinsufficiency.

MATERIALS AND METHODS

Reagents and antibodies

Unless otherwise indicated all reagents were of analytical grade and obtained from Sigma or Fischer Scientific. Cell culture media and the *E. coli* strain BL21(DE3) was purchased from were purchased from Invitrogen. All restriction endonucleases were from Thermo Scientific and *Escherichia coli* strain DH5 α was purchased from New England Biolabs. Pfu ultra DNA polymerase was purchased from Agilent. DPCPX; 8-Cyclopentyl-1,3-dipropylxanthine, endothelin-1 and adenosine were from Sigma. [γ - 32 P]GTP and [35 S]GTP γ S were from PerkinElmer Life Sciences. Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 or IRDye 800 F(ab') $_2$ were from Li-Cor Biosciences (Lincoln, NE). Rabbit antibodies raised against $G\alpha_{i3}$ (C10) and $G\beta$ (M-14) and mouse monoclonal antibodies against GFP (B-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies for myc tag were

from Sigma (C3956). Mouse monoclonal antibodies raised against tubulin (12G10) and myc tag (9E10) were from the Developmental Studies Hybridoma Bank (University of Iowa) and the mouse monoclonal antibody for HA tag (12CA5) from Roche.

Bioinformatics

Protein sequences of all 16 G α -subunits in *H. sapiens* were retrieved from Uniprot, aligned using ClustalW and shaded with Boxshade 3.21. Protein structure images were generated with PyMOL Molecular Graphics System, (Schrödinger, LLC.) using the PDB: 1GIA.

Plasmids and in vitro mRNA synthesis

Cloning of rat G α_{i3} into pcDNA3 and pET28b has been described previously (22, 24), pcDNA3-G α_q -HA [internally tagged, (40)] was from P. Wedegaertner (Thomas Jefferson University, PA). Rat G α_{i3} was cloned into the EcoRI/SalI sites of the pGBKT7 vector to generate the Gal4AD-G α_{i3} fusion protein used in yeast two-hybrid experiments. A fragment of Ric-8A corresponding to aa 12-491 was amplified from a plasmid (25) kindly provided by Stephen Sprang (University of Montana, MT) and cloned into the NdeI/EcoRI sites of the pGADT7 vector to generate the Gal4BD-Ric-8A fusion protein used in yeast two-hybrid experiments. A rat G α_{i3} construct internally tagged with YFP was generated by introducing the fluorescent protein in the b/c loop of the all-helical domain, which is a strategy previously described for G α_{i1} that preserves the native properties of the G protein (41). Briefly, YFP was inserted between S113 and A114 (b/c loop) of G α_{i3} by introducing silent mutations in pcDNA3.1(-)-G α_{i3} to create an artificial AfeI site in this location followed by digestion and ligation of the sequence encoding for the fluorescent protein. pcDNA3.1-A1R, pcDNA3.1-Venus(1-155)-G γ_2 (VN-G γ_2), pcDNA3.1-Venus(155-239)-G β_1 (VC-G β_1), pcDNA3.1-G γ_2 , pcDNA3.1-G β_1 and myc-ET $_A$ R-Rluc8 were kindly provided by N. Lambert (Georgia Regents University, GA) (20). pcDNA3.1-masGRK3ct-Nluc (21) and pcMin ET $_A$ R (42) were generous gifts from K. Martemyanov (The Scripps, FL) and P. Polgar (Boston University, MA), respectively.

Rat G α_{i3} was cloned into the EcoRI and XhoI sites of pCS2(+) to generate the DNA template for *in vitro* mRNA transcription. Briefly, pCS2(+)-G α_{i3} was linearized by digestion with NotI, and DNA purified by alkaline phenol/chloroform extraction followed by ethanol precipitation. One μ g of purified DNA was used as template for each mRNA *in vitro* transcription reaction with the SP6 mMessage mMachine Kit (Ambion). *In vitro* mRNA transcription reactions were treated by the DNase I to eliminate the template and mRNA purified by alkaline phenol/chloroform extraction, followed sequential precipitations in isopropanol and ethanol. Purified mRNAs were quantified spectroscopically and their quality checked in a 1% agarose/formaldehyde gel. mRNAs were diluted to the desired final concentrations and stored at -80°C. G α_{i3} mutants were generated using specific primers (sequences available upon request) following the manufacturer's instructions (QuickChange II, Agilent). All constructs were checked by DNA sequencing.

Xenopus laevis embryo manipulations

Frog studies were carried out with wild type animals (Nasco, MA) according to Boston University-Institutional Animal Care and Use Committee (IACUC)-approved protocol, in

compliance with the Guide for the Care and Use of Laboratory Animals. Egg laying was induced by dorsal lymph injection of human chorionic gonadotrophin (500 U) (Intervet, NJ). In vitro fertilization and embryo culture were carried out in 0.1× Marc's Modified Ringers (MMR) medium as described (43). Staging was according to Nieuwkoop and Faber. *In vitro* transcribed mRNAs (30pg or 60pg) were injected equatorially in both dorsal blastomeres of 4 or 8-cell stage embryos, which were subsequently incubated at 16°C. Embryos were fixed at late neurulation (stage 18–19) in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, 3.7% (v/v) formaldehyde), photographed and subjected to phenotypical analysis. Embryos were observed using a Leica MZ6 dissection microscope and scored as “gravitational defect” when the neural tube was facing downward (18). Sagittal bisections of MEMFA fixed embryos at stage 15 were performed with a razorblade and photographed in PBS 1X. All pictures were taken with a Canon XSi camera connected to the microscope.

For the analysis of Gα_{i3} by immunoblotting, two embryos were resuspended in 60 µl of lysis buffer [20 mM HEPES, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% Triton X-100, 1 mM DTT supplemented with a protease inhibitor cocktail (SigmaCat#S8830)] and homogenized by pipetting. After centrifugation for 10 minutes at 14,000 xg at 4°C, supernatants were supplemented with Laemmli sample buffer and boiled for 5 minutes.

Cell Culture, transfections and BRET assay

HEK293T cells were grown at 37°C in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine and 5% CO₂. All DNA plasmids were transfected using the calcium phosphate method in 6-well plates and BRET measurements were done 16–24 hours after transfection. Equal amounts (0.2µg) of masGRK3ct-Nluc, VC-Gβ₁, VN-Gγ₂ and GPCR (either A1R or ETAR) were transfected for BRET experiments. Gα_{i3} and Gα_q were typically transfected at 1µg of DNA per well. This amount of DNA was adjusted (1–4µg) in some experiments to produce similar amounts of protein among different G proteins mutants and WT. For the experiments investigating the dominant-negative effect of Gα_{i3} on Gα_q activation, the plasmids were transfected at 1:8 ratio (0.12µg: 1µg, Gα_{i3}:Gα_q). BRET experiments were carried out and analyzed as described previously (20, 21) with minor modifications. Briefly, 16–24 hours after transfection cells were gently scrapped in PBS, centrifuged and resuspended in Tyrode's solution (140mM NaCl, 5mM KCl, 1mM MgCl₂, 1mM CaCl₂, 0.37 mM NaH₂PO₄, 24 mM NaHCO₃, 10mM Hepes pH=7.4, 0.1% Glucose) at a density of 10⁶ cell/ml. Twenty-five µl of cell suspensions were added to a white opaque 96-well plate (Opti-Plate® Perkin Elmer) and mixed with an equal volume of the Nanoluciferase substrate Nano-Glo (Promega, diluted 1:50). After 2 min incubation, luminescence was measured at room temperature in a Synergy H1 plate reader (Biotek) at 460±20nm and 528±10nm. BRET signals were calculated as the ratio of the emission intensity at 528±10nm divided by the emission intensity at 460±20nm. For the kinetic experiments, BRET measurements were done every second. Basal BRET was measured for 30s, after which adenosine or endothelin (ET-1) were added to the well. With the exception of fig. S2D, kinetic BRET data are presented as the increase in BRET (BRET) for clarity. BRET was calculated by subtracting the average of BRET signal before agonist stimulation (t=0–30s) to every point of the time trace. An aliquot of the cell suspensions was processed for immunoblot analysis. Briefly, cells were pelleted and

resuspended in lysis buffer (20 mM HEPES, pH 7.2, 5 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 125 mM $\text{K}(\text{CH}_3\text{COO})$, 0.4% Triton X-100, 1 mM DTT) supplemented with a protease inhibitor cocktail (Sigma Cat#S8830) and cleared by centrifugation at 14,000 xg at 4°C for 10 minutes. After centrifugation, supernatants were supplemented with Laemmli sample buffer and boiled for 5 minutes.

***In vitro* G protein biochemistry assays**

Steady-state GTPase assays were performed using radiolabeled $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and measuring the release of $[\gamma\text{-}^{32}\text{P}]\text{Pi}$ at 30°C. GTP γS binding was determined by measuring intrinsic tryptophan fluorescence (Ex: 284 nm/ Em: 340 nm) in a Hitachi F-4500 Fluorescence Spectrophotometer or by directly measuring binding of radiolabeled $[\gamma\text{-}^{35}\text{S}]\text{GTP}\gamma\text{S}$ at 30°C. Limited proteolysis assays were carried out by incubating purified G proteins or lysates of HEK293T cells expressing different G proteins with GDP, GTP γS or GDP plus AlCl_3/NaF (AlF_4^-) at 30°C before adding trypsin. HPLC analyses of G protein nucleotide content were performed after exchanging the purified G proteins into nucleotide-free buffer and concentrating them to 50 μM . Nucleotide standards at 50 μM were prepared in the same buffer.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to PVDF membranes, which were sequentially incubated with primary and secondary antibodies (goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800, 1:10,000). The primary antibodies were used at the following dilutions: $\text{G}\alpha_{i3}$, 1:250, pan-G β , 1:250, α -tubulin, 1:2,500, HA, 1:1,000 and rabbit myc (1:1,000). Infrared imaging of immunoblots was performed according to the manufacturer's protocols using an Odyssey Infrared Imaging System (Li-Cor Biosciences). Images were processed using the Image J software (NIH) and assembled for presentation using Photoshop and Illustrator softwares (Adobe).

Protein Purification

His-tagged $\text{G}\alpha_{i3}$ proteins were expressed in *E. coli* strain BL21 (DE3) (Invitrogen) and purified as described previously (44). Briefly, bacterial cultures were induced overnight at 23 °C with 1 mM isopropyl- β -D-1-thio-galactopyranoside (IPTG). Pelleted bacteria from 1 L of culture were resuspended in 25 ml His-lysis buffer (50 mM NaH_2PO_4 (pH 7.4), 300 mM NaCl, 10 mM imidazole, 1% (v:v) Triton X-100, 25 μM GDP, 1 μM leupeptin, 2.5 μM pepstatin, 0.2 μM aprotinin and 1mM PMSF). After sonication (four cycles, with pulses lasting 30 s/cycle, and with 1 min interval between cycles to prevent heating), lysates were centrifuged at 12,000xg for 20 min at 4 °C. Solubilized proteins were affinity purified on HisPur Cobalt Resin (Pierce), eluted with imidazole, dialyzed against PBS and buffer exchanged/ concentrated in 20 mM Tris-HCl, pH 7.4, 20 mM NaCl, 1 mM MgCl_2 , 1 mM DTT, 10 μM GDP, 5% (v/v) glycerol before storage at -80 °C.

Limited proteolysis assay with purified proteins

This assay was carried out as described previously (44, 45) with minor modifications. Briefly, His- $\text{G}\alpha_{i3}$ (0.4 mg/ml) was incubated for 90 min at 30 °C in buffer (20 mM Na-

HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1 mM DTT, 0.05% (w:v) C₁₂E₁₀] supplemented with GDP (30 μM), GTPγS (30 μM) or GDP·AlF₄⁻ (30 μM GDP, 30 μM AlCl₃ and 10 mM NaF). After this incubation, trypsin was added to the tubes (final concentration 80 μg/ml) and samples were incubated for 10 min at 30 °C. Samples were rapidly transferred to ice and reactions stopped by the addition of Laemmli sample buffer and incubated at 65 °C for 5 minutes. Proteins were separated by SDS-PAGE and stained with Coomassie blue.

Limited proteolysis assay with HEK293T cell lysates

This assay was carried out as described previously (45) with minor modifications. HEK293T cells were transfected with plasmids encoding for Gα_{i3} WT or ACS mutants as described in the section “*Cell Culture, transfections and BRET assay*”. The amount of each plasmid required to achieve equal protein expression for wild-type and mutant Gα_{i3} was determined empirically in preliminary experiments. A quarter of the HEK293T cells from a well of a 6-well plate was lysed in 48 μl of buffer (20 mM HEPES, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% Triton X-100, 1 mM DTT) and supplemented with 125 μM GDP or 125 μM GTPγS. Samples were vortexed, incubated in ice for 10 min and centrifuged at 14,000g for 10 min at 4 °C. Forty μl of the supernatants were incubated for 2h 30 min at 30 °C to allow the loading of nucleotide on the G protein. Trypsin (12.5 μg/ml) was added to the tubes and incubated at 30 °C for 20 min. Reactions were stopped by addition of 12 μl of Laemmli sample buffer and boiled for 5 minutes. The “% Protection by GTPγS” was calculated by quantifying the bands corresponding to “+trypsin/+GTPγS” condition divided by the band of “-trypsin” condition and multiplying the result by 100. To determine the specific protection of each ectopically expressed Gα_{i3} protein, the quantification values of the corresponding bands (i.e., GTPγS-protected or total) of endogenous Gα_{i3} (i.e., transfected with an empty vector) were subtracted prior to the calculation described above.

Steady-state GTPase Assay

This assay was performed as described previously (44, 45). Briefly, reactions were initiated at 30 °C by mixing assay buffer [20 mM Na-HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.05% (w:v) C₁₂E₁₀] containing [γ-³²P]GTP (1 μM, ~100 cpm/fmol) with an equal volume of His-Gα_{i3} (100 nM) in the same buffer. Duplicate aliquots (50 μl) were removed at 0, 2, 4, 6, 10 and 15 min and reactions stopped with 950 μl ice cold 5% (w:v) activated charcoal in 20 mM H₃PO₄, pH 3. Samples were centrifuged for 10 min at 10,000 x g, and 500 μl of the resultant supernatant were scintillation counted to quantify the amount of [³²P] Pi released. Data are presented as raw radioactivity counts.

Measurement of GTPγS binding by intrinsic tryptophan fluorescence

This assay was performed as described previously (45). Purified His-Gα_{i3} (1 μM) was equilibrated at 30 °C in a cuvette with 1 ml of buffer [20 mM Na-HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.05% (w:v) C₁₂E₁₀]. GTPγS (1.25 μM) was added to the cuvette after ~3 min and the G protein activation rate monitored by measuring the change in intrinsic fluorescence (excitation at 284 nm, emission at 340 nm) due to structural rearrangement of the Switch II tryptophan residue W211. Data was

collected with a Hitachi F-4500 Fluorescence Spectrophotometer, background corrected (buffer fluorescence) and presented as a ratio of the basal fluorescence (F/F_0).

Measurement of radiolabeled GTP γ S binding

This assay was performed as described previously (44–46). Briefly, reactions were initiated at 30°C by mixing assay buffer [20 mM Na-HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.05% (w:v) C₁₂E₁₀], containing [³⁵S]GTP γ S (1 μ M, ~50 cpm/fmol) with an equal volume of His-G α_{i3} (100 nM) in the same buffer. Duplicate aliquots (25 μ l) were removed at the indicated time points, and binding of radioactive nucleotide was stopped by addition of 2 ml ice-cold wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂). The quenched reactions were rapidly passed through BA-85 nitrocellulose filters (Whatman). Filters were dried and subjected to scintillation counting. Data are presented as raw radioactivity counts.

Nucleotide HPLC Analysis

HPLC analyses of G protein nucleotide content were performed as previously described (47). His-G α_{i3} wild-type and His-G α_{i3} S47R mutant were exchanged into nucleotide free buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 5% (v/v) glycerol) and concentrated to 50 μ M. Nucleotide standards at 50 μ M were prepared in the same buffer. Acetonitrile was added to each protein sample or standard tube to obtain a final concentration of 7.5% (v:v), boiled for 5 minutes and centrifuged 10 minutes at 20,000xg to precipitate the denatured proteins. The supernatant was collected and used for the HPLC analysis. A Zorbax C-18 reverse phase column (4.6 x 150mm) filled with 3.5 μ m silica (Agilent) was equilibrated with 100 mM KH₂PO₄, pH 6.5, 10 mM tetrabutylammonium bromide (phase-transfer catalyst), 0.2% (w:v) NaN₃, and 7.5% (v:v) acetonitrile using an Agilent 1260 infinity quaternary pump flowing at 1 ml/min. 15 μ L of freshly prepared sample was loaded into a 20 μ L HPLC loading loop. The samples were then injected onto the C-18 column and isocratically eluted at 1 ml/min. Absorbance of 280 nm wavelength light was performed by an Agilent 1260 infinity UV detector. Control standards of GDP and GTP eluted at distinct retention times of 2.25 minutes and 2.56 minutes respectively.

Co-immunoprecipitation

This assay was performed as previously described (46). HEK293T cells were transfected in 10-cm dishes with plasmids encoding for untagged G β 1 (1.2 μ g), untagged G γ 2 (1.2 μ g), myc-ET_AR (6 μ g) and G α_{i3} -YFP (b/c loop internal) WT or ACS mutants as described in the section “*Cell Culture, transfections and BRET assay*”. The amount of plasmid required to achieve equal protein expression for wild-type and mutant G α_{i3} was determined empirically in preliminary experiments. HEK293T cells from a 10-cm plate were lysed in 1 ml of ice-cold buffer (20 mM HEPES, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% Triton X-100, 1 mM DTT), vortexed, passed through a 30G insulin syringe 5 times and incubated in ice for 10 min. After centrifugation at 14,000xg for 10 min at 4°C, the supernatant was split into two tubes for each condition. Either 2.5 μ g of anti-myc (9E10) or control mouse IgG (Santa Cruz) were added to each tube and incubated 4h at 4°C with constant rotation. Protein G agarose beads (Thermo-Scientific) were blocked with 5% BSA for 2 hours at room temperature, washed and added to each of the tubes containing lysates

and antibodies and incubated for 90 minutes at 4°C with rotation. Beads were then washed 3 times with wash buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl₂, 5 mM EDTA, 1 mM DTT) and proteins eluted by incubation in Lemmli sample buffer for 10 min at 37°C.

Immunofluorescence microscopy

This assay was performed as previously described (48). HEK293T cells were transfected with plasmids encoding for untagged Gβ₁, untagged Gγ₂ and Gα_{i3}-YFP (b/c loop internal) WT or ACS mutants as described in the section “*Cell Culture, transfections and BRET assay*” except that they were seeded on poly-L-lysine-coated glass coverslips. The amount of plasmid required to achieve equal protein expression for wild-type and mutant Gα_{i3} was determined empirically in preliminary experiments. One day after transfection, cells were fixed with 3% paraformaldehyde for 30 min, permeabilized and blocked in PBS containing 10% Normal Goat Serum and 0.1% Triton X-100 for 30 min, then sequentially incubated with primary and secondary antibodies for 1 hr at room temperature. Antibody dilutions were as follows: primary mouse anti-GFP (B-2, Santa Cruz), 1:200; secondary goat anti-mouse Alexa 488 (Lifetechnologies), 1:300. Images were acquired with a Zeiss Axiovert 200 LSM510 laser scanning confocal microscope using a 63x oil-immersion objective. All individual images were assembled for presentation using Photoshop and Illustrator software (both Adobe).

Yeast Two-Hybrid

This assay was performed using Matchmaker Gold (Promega) according to the manufacturer’s instructions. Briefly, pGADT7-Ric-8A was transformed into the *Saccharomyces cerevisiae* haploid strain Y187 and pGBKT7-Gα_{i3} (WT or mutants) into the haploid strain AH109 using the lithium acetate method (49). Transformants were selected in SD-Leu and SD-Trp media plates, respectively and mated by co-inoculation of single colonies in YPD media and overnight incubation at 30°C. Mated diploid strains were selected by inoculation of 20 µl of the overnight cultures on SD-Leu-Trp media. Individual colonies were inoculated into 3ml of SD-Leu-Trp and incubated overnight at 30°C. This starting culture was used to inoculate 20 ml of SD-Leu-Trp at 0.3 OD₆₀₀. Exponentially growing cells (~0.7–0.8 OD₆₀₀, 4–5 hours) were pelleted to prepare samples for subsequent assays (see sections “*β-galactosidase Activity Assay*” and “*Preparation of Yeast Samples for Immunoblotting*” below).

β-galactosidase Activity Assay

This assay was performed as described previously (49) with minor modifications. Pellets corresponding to 0.5 OD₆₀₀ were washed once with PBS + 0.1% (w:v) BSA and resuspended in 200 µl assay buffer (60 mM Na₂PO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂, 0.25% (v:v) β-mercaptoethanol, 0.01% (w:v) SDS, 10% (v:v) chloroform) and vortexed. 100 µl were transferred to 96-well plates and reactions started by the addition of 50 µl the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG, 100 µM final). Fluorescence (Ex 485 ± 10 nm/ Em 528 ± 10 nm) was measured every 2 min for 90 min at 30°C in a Synergy H1 plate reader (Biotek). Enzymatic activity was calculated from the slope of fluorescence (arbitrary units) vs time (min). At least 3 independent clones

determined in duplicate were measured for each condition and the results normalized (%) to the activity in cells expressing Ric-8A and G α_{i3} WT.

Preparation of Yeast Samples for Immunoblotting

This procedure was performed as described previously (49, 50) with minor modifications. Briefly, pellets corresponding to 5 OD₆₀₀ were washed once with PBS + 0.1% BSA and resuspended in 150 μ l of lysis buffer (10 mM Tris.HCl, pH 8.0, 10% (w:v) trichloroacetic acid (TCA), 25 mM NH₄OAc, 1 mM EDTA). 100 μ l of glass beads were added to each tube and vortexed in the cold room for 5 min. Lysates were transferred by poking a hole in the bottom of the tubes followed by centrifugation onto a new set of tubes. The process was repeated after the addition of 50 μ l of lysis buffer to wash the glass beads. Proteins were precipitated by centrifugation (10 min, 20,000 xg) and resuspended in 60 μ l of solubilization buffer (0.1 M Tris.HCl, pH 11.0, 3% SDS). Samples were boiled for 5 min, centrifuged (1 min, 20,000 xg) and 50 μ l of the supernatant transferred to new tubes containing 12.5 μ l of Laemmli sample buffer and boiled for 5 min.

Statistical analyses

Each experiment was performed at least 3 times. Data are presented as mean $\pm$ SEM or as one representative result from each biological replicate. The error bars of the phenotype distributions shown in Fig. 2C were calculated as follows: S.E. = Square root[P*(100-P)/n], where P is the proportion (%) of the phenotype and n is the total number of embryos analyzed. Statistical significance between various conditions was assessed with the Student's *t* test or the Fisher exact test (for the *Xenopus* embryo assays). P<0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ACS mutations affect residues that cluster around the nucleotide binding pocket of Gai3

Top, schematic diagram depicting structural elements of Gai3 and the location of all five mutations found to date in patients with Auriculo-Condylar Syndrome (ACS). Switch regions I, II and III involved in activation-induced conformational changes are depicted in grey, and the five conserved G boxes involved in nucleotide binding are shown in blue. Bottom, 3-dimensional representation of the Gai nucleotide binding pocket (PDB: 1GIA). Amino acids mutated in ACS (beige) cluster around the nucleotide binding pocket (GTP in purple).

Figure 2. ACS mutations increase the frequency of developmental defects induced by ectopic expression of $G\alpha_{i3}$ in *Xenopus laevis* embryos

(A) Validation of an assay to monitor $G\alpha_{i3}$ -induced developmental defects in *Xenopus laevis*. Left, schematic diagram illustrating the relationship between tissue remodeling defects and inversion of gravitational orientation of $G\alpha_{i3}$ -injected embryos. Top right, Representative embryos injected with mRNA encoding wild-type $G\alpha_{i3}$ (60 pg) compared with uninjected controls at the late neurula stage. Arrowhead marks inverted gravitational orientation (neural tube facing downward). Bottom right, sagittal sections of a representative control and a $G\alpha_{i3}$ -injected embryo with impaired archenteron (arc) inflation and incomplete blastocoel (bc) removal. (B, C) Assessment of the percentage of embryos displaying a gravitational defect after injection with 30 or 60pg mRNA encoding wild-type or ACS mutant $G\alpha_{i3}$. Representative images (B) are presented and marked as in (A). Quantification is shown in (C). Data are means $\pm$ S.E. of the phenotype proportion of the indicated total number (n) of embryos from each test group. **p<0.01; ***p<0.001 for $G\alpha_{i3}$ mutants compared to WT using the Fisher exact test. Immunoblots (C) confirm protein abundance in embryos injected with the indicated mRNAs.

Figure 3. ACS-associated $G\alpha_{i3}$ mutants fail to bind GTP *in vitro*

(A) Comassie blue-stained gel of purified proteins treated as indicated in a trypsin protection assay to assess whether $G\alpha_{i3}$ S47R is activated by nucleotides. Arrow marks full-length His- $G\alpha_{i3}$; * marks trypsin-resistant fragment of active His- $G\alpha_{i3}$. (B) Effect of S47R mutation on the steady-state GTPase activity of $G\alpha_{i3}$. Purified His- $G\alpha_{i3}$ proteins were incubated in the presence of radiolabeled GTP and activity determined by measuring the release of radioactive phosphate. (C, D) GTP γ S binding by mutant (S47R) and wild-type (WT) $G\alpha_{i3}$ was determined by intrinsic fluorescence measurements (Fo= basal fluorescence) (C) or radioligand binding (D). (E) Assessment of GDP occupation on wild-type and S47R $G\alpha_{i3}$. Nucleotide content of equimolar amounts of His- $G\alpha_{i3}$ WT (black) or His- $G\alpha_{i3}$ S47R (grey) (top) was compared to GDP (black) or GTP (grey) standards (bottom) by HPLC (A280= nucleotide absorbance at 280nm). (F, G) GTP γ S binding as assessed by trypsin protection assays by ACS $G\alpha_{i3}$ mutants G40R, G45V, S47R, T48N and N269Y. Representative immunoblots of $G\alpha_{i3}$ (arrow, full-length $G\alpha_{i3}$; *, trypsin-resistant fragment of active $G\alpha_{i3}$) in HEK293T cell lysates treated as indicated (F) and quantified in (G). One experiment representative of 3 is shown in (A), (B), (C), (D) and (F). Data in (G) are means $\pm$ S.E.M (n=4 experiments, *p<0.05; **p<0.01 compared to WT using Student's *t*-test).

Figure 4. ACS-associated Gα_{i3} mutants are not activated by the GPCR A₁R

(A) Schematic diagram depicting the Bioluminescence Resonance Energy Transfer (BRET) assay used to monitor the dissociation of Gα_{i3}:Gβγ trimers upon GPCR stimulation. Under resting conditions, Venus-tagged Gβγ (V-Gβγ) associates with Gα_{i3} and BRET signals are low. Upon stimulation of A₁R with adenosine, G_i trimers dissociate and free V-Gβγ binds to mas-GRK3ct-Nluc (GRK), leading to an increase of BRET signal. **(B)** Assessment of A₁R-induced dissociation of G_i trimers containing wild-type or ACS-mutant Gα_{i3} subunits. HEK293T cells were transfected with plasmids encoding for VC-Gβ₁, VN-Gγ₂, mas-GRK3ct-Nluc and A₁R along with Gα_{i3} WT or ACS mutants (G40R, G45V, S47R, T48N or N269Y) and BRET was measured every second (as described in the Materials and Methods). Equal amounts of V-Gβγ and the different Gα_{i3} proteins were verified by immunoblotting (fig. S2). After 30 s under resting conditions, cells were stimulated with adenosine (1 μM). The average of BRET signal during the first 30s (basal BRET) was subtracted from each data point to present the data as increase of BRET (ΔBRET). One representative experiment of four is shown.

Figure 5. $G_{\alpha i3}$ ACS mutations increase binding to GEFs

(A) Schematic representation of Ric-8A binding to different conformations of $G_{\alpha i3}$ during its activation cycle and description of constructs that mimic each one of these conformations. (B) Assessment of Ric-8A binding by wild-type or ACS-mutant $G_{\alpha i3}$ using yeast two-hybrid assays (schematic, top). Binding of $G_{\alpha i3}$ to Ric-8A activates the *LacZ* reporter (AD/BD= *Gal4* activation and DNA binding domains, respectively). Bottom, β -galactosidase activity in yeast strains expressing Ric-8A and the indicated $G_{\alpha i3}$ constructs. Data are mean $\pm$ SEM (n=3 experiments, *p<0.05, **p<0.01 compared to WT by Student's t test). (C) Representative immunoblot of the strains used for yeast two hybrid experiments in (B). (D) Relative affinity of ACS-associated $G_{\alpha i3}$ mutants for the ET_AR GPCR, as determined by immunoprecipitation (IP) with myc or IgG antibody in lysates of HEK293T cells transfected with myc-ETAR and the indicated $G_{\alpha i3}$ constructs followed by immunoblotting (IB). Immunoblots of equal lysate aliquots (inputs) are shown below. Blots are representative of three experiments.

Figure 6. ACS-associated $G\alpha_{i3}$ mutants prevent ET_AR-mediated activation of $G\alpha_q$
(A, B) BRET assay assessment of G_q and G_{i3} activation upon ET_AR stimulation. HEK293T cells transfected with $G\alpha_q$ (left) or $G\alpha_{i3}$ (right) along with V-G $\beta\gamma$, mas-GRK3-Nuc and ET_AR were stimulated (arrow) with the indicated concentrations of ET-1. Results were analyzed as in Fig. 4B. One representative experiment is shown in (A). Data in (B) are means $\pm$ S.E.M. (n=3 experiments). **(C)** Schematic diagram of a putative mechanism of dominant-negative action of ACS-associated $G\alpha_{i3}$ mutants on G_q activation by ET_AR as determined by BRET assays. In cells expressing $G\alpha_{i3}$ WT (top), ET_AR couples predominantly to G_q . In cells expressing ACS-associated $G\alpha_{i3}$ mutants (bottom), high affinity binding of G_{i3} ACS mutants to ET_AR precludes G_q activation. **(D)** Effect of ACS-associated $G\alpha_{i3}$ mutants on the activation of G_q and G_{i3} WT upon ET_AR stimulation. HEK293T cells transfected with $G\alpha_q$ (left) or $G\alpha_{i3}$ WT (right) (1 μg) and sub-stoichiometric amounts of $G\alpha_{i3}$ WT or an ACS mutant (0.12 μg) along with V-G $\beta\gamma$, mas-GRK3-Nuc and ET_AR were stimulated (arrow) with ET-1 ($G\alpha_q$; 0.3 μM / $G\alpha_{i3}$; 3 μM). One representative of three experiments is shown. Immunoblots of the cells used in these experiments are shown below.

Figure 7. Proposed model

Left, in healthy individuals, ET_AR couples predominantly to Gα_{q/11}, which promotes PLCβ4 activation and the expression of DLX5/6 transcription factors required for neural crest cell differentiation and normal craniofacial development. Right, in type I ACS patients, ACS-associated Gα_{i3} mutants bind with high affinity to ET_AR and form an unproductive complex. This reduces the availability of ET_AR for Gα_q activation, which blocks the signaling pathway required for normal craniofacial development.