Two missense mutations in KCNQ1 cause pituitary hormone deficiency and maternally inherited gingival fibromatosis

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Two missense mutations in KCNQ1 cause pituitary hormone deficiency and maternally inherited gingival fibromatosis

Johanna Tommiska et al. #

Familial growth hormone deficiency provides an opportunity to identify new genetic causes of short stature. Here we combine linkage analysis with whole-genome resequencing in patients with growth hormone deficiency and maternally inherited gingival fibromatosis. We report that patients from three unrelated families harbor either of two missense mutations, c.347G>T p.(Arg116Leu) or c.1106C>T p.(Pro369Leu), in KCNQ1, a gene previously implicated in the long QT interval syndrome. Kcnq1 is expressed in hypothalamic GHRH neurons and pituitary somatotropes. Co-expressing KCNQ1 with the KCNE2 β-subunit shows that both KCNQ1 mutants increase current levels in patch clamp analyses and are associated with reduced pituitary hormone secretion from AtT-20 cells. In conclusion, our results reveal a role for the KCNQ1 potassium channel in the regulation of human growth, and show that growth hormone deficiency associated with maternally inherited gingival fibromatosis is an allelic disorder with cardiac arrhythmia syndromes caused by KCNQ1 mutations.
Somatic growth mirrors nutritional status, general health, and psychosocial well-being, and is also affected by inherited and epigenetic factors. An essential role in human growth is played by growth hormone which is secreted by the anterior pituitary somatotropes, mainly under the influence of hypothalamic inputs such as growth hormone-releasing hormone (GHRH) and somatostatin. Childhood onset of growth hormone deficiency (GHD) is a clinically heterogeneous condition and defining its cause is important for diagnostics and treatment. Studying familial GHD provides an opportunity to identify genetic regulators of growth hormone secretion and pituitary function. The most common genes implicated in the genetic etiology of GHD are \( GH1 \) (MIM: 139250, Online Mendelian Inheritance in Man, http://www.omim.org/), encoding growth hormone.

![Pedigrees, gingival fibromatosis and craniofacial features, and KCNQ1 structure.](image)

**Fig. 1** Pedigrees, gingival fibromatosis and craniofacial features, and KCNQ1 structure. **a** (I) Pedigree of the large Finnish family showing autosomal dominant growth hormone deficiency and maternally inherited gingival fibromatosis. The genotype (wild-type (WT) or p.(Arg116Leu)) is given. The samples included in the linkage analysis are indicated in italics, and samples included in whole-genome sequencing are underlined. Two additional families with the same disease but another mutation in \( KCNQ1 \), p.(Pro369Leu), were identified: a Finnish trio (II) and a family (III) originating from Argentina. Note that the index patient in pedigree III has a de novo mutation. **b** Maternally inherited gingival fibromatosis is shown together with the craniofacial features of the twin boys belonging to family II. **c** Schematic of the KCNQ1 channel protein and the location of the two missense mutations in the 3D channel structure. The schematic shows the membrane domain, with helical segments S0–S6 and the intracellular domain, divided into a membrane-proximal module (helices A-B) bound by CaM and a distal module (helices C-D), responsible for tetramerization. Filled circles with labels show the positions of the mutations, Arg116Leu and Pro369Leu. The double lines depict the plasma membrane. Below the schematic is a molecular graphics depiction of the Kv7.1/CaM channel complex as based on the cryo EM Xenopus structure (PDB code: 5VMS). The channel subunits are colored green, cyan, and teal. CaM is colored pink and shown with a surface representation on the right side. Gray spheres are Ca\(^{2+}\) ions. Again, the straight lines denote the probable location of the plasma membrane. The residues that undergo mutation are drawn as CPK atoms and are labeled. One channel subunit of the tetramer and its respective CaM molecule have not been drawn in order to facilitate visualization. The helix D tetrameric coiled-coil was not observed in the cryo EM study due to flexibility in the linker between it and helix C and hence not drawn here.
Table 1 Summary of phenotypic features in patients with KCNQ1 mutations p.(Arg116Leu) or p.(Pro369Leu) and pituitary hormone deficiencies

<table>
<thead>
<tr>
<th>Subject</th>
<th>KCNQ1 mutation</th>
<th>Sex</th>
<th>QTc interval in ECG (ms)</th>
<th>Height (SDS) at the age of the onset of GH therapy</th>
<th>Brain MRI</th>
<th>Pituitary hormone deficiencies</th>
<th>Mutation inherited/gingival fibromatosis</th>
<th>Craniofacial phenotype as a child</th>
</tr>
</thead>
<tbody>
<tr>
<td>#5</td>
<td>r.R116L</td>
<td>F</td>
<td>414</td>
<td>−4.5 at 15 years</td>
<td>Normal</td>
<td>Growth hormone and gonadotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#6</td>
<td>r.R116L</td>
<td>F</td>
<td>412</td>
<td>−3.4 at 12.4 years</td>
<td>Normal</td>
<td>Growth hormone and gonadotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#7</td>
<td>r.R116L</td>
<td>M</td>
<td>391</td>
<td>−5.0 at 8.5 years</td>
<td>Small hypophysis</td>
<td>Growth hormone, gonadotropin, ACTH, and thyrotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#13</td>
<td>r.R116L</td>
<td>F</td>
<td>NA</td>
<td>−2.7 at 4.5 years</td>
<td>Normal</td>
<td>Growth hormone and thyrotropin</td>
<td>Paternally/No</td>
<td>No</td>
</tr>
<tr>
<td>#13b</td>
<td>r.R116L</td>
<td>F</td>
<td>NA</td>
<td>−2.7 at 3.7 years</td>
<td>Normal</td>
<td>Growth hormone and gonadotropin</td>
<td>Paternally/No</td>
<td>No</td>
</tr>
<tr>
<td>#8</td>
<td>r.R116L</td>
<td>M</td>
<td>398</td>
<td>−2.6 at 15.9 years</td>
<td>NA</td>
<td>Growth hormone and gonadotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#9</td>
<td>r.R116L</td>
<td>F</td>
<td>NA</td>
<td>−2.7 at 9 years</td>
<td>NA</td>
<td>Growth hormone, gonadotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#15</td>
<td>r.R116L</td>
<td>M</td>
<td>363&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−1.8 at 6 years</td>
<td>Small hypophysis with thin stalk</td>
<td>Growth hormone, gonadotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#17</td>
<td>r.R116L</td>
<td>M</td>
<td>329&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−2.2 at 5 years</td>
<td>Small hypophysis with thin stalk</td>
<td>Growth hormone, gonadotropin</td>
<td>Maternally/Yes</td>
<td>NA</td>
</tr>
<tr>
<td>#18</td>
<td>r.R116L</td>
<td>F</td>
<td>463</td>
<td>−2.3 at 13.4 years</td>
<td>Normal</td>
<td>Growth hormone, gonadotropin, ACTH and thyrotropin</td>
<td>Maternally/Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>#20</td>
<td>p.P369L</td>
<td>F</td>
<td>317&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−5.2 at 17 years</td>
<td>Normal</td>
<td>Growth hormone, gonadotropin</td>
<td>NA/No</td>
<td>No</td>
</tr>
<tr>
<td>#21</td>
<td>p.P369L</td>
<td>M</td>
<td>399</td>
<td>−3.0 at 2.7 years</td>
<td>Small hypophysis</td>
<td>Growth hormone</td>
<td>Maternally/Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>#22</td>
<td>p.P369L</td>
<td>M</td>
<td>358&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
<td>No GH therapy</td>
<td>Growth hormone (no treatment)</td>
<td>Maternally/Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>#25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p.P369L</td>
<td>F</td>
<td>349&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
<td>Normal</td>
<td>No treatment</td>
<td>De novo/Yes</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>Female, MRI magnetic resonance imaging, AF male, NA not available
<sup>b</sup>She has a de novo mutation. She has refused endocrine testing. Her two daughters have GF and craniofacial phenotype, and one of them also has microhypophysis and GH deficiency diagnosed at 5 years of age.

hormone (GH), and GHRHR (MIM: 139191), encoding the receptor for GHRH<sup>2</sup>. GHD may also result from mutations in genes that encode transcription factors involved in pituitary development: HESX1 (MIM: 601802), OTX2 (MIM: 600037), SOX2 (MIM: 184429), SOX3 (MIM: 313430), LHX3 (MIM: 600577), PITX2 (MIM: 601542), PROPI (MIM: 601538), POUIF1 (MIM: 173110), and TCF7L1 (MIM:604652).<sup>1,3</sup> Some of these mutations are associated with additional pituitary hormone deficiencies and developmental abnormalities, such as variants of septo-optic dysplasia (MIM: 182230), ocular defects, ectopic posterior pituitary, skeletal defects, and intellectual impairment<sup>1</sup>.

Occurrence of growth retardation due to GHD in combination with gingival fibromatosis (GF), described hitherto in three families<sup>7-9</sup>, suggests a previously unidentified genetic cause of GHD. Here we report that this syndrome is caused by two missense mutations, c.347G>T p.(Arg116Leu) or c.1106C>T p. (Pro369Leu), in KCNQ1, a gene that encodes the alpha subunit of the voltage-gated ion channel Kv7.1, previously implicated in cardiac arrhythmia syndromes<sup>7-9</sup>. We demonstrate the expression of Kcnq1 in hypotalamic GHRH neurons and pituitary somatotrope cells. Both mutants increase current levels in patch clamp analyses, especially when co-expressed with the auxiliary KCNE2 β-subunit. Mutant KCNQ1s also lead to reduced pituitary hormone secretion from AtT-20 cells when coexpressed with KCNE2. Taken together, our results demonstrate that two missense mutations in KCNQ1 cause pituitary hormone deficiency and maternally inherited GF in humans.

**Results**

**Pedigree and genetic analysis.** We performed genome-wide linkage analysis in a large Finnish family<sup>4</sup> (Fig. 1a) with this disease, and found statistically significant linkage, logarithm of odds (LOD) score >3.3, between the studied phenotype and a locus on 11p15. The highest LOD score, 3.54, was reached with the marker rs7947959 (hg19 chr11:4784679). The most distal marker showing statistically significant linkage was located at 286 kb and the most proximal marker at 11.36 Mb. No other chromosomal areas reached LOD scores >3.0. We then performed whole-genome resequencing (Beijing Genomics Institute) in two affected family members. Novel variants in the linkage region shared between the two genomes, and not found in any databases (dbSNP, http://www.ncbi.nlm.nih.gov/SNP; Exome Variant Server, http://evs.gs.washington.edu/EVS/; 1000 Genomes, http://www.1000genomes.org/; ExAC, http://exac.broadinstitute.org/), were annotated with SNPnexus (http://www.snp-nexus.org/). The c.347G>T p.(Arg116Leu) missense mutation in KCNQ1 (MIM: 607542), shared between the two genomes, was left in the linkage region after known variants, variants in the noncoding regions, and the synonymous changes were filtered out. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicted this mutation to be deleterious with very high probability (score 0.993). The presence or absence of the mutation was verified by Sanger-sequencing in all 16 family members, and in four additional affected family members participating in the study after completion of linkage analysis. The mutation segregated with GHD and/or short stature in the family and was
absent from the Sisu database (The Sequencing Initiative Suomi project, containing over 10,000 Finnish samples, http://www.sisuproject.fi/). Sanger sequencing of the coding exons and exon–intron boundaries of the KCNQ1 gene in a second Finnish family (mother and twin boys) with GHD and GF revealed the T p.(Pro369Leu) mutation as de novo.

Phenotypic features of KCNQ1 mutation carriers. Detailed clinical histories of mutation carriers are provided in Supplementary Note 1, and the summary of phenotypic features are given in Table 1. Patients carrying either the p.(Arg116Leu) or the p.(Pro369Leu) mutation in KCNQ1 displayed a wide endocrine phenotypic spectrum that ranged from relatively mild (only low IGF-1 levels in patients #10 and #11, or GF in subject #14) through classic GHD to multiple pituitary hormone deficiencies (Supplementary Note 1, Table 1). Several mutation carriers had GF (Fig. 1b) and mild craniofacial dysmorphic features, but only when the mutation was inherited from the mother (Table 1). One patient had retinal pigmentation (Supplementary Fig. 1). We therefore verified the expression of KCNQ1 in the developing human retina (Fig. 1b).

KCNQ1 mutations are associated with alterations in the QT interval duration. Five of the 12 mutation carriers examined (42%, 95% confidence interval 20–68%) displayed a corrected QT interval below the 2nd percentile of age- and gender-matched reference values, and two of them fulfilled the diagnostic criteria for short QT syndrome (Table 1)10–12.

Molecular modeling of KCNQ1. We mapped the location of the two mutations to the 3D channel structure (Fig. 1c). KCNQ1 has six transmembrane segments (S1–S6) and intracellular N- and C-termini (CT). Arg116 is located in the N-terminus and Pro369 at the C-terminus. Both residues are proximal to the membrane13. Arg116 interacts with and Pro369 maps to an intracellular gating module, comprised of the proximal CT and constitutively bound CaM. The gating module directly connects to and regulates the activation gate in the sixth transmembrane segment (S6) that is responsible for opening and closing the channel pore14 (Fig. 1c).

Functional channel expression requires calmodulin (CaM), the ubiquitous calcium binding protein15,16 that serves as an obligate subunit of the channel complex, bound intracellularly. The biochemical properties of the Pro369Leu mutant was tested by using a pull-down assay of the intracellular CT to test binding of CaM. However, the Pro369Leu mutation does not compromise the structural integrity of the proximal C-terminal (CT)/CaM complex (Supplementary Figs. 2 and 3).

Patch-clamp analyses and heart phenotypes of patients. The electrophysiological properties of the mutated channels were examined in whole-cell patch-clamp analyses in HEK 293 cells, in which both mutated channels (p.Arg116Leu and p.Pro369Leu)
gave higher current levels than the wild-type (WT) Kv7.1 channels when expressed alone ($P < 0.05$ – $0.001$), and notably, the activation kinetics of KCNQ1-Arg116Leu was shifted to more depolarized values (Fig. 2). Potassium channels comprised of KCNQ1 and KCNE1 subunits are responsible for one of the primary cardiac repolarizing currents, the slow delayed rectifier current ($I_{Ks}$)\textsuperscript{17}, and mutations in KCNQ1 are known to cause the long QT syndrome\textsuperscript{7,8} (LQT1, MIM: 192500) and the rare short QT syndrome\textsuperscript{8,9} (MIM: 609621). The electro-physiological properties of the mutated channels were therefore also examined when KCNQ1 was co-expressed with KCNE1. Ectopically expressed KCNQ1-Pro369Leu/KCNE1 Kv7.1 channels gave profoundly increased currents (Fig. 2), whereas the currents produced by KCNQ1-Arg116Leu/KCNE1 Kv7.1 channels at $+60$ mV did not differ from the control, but still, the voltage dependence of activation kinetics were shifted to more depolarized potentials (Fig. 2). Co-expressing KCNQ1 with KCNE2, another $\beta$ subunit expressed in a number of other tissues together with KCNQ1\textsuperscript{18,19}, showed that both KCNQ1-Pro369Leu/KCNE2 and KCNQ1-Arg116Leu/KCNE2 increased the current levels ($P < 0.05$–$0.001$) (Fig. 2). Taken together, these results suggests that the Pro369Leu and Arg116Leu mutations impart subtle but distinct conformational changes to this module that favor the open channel state, in particular when assembled with KCNE2.

Expression of KCNQ1 in mouse and human. We studied the expression of KCNQ1 in growth-regulating hypothalamic and pituitary cell types in mice by using immunofluorescence and mRNA in situ hybridization techniques. KCNQ1 expression was also examined by RT-PCR in human hypothalamic and pituitary gland cDNA. Our results show that KCNQ1 is expressed in mouse somatotrope and gonadotrope cells in the postnatal pituitary (Fig.3a, b), and in the human pituitary (Supplementary Fig. 4a). Kcnq1 was also expressed in mouse hypothalamic GHRH neurons during development, and in the human hypothalamus (Fig. 3b and Supplementary Fig. 4b). Finally, Kcnq1 was expressed along the blood vessels of the developing mouse pituitary, and KCNQ1 was detected along the postnatal pituitary capillaries by immunostaining (Fig. 3a, c). We also verified the expression of KCNE2 in human hypothalamic and pituitary (Supplementary Fig. 4c).

**ACTH secretion assay and protein expression quantification.** The impact of KCNQ1 on pituitary hormone secretion was investigated by transiently overexpressing WT or one of the mutant KCNQ1s (p.Arg116Leu, p.Pro369Leu) in the well-characterized, ACTH-secreting mouse pituitary tumor cell line AtT-20/D16v-F2, widely used to investigate the regulation of exocytosis by voltage-gated calcium entry\textsuperscript{20,21}. The LQT1-causing

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**Fig. 3** KCNQ1 is expressed in cells of the mouse hypothalamic–pituitary growth hormone axis. a Immunofluorescence staining against KCNQ1 (red) and Growth Hormone (GH, green) reveals membranous expression of KCNQ1 in a subset of GH-expressing cells of the postnatal pituitary at day 17 (P17, yellow arrowhead) as well as expression in additional pituitary cell types (white arrowhead) and surrounding blood vessels (arrows). b Sensitive RNAscope in situ hybridization detects Kcnq1 mRNA (aqua) in Ghrh-expressing neurons of the developing hypothalamus (red) at 17.5 days post coitum (dpc), and confirms the expression in Gh-expressing somatotropes (red) at P17. Kcnq1 is expressed in additional endocrine cells types such as Lhb-expressing gonadotropes (red). Examples of double-positive cells are noted by yellow arrowheads. c Kcnq1 mRNA is detected in cells surrounding blood vessels of the developing anterior pituitary at 15.5 dpc. Scalebars indicate 100 $\mu$m in low magnification images and 20 $\mu$m in high magnification images. AL anterior lobe, BV blood vessel, Ht hypothalamus, IL intermediate lobe, 3v third ventricle.
Table 2 Mixed linear regression model estimates for ACTH concentrations (ng/ml) measured in diluted medium samples from cells transfected with KCNQ1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (WT KCNQ1)</td>
<td>7.26</td>
<td>0.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>0.76</td>
<td>0.25</td>
<td>0.004</td>
</tr>
<tr>
<td>KCNQ1-Arg116Leu</td>
<td>0.32</td>
<td>0.72</td>
<td>0.659</td>
</tr>
<tr>
<td>KCNQ1-Pro369Leu</td>
<td>0.62</td>
<td>0.72</td>
<td>0.393</td>
</tr>
<tr>
<td>KCNQ1-Gly589Asp</td>
<td>1.11</td>
<td>0.72</td>
<td>0.126</td>
</tr>
</tbody>
</table>

Mixed linear regression model estimates for ACTH concentrations (ng/ml) measured in diluted medium samples (1:10) in the reference condition (WT KCNQ1 and three other KCNQ1 conditions (KCNQ1-Arg116Leu, KCNQ1-Pro369Leu, KCNQ1-Gly589Asp), their standard errors (SE), and corresponding P values. The model included both the growth time (per 1h) and the KCNQ1 conditions as fixed effects and the experiment number as a random intercept. ACTH levels produced by the cells transfected with mutant KCNQ1s did not differ from the levels produced by the WT KCNQ1. Similarly, the CRF-induced ACTH secretion did not differ between the four KCNQ1 environments (data not shown).

Table 2 depicts the mixed linear regression model estimates for ACTH concentrations (ng/ml) measured in diluted medium samples from cells transfected with KCNQ1. The model included both the growth time (per 1h) and the KCNQ1 conditions as fixed effects and the experiment number as a random intercept. ACTH levels produced by the cells transfected with mutant KCNQ1s did not differ from the levels produced by the WT KCNQ1. Similarly, the CRF-induced ACTH secretion did not differ between the four KCNQ1 environments (data not shown).

Protein–protein interaction studies. We next examined the possibility that the two KCNQ1 mutations may disrupt critical protein–protein interactions. We explored by expressing KCNQ1 in Flp-In T-Rex 293 cells, and found 68 high-confidence interacting partners that were involved in processes such as spliceosomal snRNP assembly and intracellular trafficking (Supplementary Fig. 7a). However, the protein–protein interaction heatmap did not reveal gross differences among the strongest interactors between the WT KCNQ1 and KCNQ1-Arg116Leu or KCNQ1-Pro369Leu (Supplementary Fig. 7b).

Discussion

We identified two missense mutations in KCNQ1 to underlie childhood onset of GHD and maternally inherited gingival fibromatosis. Very recently, maternally inherited variation in the KCNQ1 locus was shown to associate with reduction in adult height in the Sardinian population22. Inheritance of GHD in our families, however, followed an autosomal dominant pattern, and similarly, a lack of a parent-of-origin effect has been described in the long QT syndrome caused by loss-of-function KCNQ1 mutations23. The KCNQ1 locus resides in the imprinted region of chromosome 11p15, and during early ontogenesis, KCNQ1 is paternally imprinted. In our patients, only those with a maternally inherited KCNQ1 mutation displayed congenital GF, and several of them also had mild craniofacial dysmorphic features that were mitigated by adulthood, suggesting loosening of KCNQ1 imprinting in cranial tissues with age, similarly as reported to occur in the pancreas24. The two twin pairs (monozygotic twins #7 and #8 and dizygotic twins #21 and #22), present in our families, are prismatic for understanding the phenotypic variability: subject #21 was smaller than his brother at birth, and had a more severe endocrine phenotype (Supplementary Note 1, Table 1). Similarly, subject #7, who was born breech and smaller than his monozygotic twin brother, had more severe growth failure during childhood (Supplementary Note 1, Table 1). Thus, environmental factors are likely to modify the endocrine phenotype of this syndrome. We also found a spectrum of corrected QT intervals in the mutation carriers, which indicates incomplete penetrance and variable expressivity of the mutations in the heart, similarly as described for long QT syndrome due to loss-of-function KCNQ1 mutations25.

Our results show that KCNQ1 is expressed in mouse somatotrope and gonadotrope cells in the postnatal pituitary and in the human pituitary. Indeed, previous findings have implicated voltage-gated potassium channel currents in pituitary cells in different species26–28. KCN1 was also expressed in mouse hypothalamic GHRH neurons during development, and in the human hypothalamus. These findings raise the possibility that the Arg116Leu and Pro369Leu KCNQ1 mutations may impact hypothalamic–pituitary function at multiple levels; in the case of the growth hormone axis, both on the control of episodic GHRH secretion by hypothalamic neurons as well as on somatotroph function during growth. Kcnq1 was also expressed along the blood vessels of the developing mouse pituitary, and KCNQ1 was detected along the postnatal pituitary capillaries by immunostaining. Somatotropes are organized in the close vicinity of pituitary blood vessels29, and GHRH enhances the oxygen supply to the somatotrope network via increased capillary blood flow29,30. The anterior pituitary is richly vascularized by fenestrated capillaries emanating from the pituitary portal system, and direct arterial supply has also been demonstrated in human pituitary adenomas31–33. Although voltage-gated potassium channels, including KCNQ1, are implicated in the vascular physiology34, their possible role, if any, in the regulation of pituitary blood flow is currently unknown.

Although the exact mechanism by which the two KCNQ1 mutations cause pituitary hormone deficiency in humans is unclear, our data suggest that the KCNQ1–KCN2 complex may play a role in it. Co-expressing KCNQ1 with KCN2 β-subunit showed that both KCNQ1 mutants increased current levels in patch clamp analyses and were associated with reduced pituitary hormone secretion from AtT-20 cells. Both KCN1 and KCN2 have been implicated in exocytosis35,36, and our electrophysiological and hormone secretion data suggest that the p. Arg116Leu and p-Pro369Leu mutations increase potassium conductance, which via hyperpolarization leads to diminished hormone secretion. The presence of KCN2 expression in human hypothalamus and pituitary tissues is in agreement with the previously observed expression of Kcn2 in rodents37,38, and thus lends credence to the above hypothesis. Intriguingly, KCN2 can regulate several different Kv channels, and plays an important role in the endocrine system. Kcn2 deletion causes hypothyroidism in pregnant and lactating mice and in their pups38, and very recently Kcn2−/− mice were reported to have impaired glucose-induced insulin secretion36. In light of the mutations’ location in the 3D channel structure, the electrophysiological findings, and our observation that neither mutation compromises the integrity of the proximal CT/CaM gating module, i.e., CaM association with the CT, we conclude that the Pro369Leu and Arg116Leu mutations probably favor the open channel state especially when assembled with the KCN2 β subunit.
The protein–protein interaction heatmap did not reveal gross differences among the strongest interactors between the WT KCNQ1 and KCNQ1–Arg116Leu or KCNQ1–Pro369Leu. Thus, rather than resulting from a specific protein–protein interaction defect(s), this syndrome may primarily arise from altered potassium channel balance in different cell types, with variable manifestations depending on the interplay with environmental factors. Indeed, patients that carry mutations in other potassium channels share phenotypic similarities with our patients: a craniofacial phenotype, GHD and hypogonadotropic hypogonadism have been reported in a patient with Cantù syndrome (MIM: 239850) phenotype, GHD and hypogonadotropic hypogonadism have been reported in a patient with Cantù syndrome (MIM: 239850). 

**Linkage analysis.** We performed genome-wide single-nucleotide polymorphism (SNP) genotyping of 16 family members (Illumina HumanOmniExpress microarray, Institute for Molecular Medicine Finland) of the large Finnish pedigree. Two-point parametric linkage analysis was performed using Pseudomarker 1.0.6 software with FASTLINK 4.1P linkage package. Individuals with GF and/or GHD with short stature or low insulin-like growth factor 1 (IGF-1) levels were treated as affected; all others were considered healthy. A dominant model of inheritance with nearly complete penetrance was assumed: disease allele frequency was set to 0.0001 with a penetrance rate of 0.999 and a phenocopy rate of 0.001. Allele frequencies were calculated from the genotypes of all individuals.

**Segregation analyses and sanger sequencing of KCNQ1.** The Arg116Leu missense mutation in KCNQ1 was Sanger-sequenced in all 16 family members and in four additional affected family members participating in the study after completion of linkage analysis. The mutation was also screened in 200 healthy controls (anonymous blood donors from the Finnish Red Cross Blood Service). Additional rare variants shared between the two genomes, upstream and downstream of KCNQ1, were genotyped in the family to construct haplotypes. Coding exons and exon–intron boundaries of KCNQ1 were then sequenced in the second Finnish family (mother and twin boys), as well as in the index patient from the family originating from Argentina and her parents (Fig. 1a). The primers used in the Sanger sequencing of KCNQ1 are listed in Supplementary Table 1. PCR conditions are available upon request.

**Electrophysiological studies.** The electrophysiological properties of the Arg116Leu and Pro369Leu KCNQ1 mutants were studied by whole-cell patch clamping, as described. In brief, the two mutations were introduced into the pXOOM–hKCNQ1 expression plasmid. HEK293 cells were transiently cotransfected with 1 μg pXOOM–hKCNQ1 (WT or mutants), 1 μg pcDNA3–hKCNE1/hKCNE2 for the KCNQ1/KCNE experiments, and 0.2 μg of pcDNA3–eGFP as a reporter gene, using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Patch-clamp experiments were performed at room temperature (20–22 °C) 2–3 days after transfection. Patch-clamp recordings were conducted using an internal solution containing the following (mM): KCl (140), Na2-ATP (1), EGTA (2), HEPES (10), CaCl2 (0.1), MgCl2 (1), and D-glucose (10), pH 7.4 with KOH; external solution NaCl (140), KCl (4), CaCl2 (2), MgCl2 (1), HEPES (10), and D-glucose (10), pH 7.3 with NaOH. Cells expressing WT or mutant KCNQ1 potassium channels, detected by green fluorescence, were subjected to voltage clamping to detect potential changes in activation and inactivation kinetics. 

**Statistics for electrophysiological studies.** For electrophysiological studies, mean ± SEM values are shown. Statistical significance was evaluated by two-way analysis of variance (ANOVA) with Bonferroni correction. P < 0.05 was considered statistically significant.

**ACTH secretion studies and western blotting.** AT20–2016tvs–F2 cells (Sigma) were maintained in DMEM (Sigma) supplemented with 10% FBS (v/v) (HyClone), penicillin (25 U/mL, Sigma), streptomycin (25 μg/mL, Sigma), and 2 mM glucose (Invitrogen). At the beginning of each experiment, the cells were seeded on 24-well 

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### Table 3 Mixed linear regression model estimates for ACTH concentrations (ng/ml) measured in diluted medium samples from cells transected with KCNQ1 and KCNE2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (WT KCNQ1/KCNE2)</td>
<td>9.59</td>
<td>0.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>0.62</td>
<td>0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KCNQ1–Arg116Leu/KCNE2</td>
<td>-1.88</td>
<td>0.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KCNQ1–Pro369Leu/KCNE2</td>
<td>-1.29</td>
<td>0.42</td>
<td>0.004</td>
</tr>
<tr>
<td>KCNQ1–Gly589Asp/KCNE2</td>
<td>-0.77</td>
<td>0.42</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Mixed linear regression model estimates for ACTH concentrations (ng/ml) measured in diluted medium samples (110) in the reference condition (WT KCNQ1/KCNE2) and three other KCNQ1 conditions (KCNQ1–Arg116Leu/KCNE2, KCNQ1–Pro369Leu/KCNE2, KCNQ1–Gly589Asp/KCNE2), their standard errors (SE), and corresponding P values. The model included both the growth time (per 1 h) and the KCNQ1/KCNE2 conditions as fixed effects and the experiment number as a random intercept. Based on the regression model, the KCNQ1–Arg116Leu/KCNE2 produced 20% (~2h) or 17% (0 h) less ACTH than the reference (WT KCNQ1 with KCNE2) (P < 0.001). Similarly, the KCNQ1–Pro369Leu/KCNE2 produced approximately ~12% less ACTH (~0.001) than the reference environment. ACTH levels produced by the cells transected with the LQT1 mutant KCNQ1–Gly589Asp/KCNE2, which served as a control, did not differ significantly from the reference environment. 

The CRF-induced ACTH responses between the four KCNQ1/KCNE2 conditions did not differ (data not shown).

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**Methods**

**Ethics.** This study was approved by the ethics committees of the Oulu University Hospital, Finland, and the Helsinki University Central Hospital, Finland. The DNA from family members recruited in Switzerland was obtained by permission of the Swiss Society of Medical Genetics. All participants gave written informed consent.

**Subjects.** Members of three unrelated families were enrolled: a large Finnish family, a second Finnish family, and a Swiss family originating from Argentina. Their phenotypes were ascertained by physical examination, medical records, or, occasionally, self-report. Phenotypic information on patients 5–9 (Fig. 1) was described earlier. DNA was extracted from peripheral blood leukocytes of the subjects (once from saliva, DNA Genotek, Inc.). A 12-lead ECG was obtained from 12 mutation carriers for evaluation of conduction times. Corrected QT interval (QTc) was calculated from the QT and inter-beat (RR) interval using Bazett’s correction (QTc=QT/RR0.5). Hormone measurements were done in the clinical laboratory.
plates at a density of 90,000 cells per well. The plates were incubated overnight at 37 °C in a humidified atmosphere of 3% CO₂–97% air, and transfected using Lipofectamine 3000 transfection reagent (Invitrogen). According to manufacturer’s instructions. Each well received either 500 ng of pXOOM-hKCNQ1 (WT or mutants Arg116Leu, Pro369Leu, Gly589Asp) alone (WT KCNQ1, KCNQ1-Arg116Leu, KCNQ1-Pro369Leu, or KCNQ1-Gly589Asp), and 1:3000 goat anti-mouse IgG (H+L)-HRP conjugate secondary antibody (C4, sc-47778, Santa Cruz Biotechnology) and 1:3000 goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (#170651-S6, Bio-Rad) (Supplementary Fig. 5). To control for total protein loaded on the gel, the blot was stripped with Restore Western Blot Stripping Buffer (Millipore) and β-actin was detected with β-actin mouse monoclonal α-β-actin antibody (C4, sc-47778, Santa Cruz Biotechnology) and 1:3000 goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (#170651-S6, Bio-Rad). KCNQ1 expression was quantitated from three independent replicates by using Image Studio Lite software (LI-COR Biotechnology). Brieﬂy, the relative density of bands in relation to WT KCNQ1 was calculated, and the values were adjusted by using the relative density of β-actin of the corresponding lane.

Statistical analysis of ACTH secretion studies. Two different linear mixed models were used to explain the ACTH levels measured in diluted (1:10) cell culture media samples that were obtained after 16 h (~2 h) and 18 h (0 h) of serum starvation. In the first model, the cells had been transfected with the four different KCNQ1s alone (WT KCNQ1, KCNQ1-Arg116Leu, KCNQ1-Pro369Leu, or KCNQ1-Gly589Asp), and, in the second model, the cells had been transfected with one of the four KCNQ1 constructs along with the construct encoding the beta subunit KCN2E. In both models, the experiment number was included as a random intercept, and the KCNQ1 environment (WT KCNQ1, KCNQ1-Arg116Leu, KCNQ1-Pro369Leu, or KCNQ1-Gly589Asp), and time (~2 h, 0 h) were included as fixed effects. In both models, the WT KCNQ1 was used as the reference KCNQ1 environment. The residual plots were inspected for deviations from normality and homoscedasticity, and the model assumptions were tested. For Western blotting, cells transfected with 250 ng of pXOOM-hKCNQ1 (WT or mutants) together with 250 ng of pcDNA3-hKCN2E were collected and combined from duplicate wells 48 h after transfection and lysed in 2× Laemmli buffer and an equal volume of 8 M urea was added. Lysates were sonicated, incubated at 37 °C for 30 min, and loaded on a 4–20% gradient gel (Bio-Rad). The proteins were transferred onto a nitrocellulose membrane and detected with 1:1000 rabbit polyclonal anti-β-actin antibody (transfected Western blotting, cells), incubated at 37 °C for 30 min, and loaded on a 4–20% gradient gel (Bio-Rad). The proteins were transferred onto a nitrocellulose membrane and detected with 1:1000 rabbit polyclonal anti-β-actin antibody (transfected Western blotting, cells) and puriﬁed from the hypothalami of 30 male/female Caucasians, aged 15–68 years, (after sudden death), or, as a positive control, from the cardiac cDNA library of a 59-year-old male donor (Amosbi) with cDNA-speciﬁc primers (Supplementary Table 1, HuKCNQ1_F+R). β-actin served as a reference gene. Similarly, a 186-bp fragment of KCNQ1 cDNA was PCR-ampliﬁed from human pituitary gland cDNA (cDNAs from normal pituitary gland, pooled from 12 male/female Caucasians, aged 18–52; cause of death: trauma) using cDNA-speciﬁc primers (Supplementary Table 1, HuKCNQ1_F2+R2). GAPDH served as a reference gene. Additionally, a 172-bp fragment of KCN2 DNA was ampliﬁed from the human pituitary gland cDNA and the human hypothalamic cDNA with cDNA-speciﬁc primers. The PCR products were visualized on 1.5% agarose gel (Supplementary Fig. 4).

Cloning of the KCNQ1 expression vectors for AP-MS. To generate Gateway entry clones from Human KCNQ1 and the Arg116Leu, Pro369Leu, Gly589Asp mutants, flanking attB sites were generated using PCR, followed by Gateway BP reaction. The corresponding entry clones were then used to generate C-terminally tagged expression vectors (pcDNAs/FRT/TO/BioID/Strep/HA/GW) via Gateway LR recombination.

Affinity puriﬁcation. For in vivo puriﬁcation, tetracycline-inducible Flp-In T-Rex 293 cell lines expressing KCNQ1 wild type and other mutations were generated as described56. In brief, the cells were co-transfected with the tagged-KCNQ1 expression constructs and pOG44 vector (for Flp-recombinase expression, Invitrogen) using FuGENE 6 transfection reagent (Promega). After 2 days, transfected cells were selected in Hygromycin B (100 mg/ml; Invitrogen) containing medium for 3 weeks. The positive clones containing stable isogenic tagged KCNQ1 (wt and mutants) were expanded and for each construct ~5 × 10^7 cells (5 × 15 cm dishes) in three biological replicates were induced with 1 μg/ml doxycycline (MP Biomedicals) for 24 h. After induction, cells were washed with 0.1 mM MgCl₂, 0.1 mM EDTA and PBS, and pelleted for 10 min at room temperature. The total mRNA of the cells (500 μg) was isolated and reverse transcribed to cDNA using RevertAid Reverse Transcripase (Thermo Fisher, Cat. No. K1620) before cDNA samples were sent to Microsynth (Switzerland) for high-resolution in FTMS scan preview mode was used.

Mass spectrometry. For liquid chromatography–mass spectrometry (LC–MS/MS) samples were prepared as follows: cysteine bonds were reduced with 5 mM Tris (2-carboxyethyl)phosphine (TCEP) (Sigma–Aldrich) for 20 min at 37 °C and alkylated with 50 mM iodoacetamide (Sigma–Aldrich) for 30 min. cDNA (human hypothalamus, random brains pooled from 12 male/female Caucasians, age 15–68 years, after sudden death) was purified with Agencourt PSB for 20 min at room temperature. A total of 1 μg/μl trypsin (Promega) was added and samples digested overnight at 37 °C. Samples were quenched with 10% trifluoroacetic acid (TFA) and purified with C-18 Micro SpinColumns (The Nest Group) eluting the samples to 1% TFA in 50% acetonitrile (ACN). Samples were dried by vacuum concentration and peptides were reconstituted in 30 μl buffer A (0.1% TFA and 1% ACN in LC–MS grade water) and vortexed thoroughly. LC–MS/MS analysis was performed on an Orbitrap Elite ETD hybrid mass spectrometer using the Xcalibur version 2.2 SP 1.48 coupled to EASY-nLC II–MS (LC–MS/MS) system (all from Thermo Fisher Scientific) via a nanoelectrospray ion source. In total, 6 μl and 5 μl of peptides were loaded from StepTag and BioID samples, respectively. Samples were separated using a two-column setup consisting of a C18 trap column (EASY-Column 2 cm × 100 μm, 5 μm, 120 Å, Thermo Fisher Scientific), followed by C18 analytical column (EASY-Column 10 cm × 75 μm, 3 μm, 120 Å, Thermo Fisher Scientific). Peptides were eluted from the analytical column with a 60 min linear gradient from 5 to 35% buffer B (buffer A: 0.1% FA, 0.01% TFA in 1% acetonitrile; buffer B: 0.1% FA, 0.01% TFA in 98% acetonitrile). This was followed by 5 min 80% buffer B, 1 min 100% buffer B followed by 9 min column wash with 100% buffer B at a constant flow rate of 300 nl/min. Analysis was performed in data-dependent acquisition mode where a high resolution (m/z 500-1500, FTMS full scan (m/z 300–1700) was followed by FTMS scans (energy 35) in ion trap. Maximum fill time allowed for the FTMS was 200 ms (Full AGC target 1,000,000) and 200 ms for the ion trap (MSn AGC target 50,000). Precursor ions with more than 500 ion counts were allowed for MSn. To enable the high resolution in FTMS scan preview mode was used.

Data processing. SEQUEST search algorithm in Proteome Discoverer software (Thermo Fisher Scientific) was used for peak extraction and protein identification with the human reference proteome of UniProtKB/SwissProt database (www. uniprot.org). Allowed error tolerances were 15 ppm and 0.8 Da for the precursor.
and fragment ions, respectively. Database searches were limited to fully tryptic peptides allowing one missed cleavage (in peptide mapping semi-tryptic with one missed cleavage allowed), and carbamidomethyl +57.021 Da (C) of cysteine residue was set as fixed, and oxidation of methionine +15.995 Da (M) as dynamic modifications. For peptide identification FDR was set to <0.05.

The high-confidence protein–protein interactions were identified using stringent filtering against control contaminant database. The bait normalized relative protein abundances (% to the bait) were calculated from the spectral counts. Each average and SD was calculated from three biological replicates. The confidence protein–protein interactions data were imported into Cytoscape 3.4.0 for the visualization combined with the known protein-protein interactions from Protein Interaction Network Analysis. Expanding the spectrum of mutations in GH1 and GHRHR: genetic screening in a large cohort of patients with congenital isolated growth hormone deficiency. J. Clin. Endocrinol. Metab. 94, 3191–3199 (2009).


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