
Genetic Overlap in Kallmann Syndrome, Combined Pituitary Hormone Deficiency, and Septo-Optic Dysplasia.


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Genetic overlap in Kallmann syndrome, combined pituitary hormone deficiency, and septo-optic dysplasia


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Precis: Human mutations identified in FGFR1, FGF8, and PROKR2 support a genetic overlap between Kallmann syndrome, combined pituitary hormone deficiency, and septo-optic dysplasia.
ABSTRACT

Context: Kallmann Syndrome (KS), combined pituitary hormone deficiency (CPHD), and septo-optic dysplasia (SOD) all result from development defects of the anterior midline in the human forebrain.

Objective: To investigate whether KS, CPHD, and SOD have shared genetic origins.

Design and Participants: 104 patients with either CPHD (n=36) or SOD (n=68) were investigated for mutations in genes implicated in the etiology of KS (FGFR1, FGF8, PROKR2, PROK2 and KAL1). Consequences of identified FGFR1, FGF8, and PROKR2 mutations were investigated in vitro.

Results: Four patients with CPHD/SOD had heterozygous mutations in FGFR1; these were either predicted to affect splicing (c.336C>T, p.T112T) or were shown to alter receptor signaling (p.S450F, p.P483S, p.M532T). One patient had a synonymous change in FGF8 (c.216G>A, p.T72T) that was shown to affect splicing and ligand signaling activity. Four patients carried heterozygous rare loss-of-function variants in PROKR2 (p.R85G, p.R85H, p.R268C).

Conclusions: Mutations in FGFR1/FGF8 and PROKR2 contributed to 4.8% and 3.8% of our patients with CPHD/SOD respectively. These data suggest a significant genetic overlap between conditions affecting the development of anterior midline in the human forebrain
INTRODUCTION

In the developing vertebrate embryo, the preplacodal field arises at the edge of the neural plate adjacent to the neural crest, and its derivatives ultimately give rise to several neuronal and non-neuronal head structures (1). Cells within the preplacodal field then separate into individual placodes (epithelial thickenings), of which the most anterior are the adenohypophyseal, lens, and olfactory placodes (1). The adenohypophyseal placode gives rise to Rathke’s pouch and thus to the intermediate and anterior pituitary lobes (2). The olfactory placode gives rise to several different cell types, including vomeronasal neurons, support cells, mucous-producing cells, and gonadotropin-releasing hormone (GnRH) neurons (1). As with overall forebrain development, these developmental processes are orchestrated by strict spatiotemporal expression and regulation of multiple transcription factors and signaling molecules (2).

Mutations in the transcription factors SOX2, SOX3, HESX1, and OTX2, have been identified in patients with septo-optic dysplasia (SOD), a disorder characterized by pituitary hormone deficiencies, optic nerve hypoplasia, and midline defects including agenesis of the septum pellucidum and/or corpus callosum (3, 4). Further, mutations in transcription factors PROP1, POU1F1, LHX3, and LHX4 underlie combined pituitary hormone deficiency (CPHD) (5). However, such mutations only account for a small percentage of all CPHD/SOD cases indicating that additional genetic causes remain to be discovered.

A different set of genes have been implicated in Kallmann syndrome (KS), which is defined as idiopathic hypogonadotropic hypogonadism (IHH) and anosmia/hyposmia. These genes include KAL1, FGFR1, FGF8, PROK2, and PROKR2 (6), which play critical roles in GnRH neuron ontogeny and the developing olfactory system (7). KS manifests as absent or
incomplete puberty, sexual immaturity, and infertility, and variably with additional nonreproductive phenotypes, including midline malformations (8, 9).

FGF8/FGFR1 signaling is critical for the differentiation of the preplacodes into both the olfactory placode, and the developing pituitary gland. Further, \textit{FGFR1} and \textit{FGF8} are expressed in Rathke’s pouch and in the ventral diencephalon, respectively (10); finally, murine transcriptome data have identified members of the FGF8 signaling network during pituitary development (11). Based on these lines of evidence, we hypothesized that mutations in genes underlying KS could also underlie CPHD and/or SOD.

**SUBJECTS & METHODS**

\textit{Patients and control subjects}

A total of 104 patients with either sporadic CPHD (n=36) or sporadic SOD (n=68) were included in the study. Patients were recruited at 2 medical centers in the United States (Johns Hopkins University Medical Center, Baltimore, MD, and Massachusetts General Hospital, Boston, MA) and 2 in the United Kingdom (London Center for Pediatric Endocrinology and Diabetes based at Great Ormond Street Hospital for Children and University College London Hospitals, and Royal Victoria Infirmary, Newcastle-upon-Tyne). Patients with SOD exhibited optic nerve hypoplasia, agenesis of the corpus callosum and/or septum pellucidum on radiologic examination with or without pituitary hormone deficiencies (12). CPHD was diagnosed as a deficiency of at least two pituitary hormones. Control subjects were unaffected adults, as determined by history and physical examination (n=268). The ethics committees of participating institutions approved the study, and written informed consent was obtained prior to participation from subjects/parents/guardians.
DNA sequencing

The coding exonic regions and intron-exon boundaries of FGF8 (NG_007151.1; NM_033163), FGFR1 (NG_007729; NM_023110), KAL1 (NG_007088; NM_000216), PROK2 (NG_008275.1; NM_021935), and PROKR2 genes (NG_008132; NM_144773) were amplified by PCR and sequenced in both directions at Polymorphic DNA Technologies (Alameda, CA). Nucleotide sequence variations seen on both strands were confirmed in independent PCRs. Genetic variations are described using standard nomenclature (13). Nucleotide changes were considered mutations of potential functional importance if: (i) they were absent from the 268 genotyped unaffected control subjects, and (ii) they were absent from the database of Single Nucleotide Polymorphisms (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/) and from the 1000 genomes database (http://browser.1000genomes.org/index.html), or if their minor allele frequencies in these databases were <0.05. Proband had previously been screened for known loci for CPHD/SOD (see case descriptions in supplemental material). The potential functional impact of identified variants was assessed using 3 prediction software programs, PolyPhen-2 (14) (http://genetics.bwh.harvard.edu/pph2/), SIFT (15) (http://sift.jcvi.org/www/SIFT_BLink_submit.html), and Mutation Taster (16) (http://neurocore.charite.de/MutationTaster/index.html). The potential effect of synonymous mutations on splicing was assessed using the software Human Splicing Finder (17) (http://www.umd.be/HSF/).

Functional consequences of FGFR1 mutations

The S450F, P483S, and M532T mutations were introduced into the previously described FGFR1 expression construct (18), which incorporates an N-terminal myc-tag for antibody detection.
Expression and maturation patterns of mutant FGFR1. The cell surface expression of WT and mutant FGFR1 receptors was quantified in intact transiently transfected COS7 cells using a previously described antibody binding-assay (18). Experiments were performed in quadruplicate and were repeated 4 times. Mean expression levels of mutants were expressed as a percentage of WT and compared using Kruskal-Wallis test with Dunn’s multiple comparison post-hoc analysis. The effects of the identified FGFR1 mutations on synthesis and folding of the receptor were assayed in COS-7 cells transiently transfected with WT or mutant FGFR1 constructs, using glycosylation and Western blot analysis, as previously described (18). Total receptor expression levels were determined from PNGase F-treated samples and were normalized to β-actin levels. To quantify receptor maturation, the density of upper (Endo Hf resistant, mature) and lower (Endo Hf sensitive, immature) FGFR1 bands were determined individually from the Endo Hf-treated samples and the amount of the mature band was expressed relative to the total amount.

 Reporter gene transcription. Ligand-stimulated transcription activity downstream of WT and mutant FGFR1 receptors were assessed in L6 myoblasts (American Type Culture Collection, Manassas, VA) using the osteocalcin FGF response elements (OCFRE) firefly luciferase reporter, as previously described (18). Because constitutively active control reporters (e.g., TK-renilla luciferase) are also induced by FGFs (Orniz D., personal communication and Y. Sidis, unpublished observation), the measurements were not normalized by co-transfecting such reporters. Experiments were performed in triplicate and were repeated 4 times. Mean luciferase response values were plotted as a percentage of WT using Prism5 software (GraphPad, LaJolla, CA). Dose response curves were fitted using a 3-parameter sigmoidal model and curve tops were compared using the extra sum of squares F test.
Kinase trans-phosphorylation assay. The tyrosine trans-phosphorylation activities of the WT and M535T mutant FGFR2 (analogous to FGFR1 M532T) tyrosine kinase (TK) domains were compared using a continuous spectrophotometric assay, as previously described (19). We chose to study the impact of the M532T mutation in context of the highly homologous FGFR2 intracellular domain because the expression level of the soluble recombinant FGFR2 intracellular domain is much greater than that of FGFR1 (Mohammadi M., unpublished observation). In the assay, the consumption of the phosphate donor ATP is coupled to the oxidation of NADH to NAD⁺, measured as a reduction in NADH absorbance at 340 nm. 5 μM of recombinant WT or mutant FGFR2 TK domains were incubated in 50 μl of 100 mM Tris-HCl pH 7.5 buffer containing 1 mM ATP, 5 mM MgCl₂, 1.5 mM phosphoenolpyruvate, 1.2 mg/ml NADH, 89 units/ml pyruvate kinase, and 124 units/ml lactate dehydrogenase at 30°C, and reduction in NADH absorbance was monitored over 1 hour.

Functional consequences of FGF8 mutations

Generation of FGF8 WT and T72T mini-gene constructs. A FGF8 mini-gene was constructed in two steps. First, a human genomic DNA fragment encompassing FGF8 exons 1a, 1b and 1d (including 91bp of 5' UTR and 571bp of intron 1d, figure 3A) was amplified by PCR, using primers 5'- cgagaattCCTCCGCTCGCCCTGCTCAG -3' and 5'- gggtgcggccgcCGGCAGTAGCTCTCCGACTTGCC -3' (nucleotides added to form restriction sites are in lower case) and was cloned into the EcoRI/NotI sites of pCDNA3.1mycHis (Invitrogen Corporation, Carlsbad, California). A second fragment encompassing exons 2 and 3 (including 126bp of intron 1d, intron 2 and only the coding sequence of exon 3; Figure 3A) was similarly generated using primers 5'- ccageggeccgcAGCTGGGCTGGAGCTGGAGTCCATG-3' and 5'-
gcgtctagaTCGGGGCTCGGGGGCCCAAG -3' and was ligated into the NotI/XbaI sites of the
construct to generate FGF8MGmycWT. This was then used as template to generate a c.216
G>A (T72T) mutant version using QuikChange XLII Site-Directed Mutagenesis Kit
(Stratagene, La Jolla, CA). All constructs were confirmed by sequencing from both directions.

Reporter gene transcription. FGF8 WT or T72T mutant mini-gene construct DNA (125ng)
was transfected into HEK293 cells together with AP1-Luc reporter (5ng) (Clontech, Mountain
View, CA) in 24-well trays, and luciferase activity was assayed 42h later, as described (18).
Experiments were performed in duplicate and were repeated 3 times. Reporter activities were
normalized to WT and the FGF8 T72T and WT mini-genes were compared using Student’s t-
test.

Relative expression of FGF8 isoform transcripts. HEK293T cells were transiently transfected
with 100ng of FGF8MGmycWT or FGF8MGmycT72T constructs using Fugene6 according
to manufacturer recommendations. Total RNA was extracted using TRIZOL reagent
(Invitrogen), and purified using RNeasy Mini Kit (Qiagen) according to the manufacturers’
recommendations. 3µg of total RNA were treated with DNAsel (Life Technologies) to
remove any residual DNA, and then reverse-transcribed into cDNA using SuperScript III
First-Strand Synthesis System (Invitrogen) with oligo-(dT) priming. Relative expression
levels of FGF8 isoforms transcripts were assayed in triplicates by Real Time Quantitative
PCR using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara,
CA.) according to the manufacturer’s instructions. Isoform-specific primers used were: 5’-
GCCTCCAAGCCCAGCATG-3’, 5’-GCCTCCAAGCCCAGGTAAC-3’, 5’-
GGTGTCTCCCAACAGCATGTG-3’ and 5’-GGTGTCTCCCAACAGGTAACTG-3’ for
FGF8a, FGF8b, FGF8e, and FGF8f, respectively, each with the common reverse primer 5’-
GCGGCTGTAGAGTTGGTAGGTCC-3' (Figure 3B). To detect total FGF8 transcripts we used primers mapping to exons 2 and 3 in a region shared by all isoforms (5'-GACCCCTTCGCAAAGCTCATC-3' and 5'-GCCTTTGCCGTTGCTCTTGG-3') (Figure 3B). All primers were designed to cross exon boundaries to ensure detection of spliced transcripts only. Relative transcript levels were calculated with respect to WT by the delta-delta CT method using total FGF8 transcript as the calibrator. Experiments were performed in triplicate and were repeated 4 times. The mRNA expression levels of each FGF8 transcript were compared to WT using Student’s t-test.

**Functional consequences of R85G PROKR2 mutation**

*Mutagenesis, total and cell surface expression.* The R85G PROKR2 mutation was introduced into the previously described expression construct (20) using QuikChange XLII Site-Directed Mutagenesis Kit (Strategene) as described for FGFR1 above. Total protein expression was assessed using Western blotting following transient transfection of HEK293 cells as described (20). For cell surface expression studies, WT and R85G PROKR2 cDNAs were tagged with 3XHA epitope at the N-terminus. COS7 cells were transiently transfected with 25ng of WT or R85G construct, and the expression of the receptor at the cell surface was quantified using anti-HA antibody (Clone HA-7, Sigma) as previously described (18). Experiments were performed in quadruplicate and repeated 3 times. Mean differences between mutant and wild-type receptor expression levels were evaluated by Student’s t-test.

*Reporter gene transcription and calcium mobilization.* Receptor-mediated activation of MAPK signaling was analyzed in transiently transfected HEK293 cells using the murine early growth response-1 (Egr-1) luciferase reporter assay as previously described (20). The impact of the R85G PROKR2 mutation on intracellular calcium mobilization in response to PROK2
stimulation was assayed in HEK293 cells using a calcium-sensitive aequorin-based luminescence assay as previously described (20). Experiments were performed in duplicate and repeated 3 times. Mean values were plotted as a percentage of WT and dose response curves were fitted and analyzed using Prism5 software as described above.

**Prokr2 expression in the mouse pituitary**

Prokr2-GFP transgenic mice have been previously described (21) and Prokr2 expression was examined by immunofluorescence, as previously reported (22). Briefly, female Prokr2-GFP transgenic mice were sacrificed at diestrous stage and 10µm coronal sections of the pituitary were blocked in PBS containing 0.2% Triton X-100 (PBST) plus 10% horse serum, incubated with mouse monoclonal anti-GFP (1:1000, Invitrogen) in PBST with 3% horse serum at 4°C overnight, and then probed with Alexa488-labeled goat anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA). For arginin vasopressin (AVP) staining we used rabbit anti-AVP (1:5000, Chemicon, Temecula, CA) as primary antibody, and Alexa594-conjugated goat-anti-rabbit (1:200, Invitrogen Carlsbad, CA) as secondary antibody. Sections were counter-stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and viewed with a Zeiss (Oberkochen, Germany) fluorescence microscope.

**RESULTS**

Among 104 patients with either combined pituitary hormone deficiency (CPHD, n=36) or septo-optic dysplasia (SOD, n=68), 5 unrelated probands (4.8%) had rare sequence variants in FGFR1 or FGF8, and 4 others (3.8%) had PROKR2 variants (Table 1). All mutations were heterozygous and in most cases DNA from the parents was not available so were unable to assess if the mutations were de novo. The probands’ phenotypic data are detailed in Table 2 and their case descriptions are provided in supplemental material. Notably, most probands
exibited a reproductive phenotype consistent with HH based on their neonatal presentation, as they are yet prepubertal.

**Patients with SOD harbor mutations in FGFR1 and PROKR2**

Three FGFR1 heterozygous mutations were identified in SOD probands (Table 1). Subject 1 harbors a synonymous change (c.336 C>T, p.T112T/wt) mapping to the end of exon 3 which encodes the C-terminal end of immunoglobulin-like domain 1 (Figure 1A). This change was not observed in 268 healthy controls, in the SNP database, or in the 1,000 genomes data set. The Human Splicing Finder software predicts that this variant generates a new exonic splicing enhancer binding site (TTACTTC) for the SRp40 splicing factor (score=79.46 [0-100]) and/or disrupts an overlapping putative exonic splicing enhancer octamer (CCTACTTC) (score=31.53). The second FGFR1 variant (c.1349 C>T, p.S450F/wt) maps to the intracellular domain of the receptor upstream of the kinase domain and the amino acid (S450) is highly conserved across vertebrates (Figure 1A). The S450F mutant FGFR1 exhibited total protein (Figure 1B) and receptor cell surface expression levels (Figure 1C) similar to WT, yet downstream signaling was severely compromised (Figure 1D). The third variant identified in FGFR1 (c.1447 C>T p.P483S/wt) maps to a highly conserved residue in the TK domain. Similar to S450F, P483S also exhibits normal expression levels but disrupted downstream signaling (Figure 1D). Of note, the affected amino acid residue is also mutated in a patient with Kallmann syndrome (c.1447 C>A, P483T, Pitteloud, unpublished data). The phenotypic data of the probands are presented in Table 2.

Three SOD probands carry variants in PROKR2 (Table 1). Interestingly, 2 unrelated SOD probands, one Caucasian and one African, were found to harbor the same PROKR2 variant (c.802 C>T/wt, p.R268C) (Table 1, Figure 2A). This R268C mutation has previously been
reported in association with both normosmic IHH and KS (23, 24) and is loss-of-function in vitro (24). The other PROKR2 variant (c.253 C>G/wt, p.R85G) is also predicted to be loss-of-function (Table 1); this was confirmed by Western blotting analysis which indicated a reduced total protein expression suggesting a defect in protein folding and stability (Figure 2B). Accordingly, cell surface expression was significantly reduced (Figure 2C) accompanied by a severe decrease in signaling via both calcium (figure 2D) and MAPK (Figure 2E) (log EC50 WT=-6.944±0.155, R85G=-6.319±0.267, P<0.05) cascades.

Patients with CPHD harbor mutations in FGFR1, FGF8, PROKR2, and KAL1

A rare variant in FGFR1 (c.1595 T>C, p.M532T/wt) was identified in a patient with CPHD. This amino acid maps to the TK domain, at a highly conserved residue across species (Figure 1A) and FGFRs, and is predicted to be deleterious (Table 1). The FGFR1 M532T mutant exhibited total protein (Figure 1B) and receptor cell surface expression levels (Figure 1C) similar to WT, but led to increased ligand-dependent signaling activity in the OCFRE-luciferase reporter assay (Figure 1D). We further validated this finding with a kinase trans-phosphorylation assay: compared to WT, FGFR2 M535T (equivalent to FGFR1 M532T) displayed increased autophosphorylation activity (Figure 1E).

A synonymous change in FGF8 (c.216 G>A p.T72T/wt) was identified in a CPHD proband. This nucleotide change, which maps to exon 1D (Figure 3B), is predicted by the Human Splicing Finder program (17) to compromise an exonic splicing enhancer site for the serine/arginine rich splicing factor SF2/ASF (25). To assess the possible splicing effects of this synonymous nucleotide change, we generated a minigene expression construct, which includes the entire FGF8 gene (exons 1A to 3) except for 2.4 Kb of intron 1D sequence (Figure 3B). This minigene was used to measure relative expression levels of the four FGF8
isoforms (Figure 3C) in transfected cells using RT-qPCR. Consistent with the software prediction, the e and f isoform transcripts (which incorporate an alternatively spliced exon, 1C) expressed from the mutant construct were significantly elevated as compared to WT (Figure 3C). To further assess the biological significance of the alterations in expression we evaluated minigene-induced activity in a luciferase transcription reporter assay, and found that the mutant construct displayed significantly higher activity as compared to WT (Figure 3D).

Additionally, a CPHD proband was found to harbor a mutation in \textit{PROKR2} (c.254 G>A/wt, p.R85H) inherited from his father. This residue is highly conserved across vertebrates (Figure 2A), has been reported in patients with Kallmann syndrome (24), and is loss-of-function \textit{in vitro} (26). Notably, the proband also harbors a rare variant in \textit{KAL1} (c.1375 C>T/wt) which he inherited from his mother. Being as yet of prepubertal age, it remains to be seen whether he will have hypogonadotropic hypogonadism in adult life, but he was born with microphallus, an important neonatal cue of GnRH deficiency. Additionally, a potential role for \textit{PROKR2} in pituitary ontogeny is supported by expression studies in transgenic mice expressing GFP under control of the \textit{prok2} promoter, in which GFP immunoreactivity was clearly visualized throughout pituitary structures, and was pronounced in the pars nervosa (Figure 2F). These findings support a previously unappreciated role for the prokineticin pathway in the pituitary gland.

**DISCUSSION**

We describe 9 prepubertal patients with CPHD/SOD carrying a heterozygous mutation in either \textit{FGFR1}, \textit{FGF8}, or \textit{PROKR2} genes with altered function. Thus, mutations in genes generally associated with IHH/KS may also be implicated in patients with CPHD/SOD,
demonstrating a genetic overlap between these syndromes. A number of observations also suggest a clinical overlap in these syndromes: patients with KS often display midline defects such as cleft lip and/or palate and corpus callosum anomalies and \textit{FGF8} mutations were recently found to be associated with recessive holoprosencephaly, craniofacial defects, and hypothalamo-pituitary dysfunction (10). Additionally, as early as in 1954, de Morsier described a syndrome of “dysplasie olfacto-génitale” which included agenesis of the olfactory bulbs, corpus callosum, and anterior commissure as well as infantile genitalia (27). The hypogonadotropic hypogonadism (HH) observed in both CPHD and SOD is thought to be pituitary in origin and environmental contributions to SOD have previously been noted (12). Yet, from the little that is known about the genetic causes of CPHD/SOD, it appears that these patients can harbor mutations in genes involved in multiple developmental processes, including pituitary development and possibly also GnRH neuron ontogeny. However, in the presence of a concomitant pituitary defect, verification of hypothalamic GnRH deficiency in these patients is difficult, if not impossible.

\textit{FGFR1} mutations identified in patients with SOD are loss-of-function, as has been reported in IHH/KS. In contrast, the \textit{FGFR1} and \textit{FGF8} mutations in the two probands with CPHD show enhanced downstream receptor signaling. The M532T FGFR1 mutant has increased ligand-dependent kinase activity, and the rare synonymous change in \textit{FGF8} leads to differential expression of \textit{FGF8} isoforms compared to WT, and enhanced FGFR1 signaling \textit{in vitro}. Thus far, gain-of-function \textit{FGFR1} mutations have only been reported in osteoglophonic dysplasia (28, 29) and Pfeiffer syndrome (30, 31). However, neither of the aforementioned CPHD patients harboring gain-of-function mutations exhibit bone phenotypes. It is important to note that gain- or loss-of-function of Fgf8 signaling during zebrafish development results in qualitatively similar brain phenotypes (32, 33) suggesting that zebrafish development is
exquisitely sensitive to perturbation of FGF8 signaling. Thus, it remains unclear whether CPHD is caused by increased (and not decreased) FGF signaling or, more broadly, by any significant perturbation of FGF signaling. Interestingly, 40% of patients with Apert syndrome, which is caused by activating FGFR2 mutations, have partial or complete absence of the septum pellucidum and 23% have corpus callosum defects (34), phenotypes also observed in SOD.

There are some precedents of IHH/KS sharing the same genetic basis as another developmental disorder. Mutations in CHD7 have been shown to underlie a majority of CHARGE syndrome cases as well as a small percentage of KS patients (35, 36). Similarly, a frameshift mutation in SOX2 associated with anophthalmia/microphthalmia in siblings was also found in their mother who only manifested IHH (37). This latter finding further supports that SOD and IHH could partially share a common genetic basis. Interestingly, Sox2 and Chd7 interact physically and cooperate to regulate a set of common target genes, several of which are themselves mutated in human syndromes with malformations also present in SOX2-associated anophtalmia syndrome or in CHARGE syndrome (38). Furthermore, deletion of Otx2, a locus for SOD, targeted to GnRH neurons results in a mouse model of HH providing additional support that the genetic networks underlying IHH/KS and CPHD/SOD overlap (39).

We also evaluated the PROK2/PROKR2 pathway in patients with CPHD/SOD, as these genes underlie KS. In contrast to FGF8/FGFR1, the role of prokineticin signaling in anterior midline and pituitary development is uncharted. We found 3 loss-of-function mutations in PROKR2 in 4 unrelated CPHD/SOD probands. The PROKR2 R268C variant has been described in heterozygous state in HH/KS patients, healthy first degree relatives of KS probands, as well
as 1 in 250 healthy controls (23, 40). We therefore propose that these mutations do not cause major midline defects *per se*. Rather, they may potentially act as modifier genes that fine-tune the phenotype primarily caused by more deleterious mutations in other genes. Alternatively, they may contribute to the phenotype through digenic inheritance, as previously demonstrated in IHH/KS (41-43). Finally, we provide evidence that *prokr2* is expressed in the adult mouse pituitary (Figure 2F). Further studies are needed to elucidate the role of PROKR2 signaling in pituitary ontogeny and midline development.

We report that heterozygous mutations in genes that are known to be implicated in the etiology of KS may be identified in patients with variable hypopituitary phenotypes, including CPHD and SOD. Given the overlap in phenotypes, our data suggest that mutations in genes that are implicated in early forebrain and hypothalamo-pituitary development may lead to variable phenotypes. While the variable penetrance of such mutations remains to be explained we found one subject (#9) to harbor rare variants in both *PROKR2* and *KAL1*. As the genetic basis for CPHD/SOD continues to be unraveled, it will be interesting to see if the digenic or oligogenic architecture observed in cases of KS (41-43) may in part explain the incomplete penetrance and variable expressivity observed in CPHD/SOD.

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Figure Legends

Figure 1: Schematic of FGFR1 protein with location of mutations underlying CPHD/SOD and their conservation across vertebrates and among FGFRs (A). S=signal peptide, D1-3=Immunoglobulin like domain 1-3, A=acidic box domain, TM=transmembrane domain, TK=tyrosine kinase domain. Western blotting analysis showing similar overall expression and maturation levels of mutant FGFR1 compared to WT (B). FGFR1 was detected using an anti-myc antibody and the blot was reprobed with heat-shock protein 90 (HSP90) to demonstrate equal loading. UT=untreated, PG=PNGase F treated, EH=endoglycosidase H treated. FGFR1 cell surface expression measured by radiolabeled antibody binding assay showed no differences between mutants and WT (C). EV=empty vector. Luciferase reporter assay using osteocalcin FGF response element reporter showing increased signaling activity by M532T and decreased activity by S450F and P483S (*** P<0.001) (D). FGFR2 M535T (corresponding to FGFR1 M532T) exhibits over 2.5-fold elevated kinase activity compared to WT (E).

Figure 2: Schematic of the structure of PROKR2 showing the location of the R85 and R268 residues and their conservation across vertebrates (A). Western analysis showing the overall expression of the mutant (R85G) is slightly diminished compared to WT (B). Expression of the R85G mutant at the cell surface is significantly reduced compared to WT (**p<0.001) in a radiolabeled antibody binding assay (C). The R85G mutant receptor is loss-of-function in both the calcium and MAPK signaling pathways (D,E). Prokr2 is expressed in the adult mouse pituitary (F). Immunofluorescent staining for GFP in the pituitary of Prokr2-GFP transgenic mice shows immunoreactivity in all pituitary structures and is most pronounced in the pars nervosa. GFP=green fluorescent staining, AVP=arginin vasopressin.

Figure 3: Schematic of the FGF8 minigene construct (A) and its alternatively spliced transcripts (B). Transcripts differ according to presence/absence of exon 1C (orange) and part of exon 1D (dark gray). Black and blue arrows depict location of specific and generic qPCR primers, respectively, used to analyze relative expression of each transcript. The T72T variant is noted in red. SP=signal peptide. qPCR showing that expression of both the FGF8e and FGF8f isoforms is significantly increased compared to WT (P<0.001, P<0.05 respectively) (C). Representative experiment of a transcriptional reporter (AP1-Luciferase) assay of FGF8 WT and T72T mutant minigene showing increased signaling activity by the mutant ligand compared to WT (**P<0.001 at maximal dose) (D).
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Table 1. Rare sequence variants, functional prediction, and in vitro functionality of mutations identified in CPHD/SOD probands

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Table 2. Phenotypes of SOD and CPHD probands found to harbor gene mutations in either FGFR1, FGF8, or PROKR2

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* Hypogonadotropic hypogonadism is based on neonatal diagnosis/phenotype: cryptorchidism/microphallus, or low serum gonadotropins, or LH-RH stimulation test.

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A. SOD CPHD

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B. Cell Surface AB Binding (% of WT)

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Figure 1

C. OCFRE-LUC Activity (% of WT)

D. Fold Activity

E. OD₅₆₂ Time (secs)
Figure 2

A. Structure of PROKR2

SOD CPHD
Human RYKKL R NLTNL IRKRL R CRRKTV
Mouse RYKKL R NLTNL IRKRL R CRRKTV
Chicken RYKKL R NLTNL IRKRL R CRRKTV
Xenopus RYKKL R NLTNL IRKRL R CRRKTV
Zebrafish RYKKL R LNTNL IRKRL R RRRRTV

B. Western Blot Analysis

PROKR2
β-Actin
EV WT R85G

C. Reporter Activity

Cell Surface Exp. (% of WT)

D. Aequorin Reporter Activity

E. Egr1 Reporter Activity

F. Immunofluorescence Images

DAPI
GFP
AVP

Figure 2

Infundibulum
Pars Distalis
Pars Nervosa
Figure 3

A. ATG

B. SP Core FGF

C. AP1-Luc Activity (relative to WT)

D. AP1-Luc Activity (relative to WT)
SUPPLEMENTAL MATERIAL:

Case Descriptions of the 9 probands harboring mutations

Patient # 1: Septo-optic dysplasia with GH deficiency and possible LH & FSH deficiency
(*FGFR1* c.336C>T/wt, p.T112T). The male harboring the FGFR1 T112T rare sequence variant was born with septo-optic dysplasia and suffered repeated seizures as an infant and was hospitalized for direct hyperbilirubinemia evaluated by liver biopsy. He struggled with failure to thrive and an evaluation at 3 months of age indicated GH deficiency and low gonadotropins and testosterone in the setting of normal TSH, cortisol/ACTH, and prolactin. MRI revealed an absent corpus callosum and GH therapy was started. While his labwork was consistent with hypogonadotropic hypogonadism, he is prepubertal and as such, it remains unclear if he will have spontaneous sexual development. Previous screening conducted in this patient revealed no mutations in *HESX1, LHX3, LHX4, OTX2, PITX2, PROP1*, or *SIX6*.

Patient # 2: Septo-optic dysplasia with AVP deficiency (adipsic hypernatremia/diabetes insipidus)
(*FGFR1* c.1349 C>T/wt, p.S450F). This Caucasian male was born prematurely at 35 weeks (Birth weight 1740g, <3rd percentile, with a small ventricular-septal defect and a large atrial-septal defect which led to a hypertensive crisis and were surgically corrected. At birth he also exhibited a number of dysmorphic features including bilateral epicanthic folds, bilateral preauricular skin tags, and brachycephaly. Later he was noted to have brachydactyly, and only a single central incisor; this combination of dysmorphic features and midline defects was within the spectrum of septo-optic dysplasia. He presented again to medical attention at 19 months of age for vomiting. Labwork revealed high plasma sodium (195mmol/L) with a low potassium (2.8mmol/L) in the setting of elevated urea (26.7mmol/L) and creatinine (133nmol/L). Both rennin and aldosterone were measured and were appropriate for a hypernatremic state, yet he would not drink and was subsequently diagnosed with adipsic hypernatraemia. This was
Amenable to treatment with prescribed fluids through a gastrostomy tube. Additional evaluation included dynamic endocrine testing revealing normal GH, TSH, cortisol and prolactin responses (GH peaked at 22mU/L post glucagon, cortisol peaked at 1290 nmol/L 60 min post Synacthen, and TSH peaked at 17 mU/L, prolactin 273mU/l). Neuroimaging revealed normal optic nerves and normal pituitary but absent cavum septum pellucidum and a dysgenetic corpus callosum with thinning of the genu body and absence of the rostrum. Although he was born without microphallus or cryptorchidism, a LHRH stimulation test was performed to assess GnRH deficiency but results were deemed inconclusive (LH from <0.3 to peak of 0.3 mIU/L, FSH from <0.3 to 1.1 mIU/L) and a repeat stimulation is planned at age 12. He has exhibited slow but steady growth and had delayed language acquisition and learning difficulties. Previous screening conducted in this patient revealed no mutations in GLI2, HESX1, SHH, SIX3, TGIF, or ZIC2. The mother harbors the identical FGFR1 heterozygous change but she is phenotypically normal.

Patient #3: Septo-optic dysplasia with GH, TSH, & ACTH deficiency (FGFR1 c.1447 C>T/wt, p.P483S). This female patient was born to consanguineous Turkish parents following spontaneous pregnancy. The father had a history of oligospermia and the mother had irregular cycles as a result of polycystic ovary syndrome yet they conceived naturally. The daughter was delivered at 41 weeks of gestation via instrumental delivery with a birth weight of 2,900g and noted to have cleft lip/palate, coloboma and microphthalmia of the left eye affecting the optic disc and choroid and resulting in no vision in that eye. She developed hypoglycemia, respiratory distress and polycythemia requiring partial exchange transfusion. She was subsequently diagnosed with growth hormone, TSH and ACTH deficiency (fT4 7.3pmol/L, peak GH 0.9 ug/L, basal cortisol 201 nmol/L, peak 595 nmol/L, basal LH <0.3 mU/L, basal FSH 0.6mU/L). Her karyotype was normal (46XX) and imaging studies revealed a hypoplastic anterior pituitary and undescended posterior pituitary. She was started on GH, T4, and hydrocortisone replacement. She is yet pre-adolescent and has struggled with moderate learning difficulties during childhood.
Genetic screening in the parents indicated that she inherited the mutation from her father. Of note the father had a history of impaired fertility and had sought fertility consultation yet repeat evaluation showed a normal sperm count and motility (137 million total, 35% motile) and he has no other apparent phenotype. The proband had been was previously screened for mutations in *HESX1*, which was negative.

Patient # 4: **Combined pituitary hormone deficiency (GH & TSH)** (*FGFR1* c.1595 T>C/wt, p.M532T). This child was diagnosed neonatally and was started on hormone replacement for multiple deficiencies. Unfortunately we do not have any further medical information regarding the diagnosis of specific hormone deficiencies and the details of this child’s hormone replacement medications.

Patient # 5: **Combined pituitary hormone deficiency (GH, TSH, & ACTH)** (*FGF8* c.216 G>A/wt, p.T72T). This female harbors a synonymous T72T change in FGF8. She first presented at age 12 for short stature and was noted to have a normal 46 XX karyotype and was negative for celiac disease, yet low IGF-1, normal TSH, and undetectable gonadotropins in the setting of a delayed bone age (10 years 6 months). MRI showed a prominent posterior pituitary but was otherwise normal. She had a poor GH response to stimulation and was started on therapy. An ACTH stimulation demonstrated a poor response and the patient was diagnosed with partial ACTH-deficiency with instructions to replace cortisol during times of stress. On continued monitoring her thyroid function tests were abnormal, and less than a year later she initiated T4 therapy. She exhibited pubarche at 12, thelarche at 14 and a delayed menarche at age 16. Presently she continues on T4 therapy. Previous screening conducted in this patient revealed no mutations in *HESX1, LHX3, LHX4, OTX2, PIT-1, PITX2, PROP1*, or *SIX6*. 
Patient # 6: Septo-optic dysplasia with GH & TSH deficiency (*PROKR2* c.253 C>G/wt, p.R85G). This African American female had intra-uterine growth retardation and was born with cleft lip/palate, septo-optic dysplasia with unilateral (left) optic nerve hypoplasia, and a right club foot. She experienced neonatal hypoglycemia and a workup at 8 weeks of age identified GH deficiency with a measured GH of 3.2 ng/mL at the time of a blood glucose measured at 22 mg/dL (1.1 mmol/L). Additional workup also demonstrated both normal cortisol (30 mcg/dL [827 nmol/L]) and prolactin (34 ng/mL levels [1478 pmol/L]). Initially thyroid function tests were normal (TSH 0.8 mIU/L, free T4 7.7 ng/dL [99 nmol/L]) but with TRH stimulation she was considered to have a poor response (peak TSH: 2.5 mIU/L, peak total T4: 7.1 ng/dL [91 nmol/L]) and was started on treatment. At 11 weeks of age, LH was <0.15. A number of findings were noted on MRI examination including a thin corpus callosum, normal optic chiasm but absent optic nerve, an apparently normal pituitary, syrinx spinal cord (abnormal fluid filled cavity in the spinal cord), and schizencephaly (a rare cortical malformation of the brain). Presently the patient is maintained on T4 therapy. Previous screening conducted in this patient revealed no mutations in *HESX1, LHX3, LHX4, OTX2, PIT1, PITX2, PROP1*, or *SIX6*.

Patient # 7: Septo-optic dysplasia (*PROKR2* c.802 C>T/wt, p.R268C). This African male was born prematurely at 36.5 weeks (birth weight 2220 gr) via emergency cesarean section for maternal preeclampsia. Antenatal scans had shown oligohydramnios and intrauterine growth retardation. On initial examination he was microcephallic and had bilateral cryptorchidism without other obvious anomalies. He suffered seizures shortly after birth that were controlled with phenobarbitol. At 3 weeks of age a cranial MRI revealed a complex brain malformation consisting of incomplete corpus callosum, separation of deep gray structures by a cavum interpositum, indistinct thalamus and basal ganglia, dysplasia of the right cerebellar hemisphere and small middle cerebellar peduncles, Dandy-Walker malformation, dorsal and ventral clefts on the pons. There was no definite cortical abnormality and the pituitary gland, the optic nerves and
the optic chiasm appeared to be normal. The neurologists gave a guarded prognosis in terms of his neurodevelopmental outcome and he was discharged on day 23 under antiepileptic medication. At 15 months he was severely delayed in his developmental milestones and while he passed his neonatal hearing screening, he exhibited unusual visual behavior. He was referred for an ophthalmologic evaluation which revealed bilateral optic disc hypoplasia with an anomalous disc on the left and both electroretinogram and visual evoked response testing suggested rudimentary activation of visual pathways. Following this evaluation he was referred for a pediatric endocrine evaluation. At that time his weight was between the 25-50th percentiles but his length was at the 2nd percentile. His left testis was palpable in the inguinal canal but his right could not be detected. A pelvic ultrasound showed inguinal testes with the right (0.8x0.9x1.6 cm, volume= 0.6 cc) lying higher than the left (0.7x0.7x1.4 cm, volume=0.4 cc). His prior MRI scan was reviewed and the anterior pituitary was deemed rather small yet the posterior pituitary and the infundibulum appeared normal. His evaluation included baseline hormones, 24-hour glucose and cortisol profile, Synachten test and a 3-day hCG stimulation test were normal apart from slightly low PRL and borderline low morning ACTH (see table). He was followed and 6 months later his testes were scrotal and he has had a normal growth rate (11.25 cm/year). He continues on sodium valproate for his epilepsy, which is well controlled, and does not presently require any hormone replacement.

Patient # 8: Septo-optic dysplasia with GH, TSH, & ACTH deficiency, possible LH & FSH deficiency (PROKR2 c.802 C>T/wt, p.R268C). This Caucasian male was born via normal vaginal delivery following an uneventful pregnancy (birth weight: 3742 gr). His mother had a history of Hodgkin Disease diagnosed when she was 21 years old and she became pregnant nine years later. At 6 weeks of age he was referred for a pediatric ophthalmologic consultation regarding his odd visual behavior. He was noted to have optic disc hypoplasia and a subsequent MRI revealed bilateral optic nerve hypoplasia, thin optic chiasm, small 3rd ventricle, ectopic
posterior pituitary, and absent infundibulum. Following his diagnosis of septo-optic dysplasia, he was then referred for endocrine consultation where laboratory and dynamic testing revealed growth hormone deficiency, low free T4 (10pmol/L), and a poor cortisol response to ACTH (217 nmol/L) and he was started on replacement therapy. Of interest, at his initial endocrine consultation he was noted to have microphallus and bilateral cryptorchidism. He was given an LHRH stimulation which failed to produce any significant rise in his serum gonadotropins (peak LH 0.4 IU/L, FSH 0.7 IU/L). These results strongly suggest GnRH deficiency (he is currently of prepubertal age). During infancy, a 3-day regimen of hCG injections stimulated an appropriate rise in testosterone (7nmol/L) demonstrating a sufficient testicular Leydig cell response. Further, he received monthly depot testosterone injections for 3 months to treat his microphallus which improved slightly the size of his penis. When he presented to urology at age 2 for additional treatment for microphallus, his testes had descended and he underwent a course of topical DHT which improved the size of his phallus. During childhood his development has been only mildly delayed and he received assistance from both physical and speech-language therapy.

Patient # 9: Combined pituitary hormone deficiency (GH, TSH, & ACTH) (PROKR2 c.254 G>A/wt, p.R85H). This male patient of Turkish descent was born prematurely at 34 weeks gestation from non-consanguineous parents and initially presented with hypoglycemia, seizures, microphallus, and prolonged jaundice (unconjugated hyperbilirubinemia). Laboratory evaluation indicated low TSH (0.07 mIU/L) and initially a random low cortisol (1.54 mcg/dL [40 nmol/L]). An ACTH stimulation test demonstrated a poor cortisol response (1.54 baseline to peak of 3.44mcg/dL [40 to 95 nmol/L]). The patient had an inadequate GH level (<9 ng/,mL), but normal prolactin (36 ng/mL). Following testing he initiated T4, hydrocortisone, and GH replacement therapy. Gonadotropins were not evaluated, but the finding of microphallus at birth strongly suggests he also is gonadotropin deficient. Notably, the proband also harbors a rare variant in KAL1 (c.1375 C>T/wt, p.H459Y) and gene screening in the parents revealed that the mother
carries the identical \textit{KAL1} mutation and the father harbors the \textit{PROKR2} R85H mutation. Previous screening conducted in this proband revealed no mutations in \textit{HESX1, LHX3, LHX4, OTX2, PIT1, PITX2, PROP1,} or \textit{SIX6}.