Context: Pituitary stalk interruption syndrome (PSIS, ORPHA95496) is a congenital defect of the pituitary gland characterized by the triad of a very thin/interrupted pituitary stalk, an ectopic (or absent) posterior pituitary gland, and hypoplasia or aplasia of the anterior pituitary gland. Complex genetic patterns of inheritance of this disorder are increasingly recognized.

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Methods: Whole exome sequencing (WES) was performed using standard techniques, with prioritized genetic variants confirmed via Sanger sequencing. To investigate the effects of one candidate variant on mutant WDR11 function, Western blotting and co-immunofluorescence were used to assess binding capacity, and Leptomycin B exposure along with immunofluorescence were used to assess nuclear localization.

Results: We report a child who presented in infancy with multiple pituitary hormone deficiencies whose brain imaging demonstrated a small anterior pituitary, ectopic posterior pituitary, and a thin, interrupted stalk. WES demonstrated heterozygous missense mutations in two genes required for pituitary development, a known loss-of-function mutation in PROKR2 (c.253C>T;p.R85C) inherited from an unaffected mother, and a WDR11 (c.1306A>G;p.I436V) mutation inherited from an unaffected father. Mutant WDR11 loses its capacity to bind to its functional partner, EMX1 and to localize to the nucleus.

Conclusions: WES in a child with PSIS and his unaffected family implicates a digenic mechanism of inheritance. In cases of hypopituitarism where there is incomplete segregation of a monogenic genotype with the phenotype, the possibility that a second genetic locus is involved should be considered.
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We describe a child with digenically inherited PSIS in the setting of a maternally inherited PROKR2 mutation (c.253C>T;p.R85C) and a paternally inherited WDR11 mutation (c.1306A>G;p.I436V).
Digenic Inheritance of PROKR2 and WDR11 Mutations in Pituitary Stalk Interruption Syndrome

with Hormone Deficiencies

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Abstract

Context: Pituitary stalk interruption syndrome (PSIS, ORPHA95496) is a congenital defect of the pituitary gland characterized by the triad of a very thin/interrupted pituitary stalk, an ectopic (or absent) posterior pituitary gland, and hypoplasia or aplasia of the anterior pituitary gland. Complex genetic patterns of inheritance of this disorder are increasingly recognized.

Objective: The objective of this study was to identify a genetic etiology of PSIS in an affected child.

Methods: Whole exome sequencing (WES) was performed using standard techniques, with prioritized genetic variants confirmed via Sanger sequencing. To investigate the effects of one candidate variant on mutant WDR11 function, Western blotting and co-immunofluorescence were used to assess binding capacity, and Leptomycin B exposure along with immunofluorescence were used to assess nuclear localization.

Results: We report a child who presented in infancy with multiple pituitary hormone deficiencies whose brain imaging demonstrated a small anterior pituitary, ectopic posterior pituitary, and a thin, interrupted stalk. WES demonstrated heterozygous missense mutations in two genes required for pituitary development, a known loss-of-function mutation in PROKR2 (c.253C>T;p.R85C) inherited from an unaffected mother, and a WDR11 (c.1306A>G;p.I436V) mutation inherited from an unaffected father. Mutant WDR11 loses its capacity to bind to its functional partner, EMX1 and to localize to the nucleus.

Conclusions: WES in a child with PSIS and his unaffected family implicates a digenic mechanism of inheritance. In cases of hypopituitarism where there is incomplete segregation of a monogenic genotype with the phenotype, the possibility that a second genetic locus is involved should be considered.
We describe a child with digenically inherited PSIS in the setting of a maternally inherited \textit{PROKR2} mutation (c.253C>T;p.R85C) and a paternally inherited \textit{WDR11} mutation (c.1306A>G;p.I436V).
Introduction

Pituitary stalk interruption syndrome (PSIS, ORPHA95496) is a congenital defect of the pituitary gland that is characterized by the triad of a very thin or interrupted pituitary stalk, an ectopic or absent posterior pituitary gland, and hypoplasia or aplasia of the anterior pituitary gland. Patients with PSIS may present with heterogeneous clinical features resulting from single or multiple hypothalamic-pituitary hormone deficiencies. The rare occurrence of families in which several members have PSIS has suggested that at least in some cases PSIS may be genetic (1), and mutations in genes encoding proteins involved in the Wnt, Notch and Shh signaling pathways that are critical for hypothalamic-pituitary development have been reported in some patients with PSIS (2). Nevertheless, in most cases the etiology of PSIS remains unknown. We present a case of PSIS with multiple anterior pituitary deficiencies in which whole exome sequencing analysis identified two heterozygous mutations, thereby implicating a digenic mechanism as the basis for this condition.

Methods and Materials

Human Subject Considerations. Samples were collected under an approved Institutional Review Board protocol of the Children’s Hospital of Philadelphia; the corresponding study was conducted according to the Declaration of Helsinki. Written, informed consent or assent, as appropriate, was obtained from all participants prior to their inclusion.

Case Presentation. Relevant clinical details were abstracted from the electronic medical record.

Biochemical and Genetic Analyses. We extracted DNA from peripheral blood mononuclear cells from the proband and his unaffected parents, and performed exome capture and sequencing, as well as read processing, mapping to human genome reference (GRCh37-derived alignment set used in 1000 Genomes Project), variant calling, annotations and filtering for rare variants affecting the coding sequence and/or
consensus splice sites, as previously described (3). The family pedigree (Figure 1) did not suggest a specific mode of inheritance, so we considered nonsynonymous, splice-altering variants, and frameshift variants co-segregating with the disease under either de novo dominant or recessive inheritance models in the family with a minor allele frequency < 1% in public databases (i.e. 1000 Genomes Project and NHLBI ESP6500SI). Subsequent gene prioritization was on basis of deleterious prediction, biological and clinical relevance as suggested by existing databases (i.e., Online Mendelian Inheritance in Man (OMIM) and Human Gene Mutation Database (HGMD)). However, because we failed to identify any candidates relevant to the phenotype under either dominant or recessive models, we next focused on possible digenic mode of inheritance by identifying variants in the proband that were shared with either parent. We validated mutation candidates by Sanger sequencing and used in silico tools (4, 5) to predict their effects. Salivary DNA was collected from his unaffected sister to assess her status for the identified variants. All biochemical analyses were performed in commercial reference laboratories using standard techniques.

**Mutagenesis.** The full-length WDR11 cDNA in pcDNA-GFP or pcDEST-Myc vector was mutagenized using Q5 site-directed mutagenesis kit (New England Biolabs) to introduce c.1306A>G variant following the manufacturer’s protocol. Briefly, the nucleotide exchange was introduced using Q5 Hot Start High-Fidelity DNA Polymerase with ‘non-overlapping’ mutagenic primers (NEBase Changer) via PCR run for 25 cycles of 98°C for 30s, 98°C for 10s, 60°C for 30s, 72°C for 5min and 72°C for 2min. The products were ligated at room temperature for 5 minutes and transformed into Top10 chemically competent cells. Mutated plasmid constructs were verified by sequencing (Source Bioscience, Nottingham, U.K.). Sequences of primers used are available upon request.

**Co-immunoprecipitation and Western blot analysis.** HEK293T cells cultured in DMEM supplemented with 2mM L-glutamine, 100 µg/ml penicillin/streptomycin and 10% fetal bovine serum (Sigma-Aldrich) were transfected with plasmid constructs expressing Myc-tagged WDR11 or HA-tagged EMX1 using Fugene (Promega). At 48 hours post-transfection, total cell lysates were extracted with the lysis buffer.
(50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich). After incubation on ice for 10 min and centrifugation for 10 min at 4 °C, the pre-cleared lysate (500 µg – 1 mg protein) was incubated with anti-Myc antibody (M4439, Sigma-Aldrich) and protein A/G-agarose beads (Santa Cruz Biotechnology) overnight at 4 °C on a rotating wheel. The immune complexes on the beads were washed 4 times with the lysis buffer, separated by SDS-PAGE and transferred to Hybond-ECL membrane (Amersham) which was probed with anti-WDR11 (1:500, ab175256, Abcam) and anti-EMX1 (1:500, PA5-35373, Invitrogen) antibody diluted in blocking buffer (5% skim milk in TBS with 0.05% Tween 20).

**Immunofluorescence.** GFP-tagged wild-type and mutant WDR11 expression constructs were transfected into HEK293T. After 48 hours, cells were treated with Leptomycin B (10ng/ml) for 10 hours before being fixed with 4% paraformaldehyde. After washing three times in PBS, the nuclei were counterstained with DAPI and the cover slips were mounted in Mowiol 4-88 (Fluka). Images were analysed using Zeiss Axioplan 2 Upright fluorescence microscope and ImageJ software (http://rsbweb.nih.gov/ij/). In each experiment, approximately 200 cells were scored for the nuclear or cytoplasmic location of WDR11 based on the GFP signal at 488 nm, against the total cells in the field based on the DAPI signal at 405 nm.

**Results**

The proband was the 2.92 kg product of a 39-week gestation, born to a 45 year old G2P1A0 mother (Figure 1). He is of Ashkenazi Jewish heritage and was conceived by *in vitro* fertilization and underwent targeted pre-implantation genetic diagnosis because both parents are carriers for Gaucher disease and familial hyperinsulinism. He is a carrier of *GBA* N370S and *ABCC8* c.3989-9G>A mutations.
He developed transient, mild hypoglycemia within first 36 hours of life and was diagnosed with central congenital hypothyroidism based on low thyroid hormone levels on newborn screening. MRI of the brain showed small anterior pituitary and ectopic posterior pituitary with a thin and interrupted pituitary stalk, consistent with pituitary stalk interruption syndrome (PSIS). Hydrocortisone replacement was begun at 6 weeks of life based on low serum cortisol levels of ~1-2 mcg/dL. At birth he was thought to have a normal sized phallus, but at 8 months of life he received testosterone 25 mg IM q3weeks x 6 for small phallus.

Growth hormone status was initially normal, and his length was at the 25-50%ile until age ~16-18 months, after which he made little gain in length, such that by 2 8/12 years, his height was <1%ile. Head circumference demonstrated a similar pattern. His weight increased to 50-75%ile by around 12 months, but also faltered thereafter. He was begun on recombinant human growth hormone (GH) at 2 9/12 years for lack of statural growth (Height SD -1.8); a very low random growth hormone level of 0.93 ng/ml, low IGF-1 level of 27 ng/mL and low IGFBP3 of 0.6 mg/L, all of which in this clinical context were consistent with GH deficiency. Both height and weight responded well to GH; height increased to the 38%ile and weight to the 20%ile after ~15 months of therapy. Neurocognitive development has been normal.

Whole exome sequencing revealed heterozygous mutations in two genes known to affect hypothalamic and pituitary development, one in PROKR2 (c.253C>T; p.R85C) inherited from an unaffected mother, and one in WDR11 (c.1306A>G;p.I436V) inherited from an unaffected father, both confirmed by Sanger sequencing (Figure 1). Additional candidates were considered under de novo, X-linked and recessive models, and were excluded due to a lack of pathogenicity or relevance to the phenotype (Supplementary Table 1). A clinically unaffected sister carried only the PROKR2 missense mutation (Figure 1). Publicly available databases show that PROKR2 p.R85C is present in the population with a minor allele frequency of 0.0005024 in the ExAC database (http://exac.broadinstitute.org/gene/ENSG00000101292) and is
absent in 1,000 Genomes Project and in 6,503 exomes from the Exome Sequencing Project (ESP6500SI). Several lines of evidence indicate that PROKR2 p.R85C is pathogenic. First, the amino acid change (from basic to hydrophobic) occurs at a highly evolutionarily conserved site that is predicted to lie within an important functional GPCR domain, and in silico studies predict that this change is pathological (Polyphen2 score 1.0 predicts the change to be probably damaging; PROVEAN score -6.840 is Deleterious). Second, functional studies have indicated that the p.R85C variant has reduced activity in MAPK and/or calcium signaling pathways (6-8) without evidence of a dominant negative effect (8). Third, this mutation has previously been identified in an individual with normosomic idiopathic hypogonadotropic hypogonadism (7). Finally, all other amino acid changes reported at the same site have been also associated with hypothalamic and pituitary dysfunction. For example, heterozygous mutations including PROKR2 p.R85H, p.R85G and p.R85L have all been identified in association with pituitary stalk interruption and multiple pituitary hormone deficiencies (9, 10). On basis of the ACMG guidelines (11), this variant is therefore classified as pathogenic.

We were unable to identify a point mutation, deletion or duplication in the second PROKR2 allele, and therefore the WDR11 p.I436V variant became relevant based on the involvement of WDR11 mutations in other pituitary disorders. This variant is rare, and publicly available databases show it has a minor allele frequency of 0.001307 in the ESP6500SI (http://evs.gs.washington.edu/EVS/) and a minor allele frequency of 0.0009084 in the ExAC database (http://exac.broadinstitute.org/gene/ENSG00000120008).

Although in silico prediction tools do not indicate a high likelihood of pathogenicity (Polyphen 2 score of 0.00; PROVEAN -0.118, Neutral), its location in the functionally significant 6th WD domain, an important site for protein-protein interactions, suggested it could be pathogenic. To confirm this, we examined the behavior of this rare variant of WDR11 in two different functional assays in vitro. WDR11 binds and co-localizes with the EMX1 transcription factor in the nucleus, and this interaction must be important for its normal function, because this capacity is lost by all WDR11 mutations in patients with idiopathic hypogonadotropic hypogonadism and Kallmann syndrome (12). Our co-immunoprecipitation
Western blot assay indicated that the p.I436V variant of WDR11 was unable to bind to EMX1 (Figure 2). We also evaluated the p.I436V variant in a second functional assay that is based on the observation that wild type WDR11 can shuttle between nucleus and cytoplasm, and treatment with a nuclear export inhibitor Leptomycin B induces its accumulation in the nucleus (12). When we introduced GFP-WDR11 into HEK293T cells, both the WT and variant WDR11 proteins showed mainly cytoplasmic location, but when the cells were treated with Leptomycin B, the WT but not the p.I436V variant showed nuclear localization (Figure 3). Taken together, these studies demonstrate that the p.I436V substitution disrupts the normal function of WDR11, and provides strong evidence that this is a pathogenic variant.

Discussion

PSIS is a common finding in patients with pituitary hormone deficiency, and accounts for hypopituitarism in over 11% of adult patients (13) and in 29 of 46 children with idiopathic growth hormone deficiency (14). Although mechanical pituitary stalk rupture or pituitary stalk ischemia during breech delivery has been implicated as a major cause of PSIS, the existence of familial cases has suggested that a genetic disorder involving developmental processes underlies at least some cases of PSIS, and mutations and/or single nucleotide variants in HESX1, LHX4, PROP1, OTX2, SOX3, PROP1, PROKR2 and GPR161 have been identified in patients with this condition (2, 15). Nevertheless, most patients with PSIS do not have an identified genetic cause. Here, we have used an unbiased approach, whole exome sequencing, to identify a novel genetic basis for PSIS. The proband we studied carried heterozygous missense mutations in two different genes important for hypothalamic/pituitary function, PROKR2 and WDR11. This finding implicates a putative digenic basis for PSIS in this child, and also suggests that this unusual genetic mechanism may explain other cases of PSIS that lack conventional autosomal recessive inheritance.

A digenic disorder results from heterozygous mutations in two distinct genes that encode different proteins. Often, these proteins are both required for normal function or development of a tissue, and/or act
in the same signaling pathway. The impact of having mutations in two different genes in the same pathway can be more than additive, together producing a more severe phenotype than would be expected to occur from the simple combination of their individual effects. For a long time, the existence of digenic inheritance has been proposed as one potential explanation for why in some pedigrees, a monogenic model of inheritance suggests decreased or variable penetrance (16). In these families, a two-locus model may more accurately reflect the observed patterns. Examples of digenic inheritance include some forms of retinitis pigmentosa and facioscapulohumeral muscular dystrophy, and perhaps more relevantly, in some forms of idiopathic hypogonadotropic hypogonadism (17). The number of conditions exhibiting digenic inheritance continues to grow (18). Indeed, a curated database exists (DiGenic Diseases DAtabase, DIDA) and at the time of this writing contains 44 conditions with clear evidence of digenic inheritance (19).

Although to our knowledge this is the first report of heterozygous mutations in both PROKR2 and WDR11 in the same individual, each of these genes has previously been implicated with another gene mutation as the basis for a digenic pituitary disease. PROKR2 (OMIM #607002) encodes a 384-amino acid G-protein coupled receptor (GPCR) whose signaling activity plays a key role in both development of the olfactory bulb (20) and GnRH secretion (21). Mutations in PROKR2 have previously been associated with hypogonadotropic hypogonadism with or without anosmia and Kallmann syndrome (7). Heterozygous mutations including PROKR2 p.R85H, p.R85G and p.R85L have all been identified in association with pituitary stalk interruption and multiple pituitary hormone deficiencies (9, 10). The same heterozygous mutation on PROKR2 that was identified in the proband here, p.R85C, has also been found in a female patient with multiple pituitary hormone deficiencies, including in GH, ACTH, LH and FSH, as well as vasopressin, and an MRI demonstrating normal anterior pituitary, absent posterior pituitary and absent stalk (22). No additional mutations were found in that patient, however only two candidate genes (PROKR2 and FGFR1) were sequenced for individuals in that study. Finally, in the mouse model of PROKR2 deficiency, the Kallmann syndrome phenotype is only observed in the homozygous animals (6).
In human studies as well as in animal models, PROKR2 haploinsufficiency seems not to account for the PSIS phenotype, hence raising the need for a “second hit”, either genetic or environmental, to produce pituitary disease. This hypothesis was further supported when a patient with Kallmann syndrome was discovered to carry the same PROKR2 heterozygous mutation as our proband, p.R85C, in combination with a second heterozygous mutation in FGFR1, p.A604T, thereby providing evidence for a digenic basis for the syndrome (10). Prokineticin 2 and PROKR2 are both expressed in the hypothalamus and pituitary, and reduced expression or activity of PROKR2 is implicated in both Kallmann syndrome or PSIS, perhaps because of the important role this signaling pathway plays in endocrine angiogenesis and neuronal migration in this region of the central nervous system. Ectopic posterior pituitary has been proposed to be a consequence of defective neuronal axon projections along the pituitary stalk or defective angiogenesis of hypophyseal portal circulation. Therefore, it is reasonable to suggest that the loss of PROKR2 signaling is involved in PSIS.

Our proband carried a second heterozygous mutation in WDR11 (OMIM #606417), which encodes a protein that is a member of the WD repeat protein family that participates in a wide variety of cellular processes. The p.I436V missense mutation affects the 6th WD domain of the WDR11 protein within the evolutionarily conserved predicted propeller region that is required for interaction of WDR11 with EMX1, a homeodomain transcription factor necessary for olfactory bulb morphogenesis. We found that the p.I436V mutation, similar to other nearby amino acid substitutions that have been identified in individuals with idiopathic hypogonadotropic hypogonadism with and without anosmia (12, 23), disrupted interaction of WDR11 with EMX1. Specifically, heterozygous missense mutations in neighboring p.A435 (p.A435T) and p.R448 (p.R448Q) have been identified in each of two individuals with idiopathic hypogonadotropic hypogonadism and normosmia (12). In addition, in a different individual, the WDR11 p.A435T mutation was identified in association with a mutation in a second gene, GNRHR (c.275T>C;p.L92P), implicating digenic inheritance of this disorder as well (23). We note that this variant has been identified in homozygous form in two apparently normal subjects.
but their phenotype is unknown, and it is conceivable that one or both of these individuals has a mild pituitary phenotype.

Digenic inheritance is more likely when the functional roles of the two involved proteins affect a singular pathway and/or there are demonstrated protein-protein interactions (18). Here, both PROKR2 and WDR11 have been found to participate in key signaling processes that influence the morphogenesis of the olfactory bulb. The PROKR2 p.R85C variant has been described in heterozygous state in patients with idiopathic hypogonadotropic hypogonadism, Kallmann syndrome, healthy first-degree relatives of Kallmann probands, and in rare healthy controls. We therefore propose that p.R85C (and possibly other PROKR2 mutations) may act as modifier, and contribute to the PSIS phenotype through digenic inheritance, as previously demonstrated in idiopathic hypogonadotropic hypogonadism and Kallmann syndrome (17, 24). Further studies are needed to elucidate in more detail the role of PROKR2 signaling in pituitary and midline development.

In summary, this case adds digenic inheritance of mutations in PROKR2 and WDR11 as an additional potential cause of multiple pituitary hormonal deficiencies and PSIS, and highlights the importance of considering unconventional genetic mechanisms when there is incomplete segregation of a heterozygous mutation with the phenotype in a pedigree.
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**Figure Legends**

**Figure 1.** Pedigree and sequencing chromatograms. Panel A (top) shows the pedigree. The affected proband is indicated with an arrow (shaded). Circles are females, squares are males. The genotypes are indicated for each individual. (B) A sequence chromatogram showing the PROKR2 (CC>CT; 85 R/R>R>C) mutation. (C) A sequence chromatogram showing the WDR11 (CA>AG; 436 I/I>I/V) mutation. In the chromatograms, mutations are indicated with arrows.

**Figure 2.** Mutant WDR11 fails to bind to EMX1. (A) WDR11 fusion proteins containing a Myc epitope tag wildtype (WT) or the c.1306 A>G variant (Mutant) were co-expressed in HEK293 cells along with HA-EMX1 protein. The total cell lysates were immunoprecipitated with anti-Myc antibody, and the association of EMX1 protein was determined by immunoblot analysis using anti-EMX1 antibody. Empty pCDNA vector (-) was included as a negative control. (B) The average densitometry values of the EMX1 band intensity obtained from 3 independent experiments are shown with the standard deviations (error bars).

**Figure 3.** Mutant WDR11 fails to accumulate in the nucleus after treatment with leptomycin B. (A) HEK293 cells transfected with GFP-WDR11 (green) expression constructs were treated with an leptomycin B (Lep), an inhibitor of nuclear export, or the vehicle (solv) and analyzed by fluorescence microscopy to determine the intracellular localization of WDR11. Nuclei were stained with DAPI (blue). (B) The percentages of cells showing either nuclear or cytoplasmic localization of the mutant WDR11-GFP are shown in comparison with the WT which showed a significant increase of nuclear translocation after Lep treatment. The average data from 3 independent experiments were obtained by counting 100-200 cells in each experiment, and presented as a mean ± SEM with two-way ANOVA followed by Tukeys post-hoc test (NS, p>0.05; ****, p<0.0001).
Figure 1. Pedigree and sequencing chromatograms.

A

PROK2R: c.253 C>T (p.R85C)/+
WDR11: +/+  
PROK2R: c.1306 A>G (p.I436V)/+
WDR11: +/+  

PROK2R: c.253 C>T (p.R85C)/+
WDR11: c.1306 A>G (p.I436V)/+

B

C

PROK2R: c.253 C>T (p.R85C)/+

WDR11: c.1306 A>G (p.I436V)/+
Figure 2. Mutant WDR11 fails to bind to EMX1.

A. Myc-WDR11 -  WT  Mutant
   HA-EMX1  +  +  +
   Input
   Input
   IP: α-Myc

B. WDR11 + EMX1
   Mutant + EMX1
   pcDNA + EMX1
   Arbitrary units
Figure 3. Mutant WDR11 fails to accumulate in the nucleus after treatment with leptomycin B.