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HESX1 Mutations in Patients with Congenital Hypopituitarism: Variable Phenotypes with the Same Genotype

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Qing Fang[1], Anna Flavia Figueredo Benedetti[2], Qianyi Ma[1], Louise Gregory[3], Jun Z. Li[1], Mehul Dattani[3], Abdollah Sadeghi-Nejad[4], Ivo J.P. Arnhold[2], Berenice Bilharinho de Mendonça[2], Sally A. Camper[1*] and Luciani R. Carvalho[2*]

[1] Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI, USA

[2] Division of Endocrinology, Unit of Endocrinology and Development, Laboratory of Hormones and Molecular Genetics, Clinical Hospital of the Faculty of Medicine of the University of São Paulo, São Paulo, Brazil

[3] Developmental Endocrinology Research Group, Section of Genetics and Epigenetics in Health and Disease, Genetics and Genomic Medicine Programme, University College London, Institute of Child Health, London, UK

[4] Division of Pediatric Endocrinology, Floating Hospital for Children at Tufts Medical Center, Tufts University School of Medicine, Boston, MA, USA

**Correspondence should be addressed to:*
Sally A. Camper, Ph.D.
Department of Human Genetics
University of Michigan Medical School
Ann Arbor, MI 48109-5618, USA
Fax: 1-734-763-3784
Email: scamper@med.umich.edu

Luciani R. Carvalho, M.D., Ph.D.
Endocrinology Discipline of Internal Medicine Department
University of Sao Paulo Medical School
Sao Paulo, Brazil
Fax: 55-11-2661-7519
Email: lucianic@gmail.com

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Summary

Introduction: Mutations in the transcription factor *HESX1* can cause Isolated Growth Hormone Deficiency (IGHD) or Combined Pituitary Hormone Deficiency (CPHD) with or

without Septo-Optic Dysplasia (SOD). So far there is no clear genotype-phenotype correlation.

Patients and Results: We report three different recessive loss-of-function mutations in three unrelated families with CPHD and no midline defects or SOD. A homozygous p.R160C mutation was found by Sanger sequencing in two siblings from a consanguineous family. These patients presented with ACTH, TSH and GH deficiencies, severe anterior pituitary hypoplasia (APH) or pituitary aplasia (PA) and normal posterior pituitary. The p.R160C mutation was previously reported in a case with SOD, CPHD and ectopic posterior pituitary (EPP). Using exome sequencing, a homozygous p.I26T mutation was found in a Brazilian patient born to consanguineous parents. This patient had evolving CPHD, normal ACTH, APH and normal posterior pituitary (NPP). A previously reported patient homozygous for p.I26T had evolving CPHD and EPP. Finally, we identified compound heterozygous mutations in *HESX1*, p.[R159W];[R160H], in a patient with PA and CPHD. We showed that both of these mutations abrogate the ability of HESX1 to repress PROP1-mediated transcriptional activation. A patient homozygous for p.R160H was previously reported in a patient with CPHD, EPP, APH.

Conclusion: These three examples demonstrate that *HESX1* mutations cause variable clinical features in patients, which suggests an influence of modifier genes or environmental factors on the phenotype.

Introduction

Congenital hypopituitarism refers to the deficiency of two or more pituitary hormones, and it is caused by mutations in one of several genes implicated in pituitary development, such as *HESX1, OTX2, PROP1, POU1F1, LHX3, LHX4, SOX2, SOX3 and GLI2.*[1, 2] In some patients the hormone deficiency may present as part of a syndrome with abnormalities in structures that share a common embryological origin with the pituitary gland, such as the eye and the forebrain.

HESX1 encodes a paired-like homeobox transcription factor that was first identified in embryonic stem cells.[3, 4] HESX1 is one of the earliest known markers of the pituitary primordium. It can be detected in the anterior forebrain from 7.5 to 8.5 *days post coitum* (d.p.c.) and in the Rathke's pouch from 8.5 to 13.5 days d.p.c. Expression of HESX1 is important for the early determination and differentiation of pituitary gland,[5, 6] as well as normal forebrain formation in both mice and humans.[7, 8]

A number of autosomal dominant and recessive mutations in *HESX1* have been described in patients with a broad spectrum of phenotypes ranging from isolated growth hormone deficiency (IGHD), combined pituitary hormone deficiencies (CPHD) to septo-optic dysplasia (SOD).[6, 9-11] Magnetic Resonance Images (MRI) also reveal variable effects. The anterior pituitary can be hypoplastic or aplastic, and the posterior pituitary can be ectopic or eutopic. No clear genotype-phenotype correlation is obvious among the cases with *HESX1* mutations, but there is a trend that the recessive mutations cause more severe phenotypes and appear to be fully penetrant while heterozygous mutations may be associated with milder phenotypes and reduced penetrance.[6, 9, 10, 12-14]

The approach of Sanger sequencing of candidate genes has led to the identification of most of the known genetic causes of hypopituitarism. It is clear that hypopituitarism is a genetically heterogeneous condition. The mutations in the reported genes account for less than 20% of the cases. Thus, candidate gene screening has a low detection rate. The recent introduction of massive-parallel sequencing methods now offer the promise of detecting mutations in known candidate genes, as well as the identification of novel genes implicated in congenital hypopituitarism. In this study we report the identification of mutations in *HESX1* in patients from three unrelated families with CPHD without midline defects or SODs. This is among the first examples of applying next-generation sequencing techniques to obtain a molecular diagnosis for hypopituitarism in humans.[15, 16]

Subjects and Methods

Patients

Four patients from three unrelated families were recruited in this study (Figure 1, Table 1). Two siblings (1.1 and 1.2) in Family 1 were born to consanguineous parents of Middle-Eastern origin. Patients 1.1 and 1.2 were initially diagnosed in the Middle East and then treated at Great Ormond Street Hospital for Children, London, UK. Patient 2.1 in Family 2 was born to a Brazilian consanguineous family, and she was diagnosed and treated at the Clinical Hospital of the Faculty of Medicine of the University of São Paulo, São Paulo, Brazil. Family 3 was diagnosed and treated at Floating Hospital for Children at Tufts Medical Center, Boston, MA, USA.[17]

Patient studies were approved by the ethical committees at each institution. Patient 1.1 and 1.2 were approved by a committee functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research, issued by the Royal College of Physicians of London. Copies of the MRC recommendations can be obtained from the Medical Research Council. Patient 2.1 was approved by the National Research Ethics Commission (CONEP) and by the Ethics Committee in Research (CEP) from the University of São Paulo, Medical School, São Paulo, Brazil functioning according to the Resolution No. 466/2012 which deals with research and testing in humans adopted by the Plenary of the National Health Council (CNS) in 240ª ordinary meeting in December 2012. Exome sequencing of de-identified patient samples was approved by the IRBMED at University of Michigan.

Exome Sequencing and Variant Calling

Patient DNA samples from families 2 and 3 were subjected to whole exome sequencing. Exome capture was performed by the U-M Sequencing Core using the Nimblegen SeqCap EZ Human Exome Library v3.0, targeting a total of 64 Mb of the genome. Paired-end 100-base sequencing data were collected using an Illumina HiSeq2000 system. Exome capture and sequencing for two patients, namely 2.1 and 3.2, were performed in the same batch. BWA v0.5.9 was used to align Illumina reads to the 1000 Genomes Phase 1 reference mapped to GRCh37. Read pairs that mapped to multiple locations were removed; most of these locations contain highly repetitive sequences and are inaccessible to short-read sequencing. PICARD v1.74 was used to remove duplicate read pairs. Variant detection for both SNVs and small indels (<10 nt) were performed by the GATK Haplotype Caller v3.3. Multi-sample joint calling with 688 in-house exome samples was performed to remove the sequencing artifacts.

Sanger Sequencing

Sanger sequencing was used to analyze the *HESX1* gene in patients from Family 1. The variants identified in Family 2 and 3 by exome sequencing were confirmed by Sanger sequencing.

SNP Genotyping

To detect copy number variation, we performed genotyping on the two DNA samples for patients 2.1 using Illumina's HumanOmniExpressExome_8v1_A at the University of Michigan Sequencing Core.

Plasmids

pCMV6-Entry-human HESX1 (Myc-DDK-tagged) was purchased from OriGene (Cat. No. RC210107, OriGene Technologies, MD). The c.475C>T and c.479G>A changes were introduced into the HESX1 cDNA sequence by using QuickChange II XL Site-Directed Mutagenesis Kit (Cat. No. 200521, Stratagene). The HESX1 cDNA sequences have been checked to confirm that except for c.475C>T and c.479G>A, no other mutations were incorporated. The pGL3-$(P3)_6$E4 firefly luciferase reporter, pcDNA3.1(-)-human PROP1 have been described and used previously.[11] pcDNA3.1(-) and pRL-TK renilla luciferase reporter vectors were from Invitrogen and Promega, respectively.

Cell culture and Transfections

COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Transient transfections were carried out using FuGENE6 (Promega), following the manufacturer's protocol with modifications. Briefly, 1 x 10^5 cells were seeded into each well on a 24-well plate 24 hours before transfection. Cells were transfected with 10 ng of pRL-TK renilla luciferase vector (Promega) to control for transfection efficiency and 200 ng of $(P3)_6$E4 firefly luciferase reporter.[11] The DNA concentration of the other transfected plasmids varied depending on experimental protocol as indicated in the figure legends, but the total amount of DNA transfected per well was normalized to 400 ng by addition of the appropriate amount of empty expression vector. Cells were incubated and collected 48 hrs later and assayed for luciferase activity using the dual-luciferase protocol on GLOMAX 96 Microplate Luminometer (Promega).

Results

Clinical Characteristics

Patients 1.1 and 1.2 are consanguineous siblings who presented with hypoglycaemic seizures in the neonatal period and complete ACTH, TSH, GH and prolactin deficiencies (undetectable cortisol, free thyroxine 2.42 pmol/L in 1.2, TSH <0.005 in 1.2, undetectable IGF1 and GH 0.01 mU/L in 1.2, and prolactin <11 mU/L in 1.2). MRI revealed severe anterior pituitary hypoplasia (APH) and eutopic posterior pituitary. In both siblings, thyroxine and hydrocortisone were commenced in the neonatal period and growth hormone was commenced at the age of one year. The older sibling had hydrocephalus and required

pubertal induction at the age of 12 years. The younger sibling, currently 6 years old, is too young to assess the hypothalamo-pituitary-gonadal axis. (Figure 1, Table 1)

Patient 2.1 was the daughter of first-degree cousins. She first presented with short stature (height 125.9 cm, -6.0 standard deviation score (SDS)) at 17.2 years of age, with a weight of 32.6 kg (1.0 SD for stature age). Her bone age was delayed by 4 years. Her mid-parental height (MPH) was 150 cm (-2.03 SDS). Clinical investigations revealed GH and gonadotropin deficiencies (peak GH <0.1 ng/ml, FSH 1.3 mU/mL, LH 0.7 mU/mL) and tertiary hypothyroidism (Free T4 0.81 ng/dL, basal TSH 5.7 mU/mL with late response to TRH stimulation). She was treated with recombinant human GH and thyroxine. Puberty was induced at 21.4 years old and her final height was 151.2 cm (-1.83 SDS), well within her target height range. She has not as yet developed ACTH deficiency. MRI of the pituitary gland showed a normal stalk, anterior pituitary hypoplasia and a eutopic posterior pituitary. (Figure 1, Table 1)

Patient 3.2 was born to a non-consanguineous pedigree with a previously affected sister (Figure 1)**.** Detailed early clinical course of this patient has previously been reported.[17] Briefly, patient 3.2 is a Caucasian boy. His older sister (3.1) developed hypoglycaemia and died on the first day of life. A postmortem examination revealed absence of the anterior pituitary and atrophy of the adrenal glands. At eight hours of age, patient 3.2 became lethargic and cyanotic, and had a generalized seizure. Because of the similarity in presentation to that of his sister, a presumptive diagnosis of hypopituitarism was made, and he was treated with glucose and hydrocortisone. He was subsequently treated for hypothyroidism and growth hormone deficiency, and he achieved normal developmental milestones. He failed to develop secondary sexual characteristics as a teenager. His luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone concentrations remained in the prepubertal range on repeated tests. MRI confirmed absence of the anterior pituitary and presence of the posterior pituitary gland. His final height was 174 cm. Several years later, at the age of 30 years, he died after developing severe gastroenteritis with vomiting and diarrhoea. (Figure 1, Table 1)

Genetic Analysis

Sanger sequencing was performed to identify the homozygous p.R160C mutation in *HESX1* in both patient 1.1 and 1.2. (Figure 1)

Whole exome sequencing (WES) was performed on the genomic DNA samples from patients 2.1 and 3.2. Alignment of the reads and variant calling were performed as described in Methods. For this study, we concentrated on potentially damaging SNVs (nonsense, missense, stop loss, splicing change, frameshift, etc.) and small indels (<10 nt). Filtering steps were made according to following criteria: reading depth of variants (≥10), minor allele frequency in ExAC, ESP and 1000G public databases (≤1% for homozygous variants and ≤3% for compound heterozygous variants), prediction that the variant has a deleterious effect on the gene function by at least one software program, RVIS percentile (≤75%), (CADD Phred score (≥15) and GERP++ score (≥4) (Supplement Figure 1). Sanger sequencing was used to confirm the candidate variants.

Because patient 2.1 is from a consanguineous family, the most compelling variants are located in the runs of homozygosity (ROH) regions and transmitted in a recessive inheritance pattern. We identified two rare, homozygous variants in the ROH regions: rs28936416 (c.77T>C, p.I26T) in *HESX1* and rs141318879 (c.888G>A, p.M296I) in *HMGCLL1*. A CPHD patient homozygous for c.77T>C, p.I26T in *HESX1* gene was previously reported, and the variant was shown to impair the transcriptional repression properties of HESX1.[11] *HMGCLL1* gene encodes an isoenzyme of human HMG-CoA lyase and is located in the endoplasmic reticulum (ER).[18] There is no report on variants in *HMGCLL1* gene causing hypopituitarism. Patient 2.1 was also compound heterozygous for six genes (*ANGPTL1*, *EPHA1*, *CDCC88B*, *AGAP2*, *FASN*, *MBD1*) which carry at least one allele passing all the filtering criteria, but none of them is located in the ROH regions of patient 2.1's genome. No variants were detected in other known genes for CPHD or IGHD. Therefore, *HESX1* c.77T>C, p.I26T is the most-likely pathogenic variant for the phenotype in patient 2.1.

Patient 3.2 was the second affected child in a family with non-consanguineous parents. Therefore, we first considered a recessive inheritance pattern. We did not detect any homozygous variants, but eight compound heterozygous variants were found in four genes on the autosomal chromosomes that passed through the filtering steps. These four genes are *HESX1*, *AK9*, *H6PD*, and *CCDC168*. Among them, only mutations in *HESX1* are known to cause CPHD and/or SOD. The two variants we found in *HESX1* are c.475C>T, p.R159W and c.479G>A, p.R160H. We verified that the two variants are truly *in trans* using the Integrative Genomics Viewer (IGV) of individual reads.[19] Both p.R159W and p.R160H variants reside in the homeobox domain of the HESX1 protein. A homozygous c.479G>A,

p.R160H change was previously reported to cause CPHD.[20] This is the first report of the variant c.475C>T, p.R159W in a CPHD patient. The minor allele frequency (MAF) of the p.R159W change is less than 0.002% (ExAC database), which means this variant is extremely rare in the general population. All of the prediction software programs we used (SIFT, PolyPhen-2, Mutation Taster, Mutation Assessor and FATHMM) predict that the p.R159W change has a deleterious effect on HESX1 function. Thus, the compound heterozygous variants p.[R159W];[R160H] are the most likely pathogenic causes for the phenotype of patient 3.2.

Functional Studies

HESX1 acts as a transcriptional repressor by suppressing the activity of PROP1.[9, 11, 21] Mutations in either the homeodomain or the engrailed homology (eh) domain of HESX1 impair this repressive ability.[9, 11] To test if p.R159W and p.R160H substitutions affect the repressive ability of HESX1, Cos-7 cells were transiently transfected with plasmids expressing PROP1, normal HESX1 (HESX1-WT), HESX1-p.R159W and HESX1-p.R160H. These expression vectors were co-transfected with the reporter construct (pGL3) containing 6 tandem paired homeodomain consensus DNA binding sites $(P3)_6$ upstream of the E4 promoter activating the expression of firefly luciferase gene. As expected, PROP1 activated reporter gene expression, and HESX1-WT, HESX1-p.R159W and HESX1-p.R160H have no effect on transcription when tested individually (Figure 2). When equal amounts of HESX1-WT were co-transfected with PROP1, PROP1 activation was repressed by ~50%. Neither HESX1-p.R159W nor HESX1-p.R160H were able to repress PROP1 activity. Transfection of equal amounts of HESX1-p.R159W and HESX1-p.R160H together with PROP1 were carried out to mimic the compound heterozygous status of the HESX1 mutations in patient 3.2. The combination of p.R159W and p.R160H was also unable to repress PROP1 activation (Figure 2a). This lack of repression is not due to different expression levels of HESX1 proteins, as HESX1-WT, HESX1-p.R159W and HESX1-p.R160H were expressed at comparable levels in the cells as determined by Western Blot analysis (Figure 2b).

Discussion

Congenital hypopituitarism (CPHD) occurs in 1:4,000 to 10,000 births, and the molecular aetiology is unknown for the majority of these patients, especially the sporadic cases.[22]

Mutations in *HESX1* apparently account for 8% or less of CPHD cases.[22, 23] Mutations were initially described in patients with SOD, and later in patients presenting with non-syndromic hypopituitarism.[6, 9, 11, 24]

In this study, we identified a homozygous *HESX1* p.R160C mutation by Sanger sequencing in two CPHD patients without SOD from a consanguineous Middle Eastern pedigree. This p.R160C mutation was previously described in two siblings with CPHD and SOD from consanguineous patients of Pakistani origin.[6] The p.R160C change is located in the homeodomain, and EMSA analysis showed that it abrogates DNA binding, consistent with loss of function.[6, 9] The discrepancy in the presence of SOD in the two families with the same mutation suggests the effects of other genes, environment, or chance in enhancing or suppressing the severity of the phenotype.

We used exome sequencing to identify a homozygous p.I26T mutation in *HESX1* in a Brazilian patient who was born to consanguineous parents, and presented with CPHD and a eutopic posterior pituitary lobe. This phenotype differs from that previously reported for a homozygous p.I26T mutation in an unrelated Brazilian patient from consanguineous parents. That patient had ACTH deficiency and an ectopic posterior pituitary gland.[11] The p.I26T mutation is located in the engrailed homology domain, which is required for recruitment of the co-repressor TLE1, and functional analysis revealed that the mutation decreases the repressive function of the protein.[11, 21] The mutation p.I26T has an allele frequency at 0.002% in the general population and none in homozygous state (data from ExAC).

We identified a compound heterozygous *HESX1* mutation p.[R159W];[R160H] in a patient with CPHD, including aplastic anterior lobe but no SOD, using exome sequencing, and we demonstrated that each of these mutations impairs the repressive function of HESX1. In previous studies, two patients from different consanguineous families were reported to be homozygous for *HESX1* p.R160H, but no functional studies were done.[20, 25] Those patients presented with hypoplastic anterior pituitary with deficiencies of GH, TSH, ACTH and prolactin.

Genetically engineered mice provide an opportunity to assess the variability in presentation amongst individuals of identical genotype through generation of large cohorts and the effects of different genetic backgrounds by outcrossing to different inbred strains. A comparison of genetically engineered mice homozygous for the $Hesx1^{null}$, $Hesx1^{R160C}$ and $Hesx1^{I26T}$ mutant alleles suggested that the p.R160C mutation is a null allele, and the p.I26T

is a hypomorph.[26] Despite efforts to normalize the genetic background, there was some variability in presentation amongst animals with the same genotype. 5% of homozygous *Hesx1*^{null} mice have profound abnormalities that include absent telencephalic vesicles, eyes, olfactory placodes and Rathke's pouches.[6] The majority of *Hesx1*$^{R160C/R160C}$ and *Hesx1*$^{I26T/I26T}$ mice had eye defects and enlarged and bifurcated anterior pituitaries. Telencephalic defects were detected in nearly 80% *Hesx1*$^{R160C/R160C}$ mice, but not in *Hesx1*$^{I26T/I26T}$ mice. Interestingly, neither *Hesx1*$^{R160C/R160C}$ nor *Hesx1* $^{I26T/I26T}$ mice were deficient in the induction and differentiation of hormone-producing cells, although pituitary function could not be assessed because of lethality.[26] The phenotypic variability among mice with the same genotype could be due to chance or the action of epigenetic or environmental factors that affect how the mutations express themselves phenotypically.[27] Alternatively, the residual genetic variation in other genes and/or pathways may modify the severity of phenotypes.[28] The completely sequenced genomes of inbred mouse strains and the international Collaborative Cross (CC) project in mice would largely facilitate the mapping of the modifier genes.

To explain the phenotypic variation in humans, we need more comprehensive information about the patients' phenomes and genomes. To discover genetic modifiers of a Mendelian trait by WES, an extreme phenotype study design and/or large sample sizes are required to achieve the statistical power that is needed.[29] WES provides the ability to detect potential disease causing variants in the coding regions across the genome. Given that CPHD is a rare condition in the population and the known variants and genes only account for a minority of the cases, WES will improve the overall detection rate for CPHD mutations. In a cohort of 23 unrelated CPHD patients currently undergoing WES at University of Michigan, only the 2 cases reported in this study were found to harbor pathogenic mutations in a known gene. This detection rate is about 8.7%, which is higher than any other study screening for *HESX1* mutations. WES obviously offers the advantage of identifying novel causes of CPHD and the potential for elucidating multi-genic mechanisms similar to those that have been observed in mice.[28]

References

1 Alatzoglou, K.S. & Dattani, M.T. (2009) Genetic forms of hypopituitarism and their manifestation in the neonatal period. *Early Hum Dev* **85**, 705-712.

2 França, M.M., Jorge, A.A., Carvalho, L.R., Costalonga, E.F., Vasques, G.A., Leite, C.C., Mendonca, B.B. & Arnhold, I.J. (2010) Novel heterozygous nonsense GLI2 mutations in patients with hypopituitarism and ectopic posterior pituitary lobe without holoprosencephaly. *J Clin Endocrinol Metab* **95**, E384-391.

3 Thomas, P.Q. & Rathjen, P.D. (1992) HES-1, a novel homeobox gene expressed by murine embryonic stem cells, identifies a new class of homeobox genes. *Nucleic Acids Res* **20**, 5840.

4 Webb, G.C., Thomas, P.Q., Ford, J.H. & Rathjen, P.D. (1993) Hesx1, a homeobox gene expressed by murine embryonic stem cells, maps to mouse chromosome 14, bands A3-B. *Genomics* **18**, 464-466.

5 Hermesz, E., Mackem, S. & Mahon, K.A. (1996) Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. *Development* **122**, 41-52.

6 Dattani, M.T., Martinez-Barbera, J.P., Thomas, P.Q., Brickman, J.M., Gupta, R., Martensson, I.L., Toresson, H., Fox, M., Wales, J.K., Hindmarsh, P.C., Krauss, S., Beddington, R.S. & Robinson, I.C. (1998) Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. *Nat Genet* **19**, 125-133.

7 Martinez-Barbera, J.P., Rodriguez, T.A. & Beddington, R.S. (2000) The homeobox gene Hesx1 is required in the anterior neural ectoderm for normal forebrain formation. *Dev Biol* **223**, 422-430.

8 Dattani, M.T., Martinez-Barbera, J.P., Thomas, P.Q., Brickman, J.M., Gupta, R., Wales, J.K., Hindmarsh, P.C., Beddington, R.S. & Robinson, I.C. (1999) HESX1: a novel gene implicated in a familial form of septo-optic dysplasia. *Acta Paediatr Suppl* **88**, 49-54.

9 Brickman, J.M., Clements, M., Tyrell, R., McNay, D., Woods, K., Warner, J., Stewart, A., Beddington, R.S. & Dattani, M. (2001) Molecular effects of novel mutations in Hesx1/HESX1 associated with human pituitary disorders. *Development* **128**, 5189-5199.

10 Thomas, P.Q., Dattani, M.T., Brickman, J.M., McNay, D., Warne, G., Zacharin, M., Cameron, F., Hurst, J., Woods, K., Dunger, D., Stanhope, R., Forrest, S., Robinson, I.C. & Beddington, R.S. (2001) Heterozygous HESX1 mutations associated with isolated congenital pituitary hypoplasia and septo-optic dysplasia. *Hum Mol Genet* **10**, 39-45.

11 Carvalho, L.R., Woods, K.S., Mendonca, B.B., Marcal, N., Zamparini, A.L., Stifani, S., Brickman, J.M., Arnhold, I.J. & Dattani, M.T. (2003) A homozygous mutation in HESX1 is associated with evolving hypopituitarism due to impaired repressor-corepressor interaction. *J Clin Invest* **112**, 1192-1201.

12 Sobrier, M.L., Maghnie, M., Vie-Luton, M.P., Secco, A., di Iorgi, N., Lorini, R. & Amselem, S. (2006) Novel HESX1 mutations associated with a life-threatening neonatal phenotype, pituitary aplasia, but normally located posterior pituitary and no optic nerve abnormalities. *J Clin Endocrinol Metab* **91**, 4528-4536.

13 McNay, D.E., Turton, J.P., Kelberman, D., Woods, K.S., Brauner, R., Papadimitriou, A., Keller, E., Keller, A., Haufs, N., Krude, H., Shalet, S.M. & Dattani, M.T. (2007) HESX1 mutations are an uncommon cause of septooptic dysplasia and hypopituitarism. *J Clin Endocrinol Metab* **92**, 691-697.

14 Vivenza, D., Godi, M., Faienza, M.F., Mellone, S., Moia, S., Rapa, A., Petri, A., Bellone, S., Riccomagno, S., Cavallo, L., Giordano, M. & Bona, G. (2011) A novel HESX1 splice mutation causes isolated GH deficiency by interfering with mRNA processing. *Eur J Endocrinol* **164**, 705-713.

15 Webb, E.A., AlMutair, A., Kelberman, D., Bacchelli, C., Chanudet, E., Lescai, F., Andoniadou, C.L., Banyan, A., Alsawaid, A., Alrifai, M.T., Alahmesh, M.A., Balwi, M., Mousavy-Gharavy, S.N., Lukovic, B., Burke, D., McCabe, M.J., Kasia, T., Kleta, R., Stupka, E., Beales, P.L., Thompson, D.A., Chong, W.K., Alkuraya, F.S., Martinez-Barbera, J.P., Sowden, J.C. & Dattani, M.T. (2013) ARNT2 mutation causes hypopituitarism, post-natal microcephaly, visual and renal anomalies. *Brain* **136**, 3096-3105.

16 Karaca, E., Buyukkaya, R., Pehlivan, D., Charng, W.L., Yaykasli, K.O., Bayram, Y., Gambin, T., Withers, M., Atik, M.M., Arslanoglu, I., Bolu, S., Erdin, S., Buyukkaya, A., Yaykasli, E., Jhangiani, S.N., Muzny, D.M., Gibbs, R.A. & Lupski, J.R. (2015) Whole-exome sequencing identifies homozygous GPR161 mutation in a family with pituitary stalk interruption syndrome. *J Clin Endocrinol Metab* **100**, E140-147.

17 Sadeghi-Nejad, A. & Senior, B. (1974) A familial syndrome of isolated "aplasia" of the anterior pituitary. Diagnostic studies and treatment in the neonatal period. *J Pediatr* **84**, 79-84.

18 Arnedo, M., Menao, S., Puisac, B., Teresa-Rodrigo, M.E., Gil-Rodriguez, M.C., Lopez-Vinas, E., Gomez-Puertas, P., Casals, N., Casale, C.H., Hegardt, F.G. & Pie, J. (2012) Characterization of a novel HMG-CoA lyase enzyme with a dual location in endoplasmic reticulum and cytosol. *J Lipid Res* **53**, 2046-2056.

19 Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G. & Mesirov, J.P. (2011) Integrative genomics viewer. *Nat Biotechnol* **29**, 24-26.

20 Durmaz, B., Cogulu, O., Dizdarer, C., Stobbe, H., Pfaeffle, R. & Ozkinay, F. (2011) A novel homozygous HESX1 mutation causes panhypopituitarism without midline defects and optic nerve anomalies. *J Pediatr Endocrinol Metab* **24**, 779-782.

21 Carvalho, L.R., Brinkmeier, M.L., Castinetti, F., Ellsworth, B.S. & Camper, S.A. (2010) Corepressors TLE1 and TLE3 interact with HESX1 and PROP1. *Mol Endocrinol* **24**, 754-765.

22 De Rienzo, F., Mellone, S., Bellone, S., Babu, D., Fusco, I., Prodam, F., Petri, A., Muniswamy, R., De Luca, F., Salerno, M., Momigliano-Richardi, P., Bona, G., Giordano, M. & Italian Study Group on Genetics of, C. (2015) Frequency of genetic defects in combined pituitary hormone deficiency: a systematic review and analysis of a multicentre Italian cohort. *Clin Endocrinol (Oxf)* **83**, 849-860.

23 Coya, R., Vela, A., Perez de Nanclares, G., Rica, I., Castano, L., Busturia, M.A., Martul, P. & group, G. (2007) Panhypopituitarism: genetic versus acquired etiological factors. *J Pediatr Endocrinol Metab* **20**, 27-36.

24 Cohen, R.N., Cohen, L.E., Botero, D., Yu, C., Sagar, A., Jurkiewicz, M. & Radovick, S. (2003) Enhanced repression by HESX1 as a cause of hypopituitarism and septooptic dysplasia. *J Clin Endocrinol Metab* **88**, 4832-4839.

25 Bas, F., Uyguner, Z.O., Darendeliler, F., Aycan, Z., Cetinkaya, E., Berberoglu, M., Siklar, Z., Ocal, G., Darcan, S., Goksen, D., Topaloglu, A.K., Yuksel, B., Ozbek, M.N., Ercan, O., Evliyaoglu, O., Cetinkaya, S., Sen, Y., Atabek, E., Toksoy, G., Aydin, B.K. & Bundak, R. (2015) Molecular analysis of PROP1, POU1F1, LHX3, and HESX1 in Turkish patients with combined pituitary hormone deficiency: a multicenter study. *Endocrine* **49**, 479-491.

26 Sajedi, E., Gaston-Massuet, C., Signore, M., Andoniadou, C.L., Kelberman, D., Castro, S., Etchevers, H.C., Gerrelli, D., Dattani, M.T. & Martinez-Barbera, J.P. (2008) Analysis of mouse models carrying the I26T and R160C substitutions in the transcriptional repressor HESX1 as models for septo-optic dysplasia and hypopituitarism. *Dis Model Mech* **1**, 241-254.

27 Cooper, D.N., Krawczak, M., Polychronakos, C., Tyler-Smith, C. & Kehrer-Sawatzki, H. (2013) Where genotype is not predictive of phenotype: towards an understanding of the molecular basis of reduced penetrance in human inherited disease. *Hum Genet* **132**, 1077-1130.

28 Gaston-Massuet, C., Andoniadou, C.L., Signore, M., Sajedi, E., Bird, S., Turner, J.M. & Martinez-Barbera, J.P. (2008) Genetic interaction between the homeobox transcription

factors HESX1 and SIX3 is required for normal pituitary development. *Dev Biol* **324**, 322-333.

29 Emond, M.J., Louie, T., Emerson, J., Zhao, W., Mathias, R.A., Knowles, M.R., Wright, F.A., Rieder, M.J., Tabor, H.K., Nickerson, D.A., Barnes, K.C., National Heart, L., Blood Institute, G.O.E.S.P., Lung, G.O., Gibson, R.L. & Bamshad, M.J. (2012) Exome sequencing of extreme phenotypes identifies DCTN4 as a modifier of chronic Pseudomonas aeruginosa infection in cystic fibrosis. *Nat Genet* **44**, 886-889.

Table and Figure Legends:

Table 1: Clinical phenotype of patients harboring the *HESX1* mutations.

Figure 1: Pedigrees of families 1, 2 and 3. Filled symbols represent family members with pituitary hormone deficiency. Arrow points to the proband in family 3.

Figure 2: Functional studies reveal failure of *HESX1* compound heterozygous variants [p.R159W/p.R160H] to repress PROP1 activity. (a) Plasmid constructs carrying human PROP1, HESX1-WT, HESX1-p.R159W and HESX1-p.R160H were transfected individually or in combinations into COS-7 cells to measure the activation of firefly luciferase reporter. HESX1-WT, HESX1-p.R159W and HESX1-p.R160H have no effect on transcription by themselves. Equal amounts of HESX1-WT repress PROP1 activation by ~50%. Neither HESX1-p.R159W nor HESX1-p.R160H were able to repress PROP1 activity. The combination of p.R159W and p.R160H was also unable to repress PROP1 activation. The results represent the means of three independent experiments, each performed in triplicate. **(b)** Western blotting (WB) was performed to show that p.R159W and p.R160H mutations do not affect the protein expression level of HESX1 in the COS-7 transfected cells.

Supplementary Material Legend:

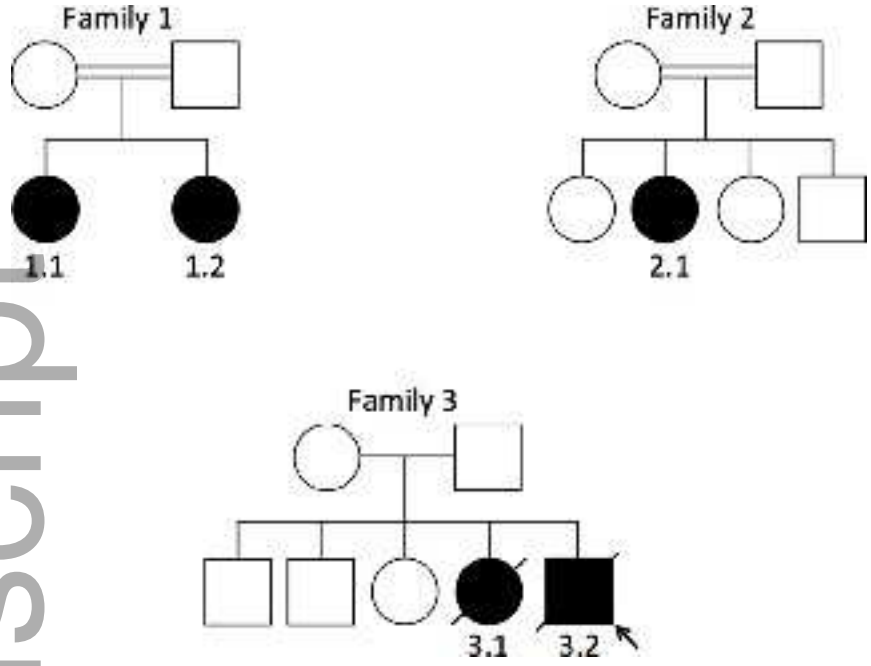
Supplemental Figure 1: Variant discovery and analysis. It shows the multi-stage filtering strategy and the number of single nucleotide variants and insertion/deletions (SNVs + Indels) remaining at each stage.

Table 1: Summary of the clinical phenotypes and MRI findings of CPHD/SOD

Patient	Sex	Clinical symptoms	Affected hormones	MRI findings	HESX1 mutation	Ref
IV-4 and IV-5	Female and male	CPHD and SOD	ACTH, GH, TSH, LH, FSH	APH, EPP, ONH, hypoplasia of the corpus callosum	p.R160C	5 & 9
1.1 and 1.2	Both females	Hydrocephalus	ACTH, TSH, GH	APH/PA, NPP	p.R160C	This study
IV-1	Female	Short stature	ACTH, GH, TSH, LH, FSH	APH, EPP	p.I26T	10
2.1	Female	Short stature	GH, TSH, LH, FSH	APH, NPP	p.I26T	This study
3.2	Male	Lethargic and cyanotic 8 hours after birth	ACTH, GH, TSH, LH, FSH	PA, PP is present and functioning	p.[R159W];[R160H]	This study

patients with HESX1 mutations related with this study

Abbreviations: CPHD, combined pituitary hormone deficiency; SOD, septo-optic dysplasia; ONH, optic nerve hypoplasia; ACTH, adrenocorticotropin; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; APH, anterior pituitary hypoplasia; PA, pituitary aplasia; PP, posterior pituitary; EPP, ectopic posterior pituitary; NPP, normal posterior pituitary; NA, not available.



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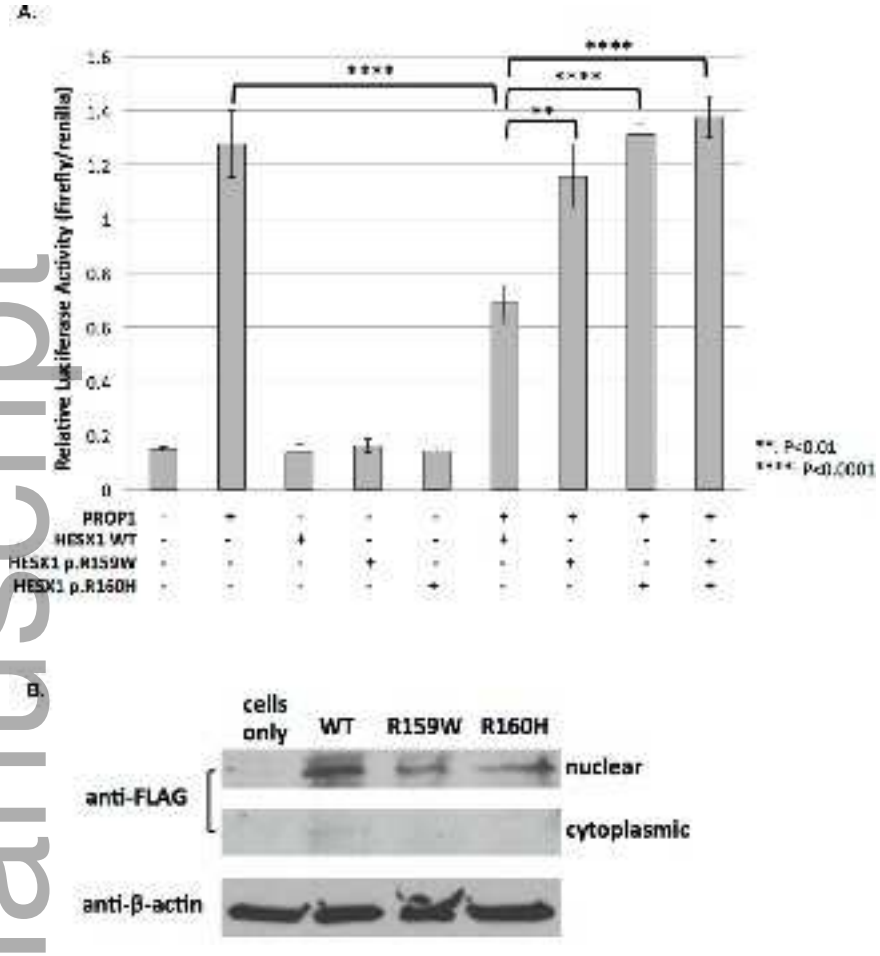

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