Truncating RAX Mutations: Anophthalmia, Hypopituitarism, Diabetes Insipidus, and Cleft Palate in Mice and Men

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Context: The transcription factor *RAX* is a paired-type homeoprotein that plays a critical role in eye and forebrain development of vertebrate species. RAX knockout mice have anophthalmia, cleft palate, and an abnormal hypothalamus and display perinatal lethality. In humans, homozygous or compound heterozygous *RAX* mutations have been reported to cause bilateral microphthalmia or anophthalmia without consistent associated features. Congenital hypopituitarism can be associated with various eye or craniofacial anomalies; however, the co-occurrence of congenital hypopituitarism, anophthalmia, cleft palate, and diabetes insipidus has been very rare.

Results: We report the case of a child with anophthalmia, congenital hypopituitarism, diabetes insipidus, and bilateral cleft lip and palate who had a homozygous frameshift truncating mutation c.266delC (p.Pro89Argfs*114) in exon 1 of the *RAX* gene. *Rax* knockout mice show loss of ventral forebrain structures, pituitary, and basosphenoid bone and palate and a misplaced anterior pituitary gland along the roof of the oral cavity.

Conclusions: Our patient's phenotype was more severe than that reported in other patients. Although most of the previously reported patients with *RAX* mutations showed either a missense or some less severe mutation in at least one of their *RAX* alleles, our patient was homozygous for truncating mutations that would yield a severe, null protein phenotype. The severity of the genetic defect, the precise match between the knockout mouse and the patient's endocrine phenotypes, and the prominent roles of RAX in eye and pituitary development and diencephalic patterning suggest that the *RAX* null mutations could fully account for the observed phenotype. **(*J Clin Endocrinol Metab* 104: 2925–2930, 2019)**

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Abbreviations: CH, congenital hypopituitarism; E, embryonic day; α-GSU, glycoprotein α-subunit.

Anophthalmia is defined as the complete absence of neuroectodermal tissue in the orbit. It can be unilateral or bilateral, and its prevalence has been reported at 1:30,000. Anophthalmia is part of the anophthalmia–microphthalmia–coloboma phenotypic spectrum. The most common causes of bilateral anophthalmia are heterozygous loss-of-function mutations in either *SOX2* or *OTX2* (1).

Congenital hypopituitarism (CH) can be associated with varied eye malformations such as microphthalmia, optic nerve hypoplasia, and coloboma. The reported genetic etiologies of this association include heterozygous loss of function mutations of *OTX2*, *SOX2*, *BMP4*, and *PAX6* (2–6). Optic nerve hypoplasia and retinal coloboma associated with CH have also been described in patients homozygous for mutations of the pituitary transcription factor *HESX1* (7, 8). CH can also be associated with median cleft lip, viewed as a mild form of holoprosencephaly, which results from an impaired cleavage of the embryonic forebrain. *GLI2* mutations are the main cause of such a phenotype (9).

Hence, clinically, although CH can be associated with various eye or craniofacial anomalies, the co-occurrence of CH, anophthalmia, cleft palate, and diabetes insipidus has seldom been described (6, 10).

We report the case of a child with bilateral anophthalmia, panhypopituitarism, diabetes insipidus, and severe bilateral cleft lip and palate. Our patient was homozygous for a truncating mutation in the *RAX* gene. To enrich the description of this hypothalamic-pituitary developmental defect newly described in humans, we have also reported on the morphological and endocrine phenotypes of *Rax* knockout mice. The human and mouse phenotypes are extremely similar.

Case Report

The reported patient was born at term after an uneventful pregnancy and delivery from a consanguineous healthy Afghan couple. He has three healthy siblings. His birthweight was 3960 g, birth length 50 cm, and head circumference 36 cm. His Apgar score was 9, 10, and 10 at 1, 5, and 10 minutes, respectively. At birth, bilateral anophthalmia, bilateral cleft lip and palate, and micropenis were noted. He presented with persistent hypoglycemia in the first few days of life that was controlled by continuous nasogastric feeding. Nausea and vomiting were at the forefront. At 6 days of age, given his midline defect, hypoglycemia, and micropenis, a laboratory assessment of his pituitary hormones was performed (Table 1), which showed panhypopituitarism but detectable levels of the pituitary hormones. Hydrocortisone and then L-thyroxine and GH substitutions were started. This immediately corrected his nausea, vomiting, and hypoglycemia. At 24 days of age, hypernatremia and polyuria were noted. A concomitant elevated plasma osmolality and low urinary osmolality confirmed the presence of diabetes insipidus, which was treated by oral desmopressin (nasal administration was not possible given the cleft lip and palate). Magnetic resonance imaging of the central nervous system (Fig. 1), pituitary, and eye showed true anophthalmia, no sella turcica, and the presence of a pituitary stalk but no visualization of the anterior and posterior pituitary glands, a patent basipharyngeal canal, and complete bilateral cleft palate. No other central nervous system anomalies were noted. Abdominal ultrasonography, echocardiography, auditory and somatosensory brain evoked potentials, and chest and abdominal radiography were performed; all

Table 1. Initial Laboratory Results of Our Patient

Laboratory Test	Result	Reference Range
At 6 d of age		
TSH, mU/L	2.66	0.9–4.3
Free T4, ng/dL	0.6	0.9–2.2
GH, ng/mL	0.38	8–27
IGF-1, ng/mL	31	27–157
ACTH, pg/mL	<5	6–60
Cortisol, μg/dL	0	7–18
LH, U/L	<0.1	0–4.7
FSH, U/L	<0.1	0–7.3
Testosterone, ng/dL	<0.1	50–300
Prolactin, μg/L	43.5	31–236
At 26 d of age		
Sodium plasma, mEq/L	150	135–144
Potassium plasma, mEq/L	4.4	3.5–4.8
Plasma osmolality, mOSM/kg H_2O	310	185–195
Urine osmolality, mOSM/kg H_2O	178	>450

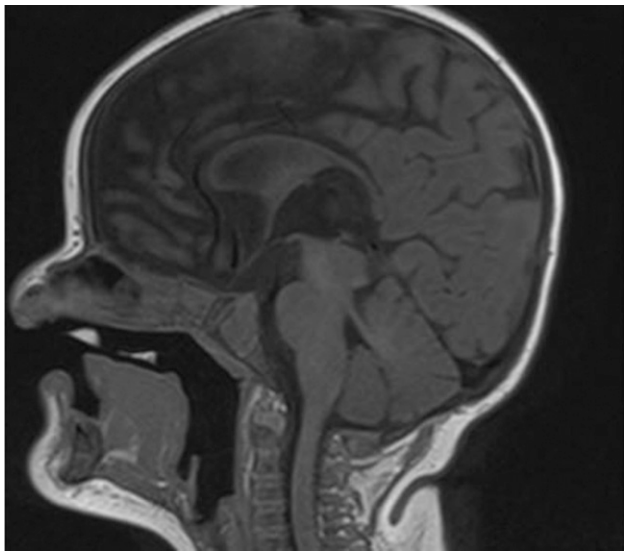

Figure 1. T1-weighted sagittal magnetic resonance imaging scan of the central nervous system showing absent anterior and posterior pituitary gland, absent sella turcica, a rudimentary pituitary stalk, and a patent basipharyngeal canal.

should normal findings. In the first 3 months of life, he presented with daily episodes of hyperthermia without evidence of either infection or inflammation, which were interpreted as hypothalamic temperature instability. In addition, at 3 months of age, he presented with bacterial meningitis secondary to otitis media. At 6 months of age, the cleft lip and palate were repaired and ear tubes inserted. Nonetheless, he presented very frequent episodes of otitis media. At 10 months of age, he could sit without support. His growth followed the 50th percentile for height, head circumference, and weight. In the context of his blindness, a circadian sleep rhythm had not been acquired by 1 year of age, and melatonin substitution was started.

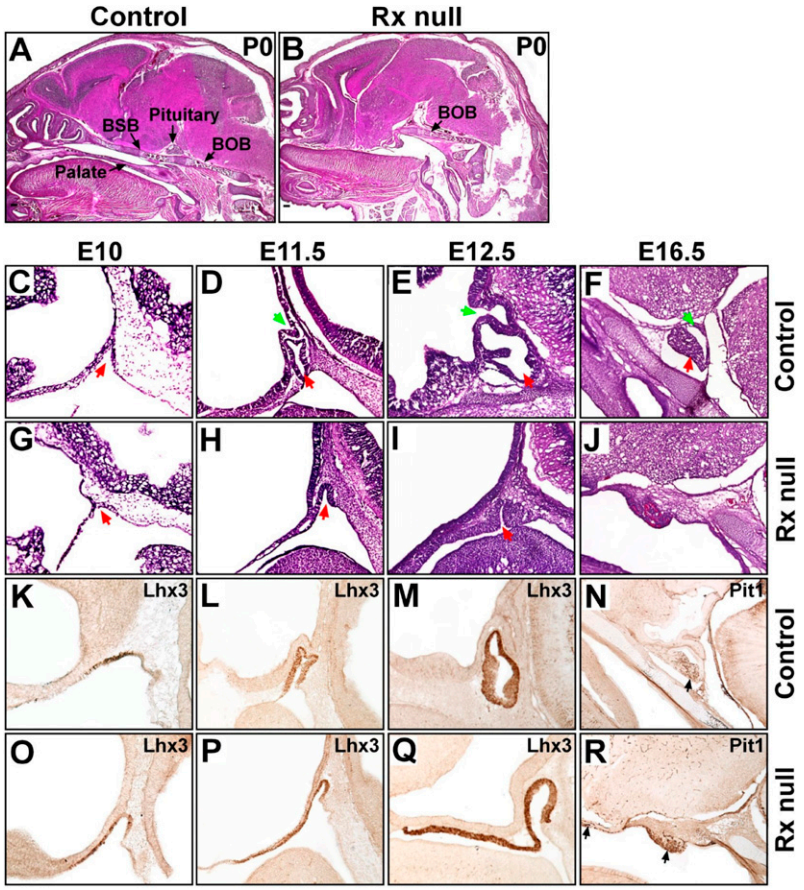

Figure 2. Rax activity is required for proper pituitary, palate, and bone formation. Midline sagittal sections of (A) Neonatal wild-type control and (B) *Rax*-null heads stained with hematoxylin and eosin. Basisphenoid bone, palate, and pituitary do not form properly in *Rax*-knockout embryos. Scale bar = 200 μm. Midline sagittal sections of (C–F, K–N) control and (G–J, O–R) *Rax*-null heads stained with hematoxylin and eosin (C–J) or processed for immunohistochemistry (K–R). (C,G) At E10.0, the Rathke pouch forms normally in *Rax*-knockout embryos (red arrows). (D,H) By E11.5, the infundibulum (green arrow in D) is absent and the Rathke pouch (red arrow) was aberrant in *Rax*-null embryos. (E,I) At E12.5, the infundibulum (green arrow) was still absent in the *Rax*-null embryos. The Rathke pouch (red arrow) has invaginated from the oral ectoderm in the (E) control but not in the (I) *Rax*-null head. (F,J) By E16.5, the three-lobed pituitary structure has not formed in the *Rx*-knockout embryos (J). (K,O) Lhx3 immunostaining at E10 demonstrated normal Rathke pouch formation in *Rax*-null embryos (O). (L,P) By E11.5, defects in Rathke pouch invagination were observed in *Rax*-null mutant embryos (P), with Lhx3 expression little changed from E10 compared with the controls (L). (M,Q) At E12.5, Lhx3-positive tissue in Rathke pouch has budded off from the oral ectoderm in control embryos (M) but has remained along the dorsal roof of the oral cavity in Rax-null embryos (Q). By E16.5, Pit1 expression marked the anterior pituitary gland in controls (arrow in N), but Pit1 expression was only seen along the roof of the oral cavity (arrows in R). BOB, basioccipital bone; BSB, basisphenoid bone.

Materials and Methods

Blood samples were obtained from the patient and his parents after provision of proper informed consent. DNA was extracted in accordance with standard procedures. Targeted exome capture (3638 genes) was achieved in the index case using the SeqCap EZ Choice XL (NimbleGen, Madison, WI). The samples were subsequently sequenced in a paired-end 125-bp run on a HiSeq 1500 instrument (Illumina, San Diego, CA) at the Brussels Interuniversity Genomics High Throughput core (BRIGHTcore, Brussels, Belgium). A bioinformatics pipeline was launched by BRIGHTcore, and filtering of the variants was accomplished using Highlander software (available at: http://sites.uclouvain.be/highlander). Details on pipeline and filtering are available on request. Analysis of the genes involved in hypopituitarism or cleft palate (*OTX2*, *SOX2*, *PAX6*, *SIX6*, *BMP4*, *KAL1*, *SMOC2*, *HESX1*, *GLI3*, *SOX3*, *PROP1*, *LHX3*, *LHX4*, *POU1F1*, *RAX*, *STRA6*, *BCOR*, *CHD7*, *GDF6*, *GLI2*, *FGFR1*, *HCCS*, *SMOC1*, *VSX2*, and *SHH*) was performed.

Animals and tissue preparation

The animal care and use committee at West Virginia University approved the use of mice in the present study. *Rx*/*Rax*-null samples were genotyped as previously described (11). Neonatal (P0) mouse brains and embryonic brain tissue were analyzed *in situ* once collected from pregnant dams after euthanasia. All brains or embryos were fixed immediately in 4% paraformaldehyde overnight at 4°C and equilibrated in 30% sucrose/PBS overnight.

Histologic examination and immunohistochemistry

For immunohistochemical analysis, tissues were sectioned at 14 μm after fixation, except for glycoprotein α-subunit (α-GSU) staining, for which the tissue was embedded fresh, sectioned, and fixed for 10 minutes at 4°C in acetone. The antibodies used in the present study were ACTH (1:1000; DAKO, Glostrup, Denmark), Pit1 (1:250; Abcam, Cambridge, MA), Lhx3 (1:250; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and GH (1:6000), α-GSU (1:200), LH-β (1:6000), TSH-β (1:6000), all from National Hormone and Pituitary Program, National Institute of Diabetes and Digestive Kidney Disease (National Institutes of Health, Bethesda, MD), and Dr. Parlow. The biotinylated secondary antibody and the Vectastain ABC detection kit were from Vector Laboratories

(Burlingame, CA). Diaminobenzidine (Sigma-Aldrich, St. Louis, MO) was used as a peroxidase substrate. Hematoxylin and eosin (Sigma-Aldrich) were used for histological staining of tissue sections.

Results

Present patient

A homozygous frameshift truncating mutation c.266delC (p.Pro89Argfs*114) and a homozygous mutation of unknown significance at position 262 (c.262G>A; p.Ala88Thr) in the *RAX* gene were found in the index case. The parents were healthy heterozygous carriers of both mutations (confirmed by Sanger sequencing). No mutations were found in the other analyzed genes.

Knockout mice

In Fig. 2A and 2B, we present sagittal sections of newborn pups, showing a loss of ventral forebrain structures and the pituitary, basosphenoid bone, and palate. A developmental series of pituitary development is presented in Fig. 2C–2J, showing that the infundibulum (the future posterior pituitary) fails to form and that Rathke pouch will fail to invaginate properly and, instead, remains along the roof of the oral cavity. To definitively identify anterior pituitary structures, we stained for two transcription factors known to be necessary for Rathke pouch and anterior pituitary development, Lhx3 and Pit1. In Rax control mice, these transcription factors will localize to the Rathke pouch at embryonic day (E)10 and E11.5 (Fig. 2K and 2L) and to the anterior pituitary (Fig. 2M and 2N). However, in Rax knockout (Rx null), the Rathke pouch never fully invaginates, leaving Lhx3-positive tissue lining the roof of the oral cavity from E10 to E12.5 (Fig. 2O–2Q). At E16.5, Pit1-positive tissue will remain along the roof of the oral cavity.

Immunohistochemistry findings

To test whether the Lhx3- and Pit1-positive tissue is competent to produce anterior pituitary hormones, we performed immunohistochemistry using antibodies against these hormones on fetal and newborn mouse brain tissue. The expression shown by immunohistochemistry of the anterior pituitary markers, ACTH, LH-β, TSH-β, GH, and α-GSU along the roof of the oral cavity in the Rax knockout mice compared with normal controls, where they all labeled the anterior pituitary is presented in Fig. 3. These results suggest that the anterior pituitary forms but is misplaced and not likely to receive innervation from the posterior pituitary, which does not form at all in the knockout mice (Fig. 2).

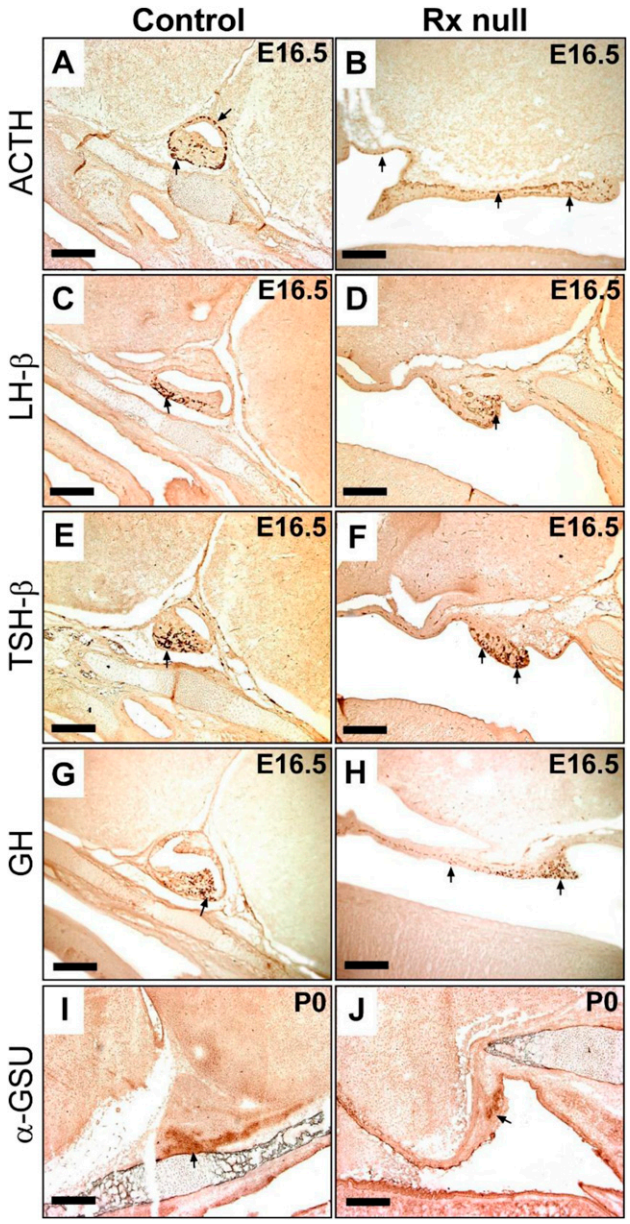

Figure 3. Anterior pituitary hormones are present in *Rax*-null embryos and pups but their location is severely perturbed. Immunostaining of (A,C,E,G,I) control and (B,D,F,H,J) *Rx*-null midline sagittal sections with antibodies for (A,B) ACTH, (C,D) LH-β, (E,F) TSH-β, (G,H) GH at E16.5, and (I,J) α-GSU at P0. Arrows point to immunopositive cells. Just as with Lhx3- and Pit1-positive cells (Fig. 2), anterior pituitary hormone expression can be seen along the roof of the oral cavity in *Rax*-null embryos. Scale bar = 200 μm.

Discussion

We present the case of a child with bilateral anophthalmia and a hypoplastic anterior pituitary gland, leading to hypopituitarism, diabetes insipidus, and median cleft lip and palate, who was homozygous for a *RAX* truncating mutation. We have also presented a complete description of the endocrine phenotype of the mouse Rax knockout model, which showed defects in the anterior and posterior pituitary and absence of the palate and basisphenoid bone. The similarity between the patient and mouse knockout phenotypes suggests a causative role of the truncating *RAX* mutations in the child's phenotype.

RAX is a homeobox gene, whose sequence is conserved among vertebrate species (11, 12). The RAX

homeoprotein is a transcription factor that is necessary for both eye and forebrain development of vertebrates, as reported by Zhang *et al.* (13) in 1997. Early in embryonic mouse development, RAX protein is present in the anterior neural region. In contrast, later, it is observed in the retina, pituitary gland, hypothalamus, and pineal gland. Both the Rax germline knockout and the conditional deletion models possess anophthalmia associated with cleft palate and an abnormal hypothalamus and display perinatal lethality (11, 14).

Establishment of the vertebrate retina follows these developmental steps, all of which are dependent on RAX and other transcription factors: specification of the anterior neural plate, evagination of the optic vesicles from the ventral forebrain, and differentiation of cells (15–17). OTX2 and SOX2 proteins coordinate *RAX* expression in eye development (18). Loss-of-function mutations of these transcription factors are well-known causes of the association between CH and eye malformations (1, 2, 4).

In vitro, RAX expression in embryonic stem cells induces their differentiation into retinal cells but also into pituitary gland and early hypothalamic precursor neurons (17). In summary, the *RAX* homeobox gene is essential for vertebrate eye development but also for the development of other tissues derived from the ventral diencephalon such as the pituitary and pineal glands.

In humans, *RAX* mutations cause microphthalmia and anophthalmia (19). In the reported data, 11 patients with two mutated *RAX* alleles (Table 2) have been described. Most of the reported patients showed either a missense or some less severe mutation in at least one of their RAX alleles. This presumably allowed for some residual activity for the RAX protein. Their eye phenotype was very severe (bilateral anophthalmia) except for the patient reported by Huang *et al.* (22), a 14-year-old boy, who had a later developmental eye anomaly (coloboma) and was homozygous for a possibly damaging missense mutation (PolyPhen2 score, 0.873). The remaining patients had a severe and early developmental eye malformation. A few of them also had extraocular signs. Thus, some patients might have had an undiagnosed endocrine condition: 2 of the 5 patients homozygous for the c.543+3A>G splicing *RAX* mutation reported by Abouzeid *et al.* (20) had died of dehydration in infancy. This could indicate either undiagnosed hypopituitarism or diabetes insipidus, or both. Another homozygous patient was reported to have polyuria or polydipsia, which was most probably the result of diabetes insipidus (21). Finally, Chassaing *et al.* (21) reported on a patient who was compound heterozygous for a gene deletion and a nonsense mutation but whose phenotype could not be detailed owing to pregnancy termination.

In conclusion, we have reported the case of a patient with an unusually severe ocular and extraocular phenotype associated with homozygous severely truncating *RAX* mutations. In addition to bilateral anophthalmia, which has already been described in patients with deleterious

Table 2. Review of Reported Data of Homozygous/Compound Heterozygous RAX Mutations or Deletions in Humans

Investigator	*RAX* Mutation	Type of Mutation	Eye Phenotype	Extraocular Phenotype
Abouzeid *et al.* (20)	Homozygous c.543+3A>G	Splicing	Bilateral anophthalmia	Died in infancy of dehydration, n = 2
		Intron 2, n = 5		Agenesis of the optic nerves, tracts, and chiasm, n = 3; cortical atrophy, n = 1
Present report	Homozygous p.Pro89Argfs*114	Frameshift truncating; exon 1, n = 1	Bilateral anophthalmia	Panhypopituitarism, diabetes insipidus, cleft lip and palate
Chassaing *et al.* (21)	Homozygous p.Arg187Gln/ p.Arg187Gln	Missense, n = 1	Bilateral anophthalmia	Polyuria-polydipsia, developmental delay
	Compound heterozygous p.Tyr160His/ p.Arg188Gln	Missense/missense	Bilateral microphthalmia	NA
	Compound heterozygous p.Ser222*/ RAXdel	Nonsense/deletion	Bilateral anophthalmia	Pregnancy termination
Huang *et al.* (22)	Homozygous p.Ile38Thr possibly damaging (PolyPhen2 score: 0.873)	Missense, n = 1	Iris coloboma left/choroid coloboma right plus retinoschisis	NA
Voronina *et al.* (19)	Compound heterozygous p.Gln147X/p.Arg192Gln	Truncating/missense	Anophthalmia/sclerocornea, persistent fetal vasculature, retinal detachment.	Abnormal EEG findings, autism
		Homeodomain, n = 1		Normal CNS MRI findings
Lequeux *et al.* (23)	Compound heterozygous p.Ser222Argfs*62/p.Tyr303*	Truncating/truncating; exon 3, n = 1	Bilateral anophthalmia	Hypoplastic optic tracts and chiasm, normal CNS MRI findings

Abbreviations: CNS, central nervous system; EEG, electroencephalography; MRI, magnetic resonance imaging; NA, not applicable.

RAX mutations, he displayed absent or hypoplastic anterior and posterior pituitary glands (hypopituitarism plus diabetes insipidus) and a median cleft lip and palate.

We believe that the severe nature of the genetic defect (homozygous frameshift truncating mutation that lies upstream to the homeodomain in exon 1) could account for the unusual severity of our patient's phenotype. In addition, we have documented that our patient's endocrine phenotype was extremely similar to the Rax knockout mouse phenotype. For these reasons, and given its prominent direct and indirect (through PAX6 and SHH) roles in eye and pituitary development and in diencephalic patterning, we believe that these deleterious *RAX* mutations were most likely the cause of the phenotype of our patient.

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