# Mutations within *Sox2*/*SOX2* are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans

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**The transcription factor *SOX2* is expressed most notably in the developing CNS and placodes, where it plays critical roles in embryogenesis. Heterozygous de novo mutations in *SOX2* have previously been associated with bilateral anophthalmia/microphthalmia, developmental delay, short stature, and male genital tract abnormalities. Here we investigated the role of *Sox2* in murine pituitary development. Mice heterozygous for a targeted disruption of *Sox2* did not manifest eye defects, but showed abnormal anterior pituitary development with reduced levels of growth hormone, luteinizing hormone, and thyroid-stimulating hormone. Consequently, we identified 8 individuals (from a cohort of 235 patients) with heterozygous sequence variations in *SOX2*. Six of these were de novo mutations, predicted to result in truncated protein products, that exhibited partial or complete loss of function (DNA binding, nuclear translocation, or transactivation). Clinical evaluation revealed that, in addition to bilateral eye defects, *SOX2* mutations were associated with anterior pituitary hypoplasia and hypogonadotropic hypogonadism, variable defects affecting the corpus callosum and mesial temporal structures, hypothalamic hamartoma, sensorineural hearing loss, and esophageal atresia. Our data show that *SOX2* is necessary for the normal development and function of the hypothalamo-pituitary and reproductive axes in both humans and mice.**

## Introduction

SOX2 is a member of the sex-determining region of the Y chromosome–related (SRY-related) high-mobility group (HMG) box (SOX) family of transcription factors, encoded by 20 genes in humans and mice, each of which carries a 79–amino acid HMG box DNA-binding domain similar to that of SRY as well as domains implicated in transcriptional regulation (1, 2). Based on HMG box homology, they are grouped into different subfamilies. *SOX1*, *SOX2*, and *SOX3* belong to the B1 subfamily and are expressed in various phases of embryonic development and cell differentiation, where they play critical roles in embryogenesis (3, 4). All 3 mark neuroepithelial progenitors and stem cells from the earliest stages of development, and there is a strong, but not absolute, tendency for them to be downregulated as cells differentiate.

In the mouse, *Sox2* RNA is first detected in cells at the morula stage (2.5 dpc) and then in the inner cell mass of the blastocyst (3.5 dpc). After implantation the gene is expressed throughout the epiblast and extraembryonic ectoderm, the multipotent precursors of all embryonic and trophoblastic cell types, and targeted mutagenesis has shown that *Sox2* plays a crucial role in both these regions (5). After gastrulation *Sox2* expression within the embryo becomes largely restricted to the presumptive neuroectoderm, and by 9.5 dpc it is expressed throughout the brain, neural tube, sensory placodes, branchial arches, and gut endoderm (6). Later, *Sox2* is expressed in neural stem cells residing in the ventricular zone of the developing neural tube and in their immediate progeny throughout embryogenesis and into adulthood, where it continues to mark stem cells and neural progenitors in the subventricular layer of the lateral ventricles and the subgranular layer of the hippocampus/dentate gyrus. It is also maintained in some differentiated cell types within the CNS.

*Sox2*$^{\beta geo}$ heterozygous mice appear relatively normal but show a reduction in size and male fertility, the extent of which is correlated with genetic background (5). *Sox2*$^{-/-}$ embryos are unaffected at blastocyst stages, but fail to survive shortly after implantation (5). By introducing a regulatory mutation in *Sox2* (*Sox2*$^{\Delta ENH}$) in addition to the *Sox2*$^{\beta geo}$ null allele, Ferri et al. accessed some later functions of SOX2 in the CNS (7). Compound heterozygosity for both alleles reduced levels of *Sox2* expression to 25–30% of wild-type levels within specific regions of the CNS. This resulted in a reduction both in the number of pups being born and in their postnatal survival, with surviving mice showing severe abnormalities including growth retardation (although this was usually compensated by 6

**Nonstandard abbreviations used:** APH, anterior pituitary hypoplasia; FSH, follicle-stimulating hormone; GH, growth hormone; GHRH, GH-releasing hormone; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; HH, hypogonadotropic hypogonadism; HMG, high-mobility group; IGFBP3, IGF-binding protein 3; LH, luteinizing hormone; NR, normal range; PRL, prolactin; rhGH, recombinant human GH; SDS, standard deviation score; SOD, septo-optic dysplasia; SOX, SRY-related HMG box; TSH, thyroid-stimulating hormone; TRH, thyrotropin-releasing hormone.

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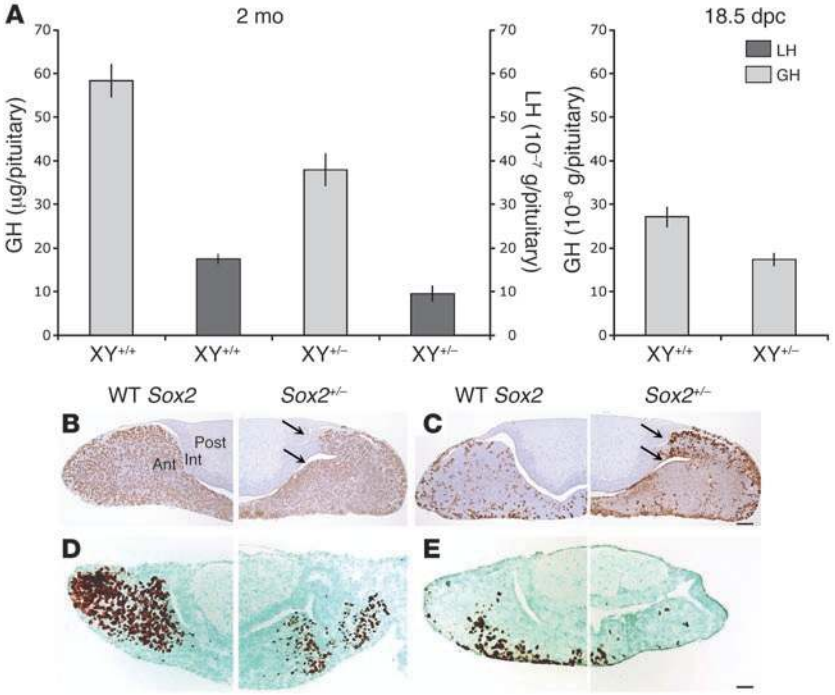


**Figure 1**
Pituitary hormone levels and immunohistochemistry in *Sox2* heterozygous mice. (**A**) GH and LH RIAs on pituitary protein extracts from 6 wild-type ($XY^{+/+}$) and 5 *Sox2* heterozygous ($XY^{+/-}$) 2-month-old littermates (GH, $P = 0.004$; LH, $P = 0.002$) and 10 wild-type and 9 *Sox2* heterozygous 18.5 dpc littermates ($P = 0.002$). Both GH and LH were affected; GH deficiency appeared before birth. (**B**–**E**) Immunohistochemistry for GH (**B** and **D**) and LH (**C** and **E**) on pituitary sections from 3-month-old (**B** and **C**) and 18.5 dpc (**D** and **E**) wild-type and *Sox2* mutant littermates. Ant, anterior lobe; Int, intermediate lobe; Post, posterior lobe. Note the presence of extra clefts in the adult sections (arrows in **B** and **C**). Staining was clearly reduced in heterozygotes at 18.5 dpc (**D** and **E**). Scale bar: 0.3 mm (**B** and **C**); 0.05 mm (**D** and **E**).

weeks of age), dystonic reactions, epileptic spikes in the cortex and hippocampus, and circling behavior. These data support a role for SOX2 in neural progenitors, as has been suggested from studies in other model systems (3), as well as highlight its importance for the maintenance of specific neuronal populations.

Like its murine counterpart, the human *SOX2* gene is a single-exon gene encoding a 317–amino acid protein containing an N-terminal domain of unknown function, a DNA-binding HMG domain, and a C-terminal transcriptional activation domain. Heterozygous mutations within *SOX2* in humans have to date been associated with bilateral anophthalmia and severe microphthalmia (8–12). Additional phenotypic abnormalities include developmental delay, learning difficulties, esophageal atresia, sensorineural hearing loss, and genital abnormalities. To date, 12 heterozygous mutations have been identified in 14 patients, all characterized by bilateral eye disease. All these mutations — 5 nonsense, 4 frameshift, 1 deletion, and 2 missense — occurred de novo. It has been speculated that the mutations are associated with loss of function, but detailed functional studies have not yet been performed.

Given the reduced size and impaired male fertility in $Sox2^{\beta geo}$ heterozygotes, we investigated the hypothalamo-pituitary axis in these animals. We found that they manifested a variable hypopituitary phenotype affecting the size and shape of the pituitary gland and causing a generalized reduction in hormone content, including a significant lowering of growth hormone (GH), luteinizing hormone (LH), ACTH, and thyroid-stimulating hormone (TSH), which would explain many aspects of their phenotype. We also report the phenotypes associated with 3 recently identified heterozygous frameshift mutations and 1 nonsense mutation that we believe to be novel, in addition to 1 previously described heterozygous frameshift mutation and 1 nonsense mutation within *SOX2* in 6 patients with anophthalmia/microphthalmia. Surprisingly, all of the affected patients manifested hypogonadotropic hypogonadism (HH), which resulted in the absence of puberty in all patients and genital abnormalities in males. We show that the 4 *SOX2* mutations we believe to be novel as well as the 3 previously described mutations (8, 9, 11) led to varying degrees of loss of transcriptional activity, revealing that haploinsufficiency for *SOX2* is likely to be the cause of the human phenotypes. There were differences in the range of other tissues affected in mice and humans; however, a common feature was the prevalence of pituitary endocrine deficits, implying a critical role for SOX2 in the development and function of the hypothalamo-pituitary axis in both species.

## Results

### *Sox2 is required for normal murine pituitary development*

In mice, homozygous deletion of *Sox2* results in peri-implantation lethality, preventing studies of its later roles, while *Sox2* heterozygous animals appear to be mostly unaffected. Some of the heterozygotes, however, are smaller than their wild-type littermates, and fertility problems are common in males (5). Moreover, since the related *Soxb1* gene, *Sox3*, has recently been shown to be necessary during development of the hypothalamic-pituitary axis (13), we explored whether there was a similar involvement for *Sox2*.

There was a moderate reduction of pituitary LH and GH content in 2-month-old *Sox2* heterozygotes compared with wild-type littermates, which was statistically significant in males but not in females (Figure 1A). Clearly, the LH deficit contributed to the fertility problems observed in males (5), while the reduction in GH was consistent with the moderate and variable reduction in size observed in the mutants. We considered the possibility that feeding difficulties could also be responsible, since esophageal atresia has been observed in some *SOX2* human patients (patient 1; see Methods) (12), and *Sox2* is expressed in the embryonic gut

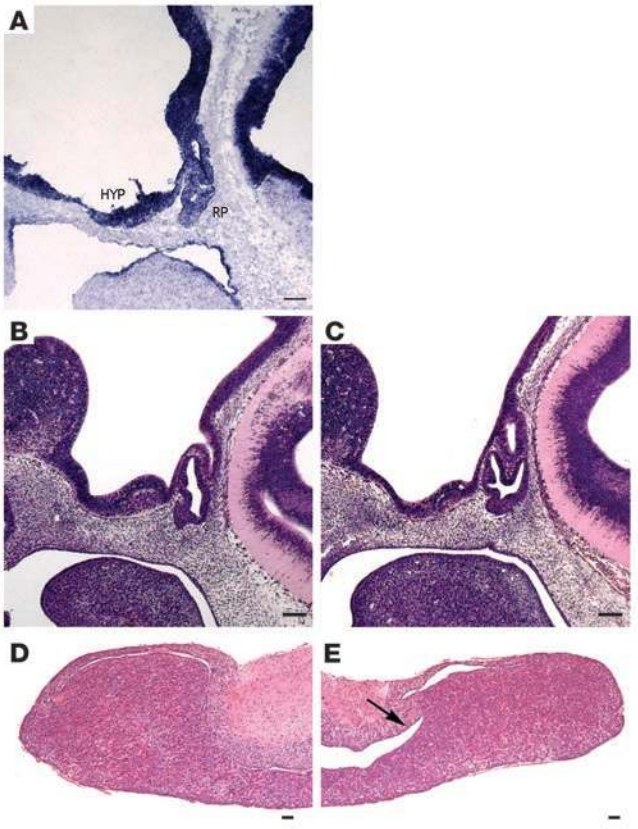


**Figure 2**
*Sox2* expression and abnormal morphogenesis of the pituitary in *Sox2* heterozygotes. (**A**) Sagittal section of an 11.5-dpc wild-type embryo hybridized to *Sox2*, showing expression in both the CNS and Rathke's pouch. (**B** and **C**) Sagittal sections of 12.5 dpc wild-type (**B**) and *Sox2* heterozygous (**C**) embryos, showing bifurcation of the pouch in the mutant embryo. (**D** and **E**) Pituitary transverse sections of 5-week-old wild-type (**D**) and *Sox2* heterozygous mice (**E**). Note the presence of an extra cleft in the *Sox2* heterozygous pituitary (arrow). HYP, presumptive hypothalamus; RP, Rathke's pouch. Scale bars: 0.1 mm.

endoderm (5, 12). However, comparison of the esophagus at 14.5 dpc in heterozygous mutants ($n$ = 5) and wild-type littermates showed no abnormalities.

Deficiencies in GH and LH may be of either hypothalamic or pituitary etiology. The regulation of pituitary hormone secretion by hypothalamic tropic factors does not become prominent until after birth, so we also examined embryonic pituitaries at 18.5 dpc (Figure 1A) and found a significant reduction in GH content in both male and female *Sox2* heterozygous embryos (LH contents at this stage were below levels detectable by RIA). We also studied pituitary somatotropes (GH) and gonadotropes (LH) by immunohistochemistry at 3 months of age (Figure 1, B and C) and at 18.5 dpc (Figure 1, D and E). In the adult, the somatotrope and gonadotrope content did not appear strikingly reduced in the mutant sections (Figure 1, B and C). However, we noted the presence of some morphological defects: extra clefts were sometimes observed in the mutants (arrows in Figure 1, B and C). Moreover, the region between the 2 clefts that histologically resembled the intermediate lobe was aberrantly positive for both somatotropes and gonadotropes. At 18.5 dpc, not only were morphological defects observed that were similar to those seen in the adults, immunohistochemistry suggested that somatotropes and gonadotropes were reduced in number in the mutants (Figure 1, D and E). We therefore counted the number of somatotropes and found that they were significantly reduced in *Sox2* heterozygous mutants (wild-type, 2,969.5 ± 456.4, $n$ = 4; *Sox2* heterozygotes, 1,230 ± 248.7, $n$ = 4; $P$ = 0.0155). Although the number of gonadotropes was also reduced (wild-type, 559.5 ± 54.2, $n$ = 4; *Sox2* heterozygotes, 378.5 ± 54.2, $n$ = 4; $P$ = 0.0534), this difference did not reach statistical significance. The anterior lobe itself was reduced in size in some mutants (Figure 1E), and the reduction in the number of endocrine cells may therefore reflect a general reduction in gland size. We then compared ratios between the number of somatotropes and the surface area of the anterior lobe. Using this approach, we found no significant difference between wild-type and *Sox2* heterozygous pituitaries (wild-type, 0.0106 ± 0.0017, $n$ = 4; *Sox2* heterozygotes, 0.0064 ± 0.0004, $n$ = 4; $P$ = 0.525). These data strongly suggest that the reduction in somatotropes (and gonadotropes) is not cell type specific, but rather reflects a general reduction in gland size, and this alone could explain the GH deficit at 18.5 dpc. Consistent with a pan-pituitary defect, other hormones (e.g., TSH and prolactin [PRL]) were also found at reduced levels in some adult pituitaries (data not shown).

It should also be noted that about one-third of heterozygous mutant animals are lost between birth and weaning (A. Avilion and R. Lovell-Badge, unpublished observations), and therefore the *Sox2* heterozygous adult population reflects surviving, mildly affected mutants. In agreement with this, the number of endocrine cells did not seem to be affected in the adults (Figure 1, B and C). Moreover, no significant difference in ACTH content was observed between wild-type and *Sox2* heterozygous

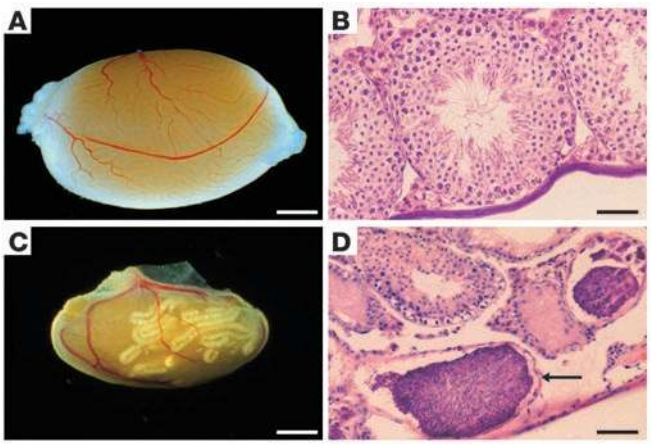


**Figure 3**
Testes morphology of *Sox2*$^{+/-}$ males. (**A**) Testis of an 11-month-old wild-type mouse. (**B**) Histological analysis of the testis shown in **A**. All the different stages of germ cells are present within the seminiferous tubules. (**C**) Testis of an 11-month-old *Sox2*$^{+/-}$ mouse showing reduced size and white opaque patches, indicative of sperm blockage. This was confirmed by histological analysis (**D**), where aberrant tubules were seen that contained very large numbers of mature sperm only, instead of a range of spermatogenic stages (arrow). The somatic cells also looked abnormal in these tubules. Scale bars: 1,260 μm (**A** and **C**); 55 μm (**B**); 42 μm (**D**).

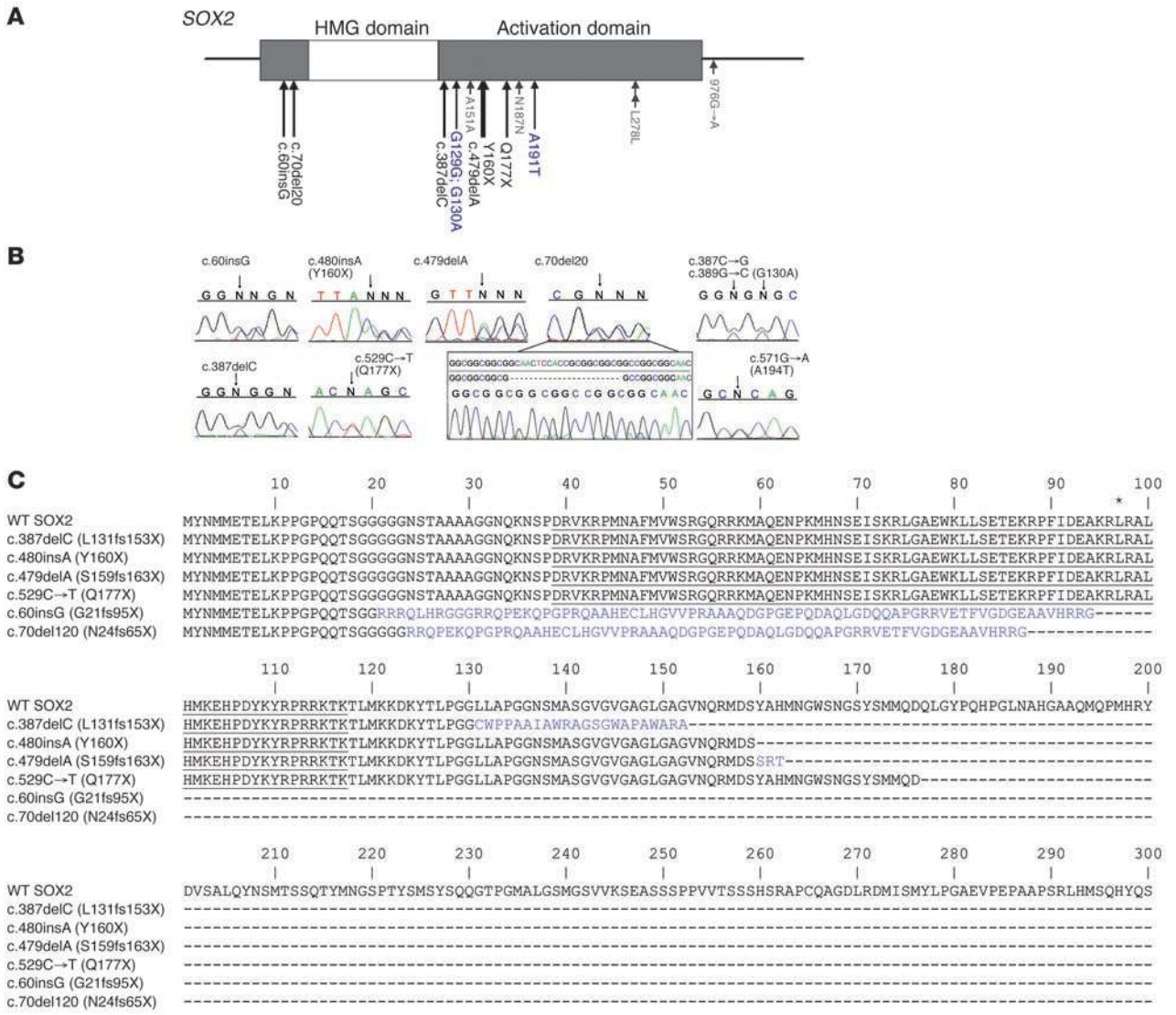


**Figure 4**

Mutation analysis of *SOX2*. (**A**) Schematic diagram of the single exon of *SOX2* showing the position of the HMG domain and transactivation domain. The position of sequence changes identified in the cohort reported here are shown with their relative positions within the gene; frameshift and nonsense mutations are shown in black, nonsynonymous changes in blue, and synonymous variants and a single nucleotide polymorphism in the 3′ untranslated region in gray. (**B**) Electropherograms of the frameshift, nonsense, and nonsynonymous coding variants with the position of each mutation marked by an arrow, demonstrating that each mutation is heterozygous. Also shown is a sequence trace from a cloned PCR product from patient 2 with the c.70del20 mutation showing the extent of the deletion by alignment with wild-type *SOX2* sequence. Note patient 7 with the G130A variant had an additional heterozygous synonymous sequence change 2 bp upstream (c.387C→G). "N" denotes heterozygous peaks in the electropherogram. (**C**) Alignment of predicted SOX2 mutant proteins with part of the wild-type SOX2 protein sequence showing the extent of the truncations. The mutations c.60insG and c.70del20 were predicted to produce proteins that completely remove the HMG box (underlined). Amino acids shown in blue result from frameshifts and do not align to the wild-type SOX2 sequence. Asterisk indicates the position of L97.

adult pituitaries, whereas a significant reduction in ACTH was observed in *Sox2* heterozygotes at P7 (wild-type, 33.6 ± 2.4, $n = 8$; *Sox2* heterozygotes, 22.5 ± 3.1, $n = 6$; $P = 0.01$), suggesting a loss of ACTH-deficient *Sox2* heterozygous pups. However, the GH deficit was comparable between 18.5-dpc and adult mice (Figure 1A) and was also observed at P7 (wild-type, 1.2 ± 0.1; *Sox2* heterozygotes, 0.72 ± 0.1; $P = 0.004$). These data suggest that in the adult, if the gland is itself less affected, then defects in the hypothalamus, where *Sox2* expression is maintained (our unpublished observations), may partially explain the occurrence of comparable deficits.

These results suggested that *Sox2* heterozygous deletion affects the development of the pituitary gland and possibly hypothalamic secretion. Hence we examined *Sox2* expression during early development of the hypothalamo-pituitary axis (Figure 2). At 11.5 dpc, *Sox2* was expressed both in the presumptive hypothalamus and in the primordium of the anterior pituitary, Rathke's pouch (Figure 2A). Later on, as cell differentiation occurs, *Sox2* expression is mostly confined to the proliferative zone, around the Rathke's pouch lumen (14), and in the adult gland, expression is maintained in some non-endocrine cell types (T. Fauquier

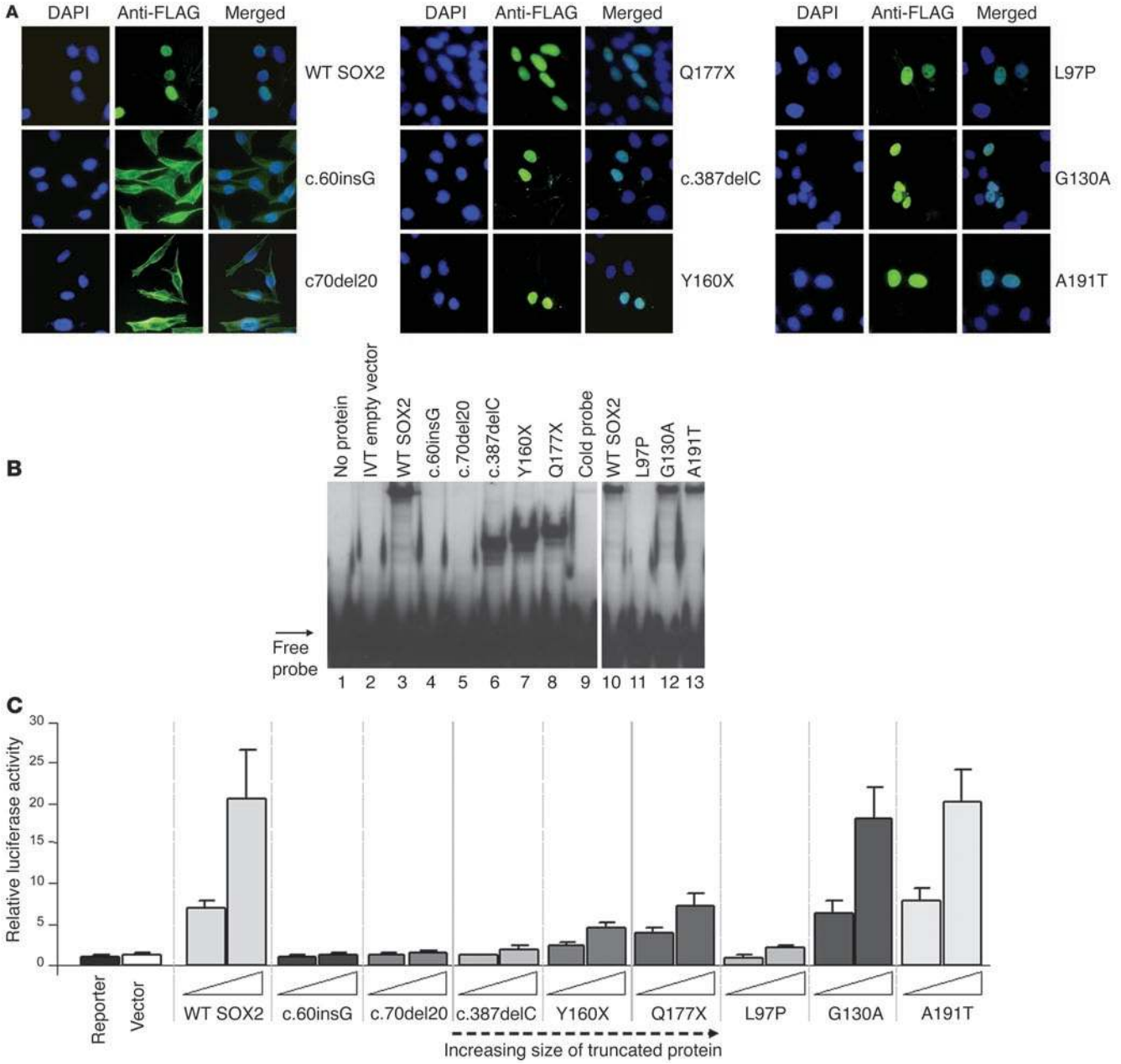


**Figure 5**

Functional analysis of SOX2 mutants. (**A**) Loss of the HMG box results in impaired nuclear localization. Cells were transfected with 50 ng plasmid construct containing wild-type or mutant *SOX2* fused to an N-terminal FLAG epitope, fixed, and stained with anti-FLAG antibody; nuclei were counterstained with DAPI. Wild-type SOX2 showed predominantly nuclear staining. Mutant proteins lacking the HMG box (c.60insG and c.70del20) showed a majority of staining in the cytoplasm and impaired nuclear localization. Mutant proteins retaining the HMG box showed staining similar to wild-type. (**B**) SOX2 mutants show variable DNA binding. Identical amounts of in vitro translated wild-type (lanes 3 and 10) or individual variant SOX2 proteins (lanes 4–8 and 11–13) were incubated with a radiolabeled DNA probe. Specific binding of SOX2 to the probe was demonstrated by duplicate experiments using in vitro translated empty vector (lane 2) or 100 times excess cold probe (lane 9). (**C**) SOX2 mutants show impaired transcriptional activation. Expression constructs containing wild-type or variant SOX2 (10–50 ng) were cotransfected with 20 ng luciferase reporter construct containing part of the proximal promoter of *HESX1*. Wild-type SOX2 led to dose-dependent activation of the reporter (up to 20-fold activation), whereas mutant truncated SOX2 proteins showed impaired activation correlating with the extent of the truncation (dashed arrow). c.60insG, c.70del20, and L97P showed similar levels of activation to empty expression vector. In all experiments described here, c.479delA showed identical results to Y160X (not shown). Results are mean ± SD of 3 independent experiments performed in triplicate.

and K. Rizzoti, unpublished observations). We then examined Rathke's pouch development in *Sox2* heterozygous embryos at 12.5 dpc (Figure 2, B and C). In one-third of the embryos ($n$ = 9) we observed the presence of a bifurcated Rathke's pouch (Figure 2C), which may explain the extra cleft that we observed in some adult mutants (arrow, Figure 2E).

**Table 1**
Endocrine phenotype in patients with *SOX2* mutations

| Pt | Mutation | Presenting age (yr) | Sex | Height (SDS) | Peak GH (ng/ml) | IGF-1 | Free T4 (ng/dl) | Peak TSH (μU/ml) | Basal PRL (ng/ml) | Peak cortisol (μg/dl) | Peak LH (IU/l) | Peak FSH (IU/l) | Age at LHRH testing (yr) | Peak testosterone 3-d HCG (ng/dl)[A] | Peak testosterone 3-wk HCG (ng/dl)[B] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | c.60insG | 12.3 | F | –2.54 | 12.9 (G) | –1 SDS | 1.1 | 10.6 | 6.45 | 33.8 | 6.1 | 5.7 | 13.0 | – | – |
| 2 | c.70del20 | 22 | F | –0.1 | 11.1 (G-A) | 148 mg/l[C] | 0.9 | N/A | 14.2 | 32.9 | 0.7 | 1.9 | 22 | – | – |
| 3 | c.387delC | 2.75 | M | –0.25 | 13.8 (G) | 0 SDS | 1.4 | 28.3 | 8.75 | 19.9 | <0.7 | <0.5 | 1.8 | <20 | 58 |
| 4 | Y160X | 9 | M | –2.8 | 11.6 (C), >36.7 (G-A) | –1 SDS | 1.1 | N/A | 9.8 | 6.9 (basal) | < 0.5 | 2.2 | 14 | – | – |
| 5 | Q177X | 9 | M | –1.2 | 10.8, 19.5 (G) | 0 SDS | 1.05 | 9.57 | 34 | 32.4 | < 0.5 | 1.5 | 9 | 26 | – |
| 6 | c.479delA | 1 | M | –3.4 | 4.2 (G), 2.6 (A) | 7.3 nmol/l[D] | 0.84 – 1.05 | N/A | 7.75 | 27.2 | 1.7 | < 0.5 | 2 | – | – |
| 7 | G130A | 11 | F | –2.5 | ND | ND | ND | ND | ND | ND | ND | ND | N/A | – | – |
| 8 | A191T | 0 | F | – | 2.3 (G) | –2 SDS | 0.7 | 16.4 | 22.4 | 9.7 | 13 | 49.1 | 0.6 | – | – |

Pt, patient; N/A, not available; ND, not done; G, glucagon; G-A, GHRH-arginine; C, clonidine; A, arginine; LHRH, LH-releasing hormone. [A]βHCG 500 U/d given i.m. for 3 days. [B]βHCG 500 U/d given twice weekly for 3 weeks. [C]NR, 116–447 mg/l. [D]NR, 4.1–26 nmol/l.

### *Sox2*$^{\beta geo}$ *heterozygous males show impaired fertility*

On an inbred 129 background, fertility in *Sox2* heterozygous males was clearly impaired. Fertile outbred males were therefore selected for and used to maintain the colony, resulting in a decreased penetrance of the infertility phenotype. However morphological analysis on outbred *Sox2* heterozygous males showed that these animals occasionally had smaller testes with sperm blockage in the seminiferous tubules (Figure 3). In these tubules, somatic cells also looked abnormal. These blockages may affect epididymal sperm count, which in some mutants was found to be approximately 50% that of wild-type animals. However, these changes should not affect fertility. The infertility is more likely a consequence of a primary defect in the developing sperm that compromises their motility and/or their ability to fertilize (A. Avilion and R. Lovell-Badge, unpublished observations). The fertility of heterozygous females, on the other hand, was not significantly reduced (A. Avilion, unpublished observations).

### *Mutation analysis of human SOX2*

Of the cohort of 235 individuals screened for variations in the *SOX2* gene, 6 unrelated individuals were found to have heterozygous mutations in *SOX2* coding regions predicted to result in the production of a truncated mutant protein (Figure 4). Two mutations were single-base deletions (c.387delC and c.479delA), one was a single-base insertion (c.60insG), and another affected individual had a deletion of 20 nucleotides (c.70del20; Figure 4, A and B). We believe three of these mutations are novel, while the fourth (c.70del20) was recently described in an 11-month-old girl with bilateral anophthalmia, rudimentary optic nerves, and partial agenesis of the corpus callosum (11). All of these mutations gave rise to a frameshift in the coding sequence. We also identified 2 nonsense mutations: what we believe to be a novel insertion at nucleotide 480 (c.480insA) resulting in the amino acid change Y160X, as well as an individual with the mutation Q177X (c.529C→T) that has been observed previously in 2 unrelated individuals (Figure 4B and ref. 8). Sequencing the parental DNAs of each of these patients (data not shown) revealed they had a normal *SOX2* sequence, indicating that all 6 mutations identified occurred de novo. Figure 4C shows the extent of the predicted truncations in the mutant proteins compared with wild-type SOX2, the 2 shortest of which (c.60insG and c.70del20) would result in proteins lacking the DNA-binding HMG domain. The other 4 mutations retain the HMG domain but would be predicted to lack variable portions of the transactivation domain.

From the rest of the cohort, 2 individuals were found to harbor heterozygous nonsynonymous amino acid variants. One of these was a G→A transition at nucleotide c.571, resulting in a substitution of a threonine for an alanine at codon 191 (A191T). A191T is conserved among human, mouse, and chicken, but is substituted by threonine in the zebrafish. The other nucleotide change (c.389G→C) resulted in a glycine to alanine substitution at residue 130 (G130A), which is highly conserved among mouse, human, zebrafish, and chicken. This individual also had a synonymous c.387G→C change 2 nucleotides upstream (G129G; Figure 4B). In both cases, these variants were inherited from the affected patients' respective fathers (data not shown), who were phenotypically normal, although the variants were not identified in 100 control chromosomes. Three other synonymous variants were also identified, A151A, N187N, and L278L; the latter was observed in 2 unrelated individuals in this cohort and has also been previously reported

**Table 2**
Clinical phenotype in patients with *SOX2* mutations

| Pt | Mutation | MRI | Ocular phenotype | Other features |
|---|---|---|---|---|
| 1 | c.60insG | Hippocampal abnormalities, small corpus callosum, hypothalamic hamartoma, APH, generalized reduction of white matter bulk, absent optic nerves | Bilateral anophthalmia | HH, learning difficulties, spastic diplegia, esophageal atresia |
| 2 | c.70del20 | Hippocampal abnormalities, abnormal anterior pituitary, absent left optic nerve | Left anophthalmia, right microphthalmia | HH, learning difficulties |
| 3 | c.387delC | Hypoplastic corpus callosum, APH, hypothalamic hamartoma, small left optic nerve and chiasm, generalized lack of white matter bulk, hippocampal abnormalities with small and rotated mesial temporal structures | Left microphthalmia, right coloboma | HH, cryptorchidism, micropenis, learning difficulties, mild spastic diplegia |
| 4 | Y160X | Partial agenesis of corpus callosum, small anterior pituitary, hippocampal abnormalities, generalized reduction in white matter | Bilateral microphthalmia | HH, cryptorchidism, micropenis, severe learning difficulties, spastic and dystonic quadriparesis |
| 5 | Q177X | Not done | Bilateral anophthalmia | HH, cryptorchidism, micropenis, severe learning difficulties, mild facial dysmorphism |
| 6 | c.479delA | APH, small hippocampus, thin corpus callosum, cavum septum pellucidum, absence of optic nerves and chiasm | Bilateral anophthalmia | HH, small testes, micropenis, learning difficulties, sensorineural deafness |
| 7 | G130A | Absent septum pellucidum, bilateral optic nerve hypoplasia, bilateral schizencephaly, right porencephalic cyst, normal anterior and posterior pituitary | Roving nystagmus with bilateral optic nerve hypoplasia | Short stature with a normal growth velocity; endocrine status not investigated |
| 8 | A191T | Absent septum pellucidum, small optic chiasm, absent infundibulum, severe APH, ectopic-undescended posterior pituitary | Roving nystagmus with bilateral optic nerve hypoplasia | GH, TSH, and ACTH deficiency |

(9). Finally, a common single nucleotide polymorphism in the 3′ untranslated region (976G→A) was detected with a frequency of approximately 5%, consistent with previous observations (8).

Since *SOX2* is a single exon gene, it is highly unlikely that the mutations described would lead to nonsense-mediated decay (NMD), because intronless genes do not undergo NMD (15–18). We therefore proceeded to study the functional consequences of the sequence variants.

***Functional analysis of mutant SOX2 proteins***

*Cellular localization*. Given that efficient transport from the cytoplasm to the nucleus plays a key role in the function of SOX proteins as transcription factors (19) and that *SOX2* is known to be regulated at this level (ref. 5 and our unpublished observations), we investigated the localization of wild-type and mutant SOX2 proteins within the cell. Wild-type SOX2 was primarily localized to the nucleus of the cell, with little cytoplasmic staining (Figure 5A). In contrast, neither mutation c.60insG nor mutation c.70del20 (both lacking the HMG domain) showed nuclear localization. The other SOX2 constructs tested showed similar results to wild-type SOX2, with localization principally within the cell nucleus (Figure 5A). The c.479delA mutation behaved in the same way as the Y160X mutation (data not shown). Although the previously described L97P missense mutation lies in the HMG domain, it does not disrupt the previously identified nuclear localization signal sequences at the N and C termini of the HMG box (20–22), and the protein was correctly targeted to the nucleus in this assay.

*DNA binding*. In order to assess the ability of the mutant proteins to bind DNA, wild-type and mutant SOX2 proteins were synthesized by in vitro transcription/translation. EMSA performed with these proteins demonstrated the ability of wild-type SOX2 to bind specifically to a radiolabeled consensus SOX sequence DNA probe (Figure 5B). Both c.60insG and c.70del20 mutations generated proteins unable to bind to DNA, as expected, given that they completely lack the DNA-binding HMG domain. The other 4 mutations (c.387delC, c.479delA [data not shown], Y160X, and Q177X) produced truncations downstream of the HMG domain, and all retained the ability to bind DNA, in complexes that migrated faster than wild-type SOX2 due to their smaller size. The L97P mutation (9) showed complete loss of DNA binding compared with the 2 missense variants identified in this study (G130A and A191T), which bound to DNA with a similar affinity to wild-type SOX2 in this assay (Figure 5B).

*Reporter assays*. We next tested the ability of the mutant proteins to activate transcription of a luciferase reporter construct downstream of the proximal promoter of the *Hesx1* gene that contains binding sites for SOX proteins and can be activated in a dose-dependent manner (23). As shown in Figure 5C, wild-type SOX2 activated transcription with a 20-fold increase in reporter expression. The mutant constructs c.60insG, c.70del20, and L97P failed to show any activation, as expected, given their inability to bind DNA (Figure 5B). The other 4 truncating mutant constructs (c.387delC, c.479delA [data not shown], Y160X, and Q177X) showed minimal activation compared with the wild-type construct, with their residual activity correlating with the length of the mutant protein. Q177X, which retained the longest fragment of the transactivation domain (see Figure 4C), showed a 7-fold increase in activation, but was clearly less effective than the wild-type SOX2. Increasing concentrations of the missense variants, G130A and A191T, reached levels of activation similar to wild-type SOX2 in this assay. These

**Table 3**
Summary of molecular and clinical features associated with *SOX2* mutations

| Pt | Mutation | Nuclear localization | DNA binding | Transactivation | Clinical phenotype |
|---|---|---|---|---|---|
| 1 | c.60insG | Impaired | Loss | Loss | Bilateral anophthalmia, HH, learning difficulties, esophageal atresia, diplegia |
| 2 | c.70del20 | Impaired | Loss | Loss | Left anophthalmia, right microphthalmia, HH, learning difficulties |
| 3 | c.387delC | Unaffected | Unaffected | Impaired | Left microphthalmia, right coloboma, HH, learning difficulties, diplegia |
| 4 | Y160X | Unaffected | Unaffected | Impaired | Bilateral microphthalmia, HH, learning difficulties, quadriparesis |
| 5 | Q177X | Unaffected | Unaffected | Impaired | Bilateral anophthalmia, HH, learning difficulties |
| 6 | c.479delA | Unaffected | Unaffected | Impaired | Bilateral anophthalmia, HH, GH deficiency, learning difficulties, sensorineural deafness |
| 7 | G130A | Unaffected | Unaffected | Unaffected | Bilateral optic nerve hypoplasia, normal growth |
| 8 | A191T | Unaffected | Unaffected | Unaffected | Bilateral optic nerve hypoplasia; GH, ACTH, and TSH deficiencies |

differences were not due to impaired synthesis of SOX2 proteins, as confirmed by Western blot using whole-cell extracts following transfection (data not shown). Cotransfection of wild-type SOX2 with mutated constructs (1:1 ratio) showed no evidence for a dominant-negative effect of mutant proteins on the ability of wild-type SOX2 to activate transcription in this assay (data not shown).

## Discussion

We have shown that the transcription factor SOX2, expressed in neuroepithelial progenitors and stem cells from the earliest stages, is implicated in the normal development of the brain and the pituitary gland in addition to that of the eyes and inner ear. The pituitary gland is a midline structure consisting of the anterior, intermediate, and posterior lobes of varying sizes and complexities in different species. Hypopituitarism may result in isolated hormone deficiency (e.g., GH, TSH, PRL, ACTH, LH and follicle-stimulating hormone [FSH]), combined pituitary hormone deficiency (CPHD) with or without involvement of the posterior pituitary, or CPHD with other congenital anomalies such as optic nerve hypoplasia, midline forebrain abnormalities (septo-optic dysplasia; SOD), and cerebellar abnormalities. The condition is associated with significant morbidity and, if undetected or inadequately treated, mortality. Its incidence is reported as ranging from 1 in 3,000 in the case of isolated GH deficiency to 1 in 50,000 for SOD, with a 2:1 male preponderance.

Several transcription factors are essential for normal pituitary development, and mutations within a number of these have been described in association with pituitary disease in humans (24, 25). Among these, *Sox3*/*SOX3* was the first member of this gene family to be implicated in X-linked hypopituitarism in both mice and humans (13, 23, 26). It is now clear that infundibular and pituitary development is critically sensitive to dosage of *SOX3*, with both duplications of *SOX3* and loss-of-function mutations being associated with anterior pituitary hypoplasia (APH), infundibular hypoplasia, an undescended/ectopic posterior pituitary, and anterior pituitary endocrine deficits (23). Given the growth and fertility defects in *Sox2* mutant mice, and given that expression domains of SOX proteins frequently overlap, we suspected that *SOX2* might also be involved in pituitary development. This was indeed the case, although there were important similarities and differences in phenotypic consequences and penetrance in humans and mice.

One obvious difference is in eye development. *SOX2* haploinsufficiency in humans is associated with bilateral anophthalmia/microphthalmia, whereas there are no ocular abnormalities in $Sox2^{\beta geo}$ heterozygous mice (5, 8). However, this is more likely due to a difference in sensitivity between the species than to a novel role for the protein in humans. Indeed it has very recently been shown in mice that further reduction of Sox2 levels (<40% of wild type) does result in a dose-dependent series of eye malformations closely related to those of the human condition (27).

In contrast, both species show abnormalities and hypoplasia of the pituitary, the details of which are different. All of our patients with *SOX2* mutations showed evidence of marked gonadotropin deficiency, but while 4 of the 6 patients (patients 1, 4, 5, and 6) were short (heights less than –2 standard deviation score [SDS]), only 2 showed evidence of blunted GH secretion (patients 5 and 6). Patient 5 in fact demonstrated a normal GH response to provocation on 2 occasions, and although the overnight GH secretory profile was suboptimal, this could simply reflect a lack of sex steroid, given that the IGF-1 and IGF-binding protein 3 (IGFBP3) concentrations have been documented as being normal (see Table 1 and "Patient phenotypes associated with de novo *SOX2* mutations" in Methods). Moreover, the 2 patients that had attained final height were within the normal range (NR; patient 2, –0.1 SDS; patient 5, –1.2 SDS). It is unusual to observe a normal GH response to provocation in the face of abnormal gonadotropin secretion and pituitary hypoplasia in patients with hypopituitarism (24). Additionally, the corticotrope, thyrotrope, and lactotrope axes appeared to be clinically normal despite the structural hypoplasia of the gland. In contrast, heterozygous loss of function of *Sox2* in the mouse was associated with a reduction in somatotrope number and GH content, as part of a general reduction in pituitary size, as early as 18.5 dpc. Moreover, in the adult, we found a general reduction of all hormones assayed. Pubertal development was not consistently affected in the mouse, but as a significant proportion of *Sox2* heterozygotes are lost postnatally, this effect may be masked. We found that some adult $Sox2^{\beta geo}$ heterozygotes were small, while others were normal, but still GH deficient (data not shown). While GH deficiency could explain the growth retardation, other aspects of the phenotype could be responsible, such as CNS defects. Some growth retardation was also observed in $Sox2^{\Delta ENH}/Sox2^{\beta geo}$ compound heterozygotes (7).

One possible explanation for the differences between the human and murine phenotypes might be the degree of redundancy that exists among members of the SoxB1 family, the extent and location of which may not be identical in both species. The phenotype resulting from mutations in each of the 3 SoxB1 genes is often limited to those sites at which only the mutant gene is expressed. For example, *Sox1* mutant mice show lens defects beginning at the time when *Sox2* is turned off and lack olfactory tubercles, an area of the

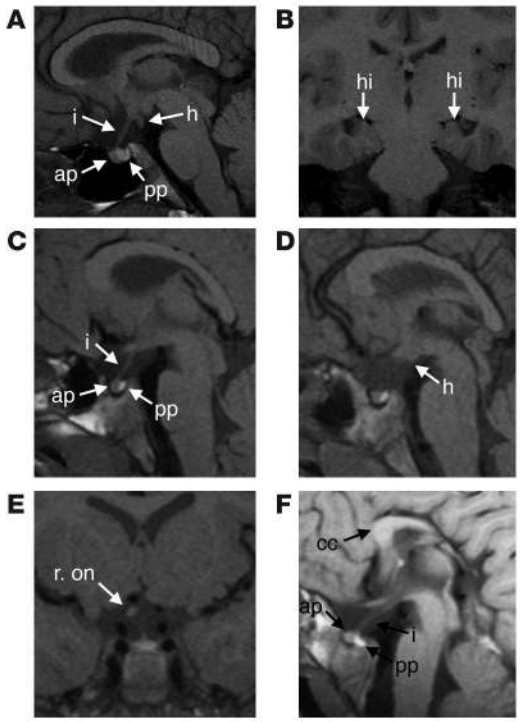


**Figure 6**
MRI scans on patients with *SOX2* mutations. (**A** and **B**) Sagittal and coronal sections from patient 1 showing APH with relatively normal posterior pituitary (pp) and infundibulum (i), a hypothalamic hamartoma (h) and a misshapen abnormal hippocampus (hi). (**C** and **D**) Sagittal sections from patient 3 showing APH and a hypothalamic hamartoma. (**E**) Coronal section in this patient revealed complete absence of the left optic nerve. r.on, right optic nerve. (**F**) Sagittal section in patient 4 showing hypoplasia of the anterior pituitary (ap) and the splenium of the corpus callosum (cc). The posterior pituitary and infundibulum were normal.

CNS where *Sox1* is uniquely expressed (28, 29). Both *Sox2* and *Sox3* are expressed in the hypothalamo-pituitary region; however, in mice, *Sox3* is expressed at a particularly high level in the infundibulum, and its expression in this region is also likely to be critical in humans, as reflected by the phenotype of infundibular hypoplasia in patients with abnormal amounts of *SOX3* (23). In the mouse, the *Sox2*$^{\beta geo}$ heterozygotes demonstrate similar defects in pituitary morphology to *Sox3* null embryos (13), although the phenotype is both less severe and less penetrant, presumably because 1 normal copy of *Sox2* remains in the former. As *Sox3* is not expressed in Rathke's pouch, it has been suggested that the morphological defects may originate from changes in the overlying infundibulum (13). A similar mechanism might be operating for *Sox2*, but unlike *Sox3*, this is also prominently expressed in Rathke's pouch, so a simpler explanation in this case could be a "pouch-autonomous" defect, not compensated for by redundancy from other *Sox* genes. The reduction in somatotrope numbers associated with anterior lobe hypoplasia at 18.5 dpc supports a pouch-autonomous function for *Sox2*. However, conditional deletion of the genes singly and in combination will be necessary to unravel this issue further. While endocrine deficits are present in both *Sox2* and *Sox3* mutant mice, they differ significantly. In contrast to *Sox2* heterozygotes, GH content is unaffected at 18.5 dpc in *Sox3* mutants (13). This suggests primary pituitary deficits in the former, while the adult endocrine deficits in *Sox3* null animals are more consistent with a hypothalamic contribution to the phenotype. Moreover, the deficits in LH are not as profound in the latter.

It is conceivable that dysregulation of hypothalamic secretion associated with *SOX2* mutations could lead to secondary hypoplasia of the anterior pituitary. This would have to be more severe for gonadotropin-releasing hormone (GnRH) in humans than in mice. Given the difficulties in measuring GnRH content in the mouse and the complexity of the GH-releasing hormone–GH (GHRH-GH) axis, it would be difficult to establish the effect of *Sox2* mutations on the hypothalamus at this stage, and further studies — possibly using conditional transgenesis — would be required to study these effects in greater detail. It is also possible, though we believe it is unlikely, that a primary abnormality in the formation of Rathke's pouch selectively affects the gonadotrope lineage in humans heterozygous for mutations in *SOX2*. On the other hand, it must be remembered that *SOX2* is expressed widely in the CNS, so the phenotype of HH might be due to a loss of function earlier in development. HH is known to be caused by genetic defects in GnRH neuronal migration (*KAL1* and *FGFR1* mutations; ref. 30), and by mutations in the GnRH receptor (*GNRHR*) or in the G protein–coupled receptor *GPR54* (30–32). A role for SOX2 in determining normal numbers of GnRH neurons and/or neuronal migration cannot be excluded. In this context, it is interesting that defects in olfactory tubercle development in *Sox1* mutant mice are due to a requirement for the protein in postmitotic neuronal precursors for their correct migration and differentiation (29).

A direct effect of *Sox2* deficiency in germ cells, where the gene is known to be expressed (5), could also contribute to the hypogonadal phenotype. The sperm blockage observed in mice could reflect a local "stem cell maintenance" role for SOX2, in parallel with its role in the CNS (for review see ref. 3). In its absence, germ cells would no longer persist but differentiate, resulting in tubules filled by sperm, as we observed. To resolve this issue and understand the potential requirement for SOX2 at different levels in the hypothalamo-pituitary-gonadal axis, conditional deletion of the gene exclusively in each compartment will be necessary. Hence, an effect of *SOX2* mutations at the additional level of the gonads cannot be completely excluded, given the coexistence of HH. This could possibly explain the complete lack of testosterone response to human chorionic gonadotropin (HCG) stimulation in patients 3 and 5 (Table 1).

It is also possible that SOX2 participates directly in the regulation of genes such as *Hesx1*, *Prop1*, and *Aes*, all of which show similar phenotypes when mutated; however, heterozygosity for *Sox2* may not be sufficient to reveal any significant reduction in expression of these putative target genes.

The endocrine status of patients 1–6 and 8 has been carefully evaluated over the last 12 months, and to date no evolution of other hormonal deficiencies has been documented. It is important to note, however, that further hormone deficiencies may evolve in patients with mutations in transcription factors that are implicated in pituitary development (33–35), and hence careful long-term follow-up is mandatory in these patients.

In addition to the endocrine abnormalities identified in our patients, we also identified a number of neurological abnormalities. Many of these could be explained by the widespread expres-

sion of *SOX2* in the developing and postnatal brain. In the mouse, significant expression is maintained in the differentiating areas of the thalamus and medial-dorsal striatum and septum during embryogenesis and postnatally (7). The CNS defects observed in our patients included abnormalities of the mesial temporal structures including the hippocampi (patients 1–4 and 6), partial agenesis or hypoplasia of the corpus callosum (patients 1, 3, 4, and 6), and hypothalamic hamartomata (patients 1 and 3). Similar anatomical defects were also previously noted in $Sox2^{\Delta ENH}/Sox2^{\beta geo}$ compound heterozygous mice (7); moreover, Sisodiya et al. recently reported hippocampal and parahippocampal malformations in 4 previously published patients with *SOX2* mutations, 2 of which had a history of epilepsy (36). None of the patients we report here with *SOX2* mutations presented with a history of epilepsy, and no association has been found between clinical epilepsy phenotypes and *SOX2* variation (36). However, mesial temporal structure abnormalities, particularly hippocampal malformations, appeared to be a common finding in association with *SOX2* mutations. The presence of hypothalamic hamartoma in 2 of our patients is interesting, and, given that *SOX2* is expressed in the developing hypothalamus, our data suggest that the gene may be implicated in some patients with a hamartoma. Usually, a hamartoma is a benign condition that is associated with precocious puberty and/or gelastic seizures. If it was not for the other neurological defects, the hamartomata may well have remained undetected in these 2 patients, given the gonadotropin deficiency.

All of our patients showed evidence of learning difficulties. This was probably to be expected given the widespread expression of *SOX2* in the CNS. Also of note, 3 of our patients had marked motor deficits in the form of spastic diplegia or quadriparesis (patients 1, 3, and 4), and this might reflect the critical nature of *Sox2* expression in the pyramidal cells of the cerebral cortex (7). One of our patients had evidence of sensorineural deafness (patient 6), similar to that in the patient described by Hagstrom et al. (10). Recently, regulatory mutations affecting *Sox2* expression in the murine inner ear, where it is the sole *Soxb1* gene to be expressed, have been associated with inner ear malformations and, in the most extreme case, complete absence of the sensory epithelium (37). Heterozygous mice are normal, however, again suggesting variable correspondence between human and mouse phenotypes.

The association between anophthalmia and esophageal atresia is well known (anophthalmia-esophageal atresia-genitalia syndrome, OMIM 600992; refs. 38–40), and mutations in *SOX2* have been described in a number of these cases (12). While one of our patients manifested esophageal atresia (patient 1), the other 5 did not; moreover we failed to find evidence for esophageal abnormalities in heterozygous $Sox2^{\beta geo}$ mice despite the expression of *Sox2* in the gut endoderm (refs. 6, 12 and our unpublished observations).

It is noteworthy that the incidence of loss-of-function mutations in the coding region of *SOX2* in our small cohort of patients with anophthalmia/microphthalmia was quite high (approximately 50%), whereas no mutations were identified in patients with hypopituitarism only or in patients with optic nerve hypoplasia. This indicates that the eye is particularly sensitive to a reduction in levels of SOX2, perhaps more so than the pituitary. Previous studies identified mutations in 4 of 102 patients with anophthalmia, microphthalmia, and coloboma (8). The majority of the mutations identified in this and other studies are nonsense or frameshift mutations, with only 2 previously reported missense mutations, both of which occurred de novo (8, 12). The 2 missense changes identified in patients 7 and 8 were in both cases inherited from their unaffected fathers. The difference in phenotype between these 2 individuals (CNS abnormalities with optic nerve hypoplasia in patient 7, and CNS abnormalities, optic nerve hypoplasia, and pituitary hypoplasia in patient 8) and the 6 harboring truncating mutations (severe CNS and ocular abnormalities with HH; Table 2), in addition to the absence of any obvious functional compromise, suggests that they may be assumed to be neutral variants, although it is interesting to note that the changes were not identified in 100 control chromosomes in our studies, nor in a previously reported screen of 176 control chromosomes (8). It is, however, important to consider the possibility that the 2 missense variants affect some hitherto uncharacterized function of SOX2 and that the mutations may be variably penetrant. Mutation in another gene implicated in CNS and pituitary development may lead to digenic inheritance, which would account for the lack of a phenotype in the parent carrying the *SOX2* variant. We have screened *HESX1* for mutations in patients 7 and 8, but failed to find a mutation in this gene.

Our data are consistent with previous findings showing that the HMG box of SOX2 is critical for its interaction with DNA (41, 42) and that 2 nuclear localization signals that lie at both the N (bipartite; KRP, RRK) and C (PRRK) termini of the HMG box in the *SRY* and *SOX9* genes (20–22) — and which are highly conserved in *SOX2* — are critical for correct nuclear translocation. As expected, truncated proteins lacking the HMG domain were associated with impairment of nuclear localization, DNA binding, and transcriptional activation, while mutant proteins retaining the HMG domain preserved DNA binding and correct nuclear transport; their residual transcriptional activation also correlated with the extent of the remaining C-terminal activation domain. We took the opportunity to assess the functional impact of 1 of only 2 missense mutations identified in *SOX2* hitherto, L97P (9). This mutation of a highly conserved residue in the HMG box did not impair nuclear localization, but led to loss of DNA binding and transcriptional activation. There was, however, no obvious phenotype-genotype correlation in our patients (Table 3), suggesting that all the mutations simply compromised overall function and that the various affected tissues were sensitive both to levels of SOX2 and to other genetic or environmental factors.

In conclusion, we have demonstrated for the first time to our knowledge that in addition to bilateral eye disease, *SOX2* mutations are commonly associated with pituitary hypoplasia and endocrine deficits. The endocrine phenotype is unusual in that the gonadotropin deficiency is profound, whereas GH secretion appears to be relatively spared. Our data suggest that haploinsufficiency of *SOX2*, as opposed to a dominant-negative effect, is the most likely cause of the phenotypes. Finally, *SOX2* is the second member of this gene family to be implicated in the development and normal function of the human hypothalamo-pituitary axis, with an important role in establishing normal reproductive development. Timely diagnosis of sex steroid deficiency in these individuals with a multitude of problems would lead to prompt treatment with the prevention of long-term morbidity, for example osteoporosis with attendant bone fractures.

## Methods

### *Patient recruitment*

Patients with congenital hypothalamo-pituitary disorders were recruited into the study from both national and international pediatric and adult endocrinology centers between 1998 and 2005. A total of 235 probands (143 male, 92 female) were screened for mutations within *SOX2*. These

included 97 patients with congenital hypopituitarism without any midline or eye defects, 126 patients with SOD (characterized by optic nerve hypoplasia in association with hypopituitarism and/or midline defects), and 12 patients with anophthalmia ($n = 9$) or microphthalmia ($n = 3$). Ethical committee approval was obtained from the Institute of Child Health/Great Ormond Street Hospital for Children Joint Research Ethics Committee (Institute of Child Health, London, United Kingdom). Informed written consent was obtained from the parents and, where applicable, the patients prior to collection of samples and genomic analysis.

### *Clinical evaluation*

Clinical details were obtained for all patients recruited into the study. These included birth details, perinatal complications, history of consanguinity, family history, and parental heights as well as the results of standard dynamic tests of pituitary function and MRI. Height and weight SDS were calculated using the UK 1990 growth standards (43). For patients from GOSH (patients 1, 3, 5, and 8), samples for GH, free T4 (NR, 12–22 pmol/l [0.9–1.65 ng/dl]), TSH, cortisol, LH, FSH, testosterone (NR adult males, 181–772 ng/dl) and PRL (NR, 100–500 mU/l [5–25 ng/ml]) were all analyzed on the DPC IMMULITE 2000 analyzer as a chemiluminescent immunometric analysis (Diagnostic Products Corp.). IGF-1 and IGFBP3 were measured in the DSL assay (Diagnostic Systems Laboratory Inc.; Beckman Coulter), and the SDS was calculated according to previously published data (44).

For patient 2, GH was measured using the DPC IMMULITE assay; serum cortisol (NR, 3.1–16.6 μg/dl), TSH (NR, 0.35–4.5 mU/l), LH (NR, 1.9–12.5 IU/l), FSH (NR, 2.5–10.2 IU/l), estradiol (NR, 40–606 pmol/l), PRL (NR, 3–31 ng/ml) and free thyroxine (NR, 0.8–1.7 ng/dl) were all measured using ADVIA Centaur immunoassays (Bayer), and IGF-1 was measured using the Nichols IRMA (Nichols Institute Diagnostics).

For patient 4, serum GH was measured by immunoradiometric assay (IRMA; Diagnostic Systems Laboratory Inc.; Beckman Coulter). Serum IGF-1 was measured by IRMA (Nichols Institute Diagnostics), as was IGFBP3 (Diagnostic Systems Laboratory Inc., Beckman Coulter). Both IGF-1 and IGFBP3 were validated in the laboratory of S. Cianfarani by measuring concentrations in 82 normal children and comparing these with the normative data provided by the manufacturer's kit. Serum TSH was measured by IRMA (NR, 0.3–4.0 mU/l; Cambridge Life Sciences), free T4 by RIA (NR, 0.6–1.8 ng/dl; DiaSorin), serum cortisol by RIA (NR, 5-25 μg/dl; DiaSorin), total and free testosterone by RIA (NR total testosterone, Tanner 1, 5–50 ng/dl; Tanner 2, 5–70 ng/dl; NR free testosterone, Tanner 1, 0.6–5.7 pg/ml; Tanner 2, 1.4–156 pg/ml; Diagnostic Systems Laboratory Inc.; Beckman Coulter), serum PRL by IRMA (NR, <24 ng/ml; Diagnostic Systems Laboratory Inc.; Beckman Coulter), and serum LH and FSH by IRMA (RADIM Diagnostics).

For patient 6, GH was measured using the IMMULITE chemiluminescent assay (Diagnostic Products Corp.), and IGF-1 was measured by RIA with acid-ethanol extraction (45). Free thyroxine (NR, 0.7–1.55 ng/dl) was measured using the Abbott Diagnostics AxSYM assay, and TSH (NR, 0.4–5.5) was measured using the Ultrasensitive hTSH II assay (Abbott Diagnostics). Cortisol, LH, FSH, testosterone (NR adult male, 2–8.4 ng/ml) and PRL were measured by immunochemiluminometric assays (NRs as listed above; ADVIA Centaur; Bayer).

For patients 1, 3–6, and 8, GH deficiency was excluded when a peak GH concentration of >6.7 ng/ml was achieved using a single provocation test (glucagon, 0.1 mg/kg i.m., or clonidine, 100 μg/m$^2$ body surface area orally) in combination with normal (>–2 SD relative to mean for age and sex) serum concentrations of GH-dependent factors (IGF-1 and IGFBP3). For patients 2 and 4 (second test of GH provocation), a peak GH response of >9 ng/ml was accepted as normal in response to the GHRH-arginine test (GHRH [1 μg/kg; GEREF diagnostic; Serono] administered as an i.v. bolus at 0 minutes followed by a 30-minute infusion of L-arginine [0.5 g/kg body weight, maximum 30 g, dissolved in 100 ml normal saline]) (46–48). ACTH deficiency was excluded when the peak serum cortisol response was >550 nmol/l (19.8 μg/dl) in response to stimulation with glucagon or Synacthen (62.5–250 μg depending on age). Gonadotropin deficiency was diagnosed with an absent serum LH and FSH response to GnRH (LH-releasing hormone, 2.5 μg/kg i.v.) in the first 2 years of life, at the expected time of puberty, or with lack of pulsatility on an overnight profile (20-minute sampling for LH and FSH).

### *Patient phenotypes associated with de novo SOX2 mutations*

*Patient 1*. A 12.3-year-old girl presented with short stature (height 130 cm, –2.9 SDS; weight 26.7 kg, –1.72 SDS), bilateral anophthalmia, learning difficulties, spastic diplegia, and pubertal delay (breast stage I with no pubic or axillary hair). She had a past medical history of esophageal atresia, and her clinical phenotype has been partially reported previously (39). Investigations performed at the age of 13 years revealed a moderate delay in the bone age (10.2 yr) with normal GH, cortisol, and TSH responses to stimulation (Table 1). GnRH stimulation revealed a LH peak of 6.1 IU/l with a FSH peak of 5.7 IU/l, both at 60 minutes. Overnight profiling revealed 4 peaks of GH secretion, 2 of which exceeded 6.7 ng/ml. These data probably reflect normal GH secretion in a prepubertal child, and in accordance with these data, the IGF-1 and IGFBP3 concentrations were within the low NR (–1 SDS). However, LH secretion was poor (1–2 IU/l with no pulsatility), and there was no pulsatile FSH secretion, with FSH concentrations ranging from 3.2 to 4.7 IU/l. The pelvic ultrasound scan revealed small ovaries (right, 0.9 ml; left, 1 ml) with 2 small follicles in the left ovary and an infantile uterus, suggesting a diagnosis of HH. MRI revealed an unusual shape to the mesial temporal structures including hippocampi, a small corpus callosum, a hypothalamic hamartoma, APH, generalized reduction of white matter bulk, and absent eyes with absent optic nerves (Figure 6, A and B). The posterior pituitary and infundibulum were both normal. She was commenced on ethinylestradiol (2 μg once daily) in order to induce her puberty and progressed to breast stage II 6 months later. She is currently on 5 μg ethinyloestradiol once daily.

*Patient 2*. A 22-year-old female presented with left anophthalmia, right microphthalmia, learning difficulties although she attended a normal school, and primary amenorrhoea. Past medical history revealed a slipped right femoral epiphysis following a fall at the age of 14 years. Her sense of smell was satisfactory. On examination at presentation, she had stage II breast development (no estrogen treatment had been given), stage III pubic hair development, and stage III axillary hair. She was 161.4 cm (–0.1 SDS) tall with a weight of 65 kg (0.8 SDS) and a BMI of 25. Her right eye was abnormal with enophthalmos and a corneal opacity. Visual assessment revealed roving eye movements with perception to light only. Investigations revealed low basal (LH, <0.1 IU/l; FSH, 0.2 IU/l) and peak (GnRH, LH, 0.7 IU/l; FSH, 1.9 IU/l) gonadotropin concentrations. PRL and thyroid function tests were normal, as were the basal and stimulated (short synacthen test) cortisol concentrations (Table 1). The estradiol concentration was undetectable. The initial basal IGF-1 concentration was low (94 μg/l; NR, 116–447 μg/l). Provocative GH testing using the GHRH-arginine test revealed a peak GH of 11.1 ng/ml, with a normal repeat IGF-1 (148 μg/l). MRI of her brain and pituitary revealed an abnormal hypoplastic pituitary gland in a small sella turcica with an absent left eye and optic nerve. Pelvic ultrasound scan revealed a tiny uterus (24 × 5 mm) with no identifiable ovaries. Pubertal induction was performed using ethinylestradiol.

*Patient 3*. A male child presented at the age of 1.7 years with severe microphthalmia on the left side that had been previously treated with a prosthesis, a large coloboma in the right eye, global developmental delay, mild diplegia, and a micropenis with a hypoplastic scrotum and bilateral cryptorchidism. His height lay just below the fiftieth percentile (83.4 cm, –0.25 SDS) with a weight on the seventy-fifth percentile (13.4 kg, +1.13 SDS). Investigations

performed at the age of 22 months revealed a normal male karyotype, no response to a 3-day HCG test (peak testosterone, <20 ng/dl), a poor response to a 3-week HCG test (peak testosterone, 58 ng/dl), and undetectable basal gonadotropins (LH, <0.7 IU/l; FSH, <0.5 IU/l), with no further response to GnRH testing. The IGF-1 (16 ng/ml; –2.5 SDS) and the IGFBP3 (–1.5 SDS) concentrations were both low, with normal thyroid function, PRL, and basal and peak cortisol concentrations (Table 1). MRI scan of the brain and pituitary revealed normal basal ganglia, hypoplasia of the splenium of the corpus callosum and the anterior pituitary (Figure 6, C and D), a small hippocampus, and small and rotated mesial temporal structures. A hypothalamic hamartoma was also present (Figure 6D). The left optic nerve was absent with a small chiasm, and there was a generalized lack of white matter bulk (Figure 6, C and E). Laparoscopy revealed no evidence of any testicular tissue. Electrophysiology revealed normal retinal responses from the right eye and an attenuated response from the left eye. Visual evoked potentials revealed postretinal dysfunction affecting the left eye with rudimentary vision, while testing in the right eye revealed good vision. Further investigations performed at the age of 4 years showed normal peak GH and cortisol responses to glucagon stimulation with a normal TSH response to thyrotropin-releasing hormone (TRH) and a normal basal PRL. The IGF-1 and IGFBP3 had improved and were at the mean for his age and sex (Table 1).

*Patient 4*. A male infant presented with bilateral microphthalmia and severe developmental delay. He was referred to the endocrinologist at the age of 8.9 years, when he was noted to have growth retardation (height 114.5 cm, –2.8 SDS; weight 19.2 kg, –1.1 SDS; annualized height velocity 3.9 cm/yr, –2 SDS). His bone age was delayed by 2 years. At the age of 10.6 years, his height was 121 cm (–2.9 SDS). He also had dysmorphic features including frontal prominence, brachydactyly, nystagmus, strabismus, and hypertelorism, with spastic and dystonic quadriparesis and a micropenis with atrophic testes (1 ml bilaterally). Endocrine evaluation on presentation revealed normal thyroid function, a normal peak GH response to clonidine stimulation, and normal IGF-1 and IGFBP3 concentrations (Table 1). Reinvestigation at the age of 10 years revealed normal thyroid function, a peak GH of >36.7 ng/ml in response to GHRH-arginine, an IGF-1 concentration of 132 ng/ml (–1 SDS) and an IGFBP3 concentration of 2.49 mg/l (–1 SDS). At the age of 14 years, he remained prepubertal with a delayed bone age (11.5 yr). Reinvestigation revealed a basal PRL concentration of 9.8 ng/ml, a fasting early morning cortisol of 6.9 μg/dl, normal thyroid function, an absent LH response to GnRH (<0.5 IU/l), and FSH that increased from 0.6 to 2.2 IU/l at 60 minutes. The testosterone was low (free, 0.7 pg/ml; total, 28 ng/dl). Serum IGF-1 was low (117 ng/ml, –3 SDS) although IGFBP3 was within the NR (3.28 mg/l, –1 SDS). MRI revealed partial agenesis of the corpus callosum, a small anterior pituitary with a normal posterior pituitary and infundibulum, mesial temporal lobe abnormalities, and generalized reduction in the white matter (Figure 6F).

*Patient 5*. A 10-month-old boy presented with bilateral anophthalmia, mild facial dysmorphism, a micropenis with bilateral cryptorchidism, neurodevelopmental delay, and abnormal movements. On examination, his length lay on the tenth percentile with a weight on the fiftieth percentile. He had bilateral anophthalmia, a prominent forehead, abnormal nares and philtrum, and global hypotonia in addition to the micropenis and bilaterally undescended testes. He also had difficulty sleeping and was treated with melatonin. Bilateral orchidopexies were performed. He was later referred to the endocrine team at the age of 9 years with short stature (height, 118 cm, SDS –2.5; weight, 23.1 kg, SDS –1.09; midparental height, 174.3 cm, –0.1 SDS) and a delayed bone age (5.7 yr). His growth velocity was suboptimal (4.3 cm/yr, –1.2 SDS). He had a small phallus with small (1 ml) testes that were identified in the scrotum. Investigations revealed normal thyroid function with a normal TSH response to TRH, normal PRL, normal peak GH and cortisol concentrations in response to glucagon stimulation, poor LH (<0.5 IU/l) and FSH (1.5 IU/l) responses to GnRH, and a poor testosterone response to HCG stimulation (peak 26 ng/dl after a 3-day HCG test). He was commenced on recombinant human GH (rhGH) at the age of 11 years, producing an excellent response in growth velocity (8.8 cm/yr; +5.5 SDS). He was commenced on testosterone replacement at the age of 12 years and progressed satisfactorily on treatment although his testes remained unchanged in size. rhGH was stopped at the age of 18 years at a final height of 166.6 cm (–1.2 SDS), and retesting off all treatment revealed a peak GH of 19.5 μg/l. The TSH and PRL responses to TRH were normal, as was the cortisol response to glucagon-induced hypoglycemia (Table 1). His peak LH response to GnRH was 0.2 IU/l, with a peak FSH of 1.0 IU/l. An overnight spontaneous GH and gonadotropin secretory profile revealed only 1 satisfactory peak of GH secretion (7.2 μg/l), whereas all the LH and FSH concentrations were undetectable (<0.7 IU/l and <0.2 IU/l, respectively), confirming gonadotropin deficiency with complete HH. Despite his low spontaneous GH concentrations, his IGF-1 (0 SDS) and IGFBP3 (+1 SDS) concentrations were well within the NR.

*Patient 6*. A male infant was born at 35 weeks gestation with a birth weight of 2.28 kg. He required ventilatory support initially. He was noted to have anophthalmia and a micropenis (stretched penile length, 2 cm), with a hypoplastic scrotum and small testes (1 ml bilaterally) located high in the scrotum. MRI scanning confirmed bilateral anophthalmia, absence of the optic nerves and chiasm, a cavum septum pellucidum, a thin corpus callosum, a hypoplastic anterior pituitary gland, and small hippocampi. Provocative testing at the age of 3.5 years revealed GH insufficiency (peak GH 4.2 and 2.6 ng/ml in response to glucagon and arginine respectively). The IGF-1 (7.3 nmol/l) concentration was within the NR, albeit at the lower end. The peak cortisol was 18 μg/dl in response to glucagon and 27.2 μg/dl in response to synacthen stimulation, and thyroid function was at the lower end of the NR (free T4, 0.84–1.05 ng/dl). Gonadotropin responses to GnRH testing were poor (peak LH, 1.7 IU/l; peak FSH, <0.6 IU/l). At the age of 4 years, he had a height of –3.4 SDS with a growth velocity of 5.3 cm/yr (–1.68 SDS). He was commenced on rhGH (0.17 mg/kg/wk) and increased his height velocity to 8.5 cm/yr (+1.64 SDS). He also suffered from feeding difficulties, for which he had a gastrostomy, and showed evidence of developmental delay with sensorineural deafness.

### *Patient phenotypes associated with inherited SOX2 missense variants*

*Patient 7*. A female infant presented with roving eye movements and generalized hypertonia noted soon after birth. Bilateral optic nerve hypoplasia was identified with severe visual impairment, and she was noted to have spastic quadriplegia. She was developmentally delayed, and an MRI scan revealed an absent septum pellucidum, bilateral schizencephaly, bilateral optic nerve hypoplasia, and a right porencephalic cyst. A brother had previously died at the age of 11 years with hydranencephaly. At the age of 11 years, her height lay on the second percentile (–2.5 SDS), with a weight on the twenty-fifth percentile (–0.4 SDS). Endocrine assessment has not been performed to date.

*Patient 8*. A female infant presented with hypoglycemia within the first 24 hours, and this was corrected with i.v. dextrose. She was later noted to have roving eye movements, and bilateral optic nerve hypoplasia was then diagnosed. Electroretinogram revealed normal retinal responses, but visual evoked potentials revealed marked postretinal dysfunction. Investigations at the age of 7 months revealed an abnormal 24-hour cortisol profile (peak, 4.9 μg/dl) with a suboptimal response to synacthen stimulation (peak, 9.7 μg/dl). The free thyroxine concentration was low (0.8 ng/dl) with a basal TSH of 4.8 mU/l. Initially, the IGF-1 (+1 SDS) and the IGFBP3 (0 SDS) concentrations were normal. She was commenced on hydrocortisone and thyroxine replacement. GnRH testing revealed a peak LH of 13 IU/l and a peak FSH of 49.1 IU/l (60 minutes). Growth deceleration was noted by 2 years of age, and glucagon testing revealed GH deficiency (peak GH, 2.3 ng/ml; peak IGF-1, 15 ng/ml, –2 SDS; peak IGFBP3, 2.06 mg/l, –1 SDS). GH treatment was commenced at that stage.

MRI revealed an absent septum pellucidum, a small optic chiasm, an absent infundibulum, severe APH, and an ectopic/undescended posterior pituitary.

### *Murine analysis*

The *Sox2*$^{\beta geo}$ mutation (5) was maintained on the MF1 (random bred) strain. All murine experiments were carried out using protocols reviewed and approved under license by the United Kingdom Home Office (Cambridge, United Kingdom) under the Animal (scientific procedures) Act 1986.

For histological analysis, embryos and adult pituitary glands were fixed in Bouin's reagent, dehydrated through graded ethanol series, and embedded in paraffin. Sections were stained with hematoxylin and eosin as described previously (5). In situ hybridization was performed on frozen sections as previously described (49) using a *Sox2*-specific probe (5) that has been previously characterized (50). Anterior pituitary glands were homogenized in phosphate-buffered saline and assayed for hormones by specific RIA using National Hormone and Pituitary Program (NHPP) reagents kindly provided by A.F. Parlow (Torrance, California, USA). For GH immunohistochemistry, a 1:2,000 dilution of a monkey anti-rat GH serum from the NHPP was used as previously described (51). The same protocol was used with a 1:500 dilution of the rabbit anti-rat βLH serum from the NHPP. Embryonic sections were counterstained with methyl green, while Carazzi hematoxylin was used on adult sections. Somatotropes or gonadotropes revealed by immunohistochemistry were counted on 5 complete pituitary sections containing the 3 lobes. Cell count numbers ± SEM are shown. Anterior lobe surface area was assessed using the ImageJ software (http://rsb.info.nih.gov/ij/) on the 5 sections that were counted. The total number of GH cells present in 5 sections was divided by the total surface area. The significance of the results was assessed by a 2-tailed Student's *t* test.

### *Mutation analysis of SOX2*

PCR primer pairs were designed to amplify the coding region of the single exon of the *SOX2* gene. Primer sequences were as follows: 1, forward, 5′-TATTCTTCGCCTGATTTTCC-3′; reverse, 5′-GGTATTTATAATCCGGGTGC-3′; 2, forward, 5′-GTGGAAACTTTTGTCGGAGAC-3′; reverse, 5′-CGAGTAGGACATGCTGTAGGT-3′; 3, forward, 5′-GTACAACTCCATGACCAGCTC-3′; reverse, 5′-CCATGCTGTTCTTACTCTCC-3′. PCR products treated with Shrimp Alkaline Phosphatase and Exonuclease I (New England Biolabs) were directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using a MegaBACE DNA Analysis System (Amersham Biosciences). Sequences were analyzed using Sequencher software (version 4.5; Gene Codes Corp.) and compared with *SOX2* reference sequence (GenBank accession no. NM_003106).

### *Plasmid constructs*

Purified PCR products comprising the entire *SOX2* coding region from both affected and unaffected individuals were subcloned downstream of the T7 promoter in the pcDNA3.1(+) vector (Invitrogen) to generate wild-type and mutant proteins for EMSA. For transient transfection and cell localization experiments, mutant and wild-type *SOX2* was subcloned into the expression vector pCMV/SV-Flag, containing an in-frame N-terminal FLAG epitope. *SOX2(L97P)* is a previously reported de novo missense mutation (9), which was utilized as a control to compare with missense sequence changes identified in this study. This mutation (c.290T→C) was introduced into wild-type *SOX2* constructs by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions.

### *Cell culture and transient transfection*

CHO cells were maintained in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Transient transfection assays were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

### *Cell localization studies*

Prior to transfection, $2 \times 10^5$ cells were seeded into each chamber of a 4-chamber tissue culture slide. Cells were transfected with 50 ng of FLAG-tagged *SOX2* expression construct, with the total amount of DNA transfected per chamber made up to 200 ng with empty expression vector. Following transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X, and blocked in blocking buffer (0.1% Triton X, 0.15% glycine, 2 mg/ml BSA) overnight. Cells were incubated with anti-FLAG antibody (1:100 dilution; Sigma-Aldrich), washed and incubated with FITC-conjugated goat anti-mouse secondary antibody (1:200 dilution; Dako). Nuclear counterstaining was performed with Vectashield containing DAPI (Vector Laboratories). Cells were visualized on a Zeiss Axioskop2 microscope, and images were captured using a Leica DC500 camera.

### *EMSA*

Wild-type and mutant SOX2 proteins were generated using the TNT Quick Coupled Transcription/Translation System (Promega). EMSAs were performed as described previously (52, 53), using a radiolabeled consensus SOX binding sequence present within the mouse *Hesx1* promoter as a probe (23, 54).

### *Dual luciferase assay experiments*

Briefly, $1 \times 10^4$ cells were seeded into each well of a 96-well tissue culture plate 24 hours prior to transfection. Cells were cotransfected with 20 ng of a firefly luciferase reporter construct containing a 570-bp fragment of the *Hesx1* promoter encompassing SOX binding sites (23), with pRL-SV40 *Renilla* Luciferase (Promega) to control for transfection efficiency and varying amounts of SOX2 expression vector as indicated in the figure legends. The total amount of transfected DNA was normalized to 120 ng/well by the addition of empty expression vector. Following transfection, cells were harvested and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

### *Statistics*

To determine significance between means of 2 samples, a 2-tailed Student's *t* test was used. A *P* value less than 0.05 was considered to be significant.

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