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Novel mutations in *LHX3* are associated with hypopituitarism and sensorineural hearing loss

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Homozygous loss-of-function mutations in the transcription factor *LHX3* have been associated with hypopituitarism with structural anterior pituitary defects and cervical abnormalities with or without restricted neck rotation. We report two novel recessive mutations in *LHX3* in four patients from two unrelated pedigrees. Clinical evaluation revealed that all four patients exhibit varying degrees of bilateral sensorineural hearing loss, which has not been previously reported in association with *LHX3* mutations, in addition to hypopituitarism including adrenocorticotropic hormone deficiency and an unusual skin and skeletal phenotype in one family. Furthermore, re-evaluation of three patients previously described with *LHX3* mutations showed they also exhibit varying degrees of bilateral sensorineural hearing loss. We have investigated a possible role for *LHX3* in inner ear development in humans using *in situ* hybridization of human embryonic and fetal tissue. *LHX3* is expressed in defined regions of the sensory epithelium of the developing inner ear in a pattern overlapping that of *SOX2*, which precedes the onset of *LHX3* expression and is known to be required for inner ear and pituitary development in both mice and humans. Moreover, we show that SOX2 is capable of binding to and activating transcription of the *LHX3* proximal promoter *in vitro*. This study therefore extends the phenotypic spectrum associated with *LHX3* mutations to encompass variable sensorineural hearing loss and suggests a possible interaction between *LHX3* and *SOX2* likely to be important for development of both the inner ear and the anterior pituitary in human embryonic development.

INTRODUCTION

The pituitary gland is a central regulator of growth, reproduction and homeostasis, mediated via hormone-signaling pathways which act to regulate the finely balanced homeostatic control in vertebrates. The gland is situated in the midline at the base of the brain and consists of the anterior and intermediate lobes, which derive embryologically from an invagination of the oral ectoderm (termed Rathke's pouch), and the posterior lobe, which develops from the neural ectoderm of the ventral diencephalon. Hormones secreted from the anterior pituitary are produced from specific cell types within the gland: somatotropes (secreting growth hormone, GH), thyrotropes (thyroid-stimulating hormone, TSH), corticotropes (adrenocorticotropic hormone, ACTH), lactotropes (prolactin, PRL) and gonadotropes (follicle-stimulating hormone, FSH, and luteinizing hormone, LH). The intermediate lobe contains melanotropes-producing pro-opiomelanocortin (POMC), which is the precursor to melanocyte-stimulating hormone in addition to endorphins. POMC is also the precursor to ACTH and is also expressed in corticotropes in the anterior pituitary.

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Congenital hypopituitarism can range from mild, involving deficiency of a single hormone, through more severe phenotypes affecting multiple pituitary hormone axes, to panhypopituitarism. Isolated growth hormone deficiency is the most common manifestation affecting between 1 in 4000 and 10 000 live births (1–5). Combined pituitary hormone deficiency (CPHD), in which GH deficiency is accompanied by insufficiency of at least one other pituitary hormone, is less common, but is associated with considerable morbidity and, if not treated promptly or adequately, occasional mortality. Recent advances in our understanding of the development of the pituitary gland have led to the identification of the etiological basis of some cases of congenital hypopituitarism. Naturally occurring and transgenic murine models have revealed that normal anterior pituitary development is dependent upon a complex genetic cascade of signaling molecules and transcription factors (reviewed in 6). Mutations in several of these genes are associated with CPHD, often with additional extra-pituitary phenotypes, including *POU1F1* (MIM 173110), *PROP1* (MIM 601538), *HESX1* (MIM 601802), *SOX3* (MIM 313430), *SOX2* (MIM 184429), *LHX3* (MIM 600577) and *LHX4* (MIM 602146) (reviewed in 7). In several cases, different mutations within the same gene have been reported to lead to variable phenotypes.

We now describe four patients from two unrelated consanguineous pedigrees with novel mutations in the LIM homeodomain transcription factor *LHX3*. All four patients presented with early-onset hypopituitarism and neonatal hypoglycemia. Subsequent clinical evaluation revealed that all four patients exhibit varying degrees of sensorineural hearing loss which has not previously been reported in association with *LHX3* mutations. We show that during human embryonic development, *LHX3* is expressed in a restricted region in the inner ear within a domain of cells expressing the transcription factor *SOX2*, which parallels the expression pattern previously observed in the mouse (8,9). Loss or downregulation of *Sox2* expression also results in pituitary abnormalities, deafness and morphological defects of the inner ear in mice (10,11), and sensorineural hearing loss has been reported as part of the phenotypic spectrum associated with *SOX2* mutations in human patients (10,12). We demonstrate that the proximal promoter region of *LHX3* contains a binding site for SOX2, which is capable of activating a luciferase reporter construct containing this region *in vitro*. Taken together, these data suggest that SOX2 is likely to play a role in the regulation of *LHX3* expression, and that both genes are potentially acting in similar molecular pathways during normal development of the anterior pituitary and the inner ear in humans.

RESULTS

Three affected siblings from family A (see pedigree, Fig. 1) exhibited an unusual phenotype consisting of panhypopituitarism, severe anterior pituitary hypoplasia, skeletal abnormalities, hyperextensible joints, loose skin and sensorineural hearing loss. In order to identify the underlying genetic defect, we adopted a homozygosity mapping strategy; affected individuals IV:2, IV:5 and IV:6, together with their unaffected sibling (individual IV:3), were genotyped for 11 555 SNP markers with an average inter-marker distance of 210 kb. The mean SNP genotype call rate obtained was 94.06% (84.59–98.36%) providing an average 10 868 genotypes per individual. Three significant regions of homozygosity shared between all three affected individuals were identified: a 2.3 Mb region on chromosome 2p24 flanked by markers rs2380657 and rs728679; a 3.9 Mb region on chromosome 12q24 (rs2013160 to rs1918210) and a 7 Mb region on chromosome 9q34-qter defined by markers rs951302 and rs1105176, the most distal marker on the array. Unaffected individual IV:3 was heterozygous for all three of these regions.

No gene in either of the loci on chromosomes 2p24 and 12q24 was a suggestive candidate for the phenotypes manifested by the patients. The region on chromosome 9q34-qter contains the transcription factor *LHX3* which presented as an excellent candidate, as mutations in the gene have previously been associated with CPHD (13–15). Sequencing of *LHX3* in the affected individuals showed that all three were homozygous for an intragenic deletion of 3088 bp (c.80-532_775+454del3088 relative to *LHX3A*; NM_178138), resulting in complete loss of exons 2–5 (Fig. 1A). The unaffected parents and siblings of the affected patients were all heterozygous with respect to the deletion (Fig. 1B).

Subsequently, we ascertained a second unrelated family (family B) with a single affected individual manifesting a very similar phenotype consisting of CPHD, pituitary and skeletal abnormalities and sensorineural deafness. Screening of the *LHX3* coding sequence in this patient revealed a homozygous transversion c.267A>T (LHX3A; NM_178138), resulting in a nonsense mutation in exon 2 (p.K50X; Fig. 1C); both unaffected parents were heterozygous carriers of the mutation.

Clinical re-evaluation of patients previously reported with *LHX3* mutations

On the basis of the finding of sensorineural hearing loss in these patients, which has not previously been associated with *LHX3* mutations, we re-investigated three patients previously reported with homozygous loss-of-function mutations in *LHX3* who were available for further evaluation (13). Two siblings (AII-1 and AII-4 from reference 13) homozygous for a p.Y116C mutation were found to exhibit moderate (60 dB) and mild (30 dB) sensorineural hearing loss. A single patient from the second family [patient BII-1 described by Netchine *et al*. (13)], harboring an intragenic deletion of 23 bp, who suffers extreme mental retardation, was diagnosed to be completely deaf on the basis of the absence of any acoustic evoked potential (AEP).

Expression of *LHX3* in the inner ear during human embryonic and fetal development

In order to investigate a potential role for *LHX3* during development of the inner ear, we investigated its expression pattern in available sections of normal human embryos. *LHX3* transcripts were detected in Rathke's pouch and the developing anterior pituitary from CS13 to CS20, in addition to discrete regions of the developing neural tube corresponding to

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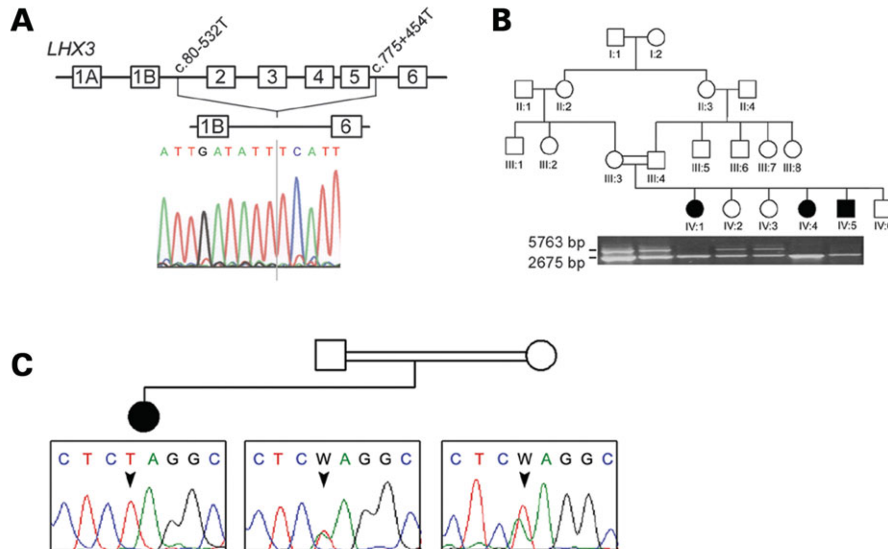

Figure 1. Mutational analysis of *LHX3*. (**A**) Schematic diagram of the intron–exon structure of *LHX3* showing the position of the breakpoints for the deletion identified in family A. Sequence trace shows sequence across the breakpoint region in an affected individual. (**B**) Pedigree of family A. PCR of LHX3 across the breakpoint region shows that affected individuals IV:1, IV:4 and IV:5 are all homozygous for the deletion; their unaffected parents and siblings are heterozygous carriers. (**C**) Electropherogram of the mutation c.267A>T (arrowhead) identified in exon 2 of *LHX3* in family B. The affected individual is homozygous for the mutation, his unaffected parents are both heterozygous with respect to the mutation. 'W' denotes the ambiguity code for adenine or thymine in heterozygous individuals.

motorneuron and interneuron subpopulations consistent with previous observations (16) (data not shown).

Expression of *LHX3* was observed within distinct domains of the developing inner ear from CS20 onwards; no expression was detected by *in situ* hybridization prior to this stage. *LHX3* transcripts were observed in defined regions of the vestibular sensory epithelium at CS20, and expression was maintained from this stage and during all fetal stages investigated (up to ~9 weeks of development; Fig. 2A). This restricted expression pattern was reminiscent to that we have previously observed for *SOX2*; however, *SOX2* is expressed prior to the onset of *LHX3* expression (data not shown). Moreover, while these studies were being performed, Hume *et al*. (8) reported that *Sox2* and *Lhx3* exhibit overlapping expression patterns during critical periods of inner ear development in the mouse. We subsequently analyzed expression of both genes in serial sections from the developing human inner ear. *LHX3* transcripts were observed in the vestibular epithelium in a defined subset of cells within the *SOX2* expression domain (Fig. 2B). In addition, these cells also express *ATOH1* (MIM 601461), an essential marker required for hair cell differentiation (17), suggesting that these correspond to the differentiating vestibular hair cells (Fig. 2B) in a pattern identical to that observed during murine vestibular morphogenesis (8).

SOX2 transcripts and protein were also detected within a restricted domain in the dorsal region of the auditory epithelium of the developing cochlear duct at CS17 (Fig. 2C). Expression is maintained within distinct regions of the developing cochlea, and by fetal stage F2, defined domains of expression are detected in the cochlea corresponding by position to regions from which the sensory hair cells will differentiate (Fig. 2C; data not shown). No expression of *LHX3* was detected by *in situ* hybridization in the cochlea sensory epithelium between CS17 and fetal stage F2 (~9 weeks of development), the latest stage for which tissue was available to study.

In the mouse, *Lhx3* is expressed exclusively within both the vestibular and cochlea hair cells in a pattern overlapping that of *Sox2*. Moreover, in the cochlea sensory epithelium, *Sox2* expression precedes that of *Lhx3*, the latter is not detectable until late embryonic stages (after E15.5) and maintained postnatally as the cochlea hair cells differentiate (8,9). Within the vestibular sensory epithelium, both *Sox2* and *Lhx3* are expressed at an earlier stage in the mouse, which parallels our observations in human development, suggesting that the pattern of *Lhx3* expression in the inner ear may also be conserved in humans. Therefore, onset of *LHX3* expression in the cochlea is likely to occur later during fetal development as the hair cells begin to differentiate, at ~11 to 12 weeks of human development (18), beyond the stage for which human fetal material was available for study.

SOX2 is capable of activating *LHX3* expression *in vitro*

Given the observation that expression of *SOX2* precedes the onset of *LHX3* in an overlapping domain of expression within both the inner ear and pituitary during human development (Fig. 2) (19), we hypothesized that SOX2 could be involved in the transcriptional regulation of *LHX3*. Analysis of the proximal promoter of *LHX3* using Matinspector software (Genomatix) identified a putative SOX-binding site at −2213 to −2224 bp relative to the start of transcription of *LHX3A* (NM_178138), to which SOX2 is capable of binding *in vitro* (Fig. 3A). The specific binding of SOX2 to this sequence is further supported by repeating the experiments using a mutated probe to which SOX2 was unable to bind (Fig. 3A). Luciferase reporter assays co-transfecting CHO cells, which do not express *Sox2*, with increasing

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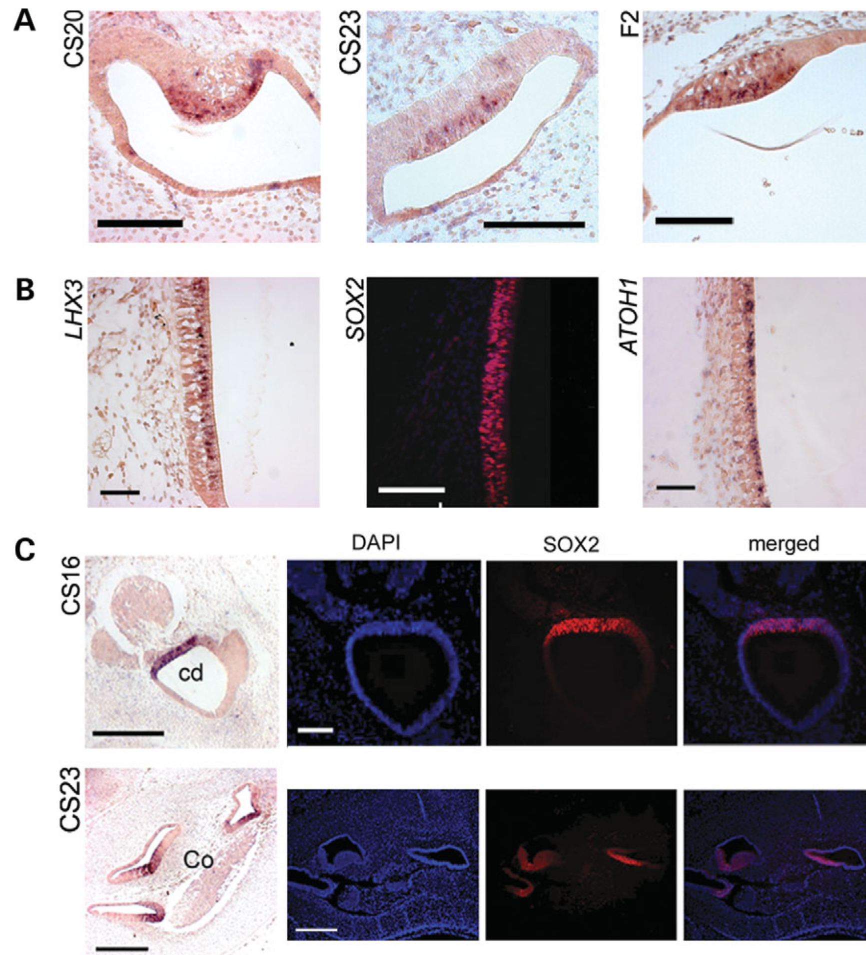

Figure 2. Expression analysis of *LHX3* and *SOX2* in the inner ear during human embryonic development. (**A**) *LHX3* transcripts were detected in defined regions of the vestibular sensory epithelium at CS20 and maintained during fetal stages of human development as shown in coronal (CS20 and CS23) and sagittal (F2) sections. No expression of *LHX3* was detected prior to CS20. (**B**) Serial sagittal sections of the vestibular sensory epithelium at fetal stage F1 showing overlapping expression of *LHX3*, as shown by *in situ* hybridization, and SOX2 protein as shown by immunohistochemistry. *ATOH1* transcripts, an essential marker of auditory sensory hair cell differentiation, were also detected within this overlapping domain. (**C**) Sagittal sections showing expression of *SOX2* mRNA and protein in the dorsal region of the developing cochlear duct at CS16 and within distinct regions of the cochlea as shown by transverse sections at later stages. cd, cochlea duct; Co, cochlea. Scale bars 300 µm (A), 100 µm (B, C).

amounts of a SOX2 expression construct together with a reporter construct comprising a 557 bp fragment of the *LHX3* proximal promoter containing the SOX2-binding site resulted in a dose dependent activation of the reporter up to a maximum of ~9-fold compared with reporter alone (Fig. 3B). Transfection of the reporter into the human EC cell line NT2/D1, which shows endogenous *SOX2* expression (20) (data not shown), led to an ~3-fold increase in reporter gene activation compared with the promoter-less reporter. A reporter construct in which the SOX2-binding site had been mutated showed significantly decreased reporter gene activation compared with the wild-type construct ($P < 0.006$; Fig. 3C). Electrophoretic mobility shift assays using nuclear extracts prepared from NT2/D1 cells showed a protein:DNA complex interacting with a probe containing the putative SOX2-binding site; addition of anti-SOX2 antibody resulted in a slower migration of the protein complex indicating the specific binding of SOX2 to the probe. No protein was observed binding to the mutated probe using NT2/D1 nuclear extracts or to either probe using CHO cell extracts (Fig. 3D; data not shown).

DISCUSSION

In the mouse, *Lhx3* expression is crucial during normal pituitary organogenesis for the commitment of the hormone-producing cell types within the anterior pituitary gland and activation of pituitary-specific genes during development (21,22). *Lhx3* null mice die before or shortly after birth, exhibiting loss of the anterior and intermediate lobes of the pituitary which form initially but then fail to proliferate and differentiate (23).

We describe two novel homozygous mutations in *LHX3*: an intragenic deletion of 3088 bp encompassing exons 2–5 and a novel nonsense mutation, p.K50X; both are predicted to result in loss of function as a result of a truncated protein or degradation of the mutant transcript by nonsense-mediated decay

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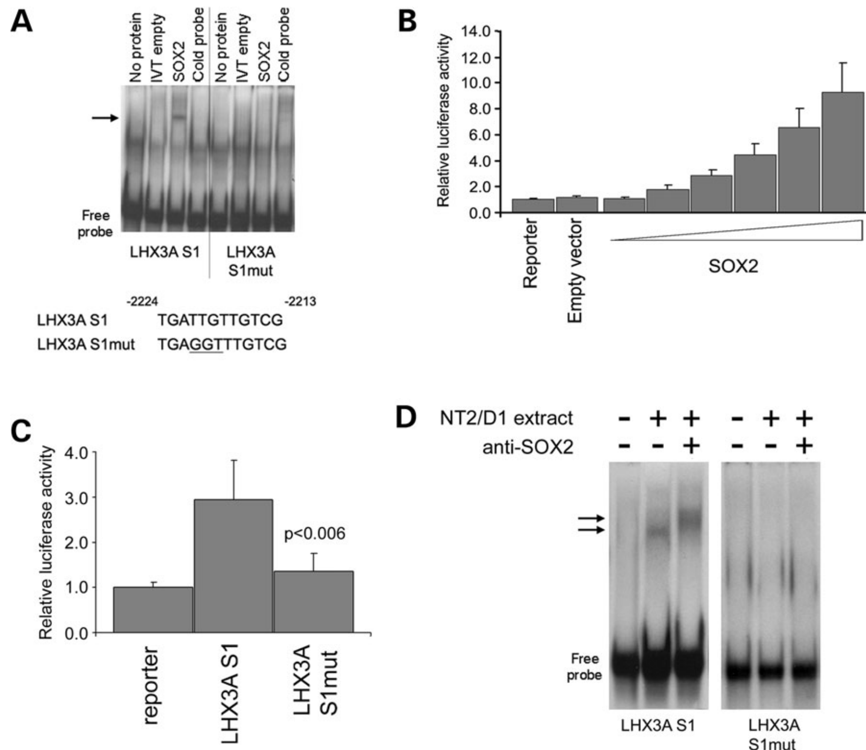

Figure 3. SOX2 is able to activate transcription of the *LHX3* promoter *in vitro*. (**A**) Electrophoretic mobility shift assay showing that SOX2 is capable of binding to a radiolabeled DNA probe encompassing a putative SOX2-binding site in the *LHX3* proximal promoter. Mutation of the SOX2-binding site (sequence shown on left) resulted in the inability of SOX2 to bind. (**B**) Luciferase reporter assay in CHO cells showing that SOX2 is capable of activating transcription of the *LHX3* proximal promoter in a dose dependent manner *in vitro*. Increasing amounts of SOX2 expression construct (1–75 ng) were co-transfected with a reporter construct comprising a 557 bp fragment of the LHX3 proximal promoter containing the SOX2-binding site identified in (A). (**C**) Luciferase reporter assay showing activation of the reporter when transfected into NT2/D1 cells which express endogenous *SOX2*; mutation of the SOX2-binding site results in significant loss of reporter gene activation. (**D**) EMSA showing that a protein complex from NT2/D1 nuclear extracts is capable of binding to a probe containing the putative SOX2-binding site. Addition of anti-SOX2 antibody results in slower migration of the protein:DNA complex. No protein:DNA complex is formed between NT2/D1 nuclear proteins and the probe containing the mutated SOX2-binding site. Transfection results are presented as mean ± SD of three independent experiments performed in triplicate.

(24). The phenotypes of the affected patients include some unusual clinical features which have not previously been described in association with *LHX3* mutations (Table 1) (13–15). The patients in family A all show signs of severe early-onset hypopituitarism with neonatal hypoglycemia. A presumptive diagnosis of ACTH deficiency was made on the basis of the clinical phenotype as well as low random cortisol concentrations, whereas previously described patients with *LHX3* mutations had preserved ACTH secretion (13,14). Unfortunately, apart from patient IV:5 who did not respond to glucagon stimulation by increasing cortisol secretion, formal provocation could not be performed to assess cortisol concentrations. ACTH stimulation performed in the proband in family B unequivocally confirmed a diagnosis of ACTH deficiency, thereby establishing this as a novel feature of LHX3 deficiency. Initial corticotrope specification is usually spared in $Lhx3^{-/-}$ mice; however, early in pituitary development, there is a severe reduction in differentiated corticotrope cells and lower expression of corticotrope transcription factors (25). In addition, it is important to note that the adrenal glands were hypoplastic, and mutants were either stillborn or died within 24 h of birth; therefore, it would not be totally unexpected to diagnose ACTH deficiency in patients with *LHX3* mutations.

We report here, for the first time to our knowledge, the association of sensorineural hearing loss in association with mutation of *LHX3* in all four patients we describe. Furthermore, clinical re-evaluation of three previously published patients has revealed that these also exhibit varying degrees of sensorineural hearing loss. This association can be explained by a potential role for *LHX3* in inner ear sensory hair cell development. In the mouse, *Lhx3* is detectable at the basal end of the cochlea specifically in differentiating sensory hair cells, beginning at embryonic day (E) 15.5, within a domain of *Sox2*-expressing cells (8). By E17, *Lhx3* begins to be expressed in the basal and middle cochlea in both inner and outer hair cells where expression levels increase until birth (8,9). Further implication of a role for *LHX3* in sensory hair cell development comes from the observation that *Lhx3* mRNA is downregulated specifically in the cochlea hair cells in the inner ears of mice with mutation of *Pou4f3*, mutations in which cause non-syndromic hearing loss in humans (DFNA15; MIM 602459), suggesting that *Lhx3* is required for cochlea hair cell differentiation and is regulated in part either directly or indirectly by *Pou4f3* specifically in the cochlea sensory epithelium (9).

In the vestibule, *Lhx3* is expressed at earlier stages of embryonic development than in the cochlea, becoming first detectable at

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Table 1. Phenotypic variability of patients previously reported with *LHX3* mutation

Mutation	Endocrine phenotype	Associated abnormalities	Deafness phenotype	Reference
p.Y116C (three patients)	GH, TSH, PRL, LH, FSH deficiency; severe anterior pituitary hypoplasia; severe growth retardation	Elevated and anteverted shoulders, restriction of cervical spine rotation; no vertebral malformation	Mild-to-moderate bilateral sensorineural hearing loss on re-investigation	(13)
23 bp deletion	GH, TSH, PRL, LH, FSH deficiency; enlargment of anterior pituitary; growth retardation	Abnormal steepness of cervical spine, no vertebral malformation	Profound sensorineural deafness on re-investigation	(13)
g.159delT	GH, TSH, PRL, LH, FSH deficiency; hypointense pituitary lesion; growth retardation	Normal alignment and configuration of cervical spine; limited neck rotation; large anterior and posterior fontanelle, hypertelorism and jaundice	Not described	(14)
p.A210V (two patients)	GH, TSH, PRL, LH, FSH deficiency; enlarged anterior pituitary; growth retardation	Short neck, elevated shoulders, limited neck rotation, loss of lordosis of cervical spine; hypoglycemia, prolonged jaundice, facial dysmorphism	Not described	(15)
p.E173X	GH, TSH, PRL, LH, FSH deficiency; anterior pituitary hypoplasia	Short neck with limited rotation, short arms, hypoglycemia, hyponatremia, dry skin, depressed nasal bridge	Not described	(15)
p.W224X	GH, TSH, PRL, LH, FSH deficiency; growth retardation	No syndromic features, normal neck rotation	Not described	(15)
LHX3 gene deletion	GH, TSH, PRL, LH, FSH deficiency; anterior pituitary hypoplasia	Short neck with limited rotation, loss of cervical lordosis; hypoglycemia, prolonged jaundice, retarded pscyhomotor development	Not described	(15)

E12 in a small subset of cells within a domain of *Sox2* expression (8). Consistent with this, we observed a restricted domain of *LHX3* expression overlapping that of *SOX2* in the vestibular epithelium; the latter being expressed prior to the onset of *LHX3* expression. Furthermore, co-expression of *LHX3* with *ATOH1* also parallels the expression pattern observed in the mouse, suggesting that expression of *LHX3* in the inner ear is highly conserved between species. This is also consistent with our observation of early expression of *SOX2* in both the cochlea and vestibular sensory epithelia. Taken together, these data suggest that *LHX3* is also likely to be expressed within the differentiating hair cells during human inner ear development. Despite the lack of any detectable *LHX3* in fetal stages we investigated, the onset of *LHX3* expression in the cochlea is likely to occur as the hair cells differentiate which begins later during fetal development, beyond the stage for which samples were available. It is unclear at present whether our patients exhibit any vestibular defects on account of the skeletal, vertebral and motor deficits in these individuals and also there is a lack of detailed imaging of the inner ear and formal vestibular function testing. However, AEP in two patients (AII-1 and AII-4 from reference 13) suggested a defect in the cochlea, rather than in the acoustic nerve. Distortion product otoacoustic emissions from the mildly affected sibling revealed a defect limited to the 1–2 kHz range, suggesting a defect of the outer hair cells within the cochlea.

It could be argued that untreated hypothyroidism in the affected individuals could be associated with hearing loss, as observed in *hyt*/*hyt* mutant mice (26). However, in family A, thyroxine was commenced by the second week of life in all three individuals, although compliance may have been sub-optimal in patient IV-1. In family B, the proband was commenced on thyroxine by the age of 2 weeks, suggesting that the deafness could not be attributed to untreated hypothyroidism. In the previously published families with *LHX3* mutations (13), AII-4 with mild sensorineural hearing loss was commenced on thyroxine within the first few days of life, whereas the moderately affected AII-1 and more severely affected BII-I were treated much later. Hence, our data suggest that the sensorineural deafness is associated with homozygous loss of LHX3, although untreated hypothyroidism may well contribute to the severity. It is also important to note that the sensorineural hearing deficit may be mild and therefore easily missed in some patients.

The ability of SOX2 to bind to a site in the promoter of *LHX3* and activate a luciferase reporter construct *in vitro*, together with the fact that *SOX2* expression in the human embryonic inner ear commences prior to the onset of *LHX3* expression, as also observed in the mouse (8,11), suggests that SOX2 is capable of directly regulating *LHX3*. This hypothesis may also be important during pituitary organogenesis, as *SOX2* and *LHX3* also show consecutive and overlapping expression domains within the developing anterior pituitary in humans (16,19). The putative SOX2-binding site that we have identified also lies within a region of the *LHX3* promoter previously shown to be involved in active *LHX3* expression (−2701 to −1581) in the mouse gonadotrope cell line, LβT2 (27). Although it is unlikely that this cell line expresses *Sox2*, this site may represent an enhancer region involved in specific temporal and/or spatial expression of *LHX3* during pituitary differentiation. Sensorineural hearing loss and anterior pituitary hypoplasia are part of the spectrum of phenotypes associated with *SOX2* mutations (10,12). Moreover, *Sox2* heterozygous null mice exhibit anterior pituitary hypoplasia (10), and regulatory mutations affecting *Sox2* can result in absent or disordered sensory hair cell formation in mice (11). Taken together, these data suggest that a genetic interaction between *SOX2* and *LHX3* may be important for normal development of the anterior pituitary and auditory hair cell differentiation during embryogenesis in both mice and humans; however, conditional deletion of these genes singly and in combination in mice will be necessary to confirm the importance of this interaction.

The intragenic deletion in *LHX3* that we report in family A is difficult to reconcile with other phenotypic features

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observed such as increased skin laxity and skeletal abnormalities. There is a distinct possibility that the deletion may have disrupted regulatory elements of another gene implicated in connective tissue formation. However, Pfaeffle *et al*. (15) recently described a case with an entire deletion of *LHX3* and no similar skin phenotype was reported. Alternatively, it is possible that a second recessive mutation may be segregating between the affected individuals in family A. This study therefore extends the phenotypic spectrum associated with *LHX3* mutations to encompass ACTH deficiency and sensorineural hearing loss; the latter can be explained by the likely role of *LHX3* in inner ear sensory hair cell development. Given the variable nature of the phenotype, particularly the mild sensorineural deafness we have observed in some patients, further careful phenotypic characterization in patients with *LHX3* mutations will shed further light on the role of this gene in human development.

MATERIALS AND METHODS

Family A

Individual IV:1. This female Middle Eastern patient was the second child of healthy consanguineous parents following an uneventful pregnancy (see pedigree, Fig. 1). Birth weight was 3.5 kg (0.22 SDS), with an occipito-frontal circumference (OFC) of 35 cm (−0.51 SDS) and a length of 51 cm (0.49 SDS). Large anterior and posterior fontanelles, coarse facies, low set ears, short neck, redundant skin folds, increased skin laxity and dislocatable hips were noted on initial examination. Hypoglycemia was apparent on the second day of life. Thyroid function was abnormal, with a total T4 of 2.28 μg/dl (normal range, NR 5–11.7 μg/dl) and TSH of 0.9 mU/l (NR 1–6 mU/l); basal cortisol was 2 μg/dl at 16 h (NR 3.5–10.9 μg/dl). Further assessment revealed low concentrations of ACTH, PRL (0.6 μg/l), LH and FSH (Table 2). GH stimulation using L-Dopa revealed severe GH deficiency (peak GH 0.33 μg/l), confirmed using insulin-induced hypoglycemia as a second test (GH peak 0.32 μg/l at the time of hypoglycemia). Treatment with thyroxine and hydrocortisone was commenced at 2 weeks of age, and recombinant hGH treatment added at the age of 4 years. Spontaneous pubertal development failed to occur and at 16 years of age, a gonadotropin-releasing hormone (GnRH) test failed to elicit a gonadotropin response (peak LH 0.1 IU/l; peak FSH 0.1 IU/l), with low estradiol concentrations (85 pmol/l; NR 231–726 pmol/l). MRI of the brain and hypothalamo-pituitary region revealed anterior pituitary hypoplasia with a normally placed posterior pituitary and no other midline abnormalities.

At the age of 17 years, the patient was short (height 118 cm; −7.4 SDS) with hyperextensible joints, soft hyperelastic skin and deep palmar and plantar creases with hypoplastic nails; she also had dental caries, short neck, broad thorax, lumbar lordosis and kyphoscoliosis and limited neck rotation. Mild flattening of the vertebral bodies, marked thoracolumbar kyphosis, hypoplastic T12 and anterior wedging of L1 were noted, together with a mild degree of spinal stenosis indicated by narrow interpedicular distances and short pedicles of the lumbar spine. Mild (30 dB) sensorineural hearing loss was diagnosed on an audiogram. Because of learning difficulties she attended a school for handicapped children.

Individual IV:4. The younger sister of patient IV:1 was born with a birth weight of 3.6 kg (0.43 SDS), OFC of 36 cm (1.25 SDS) and length of 52 cm (1.2 SDS). Poor feeding, sluggish activity, hypothermia and hypoglycemia were apparent on the second day of life. Thyroid function was abnormal at birth with a free thyroxine (FT4) of 0.2 ng/dl (NR 0.8–2.9 ng/dl) and TSH <0.2 mU/l. There was no rise in TSH after thyrotropin-releasing hormone (TRH) stimulation. A diagnosis of CPHD was confirmed with low concentrations of early morning cortisol, and treatment commenced with hydrocortisone and thyroxine. GH provocation with clonidine revealed a complete lack of GH response (<1.5 μg/l) with a low concentration of IGF-I, and treatment with rhGH was commenced. Posterior pituitary function was intact. Delayed closure of cranial fontanelles was manifest at the age of 2 years.

Examination at the age of 13 years revealed short stature (height 128 cm; −4 SDS and weight 38 kg; −0.8 SDS) and relative macrocephaly (head circumference 55 cm; 1.1 SDS) with frontal bossing, maxillary hypoplasia, a pointed chin, short neck, lumbar kyphosis and lordosis and long arms; hypermobile joints, soft hyperelastic skin, deep palmar and plantar creases and dental caries were also noted. Radiographic examination revealed slender osteoporotic bones and mild thoracic kyphoscoliosis. Narrowing of the interpedicular distances was observed in the lumbar spine with low set L-5 vertebrae between the iliac wings. She was completely prepubertal. Mild sensorineural hearing loss bilaterally was detected on audiogram (40 dB in the left ear, 30 dB right). The patient had schooling difficulties and attended a school for mentally handicapped children.

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Table 2. Endocrine phenotype in patients with *LHX3* mutations

Family, patient	Sex	Peak GH, μg/l	Total T4[a]/ FT4, ng/dl	Peak TSH, μU/ml	Basal PRL, μg/l	Basal/peak[b] cortisol, μg/dl	Peak LH, IU/l	Peak FSH, IU/l	Age at GnRH testing, years	Peak testosterone 3 day HCG, ng/ml	Peak testosterone 3 week HCG, ng/ml
A, IV:1	F	0.33[G]	2.28[a]	0.9	0.6	2.0	0.1	0.1	16	—	—
A, IV:4	F	<1.5[G]	0.2	<0.2	—	Low	—	—	—	—	—
A, IV:5	M	<1.1[G]	0.4	0.02	—	<2.5[b]	<0.1	<0.1	0.5	0.09	0.14
B, 1	M	0.08[A]	0.2	<0.1	4	10[b]	—	—	—	—	—

To convert to SI units, multiply metric units by 3 [GH (mU/l)], 12.9 [FT4 (pmol/l)], 20 [PRL (mU/l)], 27.6 [cortisol (nmol/l)], 0.0347 [testosterone (nmol/l)]. G, glucagon; A, arginine. [a]Total T4; [b] peak cortisol.

Individual IV:5. The younger brother of patients IV:1 and IV:4 was born by Caesarean section at full term. The birth weight was 3.6 kg (0.1 SDS), with an OFC of 36 cm (0 SDS) and a length of 48 cm (−1.5 SDS). Excessive skin laxity, micropenis and cryptorchidism were noted at birth. On the first day of life, he presented with mild respiratory distress, hypoglycemia and neonatal jaundice. Cord TSH was 0.02 mU/l with an FT4 of 0.4 ng/dl (NR 0.8–2.9 ng/dl). Supplementation with thyroxine and hydrocortisone was commenced in the second week of life, following documentation of low cortisol concentrations at the time of hypoglycemia. Glucagon stimulation performed after cessation of hydrocortisone treatment for 48 h at 9 months of age revealed poor GH (peak <1.1 μg/l) and cortisol (peak <2.5 μg/dl) responses. There was no evidence of impairment of posterior pituitary function. Basal testosterone at the age of 6 months was 0.09 ng/ml, with undetectable gonadotropins (LH <0.1 IU/l, FSH <0.1 IU/l) on GnRH testing. There was no testosterone response to human chorionic gonadotropin (HCG) stimulation over 3 days (testosterone 0.09 ng/ml post-stimulation) and a minimal response to 3 weeks of HCG (0.14 ng/ml).

The clinical phenotype was similar to that of patients IV:1 and IV:4, with short stature, facial dysmorphism, short broad neck and thoracolumbar kyphosis with exaggerated lumbar lordosis. Hyperelastic skin, hyperextensible wrist, elbow and knee joints, deep palmar and plantar creases, mild nail hypoplasia and dental caries were similar to his sisters. Radiological examination revealed anterior wedging of L1, pseudoepiphyses of I-IV metacarpals, narrowing of interpeduncular distances of the lumbar vertebrae, low set L5, hypoplasia of T12, anterior wedging of L1 and posterior scalloping of lumbar vertebral bodies. Neurological examination was unremarkable, with the exception of mild (30 dB) sensorineural hearing loss bilaterally. Cognitive function was normal for his age, however language development was delayed.

Family B

The single affected individual in this family, a male, was born at 41 weeks to first-degree consanguineous parents, with a birth weight of 3.77 kg (0.45 SDS). Examination at birth revealed mild dysmorphic features including macrocephaly, a short neck, crowding of facial features, large anterior and posterior fontanelles and short limbs, with no evidence of genital abnormalities, and normally descended testes. An atrial septal defect was diagnosed. Neonatal hypoglycemia was noted at 12.5 h of age. Investigations revealed a basal cortisol of 2.4 μg/dl (65 nmol/l) with a peak of 10 μg/dl (276 nmol/l) in response to synacthen stimulation, and a peak GH of 0.08μg/l in response to arginine provocation. The FT4 concentration was <0.2 ng/dl (<2.5 pmol/l), with an absent TSH response to TRH stimulation (TSH peak <0.1 mU/l). Repeat testing confirmed GH deficiency in response to arginine provocation (peak GH 0.2 μg/l, NR > 5.8 μg/l; IGF1 < 25 ng/ml, NR 40–195 ng/ml). PRL was also low (4 μg/l). He was commenced on thyroxine and hydrocortisone within 2 weeks of birth and GH at 4 weeks and responded well to treatment.

MRI revealed anterior pituitary hypoplasia with a eutopic posterior lobe and a normal infundibulum. No other intracranial abnormalities were identified. Subsequently, audiological assessment demonstrated profound bilateral sensorineural deafness (96 dB), requiring the use of hearing aids. He also had evidence of motor delay and was hypotonic. He developed the appearance of a skeletal dysplasia, with a short neck, stiff back and gibbus and prominent frontal bones of the cranial vault. Skeletal survey revealed a localized thoracic kyphosis at L1, with slight beaking of the inferior aspect of the L1 vertebral body. The interpedicular distance in the lumbar spine was slightly reduced, but was within the NR.

Hormonal testing. For the assessment of GH secretion, glucagon was administered intramuscularly at a dose of 100 μg/kg, and sampling performed at 30 min intervals over 3 h. Cortisol concentration was measured at 0, 150 and 180 min after glucagon administration. Clonidine was administered at a dose of 0.15 mg/m^2, and sampling performed every 30 min over a 2 h period. TRH was administered intravenously at a dose of 7 μg/kg, and TSH measured at 0, 20 and 60 min. GnRH was administered at a dose of 2.5 μg/kg intravenously and LH and FSH measured at 0, 20 and 60 min. HCG (500 units daily intramuscularly) was administered over a 3 day period, and testosterone measured on day 1 and day 4, 24 h after the administration of the last dose. Fasting paired plasma and urine osmolalities were measured early in the morning. GH was measured using a double radioimmunoassay (detection limit 0.9 ng/ml), and LH and FSH were measured using an automated two-step immunometric assay (Axsym, Abbot Laboratories, Maidenhead, UK). Testosterone was assayed by coated-tube radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Thyroxine and TSH were measured using chemiluminescent microparticle immunoassays. Serum cortisol was measured using a fluorescence polarization immunoassay. For family B, arginine monochloride (10% solution) was infused over 30 min at 0.5 g/kg up to a maximum of 30 g, and sampling performed at 15 min intervals for 1 h, then at 90 and 120 min. A standard synacthen test was performed, involving intravenous administration of 36 μg/kg synacthen and sampling for cortisol measurements at 0, 30 and 60 min. TRH testing was performed as described earlier. GH and IGF-1 were measured using solid-phase, two-site and enzyme-labeled chemiluminescent immunometric assays, respectively. FT4 and TSH were measured using solid-phase chemiluminescent competitive analog immunoassay and immunometric assay, respectively. Cortisol was measured using solid-phase competitive chemiluminescent enzyme immunoassay, with all assays analyzed using Immulite 2000 (Siemens Medical Solutions Diagnostic).

Genotyping and homozygosity mapping. SNP genotyping was performed using the GeneChip Mapping 10K Array and Assay kit (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Arrays were scanned using an Affymetrix Fluidics Station F450 and images obtained using an Affymetrix GeneArray scanner 3000. SNP genotype, marker order and chromosomal location were derived using GCOS/GDAS (Affymetrix), and genotype data were analyzed using ExcludeAR3 (28).

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Generation of riboprobes. A 664 bp fragment from human *LHX3* cDNA was amplified by PCR using the following primers: sense 5′-GAGGTAGACCACGCTCAGTT-3′ and antisense 5′-CCACAGGATACTCGAGAACA-3′ and cloned into the pCR4-TOPO vector (Invitrogen). Antisense and sense probes were generated by *in vitro* transcription, incorporating Digoxigenin-dUTP using the DIG RNA labeling mix (Roche). The probe for *Atoh1* has been described previously (29) and was a kind gift of Dr Jane E. Johnson.

Electrophoretic mobility shift assay. SOX2 protein was generated using the TNT Quick Coupled Transcription/Translation System (Promega). Nuclear extracts were prepared by harvesting cells in cytoplasmic lysis buffer (10 mM HEPES, 1 mM EDTA, 100 μM EGTA, 10 mM KCl, 1 mM DTT, 1 mM sodium pyrophosphate, 1 mM sodium vanadate) containing Protease Inhibitor Cocktail (Sigma), followed by cell lysis using 0.6% NP40 (Sigma). Nuclei were pelleted by centrifugation, resuspended in nuclear lysis buffer (20 mM HEPES, 0.2 mM EDTA, 0.1 mM EGTA, 25% glycerol, 20 mM NaCl, 1 mM DTT, 1 mM sodium pyrophosphate, 1 mM sodium vanadate) and lysed by repeated freeze thawing followed by centrifugation to remove nuclear debris. EMSAs were performed as described previously (30,31), using a radiolabeled DNA probe obtained by annealing the oligos 5′-ACCGGTATATTGATGTGATTGTTGTCGCTTGAGAA-AAAAAGGCAAC-3′ and 5′-ACCGGTGTTGCCTTTTTTT CTCAAGCGACAACAATCACATCAATAT-3′. A probe containing mutant SOX2-binding sites (shown in boldface) was obtained by annealing oligos 5′- ACCGGTATATTGATG TGA**GGT**TTGTCGCTTGAGAAAAAAAGGCAAC-3′ and 5′-ACCGGTGTTGCCTTTTTTTCTCAAGCGACAA**ACC**T-CACATCAATAT-3′. For nuclear lysates, EMSAs were performed using 10 μg of protein with 1 μg Poly(dI-dC) (Sigma) and 2 μg of anti-SOX2 antibody (Chemicon) as indicated in Figure 3.

Plasmid constructs. PCR products comprising a 577 bp fragment of the proximal promoter of LHX3 from −2463 to −1887 relative to the start of transcription of LHX3A (NM_178138) were generated from human genomic DNA using the following primers: sense 5′-TTTGTGGCTATTG GTGTGTC-3′ and antisense 5′-GGGTTCAAAGGCAAATAC AC-3′. Purified PCR products were cloned into pGL4.10 (Promega). Mutation of the putative SOX2-binding site within this region was performed using the Quikchange Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The expression construct containing the human *SOX2*-coding sequence has been described previously (10).

Cell culture and transient transfection. CHO and NT2/D1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C in humidified air containing 5 and 10% CO_2, respectively. Transient transfection assays were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions in a 96-well tissue culture plate. Cells were either co-transfected with 20 ng of luciferase reporter construct, together with increasing amounts of *SOX2* expression construct (1–75 ng), or 100 ng of luciferase reporter construct alone. pRLSV40 (Promega) was co-transfected in all experiments to control for transfection efficiency, and the total amount of transfected DNA was normalized to 120 ng/well for CHO cells and 100 ng/well for NT2/D1 cells by the addition of empty expression vector. Cells were harvested following transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega), using a BMG Fluostar Optima microplate reader. Transfection experiments were each repeated three times independently and performed in triplicate; data are presented as mean ± standard deviation.

In situ hybridization. Analysis of human embryonic/fetal material was performed by the MRC-Wellcome Trust Human Developmental Biology Resource *in situ* hybridization service with full ethical approval from the joint Great Ormond Street Hospital NHS Trust/Institute of Child Health ethics committee.

Human embryos/fetuses at selected stages were dissected and fixed in 4% PFA, then dehydrated and embedded in paraffin wax. Sections of 7 μm were cut using a standard microtome and mounted on Superfrost Plus slides (BDH). *In situ* hybridization was performed essentially as described by Wilkinson (32). For antibody detection, slides were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (diluted 1:1000, containing 2% FCS) overnight at 4°C. Expression patterns were visualized using the NBT/BCIP system (Roche). Sections were mounted in VectaMount (Vector Labs) and analyzed using the Axioplan2 imaging system (Zeiss).

Immunohistochemistry. Human embryo and fetal sections were de-waxed and rehydrated. Antigen retrieval was performed by boiling for 10 min in 0.01 M citric acid buffer, pH 6.0. After cooling to room temperature, slides were transferred to TBST (0.5 M Tris Base, 9% NaCl, pH 7.6, 0.025% Triton) and blocked with 10% heat-inactivated rabbit serum (HIRS) (Sigma) in TBST. All antibody incubations were carried out at room temperature in a humidified chamber. Slides were incubated with goat anti-SOX2 antibody (1:500 dilution, Immune Systems Ltd.) in TBST with 1% HIRS. Slides were washed and incubated with a polyclonal rabbit anti-goat IgG biotinylated antibody (1:100 dilution, DAKO). Biotin was detected using streptavidin-Alexa Fluor 555 conjugate in PBS (1:300 dilution, Molecular Probes). Slides were mounted in Vectashield with DAPI (Vector Laboratories) and images captured on a Leica DC500 microscope using Fire Cam Software.

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