Loss-of-function mutations in *IGSF1* cause an X-linked syndrome of central hypothyroidism and testicular enlargement

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Abstract

Congenital central hypothyroidism occurs either in isolation or in conjunction with other pituitary hormone deficits. Using exome and candidate gene sequencing, we identified eight distinct mutations and two deletions in *IGSF1* in males from eleven unrelated families with central hypothyroidism, testicular enlargement, and variably low prolactin concentrations. IGSF1 is a membrane glycoprotein highly expressed in the anterior pituitary gland and the identified mutations impair its trafficking to the cell surface in heterologous cells. *Igsf1*-deficient male mice

show diminished pituitary and serum thyroid-stimulating hormone (TSH) concentrations, reduced pituitary thyrotropin-releasing hormone (TRH) receptor expression, decreased triiodothyronine concentrations, and increased body mass. Collectively, our observations delineate a novel X-linked disorder in which loss-of-function mutations in *IGSF1* cause central hypothyroidism, likely secondary to an associated impairment in pituitary TRH signaling.

The index case in family A (A-III.11; Fig. 1a) was diagnosed with central hypothyroidism by neonatal screening for congenital hypothyroidism. His cousin (A-III.7), when referred for growth failure at 7.3 years, had central hypothyroidism, partial growth hormone (GH) deficiency and low prolactin (Table 1). In early adolescence, testicular growth advanced normally in both boys, but testes continued to grow beyond the reference range (Table 1). In contrast, serum testosterone (T) remained inappropriately low for testicular size until 15.2 and 14.2 years, respectively, leading to a late growth spurt and delayed pubic hair development. Subsequent testing of the maternal grandfather (A-I.4) showed central hypothyroidism (Table 1). X chromosome exome sequencing in the two cousins (Supplementary Table 1) uncovered a 27nt deletion, c.2137_2163del (p.Ala713_Lys721del) in Xq25-linked *IGSF1*. The same deletion was present in the grandfather (A-I.4) and another male relative (A-II.4) with the same phenotype (Table 1; Fig. 1a). Independent whole exome sequencing of two brothers (B-III.7 and B-III.8) with central hypothyroidism in family B (Table 1; Fig. 1b; Supplementary Table 1) revealed a nonsense mutation, c.2931G>A (p.Trp977X), also in *IGSF1* (Fig. 1c). B-III.7 and B-III.8 presented in infancy with prolonged neonatal jaundice, but two affected relatives harboring the same mutation were only diagnosed with central hypothyroidism following genetic screening at 65.5 (B-I.4) and 43.3 (B-II.11) years (Fig. 1b).

We found further *IGSF1* variants in seven Dutch (C-I) and two Italian (J and K) families characterized by male-specific central hypothyroidism (Fig. 1c; Table 1; Supplementary Fig. 1). Most Dutch and Italian (family J) cases were detected through neonatal screening for congenital hypothyroidism (T4 and TSH). Thyroid function tests at diagnosis are shown in Supplementary Table 2. Mean (SD) serum FT4 level was 79 (13) % of the lower limit of the reference range for age and assays. Except for two infants with lower values, all ranged between 67-94%. Serum TSH was within the reference range in all cases (range 0.8-6.0 mU/L, mean 2.6 mU/L). The FT4/TSH ratio was therefore lower than in controls (3.94 vs. 5.73, Mann-Whitney test, p=0.002). Mean (SD) serum T3 in nine 1-3 week old *IGSF1*-deficient infants was 98 (12) % of the lower limit of the age reference (range 83-121%)[1]. In seven older cases, T3 was in the lower half of the reference range. Within families, we observed considerable differences in the extent of hypothyroidism.

In eight infants (2.5-5 weeks) and eight older patients (0.4-65.5 years), standard TRH tests were conducted (Supplementary Table 2). In the infants, the TSH peak was 4.5-16.0 mU/L. A similar range was observed in previous reports on congenital central hypothyroidism[2,3], and is lower than observed in age-matched controls (14-37 mU/L)[3]. At later ages (7.3-63 years), TSH peaks were between 4.3-8.5 mU/L, which is in the lower half of the reported reference ranges for males (3.7-12.5 mU/L[4] or 4.1-13.9 mU/L[5]). In eight patients, the increment of serum FT4 [mean (SD) 14.2 (7.9)%] was lower than that reported for controls [mean (SEM) 23.9 (2.7)%], but the FT3 response to TRH was normal (36.3% vs. 41.8% in controls[6, 7] (Supplementary Table 3). We could demonstrate central hypothyroidism in five of twenty female heterozygous carriers (data not shown).

Serum prolactin concentrations were decreased in 18/26 patients (Table 1). Cases A-III.7, H-III.3, I-III.2 and K-II.3 showed growth retardation in childhood, associated with biochemical GH insufficiency, and were treated with biosynthetic GH. In young adulthood, GH stimulation tests were normal in the three patients who had discontinued treatment. Details

are presented in the Supplementary Note. In three patients, magnetic resonance imaging (MRI), performed because of macrocephaly (A-III.11), central hypothyroidism (C-III.1), and GH deficiency (K-II.3), was abnormal, showing a fronto-parietal hygroma, hypoplasia of the corpus callosum and small stalk lesion, respectively. In eight other cases, the MRI was normal.

Testicular development showed a characteristic pattern of a normal testicular volume in childhood, an increase of testicular volume from approximately 11 years onward while serum testosterone was still low, relatively large testes for serum testosterone (>2 years advance) in adolescence, and adult macroorchidism (>30 ml by Prader orchidometer, >18.3 ml by ultrasound)[8] (Table 1). No abnormalities were observed in testis morphology by ultrasound. In 10 out of 11 evaluable cases testosterone production was delayed, defined as a late rise of serum testosterone (<0.8 nmol/L = <23 ng/dL at 13.0 years[9]) and/or a pubertal growth spurt > 2 years delayed (compared with Dutch reference data[10]).

Table 2 shows the most recent data on height, body mass index (BMI), and pituitary-gonadal hormones. Mean height was close to the average of population references, but BMI was higher than 25 in 11 out of 13 adults and higher than +2 SDS in 5 out of 13 children. Plasma testosterone was normal in most cases. A-I.4, who underwent surgery for testicular torsion and in whom the remaining testis was atrophic, had low testosterone and elevated gonadotropins before the start of testosterone substitution, and currently his inhibin B and anti-Müllerian hormone (AMH) concentrations are very low. The two subjects (D-I.3 and F-II.8) with severe obesity had low plasma testosterone, but because of their low plasma SHBG, the free androgen index was normal. Plasma LH, FSH, inhibin B and anti-Müllerian hormone (AMH) were within reference ranges in the majority of cases, but plasma FSH was always higher than LH, and above the reference range in six cases. The response to GnRH (100 μg i.v.) was higher for LH than for FSH in all except two subjects. Inhibin B tended to be high (elevated in 4 subjects) and AMH was relatively low (decreased in 5 subjects). Serum HDL and LDL cholesterol, triglycerides, glucose, insulin and C-peptide did not indicate metabolic syndrome (not shown).

Figure 1c summarizes the identified *IGSF1* mutations. Families C and D harboured the same single nucleotide deletion [c.2248del (p.Glu750LysfsX28)]. In families E and F, we observed submicroscopic gene deletions of different sizes that included *IGSF1* and no other annotated genes. Affected members of families G, H, I and K bear missense or nonsense mutations and family J a single nucleotide insertion [c.3596_3597insT (p.Glu1200fsX3)]. All variants were confirmed by Sanger sequencing (Supplementary Table 4), map to conserved regions of the gene, are not present in available databases (dbSNP, 1000 Genomes Project, LOVD, HGMD, GoNL), and were not previously reported.

IGSF1 encodes a plasma membrane immunoglobulin superfamily glycoprotein[11, 12]. The canonical IGSF1 protein possesses 12 C2-type Ig loops, a transmembrane domain, and a short intracellular C-tail (Fig. 1c). A hydrophobic linker separating Ig loops 5 and 6 targets the protein for obligate co-translational proteolysis such that only the C-terminal domain (CTD) traffics to the plasma membrane[13]. *IGSF1/Igsf1* mRNA is abundantly expressed in Rathke's pouch (the developing pituitary primordium; Fig. 2a) and in adult pituitary gland and testis [13-17] (Supplementary Fig. 2). IGSF1 protein is detected in murine thyrotropes, somatotropes, and lactotropes, but not in gonadotropes (Fig. 2b) or in testis (not shown).

The phenotypes of patients with intragenic mutations are highly similar to those of individuals with complete *IGSF1* deletions (families E and F) (Table 1), suggesting loss of IGSF1 function in all cases. The identified mutations in families A-D and G-K map to the IGSF1-CTD coding region (Fig. 1c). We therefore examined expression and post-

translational regulation of IGSF1 mutants in heterologous HEK293 cells. Wild-type (WT) IGSF1 migrated as a 140-150 kDa doublet in western blot analysis (Fig. 3a, lane 2), reflecting the mature N-glycosylated or immature endoplasmic reticulum (ER) resident forms of the CTD (lanes 3-4)[13]. In contrast, IGSF1-Ala713_Lys721del (family A; for primers see Supplementary Table 5) migrated predominantly as an immature form (Fig. 3a, lanes 5-7). Similar migratory patterns were observed for IGSF1-Ser863Phe (Fig. 3a; family G; lanes 8-10) and the other two missense mutants (Supplementary Fig. 3a; Family H, Cys947Arg; Family K, Ser770Asn; lanes 6-11). The nonsense mutants in families B/I (Trp977X, Fig. 3a, lanes 11-13; and Trp1173X, Supplementary Fig. 3a, lanes 17-19) and frame-shift mutants in families C/D (Glu750LysfsX28) and J (Glu1200ArgfsX3) (Supplementary Fig. 3a, lanes 3-5, 12-14) possessed immature sugars and were truncated relative to wild-type IGSF1. Based on their patterns of glycosylation, the identified IGSF1 mutants appeared to be retained in the ER.

To assess plasma membrane trafficking, we transfected HEK293 cells with WT and mutant IGSF1 constructs and detected the expressed proteins by immunofluorescence with an antibody directed against the N-terminus of IGSF1-CTD. Membrane staining was observed in non-permeabilized cells transfected with IGSF1-WT (Fig. 3b). A similar pattern was observed with IGSF1-Ala713_Lys721del and the three missense mutants (Fig. 3b, Supplementary Fig. 3b); however, cell surface biotinylation demonstrated that mutant proteins reached the plasma membrane with poor efficiency and with distinct glycosylation patterns compared to WT (Fig. 3c). Membrane signals were never detected in non-permeabilized cells transfected with IGSF1-Trp977X (Fig. 3b) or the other truncated forms of the protein (Supplementary Fig. 3b). We did not detect secreted proteins in the culture medium (not shown). In contrast, we observed a strong intracellular staining pattern in permeabilized cells with all constructs (Fig. 3b, Supplementary Fig. 3b), confirming expression of the mutant proteins. Thus, the identified mutations block or significantly impair IGSF1-CTD plasma membrane trafficking.

To establish a causal link between loss of IGSF1 function and central hypothyroidism, we examined pituitary and thyroid function in *Igsf1*-deficient mice[17] (Supplementary Fig. 4). Messenger RNA expression for various pituitary hormone-encoding genes, including *Tshb*, did not differ between control and *Igsf1*-deficient mice (Fig. 4a, Supplementary Fig. 5a). In contrast, both pituitary and serum TSH were significantly reduced in adult *Igsf1*-deficient males (Fig. 4b,c). Pituitary prolactin content was unaffected (Supplementary Fig. 5b). Whereas circulating T3 concentrations were decreased in *Igsf1*-deficient males (Fig. 4d), T4/FT4 concentrations were similar to controls in the majority of cases and thyroid histology appeared normal (Supplementary Fig. 5c,d and not shown). Reduced TSH synthesis and secretion (Fig. 4b,c) in the face of normal *Tshb* mRNA expression (Fig. 4a) in *Igsf1*-deficient mouse pituitaries suggested impaired TRH signaling. Consistent with this idea, pituitary *Trhr* mRNA was reduced and hypothalamic *Trh* mRNA increased in *Igsf1*-deficient mice relative to controls (Fig. 4e,f). Pituitary *Gnrhr* mRNA expression was unaltered (not shown). Finally, *Igsf1*-deficient males were heavier than their control littermates (29.9 ± 0.4 vs. 28.2 ± 0.3 g at 12 weeks, p=0.004). Thus, adult male *Igsf1*-deficient mice display several characteristics of central hypothyroidism.

IGSF1 was initially hypothesized to function as a pituitary inhibin co-receptor[15, 18, 19], raising the possibility that macroorchidism in our patients might be linked to loss of inhibin action and therefore enhanced FSH secretion. However, IGSF1's putative role as an inhibin co-receptor has been challenged by more recent binding and *in vivo* data[17, 19]. In our patients, serum FSH was higher than LH but exceeded the reference range in only six cases (Table 2). Moreover, male *Igsf1*-deficient mice are fertile and have normal testicular size

and FSH ([17] and Supplementary Figs. 5a,e). Therefore, at present, the mechanisms of testicular enlargement in our patients are unresolved.

In summary, our data delineate a novel X-linked disorder in which loss-of-function mutations in *IGSF1* cause central hypothyroidism, testis enlargement, and variable prolactin and GH deficiency. The identified human *IGSF1* defects impair either expression or membrane trafficking of the IGSF1 C-terminal domain, consistent with a loss of protein function. *Igsf1* deletion decreases pituitary and circulating TSH in mice, perhaps secondary to impaired TRH receptor signaling. Loss of IGSF1 function was associated with profound hypothyroxinemia in some patients, with the attendant risk of neurodevelopmental delay, if untreated. In other patients, hypothyroxinemia was less severe, but untreated subclinical hypothyroidism is associated with adverse cardiometabolic risk, which can be reversed by thyroxine treatment[20-22]. Thus, following genetic ascertainment of future cases, biochemical screening and thyroxine treatment of affected family members will be of significant clinical benefit. Collectively, our observations uncover a completely unexpected and clinically relevant role for IGSF1 in pituitary and testicular function.

URLs

RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq/; dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/; 1000 Genomes Project, http://www.1000genomes.org/; Hapmap Project, http://hapmap.ncbi.nlm.nih.gov/; Uniprot, http://www.uniprot.org; Human Splicing Finder, http://www.umd.be/HSF/; BWA, http://bio-bwa.sourceforge.net/; Samtools, http://samtools.sourceforge.net/; GMAP, http://research-pub.gene.com/gmap/; Seattleseq Annotation, http://snp.gs.washington.edu/SeattleSeqAnnotation131/; Sift, http://sift.jcvi.org/; polyphen-2, http://genetics.bwh.harvard.edu/pph2/; LOVD IGSF1 database, www.lovd.nl/igsf1; X-chromosome exome sequencing, http://databases.lovd.nl/shared/individuals/00000209 and http://databases.lovd.nl/shared/individuals/00000208.

Methods

DNA isolation

DNA was isolated from human whole blood by salting out (Puregene, Qiagen) following manufacturer's instructions.

Exome enrichment and next generation sequencing

X-exome enrichment was carried out using Agilent's SureSelect X chromosome oligo capture library following manufacturer's protocol except DNA was hybridized with half of the suggested probe. Illumina GAII generated 51 bp paired-end reads. Reads overview in Supplementary Table 1. Whole exome sequencing was undertaken using SureSelect Human All Exon 50Mb Kit (Agilent Technologies). Sequencing was performed with SOLiD™4 System (Applied Biosystems).

Read mapping, variant calling, and annotation

For the X-exome data, reads were aligned to reference genome hg19 by BWA-0.5.8[28]. Bam file manipulation and variant (SNVs and indels) calling was done by samtools-0.1.9[29]. Deletion in family A was detected by GMAP v3. 2011-03-28(alignment)[30] and samtools-0.1.9 (variant calling). SeattleSeq Annot.131 was applied to annotate all variants. Variants in dbSNP131 and 1000 Genomes Project were filtered out for mutation screening. Whole exome experiments, sequencing and preliminary analyses were undertaken at Eastern Region Sequencing and Informatics Hub, using Genome Analysis Toolkit[31].

Filtering strategy

In family A, variants shared by both boys were classified based on their function; only exonic and splice site variants were taken. We then filtered out all the variants present in local in-house exome sequencing database, dbSNP, 1000 Genomes Project, Leiden Open Variance Database (LOVD), and Hapmap Project with an allele frequency >1% in Caucasian population, which yielded no promising results. We therefore checked the variant list generated by GMAP and samtools, as GMAP allows gap alignment and enables identification of larger insertions and deletions. The same filtering procedure was applied on those variants. For family B, we compared variants from the two brothers; only shared exonic and splice site variants were taken into the filtering step. We filtered out all variants present in databases above and the UK 10K genomes project, and focused on novel, shared homozygous or X-linked hemizygous variants, using public databases to identify plausible candidates.

Sanger sequence analysis

PCR was performed using Phire Hot Start II DNA polymerase (Finnzyme) following manufacturer's protocol (primers in Supplementary Table 4). Products were purified with QIAquick PCR purification kit (Qiagen), then mixed with 10 pmol of forward or reverse primers and sequenced by Applied Biosystems 96-capillary 3730XL system.

Microarray

We carried out a cytogenetic whole-genome 2.7M array (Affymetrix) following manufacturer's protocol. In families C/D, this revealed the same haploblock of 23 Mb around the deletion (rs929590 to rs16979902), suggesting common ancestral origin. No familial relationship is known for at least four generations.

Constructs

The IGSF1-CTD antibody epitope of human Myc-IGSF1-HA construct (from Dr. Peter Scheiffele) was modified (QuikChange, Stratagene) to match the murine protein to facilitate detection with IGSF1-CTD polyclonal antibody (Dr. Scheiffele). Mutations were introduced into the construct using QuikChange or Phusion (Thermo) (p.Ala713_Lys721del) site-directed mutagenesis (primers in Supplementary Table 5). Constructs were verified by sequencing (McGill University and Genome Québec Innovation Centre).

Cell culture

For western blotting and cell surface biotinylation, HEK293 cells were plated in 6-well plates, and transfected with 200 ng of expression vector using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. For immunofluorescence, HEK293 cells were plated in 24-well plates on glass coverslips at 50,000 cells/well and transfected with 100 ng vector.

Western blotting

Whole cell extracts were prepared from transfected cells 24 h post-transfection using RIPA buffer. Protein lysates were deglycosylated using PNGaseF and EndoH (New England Biolabs), using manufacturer's instructions. Western blots were performed as previously described[32].

Cell Immunofluorescence

Non-permeabilizing: IGSF1-CTD antiserum (1:500 in serum-free DMEM) was applied to cells for 2 h at 37°C/5% CO_2. Cells were transferred to room temperature, washed 3x with

serum-free DMEM and fixed with 4% paraformaldehyde. Cells were washed 3x with PBS, incubated in 5% BSA in PBS for 1 h, secondary antibody (1:600 in 5% BSA in PBS) was applied to cells for 1 h, cells were washed 3x with PBS and mounted using aqueous media with DAPI. Permeabilizing: cells were fixed using 4% paraformaldehyde, washed 3x in PBS, incubated in 0.3% Triton X-100 in PBS (PBSX), incubated in 5% BSA in PBSX for 1 h. IGSF1-CTD antiserum (1:500 in 5% BSA in PBSX) was applied to cells for 2 h. Cells were washed 3x with PBS, secondary antibody (1:600 in 5% BSA in PBSX) was applied for 1 h, cells were washed 3x with PBS, and mounted as above.

Pituitary immunofluorescence

The morning when a vaginal plug was detected was considered embryonic day (E) 0.5. E18.5 pregnant mice were sacrificed, embryo heads fixed overnight in 4%PFA at 4°C, embedded in paraffin, and sliced at 5 μm. Sections were treated as described before[33]. Nonspecific binding was blocked using 5% BSA in PBS with 0.2% Tween-20 (PBST). Sections were incubated with primary antibodies (IGSF1-CTD 1:500, and from Santacruz: TSH/LH 1:400, GH/prolactin, 1:200 in blocking buffer) overnight at 4°C. Sections were washed 3x with PBST, incubated in horse-anti-goat biotin antibody (1:150 in blocking buffer) for 1h, washed 3x with PBST, incubated in Streptavidin-Texas Red (1:400 in blocking buffer) and goat anti rabbit Alexa 488 (1:600) for 1h, washed 3x with PBST, and mounted as above.

Cell surface biotinylation

Transfected cells were washed 3x with PBS and incubated in 1 ml 0.5 mg/mL EZ-link-sulfo-NHS-LC-biotin (Thermo) for 30 min at 4°C, washed 3x with 100 mM glycine in PBS, harvested in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), and lysed by sonication (9W, 10 seconds). Lysates were centrifuged, supernatant collected, and immunoprecipitation was carried out using anti-HA affinity beads (Sigma) following manufacturer's instructions. Lysates were then processed for western blotting as above. After blocking, membrane was incubated in 2.5% milk in TBST with 2 drops each of A and B reagents from Vectastain kit (Vector) for 30 min, 3×10 min washes in TBST and signal visualized using Western Lightning Plus-ECL kit (Perkin-Elmer).

Animals

Igsf1-deficient mice were described previously[17]. Tissue and blood collection was performed in accordance with institutional (McGill University) and federal guidelines.

In situ hybridization

In situ hybridization was performed as previously described[34] using riboprobes from human *IGSF1* cDNA clone 30387876 (IMAGE). Human embryonic material was provided by Human Developmental Biology Resource, supported by Medical Research Council Grant G0700089 and Wellcome Trust Grant 082557.

Northern blotting

Human pituitary RNA was from Netherlands Brain Bank, NBB number 02-055, in accordance with formal permission for a brain autopsy and use of human brain material and clinical information for research purposes. Human testicular RNA was obtained from Dr. Sarah Kimmins. Thirteen μg of each RNA were blotted as previously described [17], using a probe spanning exons 18-20 of murine *Igsf1*.

Hormone measurements

At diagnosis, serum FT4, TSH, T3 and prolactin were determined in laboratories of participating departments. At follow up, samples were investigated in Endocrine Laboratory, ErasmusMC, Rotterdam (inhibin B and AMH), and Laboratory of Endocrinology and Radiochemistry, Academic Medical Center, Amsterdam (other measurements). Plasma LH and FSH were analysed on a Roche E170; intra and interassay variations: <5%. Plasma T3 was measured by in-house RIA; intra/interassay variations: 6.3%/7.8%. FT4, FT3, TSH, prolactin and GH were analysed by fluoroimmunoassay using Delfia 1232 Fluorometer (Perkin Elmer); intra/interassay variations: FT4 5.1%/6.8%, FT3 7.7%/11.3%, TSH 4.2%/5.7%, prolactin 3.4%/5.3%, and GH 3.8%/6.2%. Plasma testosterone was analysed by in-house RIA; intra/interassay variations: 9.2%/10.8%. Insulin was analysed by chemiluminescence using Immulite 2000 (Siemens); intra/interassay variations: 3.7%/5.1%. Serum inhibin B and AMH were estimated using enzyme-immunometric methods (Beckman Coulter); for AMH the Gen II assay was used; intra/interassay variation was published previously[35, 36].

Murine pituitary TSH and PRL content measurement was previously described[37], using reagents supplied by Prof. A.L. Parlow (NHPP, Torrance, CA). In Chicago, serum total T4/T3 concentrations were measured by coated tube RIAs (Siemens Medical Solutions Diagnostics) adapted for mouse. TSH was measured as described before[38]. Free T4 levels were estimated from total T4 and resin T4 uptake ratio and expressed as free T4 index (FT4I) as in [39].

Quantitative PCR

Murine RNA was extracted from whole pituitaries and 3 mm hypothalamic blocks according to following coordinates[40]: anteroposterior-interaural 3.94 to 0.94 mm, dorsoventral-interaural 2.5 mm and below, and 1.44 mm lateral. RNAs were extracted and reverse-transcribed (2 μg) as previously described[32]. qPCR was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 0.4 μmol of each primer (see Supplementary Table 6) on a Corbett Rotor-Gene 6200 HRM (Corbett Life Science), using manufacturer's protocol. Transcript levels were normalized relative to ribosomal protein L19 (*Rpl19*) and analyzed using $2^{-\Delta\Delta Ct}$ method[41, 42].

Statistics

In Figure 4 and all supplemental figures, statistical significance taken at $p < 0.05$ was determined by two-tailed Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Footnotes

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Y.S., J.T.d.D., M.K., N.S. and K.C. developed the exome sequencing protocol. A.S.P.v.T., W.O., S.G.K., N.B., N.A-D., A.M.P., M.H.B., R.C.H., M.D., N.S., L.P., I.C., M.B., P. B-P., H.D., T.D., K.C., A.C.S.H-K, D.G.D.B. and J.M.W. designed the clinical research studies. Y.S., J.F.J.L. and N.S. performed bioinformatics analyses, mutational analysis and genotyping. B.B. generated the mutant IGSF1 expression vectors and performed all associated biochemical analyses; maintained the mouse colony, collected all mouse tissues and plasma, and analyzed pituitary gene expression; and prepared figures. D.J.B. generated the mouse model; supervised all *in vitro* and mouse work; participated in data collection and construction of the figures. P.V. and M.G.W. contributed to murine phenotyping. E.C, J.K.W., and M.G.W. performed murine T4 measurements. P.L.T. performed measurements of pituitary TSH and PRL content. S.N.M.G. and J.P.M-B. carried out the IGSF1 expression studies in mouse and human embryos. C.A.L.R. and C.A.J.B. performed and analysed the microarray and hybridization experiments. A.S.P.v.T, W.O., W.H.S-B., T.V., M.K., L.P., I.C., M.B., P.B-P., H.Z., T.M.E.D., A.C.S.H-K., D.G., J.J.R., S.G.K., N.B., N.A-D., A.M.P., G.C.J.H, E.P.C, M.H.B., R.C.H., A.C.S.H-K. and M.D. contributed to clinical evaluations and the delineation of the subject phenotypes. Y.S., B.B., N.S., A.S.P.v.T., K.C., M.T.D., R.C.H., D.J.B. and J.M.W. prepared the manuscript. D.J.B., J.M.W., K.C., and M.T.D. conceived and supervised the study.

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Competing financial interests

The authors declare no competing financial interests.

Acknowledgments

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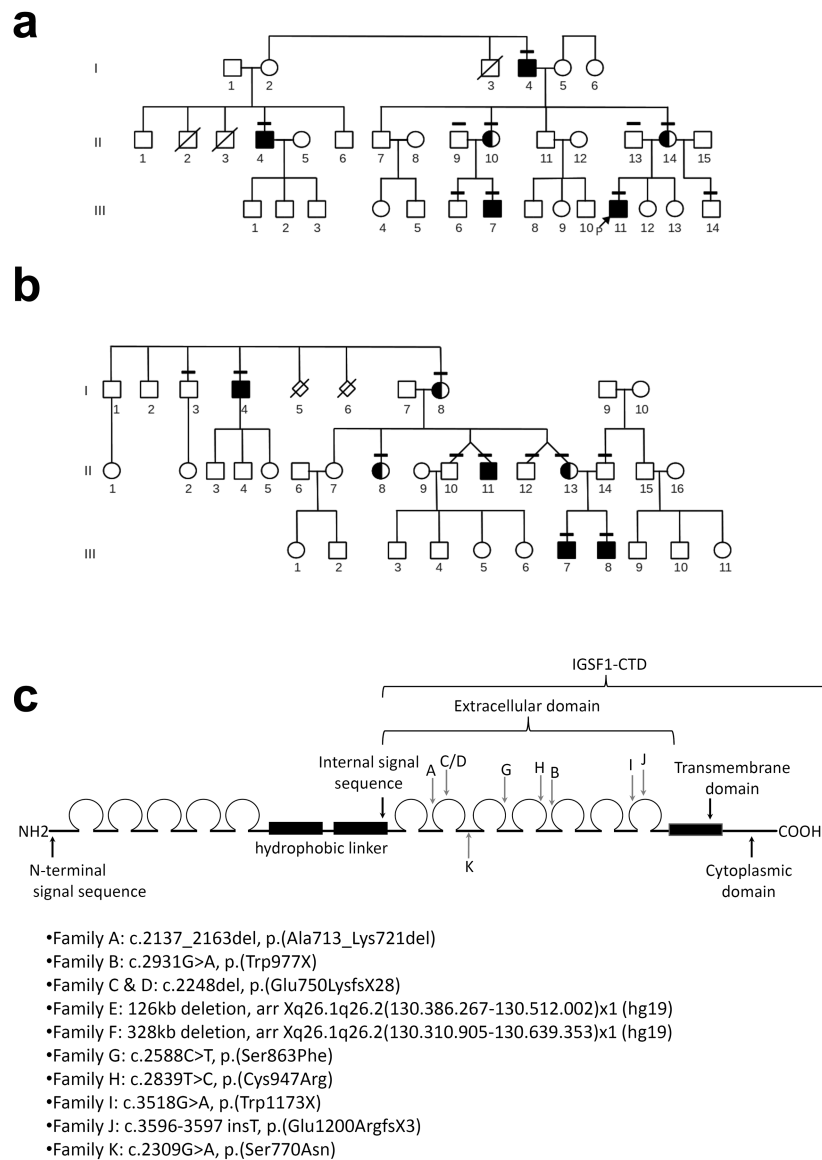
Figure 1. IGSF1 mutations identified in patients with central hypothyroidism
(a) Pedigree of family A. Small horizontal lines signify that the mutation was confirmed. (b) Pedigree of family B. (c) Schematic representation of IGSF1 protein domain structure and relative positions of identified mutations.
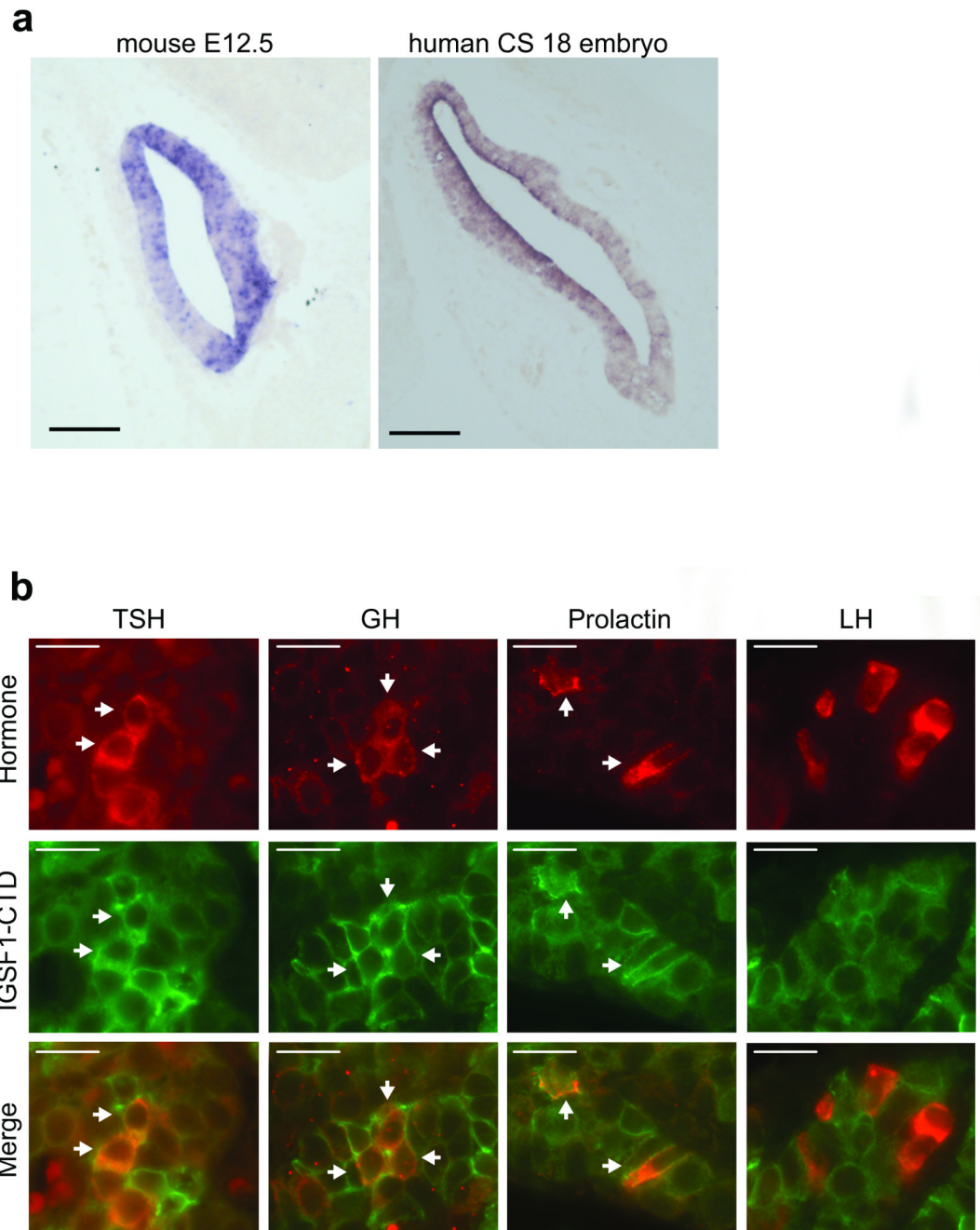
Figure 2. IGSF1 is expressed in anterior pituitary gland
(a) Expression of *IGSF1/Igsf1* mRNA in murine embryonic day 12.5 and human embryo Carnegie stage 18 Rathke's pouch progenitors as detected by *in situ* hybridization. Scale bars, 10 µm. (b) Immunofluorescence using IGSF1-CTD antibody and antibodies against the indicated anterior pituitary hormones (TSH: thyrotropes; GH: somatotropes; prolactin: lactotropes; LH: gonadotropes) was performed in WT E18.5 mouse pituitary. Scale bars, 10 µm.
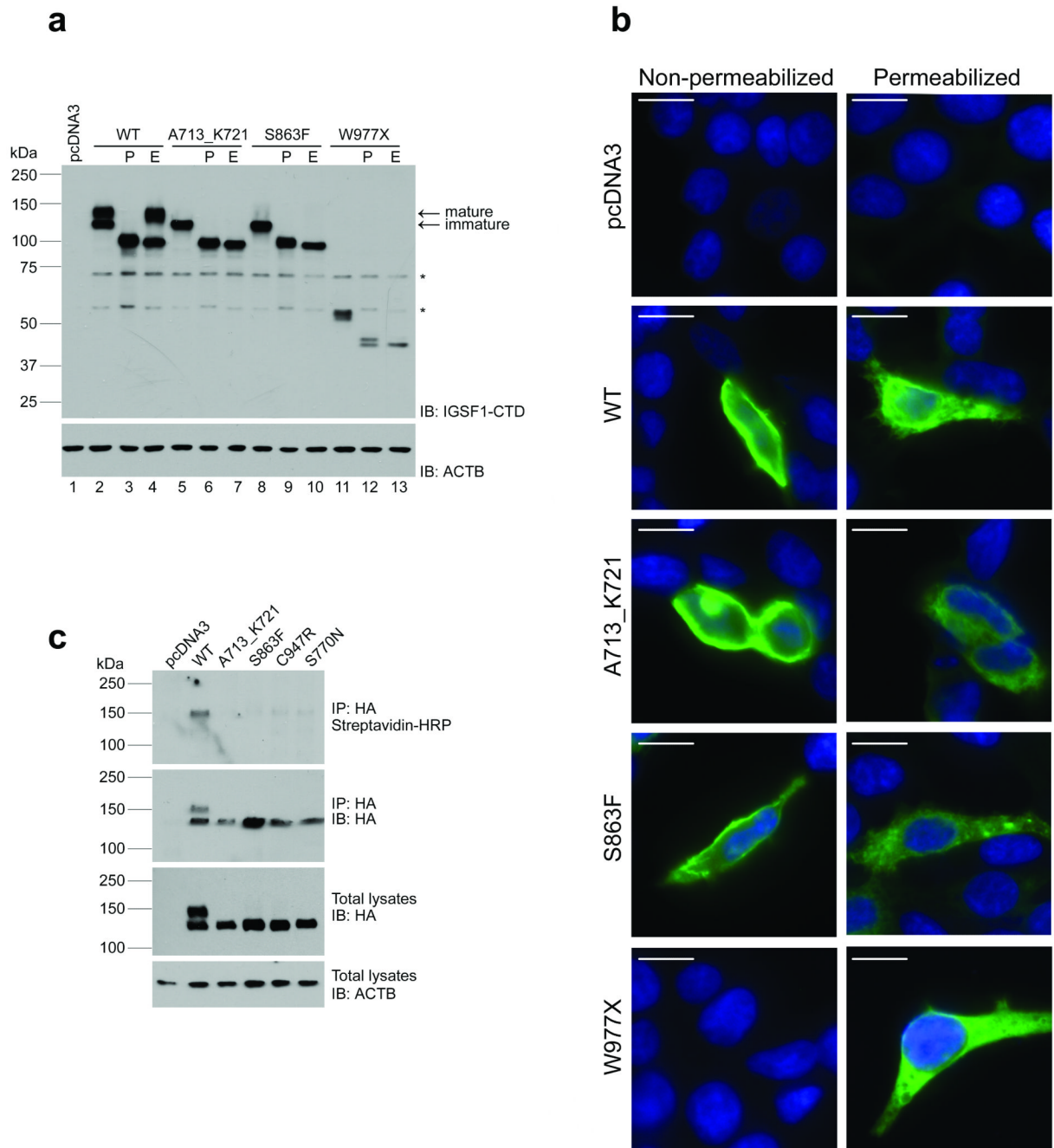
Figure 3. Mutations in IGSF1 impair its plasma membrane trafficking
(a) HEK293 cells were transfected with pcDNA3 (empty vector) or the indicated wild-type or mutant IGSF1 expression vectors. Protein lysates were deglycosylated with either PNGaseF (P) or EndoH (E), resolved by SDS-PAGE, and immunoblotted using an IGSF1-CTD antibody. Non-specific bands are indicated by *. (b) HEK293 cells were transfected with the same constructs as in (a). Expression of IGSF1-CTD was analyzed by immunofluorescence using the IGSF1-CTD antibody under non-permeabilizing and permeabilizing conditions. Nuclei were stained with DAPI (blue). Scale bars, 10 μm. (c) HEK293 cells were transfected with pcDNA3 or the indicated wild-type or mutant IGSF1

expression vectors. Membrane expression of IGSF1-CTD was analyzed by cell-surface biotinylation.
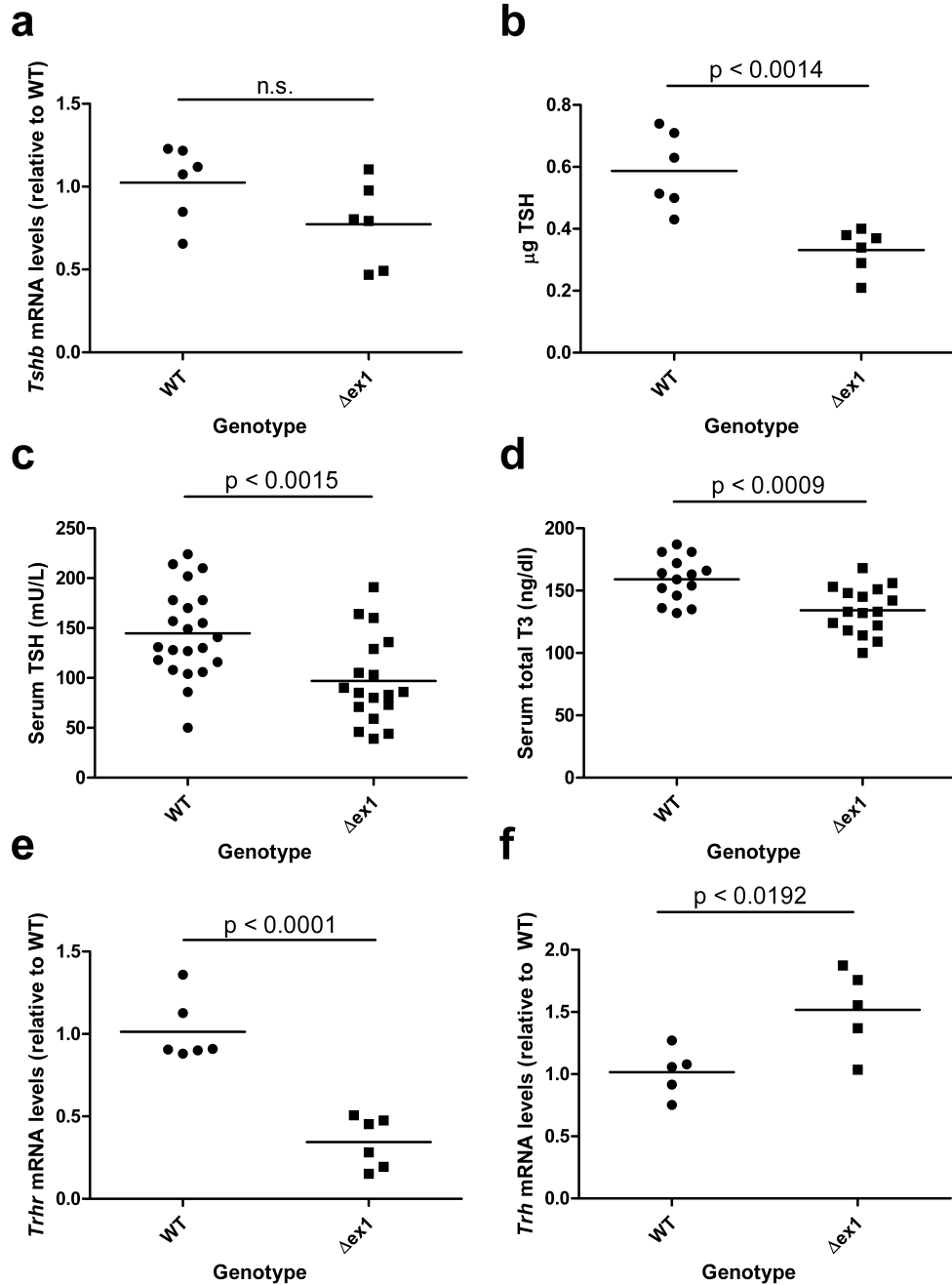
Figure 4. Igsf1^Δex1 mice have several characteristics of central hypothyroidism
(a) Pituitary *Tshb* mRNA levels in 12-week old wild-type and *Igsf1*^Δex1 mice (N=6/genotype). (b) Pituitary TSH content in male wild-type and *Igsf1*^Δex1 mice (N=6/genotype). (c) Serum TSH levels in adult wild-type and *Igsf1*^Δex1 mice(N=6/genotype). (d) Serum total T3 levels in adult wild-type and *Igsf1*^Δex1 mice (N=14-16/genotype). (e) *Trhr* mRNA levels in 12-week old wild-type and *Igsf1*^Δex1 mice (N=6/genotype). (f) *Trh* mRNA levels in 12-week old wild-type and *Igsf1*^Δex1 mice (N=5/genotype). Statistical significance was determined by two-tailed Student's t-test in each panel.

Table 1

Clinical features of cases with IGSF1 variants (all males)

Cases	Nucleotide alteration	Amino-acid alteration	Origin	Age at diagnosis of central hypothyroidism	Central hypothyroidism[1]	PRL deficiency[2]	Age (yrs)[3]	R/L testicular vol. (ml) [reference][4]
A-III.11	c.2137_2163del	p.Ala713_Lys721 del	NL	3 wks	+	−	17.64	21/20 [7.3-16]
A-III.7			NL	7.3 yrs	+	+	21.36	30/26 [8.5-18.3]
A-II.4			NL	51.5 yrs	+	−	52.41	32/29 [8.5-18.3]
A-I.4			NL	74.1 yrs	+	−	67.70	4/large [8.5-18.3][5]
B-III.7	c.2931G>A	p.Trp977X	UK	4 wks	+	+	10.52	1.1/1.0 [0.55-1.87]
B-III.8			UK	7 wks	++	−	7.95	1.8/1.5 [0.45-0.92]
B-II.11			UK	43.3 yrs	+	+	43.30	68/37 [8.5-18.3]
B-I.4			UK	65.9 yrs	+	−	66.37	19.6/21.6 [8.5-18.3]
C-III.1	c.2248del	p.Glu750LysfsX28	NL	3 wks	+	+	16.60	18/18 [7.8-16.2]6
D-III.3	c.2248del	p.Glu750LysfsX28	NL	3 wks	++	+	10.46	1/1 [0.55-1.87]
D-III.4			NL	1 wk	+	+	3.79	0.8/0.8 [0.32-0.70]
D-I.3			NL	61 yrs	+	−	62.75	21/16 [8.5-18.3]
E-IV.1	126kb deletion[7]		NL	2 wks	+	+	20.57	>>18.3 [8.5-18.3][6]
E-IV.3			NL	2.5 wks	+	−	22.37	34/25.5 [8.5-18.3]
F-IV.1	328kb deletion[8]		NL	3 wks	+	−	12.70	12.2/8.4 [4-13]
F-IV.2			NL	3 wks	+	+	9.44	1/0.9 [0.5-1.35]
F-II.8			NL	57.5 yrs	+	+	58.24	44.6/48.2 [8.5-18.3]
G-III.1	c.2588C>T	p.Ser863Phe	NL	5 wks	+	+	27.52	11.8/38 [8.5-18.3]
G-III.3			NL	2.5 wks	+	+	23.08	25.5/25.4 [8.5-18.3]
G-I.1			NL	63 yrs	+	+	87.49	>>18.3 [8.5-18.3][6]
H-III.2	c.2839T>C	p.Cys947Arg	NL	6.5 yrs	+	+	18.36	22.7/22.7 [8.5-18.3]
H-III.3			NL	3 wks	+	+	15.93	21.7/21.7 [6.7-15.3]
I-III.2	c.3518G>A	p.Trp1173X	NL	14.1 yrs	+	+	16.69	19/17 [8-16.5]
J-III.1	c.3596-3597insT	p.Glu1200fsX3	IT	3 wks	+	+	3.26	0.75/0.80 [0.32-0.70]

Cases	Nucleotide alteration	Amino-acid alteration	Origin	Age at diagnosis of central hypothyroidism	Central hypothyroidism [1]	PRL deficiency [2]	Age (yrs) [3]	R/L testicular vol. (ml) [reference] [4]
J-III.2			IT	2 wks	+	+	0.16	0.58/0.58 [0.30-0.65] [6]
K-II.3	c.2309G>A	p.Ser770Asn	IT	10.6 yrs	+	+	26.54	21.5/21.4 [8.5-18.3]

[1] + indicates serum FT4 50-99% of the lower limit of normal; ++ indicates <50% of lower limit of normal. In all cases serum TSH was normal.

[2] + indicates serum prolactin < lower limit of normal.

[3] Age at sonographic determination of testicular volume.

[4] Sonographic testicular volume right/left (ml) in comparison to age references [8]

[5] Self-reported unilateral macroorchidism, until the enlarged testis was removed after testicular torsion leading to complete infarction at 74 years; the size of the infarcted testis was 343 ml. The remaining testis was small and soft, with deficient testosterone secretion. Testosterone treatment was started at 76 years.

[6] Estimated based on Prader orchidometer (30 ml by Prader orchidometer = 18.3 ml by ultrasound, 2 ml by Prader orchidometer = 0.58 ml by ultrasound [8]).

[7] Arr Xq26.1q26.2(130.386.267-130.512.002)x1 (hg19)

[8] Arr Xq26.1q26.2(130.310.905- 130.639.353)x1 (hg19)

Table 2

Clinical and laboratory data

Case	Age (yrs)	T4 R/	Height SDS[1]	BMI SDS[2]	LH (IU/L)[3]	FSH (IU/L)[3]	LHmax to GnRH	FSHmax to GnRH	T (nmol/L)[3]	Inhibin B (ng/L)[4]	AMH (µg/L)[4]
A-III.11	17.64	+	-0.2	2.6	1.0[5]	3.8[5]	13.1[5]	8.3[5]	1.1 [1.0-16.3][5]	328 [80-300]	16.6 [10-100]
A-III.7	21.36	+	1.0	1.8	3.6	10.6	18.9	18.7	17.6	237	12.4
A-II.4	52.41	–	0.3	2.5	3	17.9	–	–	12.7	199	5.6
A-I.4	86.70	+	0.2	4.3	15[6]	54[6]	–	–	4.8[6]	<10	0.24
B-III.7	10.52	+	1.4	1.9	<1	1.2	–	–	<0.3 [0.2-1.2]	91 [20-300]	97 [30-200]
B-III.8	7.95	+	0.7	2.2	<1	3.1	–	–	<0.3 [0.070.31]	111 [20-120]	97 [100-400]
B-II.11	43.29	+	-0.2	3.3	3.7	10.1	29.6	22.6	16.8	279	7.4
B-I.4	66.37	–	-0.6	2.1	2.1	11.0	30.8	36.7	18.7	192	6.2
C-III.1	17.39	+	0.6	2.0	3.2	3.8	–	–	17.2	299 [80-300]	14.0 [10-100]
D-III.3	10.46	+	-0.4	0.8	<1	2.9	1.4	15.4	<0.3 [0.2-1.2]	97 [20-300]	35.4 [30-200]
D-III.4	3.79	+	-0.6	1.1	<1	1.1	–	–	<0.3 [0.070.28]	192 [20-100]	207 [1001000]
D-I.3	62.75	–	-1.2	7.7	2.5	6.3	22.2	14.1	10.1[7]	152	1.4
E-IV.1	20.57	+	-0.5	1.0	1.3	4.8	–	–	13.4	454	–
E-IV.3	22.37	+	1.0	2.8	4.4	6.0	38.7	13.1	19	317	26.9
F-IV.1	12.70	+	1.1	2.2	<1	2.5	9.4	5.1	4.2 [0.4-9.5]	533 [80-300]	50.2 [10-100]
F-IV.2	9.44	+	1.3	2.9	<1	<1	–	–	1.7 [0.14-0.66]	92 [20-120]	134 [100-400]
F-II.8	58.24	–	1.4	8.6	3.5	8.6	21	15.3	5.1[7]	141	5.2
G-III.1	27.52	+	-0.5	3.5	3.8	3.9	25	7.8	24	249	4.5
G-III.3	23.08	+	0.6	1.5	3.2	6.9	31.8	17.2	24	249	5.9
G-I.1	87.49	+	-1.9	2.5	–	–	–	–	–	–	–
H-III.2	20.52	+	-2.5	2.6	2.7	4.6	–	–	11.8	338	7.2
H-III.3	18.09	+	-0.7	1.7	6.5	10.7	–	–	16.9	265	6.4
I-III.2	16.69	+	-0.6	2.0	<1	3.9	14.3	9.8	9.5 [1.7-27.8]	257 [80-300]	3.3 [10-100]
J-III.1	3.26	+	0.1	1.0	–	–	–	–	–	–	–
J-III.2	0.16	+	0.5	1.4	–	–	–	–	–	–	–

Case	Age (yrs)	T4 R/	Height SDS[1]	BMI SDS[2]	LH (IU/L)[3]	FSH (IU/L)[3]	LHmax to GnRH	FSHmax to GnRH	T (nmol/L)[3]	Inhibin B (ng/L)[4]	AMH (µg/L)[4]
K-II.3	26.54	+	0.1	1.1	1.6	3.4	36.5	10.7	11.5	284	10.8

Abbreviations: T4 R/ = L-thyroxine treatment. T = testosterone. AMH = anti-Müllerian hormone.

[1] Height is expressed as standard deviation score (SDS) for national reference data for the Netherlands[23], United Kingdom[24] and Italy[25]. Median height 0.1 SDS.

[2] BMI is expressed as SDS for Dutch references obtained in 1980[26]. Median BMI 2.1 SDS.

[3] Reference ranges for males > 17 years: testosterone 11-35 nmol/L, LH <0.1-15 U/L, FSH <0.1-10 U/L. Reference ranges for testosterone in boys (P10-P90) according to Von Schnakerburg et al[27].

[4] Reference range for males >18 years: inhibin-B 150-400 ng/L, AMH 5-30 µg/L. Reference ranges for younger age groups are indicated (Dr.Y.de Rijke and Prof.F.J.de Jong, ErasmusMC, Rotterdam Netherlands).

[5] GnRH test and testosterone performed at 15.19 years, before start of testosterone substitution therapy.

[6] LH, FSH and testosterone before the start of testosterone substitution therapy (at 76 years).

[7] Because of low plasma sex hormone binding globulin (SHBG), free androgen index [FAI, (100 × testosterone)/SHBG] was high in D-I.3 (67, age reference 18-54) and normal in F-II.8 (46, age reference 30-53).