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

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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

Methods and associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession number

The IGSF1 reference sequence is deposited in the NCBI RefSeq database under the accession code NM_001170961.1.

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Author contributions

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.

Competing financial interests

The authors declare no competing financial interests.

Figure legends:

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$^{\Delta ex1}$mice have several characteristics of central hypothyroidism.* (a) Pituitary *Tshb* mRNA levels in 12-week old wild-type and *Igsf1*$^{\Delta ex1}$ mice (N=6/genotype). (b) Pituitary TSH content in male wild-type and *Igsf1*$^{\Delta ex1}$ mice (N=6/genotype). (c) Serum TSH levels in adult wild-type and *Igsf1*$^{\Delta ex1}$ mice (N=6/genotype). (d) Serum total T3 levels in adult wild-type and *Igsf1*$^{\Delta ex1}$ mice (N=14-16/genotype). (e) *Trhr* mRNA levels in 12-week old wild-type and *Igsf1*$^{\Delta ex1}$ mice (N=6/genotype). (f) *Trh* mRNA levels in 12-week old wild-type and *Igsf1*$^{\Delta ex1}$ 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_Lys721del	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]
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 [0.07-0.31]	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 [0.07-0.28]	192 [20-100]	207 [100-1000]
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	-	-	-	-	-	-	-
K-II.3	26.54	+	0.1	1.1	1.6	3.4	36.5	10.7	11.5	284	10.8

[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 x testosterone)/SHBG] was high in D-I.3 (67, age reference 18-54) and normal in F-II.8 (46, age reference 30-53).

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

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, 3x10 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 pmol 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.

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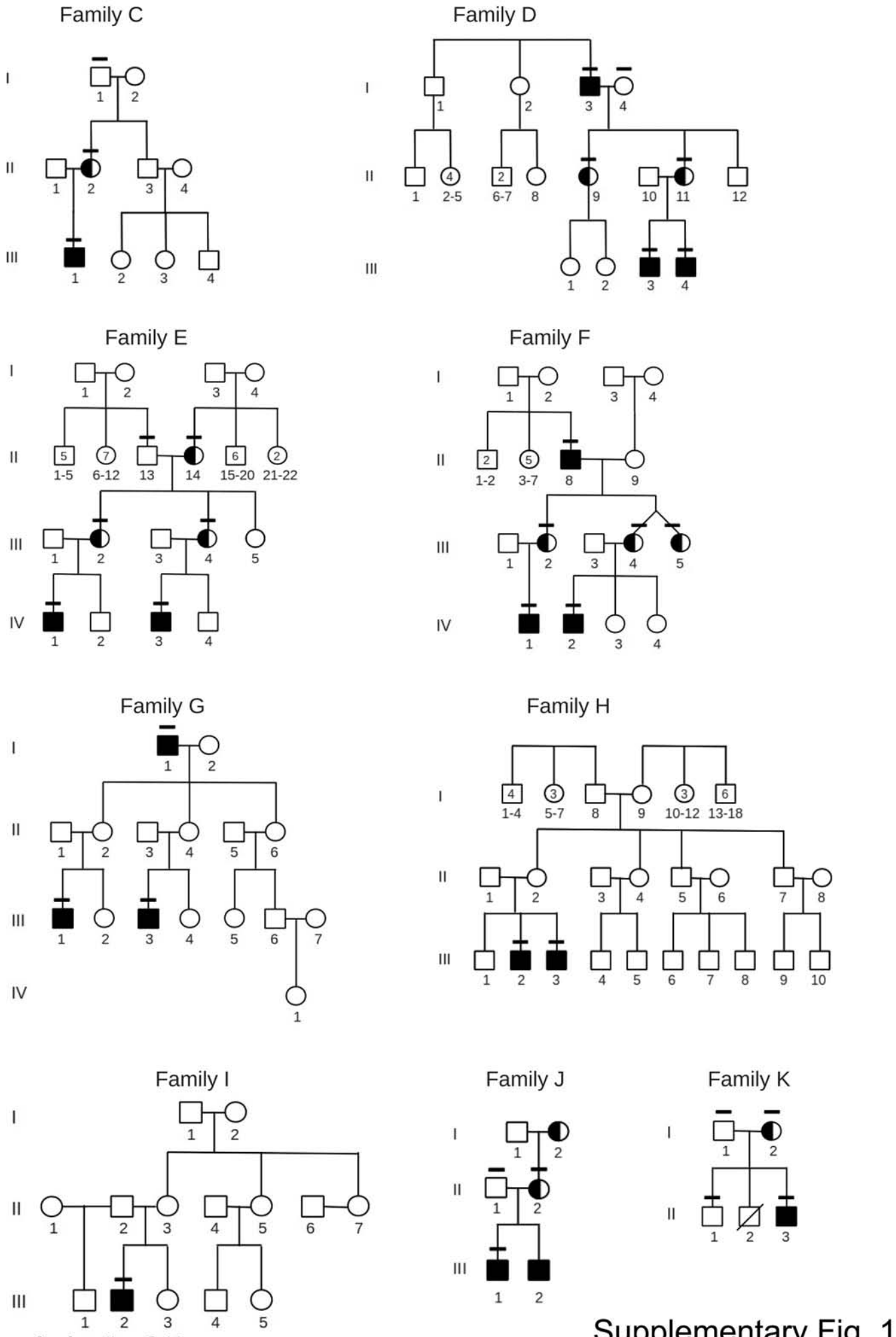
Loss-of-function mutations in IGSF1 cause an X-linked syndrome of central hypothyroidism and testicular enlargement.

Yu Sun, Beata Bak, Nadia Schoenmakers, A.S. Paul van Trotsenburg, Wilma Oostdijk, Peter Voshol, Emma Cambridge, Jacqueline K. White, Paul le Tissier, S. Neda Mousavy Gharavy, Juan P. Martinez-Barbera, Wilhelmina H. Stokvis-Brantsma, Thomas Vulsma, Marlies J. Kempers, Luca Persani, Irene Campi, Marco Bonomi, Paolo Beck-Peccoz, Hongdong Zhu, Timothy M.E. Davis, Anita C.S. Hokken-Koelega, Daria Gorbenko Del Blanco, Jayanti J. Rangasami , Claudia A.L. Ruivenkamp, Jeroen F.J. Laros, Marjolein Kriek, Sarina G. Kant, Cathy A.J. Bosch,Nienke R. Biermasz, Natasha M. Appelman-Dijkstra, Eleonora P. Corssmit, Guido C.J.Hovens, Alberto M. Pereira, Johan T. den Dunnen, Michael G. Wade, Martijn H. Breuning, Raoul C. Hennekam, Krishna Chatterjee, Mehul T. Dattani, Jan M. Wit, Daniel J. Bernard

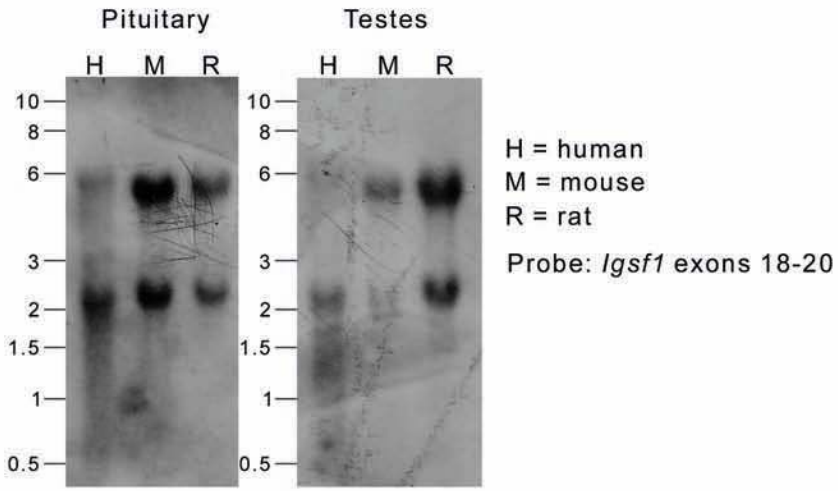
Supplementary information



Pedigrees for families C-K

Supplementary Fig. 1

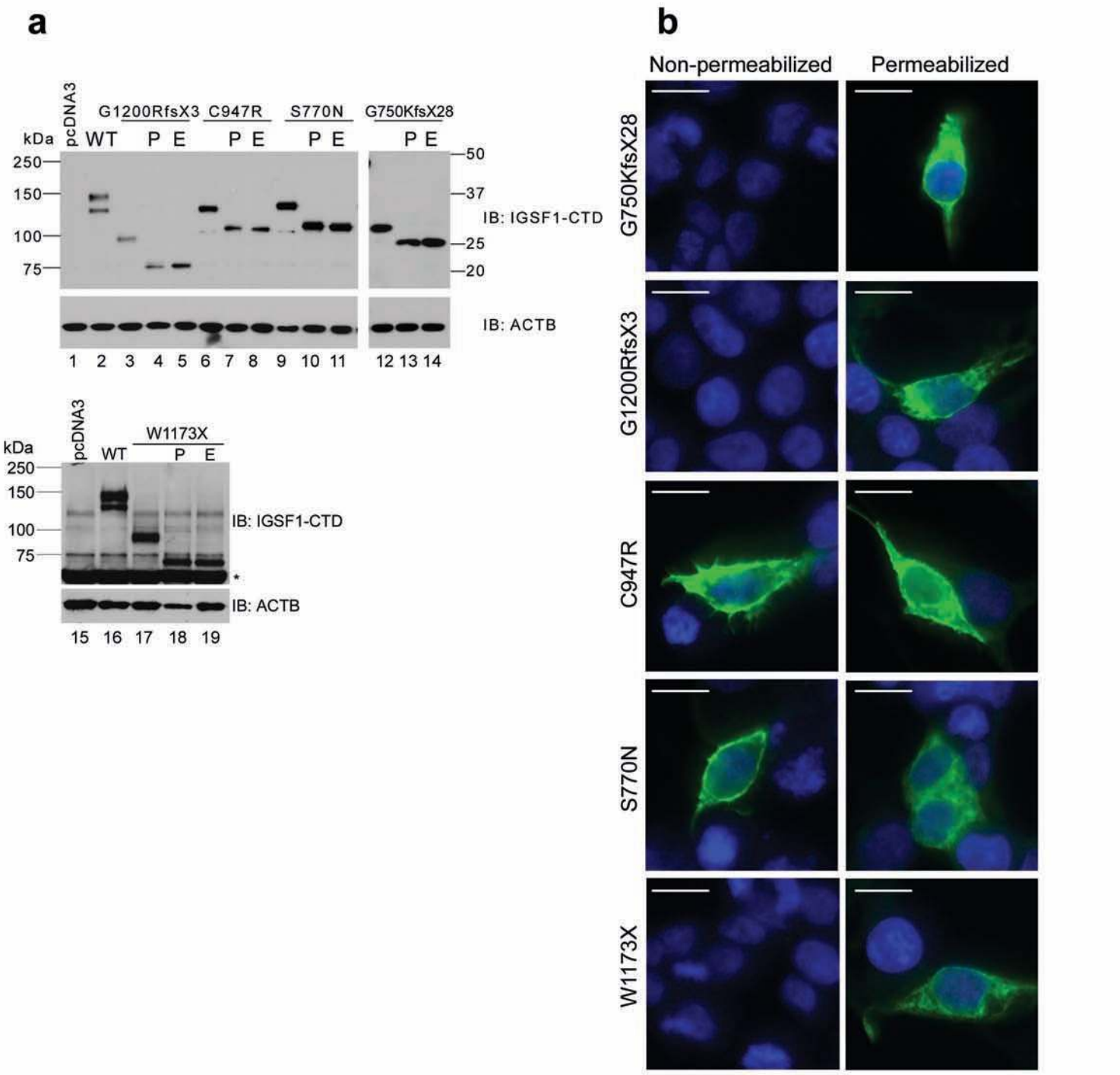
Nature Genetics: doi:10.1038/ng.2453



Northern blot comparing *IGSF1/Igsf1* expression
Northern blot comparing *IGSF1/Igsf1* expression in adult human (H), murine (M), and rat (R) pituitary and testes. Probe was directed against the 3' end of murine *Igsf1*.

Supplementary Fig. 2

Nature Genetics: doi:10.1038/ng.2453

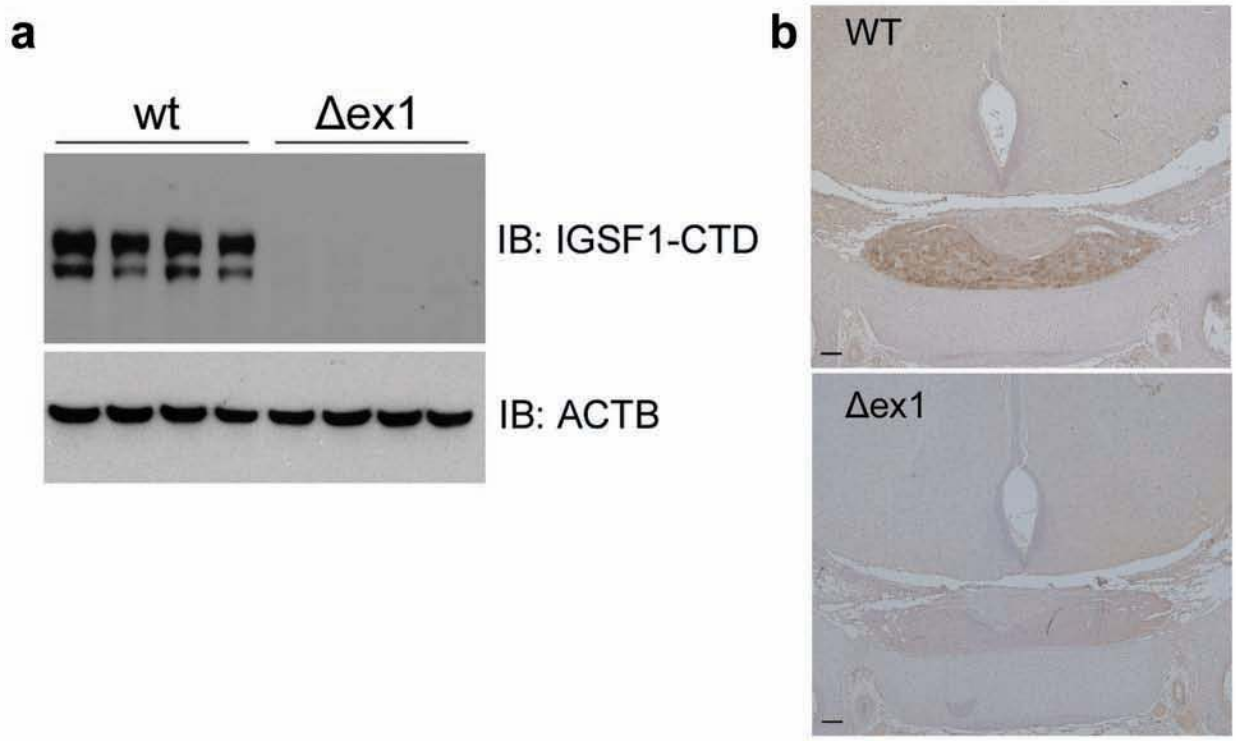


Expression of additional IGSF1 mutants

(a) HEK293 cells were transfected with 200 ng of pcDNA3 (empty vector), wild-type hIGSF1 or mutant (Glu750LysfsX28, Glu1200ArgfsX3, Cys947Arg, Ser770Asn and Trp1173X) constructs. Protein lysates were deglycosylated with either PNGaseF (P, cleaves all N-linked glycans) or EndoH (E, cleaves high-mannose glycans only), run on SDS-PAGE, and subjected to immunoblotting using αIGSF1-CTD antibody. **(b)** HEK293 cells were transfected with 100 ng of pcDNA3 (empty vector), wild-type IGSF1 or mutant (Glu750LysfsX28, Glu1200ArgfsX3, Cys947Arg, Ser770Asn, and Trp1173X) expression constructs. Expression of IGSF1-CTD was analyzed by immunofluorescence using αIGSF1-CTD antibody under non-permeabilizing and permeabilizing conditions. Nuclei are stained with DAPI (blue). Scale bars, 10 μm.

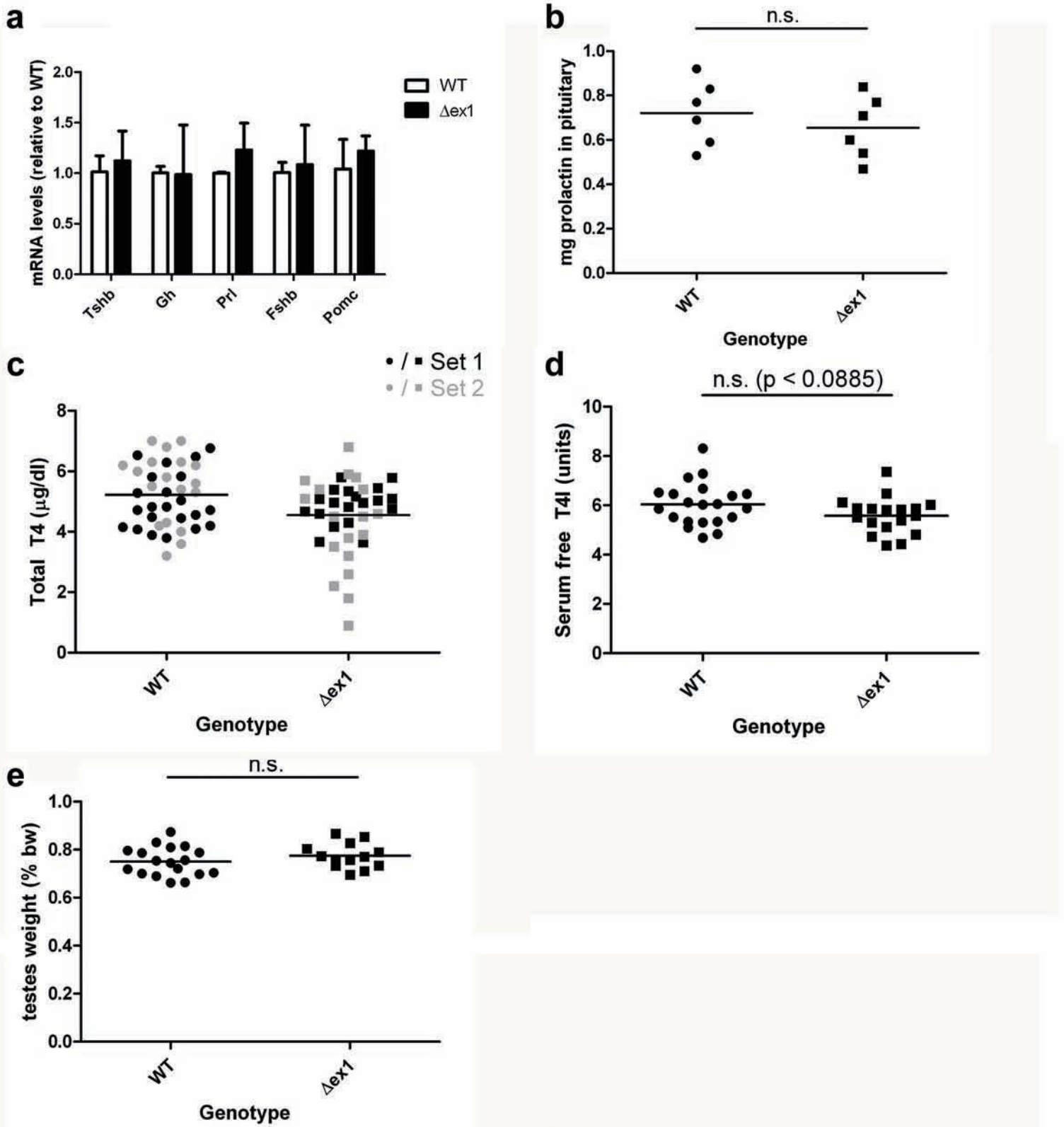
Supplementary Fig. 3

Nature Genetics: doi:10.1038/ng.2453



IGSF1-CTD expression in wild-type vs. *Igsf1*-deficient mice
(a) IGSF1-CTD protein is undetectable in pituitaries of *Igsf1*Δex1mice. Protein lysates from individual pituitaries of adult wild-type and *Igsf1*Δex1male mice were run on SDS-PAGE and subject to western blotting using IGSF1-CTD antiserum. **(b)** Immunohistochemistry for IGSF1 in WT and *Igsf1*Δex1male mice pituitary at embryonic day 18.5. Scale bars, 10 μm.

Supplementary Fig. 4



Hormone expression data and testes size in wild-type vs. *Igsf1*-deficient mice

(a) mRNA levels of anterior pituitary hormone encoding genes do not differ between adult wild-type and *Igsf1*Δex1 male mice. RNA was extracted from pools of 3 pituitaries (2 pools per genotype), reverse-transcribed, and qPCR performed using primers against *Tshb* – thyroid-stimulating hormone β subunit; *Gh* – growth hormone; *Prl* – prolactin; *Fshb* – follicle-stimulating hormone β subunit; *Pomc* – proopiomelanocortin. Data indicate mean±s.e.m., relative to wild-type for each hormone. **(b)** Pituitary prolactin content does not differ between adult male wild-type and *Igsf1*Δex1 mice. **(c)** Serum (set 1) or plasma (set 2) total T4 was measured in two different sets of animals, using two different assays. Samples from each set are indicated in black or gray. Statistics: set 1: n.s. ($p < 0.6133$); set 2: $p < 0.0094$; set 1+ set 2: $p < 0.0117$. **(d)** Serum free T4 was measured in adult wild-type and *Igsf1*Δex1 mice. **(e)** Testes weights (as a percentage of body weight) do not differ between adult male wild-type and *Igsf1*Δex1 mice.

Supplementary Table 1. Overview of exome sequencing data

Patient	*A-III.11*	*A-III.7*	*B-III.7*	*B-III.8*
Total reads	15,039,708	13,712,350	83,312,890	83,457,089
Platform	GAII, Illumina	GAII, Illumina	SOLiD 4, Applied Biosystem	SOLiD 4, Applied Biosystem
Read length	51nt, paired end	51nt, paired end	50nt, single end	50nt, single end
Aligned reads	14,865,896	13,554,229	51,718,911	46,780,793
PCT_aligned_reads	98.84%	98.85%	62.08%	56.05%
Capture kit	SureSelect Human X-exome	SureSelect Human X-exome	SureSelect Human All exon 50MB	SureSelect Human All exon 50MB
PERCENT_DUPLICATION	29.20%	21.10%	44.59%	44.36%
MEAN_BAIT_COVERAGE	76.80	73.83	17.34	15.55
ZERO_CVG_TARGETS_PCT	4.87%	4.95%	5.89%	7.28%
PCT_TARGET_BASES_10X	92.58%	91.85%	64.75%	58.0%

Supplementary Table 2. Thyroid function test results at diagnosis

Case	Age	TSH[1] (mU/L)	Free T4 (% LL)	T3 (% LL)	TSH (mU/L) response to TRH (TSH at baseline; 20 minutes)
A-III.11	3 wks	3.5	69.2	100.0	2.6; 10.4
A-III.7	7.3 yrs	1.6	88.0		0.5; 4.3
A-II.4	52.4 yrs	0.8	94.0	130.8	0.6; 4.7
A-I.4	74 yrs	0.9	90.0	145.4	
B-III.7	4 wks	1.8	79.6		
B-III.8	7 wks	2.6	46.7		
B-II.11	43.3 yrs	1.7	80.0	150.0[3]	1.7; 7.6
B-I.4	65.9 yrs	1.7	91.0	126.9	1.7; 4.5
C-III.1	3 wks	1.4	86.7	97.6	2.3; 7.8
D-III.3	3 wks	4.8	42.5		4.6; 8.4
D-III.4	1 wk	2.9	80.0	87.5	
D-I.3	61 yrs	2.9	85.0	118.2	2.2; 6.6
E-IV.1	2 wks	5.6	75.0	83.3	3.0; 8.4
E-IV.3	2.5 wks	3.7	81.7	100.0	2.8; 9.9
F-IV.1	3 wks	4.2	60.8		
F-IV.2	3 wks	1.5	78.3		
F-II.8	57.5 yrs	3.0	95.0	190.9	2.3; 8.5
G-III.1	5 wks	1.1	84.2		1.1; 4.5
G-III.3	2.5 wks	2.2	66.7	108.3	2.2; 16
G-I.1	63 yrs	1.2	88.3[2]		1.2; 5.7
H-III.2	6.5 yrs	3.6	80.0		
H-III.3	3 wks	6.0	83.3	120.9	6.3; 10.6
I-III.2	14,1 yrs	0.95	89.0		
J-III.1	3 wks	2.5	79.1	88.9[3]	
J-III.2	2 wks	3.2	74.1	93.3[3]	
K-II.3	10.6 yrs	1.2	84.1	107.7[3]	1.2; 5.7
Mean(SD)		**2.6(1.5)**	**79(13)**	**116(28)[4]**	

[1] Reference range for serum TSH: 0.8-6.0 mU/L

[2] Total T4

[3] Free T3

[4] Means (SD) in 1-3 week old infants and older individuals were 98 (12)% and 139(27)% of the lower limit (LL) of the reference range for age and assay, respectively

Supplementary Table 3. TSH, FT4 and FT3 responses to TRH

Case	Age	TSH (mU/L)		FT4 (pmol/L)		ΔFT4(%)	FT3 (pmol/L)		ΔFT3(%)
		0	**peak**	**0**	**peak**		**0**	**peak**	
E-IV.1	2 wk	3.0	8.4	8.4	9.8	16.7			
C-III.1	3 wk	2.3	7.8	9.1	9.6	5.5			
A-III.11	3 wk	2.6	10.4	8.3	9.4	13.3			
B-II.11	43.3 yr	1.7	7.6	7.2	8.0	11.1	4.5	5.1	13.3
A-II.4	52.4 yr	0.6	4.7	8.8	9.8	11.4	2.9	3.9	34.5
F-II.8	58.2 yr	2.3	8.5	8.5	10.2	20.0	3.9	6.0	53.8
D-I.3	62.8 yr	2.2	6.6	7.3	8.0	9.6	3.2	5.6	75.0
B-I.4	65.9 yr	1.7	4.5	9.1	9.9	8.8	4.0	4.8	20.0
B-I-4[3]	66.4 yr	1.3	3.8	6.6	8.7	31.8	4.3	5.2	20.9
Mean		**2.0**	**6.9**	**8.1**	**9.3**	**14.2**[1]	**3.8**	**5.1**	**36.3**[2]
SD		**0.7**	**2.2**	**0.9**	**0.8**	**7.9**	**0.6**	**0.7**	**26.4**

[1]Reference data for FT4 increment: mean (SEM) 23.9 (2.7)%

[2] Reference data for FT3 increment: mean (SEM) 41.8 (4.5)%

[3] In this subject, the TRH test was repeated

Supplementary Table 4. Primers used to amplify and sequence the *IGSF1* gene in patients in families A and C to I (Leiden)

PrimerID_lab	Sequence(5'-3')	Product size(bp)	Covered range NM_001170961.1
IGSF1_1a_F	GCGCTTTTCCAGTTTCAAAG	549	Exon 20 utr
IGSF1_1a_R	GAGCTGGGTAAAGGGGATTC		
IGSF1_1b_F	TTCATGCGCCAGTAAATCAG	581	Exon 20
IGSF1_1b_R	TGTTGTAGCCTTGGGGGTAG		
IGSF1_2_F	CACTCTTCAAGGGCAATGGT	406	Exon 19
IGSF1_2_R	AGAGTGTGGGGCAATACCAG		
IGSF1_3_F	TCCTGATTTGGGATGTCTCC	511	Exon 18
IGSF1_3_R	GTGACTCCCTGGAGATCTGG		
IGSF1_4_F	GGTTTAGGGGGCTTATCTGC	541	Exon 17
IGSF1_4_R	TCAGAGAGCTCGAGGAGAGG		
IGSF1_5_F	CCTGGGGTCTCTGCTCAATA	521	Exon 16
IGSF1_5_R	AACTACCGTGCCCAAGAGG		
IGSF1_6_F	CCATGGCTTAGGGAATGTGT	548	Exon 15
IGSF1_6_R	TGTGAGGGCTGGGTTTATCT		
IGSF1_7_F_2	GTGCATCATGGACTGGTCAC	553	Exon 14
IGSF1_7_R	AGAAGCCTCCGAGAACCAAT		
IGSF1_8_F	CCTGCCTGGCTCTAAGATTG	476	Exon 13
IGSF1_8_R	GTATCCCATCTGGGCAACTG		
IGSF1_9_F	CTCACTCCCAGGAGCAGTGT	492	Exon 12
IGSF1_9_R_2	TTCCTCCAAGCTTCTGCACT		
IGSF1_10_11_F_2	TGCAGAAGCTTGGAGGAAAT	573	Exon 10, exon 11
IGSF1_10_11_R	TGGTAAGGCCCTCAAAAAGA		
IGSF1_12_F_2	GAGACAGGTGGGGTGAGAAA	507	Exon 9
IGSF1_12_R	AGATTGTCCTCACCCCATGA		
IGSF1_13_F	CCTGCTAACTGCCTTTGAGG	510	Exon 8
IGSF1_13_R_2	CCAAGTGGAAGTGGAAAGGA		
IGSF1_14_F	GGAACAATCCTGGGAGACAA	547	Exon 7
IGSF1_14_R_2	TTAAGGAGTGGGAGCAGGAA		
IGSF1_15_F_2	AAACCACCCTTGGAAAAACC	577	Exon 6
IGSF1_15_R	CCAAGGAAGGGATAGGGAAG		
IGSF1_16a_F	TCTCCGCTAGCAAAAAGCTC	549	Exon 5 utr of NM_001170963.1
IGSF1_16a_R	GAATTGGCCACATTTGCTTT		
IGSF1_16b_F	AGGAAGCCAAAGCACAGAAA	594	Exon 5 utr of NM_001170963.1
IGSF1_16b_R	AGCAGCTTCAGCTCATCACA		
IGSF1_16c_F	CAACAAAGCTGTGATGAGCTG	618	Exon 5
IGSF1_16c_R	CTCTCCTCTGCTGCTCCCTA		
IGSF1_17_F	TTGCATGCATTTGTGAAACA	493	Exon 4
IGSF1_17_R	CCCAAGGGAAGACAAAATGA		
IGSF1_18_19_F_2	ACCATGCCATAGGAAACCAA	596	Exon 2, exon 3
IGSF1_18_19_R	GAGAGGAAGCAGCAGGTGAC		
IGSF1_20_21_F	GAGGGTTTGGAGGGAAGACT	562	Exon 1 utr, exon 1 utr NM_001170963.1
IGSF1_20_21_R	CTGAATTTGGCTCCAGCAAC		

Supplementary Table 5. Mutagenesis primers for IGSF1 expression plasmids

Mutation:	Sense Primer:	Antisense Primer:
c.2137_2163del (p.Ala713_Lys721del)	5’-GAGGGAGAGCAAGAACCTGTCCAGCA-3’	5’-CAGCCATCCTTTGCACCGGAGTTGTA-3’
c.2931G>A (p.Trp977X)	5’-ACATTCCCTAAGCCATGATTGTTTGCTGAGCCCAG-3’	5’-CTGGGCTCAGCAAACAATCATGGCTTAGGGAATGT-3’
c.2248del (p.Glu750LysfsX28)	5’-GAGAATGGAGGATAAAGACAAGGCAATTACAGCTGCC-3’	5’-GGCAGCTGTAATTGCCTTGTCTTTATCCTCCATTCTC-3’
c.2588C>T, p.(Ser863Phe)	5’-TATTATGACTTTTCTATCTGGTTTGAGCCCAGCGACC-3’	5’-GGTCGCTGGGCTCAAACCAGATAGAAAAGTCATAATA-3’
c.2839T>C, p.(Cys947Arg)	5’-GGACTCTGGGAACTATAGCCGTATCTACTATGAGACAAC-3’	5’-GTTGTCTCATAGTAGATACGGCTATAGTTCCCAGAGTCC-3’
c.3518G>A, p.(Trp1173X)	5’-ACCCTCTCTGTCAGCCTAGCCCAGCAC-3’	5’-GTGCTGGGCTAGGCTGACAGAGAGGGT-3’
c.3596-3597InsT (p.Glu1200ArgfsX3)	5’-CCAGGTGTTGAATTTGTCCTTAGAACATGATGGAGAAGA-3'	5'-TCTTCTCCATCATGTTCTAAGGACAAATTCAACACCTGG-3'
c.2309G>A, p.(Ser770Asn)	5'-GTGGTCTGAGCCCAATGAGCCGCTGGA-3'	5'-TCCAGCGGCTCATTGGGCTCAGACCAC-3'

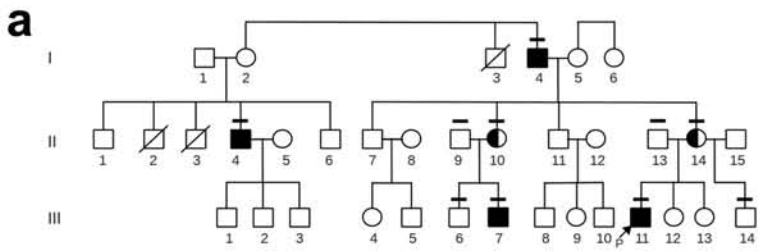
Supplementary Table 6. Primers used for quantitative RT-PCR in mouse tissues

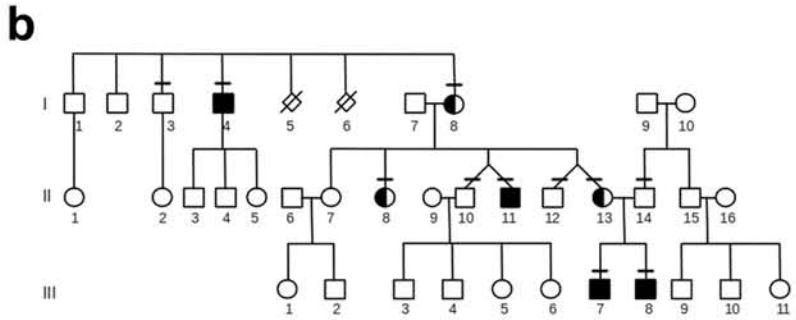
Gene:	Sense Primer:	Antisense Primer:
Tshb	5'-gaacggtggaaataccagga-3'	5'-agaaagactgcggcttggtgca-3'
Trhr	5'-ctccccaacataaccgacag-3'	5'-gcagagaaactgggctttga-3'
Fshb	5'-gtgcgggctactgctacact-3'	5'-caggcaatcttacggtctcg-3'
Gh	5'-acctcggaccgtgtctatga-3'	5'-gcagcccatagtttttgagc-3'
Prl	5'-aagcagcttcttgagggagtt-3'	5'-tgttgcgcaaagacaagatt-3'
Pomc	5'-ccaggaacagcagcagtg-3'	5'-acgttggggtacaccttcac-3'
Trh	5'- gaaagacctccagcgtgtg-3'	5'-tcttcggcttcaacgtcttc-3'
Rpl19	5'-cgggaatccaagaggattga-3'	5'-ttcagcttgtggatgtgctc-3'

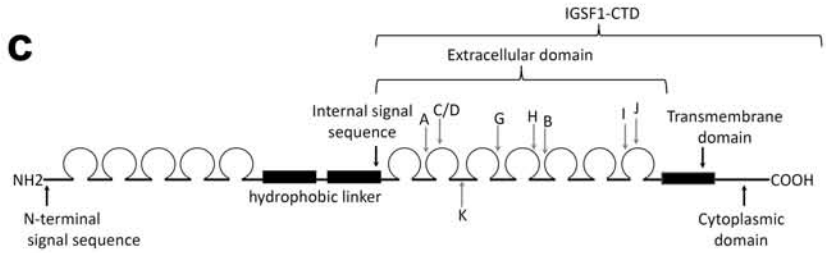
Supplementary Note

After *IGSF1* mutations were found in families A and B, all male patients with central hypothyroidism in Leiden, Amsterdam, Rotterdam and Milan of unknown genetic origin were screened for *IGSF1* variants, leading to the discovery of *IGSF1* mutations in families C through K. At a later stage, full clinical, laboratory and sonographic assessment took place. All participants provided written informed consent.

Four patients showed evidence of growth hormone (GH) insufficiency. Patient A-III.7 was referred for growth failure (height deviation from +2.6 SDS at birth down to -1.4 SDS at 7.3 years). Bone age was three years delayed, serum IGF-I was -1.0 SDS, and GH peaks in clonidine and arginine provocation tests (after correction of hypothyroidism) were 16.9 and 13.1 mU/L (5.6 and 4.4 μg/L for current standard), respectively. MRI of the brain and pituitary was normal. GH treatment was given from 8.8 to 17.5 years, which led to excellent catch-up growth (from a height of -1.4 to +1.3 SDS), and adult height was even taller than midparental height SDS. After discontinuation of GH treatment, serum IGF-I was between -1.0 and 0 SDS and the insulin tolerance test showed a normal response of 28.7 mU/L (9.6 μg/L). H-III.3 had GH peaks of 9.5 mU/L (3.2 μg/L) and 13 mU/L (4.3 μg/L) in clonidine and arginine tests and serum IGF-I was -2.0 SDS at a height of -2.1 SDS. GH treatment from 7.5 years onward led to a first year growth velocity of 8.1 cm/year and an adult height of -1.6 SDS. Brain MRI was normal but the anterior pituitary was relatively small, albeit within the normal range. After GH discontinuation, serum IGF-I was normal (0 SDS) and ATT-GHRH test showed a normal GH peak of 28 mU/L (7.3 μg/L). Patient I-III.2 was referred for short stature (age 14.1 years, height SDS -2.3 SDS), and had GH peaks after arginine and L-dopa/propranolol tests (after testosterone priming) of 11.6 and 15 mU/L (3.9 and 5 μg/L) and a serum IGF-I of -1.5 SDS. His brain MRI was normal and he responded well to GH treatment (10.9 cm/yr in the first year). Patient K-II.3 had a normal GH peak to clonidine stimulation (11 μg/L) at 11.6 years, but low spontaneous GH secretion in a 12-hour nocturnal profile (average 2.26 μg/L, reference >3.5 μg/L). Serum IGF-I was -0.9 SDS and his brain MRI showed a small pituitary stalk lesion. GH treatment led to adequate catch-up growth and normal adult height (0 SDS, 8 cm taller than midparental height SDS). After discontinuation of GH treatment, serum IGF-I, the 12-hour nocturnal profile (average GH level 4.4 μg/L) and the GH peak after GHRH and arginine (21.4 μg/L) were normal.

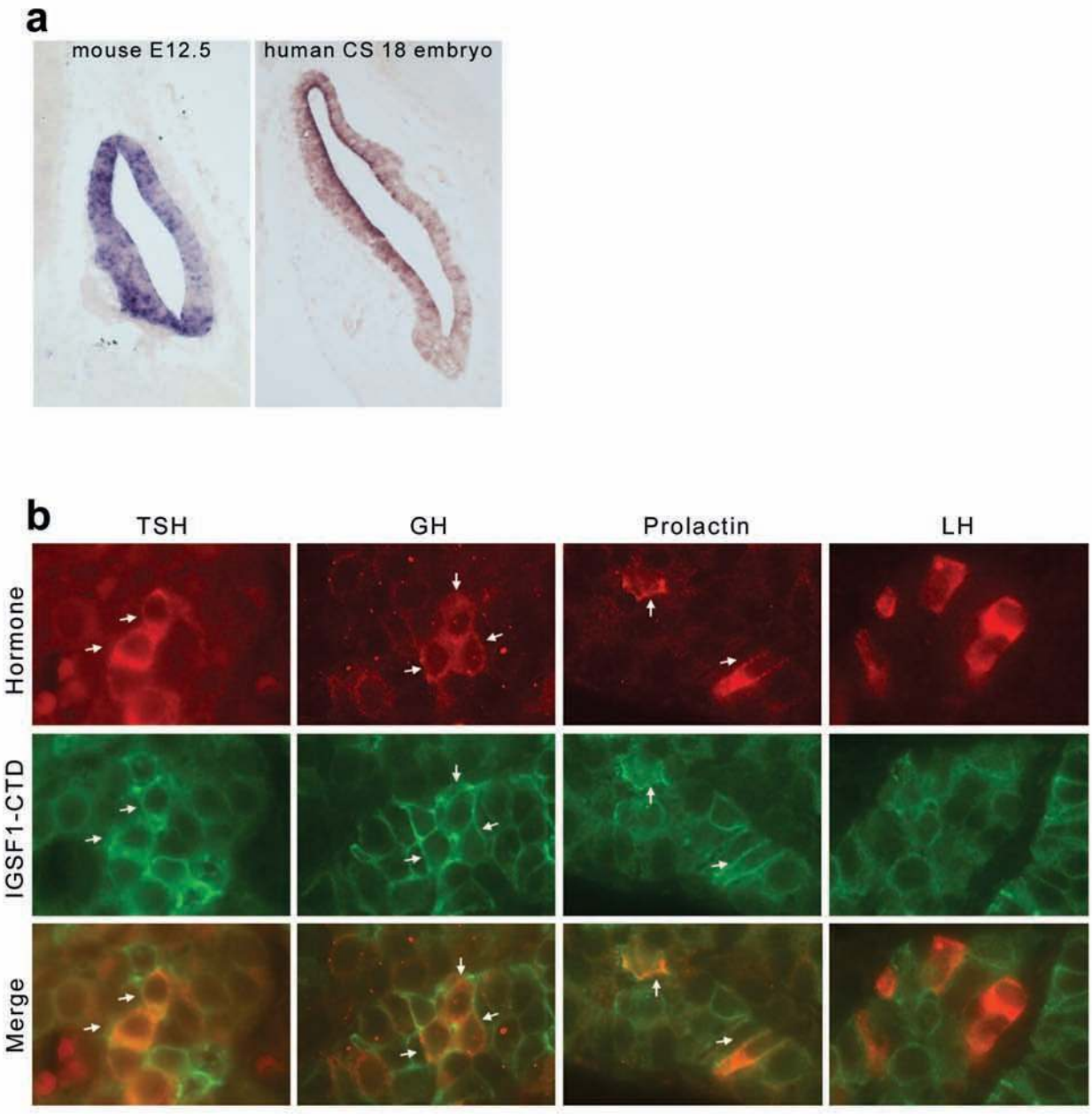






- Family A: c.2137_2163del, p.(Ala713_Lys721del)
- Family B: c.2931G>A, p.(Trp977X)
- Family C & D: c.2248del, p.(Glu750LysfsX28)
- Family E: 126kb deletion, arr Xq26.1q26.2(130.386.267-130.512.002)x1 (hg19)
- Family F: 328kb deletion, arr Xq26.1q26.2(130.310.905-130.639.353)x1 (hg19)
- Family G: c.2588C>T, p.(Ser863Phe)
- Family H: c.2839T>C, p.(Cys947Arg)
- Family I: c.3518G>A, p.(Trp1173X)
- Family J: c.3596-3597 insT, p.(Glu1200ArgfsX3)
- Family K: c.2309G>A, p.(Ser770Asn)

Fig. 1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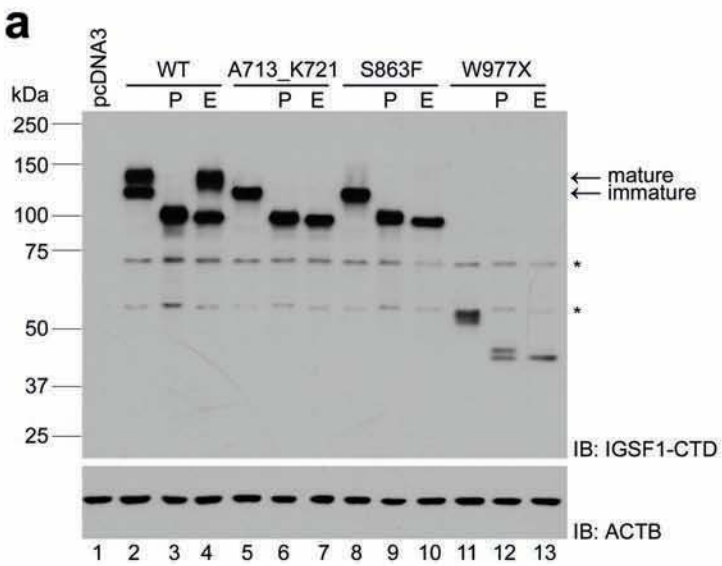
Hormone
IGSF1-CTD
Merge

Fig. 2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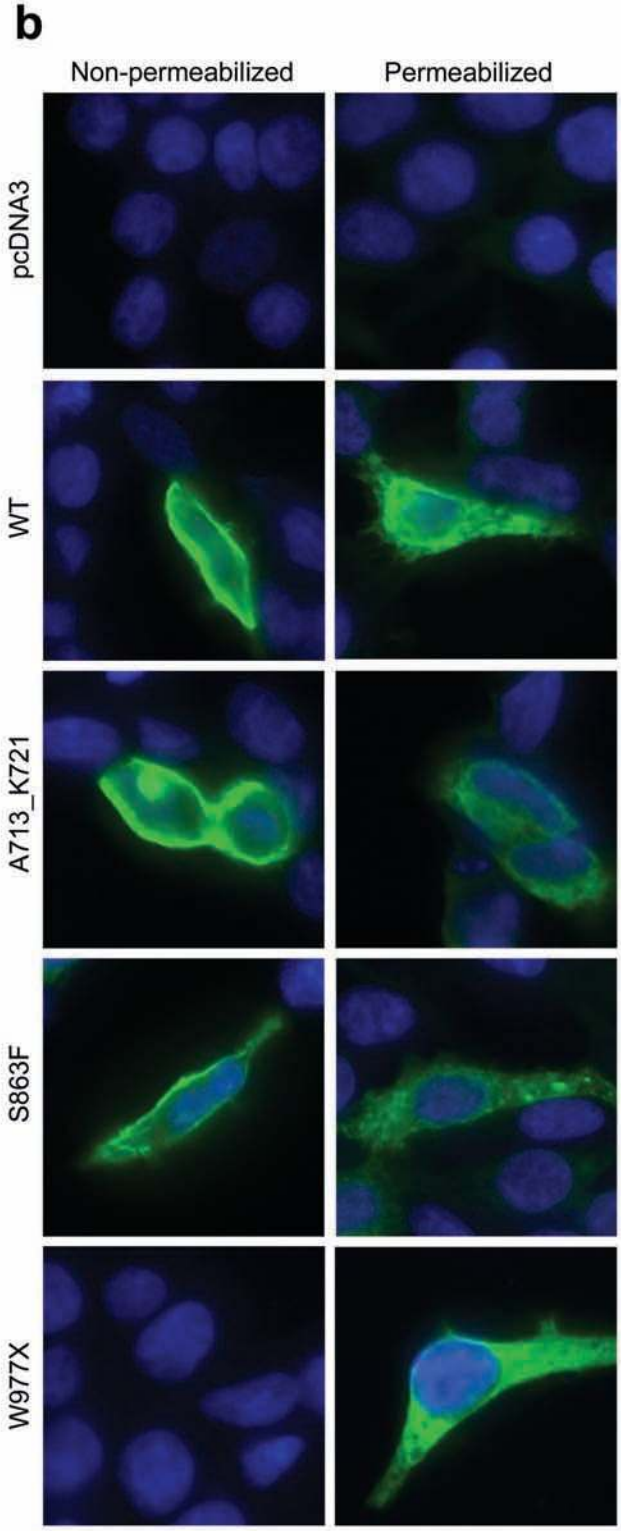
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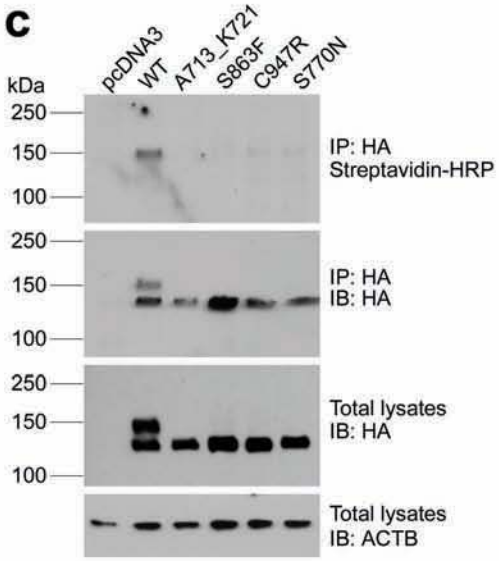
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IB: HA
Total lysates
IB: ACTB

Fig. 3

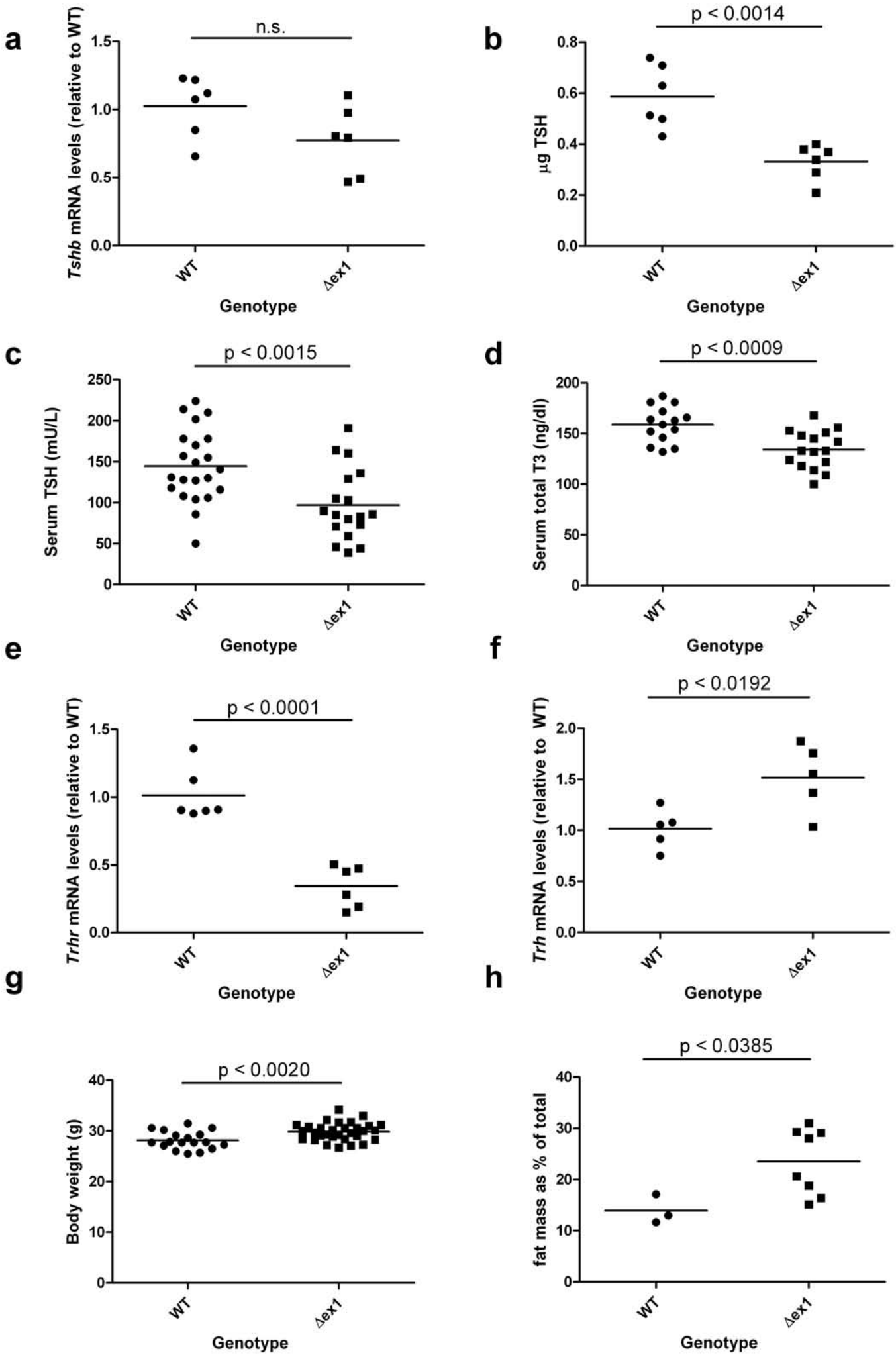
a
n.s.
Tshb mRNA levels (relative to WT)
1.5
1.0
0.5
0.0
WT
Δex1
Genotype
b
p < 0.0014
μg TSH
0.8
0.6
0.4
0.2
0.0
WT
Δex1
Genotype
c
p < 0.0015
Serum TSH (mU/L)
250
200
150
100
50
0
WT
Δex1
Genotype
d
p < 0.0009
Serum total T3 (ng/dl)
200
150
100
50
0
WT
Δex1
Genotype
e
p < 0.0001
Trhr mRNA levels (relative to WT)
1.5
1.0
0.5
0.0
WT
Δex1
Genotype
f
p < 0.0192
Trh mRNA levels (relative to WT)
2.0
1.5
1.0
0.5
0.0
WT
Δex1
Genotype
g
p < 0.0020
Body weight (g)
40
30
20
10
0
WT
Δex1
Genotype
h
p < 0.0385
fat mass as % of total
40
30
20
10
0
WT
Δex1
Genotype

Fig. 4