

The secret life of IGSF1: from consternation to revelation

By

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Abstract

Immunoglobulin superfamily, member 1 (IGSF1, formerly known as InhBP/p120) is a protein of unknown function, highly expressed in the pituitary gland. In this thesis, I characterized: 1) co- and post-translational processing of IGSF1, 2) expression of IGSF1 in several tissues, and 3) the link between IGSF1 and the hypothalamic-pituitary-thyroid (HPT) axis.

The *IGSF1/Igsf1* gene is located on the X chromosome, and multiple mRNA isoforms have been identified in both humans and mice. The canonical full-length protein is encoded by human mRNA isoforms *IGSF1-1*, *IGSF1-3*, and *IGSF1-4*, and murine *Igsf1-1*. Along with our collaborators, I demonstrated that the canonical full-length protein is co-translationally cleaved at an internal signal peptide into N-terminal (NTD) and C-terminal domains (CTD), and that N-linked glycosylation is important for trafficking of the CTD to the cell surface. I then investigated expression of IGSF1-CTD in the pituitary gland and several other tissues. Using a combination of approaches, I demonstrated *Igsf1*/IGSF1-CTD expression in anterior pituitary thyrotropes, somatotropes, and lactotropes, as well as in the murine hypothalamus and fetal liver. I also characterized the relative abundance of *Igsf1* isoforms in the pituitary and hypothalamus, and the glycosylation patterns of IGSF1-CTD in different tissues.

Finally, I sought to determine the function of IGSF1. *Igsf1*-deficient mice were previously generated by ablation of non-coding exon 1 (*Igsf1*^{Δex1}). The mice were reported to be overtly normal, although analyses were largely limited to reproductive function, as IGSF1 had been predicted to play a role in the regulation of follicle-stimulating hormone synthesis. Our clinical collaborators identified mutations in *IGSF1* in several male patients with central hypothyroidism. I showed that the disease-associated mutations lead to intracellular retention of the IGSF1-CTD protein, consistent with IGSF1 loss-of-function. Further, I analyzed HPT axis function of *Igsf1*-deficient male mice, and found that they phenocopy several features of the human disorder, thus confirming that loss of IGSF1 leads to central hypothyroidism. In addition, the mice have

decreased pituitary thyroid-stimulating hormone (TSH) content and thyrotropin-releasing hormone receptor (*Trhr*) expression, suggestive of impaired TRH signaling.

Overall, my work contributes to our understanding of IGSF1 expression, and describes a potential function for IGSF1 in regulating the HPT axis. Delineating molecular mechanisms of how IGSF1 acts in the pituitary gland to affect endocrine axes will contribute to the ongoing search for the function of this mysterious protein.

Résumé

Le membre 1 de la superfamille des immunoglobulines (IGSF1, anciennement connu sous le nom InhBP/p120) est une protéine ayant une fonction inconnue, fortement exprimé dans la glande pituitaire. Dans cette thèse, j'ai caractérisé: 1) les processus co-et post-traductionnels de IGSF1, 2) l'expression de IGSF1 dans plusieurs tissus, et 3) le lien entre IGSF1 et l'axe hypothalamo-hypophyso-thyroïdien (HPT).

Le gène *IGSF1/Igsf1* est situé sur le chromosome X, et de multiples isoformes d'ARNm ont été identifiés chez les humains et les souris. La protéine canonique complète est codée par l'ARNm des isoformes *IGSF1-1*, *IGSF1-3*, et *IGSF1-4* (homme), et *Igsf1-1* (murine). Avec nos collaborateurs, j'ai démontré que la protéine canonique est clivée durant sa traduction en deux parts: le domaine N-terminal (NTD) et le domaine C-terminal (CTD) à cause d'un peptide signal interne, et que la N-glycosylation est importante pour le transport du CTD à la surface de la cellule. J'ai ensuite étudié l'expression d'IGSF1-CTD dans la glande pituitaire et de plusieurs autres tissus. En utilisant une combinaison d'approches, j'ai démontré l'expression d'*Igsf1*/IGSF1-CTD dans les thyrotropes, somatotropes, et lactotropes de l'hypophyse antérieure, ainsi que dans l'hypothalamus murin et le foie fœtal. J'ai également caractérisé l'abondance relative des isoformes *Igsf1* dans l'hypophyse et l'hypothalamus, et les motifs de glycosylation des IGSF1-CTD dans différents tissus.

Enfin, j'ai cherché à déterminer la fonction d'IGSF1. Des souris déficientes en *Igsf1* ont été précédemment générées par l'ablation de l'exon 1 non-codant (*Igsf1*^{Δex1}). Les souris ont été ouvertement normales, même si les analyses ont été largement limitées à la fonction de reproduction, comme cela avait été prédit IGSF1 à jouer un rôle dans la régulation de la synthèse de l'hormone folliculo-stimulante. Nos collaborateurs cliniciens ont identifiés des mutations dans *IGSF1* dans plusieurs patients de sexe masculin atteints d'hypothyroïdie centrale. J'ai démontré que les mutations associées à la maladie causent la rétention intracellulaire de la protéine IGSF1-CTD, menant à une perte de fonction d'IGSF1. De plus, j'ai analysé la fonction de l'axe HPT des souris males déficientes en

Igsf1, et j'ai constaté qu'ils phénotypent plusieurs traits de la maladie humaine, confirmant ainsi la perte de IGSF1 conduisant à l'hypothyroïdie centrale. En outre, les souris ont une diminution de thyrotropine (TSH) dans l'hypophyse et une diminution des récepteurs thyroïdolibérine (*Trhr*) suggérant une détérioration de la signalisation de thyroïdolibérine (TRH).

Dans l'ensemble, mon travail contribue à notre compréhension de l'expression d'IGSF1, et décrit une fonction potentielle pour IGSF1 dans la régulation de l'axe HPT. La recherche des mécanismes moléculaires de la façon dont IGSF1 affecte les axes endocriniens dans l'hypophyse contribuera à la recherche permanente de la fonction de cette protéine mystérieuse.

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Contributions of authors

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Chapter 2: Proteolytic processing of IGSF1

Portions of this chapter, including figures 2.2B and 2.2C, were published in: Robakis T, **Bak B**, Lin SH, Bernard DJ, Scheiffele P (2008). An internal signal sequence directs intramembrane proteolysis of a cellular immunoglobulin domain protein. *J Biol Chem.* 283(52):36369-76.

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Chapter 3: Expression of *Igsf1*/IGSF1 in murine tissues

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All figures presented in this chapter were generated by B. Bak. The published paper is included as Appendix B.

Chapter 5: Loss-of-function mutations in *IGSF1* cause an X-linked syndrome of central hypothyroidism

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Abbreviations

4xY: IGSF1 internal cleavage site mutant with four tyrosines introduced

α GSU: glycoprotein hormone, alpha subunit (protein)

aa: amino acids

ACTH: adrenocorticotrophic hormone (protein)

Asn: asparagine (N)

AVP: arginine vasopressin

BCA: bicinchoninic acid

***Bcat1*:** branched chain aminotransferase 1, cytosolic (gene: mouse)

bp: base pairs

CALCRL: calcitonin receptor-like receptor (protein)

CAM: cell adhesion molecule

cAMP: cyclic adenosine monophosphate

CD: cluster of differentiation

CFTR: cystic fibrosis transmembrane conductance regulator (protein)

***CFTR*:** cystic fibrosis transmembrane conductance regulator (gene: human)

CG: chorionic gonadotropin (protein)

***CGA/Cga*:** glycoprotein hormone, alpha subunit (gene: human/mouse)

CGRP: calcitonin gene-related peptide

CHO: Chinese hamster ovary (cell line)

CNS: central nervous system

C-terminus: carboxy-terminus

CTD: C-terminal domain

C-type: constant-type immunoglobulin domain

DAPI: 4',6-diamidino-2-phenylindole, fluorescent stain used to mark nuclei

DEPC: diethylpyrocarbonate

DIO1: iodothyronine deiodinase type I (protein)

***DIO1/Dio1*:** iodothyronine deiodinase type I (gene: human/mouse)

DIO2: iodothyronine deiodinase type II (protein)

DIO2/Dio2: iodothyronine deiodinase type II (gene: human/mouse)

DIO3: iodothyronine deiodinase type III (protein)

DIO3/Dio3: iodothyronine deiodinase type III (gene: human/mouse)

DMEM: Dulbecco's Modified Eagle Medium

DAG: diacylglycerol

DIT: diiodotyrosine

E (followed by number): embryonic day of murine development

ECL: Enhanced chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

EndoH: Endoglycosidase H, a recombinant glycosidase

ER: endoplasmic reticulum

ERAD: endoplasmic reticulum-associated degradation

FACS: fluorescence-activated cell sorting

Fc receptor: fragment, crystallizable receptor, an antibody-binding receptor

FGF: fibroblast growth factor

FNIII: fibronectin type III domain

FSH: follicle-stimulating hormone (protein)

FSHB/Fshb: follicle-stimulating hormone β subunit (gene: human/mouse)

fT₃: free triiodothyronine

fT₄: free thyroxine

GDP: guanosine diphosphate

GFP: green fluorescent protein

GH: growth hormone (protein)

GH/Gh: growth hormone (gene: human/mouse)

Gln: glutamine (Q)

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

HA: hemagglutinin, epitope tag

hCG: human chorionic gonadotropin (protein)

HGVS: human genome variation society

HPT: hypothalamic-pituitary-thyroid axis

Ig: immunoglobulin

IGDC1: Immunoglobulin-like domain containing gene 1

IGF-1: insulin-like growth factor 1

IGSF1: Immunoglobulin superfamily, member 1.

***Igsf1*^{Δex1}:** *Igsf1* with deleted exon 1, alternate name for *Igsf1*-deficient mice

Igsf1-1: Immunoglobulin superfamily, member 1, transcript variant 1 (*Mus musculus*)

Igsf1-2: Immunoglobulin superfamily, member 1, transcript variant 2 (*Mus musculus*)

Igsf1-3: Immunoglobulin superfamily, member 1, transcript variant 3 (*Mus musculus*)

Igsf1-4: Immunoglobulin superfamily, member 1, transcript variant 4 (*Mus musculus*)

IGSF1-1: Immunoglobulin superfamily, member 1, transcript variant 1 (*Homo sapiens*)

IGSF1-2: Immunoglobulin superfamily, member 1, transcript variant 2 (*Homo sapiens*)

IGSF1-3: Immunoglobulin superfamily, member 1, transcript variant 3 (*Homo sapiens*)

IGSF1-4: Immunoglobulin superfamily, member 1, transcript variant 4 (*Homo sapiens*)

IGSF1-5: Immunoglobulin superfamily, member 1, transcript variant 5 (*Homo sapiens*)

IGSF1-CTD: Immunoglobulin superfamily, member 1, C-terminal domain

IGSF1-NTD: Immunoglobulin superfamily, member 1, N-terminal domain

InhBP: inhibin binding protein

IP3: inositol 1,4,5-trisphosphate

JAK1/JAK2: Janus kinase 1/ Janus kinase 2

kb: kilobases

KCl: potassium chloride

kDa: kilodaltons

Klk1b22: kallikrein 1-related peptidase b22 (gene: mouse)

LH: luteinizing hormone

LHB/Lhb: luteinizing hormone β subunit (gene: human/mouse)

MAG: myelin-associated glycoprotein

MAPK1/3: mitogen-activated protein kinase 1/3

MC2R: melanocortin 2 receptor

MCT8: monocarboxylate transporter 8

MIT: monoiodotyrosine

MOPS: 3-(N-morpholino)propanesulfonic acid

MRAP: melanocortin 2 receptor accessory protein

MSH: melanocyte-stimulating hormone (protein)

Myc: protein tag derived from c-Myc gene

NaCl: sodium chloride

NaF: sodium fluoride

NaPO₄: sodium phosphate

NBT/BCIP: solution of 18.75 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate

NCAM: neural cell adhesion molecule

NCBI: National Center for Biotechnology information, <http://www.ncbi.nlm.nih.gov/>

NTD: N-terminal domain

N-terminus: amino-terminus

N→Q: asparagine to glutamine substitution

OATP: organic anion transporting polypeptide

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline with 0.2% Tween-20

PC1/3: prohormone convertase 1/3

PFA: paraformaldehyde

PIP2: phosphatidylinositol 4,5-bisphosphate

PGSF2: pituitary gland specific factor 2

PLC: phospholipase C

PNGaseF: N-Glycosidase F

POMC/Pomc: proopiomelanocortin (gene: human/mouse)

PRL/Prl: prolactin (gene: human/mouse)

PVN: paraventricular nucleus

RAMP1: receptor activity modifying protein 1 (protein)

Ramp1: receptor activity modifying protein 1 (gene: mouse)

RIPA: radioimmunoprecipitation assay buffer

RXR: retinoid X receptor

S-100: S-100 calcium-binding protein (folliculostellate cell marker)

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser: serine (S)

SON: supraoptic nucleus

SSC: saline sodium citrate

T₃: triiodothyronine (thyroid hormone)

T₄: thyroxine (thyroid hormone)

TBS: tris-buffered saline

TBST: tris-buffered saline with 0.05% Tween-20

TGFβ: transforming growth factor β

TH: thyroid hormone

Thr: threonine (T)

THRA/Thra: thyroid hormone receptor α (gene: human/mouse)

THRB/Thrb: thyroid hormone receptor β (gene: human/mouse)

TnT: Coupled Transcription/Translation System

TR: thyroid hormone receptor

TRα1: thyroid hormone receptor α1 (protein)

TRα2: thyroid hormone receptor α2 (protein)

TRβ1: thyroid hormone receptor β1 (protein)

TRβ2: thyroid hormone receptor β1 (protein)

TRE: thyroid hormone response element

TRH: thyrotropin-releasing hormone (protein)

TRH/Trh: thyrotropin-releasing hormone (gene: human/mouse)

TRHDE: thyrotropin-releasing hormone degrading enzyme (protein)

TRHDE/Trhde: thyrotropin-releasing hormone degrading enzyme (gene: human/mouse)

TRHR: thyrotropin-releasing hormone receptor (protein)

TRHR/Trhr: thyrotropin-releasing hormone (gene: human/mouse)

TRHR2: thyrotropin-releasing hormone receptor 2 (protein)

TSH: thyroid-stimulating hormone (protein)

TSHB/Tshb: thyroid-stimulating hormone β subunit (gene: human/mouse)

TSHR: thyroid-stimulating hormone receptor (protein)

V-type: variable-type immunoglobulin domain

WT: wild-type

Y: tyrosine (Tyr)

YFP: yellow fluorescent protein

Preface

Dear reader:

Please pardon the unconventional format of this thesis, but attempting to describe my project in usual terms would not do it justice. This project has not developed in a linear, objective-oriented fashion. Instead, it has been a series of random leaps and bounds, incredible strokes of luck, and a hodge-podge of experiments in between. Accordingly, this thesis is composed of two major parts, each with its own introduction and relevant experimental chapters. Thus, I would like to present to you, in a quasi-chronological order, the story of Immunoglobulin superfamily, member 1 (IGSF1).

Part I: Consternation

Chapter 1. Introduction

1 The immunoglobulin superfamily

The immunoglobulin superfamily is a vast collection of proteins defined by one structural characteristic, referred to as the immunoglobulin (Ig) fold, or domain. This domain was first identified in the immunoglobulins, or antibodies (1). Soon thereafter, proteins that were not part of the immune system were found to contain similar domains (2), giving rise to the concept of the immunoglobulin superfamily. Proteins within the immunoglobulin superfamily contain as few as one Ig domain (2, 3) or, in extreme cases, more than one hundred and fifty (4). The superfamily is one of the largest protein families in vertebrates, with over 750 members identified in the human genome (5).

1.1 The immunoglobulin domain

Ig domains are comprised of approximately 70-110 amino acids (aa), consisting of antiparallel β -strands arranged into two β -sheets that are usually linked by a disulfide bond (6). However, Ig domains lacking the disulfide bridge have also been described (7, 8). A single Ig fold is typically encoded by a single exon, although Ig domains split by introns have also been described (9).

Ig domains can be grouped into several clearly identifiable sub-types, or 'sets', according to the number and arrangement of their β -strands. These sets are based on resemblance to the variable (V-type) and constant (C-type) domains of antibodies (9).

The V-type domains typically contain 9 β -strands, which are labeled A, B, C, C', C'', D, E, F, and G. Strands A, B, E, and D form one β -sheet, with the remaining strands (C, C', C'', F, and G) forming the other sheet. The C-terminal portion of the A strand is sometimes referred to as A' and forms hydrogen bonds with the second β sheet (10). The disulfide bond or bridge is usually formed between conserved cysteine residues in strands B and F, holding the two sheets together (5).

The C-type domains are further divided into C1- and C2-type domains. C1-type domains closely resemble the constant domain of antibodies, and are shorter than V-type domains. They lack strand C'' and have a severely shortened C' strand that is only three residues in length. As in the V-type domains, one β -sheet is composed of strands A, B, E, and D, and the other contains the remaining strands. C2-type domains are more variable than the C1-type domains, resembling C1-type domains with regard to the C' and C'' strands, but with the residues in strands E and F resembling V-type domains (9).

An additional set, termed intermediate, or I-set has been identified. Domains in this set lack the C' strand and have a shortened C'' strand like the C-type domains, but links between strands A and B, as well as E and F, resemble V-type domains (11).

1.2 Function of immunoglobulin superfamily members

It is difficult to ascribe a specific function to the Ig superfamily, as many diverse roles have been attributed to its members. However, the overarching theme of the superfamily appears to be the modulation of cell surface interaction and recognition (9). Proteins containing Ig domains are most frequently encountered in the immune system, as well as the nervous system.

1.2.1 Ig domains in the immune system

Many of the components of the immune system are immunoglobulin superfamily members. These include antibodies (6), the majority of antibody (Fc) receptors (12), antigen-presenting molecules (13), and various co-receptor and accessory proteins, including many cluster of differentiation (CD) molecules (14). These proteins contain varying numbers of Ig domains, and frequently have additional domains or features that enable activation or repression of downstream signaling cascades (15-17). As the chief role of the immune system is distinguishing between self and non-self, the array of immunoglobulin superfamily members in this system is finely tuned to enable interaction between a diverse collection of molecules, including carbohydrates, proteins, protein fragments, and other members of the immunoglobulin superfamily.

1.2.2 Ig domains in the nervous system

Immunoglobulin superfamily members are also widely expressed in the nervous system, where they are thought to play important roles in establishment and maintenance of cell-cell communication (18). Several examples of their diverse functions are summarized below:

The first member of the superfamily to be identified in the nervous system was the neural cell adhesion molecule (NCAM) (19). Like several other Ig domain-containing proteins in the nervous system (contactin (20), L1 (21), TAG-1 (22)), NCAM is a transmembrane protein with an extracellular portion that contains a combination of Ig and fibronectin type III (FNIII) domains (23). NCAM's main role appears to be cell adhesion, and its Ig domains mediate homophilic interactions between NCAMs on neighboring cells (24, 25).

Another Ig superfamily member in the nervous system, myelin-associated glycoprotein (MAG), contains 5 C2-type Ig domains, a transmembrane region, and a short cytoplasmic tail (26). This protein has been implicated in the formation and maintenance of contacts between axons and the myelin sheaths that surround them (27-29).

A family of small Ig domain-containing proteins in the fruitfly (*Drosophila melanogaster*) has been identified as important for nervous system development in the embryo. The founding member of the family, encoded by the *beaten path* (*beat-Ia*) gene, is a protein containing 2 Ig domains, which is secreted by motor neurons (30). Loss of *beat-Ia* results in failure of motor neurons to defasciculate (31). Other members of the *beat* family also contain 2 Ig domains, but are membrane-anchored, and have also been implicated in regulation of axonal fasciculation (32).

1.3 Protein of unknown function

As described above, membership in the Ig superfamily does not necessarily enable easy identification of a protein's function. Certainly, it has been a lengthy and often surprising journey in the case of IGSF1, the protein that is the focus of this thesis.

2 Discovery of *IGSF1*

Two reports emerged within a few months of each other in 1998, each reporting the existence of an Ig domain-containing gene in the Xq25 region of the human X chromosome (33, 34). Both groups noted that the gene contains 12 C2-type Ig domains divided into clusters of five and seven, separated by a hydrophobic linker region. The gene was given two different names, both reflecting its Ig domain content, with one group denominating it *Immunoglobulin superfamily gene 1* (33), and the other, *Immunoglobulin-like domain containing gene* (34). The gene is now officially designated *Immunoglobulin superfamily, member 1*, and abbreviated as *IGSF1*.

2.1 Expression of *IGSF1*

Both groups screened for *IGSF1* expression in human tissues using the same commercially available multiple tissue northern blots. According to Mazzarella et al., who used a probe within the 3' coding region of *IGSF1*, an mRNA species of approximately 4.7 kilobases (kb) was detected at high levels in pancreas, testes, and fetal liver. The same mRNA was seen at moderate levels in heart, prostate, and small intestine, and at very low levels in the brain, thymus, colon, ovary, fetal lung and fetal kidney (33). Notably, they did not observe *IGSF1* expression in adult liver, suggesting that the gene is transiently expressed in this tissue in fetal life. The authors also identified a shorter, 2.8 kb transcript in the heart, and a longer, 5.5 kb isoform in the testis, proposing that both are alternatively spliced *IGSF1* mRNA variants.

Frattoni et al. performed a similar analysis using two different probes, one mapping to the 5' end (exons 5-8, see Section 2.3.1) and the other to the 3' end (exons 18-20) of the *IGSF1* coding sequence. Using the 3' probe, they observed high levels of a 4.4 kb transcript in testis, and lower levels in brain, pancreas and heart. They also noted expression of a 1.35 kb transcript in most tissues, which was particularly abundant in heart and skeletal muscle. Using the 5' probe, they additionally observed a 2.4 kb

transcript in the pancreas (34). The group also proposed that these bands all represent alternatively processed *IGSF1* transcripts.

Two years later, during the search for an inhibin receptor, (see Section 2.4.1), abundant *Igsf1* expression was noted in bovine and rat pituitary glands. Northern blot analyses using probes mapping to the 5' end of the coding region (approximately exons 4-7) revealed transcripts of 4.6 kb and 2 kb in rat pituitary, as well as lower abundance transcripts of apparently identical size in the rat testis (35). Another group soon confirmed that the human pituitary gland is indeed the tissue with highest expression of *IGSF1* (36), suggesting that IGSF1's key function(s) may be related to the endocrine system.

2.2 *IGSF1* gene

The human *IGSF1* gene is located on the X chromosome at Xq25, on the negative strand, spanning 15.9 kb of genomic sequence. The murine *Igsf1* gene is also on the negative strand of the X chromosome, mapping to the syntenic A5 region, and spanning 15.2 kb of genomic sequence.

2.3 *IGSF1/Igsf1* mRNA isoforms and associated protein products

In line with reports in previous publications (33-36), *IGSF1/Igsf1*, like many members of the immunoglobulin superfamily, has multiple mRNA isoforms, which are chiefly products of alternative splicing.

2.3.1 Human *IGSF1* isoforms

The isoforms that have been reported in humans thus far can be divided into two categories: ones that resemble a so-called “long isoform” and ones that resemble a “short isoform”. The mRNAs and corresponding protein products for each isoform are outlined in Figure 1.1, and detailed intron/exon comparisons are summarized in Table 1.1.

Transcripts that resemble the long isoform include *Immunoglobulin superfamily, member 1, transcript variant 1* (*IGSF1-1*, NM_001555.4), *transcript variant 3* (*IGSF1-3*, NM_001170961.1), and *transcript variant 4* (*IGSF1-4*, NM_001170962.1). These are all predicted to encode a transmembrane protein with a signal sequence at the amino-terminus (N-terminus), two clusters of five and seven C2-type Ig domains separated by a linker region containing two hydrophobic domains, a transmembrane domain, and a short cytoplasmic tail at the carboxy-terminus (C-terminus).

IGSF1-1 has 20 exons, with translation beginning in exon 2 and terminating in exon 20. The predicted protein product is 1336 amino acids (aa) long with a molecular weight of 148.96 kilodaltons (kDa). *IGSF1-3* is almost identical to *IGSF1-1*, but contains an additional fifteen base pairs at the 5' end of exon 11 due to use of an earlier acceptor site, resulting in five additional amino acids in the second hydrophobic domain in the linker region. The protein product is 1341 aa long, and has a predicted molecular weight of 149.45 kDa. Both *IGSF1-1* and *IGSF1-3* have a stretch of amino acids encoded by exon 3 that forms part of the N-terminal signal peptide, including the signal peptidase cleavage site. *IGSF1-4* lacks the entirety of exon 3, which is 27 bases long. This results in a loss of 9 amino acids in the signal peptide region, and an alternate amino acid (leucine) that results from the junction of exons 2 and 4. This is predicted to result in a slightly different signal peptide cleavage site, with cleavage occurring in a stretch of amino acids encoded by exon 4 (37). This form codes for a protein product that is 1327 aa long, and has a predicted molecular weight of 147.99 kDa.

The two short isoforms, *IGSF1-2* (NM_205833.3), and *IGSF1-5* (NM_001170963.1) are identical in terms of coding sequence, but differ in their non-coding regions 5' of the translation start site. *IGSF1-2* shares the same 216 bp exon 1 as *IGSF1-1*, *IGSF1-3*, and *IGSF1-4*. In contrast, *IGSF1-5* has an alternate exon 1, which is only 40 bp, and which begins 369 bases downstream of the start site for the canonical exon 1, in what is intron 1 in the other four isoforms. Both isoforms are products of alternative splicing, with sequence that extends into, and terminates prematurely in intron 5. This results in a 242

aa, 27.21 kDa protein that contains an N-terminal signal peptide, followed by the first two Ig domains. As the protein does not have a transmembrane domain, it is predicted to be secreted, and has been purportedly found in human plasma (38).

2.3.2 Murine *Igsf1* isoforms

Unlike in human, the mouse isoforms identified so far are all distinctly different from one another. Four isoforms of *Igsf1* have been reported thus far (39), two of which have human orthologs. The mRNA and protein products for each isoform are outlined in Figure 1.2, and detailed intron/exon comparisons are summarized in Table 1.2.

Immunoglobulin superfamily, member 1, transcript variant 1 (Igsf1-1, NM_177591.4), is the canonical full-length isoform, and the murine counterpart to human *IGSF1-1*. This form spans twenty exons, with the coding sequence beginning in exon 2, and terminating in exon 20. Like its human counterpart, *Igsf1-1* is predicted to encode a protein that contains an N-terminal signal peptide, followed by five C2-type Ig domains, a hydrophobic linker region containing two hydrophobic domains, followed by seven more C2-type Ig domains, a transmembrane domain, and a short C-terminal tail. The protein is predicted to be 1317 aa, and have a molecular weight of 147.03 kDa.

Immunoglobulin superfamily, member 1, transcript variant 2 (Igsf1-2, NM_177915.4) is a product of alternative splicing, and is the murine counterpart of human *IGSF1-2*. The mRNA sequence extends into, and terminates prematurely in intron 5 (39), resulting in a 232 aa protein with a predicted molecular weight of 26.33 kDa. The protein contains only the N-terminal signal peptide, and the first two Ig domains.

Immunoglobulin superfamily, member 1, transcript variant 3 (Igsf1-3, NM_183335.2) is also a product of alternative splicing, and thus far has no described human equivalent. According to NCBI/Ensembl records, it has been reported to lack exon 1, and has only a fragment of exon 2. However, Bernard et al. proposed that the mRNA extends all the way through exon 1 (39). As this isoform appears to be deleted in *Igsf1*-deficient mice, which

have exon 1 targeted for deletion (see sections 2.5.1, 2.5.2), it is likely that *Igsf1*-3 indeed contains exon 1. *Igsf1*-3 thus has thirteen or fourteen exons (depending on the existence of exon 1), the last of which is an alternate exon 13b, which corresponds to a portion of intron 13 of *Igsf1*-1. The protein is predicted to contain the first five Ig domains, the linker region and two more Ig domains, spanning 762 aa, and having a predicted molecular weight of 86.41 kDa.

Immunoglobulin superfamily, member 1, transcript variant 4 (Igsf1-4, NM_183336.2) is the least abundant isoform in the murine pituitary and appears to be the product of an alternative promoter, located in intron 9 (39). The protein thus lacks the first five Ig loops, contains only a short portion of the linker region, corresponding to a fragment of the second hydrophobic domain, and encodes the last seven C-terminal Ig domains. The protein spans 775 aa, with a predicted molecular weight of 85.17 kDa.

Ensembl indicates an additional fifth isoform (not shown), which is alternatively spliced so that it skips part of exon 15, all of exons 16 and 17, and resumes part of the way through exon 18. This form has not been reported in the National Center for Biotechnology information (<http://www.ncbi.nlm.nih.gov>) database.

2.4 Functional significance of IGSF1

2.4.1 Inhibin receptor

In 2000, IGSF1 was identified as a putative receptor for inhibin (35), a gonadal protein hormone that negatively regulates follicle-stimulating hormone (FSH) (40, 41). FSH is a hormone produced by the gonadotrope cells of the pituitary gland, and is a key regulator of mammalian reproduction. FSH is a glycoprotein composed of a common α subunit (gene: *CGA*, protein: α GSU) which is shared with three other hormones (luteinizing hormone: LH; chorionic gonadotropin hormone: CG; and thyroid-stimulating hormone: TSH), and a unique β subunit (gene: *FSHB*, protein: FSH β), which confers biological activity (42, 43). Transcription of *FSHB* is regulated by activins and inhibins, both of which are dimeric proteins in the TGF β superfamily (44, 45), with activins stimulating

and inhibins suppressing FSH production (40, 46-48). Activins are composed of two activin β subunits, while inhibins contain one activin β and a unique α subunit. Activins propagate intracellular signals through heterotetrameric complexes of well-characterized serine/threonine receptors (45, 49). The mechanisms of inhibin action were not well understood twelve years ago, but it was thought that inhibins compete with activins for binding to activin receptor complexes, but due to the presence of only one β subunit are unable to activate the signaling pathway (50-52). The relative affinity of inhibin binding to the receptors is only about a tenth that of the activins, suggesting that a co-receptor protein which binds the inhibin α subunit may exist, resulting in increased affinity of inhibin binding, allowing it to efficiently compete with activins (52-55). IGSF1 (referred to as p120 in the initial publication, due to a misreading of its apparent size on SDS-PAGE) was identified as a potential inhibin co-receptor because it was highly expressed in the pituitary gland, and it interacted with the α subunit in affinity chromatography experiments (35). It was subsequently re-named inhibin binding protein (InhBP), and found to interact with one of the receptors in the activin receptor complex (56). However, conventional receptor binding experiments failed to demonstrate direct binding of inhibins to IGSF1 (57), leaving the protein's role in inhibin signaling uncertain.

2.4.2 IGSF1 in various pathologies

Little has been published about the role of IGSF1 outside the inhibin signaling pathway, but three reports are of note. *IGSF1* was identified as a potential marker of hepatocellular carcinoma in an analysis of microarray data from normal and tumor tissues, as it was upregulated approximately 7-fold in tumor tissues (58). This finding is interesting in light of the data that suggest *IGSF1* is expressed in fetal, but not adult liver (33). *IGSF1* was also identified as part of a gene set that differentiates normal thyroid tissue from papillary thyroid tumors, as its expression was upregulated in 15 out of 16 tumor samples, with a median change of approximately 4-fold (59). Finally, in a small study, autoantibodies against IGSF1-2/IGSF1-5 were reported in two out of four patients with idiopathic TSH deficiency, one out of five patients with lymphocytic adenohypophysitis, and one of ten patients with isolated adrenocorticotrophic hormone (ACTH) deficiency (60). These

findings suggest that IGSF1 may play important roles in a variety of organ systems in health and disease.

2.5 *Igsf1* knockout mice

2.5.1 Targeting approach

In order to clarify IGSF1's role in inhibin signaling, *Igsf1*-deficient (or *Igsf1*^{Δex1}) mice were generated (39). The targeting vector replaced a 428 bp fragment containing exon 1 of *Igsf1*, which contains the putative transcription start site for isoforms *Igsf1*-1, *Igsf1*-2, and likely *Igsf1*-3, with a positive selection cassette. The targeting strategy did not affect the transcriptional start site of *Igsf1*-4, located in intron 9, which had not been identified at the time the mice were generated.

2.5.2 Efficiency of *Igsf1* deletion

In order to assess the efficiency of *Igsf1* deletion, Bernard et al. performed northern blot analyses on wild-type (WT) and *Igsf1*^{Δex1} male and female pituitaries. Using a probe directed against the 5' end of the *Igsf1* coding sequence, they demonstrated that *Igsf1*-1, *Igsf1*-2, and *Igsf1*-3 transcripts were absent in the pituitaries of *Igsf1*^{Δex1/y} males and *Igsf1*^{Δex1/Δex1} females. They also noted that *Igsf1* is significantly more abundant in male than female pituitaries. However, when they used a probe directed against the 3' end of the *Igsf1* coding sequence, they noted a 2.7 kb transcript (*Igsf1*-4) that was retained in *Igsf1*-deficient pituitaries of both sexes. This transcript did not appear to be upregulated in *Igsf1*-deficient animals of either sex (39).

2.5.3 Fertility phenotype analysis

The original phenotype analysis focused on assessing reproductive function in *Igsf1*-deficient mice, as IGSF1 was hypothesized to play a role in regulating FSH synthesis; however, the results revealed that loss of *Igsf1* does not have an overt impact on fertility. The offspring, whether generated from pairings of heterozygous (*Igsf1*^{Δex1/+}) females with hemizygous WT, or hemizygous *Igsf1*-deficient (*Igsf1*^{Δex1/y}) males, were born at expected sex ratios and genotype distributions, indicating loss of *Igsf1* did not affect viability or

sex differentiation. Upon breeding with WT animals, *Igsf1*^{Δex1/y} males, *Igsf1*^{Δex1/+} females, and *Igsf1*^{Δex1/Δex1} females all produced numbers of pups and litters that did not differ significantly from normal. Thus, reproductive function did not appear to be affected in *Igsf1*-deficient mice (39).

The authors also sought to assess any changes in FSH synthesis. If IGSF1 played a role in inhibin signaling as originally suspected, then upon loss of the *Igsf1* gene, and thus inhibin-mediated repression, FSH levels would be predicted to be increased. They observed no difference in *Fshb* mRNA levels between WT and *Igsf1*-deficient animals of either sex. Additionally, there was no difference in mRNA levels of other anterior pituitary hormones, including luteinizing hormone β subunit (*Lhb*), thyroid-stimulating hormone β subunit (*Tshb*), growth hormone (*Gh*), prolactin (*Prl*), and proopiomelanocortin (*Pomc*) between the genotypes (39).

The authors additionally assessed serum gonadotropin levels, and found that neither serum FSH levels, nor serum LH levels differed between control and *Igsf1*-deficient animals of either sex. Finally, no overt abnormalities in the pituitaries, testes, or ovaries of *Igsf1*-deficient mice were noted (39).

2.6 A ten-year pause

The results obtained from assessment of *Igsf1*-deficient mice did not support a role for IGSF1 in inhibin signaling (39). In fact, they left IGSF1 without any clear function at all. The overtly normal phenotype of the mice, coupled with seemingly unchanged pituitary hormone transcript levels argued against IGSF1 being a major player in the endocrine system. The search for IGSF1 function was unfruitful for over a decade, before new life was breathed into it. The following chapters describe the unexpected results that emerged from studying basic IGSF1 expression, processing, and localization.

Figure legends

Figure 1.1 mRNA and predicted protein products of human *IGSF1* isoforms. Left side: identified mRNA isoforms for the human *IGSF1* gene. White boxes: non-coding exons; black boxes: coding exons; grey boxes: alternate sequence due to splicing. Right side: diagrams of predicted protein products from mRNA isoforms. Semi-circles represent Ig domains, black boxes are predicted transmembrane domains, and grey boxes are N-terminal signal peptides. Isoform *IGSF1*-3 contains an additional fifteen basepairs in exon 11 relative to *IGSF1*-1, making the predicted protein five amino acids longer. Isoform *IGSF1*-4 lacks exon 3, making it nine amino acids shorter than *IGSF1*-1. *IGSF1*-2 and *IGSF1*-5 are identical in terms of coding sequence, but *IGSF1*-5 contains an alternate exon 1.

Figure 1.2 mRNA and predicted protein products of murine *Igsf1* isoforms. Left side: identified mRNA isoforms for the murine *Igsf1* gene. White boxes: non-coding exons; black boxes: coding exons; grey boxes: alternate sequence due to splicing. Right side: diagrams of predicted protein products from mRNA isoforms. Semi-circles represent Ig domains, black boxes are predicted transmembrane domains, and grey boxes are N-terminal signal peptides.

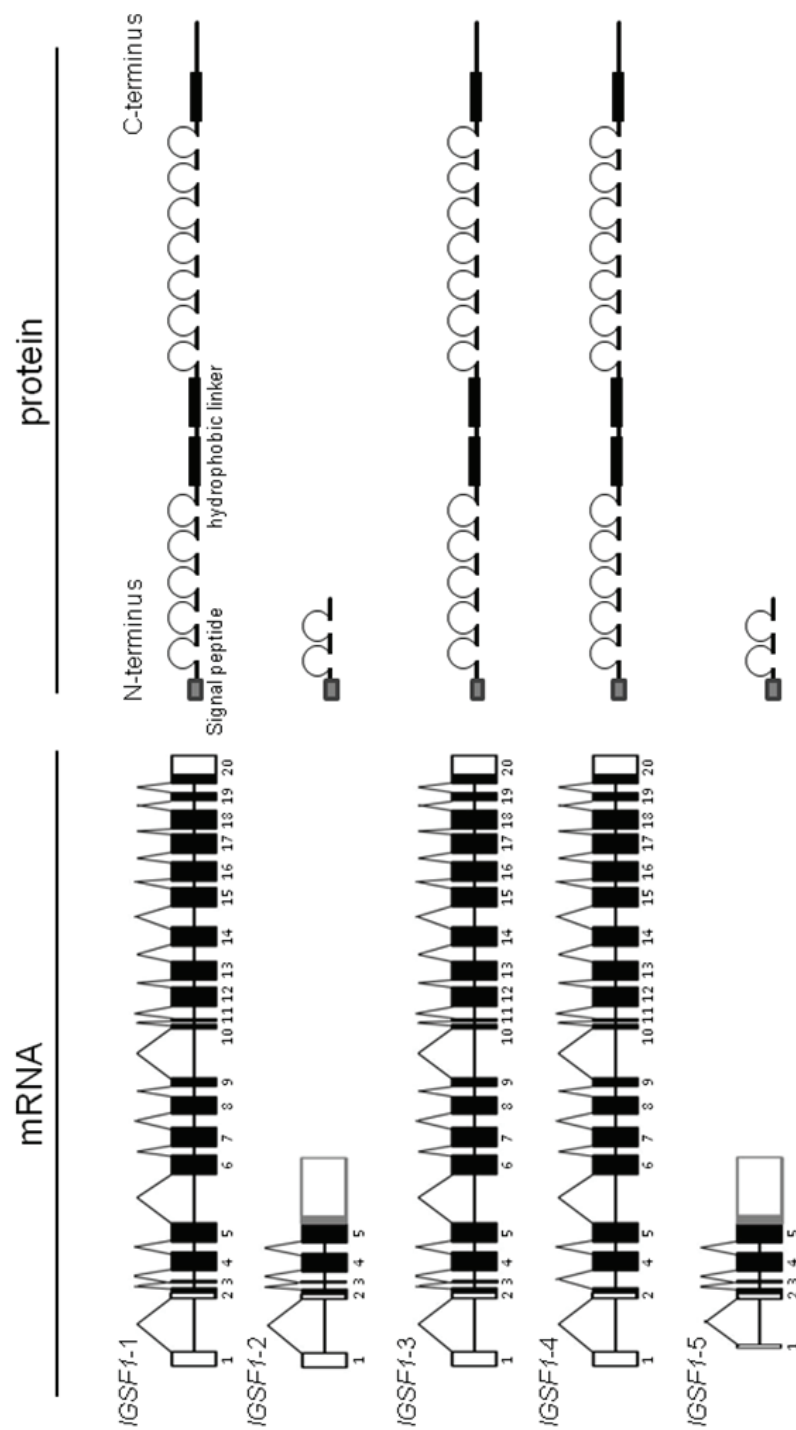


Figure 1.1

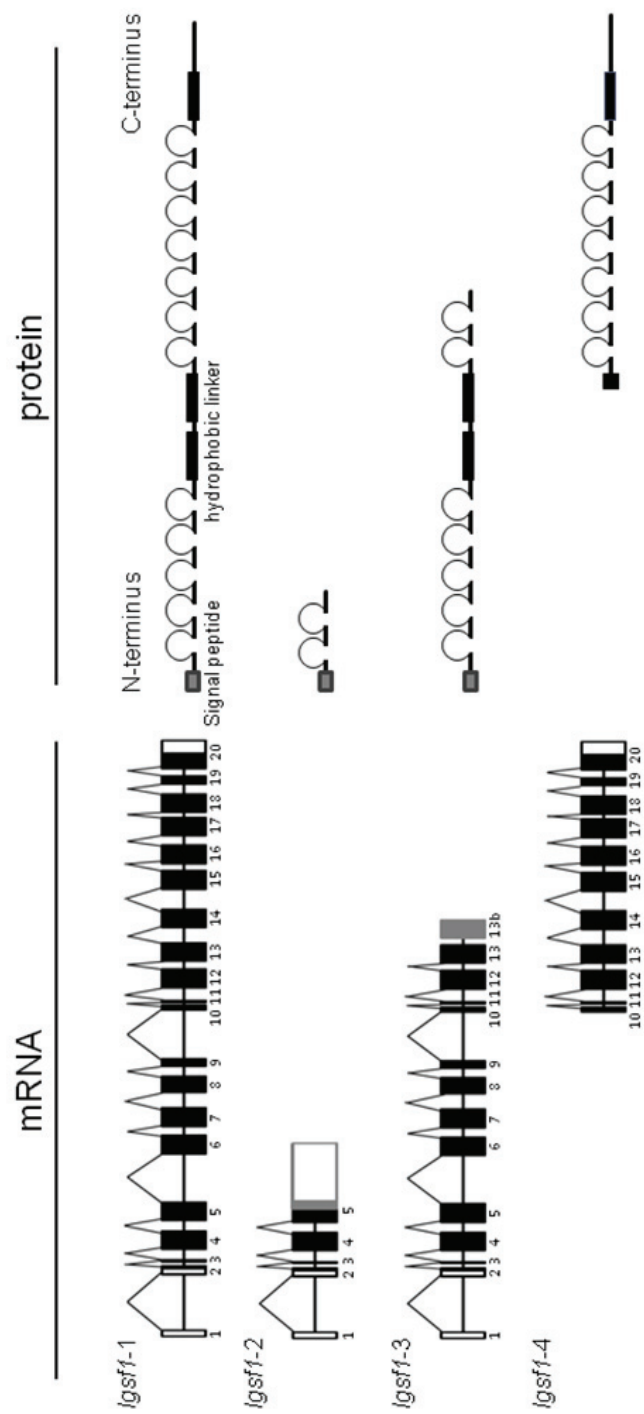


Figure 1.2

Table 1.1 Intron and exon comparisons for human *IGSF1* mRNA isoforms.

<i>IGSF1-1</i>				<i>IGSF1-3</i>				<i>IGSF1-4</i>			
Exon #	Length	Intron #	Length	Exon #	Length	Intron #	Length	Exon #	Length	Intron #	Length
1	216	1	2472	1	216	1	2472	1	216	1	2472
2	137	2	141	2	137	2	141	2	137	2	556
3	27	3	388	3	27	3	388	3		3	
4	282	4	300	4	282	4	300	4	282	4	300
5	288	5	1914	5	288	5	1914	5	288	5	1914
6	285	6	242	6	285	6	242	6	285	6	242
7	294	7	499	7	294	7	499	7	294	7	499
8	279	8	327	8	279	8	327	8	279	8	327
9	121	9	1876	9	121	9	1876	9	121	9	1876
10	71	10	95	10	71	10	80	10	71	10	95
11	33	11	391	11	48	11	391	11	33	11	391
12	291	12	326	12	291	12	326	12	291	12	326
13	279	13	629	13	279	13	629	13	279	13	629
14	288	14	690	14	288	14	690	14	288	14	690
15	288	15	195	15	288	15	195	15	288	15	195
16	288	16	191	16	288	16	191	16	288	16	191
17	288	17	121	17	288	17	121	17	288	17	121
18	279	18	392	18	279	18	392	18	279	18	392
19	124	19	151	19	124	19	151	19	124	19	151
20	426			20	426			20	426		

<i>IGSF1-2</i>				<i>IGSF1-5</i>			
Exon #	Length	Intron #	Length	Exon #	Length	Intron #	Length
1	216	1	2472	1	40	1	2280
2	137	2	141	2	137	2	141
3	27	3	388	3	27	3	388
4	282	4	300	4	282	4	300
5	1301			5	1301		

For all isoforms, translation starts at 68th base of exon 2. Major differences are highlighted in gray.

Table 1.2 Intron/exon comparisons for murine *Igsf1* mRNA isoforms.

<i>Igsf1-1</i>				<i>Igsf1-2</i>				<i>Igsf1-3</i>				<i>Igsf1-4</i>			
Exon #	Length	Intron #	Length	Exon #	Length	Intron #	Length	Exon #	Length	Intron #	Length	Exon #	Length	Intron #	Length
1	93	1	2245	1	93	1	2245	1		1		1		1	
2	129	2	129	2	129	2	129	2	86	2	129	2		2	
3	27	3	349	3	27	3	349	3	27	3	349	3		3	
4	282	4	300	4	282	4	300	4	282	4	300	4		4	
5	288	5	1804	5	1224	5		5	288	5	1804	5		5	
6	285	6	234	6		6		6	285	6	234	6		6	
7	294	7	520	7		7		7	294	7	520	7		7	
8	252	8	312	8		8		8	252	8	312	8		8	
9	121	9	1863	9		9		9	121	9	1863	9		9	
10	71	10	82	10		10		10	71	10	82	10	96	10	82
11	33	11	384	11		11		11	33	11	384	11	33	11	384
12	291	12	220	12		12		12	291	12	220	12	291	12	220
13	279	13	583	13		13		13	279	13	306	13	279	13	583
14	288	14	728	14		14		13b	204			14	288	14	728
15	288	15	178	15		15		15				15	288	15	178
16	288	16	196	16		16		16				16	288	16	196
17	288	17	131	17		17		17				17	288	17	131
18	279	18	388	18		18		18				18	279	18	388
19	124	19	147	19		19		19				19	124	19	147
20	417			20				20				20	417		

For isoforms *Igsf1-1*, *Igsf1-2*, and *Igsf1-3*, translation starts at 90th base of exon 2.

For isoform *Igsf1-4*, translation starts at 68th base of exon 10.

Major differences are highlighted in gray.

Chapter 2

Proteolytic processing of IGSF1

Portions of this chapter, including figures 2.2B and 2.2C were published in the Journal of Biological Chemistry.

Robakis T, **Bak B**, Lin SH, Bernard DJ, Scheiffele P (2008). An internal signal sequence directs intramembrane proteolysis of a cellular immunoglobulin domain protein. *J Biol Chem.* 283(52):36369-76. © the American Society for Biochemistry and Molecular Biology."

All the data presented in this chapter were generated by B Bak.

For published paper, refer to appendix A.

Chapter 2: Proteolytic processing of IGSF1

1 Introduction

The study of IGSF1 was revived when it was identified to be a potential regulator of synaptic development. In an effort to identify factors mediating synaptogenesis in the developing nervous system, Diaz et al. used cDNA microarrays to identify groups of genes upregulated at different developmental time points in the pontocerebellar projection system (61). The authors noted that *Igsf1* was upregulated during a period of synapse formation in the murine pons (62). The Scheiffele group's attempts to further investigate the function of IGSF1 led to some unexpected results that led to the discovery of a novel processing mechanism for mammalian proteins.

1.1 IGSF1 is co-translationally cleaved

Robakis et al. analyzed the expression pattern of a dually-tagged IGSF1-1 construct that contains a Myc-tag at the N-terminus (after the signal peptide), and a 3xHA tag at the C-terminus (Figure 2.1A) (63). The authors transfected heterologous cells with the dually-tagged construct, collected protein lysates, and ran them on SDS-PAGE. A single band of approximately 60-70 kDa was observed when the membrane was probed with an antibody against Myc. In contrast, a doublet around 140 kDa was seen when the membrane was probed with an antibody against HA (Appendix A, Figure 1B). This finding suggested that IGSF1-1 may be cleaved into two fragments, an N-terminal domain (IGSF1-NTD), and a C-terminal domain (IGSF1-CTD).

In order to confirm that the cleavage was a physiological phenomenon, and not just an artifact of overexpression of the protein in heterologous cells, the authors generated antisera against two different fragments of murine IGSF1-1. One antibody was raised against amino acids 21-35, just downstream of the signal peptide cleavage site (NTD antibody), while another was raised against amino acids 559-575, located in the linker region of the protein (CTD antibody) (Figure 2.1B). When used to analyze western blots of murine pituitary protein lysates, the two IGSF1 antibodies produced a similar pattern to that seen in the heterologous cells transfected with the dually-tagged construct, with

the NTD antibody detecting a single band around 60-70 kDa, and the CTD antibody detecting a doublet around 140 kDa. Both antibodies appeared to be detecting specific bands, as neither the NTD nor the CTD antibody detected the same bands in pituitary protein lysates from *Igsf1*-deficient mice (Appendix A, Figure 1B). The identical patterns produced by the dually-tagged construct and endogenous pituitary protein also suggested that the NTD and CTD antibodies are not detecting alternatively-spliced isoforms of *IGSF1* (see Chapter 1, Figure 1.2), but the same protein that has been proteolytically processed into two large fragments. Robakis et al. also performed metabolic labeling (pulse-chase) experiments to determine how soon after translation the cleavage event took place. No full-length precursor was detected, even when IGSF1 was precipitated immediately following the pulse, suggesting that cleavage takes place during, or immediately after translation (Appendix A, Figure 2C).

In order to assess subcellular localization of IGSF1-NTD and IGSF1-CTD, Robakis et al. transfected heterologous cells with the dually-tagged IGSF1-1 construct and performed immunofluorescence using the NTD and CTD antibodies under non-permeabilizing and permeabilizing conditions. Under permeabilizing conditions, a detergent was used to disrupt the plasma membrane, enabling the antibodies to access intracellular epitopes. Under non-permeabilizing conditions, the antibody could only access the epitope if it was located on the outside of the cell. The NTD antibody showed an intracellular staining pattern in permeabilized cells, and no signal under non-permeabilizing conditions. In contrast, the CTD antibody showed a robust membrane signal in intact cells, as well as intracellular staining that overlapped with the NTD signal when the cells were permeabilized (Appendix A, Figure 1C). This supported the notion that the full-length IGSF1-1 was cleaved into two distinct protein fragments that were differentially trafficked in the cell, with IGSF1-CTD being moving to the plasma membrane and IGSF1-NTD remaining intracellular, likely in the endoplasmic reticulum (ER).

1.2 Size discrepancy and glycosylation of IGSF1

If IGSF1-NTD and IGSF1-CTD were derived from the same precursor protein, why did IGSF1-CTD migrate at the predicted size of the full-length protein (approximately 150 kDa)? Analysis of the amino acid sequence revealed that IGSF1 contains fifteen potential N-linked glycosylation sites (asparagine-X-serine/threonine, where X is any amino acid except proline) (33). Upon removal of the sugar groups using the N-deglycosylating enzyme N-Glycosidase F (PNGaseF), IGSF1-CTD migrated at ~90 kDa on SDS-PAGE, again supporting the idea of an internal cleavage event (63).

1.3 Signal peptidase as a candidate enzyme for IGSF1 processing

The early timing of the cleavage event suggested that enzymes in the ER were likely responsible for IGSF1 cleavage. Based on the size of the deglycosylated IGSF1-CTD, Robakis et al. predicted that the cleavage takes place somewhere between amino acids 400 and 560 (encoded by exon 7-exon 10), which includes the linker region of IGSF1 (63). The second of the two hydrophobic domains in this region contains a stretch of amino acids that strongly resembles a signal peptide (37) (Figure 2.2A). Signal peptides are normally present at N-termini of membrane or secreted protein, and direct the nascent proteins to the ER as they are translated. They are usually around 15-30 amino acids (aa) in length, and although they do not show strict sequence conservation, they contain characteristic stretches of amino acids organized into a positively charged n-region, a central hydrophobic h-region, and a stretch of 3–8 polar and uncharged amino acids called the c-region, where the cleavage takes place (64). Intraprotein signal peptides (i.e., at sites downstream of the N-terminus) had been previously identified for viral polypeptides (65-68) but not in mammalian proteins. Signal peptides are trimmed off by signal peptidase, a resident enzyme in the ER (69, 70). Robakis et al. demonstrated that transplanting the putative IGSF1 internal signal peptide onto the N-terminus of another transmembrane protein (generating a chimeric protein) resulted in cleavage of the signal peptide in the chimera (Appendix A, Figure 4A,B). Further, mutation of the c-region of the internal signal peptide inhibited cleavage of the chimeric protein. However, a direct demonstration that IGSF1 was cleaved internally at the internal signal peptide was

lacking. Thus, the first goal of my thesis was to determine whether IGSF1 is cleaved at the putative internal signal sequence.

2 Methods

Signal Peptide Prediction

Signal peptide predictions for human IGSF1-1 (Figure 2.2A) and murine IGSF1-4 (Figure 2.4A) were carried out using SignalP 4.0 server (37) (<http://www.cbs.dtu.dk/services/SignalP/>), using default settings for eukaryotic protein. The input sequences were not truncated in order to predict potential signal peptides in the entire protein.

Constructs

A construct encoding full-length human IGSF1-1 with a Myc-tag at after the N-terminal signal peptide and a 3xHA tag at the C-terminus was a gift from Dr. Peter Scheiffele (Biozentrum, University of Basel, Switzerland, described in (62, 63)). The 4xY mutation (to disrupt the putative signal peptidase cleavage site) was introduced into the Myc-IGSF1-1-3xHA construct using primers in Table 2.1 following the QuikChange mutagenesis protocol (Stratagene, Mississauga, Ontario, Canada). Amino acids with aromatic residues are not found at positions -3 and -1 relative to the cleavage site, thus the introduction of tyrosines is likely to disrupt the recognition and/or cleavage mechanism (71). Successful introduction of the mutation was confirmed by DNA sequencing (Genewiz, South Plainfield, New Jersey). A construct encoding untagged full-length murine IGSF1-1 was generated by amplifying the coding sequence of *Igsf1*-1 using primers in Table 2.1 from murine pituitary cDNA, and ligated into pcDNA3 using restriction enzymes HindIII and XhoI. A construct encoding untagged full-length murine IGSF1-4 (in pNice vector, Clontech, Mountain View, California) was a gift from Dr. Peter Scheiffele. Asparagine to glutamine (N→Q) mutations were introduced into either the human Myc-IGSF1-1-3xHA or murine IGSF1-1 constructs using the primers listed in Table 2.2 (human) and Table 2.3 (mouse), following the QuikChange mutagenesis

protocol (Stratagene). N→Q mutations were verified by sequencing (McGill University and Genome Québec Innovation Centre, Montreal, Quebec, Canada).

Cell culture and transfection

Chinese hamster ovary (CHO) cells (72) (a gift from Dr. P. Morris, Population Council, NY) were maintained in Dulbecco's Modified Eagle Medium F-12 (Ham's) mixture (Cat# 12500-062, Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum, and 4 µg/ml gentamycin (Wisent, St. Bruno, Quebec, Canada). Cells were plated in 6-well plates, grown until 80% confluent, and transfected with 2 µg of pcDNA3 (empty vector), wild-type Myc-IGSF1-1-3xHA, 4xY mutant or human N→Q mutant constructs using Lipofectamine reagent (Invitrogen, Burlington, Ontario, Canada), following manufacturer's instructions. CHO cells were transfected with murine N→Q mutant constructs, and murine IGSF1-1 and IGSF1-4 constructs as described above, except 1 µg of indicated construct was used per well.

Protein lysates and deglycosylation

Total protein lysates were harvested 24 hr after transfection using RIPA buffer (150 mM NaCl, 50 mM NaF, 10 mM NaPO₄, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) plus protease inhibitors (CompleteMini, Roche, Indianapolis, IN). For deglycosylation experiments, protein concentration of cell lysates was determined by BCA assay (Pierce, Nepean, Ontario, Canada). For each sample, 10-20 µg of protein was denatured in 1X Glycoprotein Denaturing Buffer (New England Biolabs, Whitby, Ontario, Canada) for 10 min, followed by deglycosylation with 500 units of either Endoglycosidase H (EndoH) or PNGaseF (New England Biolabs) for 1–2 hr at 37°C, following manufacturer's protocol. Protein lysates were analyzed by western blotting.

In Vitro Translation/Translocation Assays

Wild-type and 4xY mutant IGSF1-1 proteins were synthesized *in vitro* using 500 ng of plasmid DNA and complete amino acid mix, following the TnT coupled reticulocyte lysate system protocol (Promega, Madison, Wisconsin). For translocation assays, canine

pancreatic microsomes (Promega) were included in the reactions (1.8 µl/25 µl reaction; increasing the amount of microsomes did not increase the efficiency of translocation). IGSF1-1-containing TnT lysates were deglycosylated with PNGaseF or EndoH following manufacturer's protocol, with minor alterations. Wild-type TnT-IGSF1-1 (with microsomes) samples were denatured at 100°C for 10 min, while 4xY mutant TnT-IGSF1-1 (with microsomes) samples were denatured at 70°C for 20 min to reduce aggregation of the protein.

Murine pituitary protein lysates

Mice were handled according to federal and institutional (McGill University) guidelines. Adult wild-type male mice were sacrificed by CO₂ asphyxiation, the pituitary glands dissected and immediately frozen on dry ice. Protein lysates were extracted by crushing the tissues with a hand-held homogenizer in 1 µl of RIPA buffer per mg of tissue. Tissues were then freeze-thawed in liquid nitrogen and a 37°C heat block three times, centrifuged to pellet debris, supernatant collected and protein concentration measured using Protein Assay Dye Reagent (Bio-Rad, Mississauga, Ontario, Canada) following manufacturer's instructions.

Western blotting

The protein lysates from CHO cells transfected with human Myc-IGSF1-1-3xHA and 4xY mutant were resolved on NuPAGE 4-12% BisTris gradient gels in MOPS buffer (Invitrogen, Burlington, Ontario, Canada) following manufacturer's protocol. The proteins were transferred to nitrocellulose membranes (25 V, 1.5 hr), the membranes blocked with 5% milk in TBST: 0.05% Tween-20 in Tris-buffered saline (TBS; 10 mM Tris pH 8.0, 150 mM NaCl) and incubated overnight at 4°C in 1:40,000 dilution of mouse anti-HA antibody (H9658, Sigma, St. Louis, MO) in blocking buffer. Next day, membranes were washed 3x15 min in TBST and incubated in a 1:3,000 dilution of goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Mississauga, Ontario, Canada) in blocking buffer for 1 hr at room temperature. The membranes were washed 3x15 min in TBST, followed by incubation in ECL Plus reagent

(Amersham, Baie d'Urfe, Quebec, Canada) according to manufacturer's instructions and then exposed to X-ray film (GE Healthcare, Baie d'Urfe, Quebec, Canada).

The proteins from *in vitro* translation/translocation assays were resolved on NuPAGE 4–12% BisTris gradient gels in MOPS buffer (Invitrogen, Burlington, Ontario, Canada), following manufacturer's protocol with minor alterations. Deglycosylated samples were reheated at 37°C prior to loading onto the gels. Nonglycosylated samples were heated at 70°C for 10 min. The resolved proteins were then processed as described above.

The protein lysates from wild-type male murine pituitary, CHO cells transfected with murine IGSF1-1 and IGSF1-4 constructs, as well as human and murine N→Q mutant constructs were denatured at 100°C for 5 min in Laemmli buffer and a final concentration of 2% 2-mercaptoethanol, and resolved by SDS-PAGE on 8% Tris-glycine gels. The proteins were transferred to nitrocellulose membranes (25 V, 1.5 hr), the membranes blocked with 5% milk in TBST, and incubated overnight at 4°C in 1:1,000 dilution of rabbit anti-IGSF1-CTD antiserum (a gift from Dr. Peter Scheiffele, Biozentrum, University of Basel, Switzerland) in blocking buffer. The rabbit anti-IGSF1-CTD antiserum was raised against the peptide ALTEEIEIVMPTPKPEL as described in (63). The blot was then processed as described above.

N-linked glycosylation site prediction

Prediction of N-linked glycosylation sites on murine and human IGSF1-1 proteins was carried out using the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) (73), using default settings.

Cell surface biotinylation and immunoprecipitation

Transfected CHO cells grown in 6-well plates were washed 3x with cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4) and incubated in 1 ml of 0.5 mg/ml EZ-link-sulfo-NHS-LC-biotin (Thermo, Nepean, Ontario, Canada) per well for 30 min at 4°C, with slow shaking. Cells

were then washed 3x with PBS plus 100 mM glycine and harvested in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). Cells were lysed by sonication at 9W for 10 seconds. Lysates were centrifuged and the supernatant collected. Immunoprecipitation was carried out using 40 µl of anti-HA affinity beads (Sigma, St. Louis, MO) following the manufacturer's instructions. Lysates were then processed for western blotting as described above. After the blocking step, the membrane was incubated in 2.5% milk in TBST with 2 drops each of A and B reagents from Vectastain Elite kit (Vector, Burlington, Ontario, Canada) for 30 min at 22°C, followed by three 10 min washes in TBST. Signal was visualized using ECL reagent (Amersham, Baie d'Urfe, Quebec, Canada) following manufacturer's instructions.

3 Results

In order to definitively demonstrate that the protein is cleaved at the internal signal sequence, I mutated the dually-tagged human Myc-IGSF1-1-3xHA construct in order to disrupt the predicted signal peptidase cleavage site (Figure 2.2B). The mutation altered the amino acids CNG_VL (underscore indicates cleavage site) surrounding the cleavage site to YNYYY (4xY mutant), in order to interfere with the signal peptidase recognition and cleavage mechanism (71). First, I wanted to confirm that introducing the 4xY mutation does not interfere with translation of the protein itself. I therefore used an *in vitro* transcription/translation system, in which expression constructs are transcribed and translated without additional protein processing. I observed that both the wild-type (WT) and 4xY mutant constructs, when synthesized in the *in vitro* translation system, encoded similar proteins which migrated at ~150 kDa in western blot analyses (Figure 2.3A; compare lanes 1 and 2). This suggested that the mutation did not interfere with synthesis of the protein product *in vitro*.

I next compared the migration pattern of the proteins synthesized *in vitro* to those obtained from heterologous CHO cells transfected with the same IGSF1-1 WT and 4xY expression constructs. In contrast to the proteins produced in the *in vitro* transcription/translation system, the proteins produced from the two vectors looked quite

distinct in whole cells. While the WT construct resulted in a protein product that migrated as a doublet around 140 kDa (Figure 2.3A, lane 4), the protein produced by the 4xY mutant construct migrated as a single band at an apparent weight of ~200 kDa (Figure 2.3A, lane 5). This suggested that in intact cells the two constructs are differentially processed, due to the introduced mutation.

To determine whether the cleavage event was taking place in the ER, I used the same *in vitro* translation system to synthesize the wild-type and mutant proteins, but this time with the addition of canine pancreatic microsomes. Microsomes contain signal peptidase, as well as glycosylating enzymes that catalyze the addition of immature, high-mannose sugars to proteins. Upon addition of the microsomes to the *in vitro* reaction with the WT construct, I saw the emergence of a new band of ~130 kDa (Figure 2.3B, lane 3). This band could be deglycosylated with both the deglycosylating enzyme PNGaseF (lane 4), which removes all N-linked glycans (74, 75), as well as EndoH (lane 5), which removes only immature, high-mannose sugars added in the ER (75-77). Deglycosylation of the protein with either enzyme reduced the band size to approximately 90 kDa, which is close to the predicted size of the cleaved deglycosylated protein (83.48 kDa plus 3xHA tag = 86.75 kDa). In contrast, the addition of microsomes to the reaction containing the 4xY mutant construct resulted in the emergence of a band which migrated higher than the precursor, around 200 kDa (lane 8), suggesting that the mutant protein was not cleaved. The increase in size could be explained by glycosylation of the mutant, as deglycosylation with either PNGaseF (lane 9) or EndoH (lane 10) reduced the band size to 150 kDa, the predicted size of the uncleaved protein.

I next analyzed the glycosylation pattern of IGSF1-1 WT and 4xY mutant proteins produced in CHO cells (Figure 2.3C). As mentioned above, IGSF1-1 WT migrates as a doublet around 140 kDa in cells (lane 2). Treatment of protein lysates with EndoH (lane 4), which is selective for immature glycans, revealed that the lower of the two bands was stripped of sugars, while the upper band resisted deglycosylation with this enzyme. However, the both bands could be deglycosylated with PNGaseF (lane 3). These data

indicated that the WT protein exists in two glycoforms, one immature (lower band) and the other mature (upper band). Deglycosylation of the 4xY mutant protein produced in CHO cells revealed that the single band (lane 7) can be deglycosylated by EndoH (lane 9), indicating the presence of immature glycans only. Thus, interfering with IGSF1-1 cleavage prevented maturation of the protein.

Robakis et al. demonstrated that the IGSF1-CTD is trafficked to the plasma membrane (Appendix A, Figure 1C), suggesting that it may be the functional part of the protein. The fourth murine isoform, *Igsf1-4*, is derived from an intronic promoter, and is predicted to encode a protein that is highly similar to the C-terminal portion of IGSF1-1 (39) (Chapter 1, Figure 1.2). The amino acids that function as the internal signal peptide in *Igsf1-1* are predicted to be a true signal peptide in *Igsf1-4* (Figure 2.4A). In order to demonstrate that *Igsf1-1* and *Igsf1-4* both produce IGSF1-CTD protein, I transfected murine IGSF1-1 or IGSF1-4 expression constructs into CHO cells, and ran the protein lysates on western blot along with whole cell pituitary protein lysates from wild-type mice. Immunoblotting with the CTD antibody, which recognizes an epitope located just downstream of the internal cleavage site (see Figure 2.1B), resulted in an identical band pattern for IGSF1-1 and IGSF1-4 (Figure 2.4B, lanes 2 and 4), which also co-migrated with IGSF1 from murine pituitary lysates (lane 6). Deglycosylation of all three proteins with PNGaseF resulted in single band ~90 kDa (lanes 3, 5, 7) confirming that IGSF1-1 and IGSF1-4 generate IGSF1-CTD, which is also present in murine pituitary.

As described above, the presence of both EndoH-resistant and EndoH-sensitive forms of IGSF1-CTD indicates that the protein exists in two glycoforms, one containing complex carbohydrates, and the other, high-mannose, immature sugars. This appears to be true in endogenous protein derived from the pituitary gland, as well as protein produced in heterologous cells. It had been previously reported that IGSF1 contains multiple potential N-linked glycosylation sites (33), that is, the sugar moieties are covalently attached to the protein via nitrogen atoms of asparagines (Asn) within specific glycosylation sequons. The consensus sequence for a N-glycosylation sequon is Asn-X-Ser/Thr, where X is any

amino acid other than proline (78-80). Only about a third of the predicted glycosylation sites in a given protein have sugar groups attached (81), and even within a given protein population, variability is seen in the extent of glycosylation at the same site (82). I therefore sought to characterize which predicted glycosylation sites within human and murine IGSF1-CTD are functional, and whether loss of glycans at these sites interferes with protein synthesis and trafficking.

Analysis of the amino acid sequence using NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) revealed that human IGSF1-1 contains fifteen potential N-linked glycosylation sequons. Of these, five are in the NTD and ten are in the CTD (Figure 2.5A). Of the ten IGSF1-CTD sites, seven have a high likelihood of being glycosylated (red lines in Figure 2.5A), based on the surrounding amino acid sequence. These include asparagines at positions N607, N747, N939, N986, N1027, N1082, and N1223. Using a mutagenesis approach I analyzed all identified sequons in the CTD except one, which had the lowest predicted glycosylation potential (N1147). I replaced the identified asparagines (N) with glutamines (Q) (N→Q mutations). Both asparagine and glutamine have polar, uncharged side chains, which differ only by the presence of an additional carbon in glutamine. This mutation strategy has been used previously to analyze glycosylation sites, as it is unlikely to disrupt the charge and folding of the protein, but it does affect the ability of the protein to acquire N-linked glycans (83, 84).

I compared the migration pattern of WT IGSF1-CTD with that of individual IGSF1-1 NQ mutants. Most individual mutations of the sites did not visibly alter the migration pattern of the protein, with the exception of the mutation N607Q (Figure 2.5B, lane 3), which almost completely abolished the mature glycoform. None of the mutants overtly reduced the apparent size of the immature band. I then proceeded to mutate the sites in combination (Figure 2.5C). I found that the loss of all nine sites was necessary to reduce the immature glycoform to the size of the unglycosylated protein (+N939Q mutant, lanes

21-22), suggesting that all the identified asparagines participate in glycosylation to some extent.

I then sought to determine whether loss of glycosylation leads to impaired membrane trafficking. This has previously been reported in several membrane proteins (85-90), although glycosylation is dispensable for protein trafficking in other cases (86, 91, 92). I transfected CHO cells with WT IGSF1-1, as well as the human N607Q and the nine site (+N939Q) mutant constructs. I then performed cell surface biotinylation followed by immunoprecipitation using the HA tag at the C-terminus of the protein. The extent of cell surface expression was significantly diminished in the N607Q (Figure 2.5D, lane 8) and +N939Q (lane 9) mutants compared to wild-type (lane 7), although some protein was still detectable at the cell surface, suggesting glycosylation significantly contributes to, but is not absolutely required for, plasma membrane trafficking of IGSF1-CTD.

I also performed a similar analysis of the glycosylation sites in murine IGSF1-CTD (Figure 2.6A). As in human IGSF1-1, there are 15 potential sites in the mouse protein sequence. Of those, four are in the NTD and eleven are in the CTD. Of the eleven sites in the CTD, seven are likely to be glycosylated, including N588, N728, N871, N920, N967, N1063, and N1204 (red lines in Figure 2.6A). A comparison of mouse and human glycosylation sites in IGSF1-CTD reveals they are mostly conserved in the C-terminus, with eight of the sites being identical at the protein level, two sites that differ by one amino acid each, and one novel site in the murine protein (Table 2.4).

I generated murine IGSF1-1 N→Q mutant constructs for six of the seven most likely glycosylation sites (all except N1204), transfected them into CHO cells, and analyzed the protein lysates by western blot using the IGSF1-CTD antibody (Figure 2.6B). As in the human protein, most of the mutations did not have an overt impact on the expression or size of IGSF1-CTD, with the exception of N588Q (lane 3), which resulted in the loss of the mature, but not the immature glycoform. This site is conserved between mouse and human, and corresponds to N607, which produced similar results when abolished.

4 Discussion

The discovery that IGSF1-1 is cleaved into IGSF1-NTD and IGSF1-CTD by a signal peptidase-like enzyme represents the first demonstration of an endogenous mammalian protein being co-translationally cleaved at an internal signal peptide. The mutation of the putative cleavage site completely prevents proteolysis, resulting in an IGSF1 protein that is much larger than the one expressed endogenously in the pituitary gland. As we did not detect uncleaved wild-type IGSF1 in either pituitary lysates or heterologous cells transfected with the wild-type construct, and the mutant IGSF1-1 4xY protein does not acquire mature sugars, our data suggest that the cleavage event is necessary for subsequent export of the protein out of the ER, maturation, and trafficking to the plasma membrane. Further, our data indicate that glycosylation of IGSF1-CTD is important for plasma membrane trafficking, as mutants lacking N-linked glycans are less abundant at the plasma membrane.

The human Myc-IGSF1-1-3xHA construct used in these experiments is derived from the coding sequence of the *IGSF1*-1 mRNA, and is an ortholog of the murine isoform *Igsf1*-1 (see Chapter 1, Figures 1.1, 1.2). We demonstrate that both the murine and human *Igsf1*-1/*IGSF1*-1 isoforms generate IGSF1-CTD. However, two additional isoforms that resemble *IGSF1*-1 have been identified in humans (Chapter 1, Figure 1.1). *IGSF1*-4 lacks exon 3, but encodes a linker region identical to that of *IGSF1*-1 and is likely cleaved in the same manner. The other isoform, *IGSF1*-3, encodes a protein with a five amino acid extension in the internal signal peptide. Although we have not experimentally demonstrated that IGSF1-3 is cleaved to produce IGSF1-CTD, it is likely to undergo the same processing, as the additional amino acids alter the length, but do not disrupt the hydrophobic character of the h-region (37, 64). As all *IGSF1* isoforms containing an internal signal peptide are likely cleaved, this supports the idea that the cleavage is necessary for the generation of a functional protein.

The discovery of internal cleavage of IGSF1 sheds light on results obtained during the initial characterization of IGSF1 as a putative inhibin receptor (35) (see Chapter 1,

section 2.4.1). Chong et al. performed microsequence analysis on IGSF1 derived from bovine pituitary membrane extracts. Normally, this method identifies amino acids located at the N-terminus of a protein, but the analysis by Chong et al. produced a 17 aa peptide that appeared to be derived from the middle of IGSF1. This was a surprising and, until now, unexplained result. However, in light of the discovery that IGSF1 is internally cleaved, it appears that what the authors identified was the N-terminal portion of IGSF1-CTD, as the isolated bovine peptide sequence is identical to the N-terminus of human IGSF1-CTD, just past the cleavage site. At present it is not known if the bovine IGSF1-CTD is derived from a long mRNA isoform akin to human or murine *IGSF1-1/Igsf1-1*, or from an ortholog of the murine *Igsf1-4*, that encodes only IGSF1-CTD. The only bovine *Igsf1* mRNA isoform reported in the NCBI database (accession number: NM_001105048.1) is equivalent to the human *IGSF1-2/IGSF1-5*, and the murine *Igsf1-2* isoforms (Chapter 1, Figures 1.1, 1.2). However, identification of an IGSF1-CTD-like protein in bovine pituitary lends support to the idea that this part of the protein is the mature, functional product.

Importantly, if the CTD is the functional portion of the protein, our findings suggest that *Igsf1*-deficient mice are not true knockout mice for IGSF1-CTD, as they retain isoform *Igsf1-4* (39) (see Chapter 1, sections 2.3.2 and 2.5.2). *Igsf1-4* may be able to partially compensate for the loss of *Igsf1-1* because both isoforms generate the same protein product at the cell membrane. However, as *Igsf1-4* was not upregulated in the pituitaries of *Igsf1*-deficient mice (39), and it is not readily detected at the protein level in pituitaries of these animals (63), any compensation for the loss of IGSF1-CTD derived from *Igsf1-1* would appear to be minimal. It is possible that *Igsf1-4* compensates for loss of *Igsf1-1* to a greater extent in tissues where it is more abundantly expressed relative to *Igsf1-1* than is the case in pituitary (see Chapter 3).

Our investigations focused largely on the CTD, but an equally interesting question is the fate of the NTD. Robakis et al. demonstrated that while IGSF1-CTD is trafficked to the plasma membrane, the NTD is retained intracellularly (Appendix A, Figure 1C), and

suggested that the NTD may be further processed by a second enzyme, signal peptide peptidase (63). At present, it is uncertain whether the NTD remains in the ER as a functional intracellular protein, or whether it is targeted for degradation after the cleavage event. Robakis et al. also speculated that the NTD may be trafficked to the plasma membrane under normal conditions, but the heterologous cell systems used in these studies might lack a chaperone necessary for NTD export. This last possibility could be addressed by performing immunofluorescence on cells that endogenously express IGSF1, using antibodies specific to the NTD and CTD. A limiting factor may be the quality of reagents, as in our experience, the NTD antibody described in Robakis et al. is inferior to the CTD antibody, and the NTD fragment is difficult to detect by western blot.

At least a portion of the NTD may have a function, as both mice and humans express short isoforms of *Igsf1/IGSF1*, which encode only the first two Ig domains of the NTD (see Chapter 1, Figures 1.1, 1.2), and may be secreted (38). If the entire NTD is trafficked to the cell surface, it is possible that it functions as a membrane-bound form of IGSF1-2. Many proteins have both membrane-bound and secreted isoforms, which are generated from alternative transcripts (93-96). Alternatively, the NTD could have no function, and its existence might simply be a by-product of the need to generate the short, secreted isoforms of IGSF1. At present, we can only conclude that the NTD is not strictly required for survival, as *Igsf1*-deficient mice, which lack all the NTD-containing isoforms reported thus far (*Igsf1*-1, *Igsf1*-2, *Igsf1*-3), are viable (39).

How did the internal signal peptide arise in IGSF1? Protein alignment of human NTD and CTD, using the Clustal Omega server (97) (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), where the start of CTD was defined as first amino acid of the second hydrophobic domain in the linker region, reveals significant sequence homology between the two fragments (Figure 2.7). Thus, it is possible that at some point in evolution, the sequence encoding the ancestral CTD-like protein duplicated, and was integrated upstream of itself. If the ancestral signal peptide remained intact during duplication, it maintained its function, and enabled cells to continue generating IGSF1-CTD from the

internal cleavage site. As the CTD contains seven Ig domains, and the NTD only contains five, loss of two Ig domains would have occurred at some point in evolution. Alignment of the NTD and CTD reveals two large gaps in the NTD, corresponding to the third Ig domain of the CTD (domain 8 in the full-length protein), and the second half of domain 5 to the first half of domain 6 (domains 10-11 in the full-length protein). These gaps may represent the domains that were lost during duplication. Support for IGSF1-CTD being the ancestral protein also comes from two non-mammalian species in which an IGSF1 ortholog has been identified: chicken (*Gallus gallus*, accession number: NM_001145226.2) and green anole (*Anolis carolinensis*, accession number: XM_003225852.1). Comparison of these sequences with human IGSF1 reveals these two proteins are more similar to the CTD than the NTD (not shown).

If the duplication event took place, then the fourth murine isoform, *Igsf1-4* might correspond to the ancestral form of the gene. *Igsf1-4* transcripts are generated at low levels by an intronic promoter, in contrast to the abundant *Igsf1-1* transcripts seen in the murine pituitary (39). *Igsf1-4* may thus be driven by a minimally-functional promoter that enables generation of low levels of IGSF1-CTD, while the upstream promoter which drives expression of *Igsf1-1* is highly active and subject to a more refined level of regulation. Indeed, using probes that map to the NTD-encoding portion of *Igsf1*, Bernard et al. reported that rat *Igsf1* transcript levels fluctuate across the female estrous cycle (98), suggesting the upstream promoter is regulated in the pituitary gland. Interestingly, no human ortholog to *Igsf1-4* has been reported, perhaps suggesting that the intronic promoter is no longer active in humans.

After IGSF1 is cleaved to generate IGSF1-CTD, the protein is further modified by extensive N-linked glycosylation. Our data suggest that loss of glycosylation sites, including a crucial residue that is conserved between both mouse and human, has significant impact on the abundance of the mature protein at the plasma membrane. The addition and successive trimming of sugar groups in the ER are important for interaction with molecular chaperones that play roles in protein folding and quality control; thus,

aberrant glycosylation of IGSF1 could lead to protein misfolding and targeting for degradation before it reaches the cell surface (99). Additionally, even if the immature protein reaches the cell surface, it may be rapidly degraded by peripheral quality control machinery (100). In both cases, abnormal glycosylation would lead to decreased amounts of IGSF1-CTD at the plasma membrane, likely impairing its function.

In summary, our study of IGSF1 produced some unexpected findings, including the cleavage of IGSF1 into two large protein fragments, generation of structurally-equivalent proteins from two seemingly different *Igsf1* isoforms in mice, and extensive glycosylation of the putative functional protein product, IGSF1-CTD. This newly gained insight informed the next steps in our search for IGSF1 function. However, to generate hypotheses about IGSF1 function, I first sought to delineate the tissues and cell types in which it is expressed. The results of these investigations are addressed in the next chapter.

Figure legends

Figure 2.1 Diagram of IGSF1-1. (A) Diagram of human WT IGSF1-1 construct, which is tagged at the N-terminus (after the signal peptide) with a Myc-tag, and 3xHA tag at the C-terminus. (B) Diagram of murine WT IGSF1-1 protein indicating locations of epitopes for NTD and CTD antibodies.

Figure 2.2 Internal signal peptide in IGSF1-1. (A) Signal peptide prediction analysis carried out using SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) indicates a potential internal signal peptide in the linker region of IGSF1-1. (B) The second hydrophobic loop in the linker contains a putative signal peptidase cleavage site. The 4xY mutation replaces four amino acids in the c-region with tyrosines to interfere with the cleavage event.

Figure 2.3 4xY mutation prevents cleavage of IGSF1 at internal signal peptide. (A) WT and 4xY mutant IGSF1-1 expression constructs were introduced into an *in vitro* transcription/translation system, protein lysates were run on SDS-PAGE and immunoblotted with an HA antibody (lanes 1, 2). CHO cells were transfected with pcDNA3 (empty vector), WT or 4xY IGSF1-1 expression constructs. Protein lysates were run on SDS-PAGE and immunoblotted with HA antibody (lanes 3-5). (B) WT and 4xY mutant IGSF1-1 expression constructs were translated *in vitro* in the presence of microsomes, and deglycosylating enzymes PNGaseF or EndoH, as indicated. Protein lysates were run on SDS-PAGE and immunoblotted with an HA antibody. Non-specific bands are indicated by *. (C) CHO cells were transfected with WT and 4xY mutant IGSF1-1 expression constructs. Protein lysates were deglycosylated with either PNGaseF or EndoH, run on SDS-PAGE, and immunoblotted with an HA antibody.

Figure 2.4 *Igsf1*-1 and *Igsf1*-4 encode the same protein product, IGSF1-CTD. (A) Signal peptide prediction analysis indicates a signal peptide at the N-terminus of IGSF1-4. The predicted cleavage site is the same as the internal cleavage site in IGSF1-1. (B)

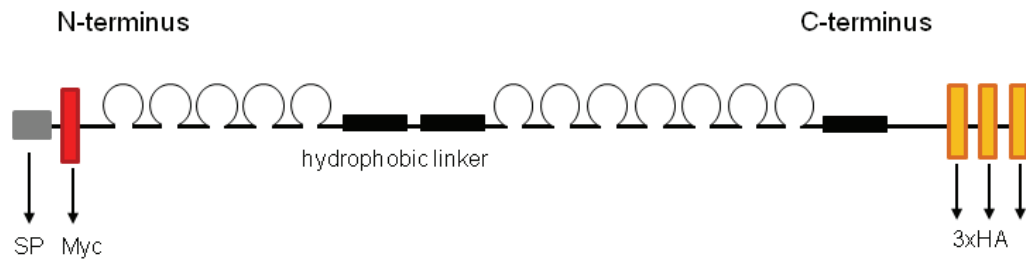
CHO cells were transfected with pcDNA3 (empty vector), murine IGSF1-1 or murine IGSF1-4 expression constructs. Protein lysates were untreated or deglycosylated with PNGaseF (+P) and run on SDS-PAGE along with adult male murine pituitary protein lysates, and immunoblotted with the IGSF1-CTD polyclonal antibody.

Figure 2.5 Human IGSF1-CTD is glycosylated at multiple sites. (A) Diagram of putative asparagine (N) residues that might be glycosylated in human IGSF1. Red lines indicate high likelihood of glycosylation, gray lines indicate low likelihood of glycosylation. (B) CHO cells were transfected with pcDNA3 (empty vector), WT human IGSF1-1 construct (with HA tag at C-terminus), or the indicated glycosylation mutants (numbers indicate glycosylation sites that were abolished by changing N→Q). Protein lysates were run on SDS-PAGE and immunoblotted with HA antibody. β -actin (ACTB) was used as a loading control. (C) CHO cells were transfected with pcDNA3 (empty vector), WT human IGSF1-1 or indicated glycosylation mutants (numbers indicate progressive addition of mutations to the construct, such that the last mutant, +N939Q, contains all 9 mutations). Protein lysates were deglycosylated with PNGaseF, and run on SDS-PAGE as in (B). (D) CHO cells were transfected with pcDNA3, WT human IGSF1-1, N607Q or +N939Q (all mutations) constructs, and subject to cell surface biotinylation. Protein lysates were then immunoprecipitated using HA antibody, and run on SDS-PAGE. Biotin signal was visualized using Streptavidin-HRP, and total and immunoprecipitated lysates were immunoblotted with HA antibody.

Figure 2.6 A specific asparagine residue is important for acquisition of mature glycosylation in mouse IGSF1-CTD. (A) Diagram of potential asparagine (N) residues that can be glycosylated in murine IGSF1. Red lines indicate high likelihood of glycosylation, orange lines indicate low likelihood of glycosylation. (B) CHO cells were transfected with pcDNA3 (empty vector), WT murine IGSF1-1 construct, or indicated glycosylation mutants (numbers indicate glycosylation sites that were abolished by changing N→Q). Protein lysates were run on SDS-PAGE and immunoblotted with IGSF1-CTD antibody.

Figure 2.7 The NTD and CTD share significant sequence homology. The NTD (amino acids 1-539 of IGSF1-1), and the CTD (amino acids 540-1336 of IGSF1-1) were aligned using Clustal Omega server (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Asterisks (*) indicate positions with a fully conserved residue, colons (:) indicate conservation between groups of strongly similar properties, and periods (.) indicate conservation between groups of weakly similar properties, as defined in (101).

A



B

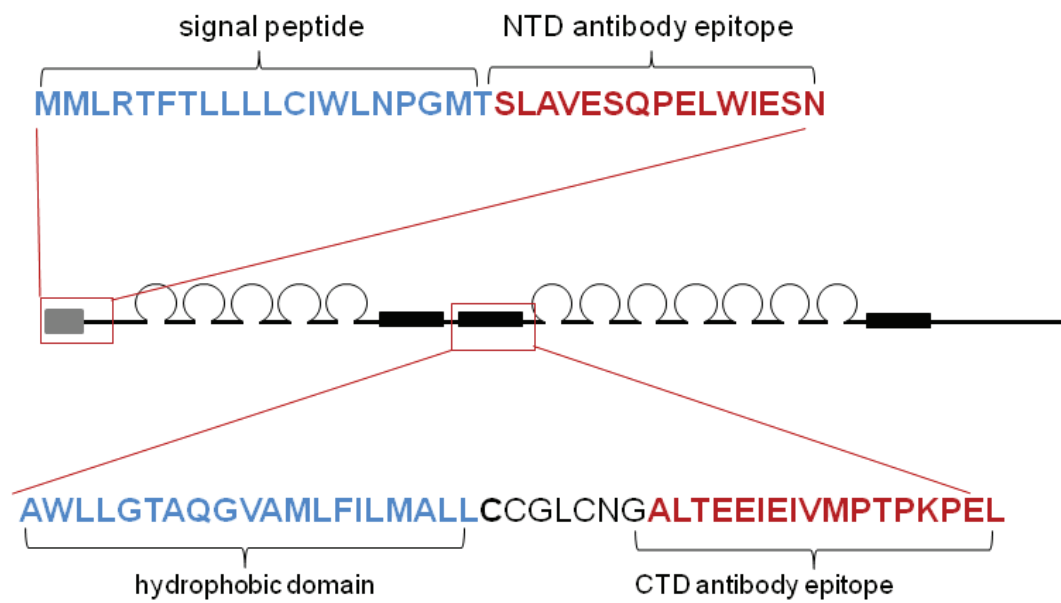
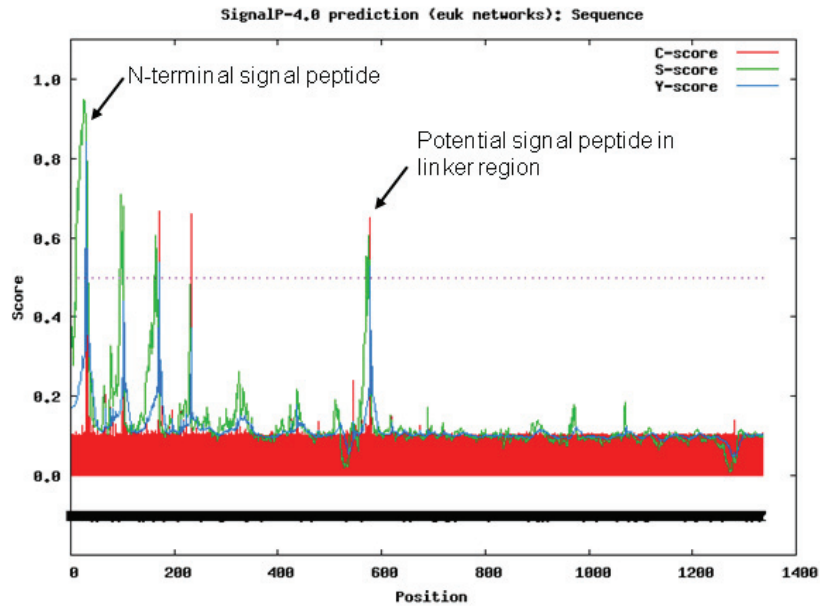


Figure 2.1

A



B

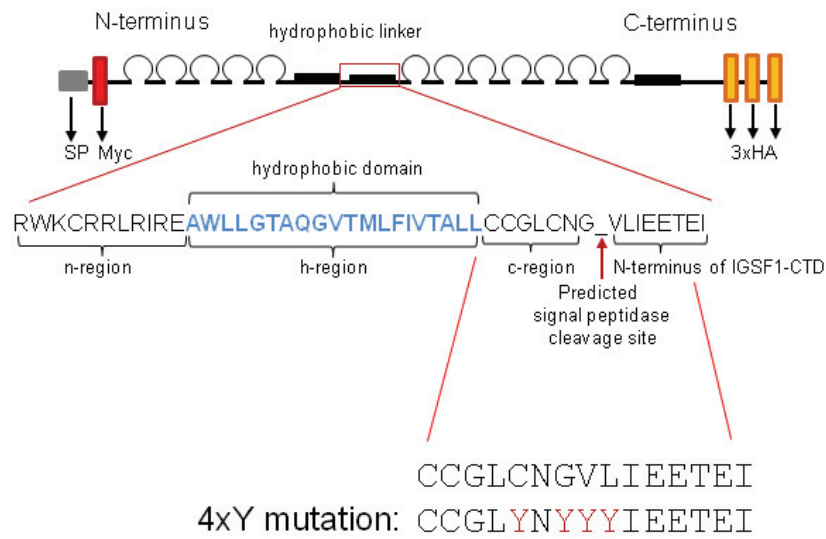


Figure 2.2

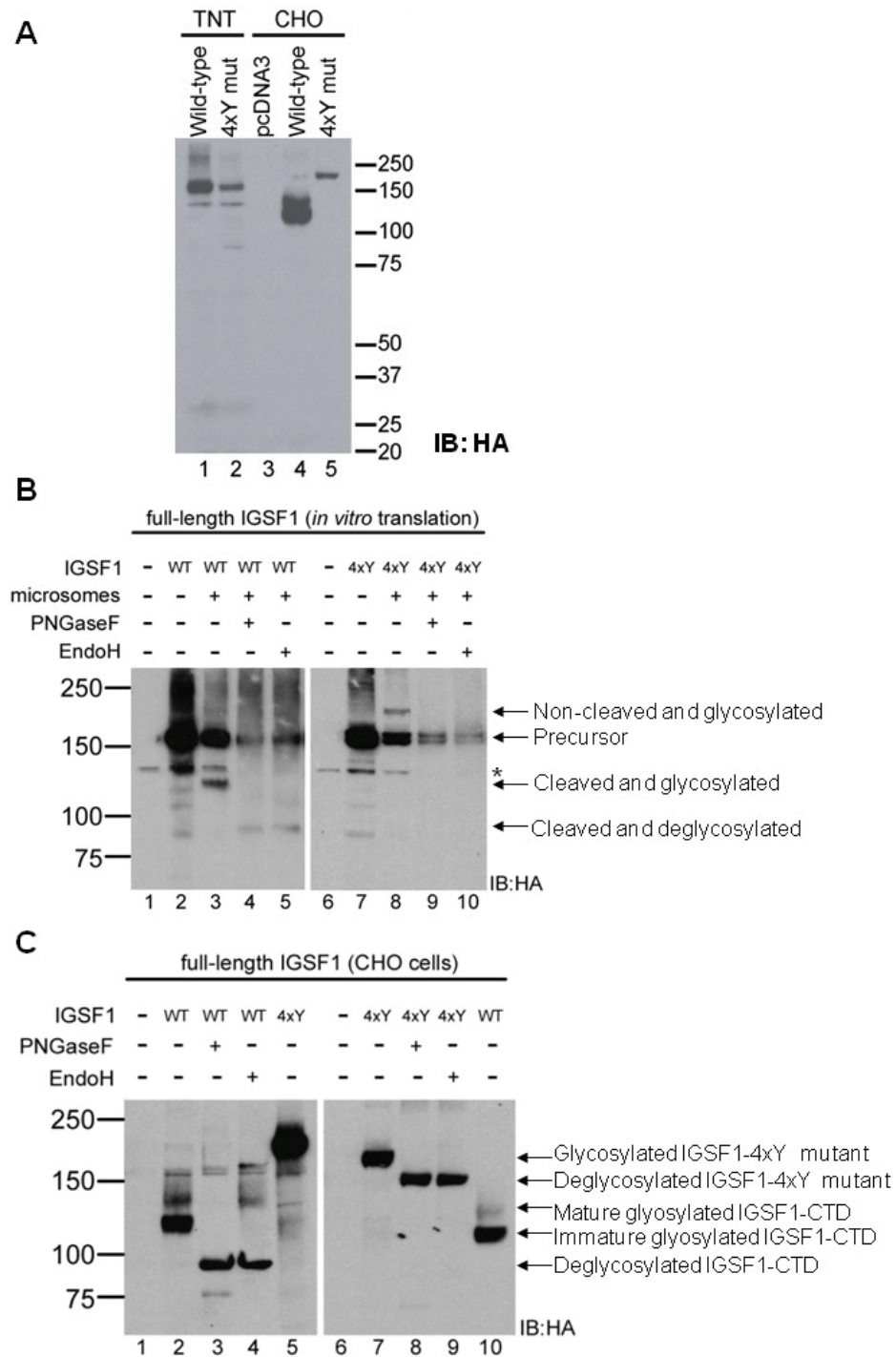
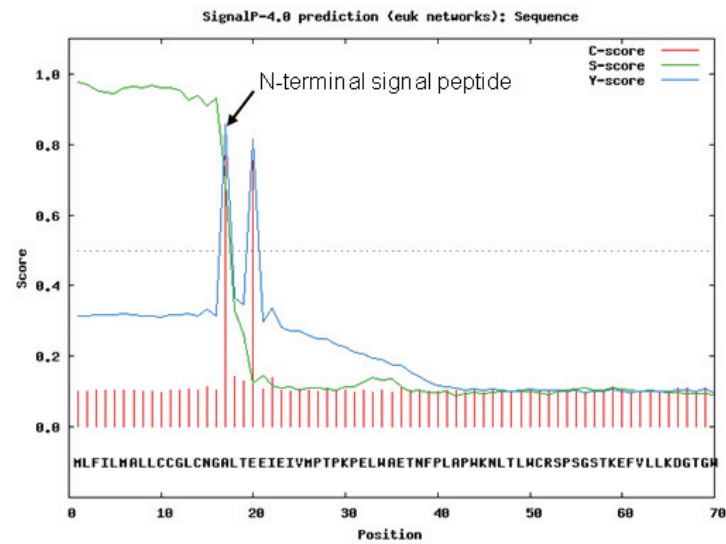


Figure 2.3

A



MLFILMALLCCGLCNG ALTEEIEIVMPTPKPEL

h-region c-region

Predicted
signal peptidase
cleavage site

B

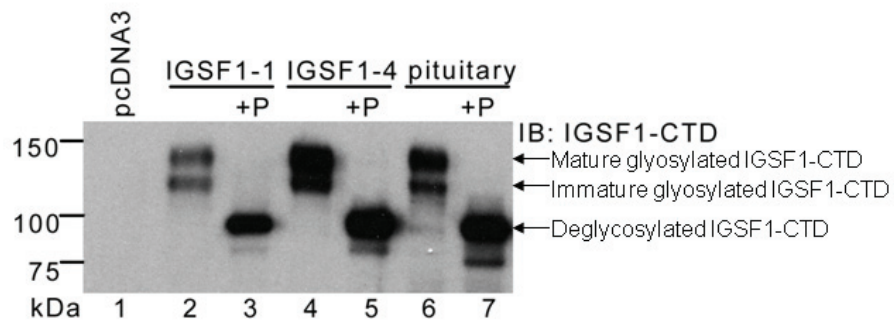


Figure 2.4

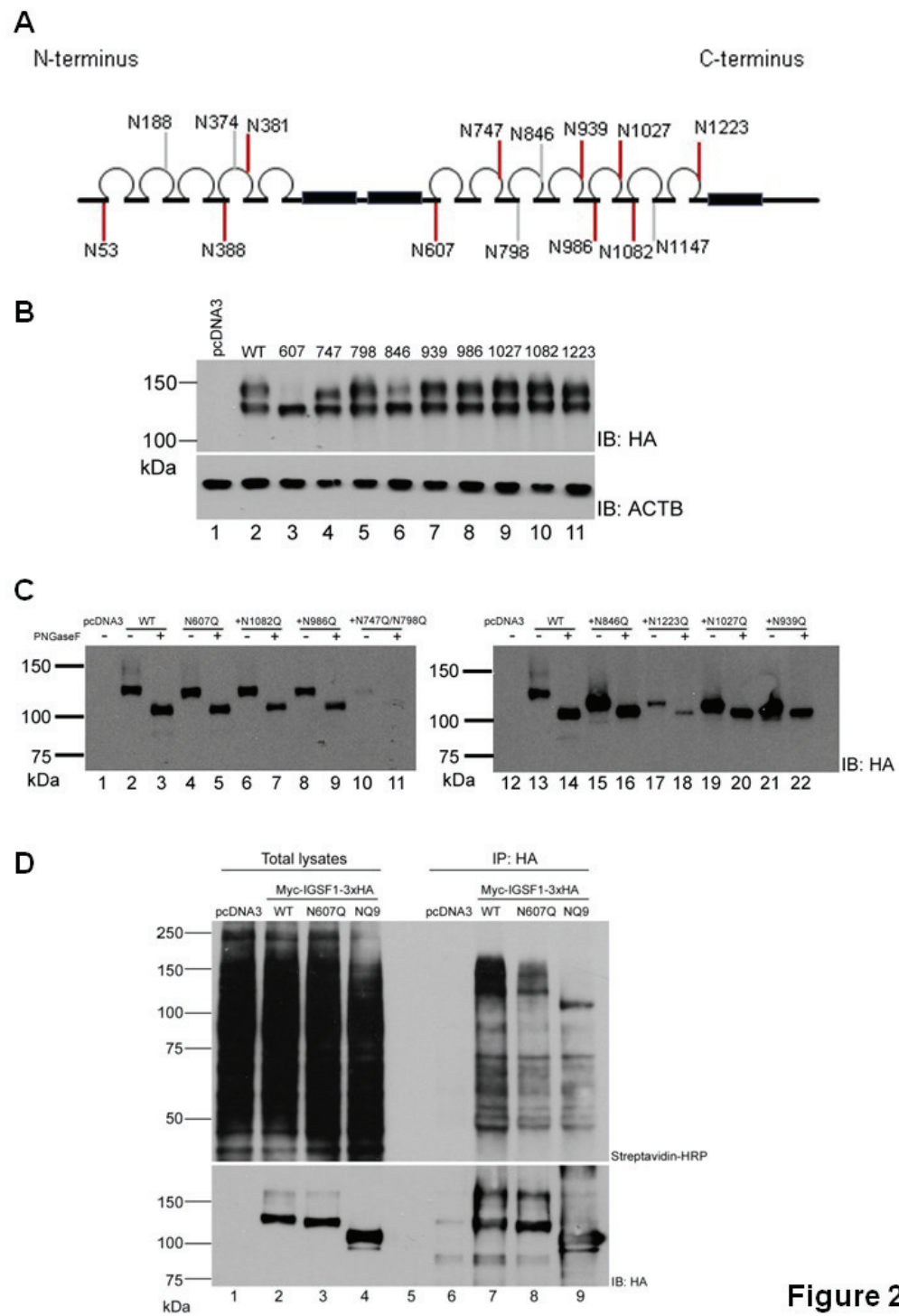


Figure 2.5

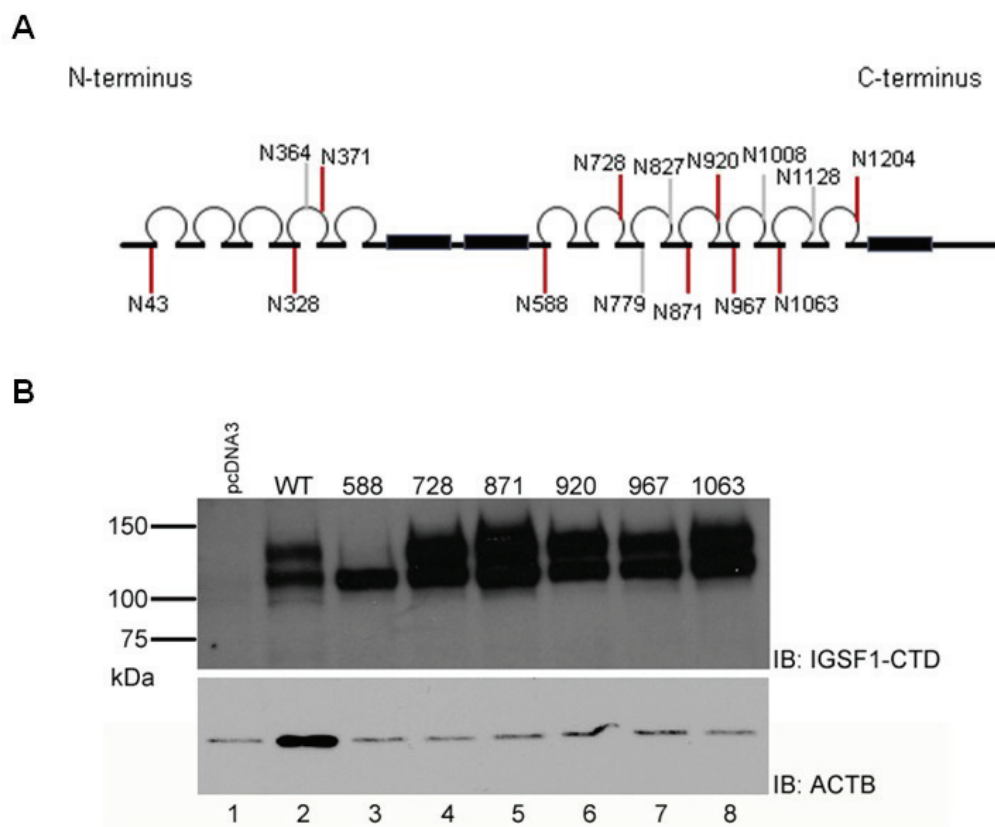


Figure 2.6

Table 2.1 Primers used to generate human IGSF1-1 4xY mutant and murine IGSF1-1 construct.

Primer name:	Sequence:
hIGSF1-4xY forward	5'-CTCTTCATAGTCACGGCCCTTCTCTGCTGTGGACTGTATAATTATTATTATATAGAAG AGACTGAAATAGTCATGCCAACCCCTAAGC-3'
hIGSF1-4xY reverse	5'-GCTTAGGGGTTGGCATGACTATTTCACTCTCTTCTATATAATAATAATTATACAGTCCA CAGCAGAGAAGGGCCGTGACTATGAAGAG-3'
mlgsf1-1 forward	5'-ACGGAAGCTTCTAAGCCATGATGCTTCGGACCT-3'
mlgsf1-1 reverse	5'-ACGGCTCGAGGAGGGTAATTATATTGGAAGTGTCACT-3'

Table 2.2 Primers used to generate human IGSF1-1 N→Q mutants.

Mutation:	Sense Primer:	Antisense Primer:
hIGSF1-N607Q	5'-CTGGCCCCGTGGAAGCAGTTAACC CTCTGGTGC-3'	5'-GCACCAGAGGGTTAACTGCTTCCAC GGGGCCAG-3'
hIGSF1-N747Q	5'-GAGGATAAAGACGAAGGCCAGTACA GCTGCCGCACTCAC-3'	5'-GTGAGTGCGGCAGCTGTACTGGCCTTCGTC TTTATCCTC-3'
hIGSF1-N798Q	5'-GCCCCGAGTGACTTTCCAGTGCTCCAC CCCCAC-3'	5'-GTGGGGGGTGGAGCACTGGAAAGTCACTC GGGC-3'
hIGSF1-N846Q	5'-GGGCATTGGTGATGGAGGGCAGTA CAGCTGCCGATATTATG-3'	5'-CATAATATCGGCAGCTGTACTGCCCTCCATC ACCAATGCCC-3'
hIGSF1-N939Q	5'-TGGAGCAGAGGACTCTGGGCAGTA TAGCTGTATCTACTATG-3'	5'-CATAGTAGATACAGCTATACTGCCCAGAGT CCTCTGCTCCA-3'
hIGSF1-N986Q	5'-GGTTCCCATGGGGCAGGTTACTCTC TGGTGCC-3'	5'-GGCACCAGAGAGTAACCTGCTGCCCCATG GGAACC-3'
hIGSF1-N1027Q	5'-GGGGCATTCCCCATCACCCAGATAT CTGGTACTAGCATG-3'	5'-CATGCTAGTACCAGATATCTGGGTGATGGG GAATGCCCC-3'
hIGSF1-N1082Q	5'-TGGTGGCCCCTGGCGAACAGATGA CTCTTCAGTGTC-3'	5'-GACACTGAAGAGTCATCTGTTGCCAGGG GCCACCA-3'
hIGSF1-N1223Q	5'-GAAGGAAAAGGCATTGGACAGTACA GCTGCAGCTACCGC-3'	5'-GCGGTAGCTGCAGCTGTACTGTCCAATG CCTTTTCCTTC-3'

Table 2.3 Primers used to generate murine IGSF1-1 N→Q mutants.

Mutation:	Sense Primer:	Antisense Primer:
mIGSF1-N588Q	5'-CTGGCCCCGTGGAAGCAGTTAACCT CTGGTGC-3'	5'-GCACCAGAGGGTTAACTGCTTCCACGGG GCCAG-3'
mIGSF1-N728Q	5'-GAGGATAAAGATGAGGGCCAGTACA GCTGCCGCACACAC-3'	5'-GTGTGTGCGGCAGCTGTACTGGCCCTCAT CTTTATCCTC-3'
mIGSF1-N871Q	5'-GTGCTTCCTGGGAAGCAGGTGACCCT GCGCTGC-3'	5'-GCAGCGCAGGGTCACCTGCTTCCCAGGAA GCAC-3'
mIGSF1-N920Q	5'-GGAGCACAGGACTTTGGGCAGTACA GCTGTGTCTACTAT-3'	5'-ATAGTAGACACAGCTGTACTGCCCAA GTCCTGTGCTCC-3'
mIGSF1-N967Q	5'-GTGGTCACCATGGGGCAGCAGGTTAC TCTATGGTGTCAA-3'	5'-TTGACACCATAGAGTAACCTGCTGCCCCA TGGTGACCAC-3'
mIGSF1-N1063Q	5'-CATGGTAGCCCCTGGAGAACAGGTGA CTCTTCAATGTC-3'	5'-GACATTGAAGAGTCACCTGTTCTCCAGGG GCTACCATG-3'

Table 2.4 Glycosylation sites comparison between human and mouse IGSF1-1.

N position in human IGSF1-1 (aa)	Sequon	N position in mouse IGSF1-1 (aa)	Sequon	Notes
N53	NITL	43	NITL	
188	NLTP			Not present in mouse
338	NVSL	328	NVSL	
374	NTSF	364	NNSF	1 aa difference
381	NVTY	371	NVTY	
607	NLTL	588	NLTL	
747	NYSC	728	NYSC	
798	NCST	779	NCST	
846	NYSC	827	NYSC	
		871	NVTL	Not present in human
939	NYSC	920	NYSC	
986	NVTL	967	NVTL	
1027	NISG	1008	NISG	
1082	NMTL	1063	NVTL	1 aa difference
1147	NHSD	1128	NHSN	1 aa difference
1223	NYSC	1204	NYSC	

Chapter 3

Expression of *Igsf1*/IGSF1 in murine tissues

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¹ These authors contributed equally to this work

² These authors jointly directed this work

All data figures presented in this chapter were generated by B. Bak.

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For published paper, refer to appendix B.

Chapter 3: Expression of *Igsf1*/IGSF1 in murine tissues

1 Introduction

One of the most challenging aspects of investigating IGSF1 function has been the lack of reliable expression data. *Igsf1*/IGSF1 mRNA is readily detectable by northern and western blotting, and while this enabled the broad identification of IGSF1-expressing tissues (33, 34, 39, 61, 63, 98), those techniques do not provide sufficient resolution to localize IGSF1 expression to specific cell types. The pituitary gland expresses the highest levels of IGSF1 of any organ (36), thus IGSF1 is likely to be important for some aspect of its function. As the pituitary contains multiple endocrine and non-endocrine cell types, the localization of IGSF1 to one or more of these cell types would facilitate the generation of hypotheses regarding its function.

1.1 The pituitary gland

The pituitary is an endocrine organ located at the base of the brain within a cavity of the sphenoid bone, known as the *sella turcica*. The pituitary is principally composed of two portions, the anterior and posterior lobes. A third zone between the two, which is prominent in rodents and absent in adult humans, is referred to as the intermediate lobe (Figure 3.1A). The pituitary is derived from two tissues of different origins, which remain in close contact with one another throughout development (102). The anterior and intermediate lobes develop from an ectodermal diverticulum of the roof of the primitive buccal cavity, referred to as the Rathke's pouch, while the posterior lobe forms from a downgrowth of the diencephalic portion of the developing forebrain (103). In mice, initiation of pituitary development is seen by embryonic day 9 (E9) with the formation of Rathke's pouch, and continues throughout embryonic life, with the various endocrine lineages emerging and/or terminally differentiating by E17.5 (102) (Figure 3.1B). Each of the lobes plays distinct roles in the regulation of multiple endocrine systems, as discussed below.

1.1.1 The anterior pituitary

The anterior pituitary, also referred to as the adenohypophysis, contains five hormone-secreting cell lineages (corticotropes, gonadotropes, somatotropes, lactotropes, and thyrotropes), as well as poorly-characterized support cells, known as folliculostellate cells, or pituicytes, which constitute up to 20% of the cell population in the pituitary (104, 105). The first terminally-differentiated cells to emerge in the developing pituitary are the corticotropes, which are detected around E12.5 in mice (106, 107). These cells, which constitute 10-20% of the pituitary, synthesize adrenocorticotrophic hormone (ACTH) from the precursor proopiomelano-cortin (POMC) (108). The next three cell types to emerge are the so-called POU1F1 lineage cells and are, in order: thyrotropes, somatotropes, and lactotropes. All three cell types require the transcription factor, POU1F1 (also known as Pit-1), for lineage commitment (109, 110). Thyrotropes, which make up about 2-5% of pituitary cells (111), also require the transcription factor GATA-2 for normal function, but not lineage commitment (112). They synthesize thyroid-stimulating hormone (TSH), starting at E14.5 (107, 113). Somatotropes and lactotropes both develop from the same immature precursor cells, referred to as somatolactotropes. Somatotropes are the most abundant cells in the pituitary, comprising up to 45% of the cell population (114). Their main product is growth hormone, which they begin to produce at E15.5 (107, 115). Lactotropes, which make up 15-25% of pituitary cells (114), begin producing prolactin, the hormone responsible for milk production, around E15.5 as well (107), although prolactin expression is largely upregulated postnatally (102, 115, 116). The last lineage to start producing its hormones is the GATA-2-dependent gonadotrope lineage (112, 117). These make up approximately 5% of the population of the pituitary, and begin synthesizing the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), at around E16.5 (107).

1.1.2 The intermediate pituitary

The intermediate lobe is made up of three cell types, only one of which, the melanotrope lineage, secretes hormones. Melanotropes express the same POMC gene as corticotropes, but it is processed into melanocyte stimulating hormone (MSH) (108, 118), which

promotes production of the skin pigment melanin. The intermediate lobe secretes MSH in human fetal life (119, 120), but is largely absent in adult humans, with few residual MSH-secreting cells (121, 122). In rodents, the intermediate lobe persists throughout adult life.

1.1.3 The posterior pituitary

The posterior lobe is composed of axon terminals of two hypothalamic nuclei, as well as pituitary glial cells known as pituicytes (123). The two hypothalamic nuclei, the supraoptic nucleus (SON) and the paraventricular nucleus (PVN), are composed of cell bodies of magnocellular neurons that synthesize arginine vasopressin (AVP) and oxytocin. The hormones are synthesized in both nuclei, and the neurons synthesize either AVP or oxytocin, but not both (124). AVP stimulates water resorption in collecting ducts of nephrons in the kidney (125), while oxytocin regulates multiple aspects of sexual and reproductive behavior, including parturition and lactation (126).

1.2 Analysis of IGSF1 expression

As the pituitary is implicated in the regulation of nearly every endocrine system, IGSF1 could play a role in any or all of these processes. In order to generate hypotheses about the potential function(s) of IGSF1, I thus attempted to localize its expression more precisely in the three tissues with highest reported levels of IGSF1: the pituitary, the brain, and fetal liver.

2 Methods

Animals

Mice were handled according to federal and institutional (McGill University) guidelines. *Igsf1*-deficient (*Igsf1*^{Δex1}) mice were described previously (39). To generate wild-type (WT) and hemizygous (*Igsf1*^{Δex1/y}) males, *Igsf1*^{Δex1} heterozygous females were mated with WT males. At specified embryonic days pregnant mice were killed by cervical dislocation, animals up to post-natal day 10 were sacrificed by decapitation and older animals by CO₂ asphyxiation. Genomic DNA was extracted by digesting tissue (5 mm of

tail for adult animals, toe for animals before post-natal day 10, or tail and lower limbs for embryos) with 100 µg/ml proteinase K (BioShop, Burlington, Ontario, Canada) in digest buffer (100 mM Tris pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.2% SDS), overnight at 55°C. Samples were centrifuged for 10 min to pellet debris, supernatant was collected, DNA was precipitated with 1 volume of isopropanol, and resuspended in 10 mM Tris pH 8.0. Genotyping and karyotyping PCR to identify WT and *Igsf1*^{Δex1/y} males was performed using primers in Table 3.1. Rat testes were provided by Dr. Bernard Robaire (McGill University). Tissues were further processed for protein lysates, immunofluorescence, or RNA extraction as detailed below.

Cell lines

Thyrotrope-like TαT1 (127, 128) and gonadotrope-like LβT2 cells (127) were a gift from Dr. Pamela Mellon (UCSD, San Diego, California), Somatolactotrope-like GH3 cells (129) were a gift from Dr. Ursula Kaiser (Brigham and Women's Hospital, Boston, Massachusetts), and corticotrope-like AtT20 cells (130) were a gift from Dr. Jacques Drouin (IRCM, Montreal, Quebec, Canada). Folliculostellate-like TtT/GF cells (131) were obtained from RIKEN cell bank (Japan). HepG2 cells (132) were purchased from American Type Culture Collection (ATCC, Rockville, MD). All cell lines were cultured in DMEM with 4.5 g/L glucose, 10% FBS (Wisent, St. Bruno, Quebec, Canada), and 1x Antibiotic-Antimycotic Solution (Wisent) at 37°C/5% CO₂. TαT1 cells were additionally cultured on a 1:20 dilution of matrigel (BD Biosciences, Mississauga, Ontario, Canada) in sterile phosphate buffered saline (PBS, Wisent). Total protein lysates were harvested in RIPA buffer (150 mM NaCl, 50 mM NaF, 10 mM NaPO₄, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) plus protease inhibitors (CompleteMini, Roche, Indianapolis, IN). Protein concentration was determined using Protein Assay Dye Reagent (Bio-Rad, Mississauga, Ontario, Canada) according to manufacturer's instructions. Protein lysates from hypothalamic cell lines mHypoE44 (133), HypoA2/12, mHypoA2/22, and mHypoA2/30 (134) were a gift from Dr. Denise Belsham (University of Toronto, Ontario, Canada).

Tissue protein extraction

All tissues were immediately frozen on dry ice following dissection. Brains regions were then sectioned on a freezing microtome according to the following coordinates (135): cortex – anteroposterior interaural 4.90 mm to 3.90 mm, above the corpus callosum; hippocampus – anteroposterior interaural 1.0 mm to 0.5 mm, borders ascertained visually; hypothalamus – anteroposterior-interaural 3.94 mm to 0.94 mm, dorsoventral-interaural 2.5 mm and below, 1.44 mm lateral on either side; thalamus – like hypothalamus but 2.5 mm and above to the corpus callosum. Frozen pituitaries and brain regions were crushed with a hand-held homogenizer. All other organs were weighed and ground up with a mortar and pestle on liquid nitrogen. 1-2 μ l of RIPA buffer was added per mg of tissue. Tissues were then freeze-thawed in liquid nitrogen and a 37°C heat block three times, centrifuged to pellet debris, supernatant collected and protein concentration measured using Protein Assay Dye Reagent (Bio-Rad, Mississauga, Ontario, Canada) according to manufacturer's instructions. Lysates were deglycosylated and western blotted as described below.

Deglycosylation

10-20 μ g of protein was denatured in 1X Glycoprotein Denaturing Buffer (New England Biolabs, Whitby, Ontario, Canada) for 10 min, followed by deglycosylation with 500 units of either Endoglycosidase H (EndoH) or PNGase F (New England Biolabs) for 1–2 hr at 37°C, following manufacturer's protocol. Protein lysates were analyzed by western blotting.

Western blotting

Protein lysates were denatured at 70°C for 20 min in Laemmli buffer and a final concentration of 2% 2-mercaptoethanol, and resolved by SDS-PAGE on 8% Tris-glycine gels. The proteins were transferred to nitrocellulose membranes (25 V, 1.5 hr), the membranes blocked with 5% milk in TBST: 0.05% Tween-20 in Tris-buffered saline

(TBS; 10 mM Tris pH 8.0, 150 mM NaCl), and incubated overnight at 4°C in rabbit anti-IGSF1-CTD antiserum (1:1,000, a gift from Dr. Peter Scheiffele, (63)) in blocking buffer. Next day, membranes were washed 3x15 min in TBST and incubated in a 1:3,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Mississauga, Ontario, Canada) in blocking buffer for 1 hr at room temperature. The membranes were washed 3x15 min in TBST, incubated in ECL Plus reagent (GE Healthcare, Baie d'Urfe, Quebec, Canada) according to manufacturer's instructions and exposed to X-ray film (GE Healthcare).

Pituitary immunofluorescence

On day E18.5, embryo heads were dissected, fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4) at 4°C, and embedded in paraffin. Postnatal animals were sacrificed by CO₂ asphyxiation, pituitaries dissected, fixed overnight in 4% PFA in PBS at 4°C, and embedded in paraffin. Five µm sections of either whole heads or isolated pituitaries were mounted on Superfrost Plus micro slides (VWR, Mississauga, Ontario, Canada). Sections were deparaffinized, rehydrated, and antigen retrieval was performed as follows: microwave treatments of 5, 4, and 3 min each in 10 mM sodium citrate (pH 6.0) at 240 W, followed by 15 min at room temperature and an additional 5 min microwaving. Nonspecific binding was blocked using 5% bovine serum albumin (BSA) in PBS with 0.2% Tween-20 (PBST). All pituitary hormone antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Primary antibodies were diluted in blocking buffer (IGSF1-CTD 1:500, TSH (sc7815) and LH (sc7824) 1:400, GH (sc10365) and prolactin (sc7805) 1:200), and sections were incubated overnight at 4°C. Next day, sections were washed 3x with PBST, incubated in horse-anti-goat biotin antibody (Vector, Burlington, Ontario, Canada) diluted 1:150 in blocking buffer for 1 hr at 22°C, washed 3x with PBST, incubated in blocking buffer containing Streptavidin-Texas Red (1:400, Invitrogen, Burlington, Ontario, Canada) and goat anti rabbit Alexa 488 (1:600, Invitrogen) for 1 hr at 22°C, washed 3x with PBST, and mounted using Pro-Long Gold reagent with DAPI (Invitrogen).

Probe generation for in situ hybridization

Digoxigenin-labeled sense and antisense probes against *Fshb* and *Igsf1* were generated using the SP6, T7, and T3 Riboprobe System (Promega), following manufacturer's instructions. The *Igsf1* probe against exons 19-20 was cloned into the pGEM-T Easy vector (Promega), which contains T7 (antisense) and SP6 (sense) polymerase promoters on either end of the multiple cloning site. The plasmid was linearized using either *SacI* (for antisense probe) or *NcoI* (for sense probe) to allow for generation of run-off transcripts. The *Fshb* probe was generated from a purified PCR product that was amplified using primers containing T7 (antisense) and T3 (sense) promoter fragments (for primers see Table 3.3). Digoxigenin label was introduced into the nucleotide mixture (3.5 mM digoxigenin-11-UTP (Roche), 6.5 mM UTP, 10 mM rATP, 10 mM rCTP, 10 mM rGTP) used in the reaction. Completed reactions were purified using G-50 columns (GE Healthcare). Probes were stored at -80°C until use.

In situ hybridization

Adult WT and *Igsf1*^{Δex1} pituitaries were dissected and snap-frozen on dry ice. Frozen pituitaries were embedded in Tissue-Tek OCT compound (Sakura Finetek, Netherlands), sliced into 20 μm sections on a cryostat, and mounted on Superfrost Plus micro slides (WVR). Slides were dried for 10 minutes at 22°C, and stored at -80°C until use. Frozen slides were thawed in DEPC-treated PBS for 3 minutes, followed by 20 minutes in 4% PFA in PBS, 3x 3 min washes in PBS, and a 10 minute wash in proteinase K solution (50 mM Tris pH 8.0, 5 mM EDTA, 1 ug/mL proteinase K). Slides were again incubated in 4% PFA for five minutes, washed 3x in PBS for 3 minutes each, incubated in acetylation solution (124 mM triethanolamine, 224 mM NaOH, 0.25% acetic anhydride) with vigorous shaking at 22°C, and washed again 3x 5 min in PBS. Slides were incubated in hybridization solution (50% formamide, 5xSSC, 5x Denhardt's, 100 ug/mL salmon sperm DNA, 375 ug/mL yeast tRNA) for 2 hr at 22°C. Note: final concentration of 5xSSC was obtained by using 20xSSC stock (3 M NaCl, 300 mM sodium citrate), and final concentration of 5x Denhardt's solution was obtained by using 50x Denhardt's stock (1% Ficoll-400, 1% polyvinylpyrrolidone, 1% Bovine serum albumin-Fraction V in

H₂O). 400 ng/mL of sense and antisense probes against *Igsf1* and *Fshb* (described above) were added to hybridization solution (100 µl solution per slide), denatured for 10 minutes at 70°C, quenched on ice, and applied to the slides. Slides were covered with cover slips, and incubated in a humidified chamber at 57°C overnight. Next day, slides were washed in 5xSSC at 60°C for 5 minutes, followed by two washes of 30 min each in 0.2x SSC at 60°C, 5 min in 0.2x SSC at 22°C, and 5 min in B1 buffer (100 mM Tris pH 7.5, 150 mM NaCl). Slides were then incubated in blocking buffer (10% normal goat serum in B1 buffer) for 1 hr at 22°C, followed by incubation in the primary antibody solution, consisting of 1:7,500 dilution of anti-Digoxigenin antibody conjugated to alkaline phosphatase (Roche, 11093274910) in blocking buffer at 4°C overnight. Next day, slides were washed 3x 20 min in B1 buffer, incubated in alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂) for 5 min at 22°C, followed by incubation in a 1:50 dilution of NBT/BCIP (Roche) in alkaline phosphatase buffer, in the dark, at 22°C, from 2 hr to overnight, until signal could be visually identified.

Fluorescence-activated cell sorting (FACS)

Whole pituitaries were collected from 8-10 week-old male and female *Gnrhr*^{GRIC/+}; *ROSA26*^{eYFP/+} mice (136-138), which selectively express YFP in gonadotrope cells of the anterior pituitary. The pituitaries were enzymatically dispersed as previously described (139), filtered with a 40 µm nylon mesh and FACS was performed on the cell suspension using a FACS Aria cell sorter (BD Biosciences, San Jose, California). Total RNA was extracted from YFP-positive (gonadotropes, YFP+) and YFP-negative (non-gonadotropes, YFP-) purified fractions using the AllPrep RNA/DNA extraction kit (Qiagen, Toronto, Ontario, Canada) according to the manufacturer's instructions. RT-qPCR was performed in order to analyze expression of *Igsf1* in the two fractions, as described below.

RNA extraction, reverse-transcription, quantitative PCR, and semi-quantitative PCR

Murine pituitary, brain, and liver RNA, and RNA from cell lines was extracted using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada), following the manufacturer's

instructions. Two µg of RNA were reverse-transcribed into cDNA using 50 ng random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wisconsin). RT- controls were processed in parallel without reverse transcriptase. Contaminating DNA was removed prior to reverse-transcription using RQ1 DNase (Promega). Four µl of cDNA was used per semi-quantitative PCR reaction, using primers listed in Table 3.2. Quantitative PCR was performed on 1 µl of cDNA using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Burlington, Ontario, Canada) and 0.4 pmol of *Igsf1* or *Rpl19* primers (Table 3.2) on a Corbett Rotor-Gene 6200 HRM (Corbett Life Science), according to Invitrogen's real-time cycling protocol. Transcript levels were normalized relative to the housekeeping gene ribosomal protein L19 (*Rpl19*). Results were analyzed using the $2^{-\Delta\Delta C_t}$ method (140, 141).

Northern blotting

Human testicular RNA was obtained from Dr. Sarah Kimmins (McGill University). RNA was extracted from murine tissues using TRIzol reagent (Invitrogen), according to manufacturer's instructions. RNA was resuspended in water and stored at -80°C. 10-20 µg of each RNA were northern blotted as previously described (39). To generate the cDNA probes, the cDNAs spanning either exons 5-6 or 18-20 of murine *Igsf1* were amplified by RT-PCR from murine pituitary using primers listed in Table 3.3. The purified PCR products were labelled with ^{32}P -dCTP (Perkin-Elmer, Montreal, Quebec, Canada) using Ready-to-Go beads (GE Healthcare, Baie d'Urfe, Quebec, Canada), following the manufacturer's instructions.

3 Results

Igsf1 expression in the pituitary gland

I detected a robust signal corresponding to IGSF1-CTD by western blot in the pituitary glands of wild-type (WT), but not *Igsf1*-deficient (*Igsf1*^{Δex1}) male mice using the IGSF1-CTD antibody (Figure 3.2A). However, several other bands (indicated by asterisks) were observed in both WT and *Igsf1*^{Δex1} pituitaries, suggesting the antibody cross-reacts with additional unidentified proteins. I next sought to determine which cell lineages in the

pituitary gland express IGSF1 by immunofluorescence using the IGSF1-CTD antibody in adult pituitary sections. I compared sections from male WT and *Igsf1*^{Δex1} mice, in order to differentiate between specific and non-specific signal in pituitary sections. I used exclusively male pituitaries, as pituitary *Igsf1* expression is higher in male than female rodents, at least at the mRNA level (39, 98). I observed some membrane-like signal in WT pituitaries (Figure 3.2B), which was also present to a similar extent in the *Igsf1*^{Δex1} mice. I also tried *in situ hybridization* in adult pituitaries, however no difference in signal intensity was detected between the two genotypes, although a positive control probe (*Fshb*) showed a robust signal (Supplementary Figure S3.1).

I then analyzed male embryonic (E18.5) pituitaries. A clear difference could be seen between WT and *Igsf1*^{Δex1} pituitaries, with intense fluorescent signal in the WT anterior lobe, and no distinct signal in *Igsf1*^{Δex1} pituitaries. No clearly identifiable signal was seen in either the intermediate or posterior lobes of WT mice (Figure 3.2C).

Having obtained a positive IGSF1-CTD signal in the anterior lobe of WT E18.5 embryos, I next attempted to co-localize IGSF1-CTD expression with different markers of the anterior pituitary lineages. I first analyzed the POU1F1 lineages (thyrotropes, somatotropes, and lactotropes), for reasons that will be discussed in more detail in **Part II** of the thesis. IGSF1-CTD signal was present in some thyrotropes, although most TSH-positive cells did not have an intense IGSF1-CTD signal (Figure 3.3A). IGSF1-CTD co-localized extensively with GH, the marker for somatotropes. However, as with thyrotropes, I could also identify cells that expressed GH but not IGSF1-CTD (Figure 3.3A). I also attempted to co-localize IGSF1-CTD with lactotropes, using prolactin as a lactotrope marker. Because prolactin is largely upregulated postnatally (115, 116), very few lactotropes could be detected in E18.5 pituitaries; however, the few lactotropes I could detect co-localized with IGSF1-CTD (Figure 3.3A). I also analyzed IGSF1-CTD expression in gonadotropes using LH as a marker, but did not observe any co-localization between IGSF1-CTD and LH (Figure 3.3A). I attempted to localize IGSF1-CTD to folliculostellate cells using the marker protein S-100 (142), but failed to detect S-100

signal in E18.5 pituitaries, likely because S-100 is largely expressed postnatally (143). Corticotropes were not analyzed.

IGSF1 had previously been localized to gonadotropes (35, 98). As I did not observe colocalization of LH with IGSF1-CTD, I employed an alternate approach to determine whether *Igsf1* is expressed in gonadotropes. I performed RT-qPCR on purified gonadotrope cells from *Gnrhr*^{GRIC/+}; *ROSA26*^{eYFP/+} mice (136), and found that *Igsf1* was not enriched in the gonadotrope population relative to non-gonadotropes (Figure 3.3B).

Further support for expression of IGSF1 in thyrotropes emerged from cell lines. Several cell lines have been developed that are representative of the various anterior pituitary lineages. These include the gonadotrope-like cells (LβT2), thyrotrope-like cells (TαT1), somatolactotrope-like cells (GH3), corticotrope-like cells (AtT20), and folliculostellate-like cells (TtT/GF). Of these, only the TαT1 cells produced a clear, typical IGSF1-CTD-like signal in western blotting (Figure 3.4A, lane 2), with two bands that could be deglycosylated to reveal the unglycosylated protein at approximately 90 kDa (Figure 3.4B, lane 2). The antibody cross-reacted with proteins in the GH3 and AtT20 lysates (Figure 3.4A, lanes 4-5), although they did not resemble the typical IGSF1-CTD banding pattern. Upon deglycosylation, the bands in GH3 and AtT20 lysates did not migrate at the predicted size of IGSF1-CTD (Figure 3.4B, lanes 3-6), suggesting they were non-specific. Thus, in the pituitary gland, IGSF1-CTD was only detected in embryonic cells of the POU1F1 lineage.

Igsf1 in the brain

As the IGSF1-CTD antibody cross-reacts with multiple unspecific proteins, and does not appear to be suitable for immunofluorescence in adult tissues, I first analyzed expression of IGSF1-CTD in the brain using western blotting and semiquantitative RT-PCR in WT and *Igsf1*^{Δex1} male mice. Using primers directed against exons 18-20, which amplify both *Igsf1*-1 and *Igsf1*-4, I found *Igsf1* was detectable in whole brain as early as E13.5 (the earliest time point examined), and was detectable in both WT (Figure 3.5A, lane 1) and

Igsf1^{Δex1} animals (Figure 3.5A, lane 3). The signal appeared weaker in the latter, but given the semi-quantitative nature of the analysis, these data do not accurately portray the extent of the difference between genotypes. The IGSF1-CTD protein was only clearly observable in whole brain of WT mice starting at post-natal day 11, and increased in abundance as the animals matured (Figure 3.5B). Because IGSF1-CTD in the brain co-migrates at the same size as another protein that is recognized by the antibody (e.g., see lanes 7-12 in Fig. 3.5B), I used the deglycosylating enzyme PNGaseF to strip IGSF1-CTD of sugars in order to detect the unmodified IGSF1-CTD protein, which does not co-migrate with any non-specific bands (Figure 3.5B, lanes 8,10,12).

I then attempted to localize IGSF1-CTD expression to specific brain regions by sectioning adult brains into sub-regions, and extracting either RNA or protein. At the RNA level, I noted high *Igsf1* expression in the hypothalamus (Figure 3.6A, lane 5), thalamus (lane 9), and hippocampus (lane 13), and a barely detectable signal in the cortex (lane 1) of WT mice. Hypothalamic expression of *Igsf1* in mouse was previously reported during a search for genes implicated in regulating body weight (144). *Igsf1*^{Δex1} mice showed a reduction in signal intensity in each of those areas, although residual signal was still present in hypothalamus (lane 7) and thalamus (lane 11). At the protein level, I observed a robust IGSF1-CTD signal in the hypothalamus (Figure 3.6B, lanes 4-6), lower levels in the thalamus (lanes 7-9) and the hippocampus (lanes 10-12), and no detectable IGSF1-CTD in the cortex of WT mice (Figure 3.6C, lanes 1, 2). *Igsf1*^{Δex1} mice showed significantly diminished levels of IGSF1-CTD in the hypothalamus and thalamus compared with WT mice (Figure 3.6C, compare lanes 5-6 with 7-8, and lanes 9-10 with 11-12). Hypothalamic expression of IGSF1-CTD was also supported by cell line data. A screen of eleven murine hypothalamic cell lines revealed that three adult hypothalamic cell lines, mHypoA-2/12, mHypoA-2/22 and mHypoA-2/30, express IGSF1-CTD (Figure 3.6D, lanes 3-8). A non-expressing cell-line mHypoE-44 (embryonic, lanes 9-10) was included to distinguish between specific and non-specific bands.

I wanted to confirm and extend the western blot results using immunofluorescence on brain sections. Unfortunately, no identifiable signal was seen in the hypothalamus or thalamus in either adult or E18.5 WT brain sections (not shown). However, I found strong IGSF1-CTD signal in E18.5 choroid plexus (Figure 3.7). Importantly, the signal was reduced in *Igsf1*^{Δex1} mice, suggesting the staining is specific for IGSF1-CTD.

Comparison of pituitary and brain Igsf1 mRNA isoforms

I next analyzed whether the various *Igsf1* isoforms are differentially expressed between the pituitary gland and the hypothalamus. I performed northern blotting using probes against various *Igsf1* exons. Using a probe against exons 18-20, which should detect isoforms *Igsf1*-1 and *Igsf1*-4, I found that, consistent with previous reports (35, 39), the pituitary gland contains predominantly *Igsf1*-1 (Figure 3.8A, band a). In contrast, the hypothalamus contains a large proportion of a transcript that migrates at the size of isoform *Igsf1*-4 (band b). If the transcript corresponds to *Igsf1*-4, it would be predicted to be present in *Igsf1*^{Δex1} hypothalami to the same extent as in WT tissue, as *Igsf1*-4 was not ablated in *Igsf1*^{Δex1} mice (39). However, I noted that the transcript is greatly reduced in abundance, although not completely absent in the brain. Similarly, a transcript that is predicted to be *Igsf1*-4 in the pituitary gland, is reduced in intensity, although not completely absent in the *Igsf1*^{Δex1} mice (Figure 3.8A, band b). Finally, a third band around 2 kb is seen in both pituitary and hypothalamus (Figure 3.8A, band c). Although similar in size to *Igsf1*-2, this band cannot correspond to it as the *Igsf1*-2 transcript terminates in intron 5, whereas the probe is specific to exons 18-20. This suggests it may represent a previously unreported isoform.

Igsf1 is predicted to have multiple mRNA isoforms, largely due to alternative splicing. I have detected and cloned an alternate mRNA isoform in the pituitary gland that is alternatively spliced resulting in the deletion of most of exon 12, all of exons 13-17, and the beginning of exon 18 (Figure 3.8B). It is predicted to code for a protein which contains the first five Ig domains, the linker region, and one Ig domain in the CTD. This novel mRNA, if it begins at exon 1, would be predicted to be approximately 2.4 kb in

size. However, attempts to detect the protein product of this isoform have been unsuccessful in heterologous cells. Additional isoforms also likely exist, as performing northern blotting on pituitary RNA using a probe against exons 5-6 reveals the presence of additional bands, as well as the already identified forms *Igsf1*-1, *Igsf1*-2, and *Igsf1*-3 (Figure 3.8C).

Igsf1 in the liver

IGSF1 had previously been reported to be expressed in human fetal liver (33). In order to determine whether these findings extended to mice, I collected murine livers at various ages from E13.5 to adult, and analyzed IGSF1-CTD protein levels by western blot. I observed IGSF1-CTD expression as early as E13.5 (the earliest time point examined) in WT liver (Figure 3.9A, lane 1), noted a significant decrease by postnatal day 10 (lanes 9, 10), and a complete absence of signal in adult liver (lane 13). IGSF1-CTD signal was decreased, but not completely absent in *Igsf1*^{Δex1} mice (Figure 3.9A, lanes 4, 6). Expression of IGSF1-CTD in the liver is also supported by cell line data. The human hepatocellular carcinoma cell line HepG2 was assessed for *IGSF1* mRNA and IGSF1-CTD expression. The IGSF1-CTD antibody weakly cross-reacts with the human epitope, making it difficult to study IGSF1-CTD expression in human tissues and cells in detail. However, it is possible to detect a band of the correct size in HepG2 lysates, which is reduced to approximately 90 kDa upon deglycosylation with PNGaseF. *IGSF1* is also detectable in HepG2 cells by RT-PCR (Figure 3.9 B,C).

Glycosylation pattern of Igsf1

IGSF1 is heavily glycosylated, with multiple N-linked glycans (see Chapter 2, Figures 2.5 and 2.6). I compared the glycosylation pattern of IGSF1-CTD in the pituitary, hypothalamus, and fetal liver, and found that IGSF1-CTD migrates differently on SDS-PAGE depending on the source tissue (Figure 3.10). Pituitary-derived IGSF1-CTD migrates as a doublet around 140 kDa (lane 1). The upper band is EndoH-resistant (lane 3), and is the mature glycoform, whereas the lower band is EndoH-sensitive (lane 3), and contains high-mannose glycans. IGSF1 from the hypothalamus migrates as a single band,

which is slightly smaller than the upper band of pituitary-derived IGSF1-CTD (lane 4). Hypothalamic IGSF1-CTD can be partially deglycosylated with EndoH (lane 6), and completely deglycosylated with PNGaseF (lane 5), suggesting that the hypothalamus contains a ‘hybrid’ IGSF1-CTD glycoform, with a combination of high-mannose and complex sugars. Finally, embryonic liver-derived IGSF1-CTD migrates as a single band roughly the size of the upper band of pituitary-derived IGSF1-CTD (lane 7). This band is EndoH-resistant (lane 9), indicating that the liver contains only a mature glycoform of IGSF1-CTD.

4 Discussion

Using various approaches, I demonstrated that *Igsf1*/IGSF1-CTD is expressed in several murine tissues, including the pituitary gland, hypothalamus and thalamus, and fetal liver. Further, I localized IGSF1-CTD expression to the POU1F1 lineages of the anterior pituitary gland: the thyrotropes, somatotropes, and lactotropes. I also noted a difference in the abundance of different *Igsf1* mRNA isoforms between pituitary and hypothalamus, and a differential glycosylation pattern of IGSF1-CTD between pituitary, hypothalamus, and fetal liver, the three tissues that express IGSF1-CTD at the highest levels.

IGSF1-CTD is highly expressed in WT adult pituitary, and virtually absent in pituitaries of *Igsf1*^{Δex1} mice, as shown by western blotting. Thus, it is surprising that we did not observe a difference in signal intensity between the two genotypes by immunofluorescence. A weak membrane-like signal is present in both genotypes to a similar extent (Figure 3.2). It is likely non-specific, as the antibody cross-reacts with multiple unidentified proteins in western blots, or, as *Igsf1*^{Δex1} mice retain the *Igsf1*-4 isoform (39), the antibody could recognize residual IGSF1-CTD.

In contrast to the adult tissues, we observed a clear difference in IGSF1-CTD immunoreactivity between pituitaries of E18.5 WT and *Igsf1*^{Δex1} mice. The fact that IGSF1-CTD is detectable by immunofluorescence in embryonic, but not adult pituitary, although it is detectable by western blot in the latter, suggests that as pituitary

development progresses, the epitope that is recognized by the CTD antibody becomes inaccessible (i.e. masked). Notably, while I could not detect IGSF1-CTD by immunofluorescence in WT E18.5 hypothalami, the signal in the choroid plexus was not masked in those same sections, suggesting the epitope masking process might be specific to individual tissues, regions, or cells. We have not confirmed IGSF1-CTD expression in the choroid plexus by western blot, thus it is also possible the signal is non-specific.

Although we localized IGSF1-CTD to the POU1F1 lineages of the pituitary, we also noted many TSH, GH, and prolactin-expressing cells that lacked a strong membrane IGSF1-CTD signal at E18.5. The partial co-localization of IGSF1-CTD to these cell types suggests one of two possibilities. The first is that IGSF1-CTD is only expressed in a sub-population of these cells. The second, perhaps more likely possibility, is that the CTD epitope has already become masked in some of these cells, precluding its recognition by the antibody. To address this, I tried double-label immunofluorescence with IGSF1-CTD and TSH antibodies at an earlier time point (E15.5), but found it difficult to detect TSH. Indeed, the limitation of this approach is that most of the markers of terminal differentiation and cellular maturation (i.e. their principal hormone products) are not expressed before E15.5 in mice (106, 107, 113, 115). An alternative approach would be to co-localize IGSF1 with POU1F1 (Pit-1), which is expressed as early as E13.5 (145) or, at even earlier time points, with PROP1. PROP1 (Prophet-of-Pit-1) is transiently expressed between E10.5-14.5 in the same lineages (146) and is required for POU1F1 expression (146-149). The putative masking of the IGSF1-CTD epitope also makes it difficult to conclude with certainty that the other pituitary lineages (gonadotropes, folliculostellate cells) do not also express the protein. Ultimately, this could be assessed by purifying different pituitary cell types through genetic labeling and sorting (136).

The CTD epitope could be masked due to interaction with another protein, or perhaps with IGSF1 itself (homophilic interaction) (56), preventing the antibody from accessing the epitope in its native state. IGSF1 is also extensively glycosylated (see Chapter 2, Figures 2.5, 2.6), and changes in glycan composition throughout development have been

documented for multiple proteins (150-154). Access of the antibody to the epitope may thus be hampered by changes in the glycosylation pattern of IGSF1 (155-159). While I assessed other commercially-available antibodies, the IGSF1-CTD antibody is the only one to detect proteins of the correct size on western blot. Certain commercial antibodies for IGSF1 target epitopes in the NTD, which of course precludes the analysis of the CTD. In our experience, the NTD is much more difficult to detect, and at least two commercial antibodies failed to detect proteins of the predicted molecular weight in western blots performed with murine pituitary lysates. One strategy that might permit detection of IGSF1-CTD in adult tissues is to generate an antibody against the short C-terminal intracellular tail, as IGSF1-CTD can be detected by immunofluorescence using an antibody against a C-terminal 3xHA epitope tag in heterologous cells transfected with the Myc-IGSF1-1-3xHA construct. We also attempted *in situ* hybridization to localize *Igsf1* to specific cell types and brain areas, and found that, as in the case of the protein, the signal was not detectable in the adult tissues, despite robust *Igsf1* expression seen by northern blot. Interestingly, a collaborator has noted that while his lab detected *Igsf1* mRNA in murine Rathke's pouch at (E12.5) using *in situ* hybridization, the signal was not detected after E18.5; the reasons for this are unclear at present time (J.P. Martinez-Barbera, personal communication). Another approach to bypass the difficulties associated with indirect methods of protein or RNA detection is to generate transgenic reporter mice, whereby we could, for example, detect the expression of a fluorescent protein driven by the *Igsf1* promoter (160).

Consistent with previous reports, expression of *Igsf1* isoforms differs depending on the tissue (35, 98). Our data suggest that, in contrast to the pituitary where *Igsf1*-1 predominates, the major transcript in the hypothalamus is a shorter isoform, which migrates at a similar size to *Igsf1*-4 (Figure 3.8A). Interestingly, the expression of this isoform is reduced, but not completely absent in *Igsf1*-deficient mice. *Igsf1*-4 is not predicted to be ablated by the deletion of the first exon, the strategy that was used to generate these mice (39). Bernard et al. previously reported that its expression was comparable in pituitaries of WT and *Igsf1*-deficient mice, although given the semi-

quantitative nature of the analyses, subtle downregulation of *Igsf1*-4 may have been missed (39). If *Igsf1*-4 is the most abundant isoform in the hypothalamus, then it is downregulated in hypothalami of *Igsf1*-deficient mice, although the residual signal is more abundant than in the pituitary gland. Indeed, while IGSF1-CTD is almost undetectable by western blot in pituitaries of *Igsf1*^{Δex1} mice, there is some detectable IGSF1-CTD in their hypothalami (Figure 3.6C). Thus, it may not be possible to assess IGSF1 function in the brain using this particular mouse strain. Alternately, this transcript may be a novel, previously unreported *Igsf1* isoform that encodes IGSF1-CTD. Cloning of this isoform will be necessary to determine its identity.

The likely existence of novel *Igsf1* transcripts (see Figure 3.8) is not surprising, as *Igsf1*, like other members of the immunoglobulin superfamily, is predicted to be extensively alternatively spliced (http://www.fruitfly.org/seq_tools/splice.html) (161). In addition to identifying the four known *Igsf1* isoforms in mice (39), Bernard et al. also identified a unique *Igsf1* transcript in rat testes which is predicted to contain additional sequence 5' of the canonical exon 1, and observed other isoforms in both pituitary and testes, which they did not characterize (98). The existence of multiple *Igsf1* isoforms, which could be differentially regulated depending on the tissue, developmental stage (162), and physiological or pathological conditions (163), may reflect the diverse roles IGSF1 plays in the lifetime of an organism. In the future, each of these transcripts will have to be studied in detail to uncover the complete array of IGSF1 forms and functions.

Finally, I noted that the pituitary, hypothalamus, and fetal liver each exhibit distinct IGSF1-CTD glycosylation patterns. Glycosylation has been implicated in multiple processes, including quality control during protein synthesis and folding (99, 164, 165), oligomer assembly (166), both global and local protein stability (167, 168), and protein-protein interaction (169-171). It is tempting to speculate that differentially glycosylated forms of IGSF1-CTD reflect adjustments for optimal protein function in each tissue, or perhaps for enhancing protein-protein interactions.

In summary, our data localize IGSF1 expression to the POU1F1 lineages in the murine pituitary gland, as well as the hypothalamus and fetal liver. It also reveals the diversity of transcripts that can be generated from the *Igsf1* gene via alternative splicing and alternative promoters, with added complexity generated by differential glycosylation, which indicates that IGSF1 may have multiple, complex functions that await unveiling.

Figure legends

Figure 3.1 Diagram of murine pituitary at embryonic day 18.5 (E18.5). (A) Coronal section of murine pituitary shows the three major lobes and remnants of Rathke's pouch. (B) Pituitary lineages of the anterior and intermediate lobes emerge at specific time points during murine embryonic development. The final hormone products are shown in bold. *Pou1f1*, a transcription factor required for development of thyrotropes, somatotropes, and lactotropes is indicated.

Figure 3.2 IGSF1-CTD is detectable in embryonic, but not adult tissue sections. (A) Protein lysates were harvested from individual WT and *Igsf1*^{Δex1} male pituitaries, run on SDS-PAGE, and immunoblotted with IGSF1-CTD antibody. β-actin (ACTB) was used as a loading control. Non-specific bands are indicated by *. (B,C) Immunofluorescence was performed using the IGSF1-CTD antibody on sections from (B) adult WT and *Igsf1*^{Δex1} male pituitaries, and (C) E18.5 WT and *Igsf1*^{Δex1} male pituitaries. Control sections were processed without inclusion of the primary antibody.

Figure 3.3 Localization of IGSF1-CTD expression in specific anterior pituitary cell types. (A) Immunofluorescence was performed on E18.5 WT male pituitary sections to detect IGSF1-CTD (green) and various anterior pituitary hormone markers (red: TSH, GH, prolactin, or LH). Arrows indicate colocalization. (B) RNA was extracted from YFP+ (gonadotrope) and YFP- (non-gonadotrope) cells, reverse-transcribed, and subject to RT-qPCR analysis to assess levels of *Igsf1* in each population. Data reflect mean of two experiments, relative to *Igsf1* levels in YFP+ cells.

Figure 3.4 IGSF1-CTD is expressed in thyrotrope-like TαT1 cells. (A) Protein lysates from cell lines representative of various anterior pituitary lineages (TαT1 – thyrotrope-like; LβT2 – gonadotrope-like; GH3 – somatolactotrope-like; AtT20 – corticotrope-like; TtT/GF – folliculostellate) were run on SDS-PAGE and immunoblotted with the IGSF1-CTD antibody. Tubulin was used as a loading control. (B) GH3 and AtT20 lysates were

deglycosylated to determine whether the bands seen in (A) are alternately-glycosylated forms of IGSF1-CTD. TaT1 cells were included in order to compare the banding pattern.

Figure 3.5 *Igsf1*/IGSF1-CTD is expressed in the murine brain. (A) RNA was extracted from embryonic day 13 and 18 brains from WT and *Igsf1*^{Δex1} mice. Semiquantitative RT-PCR was performed to assess *Igsf1* expression using primers in exons 18 and 20. RT+: reverse-transcriptase was included in reaction. RT-: reactions lacking reverse-transcriptase were used as controls for genomic DNA contamination. *Rpl19* was used as a loading control. (B) Protein lysates were extracted from WT brains at E13, E19, and post-natal days 5, 11, 19 and in adulthood. Lysates were deglycosylated with PNGaseF, run on SDS-PAGE and immunoblotted with the IGSF1-CTD antibody.

Figure 3.6 *Igsf1*/IGSF1-CTD is highly expressed in the hypothalamus. (A) Cortices, hypothalami, thalami and hippocampi were dissected from adult WT and *Igsf1*^{Δex1} male mice and RNA was extracted. Semiquantitative RT-PCR was performed to assess *Igsf1* expression using primers in exons 18 and 20. (B, C) Protein lysates were extracted from adult pituitaries, hypothalami, thalami, hippocampi, and cortices. Lysates were deglycosylated with PNGaseF or EndoH, run on SDS-PAGE and immunoblotted with the IGSF1-CTD antibody. (D) Protein lysates from murine hypothalamic cell lines (mHypoA 2/12, mHypoA 2/22, mHypoA 2/30 and mHypoE 44) were processed as in (B). CHO cells transfected with murine IGSF1-1 construct were included in order to compare the banding patterns.

Figure 3.7 IGSF1 is expressed in embryonic choroid plexus. Immunofluorescence was performed on brain sections from E18.5 male WT and *Igsf1*^{Δex1} mice using the IGSF1-CTD antibody. Control sections were processed without inclusion of the primary antibody.

Figure 3.8 Expression of *Igsf1* isoforms differs between pituitary and hypothalamus. (A) Northern blotting was performed on pituitary and hypothalamic RNA from adult WT

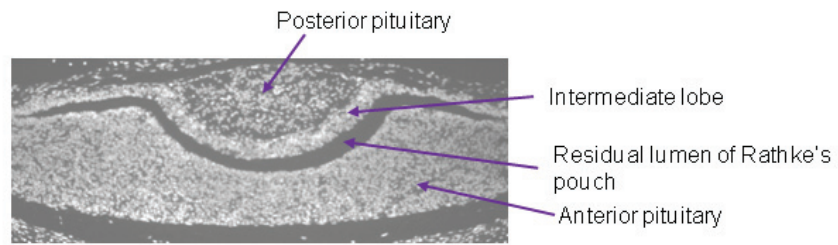
and *Igsf1*^{Δex1} mice using a probe corresponding to exons 18-20. To equalize signal intensity, 10 μg of pituitary RNA and 20 μg of hypothalamic RNA were loaded onto the gel. *Rpl19* was used as a loading control. Band a is predicted to correspond to *Igsf1*-1, band b to *Igsf1*-4, and the identity of band c is unknown. **(B)** Primers used to clone full-length *Igsf1*-1 coding sequence from murine pituitary cDNA also amplified a smaller band. The band was cloned and sequenced. Diagram illustrates mRNA and predicted protein structure of the novel isoform. Question mark at 5' end indicates uncertainty as to the transcriptional start site of the novel isoform. **(C)** Northern blotting was performed on pituitary RNA from adult WT and *Igsf1*^{Δex1} mice using a probe targeted against exons 5-6. Arrows indicate isoforms *Igsf1*-1, *Igsf1*-2, and *Igsf1*-3, as well as two additional isoforms.

Figure 3.9 IGSF1 is expressed in fetal, but not adult liver. **(A)** Protein lysates were extracted from WT and *Igsf1*^{Δex1} livers at E13, E19, and post-natal days 1, 5, 10 and in adulthood. Lysates were run on SDS-PAGE and immunoblotted with IGSF1-CTD antibody. **(B)** Lysates from HepG2 cells were processed as in (A). **(C)** RNA was extracted from HepG2 cells, and semiquantitative RT-PCR was performed to assess *Igsf1* expression using primers in exons 1 and 4.

Figure 3.10 IGSF1-CTD has distinct glycosylation patterns in different tissues. Protein lysates from WT adult male pituitary, hypothalamus, and E13 liver were deglycosylated with PNGaseF or EndoH, run on SDS-PAGE and immunoblotted with IGSF1-CTD antibody.

Supplementary Figure S3.1 *Igsf1* was not detected in adult pituitary glands using *in situ* hybridization. **(A)** *In situ* hybridization was performed on WT and *Igsf1*^{Δex1} adult pituitary sections using antisense and sense (negative control) probes against exons 19-20 of *Igsf1*. **(B)** A probe against *Fshb* was used on WT pituitaries as a positive control.

A



B

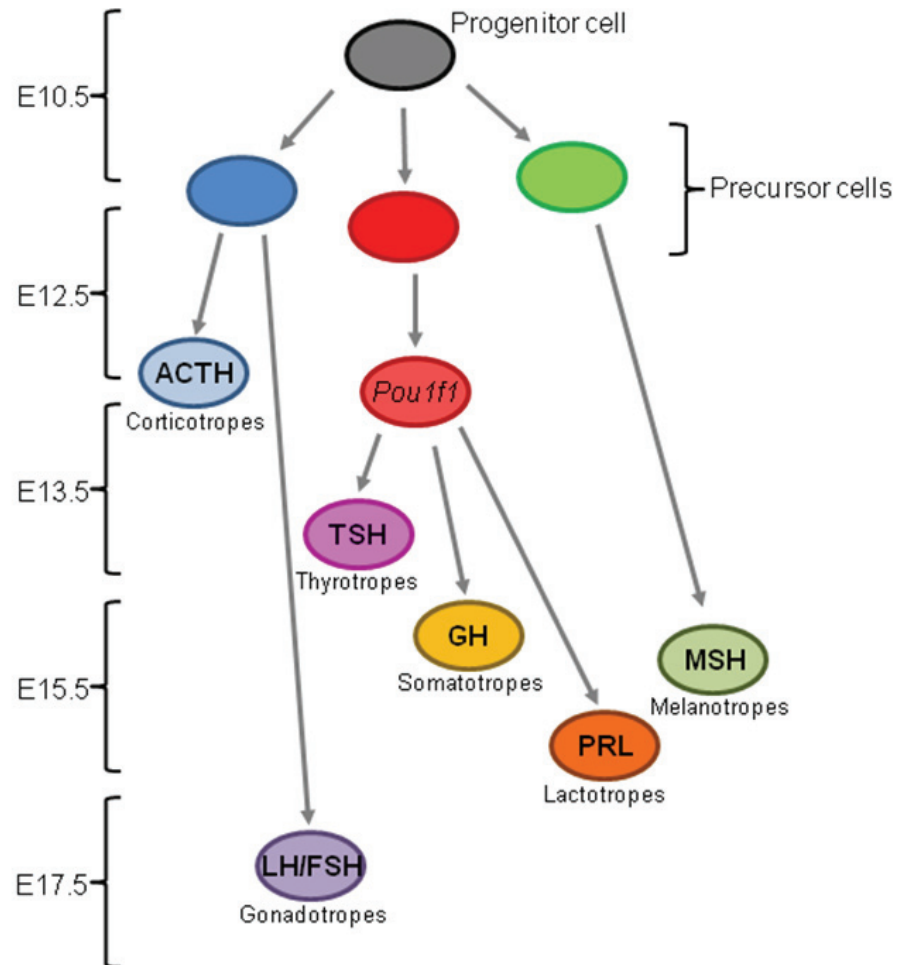


Figure 3.1

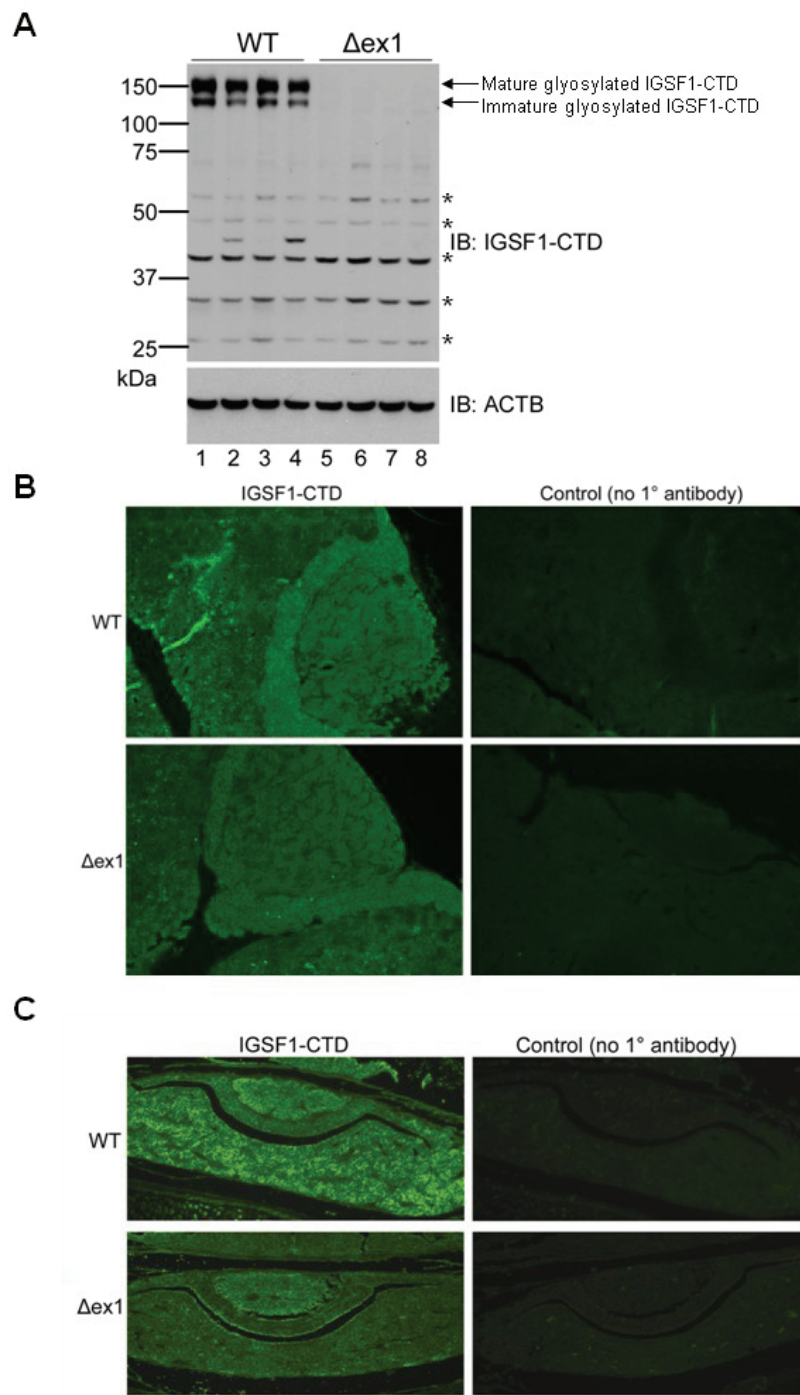
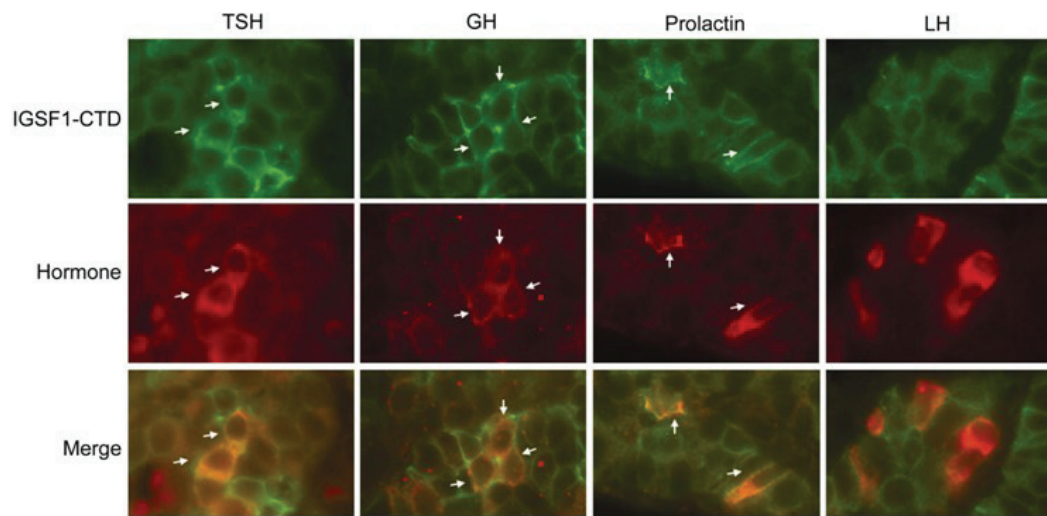


Figure 3.2

A



B

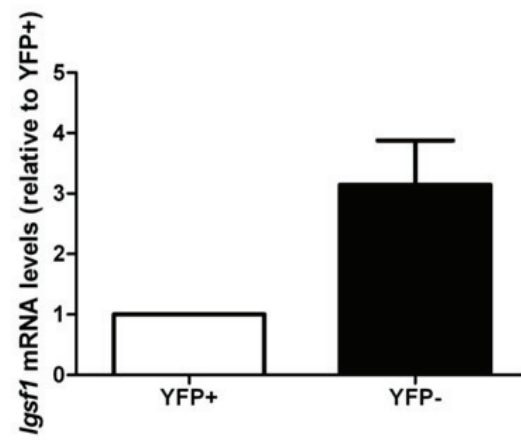
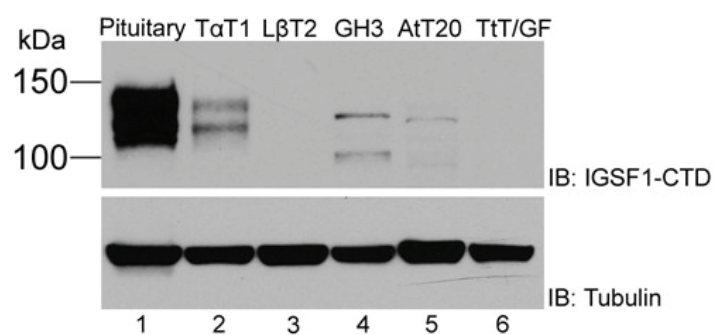


Figure 3.3

A



B

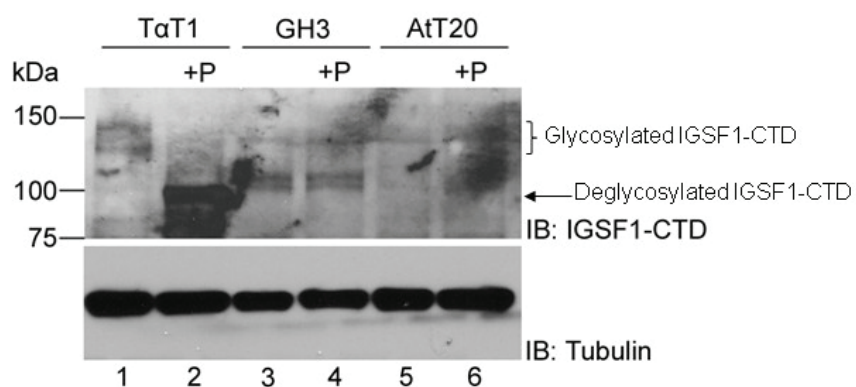


Figure 3.4

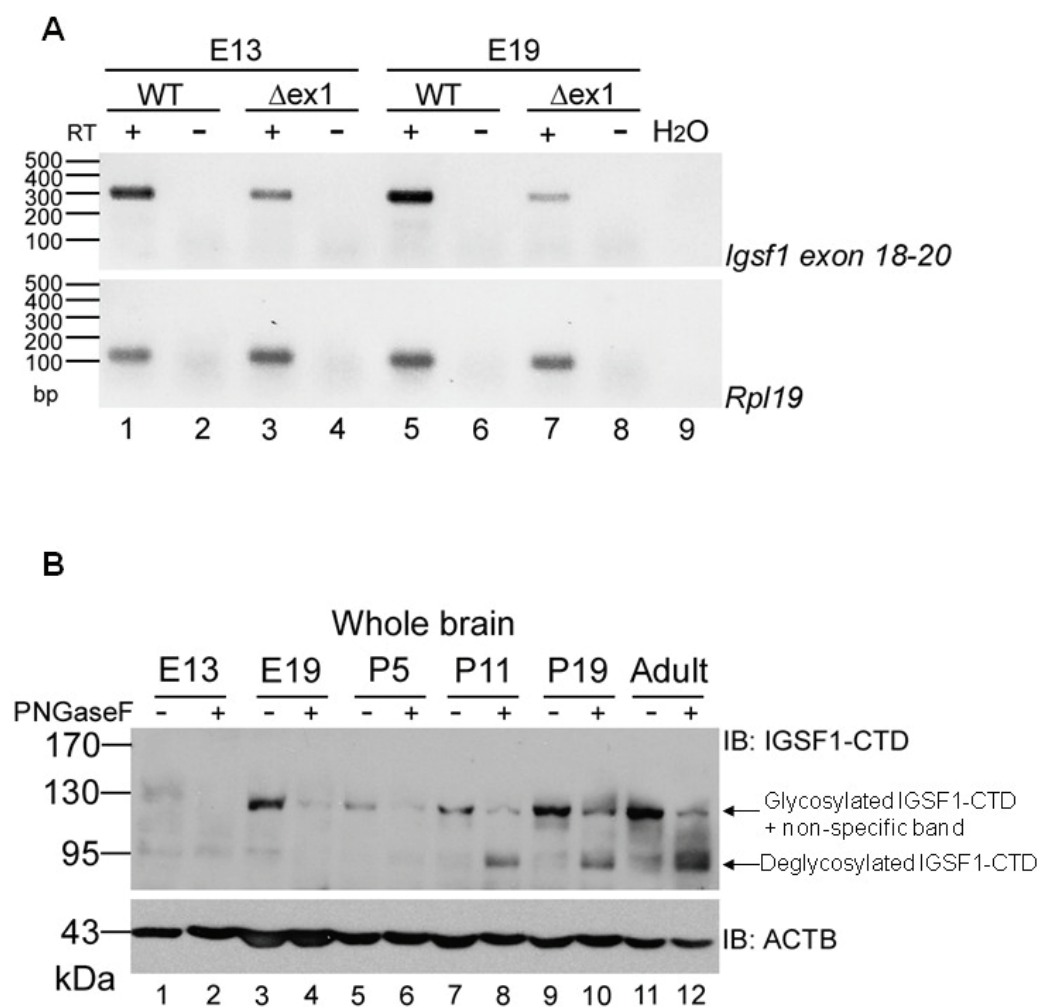


Figure 3.5

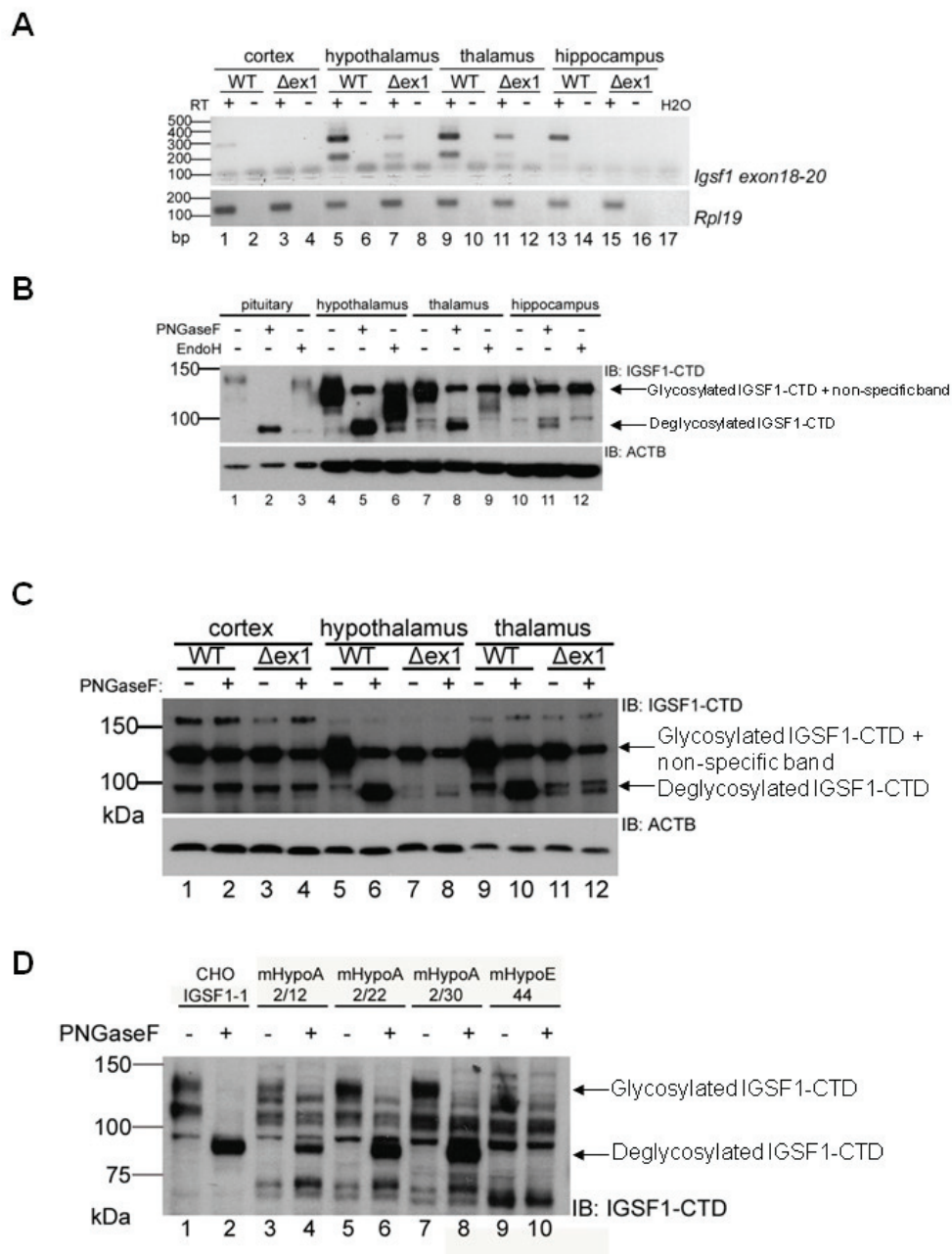


Figure 3.6

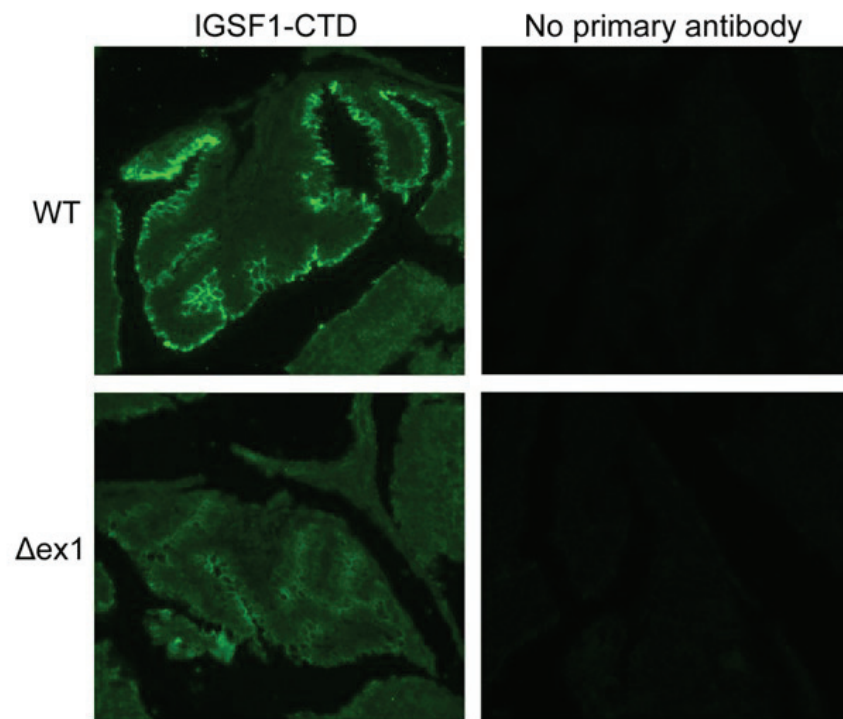


Figure 3.7

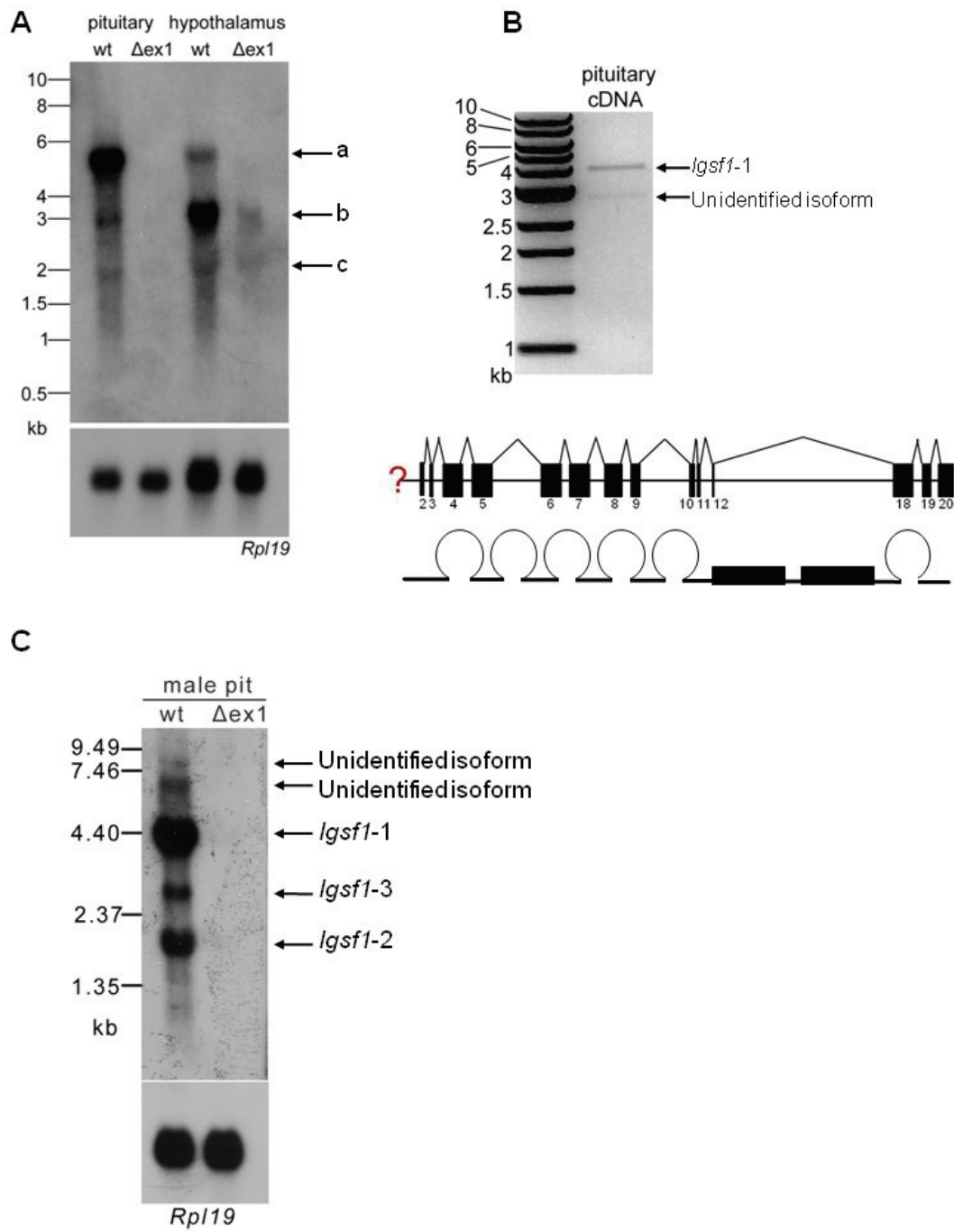
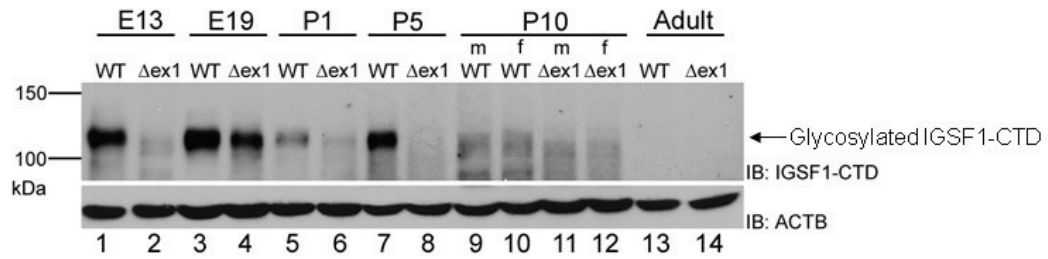
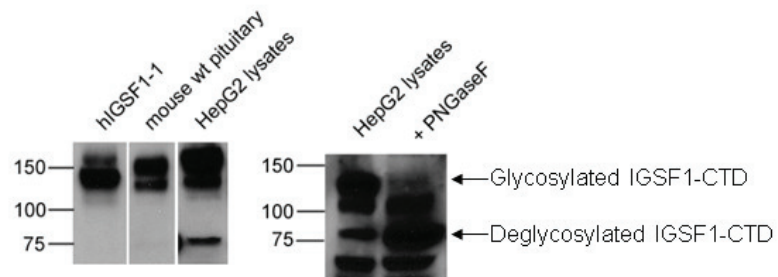


Figure 3.8

A



B



C

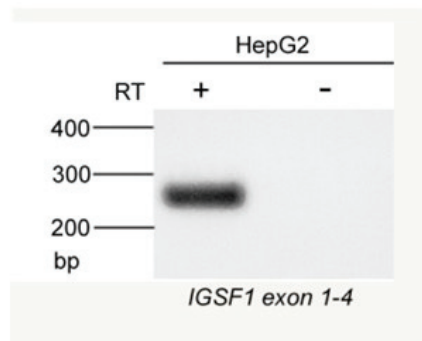


Figure 3.9

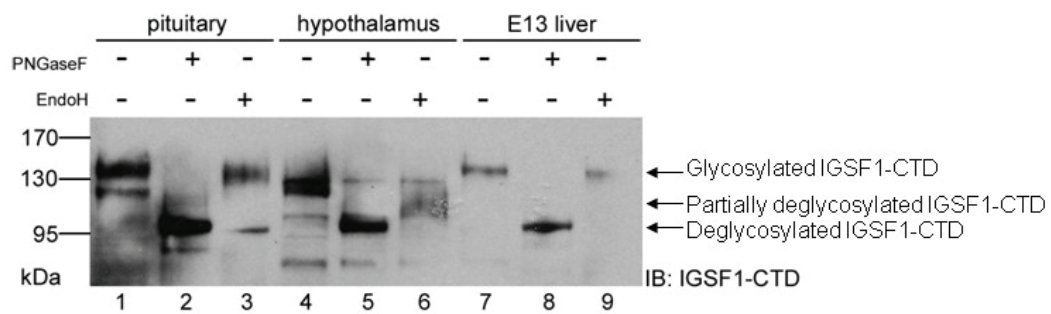
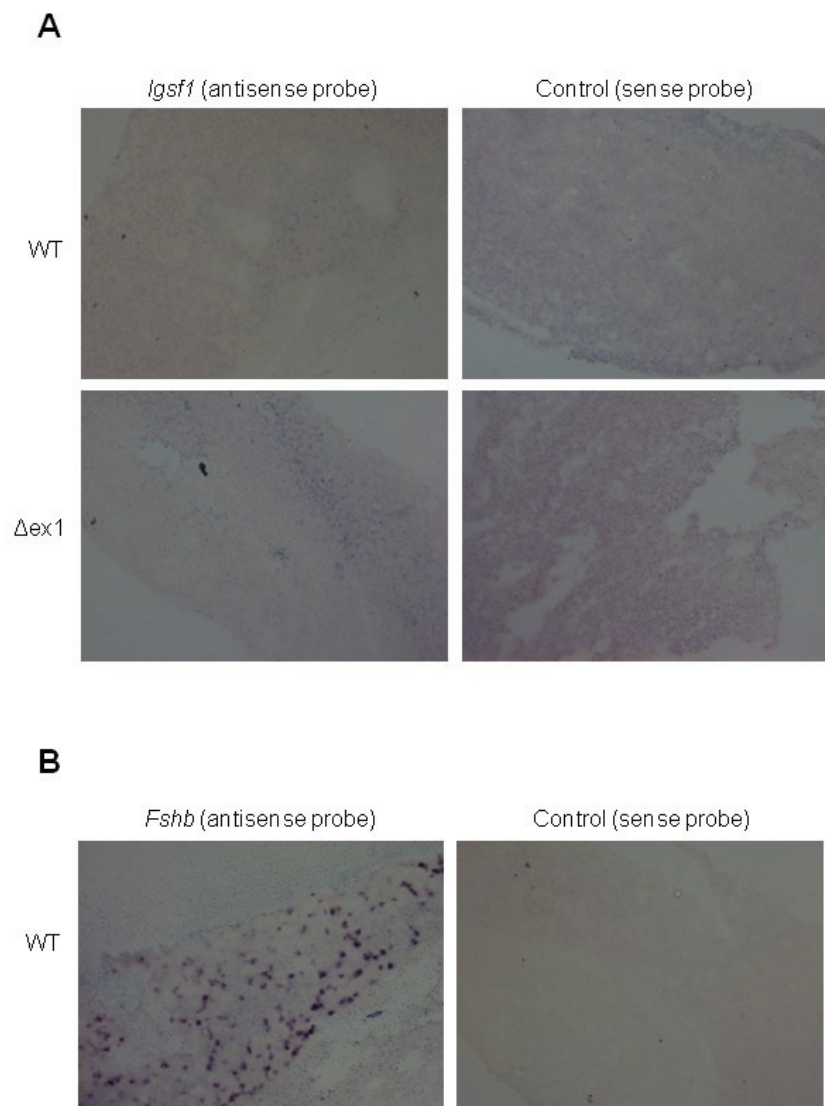


Figure 3.10



Supplementary Figure S3.1

Table 3.1 Primers for genotyping mice.

Fragment name:	Sense Primer:	Antisense Primer:
<i>lgsf1</i> exon1-intron 1 (present in WT, absent in <i>lgsf1</i> ^{Δex1} mice)	5'-TTTGCGGCATCTGGAGGAG-3'	5'-GGGAGCAGTTTGATTACGGC-3'
<i>Hprt</i> cassette (absent in WT, present in <i>lgsf1</i> ^{Δex1} mice)	5'-TGTTCTCCTCTTCCTCATCTCC-3'	5'-ACCCTTTCCAAATCCTCAGC-3'
<i>Zinc-finger protein 1, Y-linked (Zfy)</i> (present in males, absent in females)	5'-TGCAGCTCCCCTTCAGCTCTTCCTTTC-3'	5'-AATCCCAAAACCTGCTTGTCCTCCAG-3'

Table 3.2 PCR primers for semiquantitative (SQ) and quantitative (Q) PCR.

Gene name:	Sense Primer:	Antisense Primer:
<i>mIgsf1</i> exon 18-20 (SQ)	5'-GGAAGGGAAAGGCATTGGAAAC-3'	5'-TGGTTACACTCTTCAAAGACTACGG-3'
<i>mRpl19</i> (SQ and Q)	5'- CGGGAATCCAAGAGGATTG-3'	5'-TTCAGCTTGTGGATGTGCTC-3'
<i>hIGSF1</i> exon 1-4 (SQ)	5'-CAACTACCATCCCACCACCT-3'	5'-ACCAAAGCGTGATGTTCTCC-3'

Table 3.3 Primers used to generate *in situ* hybridization (ISH) and northern probes (NP).

Probe name:	Sense Primer:	Antisense Primer:
<i>mIgsf1</i> ISH exon 19-20	5'-CCGAAGTACCCTGATTGTGG-3'	5'-AACAGAATTGCATGCACCAG-3'
<i>mFshb</i> ISH	5'-TAATACGACTCACTATAGGGAGAACT GAAGGAGCAGTAGCTGGGT-3'	5'-AATTAACCCTCACTAAAGGGAGAGCAT CTTATTCTGGTGCTGGAG-3'
<i>mIgsf1</i> NP exon 5-6	5'-GCCAACTCCCTAAACCCATC-3'	5'-CAGTCACCCAGATTTTCAGGA-3'
<i>mIgsf1</i> NP exon 18-20	5'-CCCAGCACCATCTTCAAAC-3'	5'-CCAGTAAATCAGTACAGTGAGGAGTT-3'
<i>mRpl19</i> NP	5'-CTGAAGGTCAAAGGGAATGTG-3'	5'-GGACAGAGTCTTGATGATCTC-3'

Consternation to revelation

Although we had gained understanding of the expression and processing of IGSF1, the absence of insight into its function was a source of frustration for student and supervisor alike. The breakthrough came in the form a serendipitous finding by two independent groups of clinical investigators, both linking mutations in *IGSF1* to the same unusual clinical syndrome. Within a year, the clinical and molecular evidence gathered would lead to the identification of a novel syndrome caused by *IGSF1* loss-of-function. Part II of my thesis describes recent advances stemming from this revelation.

Part II: Revelation

Chapter 4. Introduction

1 A change of direction

In the summer of 2011, two clinical groups (now our collaborators) independently identified a subset of male patients with hypothyroidism (low levels of thyroid hormone) and associated mutations in *IGSF1*, shifting the focus of our investigation to IGSF1 function in the context of the hypothalamic-pituitary-thyroid (HPT) axis.

1.1 Hypothalamic-pituitary-thyroid axis

The HPT axis is the major endocrine system regulating metabolic rate, as well as having a profound impact on cardiac function, growth, and development. A general overview of the axis is presented in Figure 4.1. In brief, the hypophysiotrophic thyrotropin-releasing hormone (TRH) neurons are located in the medial and periventricular parvocellular portions of the hypothalamic paraventricular nucleus (PVN) (172). Their axon terminals project to the external layer of the median eminence, and release TRH into the hypophysial portal system (173, 174). TRH acts on the TRH receptor (TRHR) on the thyrotrope cells of the anterior pituitary, activating a signaling cascade that leads to increased production and release of thyroid stimulating hormone (TSH) (175-179). TSH is released into the circulation and binds the TSH receptor (TSHR) on the surface of thyroid epithelial cells (180-182) stimulating production and release of the thyroid hormones, thyroxine (T_4) and its highly-active derivative, triiodothyronine (T_3). These hormones are released into the general circulation and travel to their target tissues, where they are transported into cells. T_4 is deiodinated in target tissues to the more biologically-active T_3 , which then binds thyroid hormone receptors (183). The ligand-bound receptors bind to DNA to regulate transcription of their target genes. T_4 and T_3 also regulate their own production by negative feedback at the level of the pituitary, as well as the hypothalamus (184), inhibiting production and release of TRH and TSH. Although thyroid hormone feedback at the pituitary was long believed to be the crucial determinant of circulating TSH levels (185-187), TRH was later shown to be the dominant regulator

of the HPT axis (188). The sections below provide more details about the major players and modulators of the axis.

1.1.1 TRH

Thyrotropin-releasing hormone, or TRH, is a tripeptide, (pyro)-Glu-His-Pro-NH₂ (189, 190), encoded by the *TRH/Trh* gene. The human *TRH* gene codes for a progenitor sequence, prepro-TRH, which contains six repeats of the TRH precursor peptide Gln-His-Pro-Gly (191), while the murine (192) and rat (193) prepro-TRH contain five repeats. The precursor peptides are flanked by dibasic peptides on either side, typically Lys-Arg, or Arg-Arg. Prepro-TRH also encodes additional peptides that are not related to TRH, which lie between the TRH precursor repeats.

Prepro-TRH undergoes extensive processing to generate mature TRH (reviewed in (194)). The 29 kDa prepro-TRH is first cleaved in the ER to eliminate the N-terminal signal peptide. The resulting 26 kDa peptide, pro-TRH, is then cleaved in the *trans*-Golgi network by prohormone convertase 1/3 (PC1/3) at one of two mutually-exclusive sites, generating intermediate peptides of either 15 and 10 kDa, or 9.6 and 16.5 kDa (195, 196). The intermediate peptides are then further sorted into immature secretory granules (197-199), where they are cleaved by PC1/3 and to a lesser extent, prohormone convertase 2, at the dibasic residues to liberate the TRH precursor peptides, followed by cleavage by carboxypeptidase E and D (200, 201) to excise the C-terminal basic residues, amidation of the proline by peptidyl glycine α -amidating monooxygenase (202, 203), and cyclization of the glutamine to pyroglutamate, likely by glutaminyl-peptide cyclotransferase (204) to generate the mature TRH hormone. Secretory granules containing mature TRH are stored in the axon terminals in the median eminence.

1.1.2 TRH signaling through the TRHR

Once TRH is secreted into the portal vessels and reaches the pituitary, it binds to the TRH receptor (TRHR), which is expressed by thyrotropes, somatotropes, and lactotropes (205, 206). The receptor is also expressed in extra-pituitary sites, including the central

nervous system (CNS), pancreas, adrenal gland, and testis (207-211). TRHR belongs to Class A of the G protein-coupled receptor (GPCR) superfamily (212-215). Like other members of the superfamily, it is an integral membrane protein, with an extracellular N-terminus, seven transmembrane helices, and a cytoplasmic C-terminal tail. TRHR couples to the $G_{\alpha q/11}$ subclass of G protein, which uses phospholipase C (PLC) as its effector (216-221). Ligand binding to the GPCR induces a conformational change that is propagated to the associated G protein, enabling it to exchange GDP for GTP. GTP-bound G_{α} then activates PLC, which in turn catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 diffuses to the ER, where it binds the IP3 receptor and induces the release of internal calcium ion (Ca^{2+}) stores, activating transport of secretory granules to the cell surface where they release their cargo (222-225).

Although TRH is best known as a potent TSH secretagogue as well as a regulator of TSH production and maturation (see 1.1.3, TSH), TRH also stimulates prolactin release from the pituitary gland (226, 227) at the same concentrations that promote TSH secretion (228). In addition, TRH may stimulate prolactin transcription (229). However, TRH is not a crucial physiological regulator of prolactin, as demonstrated in *Trh* and *Trhr* knockout mice (230-232). That is, male and female *Trh* knockout mice have normal serum prolactin concentrations and pituitary prolactin content. Interestingly, they exhibit reduced pituitary prolactin mRNA levels, which can be reversed by thyroid hormone treatment indicating that the decrease in prolactin mRNA is a result of hypothyroidism, rather than TRH deficiency per se (230, 231). Lactating *Trh* knockout female mice have decreased serum prolactin concentrations that could be rescued by TRH, but not thyroid hormone replacement, indicating a direct effect of TRH, but the decrease in prolactin did not overtly affect milk production, as demonstrated by normal growth of pups nourished by *Trh* knockout females (231). Slightly different results were observed in the *Trhr* knockout mice, which show a decrease in both serum prolactin and pituitary *Prl* mRNA in both males and females. The decrease in *Prl* mRNA could not be rescued by administration of thyroid hormone. However, as with the TRH-deficient mice, lactation

was not affected, and pups nourished by *Trhr* knockout females exhibited normal weight gain (232).

TRH has also been reported to induce growth hormone (GH) release in pathological, but not physiological conditions, including liver disease, depression, acromegaly, status epilepticus, and renal failure (233-240). GH release in response to TRH has also been reported in rats (241, 242) and chickens (243-245). It has been proposed that subtle, sub-clinical hypothyroidism may enable the paradoxical stimulation of GH release by TRH (246).

In addition to being highly expressed in the PVN (172, 247), TRH or its precursor, proTRH, as well as its receptors have been found widely distributed in the central nervous system (193, 207, 209, 247-251) suggesting roles outside the pituitary, including modulation of anxiety and fear behavior (252-255). In addition, the non-TRH peptides generated during processing of pro-TRH may act as modulators of TRH signaling (256). TRH or pro-TRH are also expressed outside the central nervous system, notably in the pancreas (257, 258), heart (259), and testes (260, 261). Pancreatic TRH is expressed in insulin-secreting β -cells (262), and TRH signaling has been implicated in regulation of blood glucose levels (230, 263).

Unlike humans, rodents express an additional form of the TRH receptor, TRHR2 (264, 265), which is encoded by a different gene, and has a different tissue expression pattern than the canonical TRHR (209, 266). TRHR2 does not appear to be expressed in the pituitary gland, and mice lacking the receptor do not exhibit defects in their HPT axis. However, they do show alterations in anxious and depressive behaviors, suggesting that in rodents TRHR2 may mediate some of the extra-pituitary effects of TRH (255).

1.1.3 TSH

TSH is a dimeric glycoprotein, composed of the common α GSU (shared with FSH, LH, and hCG) and a unique TSH β subunit, which is expressed in thyrotropes. TSH β subunit

is specific to TSH, and confers biological specificity to the protein. The two subunits, which are encoded by different genes, are transcribed in a coordinated manner.

The human *TSHB* gene is located on chromosome 1p13 (267), and contains three exons, with the coding region of the β subunit spanning the second and third exons (268, 269). This is similar to the rat (270), but not the murine *Tshb* gene, which contains two additional non-coding exons upstream of the coding sequence (271, 272). Notably, the human gene has a single transcriptional start site (268), while both murine and rat *Tshb* genes have two transcriptional start sites, located 43 bp apart (271, 273). The downstream transcriptional start site appears to be the preferentially-used site in both species (273, 274).

TSHB/Tshb transcription is regulated by TRH (275), with two TRH-responsive regions identified in the human *TSHB* promoter (at bp -128 to -61, and -28 to +8 relative to transcription start site) (276), and similar TRH-responsive regions in the rat promoter (277, 278). TRH activates an AP-1-like transcription factor, which induces *TSHB* promoter activity (279), and stimulates transcription of *mitogen-activated protein kinase 8 interacting protein 1* (*Mapk8ip1*, also known as *Ib1/Jip-1*), which in turn stimulates the *Tshb* promoter (280). The nuclear receptor NR4A1 has also been implicated in mediating TRH-induced *Tshb* transcription (281). However, TRH is certainly not an essential regulator of *Tshb* transcription, as mice deficient in either TRH or its receptor (TRHR) have only slightly reduced, or normal *Tshb* mRNA levels (230, 232).

Additional factors implicated in *Tshb/TSHB* regulation include transcription factors POU1F1 and GATA2. Mice deficient in either factor have decreased numbers of thyrotropes and fail to upregulate *Tshb* in response to thyroidectomy (109, 112). In addition, POU1F1 and GATA2 can activate the *Tshb* promoter *in vitro* by binding to adjacent sequences within the -135 to -88 region (relative to the downstream transcriptional start site) (282)(274). The transcription factor MED1 potentiates the POU1F1/GATA2 effect on the *Tshb* promoter (283).

Dopamine has been implicated in negative regulation of *Tshb* transcription by decreasing intracellular cyclic adenosine monophosphate (cAMP) concentrations (284). However, the most important negative regulator of *Tshb/TSHB* transcription is thyroid hormone (described in sections 1.1.6 and 1.1.7).

The human gene encoding the common α GSU subunit (*CGA*) is on chromosome 6 (285), and is differentially regulated depending on cell type. Its promoter contains distinct regions that enable cell-specific transcription. Thus, expression of *CGA* in the placenta requires only the promoter fragment downstream of -178 bases (286), which contains cAMP-responsive elements (287, 288). Expression of *CGA* in gonadotropes is enhanced by two regions, one located between -224 to -268 (289), which contains a binding site for steroidogenic factor 1 (SF-1, encoded by *NR5A1*) (290), and a second site from -442 to -391 bases (289). Thyrotrope-specific expression requires regulatory sequences located even further upstream, between -480 and -254 bases (291-293). TRH mildly increases transcription of *CGA* (284).

As the *Tshb/TSHB* and *Cga/CGA* mRNAs are translated, they are imported into the ER. Both TSH β and α GSU are then co-translationally glycosylated, which is necessary for their proper protein folding and dimeric assembly (294, 295). TSH β in both humans and rodents has a single N-linked glycosylation site, while α GSU is N-glycosylated at two sites, although the second site is glycosylated post-translationally (296, 297). In addition, excess free α GSU monomers are O-glycosylated at a single residue (298, 299). TSH β and α GSU (along with the other glycoprotein hormone subunits, LH β , FSH β , and hCG β) have similar predicted structures, with each subunit made of two β -hairpin loops on one side of a so-called “cysteine knot”, made by three intramolecular disulfide bonds (300, 301). The two subunits are combined by virtue of threading of the “seatbelt region” of α GSU (300) through a hole in TSH β (302) to form the intact TSH molecule. Subunit combination begins in the rough ER, and continues in the smooth ER and Golgi apparatus (297). Amino acids 47-51 (27-31 after cleavage of the signal peptide) in the

TSH β subunit have been implicated in the ability of TSH β to combine with α GSU (303). The sugar groups on TSH are then modified in the *trans*-Golgi by sulfation and/or sialylation (304), resulting in a mature TSH molecule that is stored in secretory granules until it is ready to be secreted (305, 306).

Glycosylation of TSH is an important determinant of its biological activity. Deglycosylated TSH has reduced ability to stimulate cAMP production (307), and recombinant synthesis of exclusively sialylated TSH in heterologous CHO cells results in the production of hormone with attenuated bioactivity *in vitro* (308). Alterations in the glycosylation status of TSH have been noted in various pathological states, including primary and central hypothyroidism, pituitary tumors, euthyroid sick syndrome, and thyroid hormone resistance (309-314). The key regulator of TSH glycosylation is TRH, which promotes secretion of TSH with increased carbohydrate content and biological activity (315, 316). Lesions to the PVN in rats impair TSH glycosylation (317), but this can be reversed by exogenous TRH administration (318). Finally, *Trh* knockout mice secrete increased levels of TSH relative to controls upon bolus administration of exogenous TRH, but have a decreased serum thyroid hormone response, indicative of decreased TSH biological activity (230).

TSH is released from the anterior pituitary in pulses that are superimposed on a basal level of TSH secretion (319). The pulses occur approximately every 1.5-3 hr in both men and women, with increased release of TSH at night (320, 321). TRH is a potent TSH secretagogue (230, 322), and has been proposed to regulate TSH pulsatility (323); however, it appears to affect TSH pulse amplitude rather than frequency (324). Accordingly, patients with lesions of the pituitary or hypothalamus do not show alterations in pulse frequency, but failed to show the typical nocturnal increase in TSH (321). TSH secretion is modulated by several additional factors. It can be inhibited by somatostatin (325, 326), dopamine (327, 328), and neuromedin B (329), as well as fasting (330, 331). Additionally, increased GH levels in acromegaly are associated with a

decrease in TRH-stimulated TSH secretion (332), while GH deficiency is associated with increased TSH response to TRH (333).

1.1.4 TSH action on the thyroid gland

Once TSH is released into the circulation, it can bind to the TSH receptor (TSHR), which is highly expressed in the thyroid gland (180-182), as well as in adipose tissue (334), brain (335), erythrocytes (336), liver, (337), ovary (338), and hair follicles (339). The receptor belongs to the GPCR superfamily, and its activation induces production of cAMP, as well as IP₃ generation by PLC (340-342). TSH has also been reported to induce phosphorylation of Janus kinases 1 and 2 (JAK1/JAK2), leading to their activation (343). TSH signaling has a number of effects on the thyroid gland, which serve to increase production and release of thyroid hormones, T₄ and T₃.

Thyroid hormones are synthesized from a precursor glycoprotein known as thyroglobulin, which is produced in the thyroid epithelial cells. The epithelial cells surround hollow structures known as colloid, where the synthesized thyroglobulin is secreted and stored. In the colloid, tyrosyl residues (derivatives of tyrosine) on thyroglobulin are iodinated by the action of thyroperoxidase, producing either moniodotyrosine (MIT), which is iodinated at one position, or diiodotyrosine (DIT), which is iodinated at two positions. Thyroperoxidase also combines MIT and DIT residues, forming either the active thyroid hormones T₄ (DIT + DIT) and T₃ (MIT + DIT), or the inactive reverse-T₃ (DIT + MIT) (Figure 4.2A). The thyroid hormones are then transported back into the epithelial cells by endocytosis, released from the thyroglobulin by proteolysis, and released into the circulation (344).

TSH signaling is required for expression of the sodium/iodide symporter and thyroperoxidase (345, 346), which are important for iodide import into the thyroid gland, and synthesis of thyroid hormones. TSH signaling also regulates the subcellular distribution of the sodium/iodide symporter, targeting it to the plasma membrane, and prolonging its half-life (347). TSH signaling stabilizes thyroglobulin mRNA (348), and

increases expression and activation of RAB5A and RAB7 (349, 350), which are rate-limiting catalysts for endocytosis of thyroglobulin and its transfer to lysosomes. Both T_4 and T_3 are released from the thyroid gland upon TSH stimulation, although T_4 constitutes the majority of released hormone (351-353).

1.1.5 Thyroid hormone action

Thyroid hormones in the circulation are bound by a variety of proteins, with less than 1% circulating as free hormone (fT_4 or fT_3) (344). In humans, the major binding protein is thyroxine-binding globulin (354), while in adult rodents transthyretin is the major carrier (355, 356). Thyroid hormones are transported into target cells via the monocarboxylate transporter 8 (MCT8) (357), and several members of the organic anion transporting polypeptide (OATP) family (358-361). Once the hormones are imported into cells, they can be deiodinated by a family of iodothyronine deiodinases (DIO1, DIO2, DIO3), which can activate or inactivate the hormones in a tissue and time-dependent manner (183) (Figure 4.2B). Deiodination of T_4 by DIO2 leads to its conversion to the more biologically active T_3 , while deiodination of T_4 by DIO3 results in formation of inactive reverse- T_3 . DIO1 can convert T_4 to either T_3 or reverse- T_3 , as well further deiodinate them to diiodothyronine (T_2) (362). In cells, DIO2 is localized to the ER, while DIO1 and DIO3 are in the plasma membrane (363-366). Once T_3 is imported or generated in the cell, it can exert its actions by binding to its receptors.

Thyroid hormone receptors (TR) belong to the family of nuclear receptors (367). Two genes code for TRs: the *THRA* gene encodes $TR\alpha_1$, as well as a splice variant $TR\alpha_2$, which does not bind T_3 (368-370); the *THRB* gene encodes $TR\beta_1$ and $TR\beta_2$, both of which bind T_3 (371). $TR\alpha_1$ and $TR\beta_1$ are widely expressed, while $TR\beta_2$ expression is more restricted, with receptors found predominantly in the hypothalamus and pituitary gland (372-375). The receptors can function as monomers (376), or dimerize with each other, or retinoid X receptor (RXR) (377-379), and exert their effects by binding to thyroid hormone response elements (TRE) in DNA, and acting as repressors or activators of transcription. In the absence of ligand, they tend to act as repressors, and in the

presence of ligand, as activators (380-383). Generally, thyroid hormone-mediated signaling increases metabolic rate, carbohydrate and lipid metabolism, heat production, activity of the sympathetic nervous system, as well as having profound effects on growth and development, including normal development of the central nervous system (344).

1.1.6 Mechanism of negative feedback by thyroid hormones

Thyroid hormones negatively regulate their own synthesis by acting at the hypothalamus and pituitary gland to repress TRH and TSH production. These effects are mediated by the TR β 2 isoform (384). However, the precise mechanism of thyroid hormone feedback is not clear. Negative regulation appears to require an intact DNA binding domain of the receptor (385-387), thus it is thought that ligand-bound TR β 2 binds to DNA and recruits co-repressors rather than co-activators in negatively-regulated genes (388). However, conflicting data have thus far precluded confirmation of this hypothesis. Whereas T₃ stimulated increased histone deacetylase 1 (a putative repressor) recruitment to the *Tshb* promoter (389), it also caused release of histone deacetylase 3 (also a putative repressor) from the *Cga* promoter (390), which is usually negatively regulated by T₃ (391-393). In mice, a mutation in *Thrb* that prevents interaction with co-activators, but not co-repressors, impaired negative regulation of *Tshb* by T₃, suggesting that, paradoxically, co-activator recruitment may be important for repression of *Tshb* by thyroid hormones (394). Similarly, overexpression of co-repressors enhanced basal transcriptional activation of negatively-regulated genes (*TSHB*, *CGA*, *TRH*) *in vitro*, and addition of T₃ reversed this basal activation, suggesting canonical co-repressors may act as co-activators in negatively-regulated genes (395). In addition, a mutation in the *THRB* gene that impairs the ability of the receptor to induce co-repressor release from the *TRH* promoter leads to thyroid hormone resistance in patients (high TSH levels in the presence of high thyroid hormone levels) (396), suggesting presence of co-repressors on the promoters of negatively-regulated genes may lead to increased transcriptional activation.

1.1.7 Negative feedback by thyroid hormone at the pituitary gland

T₃ is a crucial negative regulator of TSH production. In addition to suppressing TSH secretion (397, 398), thyroid hormone directly suppresses *Tshb*/*TSHB* transcription (391, 392, 399, 400). The transcriptional activity of the promoter depends on the degree of nuclear receptor occupancy (401, 402), and TREs have been identified in both *Tshb* and *Cga* genes (403, 404). The ligand-receptor complex has been proposed to interfere with the binding of the *Tshb* transcriptional activator GATA2 to the promoter (405). In addition, it has been shown to decrease *Tshb* mRNA stability (406, 407).

1.1.8 Negative feedback by thyroid hormone at the hypothalamus

Thyroid hormone feedback at the level of the pituitary gland was widely believed to be the key determinant of TSH production (187). However, the demonstration that mice deficient in both TRH and TR β have low serum TSH levels rather than thyroid hormone resistance, revealed that hypothalamic TRH plays a dominant role in regulating circulating TSH levels (188). Thus, feedback of thyroid hormone on the hypothalamus is also important in maintaining proper function of the HPT axis. Low levels of thyroid hormones in rats increase of *Trh* mRNA levels in the PVN (172), and T₃ replacement restores them to normal levels (408, 409). These effects are mediated by TR β 2, as TR β 2-deficient mice showed elevated levels of *Trh* in the PVN, which cannot be normalized by T₃ treatment (410).

1.1.9 Thyrotropin-releasing hormone degrading enzyme (TRHDE)

One additional notable way of regulating TRH signaling is through its degradation. A specialized ecto-enzyme, TRHDE (formerly known as pyroglutamyl-peptidase II) has been shown to specifically degrade TRH in the extracellular space (411, 412). *Trhde*/TRHDE has also been shown to be highly expressed in the brain, and at low levels in the anterior pituitary (413, 414). Interestingly, TRHDE expression and activity in the pituitary and mediobasal hypothalamus are upregulated by T₃ (415-417), indicating another potential mode of negative feedback by thyroid hormones in both the pituitary and hypothalamus. However, analysis of anterior pituitary *Trhde* revealed that only about

5% of anterior pituitary cells express *Trhde*, with the majority of these being lactotropes, and no apparent *Trhde* expression in thyrotropes (413, 418), suggesting pituitary TRHDE may be more important for regulating TRH-induced prolactin release.

1.2 Hypothyroidism: a defect of thyroid hormone production

Hypothyroidism is a condition in which the thyroid gland does not produce sufficient quantities of thyroid hormone, and is the most common disorder of thyroid function (419). Symptoms of hypothyroidism reflect the importance of thyroid hormone in regulating metabolic rate, and include fatigue, weight gain, depression, and cold intolerance. In addition, neonatal hypothyroidism can lead to permanent physical and mental impairment if not treated. Most cases of hypothyroidism are caused by dysfunction of the thyroid gland itself, and are referred to as primary hypothyroidism. It is found in 1-2% of the general population, with women representing the majority of the affected patients (419). In industrialized nations, the most common cause of primary hypothyroidism is autoimmune destruction of the gland, while dietary iodine deficiency is a concern for approximately 2 billion individuals worldwide (420).

1.2.1 Central hypothyroidism

Hypothyroidism can also be caused by dysfunction at the level of the pituitary gland or the hypothalamus, collectively referred to as central hypothyroidism. It is a relatively rare disorder (estimated at 0.0002-0.005% of the population), and most often caused by tumors of the pituitary gland, but can also result from head trauma, radiotherapy or surgery, infectious diseases, or congenital malformations. In these cases, hypothyroidism is often associated with additional hormone deficiencies (419). Congenital central hypothyroidism can also be due to genetic defects, which can be associated with additional hormone deficiencies (panhypopituitarism), or occur as isolated TSH deficiency (see section.1.2.2).

Several mutations have been reported that lead to pituitary hormone deficiencies. One of the most common genetic causes of multiple pituitary deficiencies is loss-of-function

mutations in the gene *PROP1* (421-428), which is required for differentiation of the somatotrope, lactotrope, and thyrotrope lineages. Similarly, mutations leading to multiple pituitary deficiencies have been described in the *POU1F1* gene, whose expression is activated by *PROP1* (429-432). Mutations in *LIM homeobox 3* (*LHX3*) and *LIM homeobox 4* (*LHX4*) genes, which are involved in development of the pituitary gland, also result in multiple pituitary hormone deficiencies (433, 434), and mutations in the transcription factor *HESX homeobox 1* (*HESX1*) gene have been linked to septo-optic dysplasia, a disorder of optic nerve, brain midline, and pituitary development (435, 436).

1.2.2 Isolated TSH deficiency as a cause of central hypothyroidism

Genetic causes of isolated TSH deficiency are rare, and thus far have only been attributed to loss of function in two genes directly involved in the HPT axis: *TSHB* and *TRHR*. Multiple inactivating mutations have been described in *TSHB* (summarized in Table 4.1), of which seven map to the coding region of the gene, (303, 437-456). One intronic mutation leads to skipping of exon 2 (457, 458). The affected patients are either homozygous, or compound heterozygous for the mutations, which include premature truncations, frameshift mutations, and single nucleotide substitutions. Two mutations have been described in *TRHR*, one of which is a premature truncation, and the other a three amino-acid deletion and a single nucleotide substitution (summarized in Table 4.1) (459, 460).

Patients with isolated TSH deficiency due to *TSHB* mutations are usually diagnosed before one year of age, presenting with a variety of symptoms, including persistent constipation, jaundice, anemia, feeding problems, and growth delay (455). Patients with *TRHR* mutations tended to be diagnosed later in childhood, with growth retardation and fatigue. TRH stimulation tests, where a single dose of TRH is administered, and circulating TSH, fT₄ and/or fT₃ levels are measured at different intervals following injection, showed abnormally low (or absent) TSH and thyroid hormone responses in patients with either *TSHB* or *TRHR* mutations. In addition, patients with *TRHR* mutations also showed abnormally low prolactin release upon TRH stimulation.

1.3 A link between *IGSF1* and central hypothyroidism

Recently, the identification of male patients with central hypothyroidism, in the absence of *TSHB* or *TRHR* mutations, suggested the involvement of a novel, X-linked gene in regulation of the HPT axis. The advent of whole exome sequencing (461-463) allowed rapid, relatively inexpensive screening of the coding regions of genes in individual patients, enabling the identification of mutations in *IGSF1* in the two index families. The following chapter describes the discovery of this X-linked syndrome of central hypothyroidism due to mutations in *IGSF1*.

Figure legends

Figure 4.1 Hypothalamic-pituitary-thyroid axis. A simplified diagram of the hypothalamic-pituitary-thyroid axis. Green arrows indicate stimulatory effects, red arrows indicate negative feedback.

Figure 4.2 Assembly and deiodination of thyroid hormones, thyroxine and triiodothyronine. (A) Monoiodothyronine (MIT) and diiodothyronine (DIT) undergo coupling by thyroxine peroxidase to produce either T_4 (DIT+DIT), T_3 (MIT+DIT), or reverse- T_3 (DIT+MIT) (B) T_4 is peripherally deiodinated by the enzymes DIO1, DIO2, or DIO3 to produce either the highly biologically-active T_3 , or the inactive reverse- T_3 . Both can be further deiodinated to T_2 .

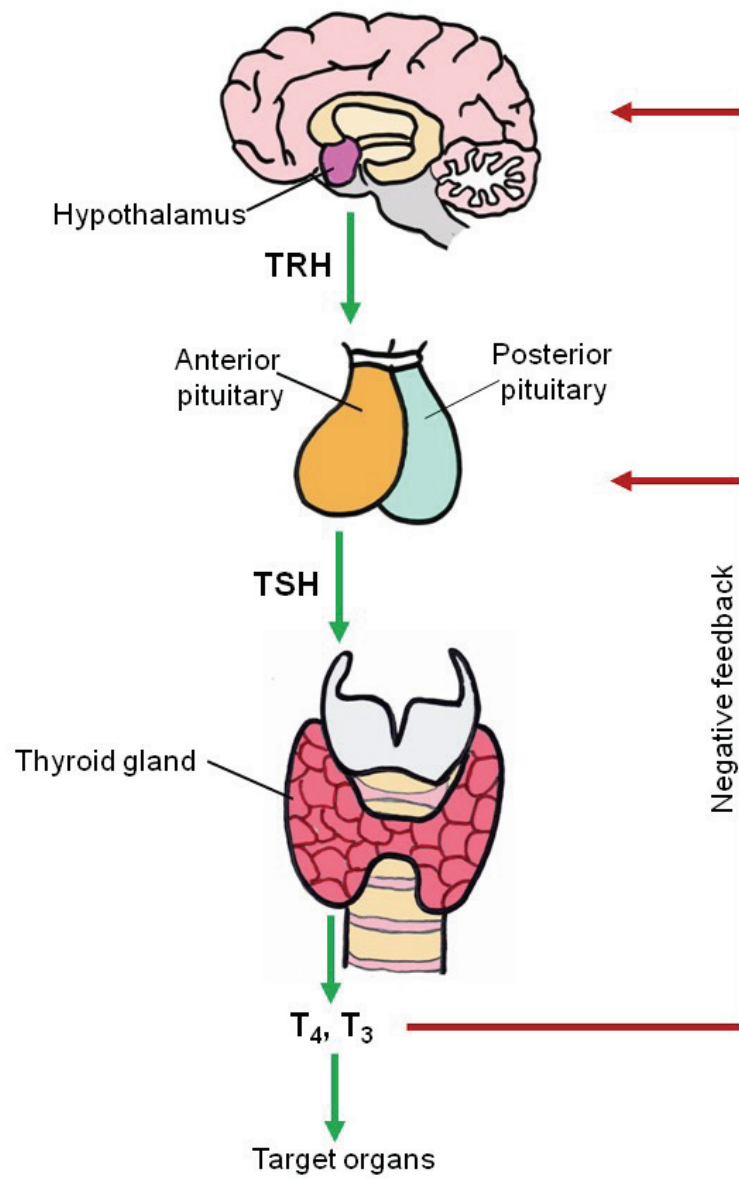
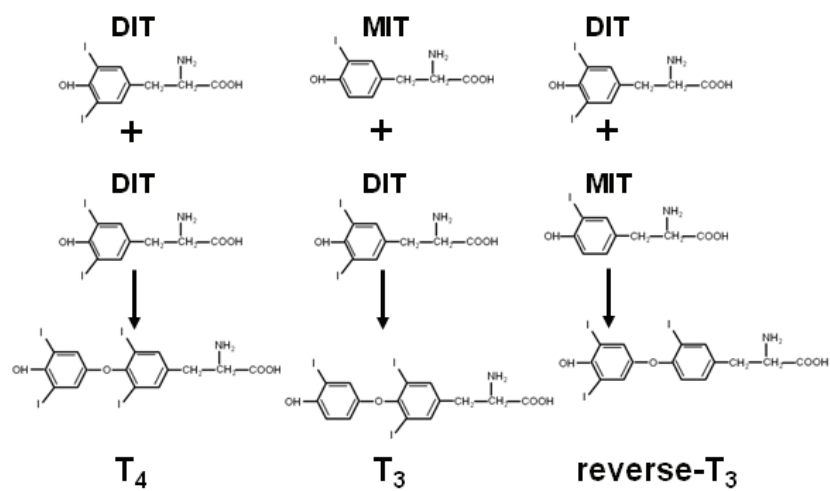


Figure 4.1

A



B

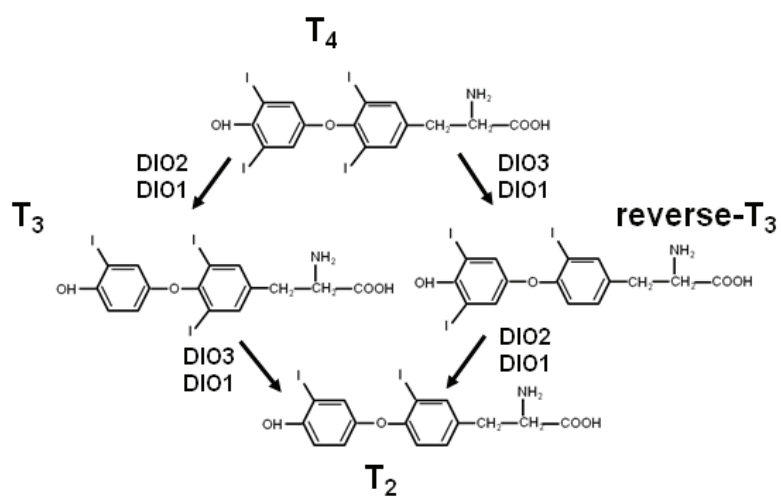


Figure 4.2

Table 4.1 Mutations found in patients with central hypothyroidism.

<i>TSHB</i> mutations found in patients with central hypothyroidism				
Mutation	Nucleotide alteration	Amino acid alteration	Alleles affected	References
I	c.94G>T	p.(Glu32X)	Homozygous	(438)
II	c.145G>A	p.(Gly49Arg)	Homozygous	(303) (439)
III	c.373del	p.(Cys125ValfsX10)	homozygous (442) Homozygous (449) Homozygous (450) Compound heterozygous (with VI) (453) Compound heterozygous (with VIII) (456) Compound heterozygous (with IV) (451) Homozygous (454)	(441-443) (440, 446, 448-453) (454-456)
IV	c.205C>T	p.(Gln69X)	Homozygous (445) Homozygous (447) Compound heterozygous (with III) (451)	(445) (444, 447, 451, 455)
V	c.313T>C	p.(Cys105Arg)	Homozygous	(447)
VI	c.229 or 230del	p.(Phe57SerfsX6)	Compound heterozygous (with III)	(453)
VII	c.162+5del	Skips exon 2	Homozygous	(457, 458)
VIII	c.323G>A	p.(Cys108Tyr)	Compound heterozygous (with III)	(456)
IX	c.162G>A	Skips exon 2	Homozygous	(456)
<i>TRHR</i> mutations found in patients with central hypothyroidism				
I	c.49C>T	p.(Arg17X)	Compound heterozygous (with II) (459); Homozygous (460)	(459, 460)
II	c.(343_351del;352G>A)	p.(Ser115_Thr117;Ala118Thr)	Compound heterozygous (with I)	(459)

Chapter 5

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

Portions of this chapter, including figures 5.1, 5.2, 5.3, 5.4, 5.5A,B,D, 5.6A,B,C, 5.8A,B, and 5.10B,E were published in:

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¹ These authors contributed equally to this work. ² These authors jointly directed this work.

Data in figures 5.2, 5.3, 5.4, 5.6A, 5.7A, 5.8A,B, 5.9, and 5.10A,C,E were generated by Bak B.

Data in figures 5.6B, and 5.10B,D were generated by Dr. Paul le Tissier (University College London (UCL) Institute of Child Health, London, UK; National Institute for Medical Research, Mill Hill, London, UK) from samples collected by Bak B.

Data in figures 5.5C, 5.7B, and 5.8C,D were generated by Dr. Mike G. Wade (Environmental Health Science and Research Bureau, Health Canada, Ottawa, Ontario, Canada) from samples collected by Bak B.

Data in figures 5.5A,B,D, 5.6C, and 5.8E,F were generated by Dr. Samuel Refetoff and Xiao-Hui Liao (University of Chicago, Illinois), from samples collected by Bak B (with assistance from Stella Tran and Xiang Zhou).

Part of the data set in figure 5.5A were generated by Dr. Nadia Schoenmakers (Institute of Metabolic Science, Metabolic Research Laboratories, Addenbrooke's Hospital, University of Cambridge, Cambridge, UK) from samples collected by Bak B.

For published paper, refer to appendix B.

Chapter 5. Loss-of-function mutations in *IGSF1* cause an X-linked syndrome of central hypothyroidism

1 Introduction

IGSF1 is a protein of unknown function, which is highly enriched in the anterior pituitary gland. It can be detected in thyrotropes, somatotropes, and lactotropes of mice (Chapter 3, Figure 3.3), suggesting it may play a role in one or more of the endocrine axes associated with these cell types. However, *Igsf1*-deficient (*Igsf1*^{Δex1}) mice did not display overt physical defects (39), indicating that loss of IGSF1 is not crucial for normal development, and that any associated phenotype associated with its absence may be subtle. The first indications of IGSF1's function emerged from whole exome sequencing of DNA from two families with central hypothyroidism.

1.1 Index families with male-specific central hypothyroidism

The index cases were identified by our collaborators in two families from the Netherlands (family A) and the UK (family B). In family A, the index patient was diagnosed with central hypothyroidism during neonatal thyroid function screening, presenting with low free T₄ levels, and normal TSH. The patient's cousin was diagnosed with central hypothyroidism, as well as prolactin deficiency at the age of seven. In family B, two brothers were diagnosed with central hypothyroidism in infancy, after presenting with jaundice and low free T₄ levels. The patients were screened for mutations in *TRH*, *TRHR*, and *TSHB*, but no abnormalities were detected. Also, the fact that only males were affected suggested an X-chromosome defect. Subsequently, exome sequencing was carried out in families A (X-chromosome only) and B (whole exome), to identify candidate genes with mutations that might cause central hypothyroidism.

1.2 Mutations in *IGSF1*

Exome sequencing in family A revealed a 27-nucleotide deletion in *IGSF1*, predicted to lead to an in-frame loss of nine amino acids (c.2137_2163del, p. (Ala713_Lys721 del)) (Figure 5.1). The mutation was present in both affected cousins, as well as their maternal

grandfather. In addition, both mothers were heterozygous carriers of the mutation. Exome sequencing in family B revealed a single nucleotide substitution (c.2931G>A) in *IGSF1*, changing a tryptophan to a stop codon (p.Trp977X) in both brothers and in one of the maternal alleles. These initial discoveries led to the identification of eight additional *IGSF1* mutations in nine other families with males with unexplained central hypothyroidism (summarized in Figure 5.1). Mutations in families A-D and G-K localize to the portion of the gene encoding IGSF1-CTD, while families E and F have large deletions that encompass the whole gene. The mutations in families A-D and G-K can be divided into two categories: those causing premature truncation of the protein, and in-frame mutations. Mutations that lead to a premature truncation include frameshift mutations (families C/D and J) and single nucleotide substitutions that introduce stop codons (nonsense mutations in families B, I). In-frame mutations include single nucleotide substitutions (families G, H and K), and the 27-nucleotide deletion in family A. The phenotype of the patients with whole gene deletions was highly similar to those with premature truncations and single nucleotide substitutions, suggesting that loss of IGSF1 function leads to central hypothyroidism in all of these patients.

1.3 Summary of patient phenotypes

In total, 26 male patients with central hypothyroidism and concomitant *IGSF1* mutations were identified. The degree of hypothyroidism varied from severe, presenting in infancy, to mild, discovered only after screening of family members of probands. Phenotype did not correlate with the type of mutation, as some patients from the same family were diagnosed as children, while others were not identified until late adulthood. Serum prolactin deficiency was present in 18 of the 26 patients; again, there was no correlation between type of mutation and prolactin deficiency, as some patients from the same family presented with prolactin deficiency, while others did not. In addition, 20 of 25 clinically evaluated patients had testicular volumes above the reference range for their age group, with all adult patients presenting with macroorchidism. Four patients also presented with growth hormone deficiency in childhood, and were treated accordingly.

In order to determine whether mutations in *IGSF1* caused central hypothyroidism, I assessed the effects of the mutations on IGSF1-CTD expression, maturation, and cell surface trafficking, as well as the function of the HPT axis in *Igsf1*^{Δex1} mice.

2 Methods

Reagents

Rabbit antiserum against murine IGSF1-CTD was generated and provided by Dr. Peter Scheiffele (63). Antibodies against β -actin (A5316) and HA (H9658), Nytran SuPerCharge nylon membrane (Z670405), TRH (P1319), EZview anti-HA affinity gel (E6779), and HA peptide (I2149) were obtained from Sigma (St. Louis, Missouri). Secondary antibodies for western blotting (goat anti-mouse 170-6516, rabbit anti-goat 172-1034, goat anti-rabbit 170-6515) and Protein Assay Dye Reagent Concentrate (500-0006) were from BioRad (Mississauga, Ontario, Canada). Secondary antibody for immunofluorescence (Goat anti rabbit Alexa 488: A-11008), Pro-Long Gold antifade reagent with DAPI (P36935), antibody against β -tubulin (32-2600), TRIzol reagent (15596-026) and Lipofectamine 2000 were from Invitrogen (Burlington, Ontario, Canada). PNGase F (P0704S) and Endo H (P0702L) were purchased from New England Biolabs (Pickering, Ontario, Canada). Matrigel (354234) was from BD (Mississauga, Ontario, Canada). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). *Pfu* Turbo enzyme (600252-52) for QuikChange mutagenesis was from Agilent (Mississauga, Ontario, Canada). Protease inhibitor tablets (CompleteMini) were from Roche (Indianapolis, Indiana). Vectastain ABC kit (PK 6100) and biotinylated horse-anti-goat antibody (BA-9500) were from Vector labs (Burlington, Ontario, Canada). Western Lightning Plus-ECL (NEL105001EA) was purchased from Perkin Elmer (Waltham, MA). EZ-Link Sulfo-NHS-LC-Biotin (21335) and Phusion site-directed mutagenesis kit (F-541) were from Thermo Scientific (Nepean, Ontario, Canada). ³²P -dCTP (BLU513H250UC) was purchased from Perkin-Elmer (Woodbridge, Ontario, Canada). Ready-to-Go beads were from GE Healthcare (Baie d'Urfe, Quebec, Canada).

Constructs

The human Myc-IGSF1-3xHA expression construct was obtained from Dr. Peter Scheiffele (Biozentrum, University of Basel, Switzerland), and the IGSF1-CTD antibody epitope therein was modified using the QuikChange method (Stratagene) to match that of the murine protein to facilitate detection with the anti-murine IGSF1-CTD polyclonal antibody, using primers listed in Table 5.1. Mutations found in patients from families B-D and G-K were introduced into the construct using QuikChange or Phusion (Thermo) site-directed mutagenesis (Family A; p.Ala713_Lys721del) using the primers listed in Table 5.1. All constructs were verified by sequencing (McGill University and Genome Québec Innovation Centre, Montreal, Quebec, Canada). Although the mutations in patients are named according to the coding sequence of the longest isoform (*IGSF1-3*), I generated the mutants in an expression vector for IGSF1-1. See Table 5.2 for human mutation nomenclature according to *IGSF1-3*, and equivalent *IGSF1-1* changes.

Cell culture and transfections

For western blotting and cell surface biotinylation, HEK293 cells (a gift from Dr. Terrence Hébert, McGill University, Montreal, Quebec, Canada) were plated in 6-well plates, grown until 80% confluent, and transfected with 200 ng of expression vector using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For immunofluorescence, HEK293 cells were plated on glass coverslips at 50,000 cells/well and transfected with 100 ng of expression vector using 2 µl Lipofectamine 2000 per well.

Deglycosylation and western blotting

Whole cell protein extracts were prepared from transfected HEK293 cells 24 hr post-transfection using RIPA buffer (150 mM NaCl, 50 mM NaF, 10 mM NaPO₄, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors (Roche). Protein lysates were deglycosylated using PNGaseF and EndoH (NEB), according to manufacturer's instructions (2 hr at 37°C). The proteins were denatured at 100°C for 5 min in Laemmli buffer containing 2% 2-mercaptoethanol, and resolved by SDS-PAGE on 8% Tris-glycine gels. The proteins were transferred to nitrocellulose

membranes (25 V, 1.5 hr), the membranes blocked with 5% milk in TBST (0.05% Tween-20 in TBS), and incubated overnight at 4 °C in rabbit anti-IGSF1-CTD antibody (1:1,000) in blocking buffer. Next day, membranes were washed 3x15 min in TBST and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad; 1:3,000 in blocking buffer) for 1 hr at room temperature. The membranes were washed 3x15 min in TBST, followed by incubation in ECL Plus reagent (Perkin-Elmer) according to manufacturer's instructions and then exposed to x-ray film (GE Healthcare).

Cell Immunofluorescence

Transfected HEK293 cells grown on glass coverslips in 24-well plates were subjected to immunofluorescence. In brief, under non-permeabilizing conditions, IGSF1-CTD antiserum was diluted 1:500 in serum-free DMEM and applied to the cells for 2 hr at 37°C/5% CO₂. Cells were washed 3x with serum-free DMEM and fixed with 4% paraformaldehyde. Cells were washed 3x with PBS, then incubated in 5% BSA in PBS for 1 hr at 22°C. Secondary antibody (goat anti-rabbit Alexa 488, Invitrogen) was diluted 1:600 in 5% BSA in PBS and applied to cells for 1 hr at 22°C. Cells were washed 3x with PBS and coverslips mounted on microscope slides using Pro-Long Gold reagent with DAPI. Under permeabilizing conditions, cells were fixed using 4% paraformaldehyde, washed 3x in PBS, and incubated in 0.3% Triton X-100 in PBS. Cells were then incubated in 5% BSA in PBS for 1 hr at 22°C. IGSF1-CTD antiserum was diluted 1:500 in 5% BSA in PBS with 0.3% Triton X-100, and was applied to the cells for 2 hr at 22°C. Cells were washed 3x with PBS, and secondary antibody was applied at a dilution of 1:600 in 5% BSA in PBS with 0.3% Triton X-100 for 1 hr at 22°C. Cells were washed 3x with PBS, and coverslips mounted as above.

Cell surface biotinylation and immunoprecipitation

Transfected HEK293 cells grown in 6-well plates were washed 3x with PBS and incubated in 1 ml 0.5 mg/ml EZ-link-sulfo-NHS-LC-biotin (Thermo) per well for 30 min at 4°C, with slow shaking. Cells were then washed 3x with PBS plus 100 mM glycine and harvested in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1%

Triton X-100). Cells were lysed by sonication at 9W for 10 sec. Lysates were centrifuged and the supernatant collected. Immunoprecipitation was carried out using 40 µl of anti-HA affinity beads (Sigma) following the manufacturer's instructions. Lysates were then processed for western blotting as described above. After the blocking step, the membrane was incubated in 2.5% milk in TBST with 2 drops each of A and B reagents from a Vectastain kit (Vector) for 30 min at 22°C, followed by three 10 min washes in TBST. Signal was visualized using Western Lightning Plus-ECL kit (Perkin-Elmer), following the manufacturer's instructions.

Animals

Igsl-deficient mice were described previously (39). The majority of mice used in the studies were crossed onto a C57BL/6 background (from Charles River, Quebec, Canada) over 7-10 generations, and generated from heterozygous mothers. No overt differences were noted in any of the parameters measured between mixed background and pure background mice. Genotyping was carried out as described in chapter 3.

Reverse-transcription and quantitative PCR

Murine hypothalamic RNA was extracted from 3 mm thick hypothalamic blocks from 12-18 week old males (5 per genotype) prepared using a freezing microtome, according to coordinates from (135). The coordinates were as follows: anteroposterior-interaural 3.94 to 0.94 mm, dorsoventral-interaural 2.5 mm and below, and 1.44 mm lateral on either side. Pituitary and hypothalamic RNAs were extracted using TRIzol. Two µg of RNA were reverse-transcribed into cDNA using 50 ng random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega). Contaminating genomic DNA was removed using RQ1 DNase (Promega). Each reverse transcription reaction was diluted 1:5 for qPCR analysis. The qPCR was performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 0.4 pmol of each primer (see Table 5.3 for primers) on a Corbett Rotor-Gene 6200 HRM (Corbett Life Science), according to Invitrogen's real-time cycling protocol. Transcript levels were normalized relative to

housekeeping gene ribosomal protein L19 (*Rpl19*). Results were analyzed using $2^{-\Delta\Delta Ct}$ method (140, 141).

Hormone measurements

In the UK, the murine T₄ immunoassay was designed and optimised in-house and performed using the AU400 system (Beckman Coulter) and reagents (Beckman Coulter ref 445995). In Chicago, serum total T₄ and T₃ concentrations were measured by coated tube RIAs (Siemens Medical Solutions Diagnostics) adapted for mouse. TSH was measured as described in (464). Free T₄ levels were estimated from total T₄ and resin T₄ uptake ratio and expressed as free T₄ index (FT₄I) as described in (465). Murine pituitary TSH, PRL, and GH content measurement was previously described (466), using reagents supplied by Prof. A.L. Parlow (NHPP, Torrance, California). In Ottawa, serum T₄ was assayed using a commercial RIA kit (MP Biomedicals, Lachine, Quebec, Canada), according to the manufacturer's instructions.

TRH simulation in mice

Mice were injected with TRH (5 µg/kg i.p.). Blood was collected by submandibular venipuncture 15 min before TRH injection (baseline) and at 30 min post-injection (for TSH), and by cardiac puncture 3 hr after injection (for T₄ and T₃ measurements). Blood was allowed to clot for 30 min at room temperature and was then centrifuged at 3000 rpm for 10 min. Serum was collected for hormone measurement. In trial 1, T₄ was measured at baseline and 2 hr post-TRH in Ottawa, as described above. In trial 2, T₄ and T₃ were measured at baseline and 3 hr post-TRH in Chicago, as described above. TSH was measured in Chicago at baseline and 30 min as described above.

Body weight assessment

Animals were weighed at weekly intervals on a precision balance, starting at week 1, and continuing to week 12.

Thyroid gland histology

Thyroid glands *in situ* on trachea were fixed in 4% paraformaldehyde for at least 48 hr and transferred to 70% ethanol until embedded in paraffin. Sections (5 μ m) were stained with Periodic acid-Schiff (PAS) stain and assessed by a single observer who was blinded to the genotype of the specimens (Health Canada, Ottawa, Ontario, Canada).

Statistical analyses

In most figures, statistical significance was determined by two-tailed Student's t-test. Significance was taken at $p < 0.05$. In Figure 5.7, multiple t-tests were performed, using the Bonferroni correction ($p < 0.005$).

3 Results

Effects of mutations on IGSF1-CTD maturation and trafficking

As IGSF1 function is presently unknown, I analyzed the effects of the *IGSF1* mutations on IGSF1-CTD expression. I introduced the mutations found in families A-D and G-K into a human Myc-IGSF1-3xHA construct, transfected the wild-type (WT) and mutant constructs into heterologous HEK293 cells and performed SDS-PAGE and western blotting on the protein lysates. We compared the migration patterns of the various mutants to the migration pattern of wild-type IGSF1-CTD by blotting with the IGSF1-CTD antibody, in order to detect both premature truncation mutants and in-frame mutations. Wild-type IGSF1-CTD migrates as a doublet around 140 kDa (Figure 5.2A, lane 2), with the upper band representing the mature glycoform, which is resistant to deglycosylation by the enzyme EndoH, and the lower band composed of immature glycoprotein, containing EndoH-sensitive high-mannose sugars (lane 4). Both glycoforms can be deglycosylated with PNGaseF, which strips the protein of all N-linked glycans, and reveals its unmodified size to be ~90 kDa (lane 3). In contrast to the WT protein, all the protein products of the mutant constructs migrated predominantly as single bands (Figures 5.2A, lanes 5, 8, 11; 5.2B, lanes 3, 6, 8, 12; 5.2C, lane 3) that could be deglycosylated to an equal extent by EndoH and PNGaseF, indicating they contained mostly high-mannose sugars (Figures 5.2A, lanes 7, 10, 13; 5.2B, lanes 5, 8, 11, 14; 5.2C,

lane 5). Upon deglycosylation with PNGaseF, the mutant proteins migrated at their predicted size (Figures 5.2A, lanes 6, 9, 12; 5.2B, lanes 4, 7, 10, 13; 5.2C, lane 4).

The predominance of EndoH-sensitive glycoforms suggested that the mutant proteins may not be trafficked out of the ER. To determine whether the mutants could be transported to the cell surface as immature glycoproteins, or if they were trapped intracellularly, I transfected HEK293 cells with the WT and mutant constructs and detected the protein products by immunofluorescence under non-permeabilizing and permeabilizing conditions. The IGSF1-CTD epitope is located at the N-terminal portion of the C-terminal domain (CTD); thus, when the protein is inserted into the plasma membrane, the epitope is accessible outside the cell. Under non-permeabilizing conditions, I incubated the cells with antibody while they were still in culture with their plasma membranes intact. As the IGSF1-CTD antibody could only access extracellular epitopes under those conditions, IGSF1-CTD signal should only be detectable if the protein is present in the plasma membrane. Under permeabilizing conditions, I perforated the plasma membranes using a detergent, enabling the antibody to access intracellular proteins. Under non-permeabilizing conditions, I saw a robust wild-type IGSF1-CTD signal at the cell surface. I also detected signal at the plasma membrane in cells transfected with the in-frame mutants Ser863Phe, Cys947Arg, Ser770Asn, and to a lesser extent in the Ala713_Lys721 mutant. In contrast, I did not detect any cell surface signal under non-permeabilizing conditions in cells transfected with the premature truncation mutants Trp977X, Trp1173X, Gly750LysfsX28 and Gly1200ArgfsX3 (Figure 5.3). As the truncation mutants all terminate before the transmembrane domain that normally anchors the protein in the plasma membrane, the complete absence of signal was not surprising. Under permeabilizing conditions, I detected signal in cells transfected with all the truncation mutant constructs (Figure 5.3), indicating the protein products were expressed, but not trafficked to the cell surface.

Although I detected some membrane signal in cells transfected with in-frame mutants, the signal intensity was variable, suggesting the mutants may not be trafficking as

efficiently to the surface as the wild-type protein. In order to more quantitatively examine the cell-surface signal produced by the in-frame mutants, I used a cell-surface biotinylation approach. I transfected HEK293 cells with wild-type or in-frame mutant constructs Ala713_Lys721, Ser863Phe, Cys947Arg, and Ser770Asn, and labeled all cell surface protein with biotin. I then immunoprecipitated the proteins using an HA-antibody, as all the in-frame mutants contained the same C-terminal HA epitope tag as in the wild-type protein. I ran the protein lysates on SDS-PAGE, and detected biotin signal with streptavidin-HRP, in order to determine the extent to which the precipitated IGSF1-CTD was biotinylated, reflecting the amount that was present at the cell surface. Wild-type IGSF1-CTD produced a robust HRP signal corresponding to the mature glycoform only, whereas all the in-frame mutant proteins produced a barely-detectable signal (Figure 5.4). Thus, all of the *IGSF1* mutations identified in patients with central hypothyroidism resulted in protein products that are either less abundant at the plasma membrane or not trafficked out of the ER at all. It is likely that that reduced IGSF1-CTD expression at the plasma membrane in these patients reflected impaired, if not complete loss of IGSF1 function.

Effects of loss of Igsf1 in mice

To confirm that loss of IGSF1 function causes central hypothyroidism, we analyzed HPT axis function in *Igsf1*-deficient animals. We limited our analyses to male mice, as the human phenotype was only observed in male members of the affected families. Additionally, male mice have higher levels of pituitary *Igsf1* mRNA than females, and the extent of *Igsf1* deficiency is more dramatic in *Igsf1*^{Δex1} males than females (39). Thus, we reasoned that it might be easier to detect a subtle phenotype in *Igsf1*-deficient males than females.

We began by measuring thyroid hormone levels in adult wild-type and *Igsf1*^{Δex1} males. We observed a large degree of variability in the extent of hypothyroidism, with some *Igsf1*^{Δex1} mice having drastically lower levels of total serum T₄, while others were euthyroid and indistinguishable from controls. In addition, results from assays performed

on plasma showed greater variability than results obtained from serum measurements (Figure 5.5A). We also found that *Igsf1*^{Δex1} males showed a non-significant trend for decreased free T₄ index relative to controls ($p < 0.0885$) (Figure 5.5B). We did not observe any progressive increases or decreases of T₄ with age up to 20 weeks in either WT or *Igsf1*^{Δex1} males (Figure 5.5C), although the values tended to vary more with increasing age in both groups. We also measured total serum T₃, and found that it was mildly, though consistently decreased in *Igsf1*^{Δex1} mice (Figure 5.5D).

We then analyzed *Tshb* mRNA levels and TSH protein content in the pituitary. Consistent with previous reports (39), we saw no significant difference between *Tshb* mRNA levels between WT and *Igsf1*^{Δex1} mice (Figure 5.6A); however, the pituitary TSH protein content was significantly decreased in the knockout animals (Figure 5.6B). Although variable, serum TSH levels were also decreased in *Igsf1*^{Δex1} mice (Figure 5.6C). Thyroid hormone levels did not correlate with serum TSH or pituitary TSH content (Figure 5.6D, Figure 5.6E).

As hypothyroidism is characterized by a lower metabolic rate, and hypothyroid patients tend to be overweight, we also analyzed the body weight of our animals. We weighed the animals at weekly intervals starting at 1 week after birth, and continuing to 10 weeks. We saw that *Igsf1*^{Δex1} mice exhibited increased body weight starting post-weaning (about 4 weeks) compared to WT littermates; the difference was statistically significant at 7 weeks (Figure 5.7A). We also analyzed thyroid histology and found that, consistent with the mild phenotype, there were no dramatic effects on thyroid gland size and morphology, although thyroid epithelium thickness was slightly increased in *Igsf1*^{Δex1} males (Figure 5.7B).

The decrease in circulating TSH and pituitary TSH content without a concurrent decrease in *Tshb* mRNA suggested that the defect may be related to TRH signaling. It has been reported that TRH plays an important role in maturation and glycosylation of TSH, and a comparatively lesser role in *Tshb* transcription (see Chapter 4, section 1.1.3). I analyzed

pituitary *Trhr* and hypothalamic *Trh* mRNA levels, and found a greater than 50% decrease in *Trhr* (Figure 5.8A), along with a mean increase of 50% in hypothalamic *Trh* in *Igsf1*^{Δex1} mice (Figure 5.8B). The increase in *Trh* is consistent with reduced thyroid hormone feedback in the hypothalamus in a hypothyroid state. The decrease in pituitary *Trhr* levels suggested *Igsf1*-deficient mice may have an impaired response to TRH. In order to determine whether TRHR signaling is impaired in *Igsf1*^{Δex1} mice, we measured hormone release following exogenous TRH treatment. Whereas four of the six WT males exhibited TRH-induced increases in serum T₄, the response was virtually absent in all six *Igsf1*^{Δex1} mice (Figure 5.8C,D). The difference between genotypes, however, was not statistically significant, prompting us to repeat the experiment on a larger cohort of mice, and measure both TSH and thyroid hormone release. Unfortunately, for reasons that are unclear, neither the WT nor *Igsf1*^{Δex1} mice showed increased T₄ levels following TRH administration (Figure 5.8E), despite a robust TSH response in both genotypes (Figure 5.8F). The *Igsf1*^{Δex1} mice did show decreased release of TSH in response to TRH administration in the trial compared to WT mice (Figure 5.8F); however, as their pituitary TSH content is lower at baseline, the decreased response to TRH may reflect lower TSH reserves, rather than a TRH signaling defect.

I also analyzed additional components of the HPT axis in the *Igsf1*-deficient mice by RT-qPCR, including the expression of deiodinating enzymes *Dio1*, *Dio2*, and *Dio3* in the pituitary gland. Both *Dio2* and *Dio3* mRNA levels were significantly lower in *Igsf1*^{Δex1} mice compared to controls; *Dio1* was normal (Figure 5.9A,B,C). Additionally, *Thrb2*, the main thyroid hormone receptor isoform responsible for negative feedback, was significantly decreased in pituitaries of *Igsf1*^{Δex1} mice (Figure 5.9D). No differences in *Dio2*, *Dio3*, or *Thrb2* were observed in the hypothalami of *Igsf1*^{Δex1} mice (not shown).

Because over half of the patients with *IGSF1* mutations had prolactin deficiency in addition to central hypothyroidism, we measured *Prl* mRNA and prolactin protein content in the pituitary glands of the mice, but neither differed between control and *Igsf1*-deficient mice (Figure 5.10A,B). We also assessed growth hormone (*Gh*) mRNA and

pituitary protein content, and found a significant increase in growth hormone protein content in *Igsf1*^{Δex1} mice, in the presence of unchanged *Gh* mRNA levels (Figure 5.10C,D).

Finally, whereas most of the patients with *IGSF1* mutations present with macroorchidism, testes were not enlarged in *Igsf1*^{Δex1} mice, consistent with previous reports (Figure 5.10E) (39).

4 Discussion

In this study, we demonstrated that loss-of-function mutations in *IGSF1* cause central hypothyroidism in humans. All the identified mutations abolish or decrease the presence of IGSF1-CTD at the plasma membrane, where it presumably needs to be trafficked to carry out its function. Further, *Igsf1*-deficient mice phenocopy key aspects of central hypothyroidism phenotype in humans, including low thyroid hormone and TSH levels. However, many questions as to how loss of IGSF1 leads to hypothyroidism remain unanswered, as discussed below.

Strikingly, all identified in-frame mutants show impaired trafficking to the plasma membrane, and both in-frame and prematurely truncated mutant proteins acquire predominantly immature glycans, suggestive of their retention in the ER. Although the prematurely truncated proteins could also be secreted, as they all terminate before the transmembrane domain, we did not detect them in culture media collected from transfected cells (not shown). The most plausible explanation is that the mutations lead to protein misfolding, initiating a process known as endoplasmic reticulum-associated degradation (ERAD). ERAD allows cells to recognize and degrade proteins that have failed to fold correctly, preventing their accumulation in the ER (467). One of the best-studied examples of proteins that undergo this process is the cystic fibrosis transmembrane conductance regulator (CFTR). The most frequently encountered pathogenic mutation in the human *CFTR* gene is a deletion of a phenylalanine at position 508 (ΔF508), which leads to misfolding of the protein (468), but only partially reduces its

function (469-471). Like IGSF1, CFTR is a large protein that exists in both immature and mature glycoforms, with the mature form found at the plasma membrane (472). In contrast, the CFTR Δ F508 mutant only acquires immature glycans (468), and is degraded by ERAD shortly after translation (473). Thus, it is conceivable that mutant forms of IGSF1 undergo the same degradation process. Pulse-chase analyses are required to determine whether the mutant proteins are trafficked out of the ER, and whether inhibiting the ERAD-associated proteasomal degradation pathway results in increased protein content in the cells. Interestingly, the CFTR Δ F508 folding defect can be repaired *in vitro* by chemical chaperones that enable the mutant protein to fold correctly, restoring CFTR expression at the cell surface (474-476), suggesting molecular chaperones are a viable therapeutic strategy to rescue protein folding defects (477, 478). However, the development of these compounds to treat patients with central hypothyroidism associated with IGSF1 mutations is unlikely, as thyroid hormone replacement therapy is a safe, effective, and established method to treat central and other forms of hypothyroidism (479).

We observed that the hypothyroidism phenotype in *Igsf1*-deficient mice is relatively mild. The mice have increased body weight, but otherwise appear overtly normal. Serum/plasma T₄ values were highly variable across individuals, with most mice having normal or near-normal values, although a few mice had very low levels. T₃ levels were more consistent within genotypes, and significantly decreased in *Igsf1*-deficient animals relative to controls. Thyroid histology was largely normal, although thyroid epithelium was approximately 15% thicker in *Igsf1*-deficient mice. Thyroid enlargement (goiter) is associated with primary hypothyroidism, as high levels of TSH stimulate thyroid growth (480), but has not been reported in central hypothyroidism. However, additional factors can stimulate proliferation of thyroid cells, including growth hormone, via the mediator insulin-like growth factor 1 (IGF-1) (481-484), and fibroblast growth factor (FGF) (485). As *Igsf1*-deficient mice have increased pituitary growth hormone content, it will be interesting to determine whether these mice have elevated serum growth hormone and/or IGF-1, leading to increased thyroid growth.

It is possible that the phenotype is mild in *Igsf1*-deficient mice due to retention of *Igsf1*-4, an mRNA isoform that is not ablated in pituitary by the gene targeting strategy, and which encodes IGSF1-CTD (Chapter 1, section 2.5.2; Chapter 2, Figure 2.4). However, the human phenotype is also highly variable, with the extent of hypothyroidism in patients within the same families (i.e. same mutations) ranging from overt neonatal thyroid hormone deficiency to mild, subclinical hypothyroidism detected only in adulthood (Appendix B, Table 1). The variability seen in both mice and patients suggest that genotype alone does not determine the severity of the disorder. Additional factors, such as epigenetic variations (486, 487) or polymorphisms in genes involved in regulation of HPT axis function, including *TSHR*, thyroid hormone transporters, or deiodinase genes (488-490) can likely alter the extent of central hypothyroidism caused by loss of IGSF1 function. These changes could result in differential sensitivity of the thyroid to TSH, or alterations in the thyroid hormone feedback mechanism at the level of the pituitary, determining the extent of hypothyroidism in individuals.

The mechanism behind the central hypothyroidism in patients with *IGSF1* mutations and *Igsf1*-deficient mice is unknown at present. The decrease in pituitary TSH content in the absence of a decrease of *Tshb* mRNA, combined with a decrease in *Trhr* mRNA levels, suggests an impairment in TRH signaling. TRH signaling is important for TSH maturation and acquisition of bioactivity (316, 318), thus we might also expect to see a decrease in TSH biological activity in *Igsf1*-deficient mice, and patients with mutations in *IGSF1*. Indeed, mice deficient in either TRH or TRHR have either normal or only slightly (79%) decreased *Tshb* levels, but significant impairments in pituitary TSH content and/or bioactivity, though not serum TSH levels (230, 232). Upon TRH stimulation, *Igsf1*-deficient mice had lower serum TSH relative to control animals, although it is difficult to determine whether it was due to decreased TRH signaling, or due to depletion of TSH reserves in the thyrotropes. Similarly, TRH challenge in patients with *IGSF1* mutations produced mixed results, with younger patients (2.5-5 weeks) having lower TSH levels than the reference range, while older patients (0.4-65.5 years) were within the lower half

of the reference range (see Appendix B, Supplementary Table 2). Thus, IGSF1 deficiency in both mice and patients may result in decreased TSH release, but not reduced TSH bioactivity. Our collaborators attempted to directly assess TSH bioactivity in serum collected from patients; however, the amount of serum needed to purify sufficient TSH for bioactivity testing was not available at the time of publication (see Appendix B). I attempted to test TSH bioactivity in whole serum directly in the mice (491, 492), but the assay was not sensitive enough to detect changes in bioactivity. In the future, the assay will likely have to be carried out with purified TSH. Therefore, at this time we are unable to determine whether TSH secreted from the pituitaries of *Igsf1*-deficient mice shows a decrease in bioactivity.

We noted decreased pituitary *Thrb2*, *Dio2*, and *Dio3* expression in *Igsf1*-deficient mice. *Thrb2* is downregulated by TRH in GH3 cells *in vitro* (493), suggesting that increased hypothalamic *Trh* (and by extension peptide synthesis and secretion) in *Igsf1*-deficient mice could lead to decreased *Thrb2* expression. To address whether the downregulation of *Thrb2* is driven by hypothalamic TRH, we assessed *Thrb2* levels in primary pituitary cultures, which are removed from the influence of extrapituitary hormones. We did not see a difference between the genotypes under these conditions, suggesting the difference is due to an extrinsic factor; however, TRH treatment of up to 24 hours did not significantly downregulate *Thrb2* expression in either WT or *Igsf1*-deficient cultures (not shown), indicating another hormone or factor may be responsible for *Thrb2* downregulation in *Igsf1*-deficient mice. The decreases seen in mRNA levels of both the activating (*Dio2*), and inactivating (*Dio3*) deiodinase mRNA levels in the pituitary gland are interesting, because rather than altered processing of thyroid hormone in one direction (increased activation *or* increased deactivation), it suggests dampening of both processes in the pituitary glands of *Igsf1*-deficient mice. However, as we do not know which cell type(s) in the pituitary exhibit decreased *Dio2* and/or *Dio3*, the effects, if any, of these changes are difficult to predict. Further, DIO2 activity is extensively regulated by ubiquitination, making *Dio2* mRNA levels and enzymatic activity hard to correlate (494-496). DIO2 is crucial for conversion of T₄ to T₃ in the pituitary, as *Dio2*-deficient mice

display pituitary resistance to T_4 and increased serum TSH (497). Thus, if the decrease in *Dio2* mRNA leads to decreased DIO2 activity in thyrotropes of *Igsf1*-deficient mice, this might lead to decreased T_3 feedback in the pituitary, perhaps as an adaptive response to hypothyroidism. In contrast, *Dio3*-deficient mice have high perinatal serum T_3 , followed by hypothyroidism in adulthood, as a result of multiple defects in the HPT axis (498, 499). Thus, if pituitaries of *Igsf1*-deficient mice have decreased *Dio3*, we might expect increased negative feedback as a result of decreased clearance of T_3 , leading to hypothyroidism. Ultimately, the balance of DIO2 and DIO3 may determine the outcome in *Igsf1*-deficient mice. Addressing these possibilities will require analysis of deiodinase activities (500, 501) in pituitaries from *Igsf1*-deficient mice. We do not know if patients with *IGSF1* mutations exhibit similar alterations in pituitary *DIO2* and *DIO3* levels. Interestingly, loss of DIO2 activity occurs during ER stress, associated with increased activity of the ERAD pathway (502). Thus, in some patients with *IGSF1* mutations, accumulation of mutant IGSF1 protein products could decrease DIO2 activity, leading to reduced conversion of T_4 to T_3 , resulting in diminished negative feedback in these individuals.

One feature of the disorder that remains elusive is the macroorchidism seen in patients with *IGSF1* mutations. It is possible that macroorchidism seen in patients with *IGSF1* mutations is secondary to their hypothyroidism, as macroorchidism has been reported in some cases of prepubertal thyroid dysfunction (503). However, the high incidence of macroorchidism (nearly 100%) in our patients, including in those that have been treated with thyroid hormone from infancy (Appendix B, Tables 1,2), argues against this possibility. Because IGSF1 was originally identified as a potential inhibin receptor (35), it is tempting to speculate that the macroorchidism reflects impaired inhibin function, and a resulting increase in FSH. FSH is important for germ cell development and Sertoli cell proliferation in the testes, particularly during fetal life (504-508). Thus, increased FSH signaling could result in increased Sertoli cell proliferation and macroorchidism. However, inhibin does not bind IGSF1 *in vitro* (57), and *Igsf1*-deficient mice show no changes in either pituitary *Fshb* mRNA or serum FSH levels, casting doubt on its role as

an inhibin receptor. In addition, most of the patients had normal serum FSH levels, again arguing against loss of inhibin action in the absence of functional IGSF1. Thus, how the loss of IGSF1 function leads to macroorchidism in humans remains to be determined. Unfortunately, the *Igsf1*-deficient mice do not appear to be a suitable model to study the role of IGSF1 in testes, as they do not show an increase in testes size. This could be a reflection of differential expression of IGSF1 between humans and mice in various tissues, including testis. Although we can detect *Igsf1* mRNA in both murine and human testes (Appendix B, Supplementary Figure 2), we have not been able to detect the protein in the former by western blot using the IGSF1-CTD antibody, which has been used successfully in other murine tissues (data not shown).

Collectively, our data demonstrate that loss of IGSF1 function, whether due to inactivating mutations or deletion of the gene, leads to central hypothyroidism in both mice and humans. However, the phenotype does not enable us to determine the function of IGSF1. The decrease in *Trhr* expression in the pituitary, decreased TSH response to TRH, and the fact that IGSF1 is most abundantly expressed in the pituitary gland argue in favor of a pituitary-specific defect. However, as IGSF1 is also expressed in the hypothalamus, it is possible the disorder stems from a combination of pituitary and hypothalamic defects. Our data suggest IGSF1 may play a role in propagation or modulation of TRH signaling; further investigation is necessary to test this hypothesis, and to delineate IGSF1's function.

Figure legends

Figure 5.1 Diagram of IGSF1 protein domain structure and relative positions of identified mutations in 11 families with central hypothyroidism. Nucleotide and amino acid alterations are indicated below diagram. The mutations are named according to coding sequence of the longest isoform, *IGSF1-3* (Chapter 1, Figure 1.1), using Human Genome Variation Society (<http://www.hgvs.org>) nomenclature.

Figure 5.2 IGSF1 mutant proteins acquire only core glycosylation. HEK293 cells were transfected with 200 ng of pcDNA3 (empty vector) or indicated wild-type or mutant human IGSF1-1 expression vectors. Protein lysates were deglycosylated with either PNGaseF (P) or EndoH (E), resolved by SDS-PAGE, and immunoblotted using IGSF1-CTD antibody. β -actin (ACTB) was used as a loading control. Asterisks indicate non-specific bands. **(A)** Western blot shows results from family A (A713_K721), family G (S863F), and family B (W977X). **(B)** Western blot shows results from family J (G1200RfsX3), family H (C947R), family K (S770N), and families C/D (G750KfsX28). **(C)** Western blot shows results from family I (W1173X).

Figure 5.3 Prematurely truncated IGSF1 mutant proteins do not traffic to the plasma membrane. HEK293 cells were transfected with 100 ng of pcDNA3 (empty vector) or the indicated WT or mutant human IGSF1-1 expression constructs. Expression of WT or mutant protein products was analyzed by immunofluorescence using IGSF1-CTD antibody under non-permeabilizing and permeabilizing conditions. Scale bars 10 μ m. DAPI was used as a nuclear marker (blue).

Figure 5.4 In-frame IGSF1 mutant proteins show impaired trafficking to the plasma membrane. HEK293 cells were transfected with 200 ng of pcDNA3, WT or indicated in-frame mutant human IGSF1-1 expression constructs, and subjected to cell surface biotinylation. Protein lysates were then immunoprecipitated using an HA antibody, and run on SDS-PAGE. Biotin signal was visualized using Streptavidin-HRP, and total and

immunoprecipitated lysates were immunoblotted with HA antibody. β -actin (ACTB) was used as a loading control.

Figure 5.5 *Igsf1* ^{Δ ex1} males have variable thyroid hormone deficiency. (A) Serum (set 1, n = 19-22 per genotype), or plasma (set 2, n = 18 per genotype) total T₄ was measured in two different sets of WT and *Igsf1* ^{Δ ex1} adult males, 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. Individual samples are represented by circles (WT) or squares (*Igsf1* ^{Δ ex1}). Horizontal line indicates the mean. (B) Serum free T₄ was measured in WT and *Igsf1* ^{Δ ex1} adult males (n = 18-21 per genotype). (C) Plasma T₄ levels were measured at 4, 8, 12, 16, and 20 weeks of age in WT (black circles) and *Igsf1* ^{Δ ex1} (white circles) males. (D) Serum total T₃ was measured in WT and *Igsf1* ^{Δ ex1} adult males (n = 14-16 per genotype).

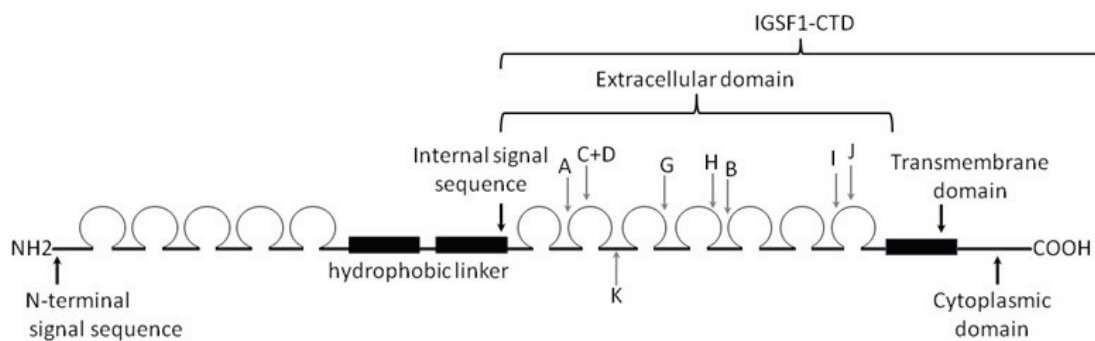
Figure 5.6 *Igsf1* ^{Δ ex1} males have decreased pituitary and serum TSH, but not *Tshb* mRNA. (A) RNA was extracted from individual pituitaries and RT-qPCR was performed to assess *Tshb* mRNA levels in WT and *Igsf1* ^{Δ ex1} adult males (n = 6 per genotype). (B) TSH protein content was measured in individual pituitaries from WT and *Igsf1* ^{Δ ex1} adult males (n = 6 per genotype). (C) Serum TSH was measured in WT and *Igsf1* ^{Δ ex1} adult males (n = 18-22 per genotype). (D) Serum TSH vs. serum total T₄ in WT (black) and *Igsf1* ^{Δ ex1} (gray) adult males (n = 18-22 per genotype). (E) Pituitary TSH protein content vs. plasma total T₄ in WT and *Igsf1* ^{Δ ex1} adult males (n = 6 per genotype).

Figure 5.7 *Igsf1* ^{Δ ex1} males have increased body weight, and normal thyroid histology. (A) WT and *Igsf1* ^{Δ ex1} adult males (n = 6-24 per genotype) were weighed weekly starting at 1 week after birth. Circles indicate mean for each genotype (WT: white; *Igsf1* ^{Δ ex1}: black). Error bars = s.e.m. (B) Thyroid gland morphology and epithelium thickness was assessed in WT and *Igsf1* ^{Δ ex1} adult males.

Figure 5.8 *Igsf1*^{Δex1} males may have alterations in TRH signaling. (A) RNA was extracted from individual pituitaries and RT-qPCR was performed to assess *Trhr* mRNA levels in WT and *Igsf1*^{Δex1} adult males (n = 6 per genotype). (B) RNA was extracted from individual hypothalami and RT-qPCR was performed to assess *Trh* mRNA levels in WT and *Igsf1*^{Δex1} adult males (n = 5 per genotype). (C, D) 5 μg/kg of TRH was administered i.p. to WT and *Igsf1*^{Δex1} adult males. Serum T₄ was measured before and 2 hr after TRH administration (n= 6 per genotype). Individual responses (C) and percent change (D) are shown (n.s., p < 0.0835). (E, F) 5 μg/kg of TRH was administered i.p. to WT and *Igsf1*^{Δex1} adult males. Serum T₄ (E) was measured before and 3 hr after TRH administration (n = 14-16 per genotype). Serum TSH (F) was measured before and 30 min after TRH administration (n = 7-9 per genotype). Error bars in D, E, F = s.e.m.

Figure 5.9 *Igsf1*^{Δex1} males have altered levels of *Dio2*, *Dio3* and *Thrb2* mRNA. RNA was extracted from individual pituitaries and RT-qPCR was performed to assess (A) *Dio1*, (B) *Dio2*, and (C) *Dio3* mRNA levels in WT and *Igsf1*^{Δex1} adult males (n = 5-6 per genotype). (D) RNA was extracted from individual pituitaries and RT-qPCR was performed to assess *Thrb2* mRNA levels in WT and *Igsf1*^{Δex1} adult males (n = 5-6 per genotype).

Figure 5.10 *Igsf1*^{Δex1} males have normal pituitary *Prl* mRNA and prolactin protein content, normal testes weight, but increased pituitary GH content. (A) RNA was extracted from individual pituitaries and RT-qPCR was performed to assess *Prl* mRNA levels in WT and *Igsf1*^{Δex1} adult males (n = 5-6 per genotype). (B) Prolactin protein content was measured in individual pituitaries from WT and *Igsf1*^{Δex1} adult males (n = 6 per genotype). (C) RNA was extracted from individual pituitaries and RT-qPCR was performed to assess *Gh* mRNA levels in WT and *Igsf1*^{Δex1} adult males (n = 5-6 per genotype). (D) Growth hormone protein content was measured in individual pituitaries from WT and *Igsf1*^{Δex1} adult males (n = 6 per genotype). (E) Testes weights (relative to body weight) from adult WT and *Igsf1*^{Δex1} adult males (n = 13-18 per genotype).



- Family A: c.2137_2163del, p.(Ala713_Lys721del)
- Family B: c.2931G>A, p.(Trp977X)
- Family C & D: c.2248delG, p.(Glu750LysfsX28)
- Family E: 126kb deletion, arr Xq26.1q26.2(130.386.267-130.512.002)x0 (hg19)
- Family F: 328kb deletion, arr Xq26.1q26.2(130.310.905-130.639.353)x0 (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.3596dupT, p.(Glu1200ArgfsX3)
- Family K: c.2309G>A, p.(Ser770Asn)

Figure 5.1

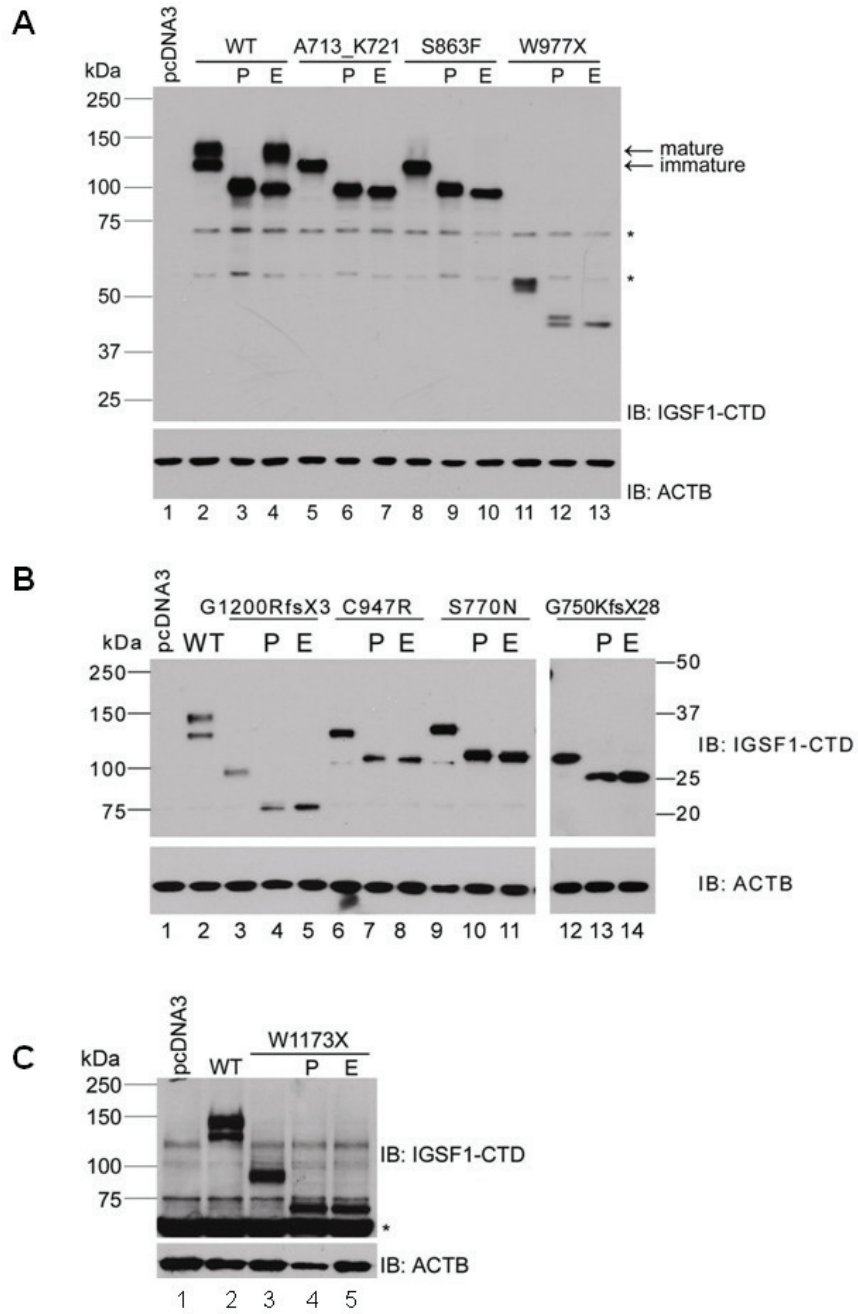


Figure 5.2

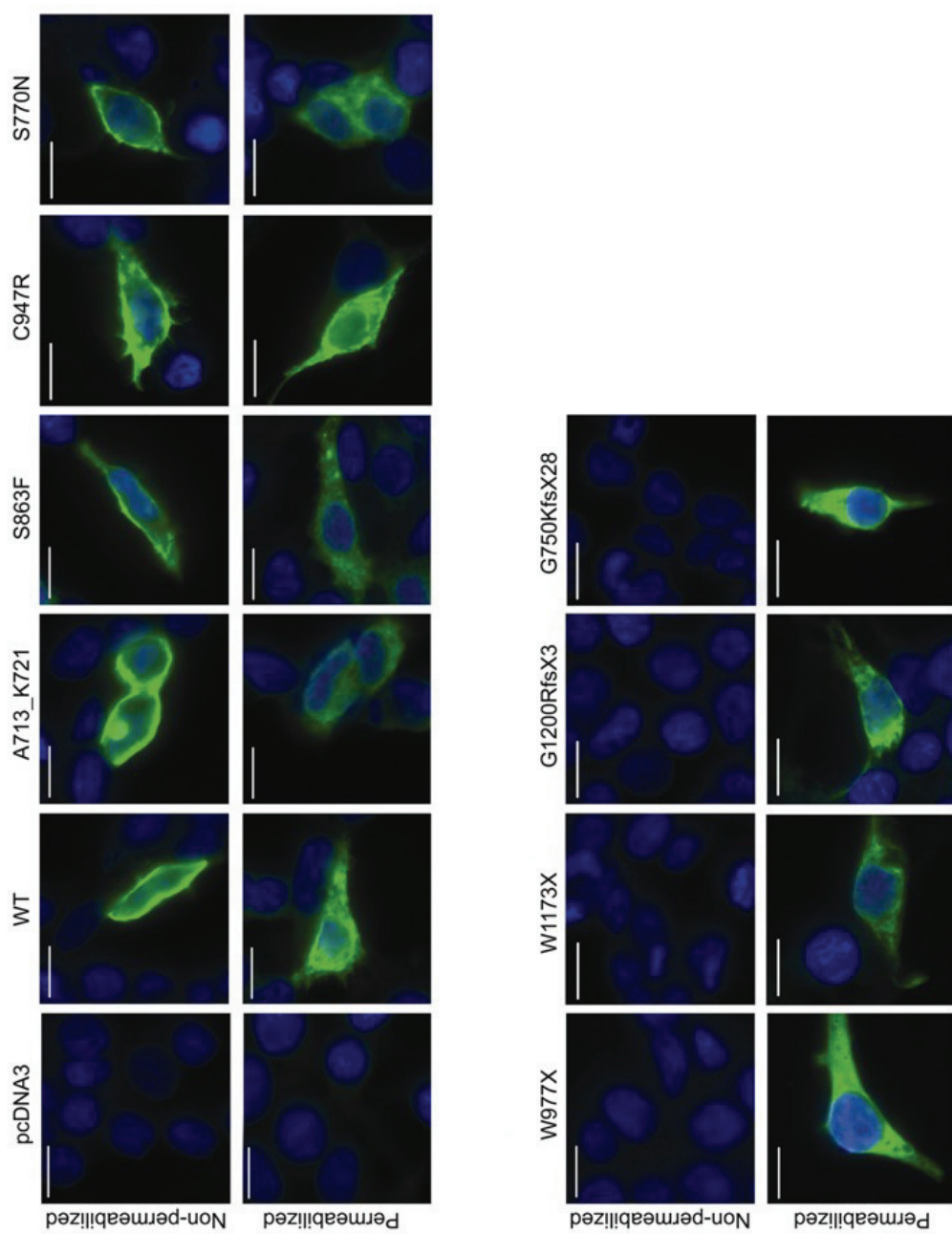


Figure 5.3

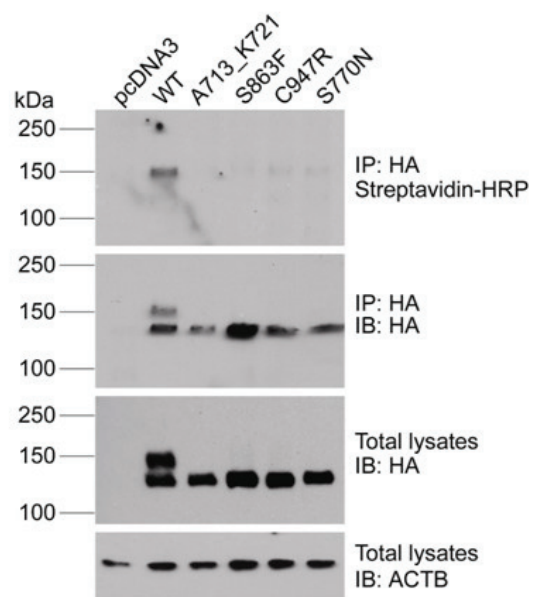


Figure 5.4

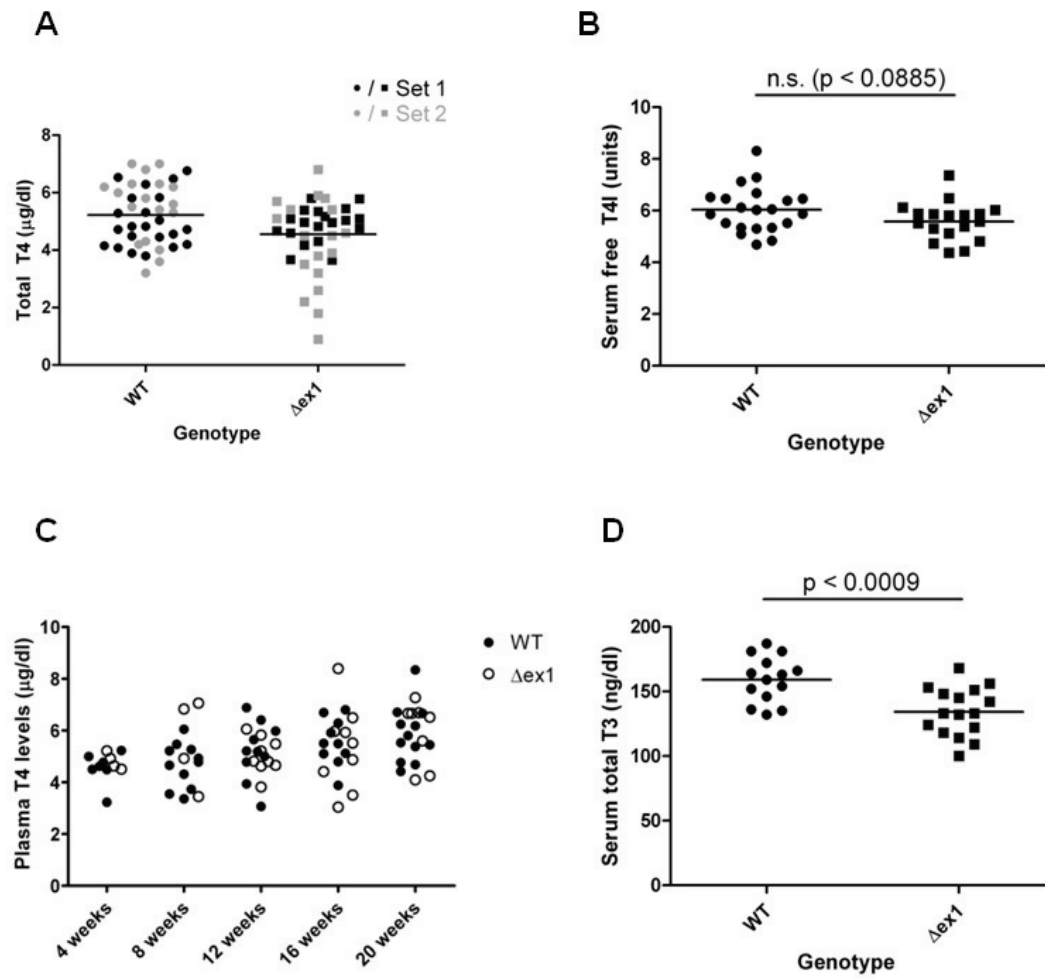


Figure 5.5

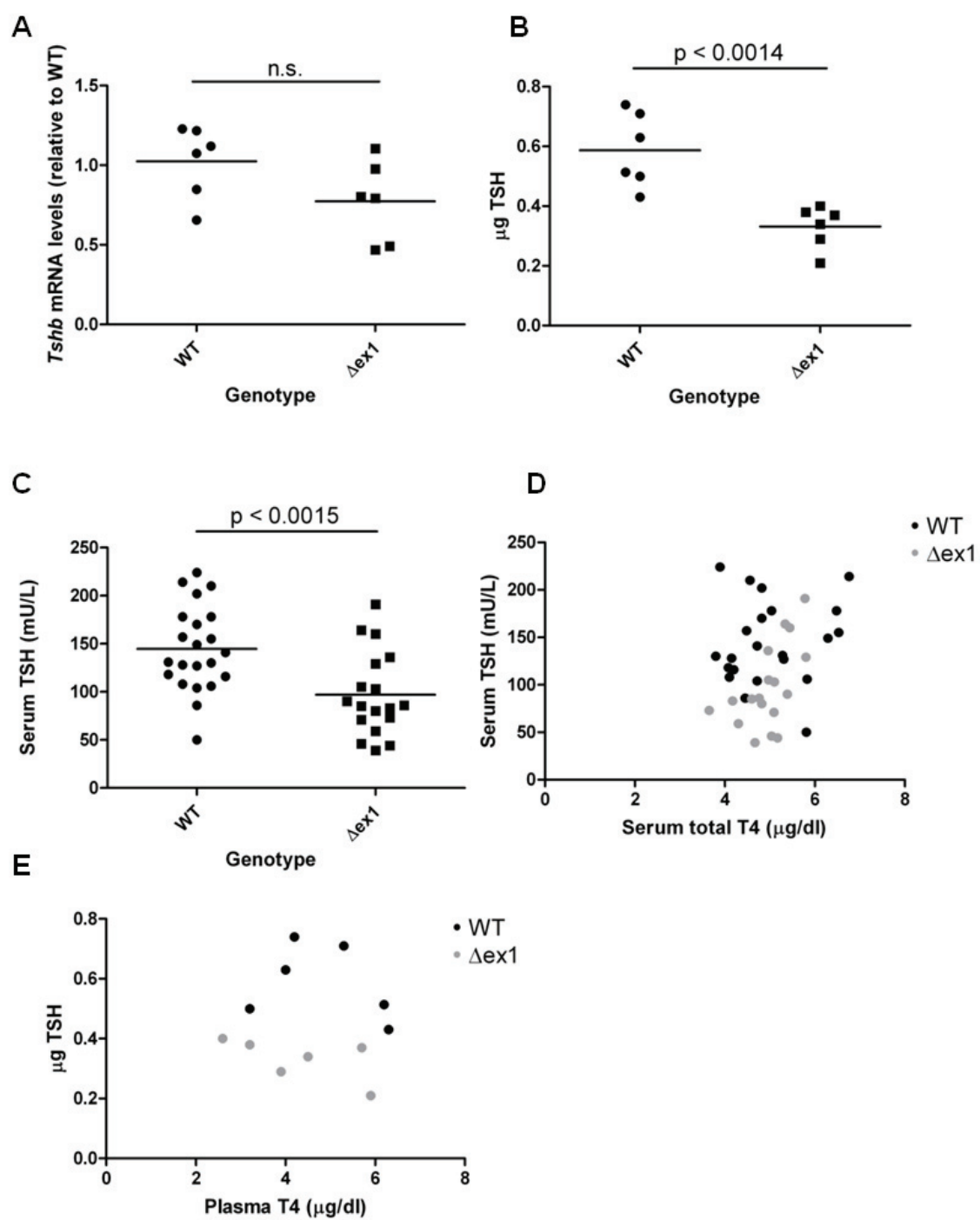
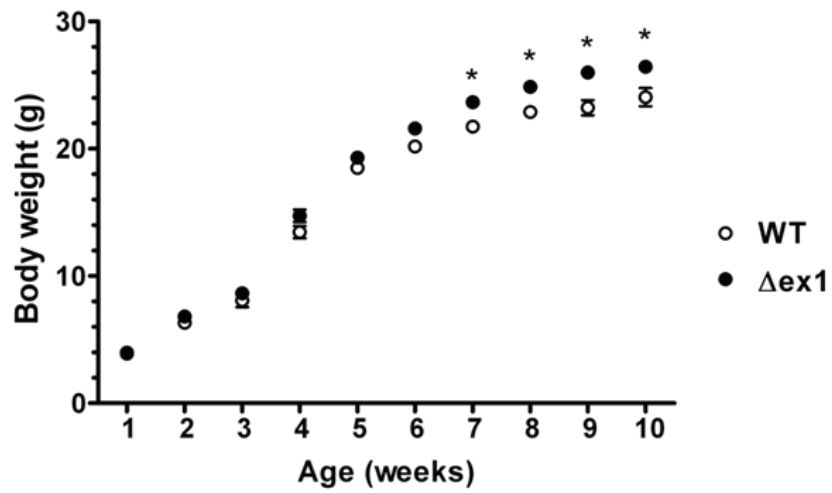


Figure 5.6

A



B

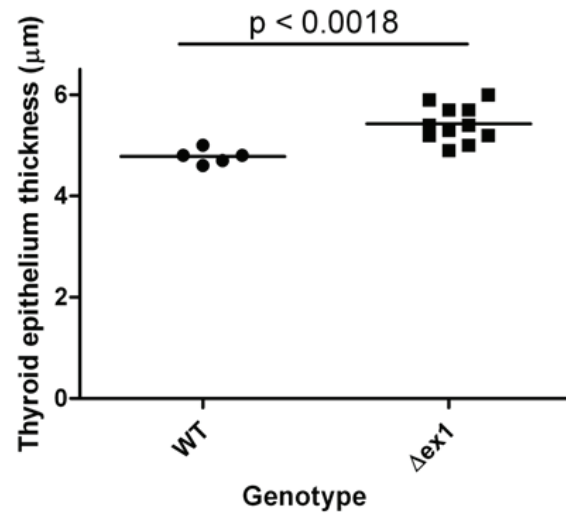


Figure 5.7

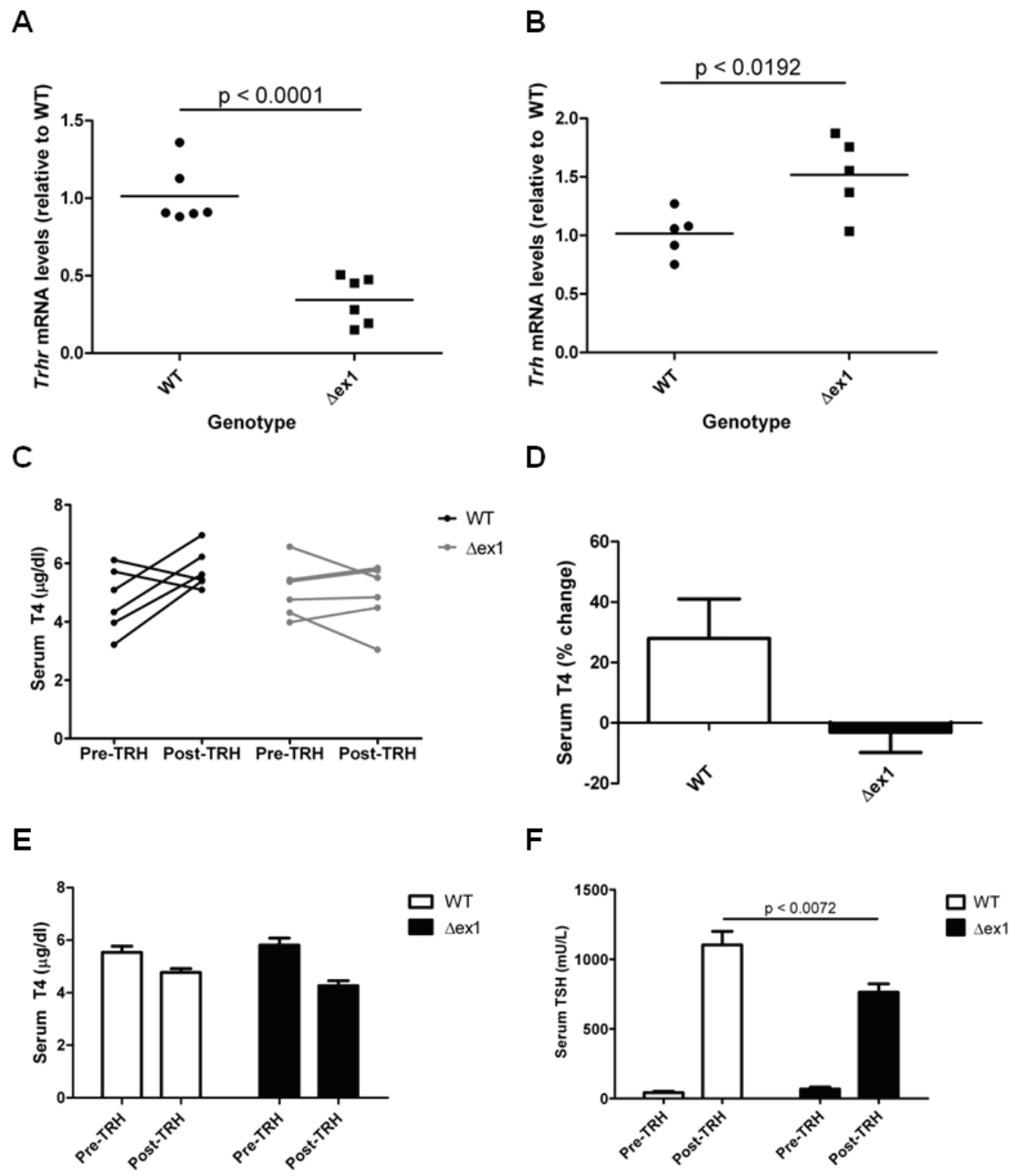


Figure 5.8

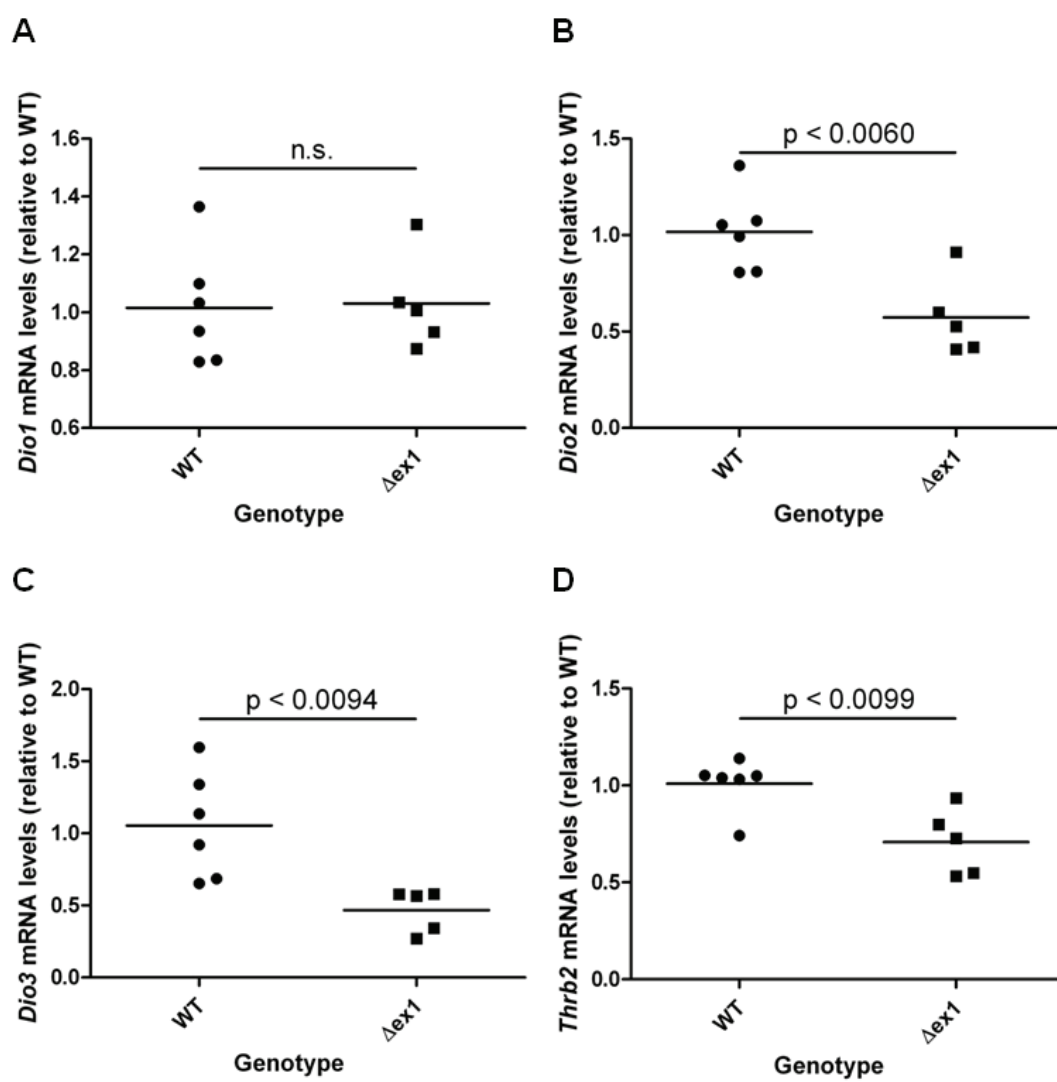


Figure 5.9

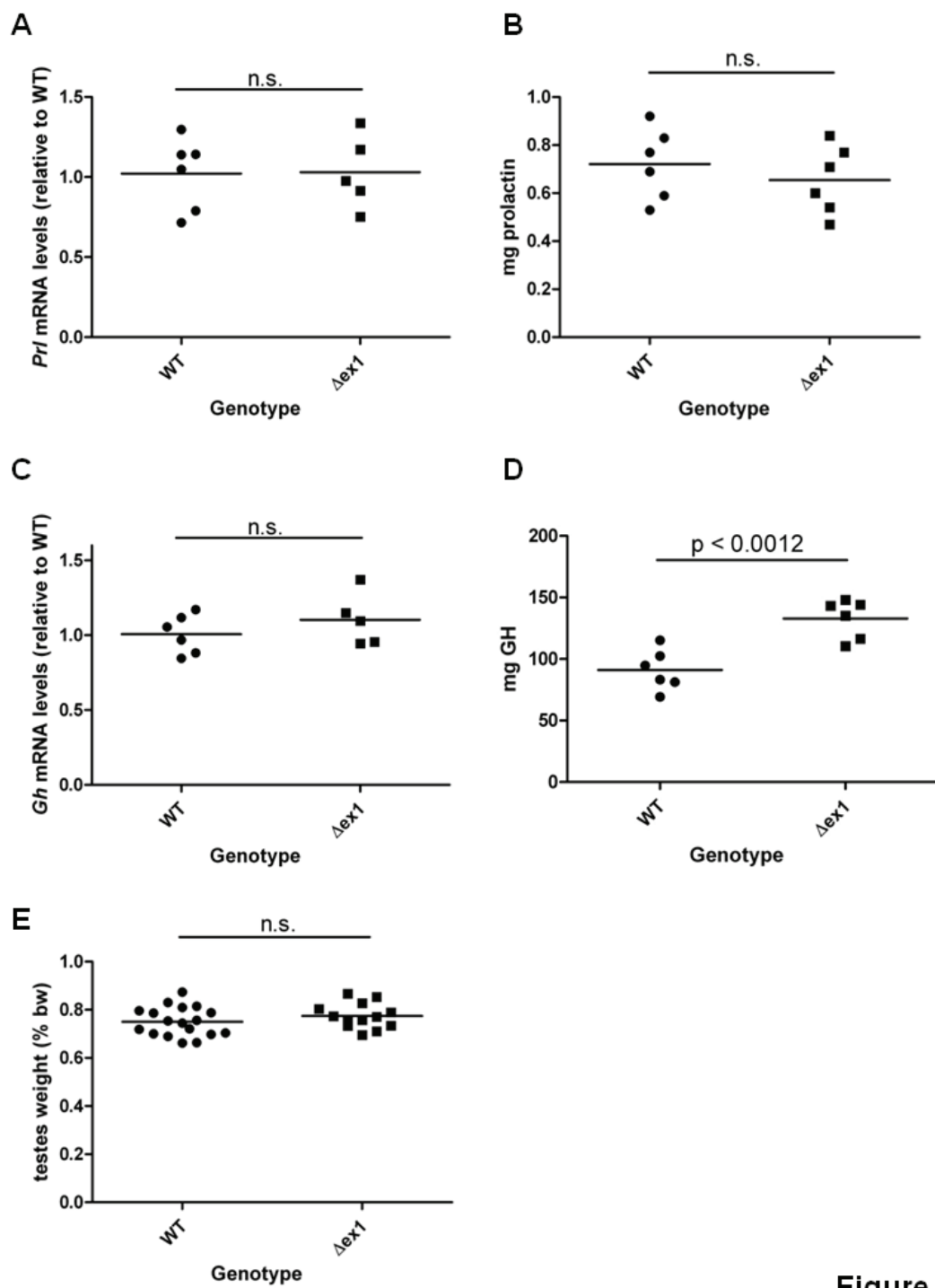


Figure 5.10

Table 5.1 Primers used to introduce mutations into the Myc-IGSF1-1-3xHA vector.

Mutation:	Sense Primer:	Antisense Primer:
Primers to change IGSF1-CTD epitope	5'-GTGGACTGTGCAATGGGGCATTGACA GAAGAGATTGAAATAGTCATGCCAAC-3'	5'-GTTGGCATGACTATTTCAATCTCTTCT GTCAATGCCCCATTGCACAGTCCAC-3'
c.2137_2163delG p.(Ala713_Lys721del)	5'-GAGGGAGAGCAAGAACCTGTCCAG CA-3'	5'-CAGCCATCCTTTGCACCGGAGTTG TA-3'
c.2931G>A p.(Trp977X)	5'-ACATTCCCTAAGCCATGATTGTTTGC TGAGCCCAG-3'	5'-CTGGGCTCAGCAAACAATCATGGCTT AGGGAATGT-3'
c.2248del p.(Glu750LysfsX28)	5'-GAGAATGGAGGATAAAGACAAGGCA ATTACAGCTGCC-3'	5'-GGCAGCTGTAATTGCCTTGTCTTTATCC TCCATTCTC-3'
c.2588C>T p.(Ser863Phe)	5'-TATTATGACTTTTCTATCTGGTTTGAG CCCAGCGACC-3'	5'-GGTCGCTGGGCTCAAACCAGATAGAA AAGTCATAATA-3'
c.2839T>C p.(Cys947Arg)	5'-GGACTCTGGGAACATAGCCGTATCT ACTATGAGACAAC-3'	5'-GTTGTCTCATAGTAGATACGGCTATA GTTCCCAGAGTCC-3'
c.3518G>A p.(Trp1173X)	5'-ACCCTCTCTGTCAGCCTAGCCCAGCA C-3'	5'-GTGCTGGGCTAGGCTGACAGAGAG GGT-3'
c.3596dupT p.(Glu1200ArgfsX3)	5'-CCAGGTGTTGAATTTGCCTTAGAACA TGATGGAGAAGA-3'	5'-TCTTCTCCATCATGTTCTAAGGACAAA TTCAACACCTGG-3'
c.2309G>A p.(Ser770Asn)	5'-GTGGTCTGAGCCCAATGAGCCGCT GGA-3'	5'-TCCAGCGGCTCATTGGGCTCAGA CCAC-3'

Table 5.2 Human mutations according to coding sequences of isoforms *IGSF1-3* and *IGSF-1*.

Mutation in <i>IGSF1-3</i>	Equivalent mutation in <i>IGSF1-1</i>
c.2137_2163del, p.(Ala713_Lys721del)	c.2122_2148del, p.(Ala708_Lys716del)
c.2931G>A, p.(Trp977X)	c.2916G>A, p.(Trp972X)
c.2248delG, p.(Glu750LysfsX28)	c.2233delG, p.(Glu745LysfsX28)
c.2588C>T, p.(Ser863Phe)	c.2573C>T, p.(Ser858Phe)
c.2839T>C, p.(Cys947Arg)	c.2824T>C, p.(Cys942Arg)
c.3518G>A, p.(Trp1173X)	c.3503G>A, p.(Trp1168X)
c.3596dupT, p.(Glu1200ArgfsX3)	c.3581dupT, p.(Glu1195ArgfsX3)
c.2309G>A, p.(Ser770Asn)	c.2294G>A, p.(Ser765Asn)

Table 5.3 Primers used for quantitative RT-PCR in mouse tissues.

Gene:	Sense Primer:	Antisense Primer:
<i>Tshb</i>	5'-GAACGGTGGAAATACCAGGA-3'	5'-AGAAAGACTGCGGCTTGGTGCA-3'
<i>Trhr</i>	5'-CTCCCCAACATAACCGACAG-3'	5'-GCAGAGAAACTGGGCTTTGA-3'
<i>Fshb</i>	5'-GTGCGGGCTACTGCTACACT-3'	5'-CAGGCAATCTTACGGTCTCG-3'
<i>Gh</i>	5'-ACCTCGGACCGTGTCTATGA-3'	5'-GCAGCCCATAGTTTTTGAGC-3'
<i>Prl</i>	5'-AAGCAGCTTCTTGAGGGAGTT-3'	5'-TGTTGCGCAAAGACAAGATT-3'
<i>Pomc</i>	5'-CCAGGAACAGCAGCAGTG-3'	5'-ACGTTGGGGTACACCTTCAC-3'
<i>Trh</i>	5'- GAAAGACCTCCAGCGTGTG-3'	5'-TCTTCGGCTTCAACGTCTTC-3'
<i>Dio1</i>	5'- GGCAGAGACTGGAAGACAGG-3'	5'-AGGTGCAACTGCCAAAGTTC-3'
<i>Dio2</i>	5'-TCTGAGCCGCTCCAAGTC-3'	5'-GGAGCATCTTCACCCAGTTT-3'
<i>Dio3</i>	5'-ACAGATGAGCACAGCCACAG-3'	5'-GGTAAACTCCAGCAGGGACA-3'
<i>Thrb2</i>	5'-CAGTGGTCGTGATGTCCAAG-3'	5'-GGCCATGTCCAAGTCAGAG-3'
<i>Rpl19</i>	5'-CGGGAATCCAAGAGGATTGA-3'	5'-TTCAGCTTGTGGATGTGCTC-3'

Chapter 6. General Discussion

The search for IGSF1 function has been an active area of research in our laboratory since its presumptive role in inhibin signaling was discredited in 2002 and 2003 (39, 57). Despite a wealth of data about *Igsf1*/*IGSF1* expression in tissues (33-35, 98), it has been difficult to assign a particular role to the protein. Data contained in this thesis have contributed to establishing that: 1) IGSF1 is co-translationally cleaved by a signal peptidase-like enzyme into N-terminal and C-terminal domains; 2) IGSF1 is expressed in the POU1F1 lineage of the anterior pituitary (thyrotropes, somatotropes, lactotropes); as well as in the murine hypothalamus and fetal liver; and 3) Loss of IGSF1 function causes central hypothyroidism, possibly due to an impairment in TRH signaling.

1 Thesis summary

In **part I** of my thesis, I explored IGSF1 expression (Chapter 3), as well as protein processing and glycosylation (Chapter 2). I demonstrated that IGSF1 is cleaved at an internal signal peptide, leading to production of IGSF1-CTD, and showed that both *Igsf1*-1 and *Igsf1*-4 mRNA isoforms in mouse encode the same IGSF1-CTD protein. I localized IGSF1-CTD expression to several brain sub-regions, though not to specific nuclei or cell types. I demonstrated expression of IGSF1 in fetal liver, and gradual loss of IGSF1 expression postnatally. I observed differences in relative mRNA isoform abundance between the pituitary and hypothalamus, and noted the existence of potential novel *Igsf1* isoforms in the two tissues. Most importantly, for the first time, I identified several pituitary lineages that express IGSF1-CTD. The localization of IGSF1 to specific cell types is of paramount importance, as it enables us to speculate on potential protein functions, and search for specific phenotypes in *Igsf1*-deficient mice. The knowledge gained in **part I** of the thesis has provided us with a solid basis for understanding the expression pattern and processing of *Igsf1*/*IGSF1*-CTD in various tissues, and clarified some of the previous inconsistencies in published works.

In **part II** of my thesis, I focused on a potential function of IGSF1 in regulating the HPT axis, and explored the connection between loss-of-function mutations in *IGSF1* and central hypothyroidism, both *in vitro* and *in vivo*. All the identified mutations cluster to

the portion of the gene encoding IGSF1-CTD, consistent with it being the mature, functional portion of the protein, as discussed in Chapter 2. I demonstrated that *IGSF1* mutations identified in patients with central hypothyroidism result in the synthesis of protein products that either do no traffic to the cell surface or do so inefficiently. I also showed that *Igsf1*-deficient mice recapitulate key features of the human disorder, including TSH and thyroid hormone deficiency. Importantly, I found that loss of IGSF1 may lead to central hypothyroidism by interfering with TRH signaling through its receptor, TRHR. Although these data provide a firm foundation for the hypothesis that IGSF1 plays an important role in the regulation of the HPT axis at the level of the pituitary, a specific function for the protein is still lacking. Additionally, expression of IGSF1 in non-thyrotrope lineages suggests additional roles for the protein in regulation of other endocrine axes. Multiple aspects of IGSF1 expression and function remain unresolved, several of which are described below.

2 TRH signaling in *Igsf1*-deficient mice

Igsf1-deficient mice have significantly decreased pituitary *Trhr* mRNA levels, and increased hypothalamic *Trh*, suggestive of an impairment in TRH signal propagation at the level of pituitary thyrotropes. Increased hypothalamic *Trh* synthesis is likely due to diminished negative feedback from the thyroid hormones in the hypothyroid state (172, 408, 409). It is also possible that the decrease in *Trhr* mRNA is due to increased TRH stimulation, as TRH treatment of pituitary tumor cell lines decreases *Trhr* mRNA levels (509, 510). To address this possibility, I compared *Trhr* mRNA levels in primary pituitary cultures from male WT and *Igsf1*-deficient mice in the absence and presence of TRH (Figure 6.1). Under control conditions, *Trhr* was decreased by more than 50% in *Igsf1*-deficient cultures compared to WT, recapitulating the *in vivo* data and indicating a pituitary-intrinsic effect of *Igsf1* deficiency on *Trhr* expression, rather than a decrease due to increased TRH signaling. Interestingly, in a preliminary experiment, *Trhr* mRNA levels were downregulated by TRH treatment in WT, but not in *Igsf1*-deficient cultures. The most parsimonious explanation is that TRH signals through the TRHR to induce its

downregulation. Thus, in *Igsf1*-deficient cultures, low *Trhr* expression may result in decreased sensitivity to TRH, preventing further downregulation of the receptor.

It is possible that downregulation of *Trhr* due to loss of *Igsf1*, and resulting impairment of TRH signaling may be sufficient to explain the central hypothyroidism observed in *Igsf1*-deficient mice and patients with *IGSF1* mutations. However, we have not assessed whether the decrease in *Trhr* mRNA is mirrored by a decrease in levels of TRHR protein at the cell surface of thyrotropes or any other cell type. In the absence of reliable antibodies, this could be accomplished using radioligand binding assays in primary pituitary cultures from WT and *Igsf1*-deficient mice.

We attempted to assess TRH signaling *in vivo* by measuring thyroid hormone release upon administration of TRH in WT and *Igsf1*-deficient mice. However, on our second of two experiments, TRH failed to induce thyroid hormone release in both WT and *Igsf1*-deficient mice, despite prominent TSH release (Figure 5.8E,F). Similarly, in the first experiment, the two WT mice with highest baseline T₄ levels also failed to increase T₄ secretion in response to TRH (Figure 5.8C). High levels of thyroid hormone can decrease responsiveness of the thyroid gland to TSH (511-513), thus it is possible that the mice already had elevated thyroid hormone levels, and TSH stimulation could not increase them further. However, the baseline values reported in the assays (3.73 to 8.19 µg/dl) are largely within normal range for C57BL/6 mice (514). In the first experiment, blood was collected 2 hours after TRH administration, and in the second experiment at 3 hours post-TRH. Thus, the assay needs to be optimized in terms of collection time, as well as dose of TRH, and route of administration. Also, to rule out a defect at the level of the thyroid gland, we could treat WT and *Igsf1*-deficient mice with TSH and measure thyroid hormone release (230); the response is predicted to be similar between the genotypes. *In vivo* demonstration of impaired TRH signaling will further clarify the mechanism behind central hypothyroidism in patients with *IGSF1* mutations.

We also have not determined how loss of IGSF1 leads to decreased *Trhr* expression. I tried to determine if loss of IGSF1 *in vitro* leads to a decrease in *Trhr* mRNA using thyrotrope-like TαT1 cells. These cells are difficult to transfect using conventional lipid-based methods, thus I used lentiviral vectors expressing anti-*Igsf1* shRNAs to knock down *Igsf1* expression. Although expression of both *Igsf1* mRNA and IGSF1-CTD protein expression were efficiently reduced in shRNA-expressing TαT1 cells (Figure 6.2A,B), I did not observe an associated change in *Trhr* expression (Figure 6.2C). However, the experiment lacked a true control group (i.e. cells infected with empty vector), and comparing virus-infected to uninfected cells may not accurately portray changes in gene expression due to loss of *Igsf1*. In addition, TαT1 cells were developed from pituitary tumors resulting from the expression of the SV40 T-antigen driven by the 5' promoter region of the human *CGA* gene (127). *Cga* is first expressed in mice at E11.5 (107), therefore these cells may be arrested at a relatively early stage of development/differentiation. Thus, they could lack signaling molecules or transcription factors that are necessary for downregulation of *Trhr* by loss of *Igsf1*. Ideally, we would knock down *Igsf1* expression in purified thyrotropes to determine whether loss of *Igsf1* is sufficient to induce *Trhr* downregulation. This experiment would necessitate crossing mice expressing Cre recombinase selectively in thyrotropes, such as the *Tg(Tshb-cre)* strain (515), with a reporter mouse strain, like the *ROSA26^{eYFP}* mice (137). The resulting mice would express yellow-fluorescent protein (YFP) only in thyrotropes, due to Cre-mediated excision of a termination sequence that prevents expression of YFP under normal circumstances. The thyrotropes could then be isolated from dispersed pituitaries by fluorescence-activated cell sorting (FACS), followed by infection of the cells with lentiviral vectors expressing anti-*Igsf1* shRNAs. However, this approach is technically challenging, and as thyrotropes account for less than 5% of cells in the pituitary gland, it would require large amounts of resources and animals to generate sufficient quantities of cells for experimental purposes. In addition, although *Trhr* is downregulated in *Igsf1*-deficient primary pituitary cultures, these cultures contain a mixture of cells, some of which may produce paracrine factors that contribute to *Trhr* downregulation; thus, we may not be able to demonstrate *Trhr* knockdown in a pure

thyrotrope population. It is also possible that that reduced *Trhr* expression may not be thyrotrope-specific.

TRHR is expressed in thyrotropes, somatotropes, and lactotropes (205, 206). *Trhr* is reduced by over 50% in *Igsf1*-deficient pituitaries, suggesting that loss of *Trhr* in thyrotropes alone (2-5% of the pituitary population (111)) is unlikely to explain the large reduction observed. Thus, we need to assess the extent of *Trhr* downregulation in each of these cell lineages. This could be accomplished by RT-qPCR analyses on sorted pituitary lineages, or a less quantitative method, such as *in situ* hybridization combined with immunofluorescence in WT and *Igsf1*-deficient pituitaries. Additional analyses will also be required to determine the impact of *Trhr* downregulation on TRH signaling in each of these cell types.

The role IGSF1 plays in modulating HPT axis function in the pituitary gland is unknown. Hypothalamic TRH signal is transduced through the TRHR, which is a G protein-coupled receptor (GPCR). GPCRs can associate with accessory proteins, modulating their ligand interactions, signaling pathways, and receptor trafficking (516, 517). Specific chaperones for individual GPCRs have been identified. For example, the calcitonin receptor-like (CALCRL) receptor requires one of three receptor activity modifying proteins (RAMPs) to reach the cell surface (518), while the melanocortin 2 receptor (MC2R) requires the chaperone MRAP for trafficking and responsiveness to ACTH (519, 520). TRHR can be differentially trafficked in different cell types, with pituitary-derived cell lines preferentially trafficking the receptor to the surface (521), suggesting that TRHR may also require a chaperone for export to the plasma membrane. We speculated that IGSF1-CTD may be a pituitary-specific chaperone for TRHR, as it is expressed in all three pituitary lineages that express the receptor. I sought to determine whether overexpression of IGSF1 in the non-pituitary cell line HEK293 would lead increase TRHR at the cell surface. I did not see an overt difference in the abundance of TRHR at the cell surface in the presence or absence of IGSF1 (Figure 6.3), however, I did not perform any quantitative analyses. I also did not detect any interaction between TRHR and IGSF1-

CTD in co-immunoprecipitation experiments in heterologous cells (not shown). Thus, a role for IGSF1 in trafficking of TRHR to the cell surface appears unlikely.

IGSF1 appears to modulate TRHR signaling at the cell surface. TRHR is coupled to the $G_{\alpha q/11}$ subclass of G protein (218), thus I attempted to assess TRH signaling in presence and absence of IGSF1 in T α T1 cells using a G_q -responsive luciferase-reporter. Unfortunately, the reporter had very low activity in these cells (data not shown), precluding its use in assessing TRH signaling. TRH signaling can also be assessed in T α T1 cells using an alternative readout method, such as phosphorylation of the mitogen-activated protein kinases 1 and 3 (MAPK1 and MAPK3, also known as ERK1/2), which are activated by TRH (522), or by measuring intracellular calcium release in response to TRH. Determining if loss or overexpression of IGSF1 *in vitro* alters TRH responsiveness will aid us in establishing whether IGSF1 modulates TRHR function.

Finally, IGSF1-CTD may not need to be expressed in thyrotropes to play a role in regulating the HPT axis. It is possible that IGSF1-CTD expressed on the surface of surrounding cells, including somatotropes and lactotropes, might interact with an as-of-yet unidentified membrane protein on thyrotropes. One of the best-known examples of interactions of membrane-anchored proteins on adjacent cells is the Notch signaling pathway, first identified in *Drosophila* (523). Upon interaction of Notch with its cell-bound ligands (Delta-Serrate-Lag2, collectively known as DSL ligands), the intracellular domain of Notch is cleaved, and translocates to the nucleus, to activate transcription of its target genes (reviewed in (524)). Many members of the immunoglobulin superfamily are involved in cell adhesion and communication (18), thus it is conceivable that IGSF1 could participate in a Notch-like signaling pathway. However, the search for the missing binding partner may be complicated, particularly if the interaction depends on the proper folding and glycosylation of IGSF1, as conventional high-throughput methods to detect protein-protein interaction (phage-display, yeast two-hybrid screen) do not accurately emulate mammalian protein processing. Alternatively, a tagged IGSF1 construct can be expressed in a mammalian cell line, retrieved by immunoprecipitation or tandem affinity

purification, and mass spectrometry used to identify proteins that were pulled down with IGSF1. However, this approach may not be sensitive enough to detect transient or weak interactions, and may produce many false-positive results (525).

3 IGSF1 and prolactin

Given that over half of the patients with *IGSF1* loss-of-function mutations have prolactin deficiency, we might have expected to see a similar phenotype in *Igsf1*-deficient mice. Although we did not see a difference in pituitary *Prl* mRNA levels or pituitary prolactin content between wild-type and *Igsf1*-deficient male mice, it is necessary to measure serum prolactin to determine if the mice are prolactin deficient or not. Prolactin is chiefly synthesized in the pituitary, but is also locally produced in other tissues, including the placenta, myometrium, mammary glands, and lymphocytes (526). In addition, prolactin receptors have been found in nearly every tissue (527), suggesting a role for prolactin in many biological processes, including immune system function (528, 529). However, it is best known for its role in female reproduction and milk production in mammals (527). Indeed, while female mice with targeted disruption of the *Prl* gene are infertile and have abnormal mammary gland morphology, *Prl*-deficient males are fully fertile (530), and neither male nor female *Prl*-deficient mice display overt metabolic (531) or hematopoietic (530) phenotypes. Thus, pathological consequences of prolactin deficiency in *Igsf1*-deficient male mice or male patients with *IGSF1* mutations may be difficult to ascertain.

To determine whether loss of IGSF1 has an impact on prolactin function, we need to assess *Igsf1*-deficient mice at the times when prolactin is most crucial: pregnancy and lactation. Confirming prolactin deficiency in human female patients carrying *IGSF1* mutations may be difficult as the *IGSF1* gene is X-linked; thus, females might need to carry mutations on both alleles to be affected. Heterozygous females may display a mosaic phenotype, depending on whether the wild-type or mutant X-chromosome was inactivated and to what relative extents (532). Additionally, loss of *Igsf1* in female mice may have less impact on pituitary function than in males, as *Igsf1* mRNA levels are

considerably lower in female mouse pituitaries compared to males (39); the sex difference is not as dramatic at the protein level (not shown). However, to our knowledge neither *Igsf1* mRNA nor IGSF1-CTD protein levels have been assessed during pregnancy or lactation. *Igsf1* mRNA levels are dynamically regulated across the estrous cycle in rats (98), and preliminary gene array data indicates the gene may be upregulated in lactotropes of lactating mice (P. le Tissier, personal communication), suggesting changes in *Igsf1* expression, at least at the mRNA level, during the female reproductive lifespan. If IGSF1 is important for regulating prolactin production or secretion, we might expect its expression to be increased at those times, thus we need to assess *Igsf1*/IGSF1-CTD expression in WT and *Igsf1*-deficient virgin and pregnant females.

If loss of IGSF1 leads to decreased prolactin, we will need to assess the functional consequences of prolactin deficiency in *Igsf1*-deficient mice. Although *Prl* (530) and prolactin receptor (533) knockout females are infertile, *Igsf1*-deficient females have normal-sized litters (39); thus, if loss of IGSF1 results in prolactin deficiency, it is not sufficient to affect fertility. Prolactin is also crucial for production of milk proteins and fats in the mammary gland (534), and mammary gland development and proliferation (535). A commonly used indicator of sufficient milk production is pup weight gain during nursing (231, 533, 536). Thus, we could compare weights of pups nursed by either wild-type or *Igsf1*-deficient females to determine whether the loss of *Igsf1* impairs prolactin production sufficiently to impact lactation. It is possible that we will not see a difference in pup weight, as neither *Trh*- nor *Trhr*-deficient mice show impairments in their ability to nurse their pups, despite having significantly decreased *Prl* mRNA and serum prolactin levels (231, 232). However, in these studies, prolactin deficiency was proposed to be an indirect consequence of central hypothyroidism whereas it is possible that loss of *Igsf1* in lactotropes in our model might have a more direct and perhaps more pronounced effect on prolactin production. It remains to be determined whether prolactin deficiency in patients with *IGSFI* mutations is a result of decreased TRH signaling, or whether it is mediated through an independent mechanism.

4 Additional consequences of loss of IGSF1

Long before we began focusing on the HPT axis, I used another approach to identify potential IGSF1 functions. I extracted RNA from adult male WT and *Igsf1*-deficient pituitaries, and performed a microarray analysis in order to identify differentially expressed genes (Figure 6.4). Using 2-fold change in either direction as the cutoff, I identified four genes that were upregulated, and six genes (including *Igsf1*) that were downregulated in *Igsf1*-deficient males relative to controls (Table 6.1). Of these, I could reliably reproduce the results in independent samples for the upregulated gene *Ramp1*, and downregulated genes *Klk1b22* and *Bcat1* by RT-qPCR (Figure 6.5). Of note, the microarray also identified *Trhr* as downregulated in *Igsf1*-deficient males, but it was below the cutoff at 0.8-fold of wild-type levels, thus I did not pursue the analysis at the time.

Relatively little is known about the two downregulated genes, *Klk1b22* (kallikrein 1-related peptidase b22) and *Bcat1* (branched chain aminotransferase 1, cytosolic), so I chose to focus on *Ramp1*, as it has been well-characterized in the literature. The gene encodes the receptor (G protein-coupled) activity modifying protein 1 (RAMP1), which is a single-pass membrane protein. It functions as a co-receptor for the G-protein coupled CALCRL receptor, enabling high-affinity binding of the calcitonin gene-related peptide (CGRP) to the CALCRL-RAMP1 complex, and as a chaperone for CALCRL trafficking to the cell surface (537, 538). CGRP is widely expressed in neurons of the central and peripheral nervous systems, and its expression has also been reported in a variety of other organs, including skin, cardiovascular system and gastrointestinal tract (539-541). It is best known for its role as a vasodilator (542), but has also been implicated in immune function (543-546) and pain signaling (547), including in migraine headaches (548, 549).

Consistent with its role as the ligand-binding component of the CALCRL-RAMP1 complex, *Ramp1* knockout mice have high blood pressure due to decreased sensitivity to CGRP. The mice also show elevated proinflammatory cytokine levels in response to an inflammatory stimulus (550). In contrast, transgenic mice that overexpress human

RAMP1 in all tissues have a lower heart rate, and an attenuated response to angiotensin-induced hypertension (551). Interestingly, CGRP may also be important for regulating energy expenditure, as transgenic mice that overexpress human RAMP1 in the central nervous system have decreased body weight and fat mass, and increased body temperature and oxygen consumption (552).

The role of RAMP1 in the pituitary gland is unknown. I attempted to confirm whether RAMP1 is increased in pituitaries of *Igsf1*-deficient mice at the protein level, but the commercial antibody used (sc-8851, Santa Cruz, California) was not suitable for the analysis. In the absence of a reliable antibody, we can confirm increased RAMP1 expression at the cell surface by performing binding assays using radiolabeled CGRP. We hypothesized that *Ramp1* may be upregulated to compensate for loss of *Igsf1* if the two proteins perform a similar function (e.g. if IGSF1 is also a co-receptor or a chaperone for CALCRL), or if IGSF1 functionally or physically interacts with RAMP1 at the plasma membrane. To determine whether IGSF1 physically interacts with either RAMP1 or CALCRL, I transfected HEK293 cells with expression constructs for murine IGSF1-1, human Myc-RAMP1, and human Myc-CALCRL, and used a Myc antibody to pull down RAMP1 or CALCRL. I did not detect any immunoprecipitated IGSF1-CTD in cells transfected with IGSF1 and RAMP1 or with IGSF1 and CALCRL. However, I observed IGSF1-CTD signal in cells expressing IGSF1, RAMP1, and CALCRL (Figure 6.6A). The band was very faint in comparison to the level of IGSF1 expression in total lysates, but suggests that IGSF1 may interact with the receptor complex. The size at which IGSF1-CTD migrates on gel corresponds to the immature IGSF1-CTD glycoform, implying the interaction, if real, takes place in the ER. Thus, a role, if any, for IGSF1 in CALCRL signaling at the cell surface remains to be elucidated. Additionally, if *Ramp1* is upregulated in order to compensate for loss of *Igsf1*, it would be interesting to determine whether loss of *Ramp1* results in reciprocal *Igsf1* upregulation in *Ramp1* knockout mice.

Another possibility is that *Ramp1* expression is normally repressed by a signal mediated by IGSF1 in wild-type pituitaries, and in the absence of IGSF1, *Ramp1* expression is

increased. I confirmed that *Ramp1* is upregulated in male *Igsf1*-deficient primary pituitary cultures (Figure 6.6B), suggesting that no extra-pituitary signal is required for elevated *Ramp1* expression in *Igsf1*-deficient pituitaries. To address whether loss of *Igsf1* alone can lead to upregulation of *Ramp1*, I compared *Ramp1* levels in TαT1 cells stably expressing shRNA directed against murine *Igsf1* versus uninfected TαT1 cells. *Ramp1* mRNA levels were not upregulated in *Igsf1*-deficient cells (Figure 6.6C), suggesting that loss of IGSF1 alone is not sufficient to induce *Ramp1* expression in these cells. However, for reasons mentioned in **section 2** of the discussion, TαT1 cells may not be a good model to study the interplay between *Igsf1* and *Ramp1*. Further investigations are required to determine in which pituitary cell type(s) RAMP1 is expressed and upregulated, whether IGSF1 and RAMP1 are co-expressed, and if loss of *Igsf1* has any impact on CGRP signaling *in vitro* and *in vivo*.

Conclusion

Though we have made much progress in advancing our understanding of IGSF1 expression and function in the last 5 years, we have really only scratched the surface. The work presented in this thesis establishes for the first time that IGSF1 modulates the HPT axis. However, IGSF1 is expressed in multiple pituitary cell types, and several other tissues including the hypothalamus. The challenge going forward will be to discern the protein's function in these different organs and cell types. Although it is tempting to say that the road to discovering the function of IGSF1 has been long, the truth is that the data presented in this thesis represent the beginning of what will no doubt be a much longer, but ultimately much more rewarding journey.

Figure Legends

Figure 6.1 TRH does not induce upregulation of *Trhr*. Primary pituitary cultures from WT and *Igsf1*-deficient adult males were prepared as described in (139) (n = 6 pituitaries per genotype). Note: one WT pituitary was erroneously included in *Igsf1*-deficient cultures. 24 hr after plating, wells were treated with 100 nM TRH for 1, 2, or 24 hr. Treatment was carried out in serum-free media for the 1 and 2 hr timepoints, and in media with 2% fetal bovine serum for the 24 hr timepoint to minimize cell death. RNA was harvested and RT-qPCR was performed to assess *Trhr* mRNA levels. Pooled results of 2 samples per treatment are shown (mean $\pm$ s.d.).

Figure 6.2 Knockdown of *Igsf1* does not decrease *Trhr* expression in T α T1 cells. (A) T α T1 cells were infected with lentivirus expressing shRNA targeted against *Igsf1*. RNA was harvested from WT and Lenti-*Igsf1* shRNA #5 cells and RT-qPCR was performed to assess *Igsf1* mRNA levels. Pooled results of 3 independent qPCR runs are shown. **(B)** Protein lysates from WT and Lenti-*Igsf1* shRNA-infected T α T1 cells were run on SDS-PAGE and immunoblotted using IGSF1-CTD antibody. **(C)** RNA was harvested from WT and Lenti-*Igsf1* shRNA #5 cells and RT-qPCR was performed to assess *Trhr* mRNA levels. Pooled results of 2 independent qPCR runs are shown. Error bars in A, C= s.e.m.

Figure 6.3 TRHR does not appear to require IGSF1 to traffic to the cell surface. HEK293 cells were transfected with pcDNA3 (empty vector), HA-TRHR or TRHR+IGSF1-1 expression vectors. TRHR trafficking to the cell surface was assessed by immunofluorescence under non-permeabilizing conditions. TRHR was detected using an HA antibody, and IGSF1 was detected using IGSF1-CTD antibody. Although the IGSF1 construct has an HA tag, it is intracellular, and thus not accessible under non-permeabilizing conditions.

Figure 6.4 Gene array of genes up- or downregulated in *Igsf1* ^{Δ ex1} male mice. 10 μ g of RNA was extracted from two groups of 3 pituitaries per genotype, and a microarray

analysis was performed using Illumina MouseWG-6 v2.0 Expression BeadChip (Illumina, San Diego, California). Changes in the expression of ~45,000 probesets were analyzed. Data were analyzed using Flexarray software (version 1.4.1), and the results presented as a volcano plot. The vertical lines on the X-axis at +1 and -1 (log2) represent cutoff values for genes that were upregulated or downregulated by twofold, respectively. Statistical significance ($p < 0.05$) is indicated by the horizontal line at 4.5 ($-\log_2 p$ -value) on the Y-axis. Thus, the genes indicated by name were either significantly up- or downregulated by at least two-fold.

Figure 6.5 Altered expression of *Ramp1*, *Klk1b22* and *Bcat* in *Igsf1*^{Δex1} male mice. RNA was extracted from pools of 3 pituitaries per genotype and RT-qPCR using primers in Table 6.2 was performed to assess (A) *Ramp1* mRNA levels in WT and *Igsf1*^{Δex1} adult males (pooled results of 4 independent qPCR runs are shown); (B) *Klk1b22* mRNA levels (pooled results of 2 independent qPCR runs are shown); (C) *Bcat1* mRNA levels (pooled results of 2 independent qPCR runs are shown). Error bars for A, B, C = s.e.m.

Figure 6.6 RAMP1/CALCRL may interact with IGSF1, but loss of IGSF1 *in vitro* does not result in decreased *Ramp1* mRNA levels. (A) HEK293 cells in 10 cm plates were transfected with 5 ug of pcDNA3.1, pcDNA3, Myc-RAMP1-GFP (in pcDNA3.1), Myc-CALCRL-GFP (in pcDNA3.1), or mouse IGSF1-1 (in pcDNA3) as indicated. Protein lysates were harvested, immunoprecipitated using Myc antibody, run on SDS-PAGE, and immunoblotted with either IGSF1-CTD antibody, or Myc antibody. β -actin (ACTB) was used as a loading control. (B) Primary pituitary cultures from WT and *Igsf1*-deficient adult males were plated for 72 hrs, followed by RT-qPCR to assess *Ramp1* expression. Results from one experiment are shown. (C) T α T1 cells were infected with lentivirus expressing shRNA targeted against *Igsf1*. RNA was harvested from WT and Lenti-*Igsf1* shRNA #5 cells and RT-qPCR was performed to assess *Ramp1* mRNA levels, relative to WT cells. Pooled results of 2 independent qPCR runs are shown. Error bars = s.e.m.

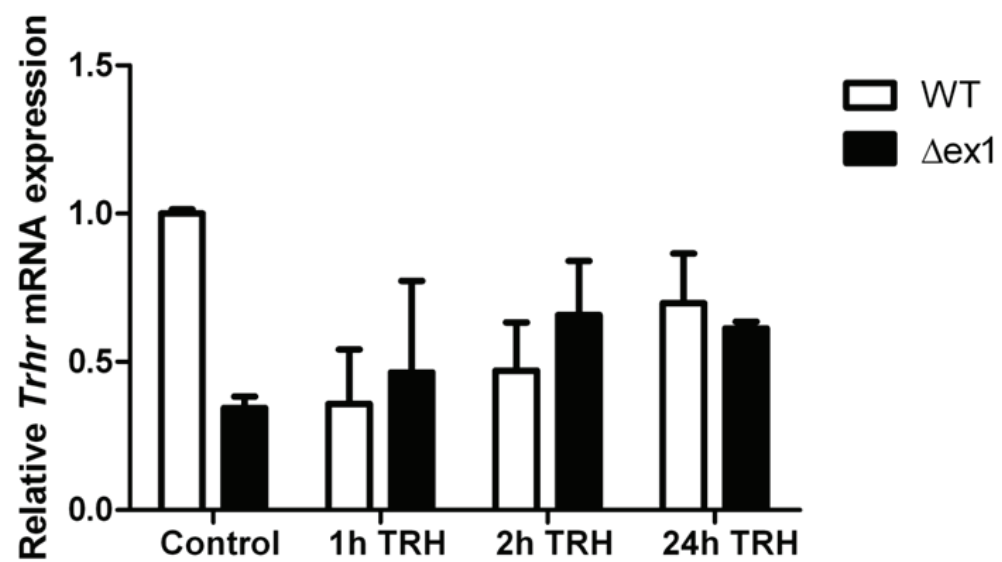


Figure 6.1

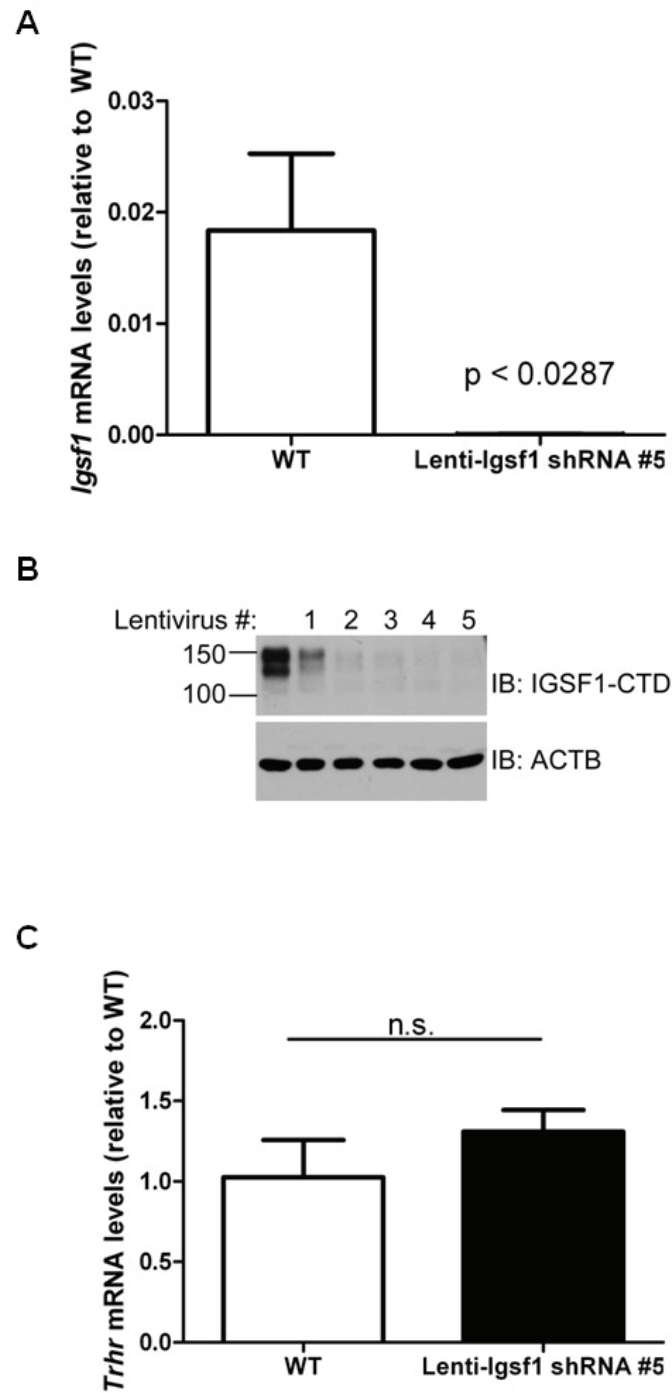


Figure 6.2

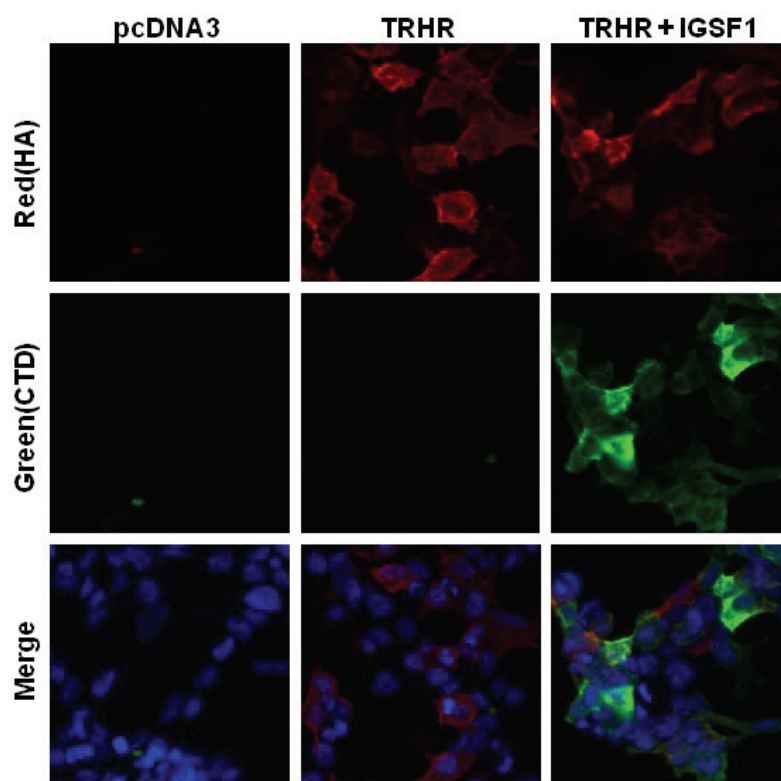


Figure 6.3

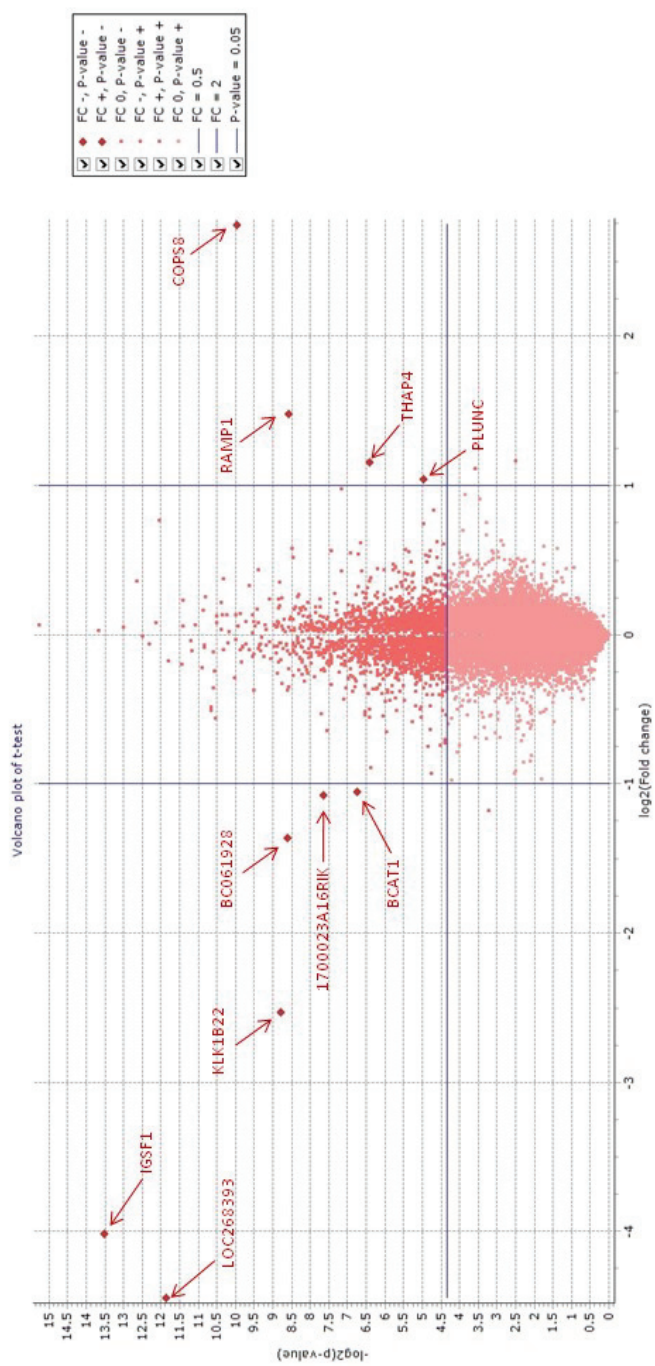


Figure 6.4

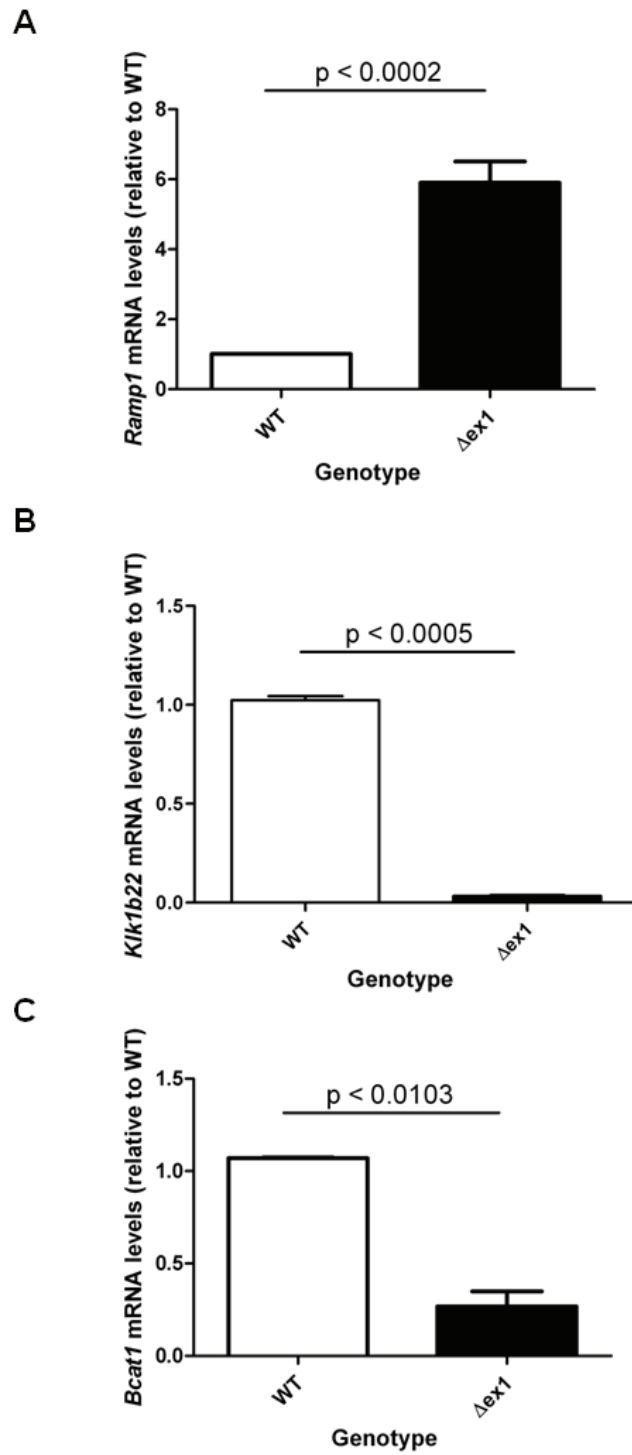


Figure 6.5

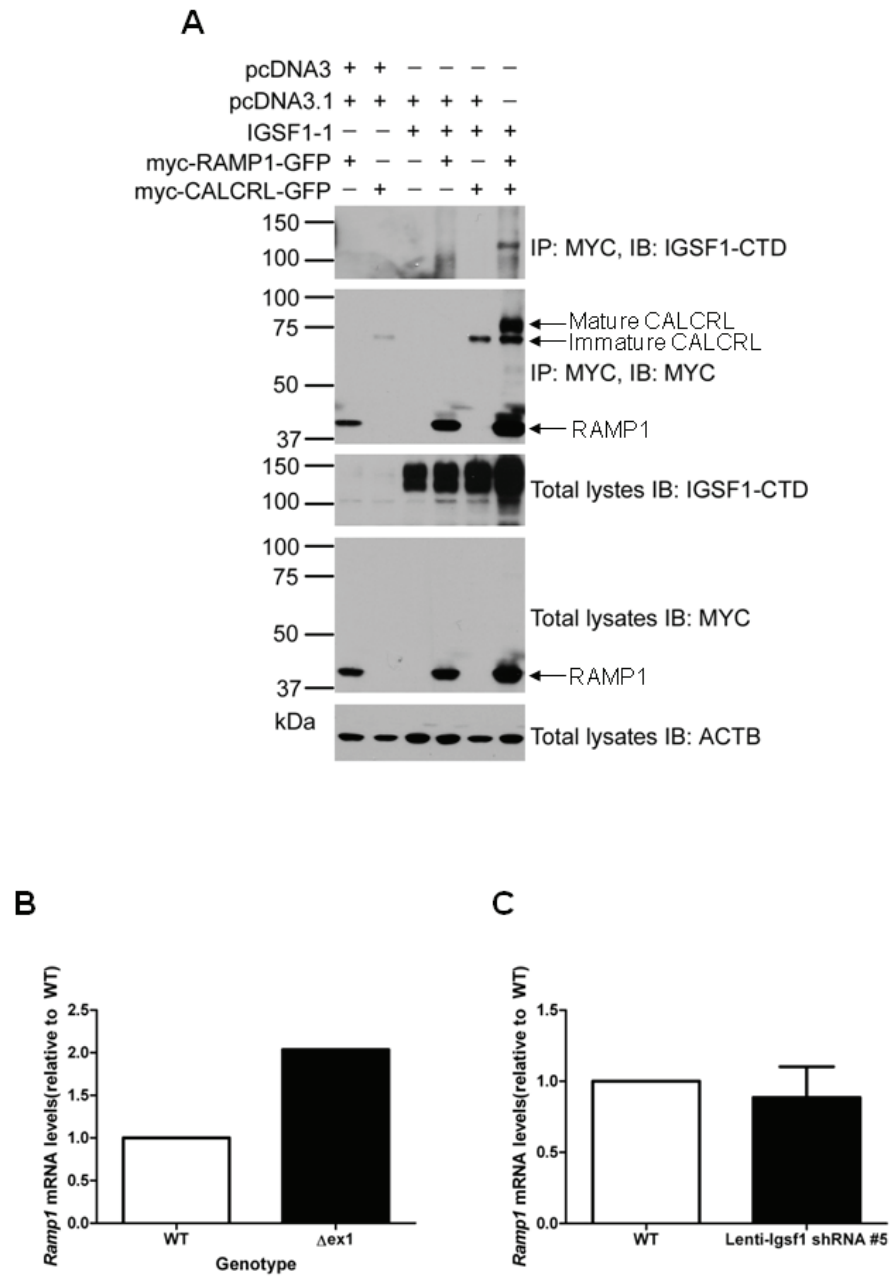


Figure 6.6

Table 6.1 Changes in gene expression in murine male *Igsf1*-deficient pituitaries.

Upregulated genes:		
Gene name:	Fold change:	p-value:
<i>Cops8</i>	6.718	0.001
<i>Ramp1</i>	2.786	0.002
<i>Thap4</i>	2.236	0.012
<i>Plunc</i>	2.062	0.032
Downregulated genes:		
Gene name:	Fold change:	p-value:
<i>Gm12091</i>	0.046	<0.001
<i>Igsf1</i>	0.062	<0.001
<i>Klk1b22</i>	0.173	0.002
<i>BC061928</i>	0.389	0.002
<i>1700023A16RIK</i>	0.474	0.005
<i>Bcat1</i>	0.483	0.009

Table 6.2 Primers to confirm gene expression changes from gene array.

Gene:	Sense Primer:	Antisense Primer:
<i>Ramp1</i>	5'-TGACTATGGGACTCTCATCCAG-3'	5'-GCCAGAAACAGCCAATCGT-3'
<i>Bcat1</i>	5'-CCAGTATGGAAGGGTGGAGA-3'	5'-ACGCGACTACAATGTGCAAG-3'
<i>Klk1b22</i>	5'-GAGTACCTATGCGGGGGAGT-3'	5'-GATGAGGGAAGCTTTTGCTG-3'

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Appendix A

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An Internal Signal Sequence Directs Intramembrane Proteolysis of a Cellular Immunoglobulin Domain Protein^{*[S]}

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Precursor proteolysis is a crucial mechanism for regulating protein structure and function. Signal peptidase (SP) is an enzyme with a well defined role in cleaving N-terminal signal sequences but no demonstrated function in the proteolysis of cellular precursor proteins. We provide evidence that SP mediates intraprotein cleavage of IgSF1, a large cellular Ig domain protein that is processed into two separate Ig domain proteins. In addition, our results suggest the involvement of signal peptide peptidase (SPP), an intramembrane protease, which acts on substrates that have been previously cleaved by SP. We show that IgSF1 is processed through sequential proteolysis by SP and SPP. Cleavage is directed by an internal signal sequence and generates two separate Ig domain proteins from a polytopic precursor. Our findings suggest that SP and SPP function are not restricted to N-terminal signal sequence cleavage but also contribute to the processing of cellular transmembrane proteins.

Proteolysis is one of the central mechanisms regulating tissue morphogenesis and cellular functions. Proteolytic processing underlies protein degradation and the regulated release of functionally active protein domains. Classic experiments performed in the 1970s by Blobel *et al.* (see Ref. 1 for review) showed that signal peptidase (SP)⁴, an enzyme of the endoplasmic reticulum (ER), co-translationally cleaves the short leader chains that direct transmembrane and secreted proteins through the ER membrane. SP is an integral membrane protease that cleaves in

the juxtamembrane region of its substrate, removing the membrane-bound signal sequence from its parent protein (2). SP recognizes small N-terminal sequences that display well characterized patterns of hydrophobicity and charge (3).

Recently, a companion enzyme to SP was identified by its ability to cleave membrane-embedded signal peptides previously processed by SP in the ER (4). This enzyme was named signal peptide peptidase (SPP) and later shown to be the founding member of a larger protease family.

Only a subset of SP substrates are also substrates for SPP; cleavage requires the presence of polar amino acids in the membrane-spanning sequence (5). The primary purpose of this intramembrane cleavage event was thought to be the clearance of signal peptides from the ER membrane, but cytoplasmic signaling functions of the released peptides have also been proposed (6, 7). Ablation of SPP in *Caenorhabditis elegans*, *Drosophila*, and zebrafish is lethal (8–10). However, the reason for the lethality is unknown, and to date no endogenous nonsignal sequence substrates for SPP cleavage have been identified.

The SPP family of enzymes represents the most recent addition to a class of structurally related intramembrane proteases, including rhomboids, the Site-2-Protease S2P, and presenilins, which mediate regulated intramembrane proteolysis, an important theme in proteolysis-dependent cell signaling (11–13). Through cleavage within transmembrane domains, these proteases trigger the release of extracellular and intracellular protein domains. Many of these fragments then exert signaling functions that can contribute to a diverse array of processes including cell differentiation, lipid metabolism, cytokine production, and the response to unfolded proteins (14–17).

SPP and the several known signal peptide peptidase-like proteins (SPPLs) localize to distinct subcellular compartments where they may target different substrates (10, 18). In contrast to presenilins and rhomboids, which cleave type I oriented transmembrane domains, SPP family proteins target type II-oriented membrane domains (15, 18, 19). For example, the transmembrane domain of the type II-oriented tumor necrosis factor α is a substrate for cleavage by SPP-like proteins SPPL2a and SPPL2b in endosomes and the plasma membrane (20, 21).

Some viruses utilize the proteolytic activity of SP and SPP in the ER for viral polypeptide biosynthesis (22–24). For example, membrane proteins of Semliki forest virus are translated as one large polypeptide precursor from which mature p62, 6k, and E1 proteins are released by SP cleavage (25). In contrast to signal peptides in cellular proteins, the SP cleavage sites are found not at the N terminus but rather internally within the polypeptide sequence. Similarly, an internal signal sequence targets the hep-

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⁴ The abbreviations used are: SP, signal peptidase; SPP, signal peptide peptidase; ER, endoplasmic reticulum; SPPL, signal peptide peptidase-like protein; HA, hemagglutinin; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PNGase, peptide N-glycosidase; EndoH, endoglycosidase H; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TNT, transcription/translation; MOPS, 4-morpholinopropanesulfonic acid; NTD, N-terminal domain; CTD, C-terminal domain; EGFP, enhanced green fluorescent protein.

atitis C virus polyprotein to the ER (26), where the core and envelope proteins are formed by the sequential action of SP and SPP. The envelope protein E1 becomes a transmembrane protein that is targeted to the plasma membrane, whereas the core protein is released into the cytoplasmic matrix where it associates with lipid droplets (27). Despite the small size of the peptide fragment released by SPP, functional SPP cleavage is necessary for productive viral infection *in vivo* (28). Because internal cleavable signal sequences are thought to be restricted to viral polyproteins, it has been assumed that polyprotein precursors of cellular proteins cannot be similarly converted into functionally distinct proteins by SP and SPP processing.

We investigated the biosynthesis and processing of IgSF1 (p120/inhibin-binding protein), an Ig domain protein that is highly expressed in the pituitary and brain (29–32). Several previous findings highlighted potential functions of IgSF1 in cell signaling. Pituitary *IgSF1* mRNA levels are dynamically regulated during the rat estrous cycle (29), and the IgSF1 protein has been proposed to associate with transforming growth factor β receptors (33). Moreover, in recent studies we observed that IgSF1 expression was dramatically up-regulated in the developing pontocerebellar system during neuronal synapse formation (34). Sequence analysis suggested that IgSF1 is a type I transmembrane protein with an N-terminal signal sequence, followed by 11 Ig domains, a single transmembrane domain, and a short cytoplasmic tail (30–32) (see Fig. 1A). We show here that two additional hydrophobic domains in the center of the IgSF1 polypeptide act as stop transfer and internal signal sequences. Sequential proteolytic processing by SP and SPP generates two Ig domain proteins, each with a single transmembrane domain. Our results demonstrate that processing of internal signal sequences is not restricted to viral polyproteins and expand SP and SPP function to the generation of cellular proteins.

EXPERIMENTAL PROCEDURES

DNA Constructs and Antibody Reagents—A construct encoding full-length human IgSF1 with a triple HA tag on the C terminus was a gift of Dr. Teresa Woodruff (Northwestern University). Mouse SHPS1 and CD47 cDNAs were isolated by reverse transcription-PCR from hippocampal total RNA.⁵ An amyloid precursor protein expression construct was kindly provided by Dr. Tae-Wan Kim. All of the expression constructs contained the cytomegalovirus promoter for eukaryotic expression. See the supplemental material for details on the construction of DNA vectors.

Two anti-peptide antibodies were raised for detection of IgSF1. N-terminal domain antibodies were generated in guinea pigs using a synthetic peptide representing amino acids 21–35 (SLAVESQPELWIESN), and central domain antibodies were generated against a synthetic peptide representing amino acids 559–575 (ALTEEIEIVMPTKPEL). Each peptide was synthesized with an additional cysteine at its C terminus for coupling to keyhole limpet hemocyanin, and antibodies were affinity-purified on the antigen coupled to Sulfo-link beads (Pierce). Rat anti-HA and mouse anti-Myc antibodies were obtained from Roche Applied Science and Zymed Laboratories Inc., respectively. Mouse anti-actin antibodies were from Sigma. Second-

ary antibodies raised in donkey were obtained from Jackson ImmunoResearch.

Cell Culture, Transfection, and Tissue Preparation—Human embryonic kidney (HEK293), Chinese hamster ovary (CHO), and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin (Invitrogen). Transfections were performed using Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen).

All animal use was approved by and according to the Institutional Animal Care and Use Committee at Columbia University. IgSF1^{-/-} mutant mice were bred and genotyped as described previously (35). Adult mice were sacrificed by CO₂ inhalation. Fresh pituitaries were dissected on ice and solubilized in phosphate-buffered saline (PBS) supplemented with 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 2 mM dithiothreitol, and complete protease inhibitor mixture (Roche). Protein concentration was determined by BCA assay (Pierce) prior to SDS-PAGE analysis.

Protein Analysis Methods—For deglycosylation experiments, the protein concentration of cell lysates was determined by BCA assay (Pierce). For each sample, lysate containing 20 μ g of protein was denatured in SDS-containing buffer (New England Biolabs) for 10 min. The lysates were then treated with 500 units of endoglycosidase H or PNGase F (New England Biolabs) for 1–2 h at 37 °C and analyzed by Western blotting. Because the N-terminal IgSF1 fragment tends to aggregate upon boiling, the protein samples were only heated to 37 °C before analysis by SDS-PAGE.

(ZLL)₂-ketone and Compound E (Calbiochem) were added to cells 10–12 h before analysis at 100 μ M and 10 nM, respectively. To separate SPP-cleaved and uncleaved N-terminal fragments, the protein samples were separated on 15% Tris-Tricine gels.

For pulse-chase analysis, transfected HEK293 cells were starved for 2 h in cysteine/methionine-free Dulbecco's modified Eagle's medium (Invitrogen) and then pulsed for 10 min with 14.3 μ Ci of ³⁵S-radiolabeled cysteine/methionine (Pro-Mix; Amersham Biosciences). The cells were washed once and left in media supplemented with 40 μ g/ml each of unlabeled cysteine and methionine. After the indicated chase times, the cells were washed once in ice-cold PBS and collected in lysis buffer (PBS, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 2 mM dithiothreitol; Complete protease inhibitor mixture). Proteins were immunoprecipitated from cleared lysates with control or anti-HA antibodies, antibody complexes were collected with protein G beads (Amersham Pharmacia Biosciences), beads were washed five times in lysis buffer, and precipitated proteins were eluted in Laemmli buffer and separated by SDS-PAGE. The proteins were visualized by autoradiography.

For immunofluorescence analysis, the cells were fixed for 10 min with phosphate-buffered 4% paraformaldehyde. The cells were permeabilized with 0.1% Triton X-100 or left unpermeabilized, blocked for 1 h with PBS containing 10% goat serum, and incubated with primary antibodies overnight at 4 °C. For detection, cross-absorbed CY2- and CY3-conjugated secondary antibodies were used (Jackson ImmunoResearch),

⁵ B. Chih, unpublished observations.

and the cells were imaged on a Zeiss LSM 510meta confocal microscope.

In Vitro Translocation Assays—Wild-type and 4xY mutant IgSF1, in which four residues near the SP cleavage site have been replaced by tyrosines (see supplemental material for details) were synthesized *in vitro* using 500 ng of plasmid DNA and complete amino acid mix, following the TNT coupled reticulocyte lysate system protocol (Promega). For translocation assays, canine pancreatic microsomes (Promega) were included in the reactions (1.8 μ l/25- μ l reaction; increasing the amount of microsomes did not increase the efficiency of translocation; data not shown). IgSF1-containing TNT lysates were deglycosylated with PNGaseF or EndoH following the manufacturer's protocol, with minor changes. Wild-type TNT-IgSF1 (with microsomes) samples were denatured at 100 °C for 10 min in the denaturation buffer provided by the manufacturer. 4xY mutant TNT-IgSF1 (with microsomes) samples were denatured at 70 °C for 20 min to reduce aggregation of the protein.

The proteins were resolved on NuPAGE 4–12% BisTris gradient gels in MOPS buffer (Invitrogen), following the manufacturer's protocol with minor alterations. Deglycosylated samples were reheated at 37 °C prior to loading onto the gels. Nonglycosylated samples were heated at 70 °C for 10 min. The proteins were transferred to nitrocellulose membranes (30 V, 1 h), and the membranes were blocked with 5% milk in TBST (0.05% Tween in TBS). The blots were incubated overnight at 4 °C in mouse anti-HA (1:40,000, Sigma). The following day, the membranes were washed three times for 15 min in TBST and incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature (Bio-Rad; 1:3,000 in 5% milk). The membranes were washed three times for 15 min in TBST, followed by incubation in Amersham ECL Plus reagent and exposure to x-ray film (GE Healthcare).

All of the experiments described in this study were repeated at least three times. The repetitions yielded highly similar results, and the data included in the manuscript are representative for these independent experiments.

RESULTS

IgSF1 Is Subject to Internal Proteolytic Cleavage—We raised polyclonal antibodies to study the biosynthesis and processing of IgSF1. Specific antisera were obtained for two peptide epitopes: one located at the mature N terminus of the protein and one located in the presumptive center of the coding sequences. Surprisingly, affinity-purified antisera detected proteins of different molecular masses from mouse pituitary. The N-terminal domain (NTD) antibody detected a protein migrating at ~60–70 kDa, whereas the C-terminal domain (CTD) antibody detected a doublet migrating at ~130–150 kDa. These apparent molecular masses reflect the masses of glycosylated proteins and thus are larger than the predicted molecular masses as illustrated in the diagram (Fig. 1A). This fact will be further examined below (see Fig. 3).

Immune reactivity for both products was absent or strongly reduced in lysates of pituitary tissue from knock-out mice lacking the first (noncoding) exon of the *IgSF1* gene (35), demonstrating the specificity of the antibody detection (Fig. 1B). Mul-

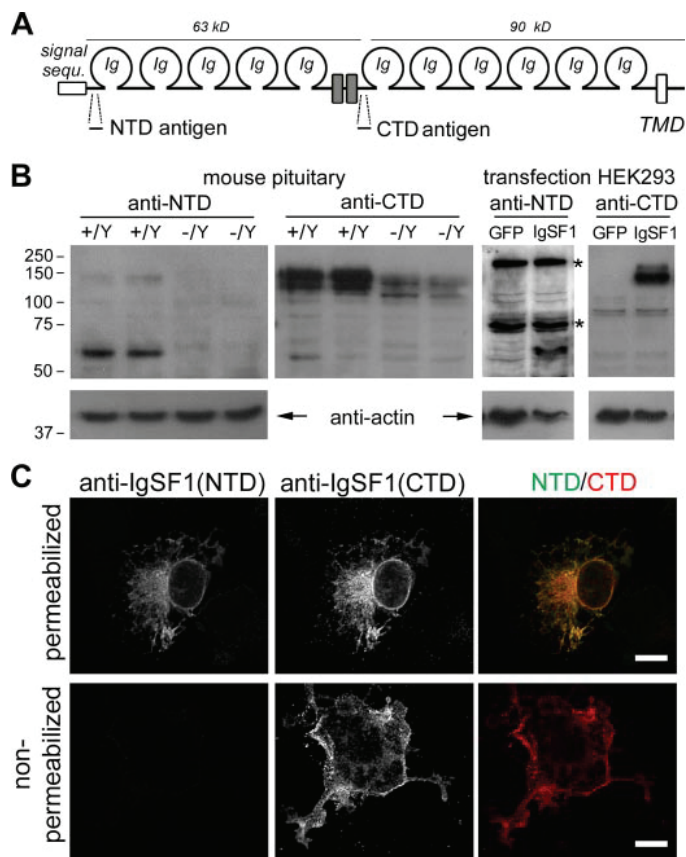


FIGURE 1. Endogenous IgSF1 is proteolytically processed. A, predicted domain organization of IgSF1. Open circles indicate Ig domains; rectangles indicate hydrophobic segments. Previous work suggested the presence of an N-terminal signal sequence and a single transmembrane domain (TMD). Anti-peptide antibodies were generated against epitopes located at the NTD and CTD of the protein. B, lysates were obtained from pituitaries of adult wild-type (+/Y) and mutant mice (lacking exon 1 of the *IgSF1* gene located on the X chromosome (–/Y)). In addition, HEK293 cells transfected with EGFP (GFP) or with a full-length IgSF1 cDNA (IgSF1) were analyzed. Western blots were probed with affinity-purified antibodies directed against the NTD (left) or the CTD (right) of the IgSF1 protein. With the NTD antibodies, two nonspecifically cross-reacting bands are observed in the EGFP and IgSF1-transfected cells (marked with asterisks). Equal sample preparation and loading was confirmed by reprobing of the nitrocellulose membranes with anti-actin antibodies (bottom). C, antibodies to the NTD and CTD of IgSF1 detect protein in different cellular compartments. Permeabilized (top) or nonpermeabilized (bottom) COS7 cells transiently transfected with full-length IgSF1 expression constructs were double-labeled with guinea pig anti-NTD (green, left) and rabbit anti-CTD antibodies (red, middle). Untransfected cells showed no specific staining with either antibody. Overlays are at the right. Scale bars, 10 μ m.

tipule IgSF1 transcripts have been identified in pituitary (29, 35). To examine whether the NTD and CTD proteins are the products of separate transcripts or processing of a single polypeptide, we probed cell lysates of HEK293 cells transfected with an expression construct encoding the full-length human IgSF1 mRNA (Fig. 1B). As observed for the endogenous protein, two protein species were generated from this cDNA, strongly supporting the idea that proteolytic processing of an IgSF1 precursor gives rise to the two protein isoforms. We further probed the subcellular localization of the IgSF1 products by immunofluorescence analysis of COS7 cells transfected with the same full-length IgSF1 expression construct (Fig. 1C). Staining of permeabilized cells with the NTD and CTD antibodies revealed partially nonoverlapping localization of these proteins. Whereas both antibodies detected intracellular pools of

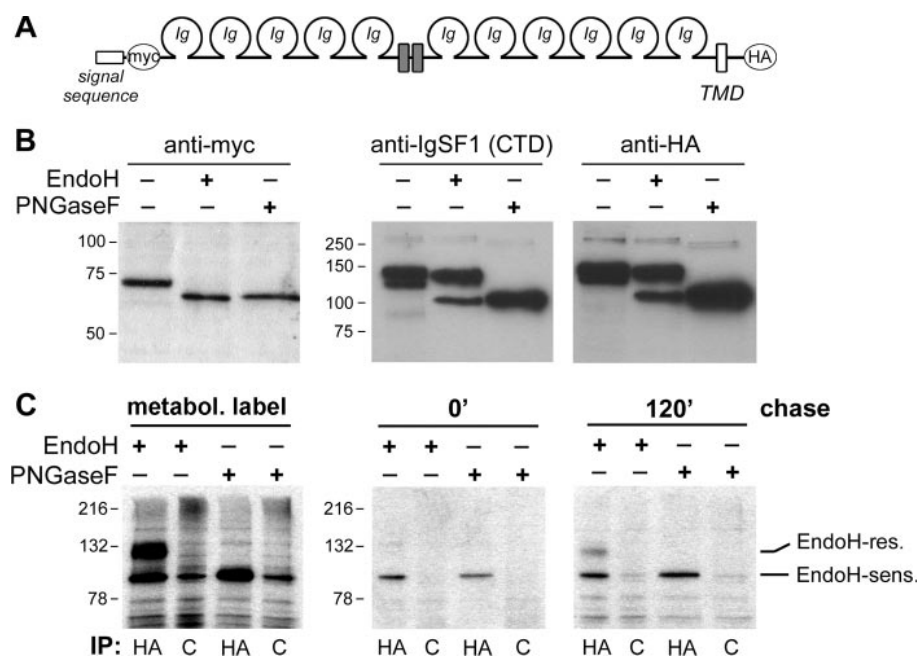


FIGURE 2. Cleavage of IgSF1 occurs in the endoplasmic reticulum. *A*, diagram of dually epitope-tagged IgSF1 containing an N-terminal Myc tag and a C-terminal HA tag (Myc-IgSF1-HA). *B*, lysates from HEK293 cells transiently expressing Myc-IgSF1-HA were treated with either vehicle (–), EndoH, or PNGase F. The proteins were analyzed by Western blotting with anti-IgSF1 (CTD) (*left*), anti-HA (*middle*), or anti-Myc (*right*) antibodies. Note the different molecular mass markers in the *left* panel and the *two right* panels. *C*, HEK293 cells expressing Myc-IgSF1-HA were radiolabeled overnight (*metabol. label*, *left*) or were pulse-labeled for 5 min with [³⁵S]cysteine and methionine and chased in presence of excess unlabeled cysteine and methionine for 0 or 120 min (*right*). IgSF1 protein was immunoprecipitated with anti-HA (HA) or control antibodies (C). Immunoprecipitates were treated with EndoH or PNGase F as indicated and analyzed by autoradiography.

the protein in the ER, only the CTD antibody detected protein in nonpermeabilized cells. This suggests that the N-terminal protein product might be retained intracellularly, whereas the CTD protein is targeted to the plasma membrane.

The apparent processing of IgSF1 was further examined using a dually tagged IgSF1 cDNA (Fig. 2*A*). A Myc tag was introduced after the N-terminal signal sequence, and a triple HA epitope was inserted at the C terminus. Immunoblotting with anti-Myc and anti-HA antibodies confirmed that IgSF1 underwent proteolytic cleavage (Fig. 2*B*). The HA-tagged (C-terminal) fragment had the same molecular mass as the protein detected with the CTD antibody, whereas the Myc-tagged fragment corresponded in size to the protein detected by the NTD antibody. Taken together with the findings on the endogenous protein, this indicates that IgSF1 is cleaved into at least two fragments, one containing the C terminus and the other containing the N terminus of the original polypeptide. Interestingly, the protein fragments also differed in their carbohydrate modifications (Fig. 2*B*). A substantial portion of the HA-tagged (C-terminal) protein acquired EndoH-resistant N-glycans, whereas the glycans on the Myc-tagged (N-terminal) protein remained fully EndoH-sensitive consistent with its predicted retention in the ER.

The retention of the NTD in the ER (Fig. 1*C*) and the lack of EndoH-resistant carbohydrates suggested that cleavage of the IgSF1 precursor protein might occur early in the secretory pathway. To explore the kinetics of IgSF1 precursor cleavage, transfected HEK293 cells were pulsed with radiolabeled methionine and cysteine for 5 min, and IgSF1 was immunoprecipi-

tated either with antibodies against the C-terminal HA tag or with a control antibody (Fig. 2*C*). Precipitates were treated with either EndoH or PNGase F to discriminate size variations caused by changes in the protein backbone and N-glycosylation. Even when proteins were precipitated immediately after the pulse (0 min), no IgSF1 precursor was recovered. After the 120-min chase, 30% of IgSF1 had acquired complex carbohydrates, indicating transport to the Golgi complex, and ~75% had acquired EndoH resistance in samples metabolically labeled overnight (Fig. 2*C*). These findings strongly suggest that proteolytic cleavage of IgSF1 occurs co-translationally or very soon after translation of the full-length protein.

IgSF1 Contains an Internal Signal Sequence—Based on the apparent molecular masses of the N- and C-terminal fragments, as well as the location of the epitope localized by the CTD antibody, we predicted that the cleavage site should be

located within amino acids 400–560 of full-length IgSF1. This region contains two hydrophobic domains, the second of which bears a strong similarity to that of an N-terminal signal peptide. It contains a positively charged n-region, a central hydrophobic h-region, and a polar C-terminal c-region (predictions with Signal P 3.0; Fig. 3*A*) (3, 36, 37). To test whether this hydrophobic region was indeed capable of functioning as a signal peptide, we constructed a truncated IgSF1 expression construct (IgSF1-ΔN) where the N-terminal signal sequence, the first five Ig domains, and the first hydrophobic domain were deleted (Fig. 3*B*). When expressed in COS7 cells, IgSF1-ΔN acquired complex EndoH-resistant carbohydrate modifications and was efficiently transported to the plasma membrane (Fig. 3, *B* and *C*). This demonstrates that the second internal hydrophobic domain in IgSF1 represents a functional signal sequence when placed at the N terminus of a protein and is sufficient to direct translocation and subsequent cleavage by SP. We speculated that this sequence also might direct cleavage of the full-length protein.

The Internal Signal Sequence in IgSF1 Is Sufficient to Direct Site-specific Cleavage of Membrane Proteins—To test directly whether the internal signal sequence in IgSF1 is sufficient to confer proteolytic cleavage, we generated a series of chimeric molecules using sequences from IgSF1 and SHPS-1 (SHP substrate 1), a type I transmembrane protein with an N-terminal signal sequence, three extracellular Ig domains, and a single transmembrane domain. In the chimera SHPS1-IgSF1, the two hydrophobic segments and the adjacent c-region of IgSF1 were fused into SHPS-1 (Fig. 4*A*). As a negative control, we trans-

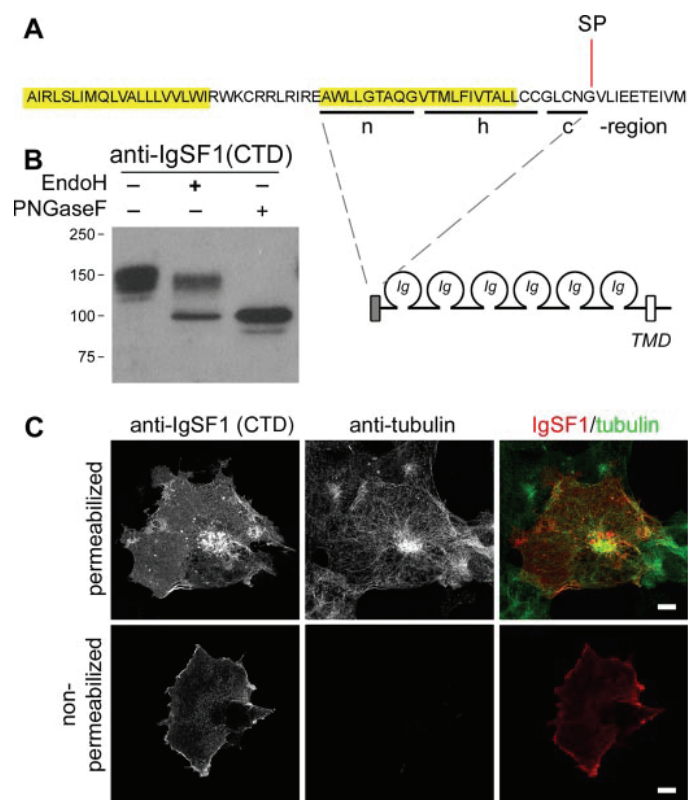


FIGURE 3. An internal peptide sequence in IgSF1 represents a functional signal peptide. **A**, analysis of internal IgSF1 sequences with SignalP reveals a candidate internal signal peptide. The amino acid sequences of the two internal hydrophobic domains are marked in yellow, predicted n-, h-, and c-regions are underlined, and the predicted SP cleavage site is marked. **B**, diagram of a truncated IgSF1 construct where the first five Ig domains and first hydrophobic domain were deleted, placing the predicted internal signal sequence at the N terminus. The gray box represents the n-, h-, and c-regions shown in **A**. HEK293 cells transiently transfected with this construct were lysed, treated with vehicle, EndoH, or PNGase F, and analyzed by Western blotting with anti-IgSF1 (CTD) antibodies. **C**, permeabilized or nonpermeabilized COS7 cells transiently transfected with the truncated IgSF1 expression construct were double-labeled with rabbit anti-IgSF1CTD (red) and mouse anti-tubulin antibodies (green). Scale bar, 10 μ m.

planted two hydrophobic segments (a stop transfer and a start transfer sequence) of the polytopic plasma membrane protein CD47 in the same position (SHPS1-CD47). The hydrophobic domains of IgSF1 were further dissected in three additional chimeras. In CD47/IgSF1, the second hydrophobic domain of IgSF1 and the adjacent c-region were combined with the stop transfer sequence of CD47. In IgSF1 Δ c the c-region of IgSF1 was deleted, and in IgSF1 4xY four amino acids near the predicted SP cleavage site were mutated to tyrosines.

HEK293 cells were transfected with these constructs, and processing of their protein products was monitored using a C-terminal HA tag for detection. All of the samples were deglycosylated with PNGaseF to eliminate any size differences caused by alterations in carbohydrate modifications. Using this detection system, noncleaved proteins have an apparent molecular mass of 70–80 kDa, whereas proteolytic cleavage produces C-terminal fragments of about 55 kDa (Fig. 4B).

Analysis of the chimeric molecules strongly supported the idea that the internal signal sequence in IgSF1 directs site-specific protein processing. Transfer of the two hydrophobic domains and the c-region of IgSF1 into SHPS1 was sufficient to

convert SHPS1 into a cleavage substrate. Whereas the first hydrophobic domain of IgSF1 could be replaced by the stop transfer sequence of CD47 without affecting processing (CD47/IgSF1), cleavage was abolished in chimeras that contained only CD47 transmembrane domains (CD47) or that lacked the c-region of the internal IgSF1 signal peptide (IgSF1 Δ c). Mutation of amino acids in the c-region of signal sequences has been demonstrated to block processing by SP in several proteins (3, 25). Importantly, similar mutations in the internal IgSF1 c-region strongly reduced internal cleavage (IgSF1 4xY), supporting the idea that this site is processed by SP or by a closely related enzyme.

IgSF1 Is Cleaved Internally by an ER-localized Protein—To test whether the SP cleavage site in the internal signal sequence was required for processing of full-length IgSF1, we introduced the 4xY mutation into the human Myc-IgSF1-HA expression vector. The wild-type and mutant constructs were then transfected into CHO cells, and products were analyzed by Western blotting with antibodies to the C-terminal HA epitope tag. The wild-type construct resulted in a product of $\sim$ 130 kDa (Fig. 4C), a size similar to the endogenous protein detected with a C-terminal antibody (Fig. 2B). In contrast, the 4xY mutant yielded a protein of $\sim$ 180–200 kDa, suggesting that the mutation of four amino acids in the internal signal sequence indeed resulted in an inhibition of precursor cleavage. Importantly, the differences in electrophoretic mobility were not due to differences in carbohydrate modifications. Deglycosylated forms of the wild-type protein had an apparent molecular mass of $\sim$ 90 kDa, whereas the 4xY mutant migrated at a size of $\sim$ 160 kDa (Fig. 4C), sizes that are in good agreement with the molecular masses predicted from the amino acid sequence of the protein (Fig. 1A).

To further examine whether processing of IgSF1 occurs during or soon after ER translocation, we employed *in vitro* TNT assays in the presence and absence of microsomes. The products were run on SDS-PAGE and subjected to Western blotting with an anti-HA antibody. In the absence of microsomes, wild-type IgSF1 generated a protein precursor of $>$ 150 kDa (Fig. 4D, lane 2). When microsomes were included in the TNT reaction, we observed a novel band at $\sim$ 130 kDa (Fig. 4D, lane 3), with equivalent mobility to the form of the protein observed in CHO cells transfected with the wild-type construct (Fig. 4C, lane 2). Importantly, treatment of the proteins from the TNT/microsome assays with EndoH or PNGaseF (Fig. 4D, lanes 4 and 5) caused the disappearance of the $\sim$ 130-kDa band and the emergence of the $\sim$ 90-kDa band that was observed with deglycosylated IgSF1 from transfected cells (Fig. 4C, lanes 3 and 4). These data confirm that in the presence of microsomes (and resident SP), the IgSF1 precursor (lane 2, $\sim$ 160 kDa) can be cleaved and glycosylated, yielding a $\sim$ 130-kDa protein ($\sim$ 90 kDa after deglycosylation).

Expression of the IgSF1 4xY mutant protein in the absence of microsomes yielded a precursor protein of similar mobility as the wild-type protein. However, inclusion of microsomes caused a significant size shift to $\sim$ 180–200 kDa (Fig. 4D, compare lanes 7 and 8), consistent with the carbohydrate modification of the protein in microsomes. This protein co-migrated with IgSF1 4xY protein detected in transfected CHO cells (Fig.

Internal Cleavage of a Cellular Protein by Signal Peptidase

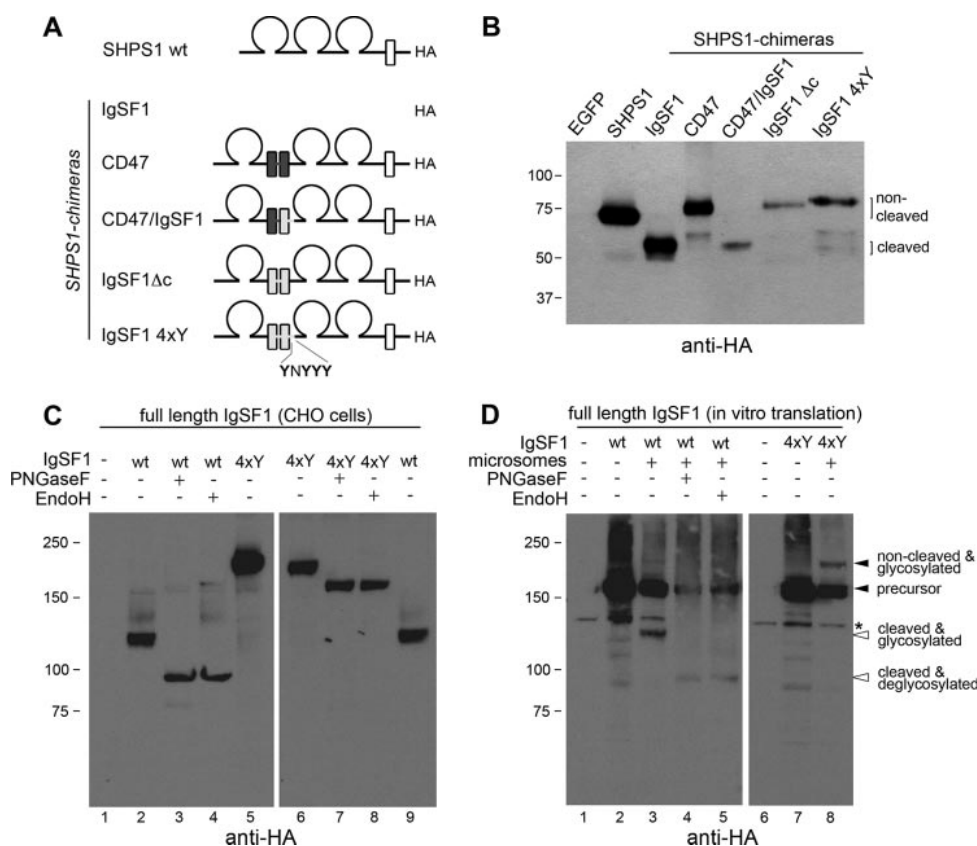


FIGURE 4. The internal signal sequence in IgSF1 directs protein processing by a signal peptidase-like factor. A, diagram of chimeric proteins. Internal hydrophobic sequences of IgSF1 (light gray) or CD47 (dark gray) were introduced into SHPS1 (black), a type I transmembrane protein with three extracellular Ig domains. In IgSF1Δc the two hydrophobic domains were included, but the c-region of the predicted internal signal peptidase cleavage site of IgSF1 was removed. In IgSF1 4xY four residues within the putative SP cleavage site were mutated to tyrosines. All of the constructs contained a C-terminal HA tag. B, HEK293 cells were transfected with cytomagalovirus-EGFP or the constructs diagrammed in A. All of the lysates were treated with PNGaseF and analyzed by Western blotting with anti-HA antibodies. The positions of cleaved and noncleaved products are indicated. C, proteins derived from transfected CHO cells were run on 4–12% gels and probed with anti-HA antibodies in Western blot analyses. Wild-type (wt) or 4xY mutant IgSF1 (4xY) expression vectors and deglycosylating enzymes (EndoH or PNGaseF) were included as indicated. We consistently noted lower expression from the 4xY construct in transfected cells. Therefore, 6 μg of whole cell extract from control transfected cells were run in lane 1, 3 μg of wild type in lanes 2–4 and lane 9, and 15 μg of 4xY in lanes 5–8. Molecular mass markers (in kDa) are at the left. D, proteins derived from TNT assays were run on 4–12% gels and probed with anti-HA antibodies in Western blot analyses. Wild-type and 4xY mutant expression vectors, microsomes, and deglycosylating enzymes were included as indicated. Lane 1 contains TNT lysate without DNA template. 10% (2.5 μl) of each TNT lysate was loaded in the indicated wells. The asterisk denotes a nonspecific band detected in TNT lysates. Molecular mass markers (in kDa) are at the left. The different forms of IgSF1 are marked by arrowheads on the right. Note that the protein sizes observed in the TNT assay correspond in molecular mass to the equivalent forms observed in C for transfected CHO cells.

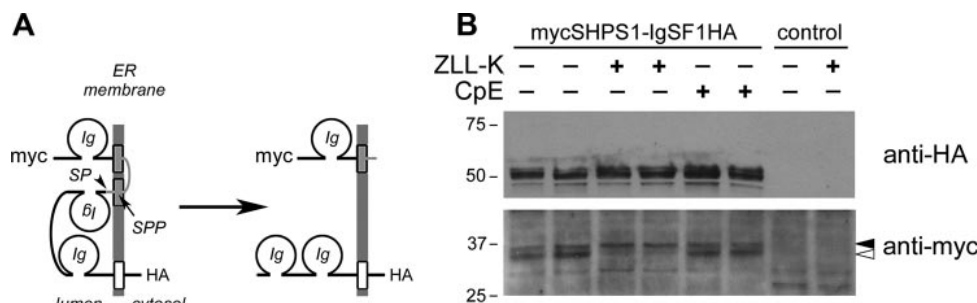


FIGURE 5. The IgSF1 N-terminal domain is processed by SPP-like activity. A, hypothetical model of sequential processing of the SHPS1-IgSF1 chimeric protein by SP and SPP. The positions of the N-terminal Myc and C-terminal HA epitopes are marked. B, HEK293 cells transfected with a construct encoding the Myc-SHPS1-IgSF1-HA or EGFP (control) were treated overnight with 100 μM (ZLL)₂-ketone, 10 nM compound E (CpE), or vehicle. The cells were lysed and treated with PNGaseF, and duplicate samples were separated on 15% Tris-Tricine gels and immunoblotted with anti-HA and anti-Myc antibodies to detect C-terminal and N-terminal fragments, respectively. The (ZLL)₂-ketone-sensitive Myc-tagged product is marked with a white arrowhead, and the (ZLL)₂-ketone-resistant form is marked with a black arrowhead.

4C, lane 6). These data show that the mutation (4xY) in the putative SP cleavage site inhibits proteolytic processing of full-length IgSF1. Collectively, these data support the hypothesis that IgSF1 is cleaved at an internal site by SP or an SP-like protein within the ER.

IgSF1 Is a Substrate for Intramembrane Proteolysis by SPP—Cleavage of the internal signal sequence in IgSF1 is predicted to leave the NTD of the protein attached to two transmembrane domains connected through a short cytoplasmic loop (Fig. 5A). Recent work on SP processing of N-terminal signal sequences and viral polypeptide precursors revealed a second intramembrane cleavage event mediated by SPP (4, 5). Interestingly, the second hydrophobic domain of IgSF1 contains several polar amino acids, similar to N-terminal signal sequences that are substrates of SPP (Fig. 3A). To test whether IgSF1 sequences would undergo sequential processing by SP followed by SPP, we incubated SHPS1-IgSF1 expressing cells with (ZLL)₂-ketone, a specific inhibitor of SPP (38, 39). As a negative control, the cells were treated with Compound E, an inhibitor of intramembrane proteolysis by the presenilin-γ-secretase complex.

Analysis of the N-terminal fragments generated by processing of SHPS1-IgSF1 revealed two specific bands that differed in apparent molecular mass by ~1 kDa (Fig. 5B). (ZLL)₂-ketone, but not Compound E, resulted in a significant loss of the smaller product, strongly supporting the idea that generation of this smaller protein product requires an SPP-like activity. However, blocking SPP activity had no visible effect on migration of the C-terminal (HA-tagged) fragment of the SHPS1-IgSF1 chimera. Similarly, we found that cleavage of full-length IgSF1 into N- and C-terminal fragments was not significantly altered by (ZLL)₂-ketone (supplemental Fig. S1). Interestingly, on overexposed Western blots, we could detect a small

amount of apparent full-length IgSF1 (less than 1% of the total protein), which might accumulate because of a back-up of protein in the ER membrane. This indicates that the initial cleavage step does not require SPP activity but that an SPP-like activity is required for a subsequent cleavage event in the NTD. In summary, this work reveals sequential processing of a cellular transmembrane protein by SP and SPP activities, which generates two independent Ig domain proteins from one polytopic transmembrane precursor.

DISCUSSION

This study provides evidence for a novel role for SP activity in the biosynthesis of a cellular Ig domain protein. The IgSF1 full-length transcript encodes an 11 Ig domain precursor protein that is targeted to the ER by an N-terminal signal sequence. We demonstrate that a stop transfer sequence and internal signal sequence located after the first five Ig domains then direct proteolytic cleavage of the IgSF1 polypeptide, separating the first five from the remaining six extracellular Ig domains. The internal signal sequence subsequently undergoes intramembrane proteolysis mediated by SPP or an SPP-like protein in the ER. This second cleavage event presumably releases the cleaved signal peptide into the cytoplasm, leaving the stop transfer sequence as a single transmembrane domain of the N-terminal IgSF1 isoform. This sequential enzymatic processing event ultimately converts one large polytopic membrane protein into two type I proteins that are further transported independently of each other.

A role for SP and SPP in this sequential proteolytic processing is supported by the following observations. First, immunohistochemical and carbohydrate analyses demonstrate that cleavage occurs in the ER. Second, pulse-chase analysis and *in vitro* translocation assays indicate that cleavage occurs rapidly after synthesis or co-translationally. Third, when placed at the N terminus of the central domain, the internal cleavage site of the protein acts like a functional signal sequence. Fourth, mutations that abolish SP cleavage in other proteins also abrogate internal processing of the IgSF1 precursor protein. Interestingly, the sequence of the predicted internal SP cleavage site (Fig. 3A) matches exactly the sequence of an IgSF1 N-terminal peptide isolated from bovine pituitary membranes (30). Chong *et al.* (30) obtained the sequence NH₂-VLIETEIVMPTPTK-PEL-COOH by Edman degradation and assumed that this internal sequence was recovered because of nonphysiological fragmentation of the protein. Our results strongly suggest that this sequence is in fact the N terminus of the C-terminal IgSF1 product that results from internal SP cleavage.

The hypothesis that a second cleavage event is mediated by SPP is based on the fact that it occurs after the SP cleavage event and is inhibited by (ZLL)₂-ketone. SPPL3 is another ER SPP-like protein that is inhibited by (ZLL)₂-ketone (10, 40). Therefore, it is possible that SPPL3 and not SPP is involved in processing of IgSF1 or that both enzymes participate.

Although the C-terminal IgSF1 cleavage product was transported to the cell surface, the N-terminal fragment was primarily detected in the ER. ER retention might be due to slower processing of the N-terminal fragment. Alternatively, this fragment might require association with additional membrane pro-

teins for surface transport. In this case, ER retention would reflect an inability of the protein to pass ER quality control in transfected cells. Recent studies revealed that SPP can associate with misassembled transmembrane domain sequences (41) and that SPP is involved in the dislocation of cellular proteins from the ER (42). Our analysis of IgSF1 expression in pituitary tissue demonstrates that proteolytic processing of IgSF1 is a normal step in its biogenesis *in vivo* rather than a response to misfolding, although the limited quality of our antibody reagents has so far precluded histological studies to determine the localization of the N- and C-terminal domains in the pituitary.

Our data on internal signal sequence processing of IgSF1 reveal clear parallels with intramembrane cleavage events in other cellular compartments (11). Intramembrane cleavage of type I oriented cell surface proteins such as Notch and amyloid precursor protein by presenilin/ γ -secretase, as well as type II oriented sterol regulatory element-binding protein by S2P and tumor necrosis factor α by SPPL2a and SPPL2b, requires an initial cleavage by a shedding protease that removes the extracellular domain. Intramembrane proteolysis follows release of the extracellular domains and is dependent on it (5, 11, 20, 21). Similarly, full-length IgSF1 only becomes a substrate for SPP after SP cleavage of the internal signal peptide in the luminal face of the ER membrane. Consequently, the addition of the SPP inhibitor (ZLL)₂-ketone was not sufficient to prevent separation of the N- and C-terminal domains of IgSF1 but only inhibited the second processing step of the N-terminal fragment (Fig. 5 and supplemental Fig. S1).

The purpose of the novel cleavage event identified here remains speculative, because the biological function of IgSF-1 is poorly defined at present. The presence of the C-terminal domain at the cell surface suggests a possible function in cell-cell adhesion or signaling. The confinement of the N-terminal domain to the ER membrane in our experiments could suggest any of several possibilities. First, the N-terminal domain could, as a resident transmembrane ER protein, have ER functions. Second, it could be that, under physiological conditions, the N terminus is transported out of the ER to the cell surface or to another membrane-bound organelle. Signals that would normally mediate this process *in vivo* might be lacking in the cultured cell lines used for our studies. Both of these cases postulate an independent function of the isolated N terminus, a possibility we consider likely in view of its size, its status as a substrate for SPP family cleavage, and its stability sufficient for detection in our experiments. Either of these possibilities would reflect a novel activity of SP in processing a cellular polyprotein. It should be noted that the lack of an obvious phenotype in mice lacking upstream DNA sequences of *IgSF1* (35) implies that the function of the N-terminal protein is not required for grossly normal development and reproduction. Investigations directed toward uncovering more subtle phenotypes in these mice are ongoing.

An interesting question emerging from this work is whether internal signal sequence cleavage might be regulated in response to physiological signals. Whereas regulated cleavage of signal sequence substrates has not been described, it has been proposed that subcompartmentalization of the ER as well as differential use of signal peptides for translocation could yield functionally distinct protein pools (43, 44). In our experiments,

both for the endogenous protein *in vivo* and the protein expressed from cDNAs *in vitro*, we always observed processing of the majority of IgSF1 and did not recover a significant non-cleaved pool of full-length IgSF1. This indicates that under our experimental conditions, processing is constitutive. However, further work will be required to clarify whether regulation might occur under different conditions.

In summary, our work highlights an unexpected proteolytic processing of a cellular transmembrane protein dependent on an internal signal sequence. It is plausible that other proteins might be modified by a similar mechanism. Considering the extensive knowledge and powerful computational prediction methods for N-terminal signal sequences (36), new candidate substrates for internal signal sequence cleavage could be identified using bioinformatic approaches.

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Appendix B

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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 8 distinct mutations and 2 deletions in *IGSF1* in males from 11 unrelated families with central hypothyroidism, testicular enlargement and variably low prolactin concentrations. *IGSF1* is a membrane glycoprotein that is 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 new 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 of age, had central hypothyroidism, partial growth hormone (GH) deficiency and low prolactin concentration (**Table 1**). In early adolescence, testicular growth advanced normally in both boys, but testes continued to grow beyond the reference range of testis volume (**Table 1**). In contrast, serum testosterone concentration remained inappropriately low for testicular size until 15.2 and 14.2 years of age, respectively, leading to a late growth spurt and delayed pubic hair development in each. 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**) identified a 27-nt deletion, c.2137_2163del (p.Ala713_Lys721del) in *IGSF1* at Xq25 (*IGSF1* reference sequence, [NM_001170961.1](#)). The same deletion was present in the grandfather (A-I.4) and another male relative (A-II.4) with the same phenotype (**Fig. 1a** and **Table 1**). Independent whole-exome sequencing

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of two brothers (B-III.7 and B-III.8) with central hypothyroidism in family B (**Fig. 1b**, **Table 1** and **Supplementary Table 1**) identified a nonsense mutation, c.2931G>A (p.Trp977*), 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 after genetic screening at 65.5 (B-I.4) and 43.3 (B-II.11) years of age (**Fig. 1b**).

We found additional *IGSF1* variants in seven Dutch families (families C–I) and two Italian families (families J and K) characterized by male-specific central hypothyroidism (**Fig. 1c**, **Table 1** and **Supplementary Fig. 1**). Most Dutch and Italian (family J) cases were detected through neonatal screening for congenital hypothyroidism (on the basis of thyroxine (T4) and TSH concentrations). Thyroid function tests at diagnosis are shown in **Supplementary Table 2**. Mean (s.d.)

Case	Nucleotide alteration	Amino-acid alteration	Origin	Age at diagnosis of central hypothyroidism	Central hypothyroidism ^a	Prolactin deficiency ^b	Age (years) ^c	Right/left testicular volume (ml) (reference) ^d
A-III.11	c.2137_2163del	p.Ala713_Lys721del	Netherlands	3 weeks	+	—	17.64	21/20 (7.3–16)
A-III.7			Netherlands	7.3 years	+	+	21.36	30/26 (8.5–18.3)
A-II.4			Netherlands	51.5 years	+	—	52.41	32/29 (8.5–18.3)
A-I.4			Netherlands	74.1 years	+	—	67.70	4/large (8.5–18.3) ^e
B-III.7	c.2931G>A	p.Trp977*	UK	4 weeks	+	+	10.52	1.1/1.0 (0.55–1.87)
B-III.8			UK	7 weeks	++	—	7.95	1.8/1.5 (0.45–0.92)
B-II.11			UK	43.3 years	+	+	43.30	68/37 (8.5–18.3)
B-I.4			UK	65.9 years	+	—	66.37	19.6/21.6 (8.5–18.3)
C-III.1	c.2248delG	p.Glu750Lysfs*28	Netherlands	3 weeks	+	+	16.60	18/18 (7.8–16.2) ^f
D-III.3	c.2248delG	p.Glu750Lysfs*28	Netherlands	3 weeks	++	+	10.46	1/1 (0.55–1.87)
D-III.4			Netherlands	1 week	+	+	3.79	0.8/0.8 (0.32–0.70)
D-I.3			Netherlands	61 years	+	—	62.75	21/16 (8.5–18.3)
E-IV.1	126-kb deletion ^g		Netherlands	2 weeks	+	+	20.57	>18/>18 (8.5–18.3) ^f
E-IV.3			Netherlands	2.5 weeks	+	—	22.37	34/25.5 (8.5–18.3)
F-IV.1	328-kb deletion ^h		Netherlands	3 weeks	+	—	12.70	12.2/8.4 (4–13)
F-IV.2			Netherlands	3 weeks	+	+	9.44	1/0.9 (0.5–1.35)
F-II.8			Netherlands	57.5 years	+	+	58.24	44.6/48.2 (8.5–18.3)
G-III.1	c.2588C>T	p.Ser863Phe	Netherlands	5 weeks	+	+	27.52	11.8/38 (8.5–18.3)
G-III.3			Netherlands	2.5 weeks	+	+	23.08	25.5/25.4 (8.5–18.3)
G-I.1			Netherlands	63 years	+	+	87.49	>18/>18 (8.5–18.3) ^f
H-III.2	c.2839T>C	p.Cys947Arg	Netherlands	6.5 years	+	+	18.36	22.7/22.7 (8.5–18.3)
H-III.3			Netherlands	3 weeks	+	+	15.93	21.7/21.7 (6.7–15.3)
I-III.2	c.3518G>A	p.Trp1173*	Netherlands	14.1 years	+	+	16.69	19/17 (8–16.5)
J-III.1	c.3596dupT	p.Glu1200Argfs*3	Italy	3 weeks	+	+	3.26	0.75/0.80 (0.32–0.70)
J-III.2			Italy	2 weeks	+	+	0.16	0.58/0.58 (0.30–0.65) ^f
K-II.3	c.2309G>A	p.Ser770Asn	Italy	10.6 years	+	+	26.54	21.5/21.4 (8.5–18.3)

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serum free thyroxine (FT4) concentration 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% of the reference range. Serum TSH concentrations were within the reference range in all cases (range of 0.8–6.0 mU/l, mean 2.6 mU/l). The FT4/TSH ratio was therefore lower than in controls (3.94 versus 5.73; Mann-Whitney test $P = 0.002$). Mean (s.d.) serum triiodothyronine (T3) concentration in nine 1- to 3-week-old *IGSF1*-deficient infants was 98% (12%) of the lower limit of the reference range for age (range of 83–121%)¹. In seven older cases, T3 concentration 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 old) and eight older subjects (0.4–65.5 years old), standard TRH tests were conducted (**Supplementary Table 2**). In the infants, the peak TSH concentration was 4.5–16.0 mU/l. A similar range was observed in previous reports on congenital central hypothyroidism^{2,3}, and concentrations are lower than observed in age-matched controls (14–37 mU/l)³. At later ages (7.3–63 years), peak TSH concentrations were between 4.3 and 8.5 mU/l, which is in the lower half of the reported reference ranges for males (3.7–12.5 mU/l (ref. 4) or 4.1–13.9 mU/l (ref. 5)). In eight subjects, the increment of serum FT4 (mean (s.d.) = 14.2% (7.9%)) was lower than that reported for controls (mean (s.e.m.) = 23.9% (2.7%)), but the FT3 response to TRH was normal (36.3% versus 41.8% in controls)^{6,7} (**Supplementary Table 3**). We could demonstrate central hypothyroidism in 5 of 20 female heterozygous carriers (data not shown).

Serum prolactin concentrations were decreased in 18 of 26 cases (**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 subjects who had discontinued treatment. Clinical and biochemical details are presented in the **Supplementary Note**. In three cases, 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 frontoparietal hygroma, hypoplasia of the corpus callosum and small stalk lesion, respectively. In eight other cases, MRIs were normal.

Testicular development showed a characteristic pattern involving normal testicular volume in childhood, increase in testicular volume from approximately 11 years of age 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 testicular morphology by ultrasound. In 10 out of 11 evaluable cases, testosterone production was delayed, defined as a late increase in serum testosterone concentration (<0.8 nM = <23 ng/dl at 13.0 years)⁹ and/or a pubertal growth spurt that was >2 years delayed (compared to Dutch reference data¹⁰).

The most recent (July 2012) clinical data on height, body mass index (BMI) and pituitary-gonadal hormones for cases are shown in **Table 2**. Mean height was close to the average for population references, but BMI was higher than 25 in 11 out of 13 adults and higher than +2 s.d. score (SDS) in 5 out of 13 children. Plasma testosterone concentration was normal in most cases. A-I.4, who underwent surgery for

Table 2 Clinical and laboratory data

Case	Age (years)	T4 R/	Height SDS ^a	BMI SDS ^b	LH (IU/l) ^c	FSH (IU/l) ^c	LH _{max} to GnRH	FSH _{max} to GnRH	Testosterone (nM) ^c	Inhibin B (ng/l) ^d	AMH (μg/l) ^d
A-III.11	17.64	+	-0.2	2.6	1.0 ^e	3.8 ^e	13.1 ^e	8.3 ^e	1.1 (1.0–16.3) ^e	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 ^f	54 ^f	-	-	4.8 ^f	<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 (100–1,000)
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 ^g	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

T4 R/, L-thyroxine treatment; IU, International Units.

^aHeight is expressed as s.d. score (SDS) for national reference data for The Netherlands²³, UK²⁴ and Italy²⁵. The mean (s.d.) height is 0.0 (1.0) SDS. ^bBMI is expressed as SDS for Dutch references obtained in 1980 (ref. 26). Median BMI = 2.1 SDS. ^cReference ranges for males of >17 years: testosterone of 11–35 nM, LH < 0.1–15 IU/l and FSH < 0.1–10 IU/l. Reference ranges for testosterone in boys (10th to 90th percentile) according to von Schnakerburg *et al.*²⁷. ^dReference range for males of >18 years: inhibin B of 150–400 ng/l and AMH of 5–30 μg/l. Reference ranges for younger age groups are indicated. ^eGnRH test and testosterone performed at 15.19 years, before start of testosterone substitution therapy. ^fLH, FSH and testosterone concentrations before the start of testosterone substitution therapy (at 76 years). ^gBecause of low plasma SHBG concentration, free androgen index (FA = (100 × testosterone)/SHBG) was high in D-I.3 (67 years, age reference 18–54 years) and normal in F-II.8 (46 years, age reference 30–53 years).

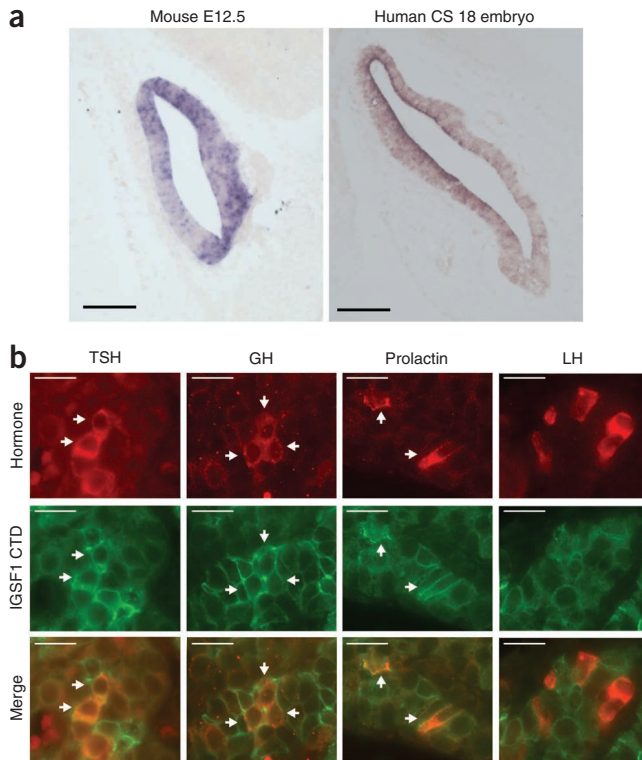


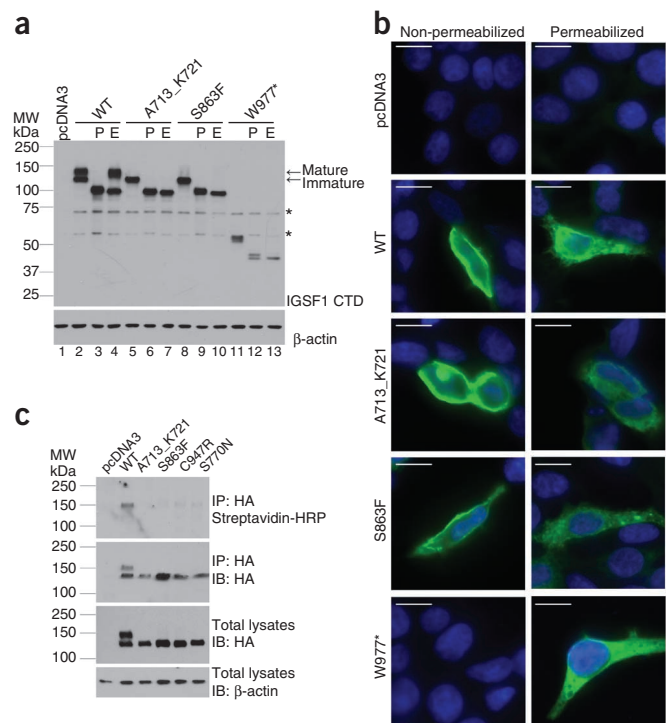
Figure 2 IGSF1 is expressed in anterior pituitary gland. (a) Expression of *Igsf1* mRNA in mouse embryonic day (E) 12.5 embryo and *IGSF1* mRNA in human embryo Carnegie stage 18 Rathke's pouch progenitors as detected by *in situ* hybridization. Scale bars, 10 μ m. (b) Immunofluorescence using antibodies against the IGSF1 CTD and the indicated anterior pituitary hormones (TSH, thyrotropes; GH, somatotropes; prolactin, lactotropes; LH, gonadotropes) was performed in wild-type E18.5 mouse pituitary. Arrows indicate colocalization of IGSF1 CTD and hormone signal. Scale bars, 10 μ m.

The identified *IGSF1* mutations are summarized in **Figure 1c**. Families C and D harbored the same single-nucleotide deletion (c.2248delG (p.Glu750Lysfs*28)). In families E and F, we observed submicroscopic gene deletions of different sizes that affected *IGSF1* and no other annotated gene. Affected members of families G–I and K had missense or nonsense mutations, and there was a single-nucleotide duplication (c.3596dupT (p.Glu1200Argfs*3)) in family J. 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, the Leiden Open Variance Database (LOVD), the Human Gene Mutation Database (HGMD) and Genome of the Netherlands (GoNL, pilot data of 500 unrelated individuals)) and were not previously reported.

IGSF1 encodes a plasma membrane immunoglobulin superfamily glycoprotein^{11,12}. The canonical IGSF1 protein has 12 C2-type immunoglobulin loops, a transmembrane domain and a short intracellular C tail (**Fig. 1c**). A hydrophobic linker separating immunoglobulin loops 5 and 6 targets the protein for obligate cotranslational proteolysis, such that only the C-terminal domain (CTD) traffics to the plasma membrane¹³. Human *IGSF1* and mouse *Igsf1* mRNAs are 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 mouse thyrotropes, somatotropes and lactotropes but not in gonadotropes (**Fig. 2b**) or in testis (data not shown).

testicular torsion and in whom the remaining testis was atrophic, had low testosterone concentration and elevated gonadotropins before the start of testosterone substitution, and, currently, at the age of 86.7 years, his inhibin B and anti-Müllerian hormone (AMH) concentrations are very low. The two subjects with severe obesity (D-I.3 and F-II.8) had low plasma testosterone concentrations, but, because of low plasma sex hormone-binding globulin (SHBG) concentration, the free androgen index was normal. Plasma luteinizing hormone (LH), follicle stimulating hormone (FSH), inhibin B and AMH concentrations were within reference ranges in the majority of cases, but plasma FSH concentration was always higher than LH concentration and was above the reference range in six cases. The response to gonadotropin-releasing hormone (GnRH) (100 μ g administered intravenously) was higher for LH than for FSH concentration in all except two subjects. Inhibin B concentration tended to be high (elevated in four subjects), and AMH concentration was relatively low (decreased in five subjects) relative to the reference range. Serum high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglyceride, glucose, insulin and C-peptide concentrations did not indicate the presence of a metabolic syndrome (data not shown).

Figure 3 Alterations in IGSF1 impair its plasma membrane trafficking. (a) HEK293 cells were transfected with pcDNA3 (empty vector) or with vectors expressing the indicated wild-type (WT) and mutant IGSF1 proteins. Protein lysates were deglycosylated with either PNGaseF (P) or EndoH (E), resolved by SDS-PAGE and immunoblotted using an antibody specific to the IGSF1 CTD. Non-specific bands are indicated with an asterisk. MW, molecular weight. (b) HEK293 cells were transfected with the same constructs as in a. Expression of the IGSF1 CTD was analyzed by immunofluorescence using the antibody against IGSF1 CTD under non-permeabilizing and permeabilizing conditions. Nuclei were stained with DAPI (blue). Scale bars, 10 μ m. (c) HEK293 cells were transfected with pcDNA3 or with vectors expressing the indicated wild-type and mutant IGSF1 proteins. Membrane expression of IGSF1 CTD was analyzed by cell surface biotinylation. IB, immunoblot; IP, immunoprecipitation; HRP, horseradish peroxidase.



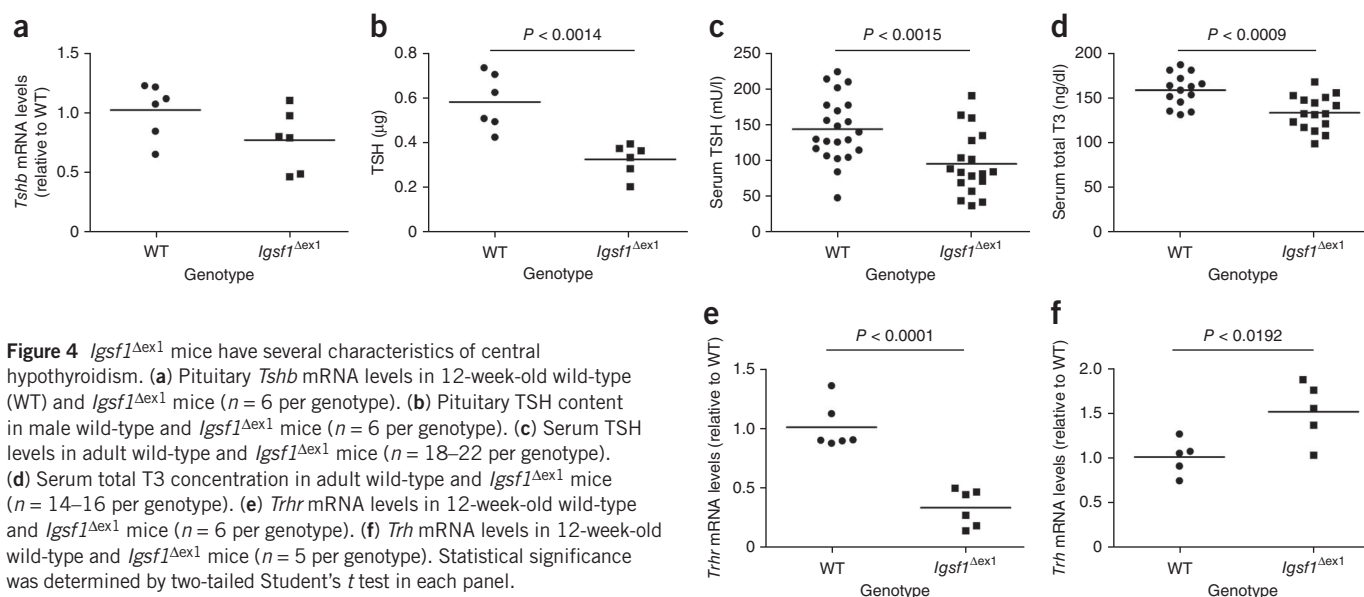


Figure 4 *Igsf1*^{Δex1} mice have several characteristics of central hypothyroidism. (a) Pituitary *Tshb* mRNA levels in 12-week-old wild-type (WT) and *Igsf1*^{Δex1} mice ($n = 6$ per genotype). (b) Pituitary TSH content in male wild-type and *Igsf1*^{Δex1} mice ($n = 6$ per genotype). (c) Serum TSH levels in adult wild-type and *Igsf1*^{Δex1} mice ($n = 18$ – 22 per genotype). (d) Serum total T3 concentration in adult wild-type and *Igsf1*^{Δex1} mice ($n = 14$ – 16 per genotype). (e) *Trhr* mRNA levels in 12-week-old wild-type and *Igsf1*^{Δex1} mice ($n = 6$ per genotype). (f) *Trhr* mRNA levels in 12-week-old wild-type and *Igsf1*^{Δex1} mice ($n = 5$ per genotype). Statistical significance was determined by two-tailed Student's *t* test in each panel.

The phenotypes of cases with intragenic mutations are highly similar to those of individuals with complete *IGSF1* deletion (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 IGSF1 migrated as a doublet of 140–150 kDa in protein blot analysis (Fig. 3a, lane 2), reflecting the mature N-glycosylated and immature endoplasmic reticulum (ER)-resident forms of the CTD (Fig. 3a, lanes 3 and 4)¹³. In contrast, IGSF1 Ala713_Lys721del (family A; primer sequences are given in Supplementary Table 5) migrated predominantly as an immature form (Fig. 3a, lanes 5–7). Similar migratory patterns were observed for IGSF1 Ser863Phe (family G; Fig. 3a; lanes 8–10) and the other two missense mutants (Cys947Arg (family H) and Ser770Asn (family K); Supplementary Fig. 3a, lanes 6–11). The nonsense mutants in families B and I (p.Trp977*; Fig. 3a, lanes 11–13; p.Trp1173*, Supplementary Fig. 3a, lanes 17–19) and frameshift mutants in families C and D (p.Glu750Lysfs*28) and J (p.Glu1200Argfs*3) (Supplementary Fig. 3a, lanes 3–5 and 12–14) possessed immature sugars and were truncated relative to wild-type IGSF1. On the basis of 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 constructs encoding wild-type and mutant IGSF1 and detected the expressed proteins by immunofluorescence with an antibody directed against the N terminus of the IGSF1 CTD. Membrane staining was observed in non-permeabilized cells transfected with the construct for wild-type IGSF1 (Fig. 3b). A similar pattern was observed with IGSF1 Ala713_Lys721del and the three missense mutants (Fig. 3b and Supplementary Fig. 3b); however, cell surface biotinylation showed that mutant proteins reached the plasma membrane with poor efficiency and with distinct glycosylation patterns compared to wild-type protein (Fig. 3c). Membrane signals were never detected in non-permeabilized cells transfected with vectors expressing IGSF1 p.Trp977* (Fig. 3b) or the other truncated forms of the protein (Supplementary Fig. 3b). We did not detect secreted proteins in the culture medium (data not shown). In contrast, we observed a strong intracellular staining pattern in permeabilized cells with all constructs (Fig. 3b and Supplementary Fig. 3b), confirming expression of the mutant

proteins. Thus, the identified mutations block or substantially 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 mice¹⁷ (Supplementary Fig. 4). mRNA levels for various pituitary hormone-encoding genes, including *Tshb*, did not differ between control and *Igsf1*-deficient mice (Fig. 4a and Supplementary Fig. 5a). In contrast, both pituitary and serum TSH content was 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 those of controls in the majority of cases, and thyroid histology appeared normal (Supplementary Fig. 5c,d and data not shown). Reduced TSH synthesis and secretion (Fig. 4b,c) in spite of normal *Tshb* mRNA expression (Fig. 4a) in *Igsf1*-deficient mouse pituitaries suggested impaired TRH signaling. Consistent with this idea, pituitary *Trhr* mRNA levels were reduced, and hypothalamic *Trhr* mRNA levels were increased, in *Igsf1*-deficient mice relative to controls (Fig. 4e,f). Pituitary *Gnrhr* mRNA expression was not altered (data not shown). Finally, *Igsf1*-deficient males were heavier than their control littermates (29.9 ± 0.4 versus 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 coreceptor^{15,18,19}, raising the possibility that macroorchidism in our cases might be linked to loss of inhibin activity and, therefore, enhanced FSH secretion. However, the putative role of IGSF1 as an inhibin coreceptor has been challenged by more recent binding and *in vivo* data^{17,19}. In our cases, serum FSH concentration was higher than that of 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 concentration (ref. 17 and Supplementary Fig. 5a,e). Therefore, at present, the mechanisms of testicular enlargement in our cases are unresolved.

In summary, our data delineate a new X-linked disorder in which loss-of-function mutations in *IGSF1* cause central hypothyroidism, testicular enlargement and variable prolactin and GH deficiency. The identified human *IGSF1* defects impair either expression or membrane trafficking of the IGSF1 CTD, consistent with a loss of protein function.

Igsf1 deletion decreases pituitary and circulating TSH content in mice, perhaps secondary to impaired TRH receptor signaling. Loss of IGSF1 function was associated with profound hypothyroxinemia in some cases, with the attendant risk of neurodevelopmental delay, if untreated. In other cases, hypothyroxinemia was less severe, but untreated sub-clinical 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 substantial 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, <http://www.lovd.nl/igsf1>.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Accession codes. X-chromosome sequencing data were deposited at the LOVD3 database: <http://databases.lovd.nl/shared/individuals/00000208> and <http://databases.lovd.nl/shared/individuals/00000209>.

Note: Supplementary information is available in the online version of the paper.

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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.R.B., N.M.A.-D., A.M.P., M.H.B., R.C.H., M.T.D.,

N.S., L.P., I.C., M.B., P.B.-P., H.Z., T.M.E.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 vectors expressing mutant IGSF1 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 and participated in data collection and construction of the figures. P.V. and M.G.W. contributed to mouse phenotyping. E.C., J.K.W. and M.G.W. performed mouse T4 measurements. P.I.T. performed measurements of pituitary TSH and prolactin 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 analyzed the microarray and hybridization experiments. A.S.P.v.T., W.O., W.H.S.-B., T.V., M.J.K., L.P., I.C., M.B., P.B.-P., H.Z., T.M.E.D., A.C.S.H.-K., D.G.D.B., J.J.R., S.G.K., N.R.B., N.M.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.T.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.

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ONLINE METHODS

DNA isolation. DNA was isolated from human whole blood using the Autopure LS system (Qiagen) and associated reagents, following the manufacturer's protocol. All participants provided written informed consent. The study was approved by the ethics committees of Leiden University Medical Center (Leiden, The Netherlands), Academic Medical Center (Amsterdam, The Netherlands), Anglia and Oxford MREC (Cambridge, UK), Great Ormond Street Hospital (London, UK), Erasmus University Medical Center (Rotterdam, The Netherlands) and the IRCCS Istituto Auxologico Italiano (Milan, Italy).

Exome enrichment and next-generation sequencing. X-chromosome exome enrichment was carried out using Agilent's SureSelect X chromosome oligonucleotide capture library according to the manufacturer's protocol, except that DNA was hybridized with half of the amount of suggested probe. Illumina Genome Analyzer IIx sequencing generated 51-bp paired-end reads. An overview of reads is shown in **Supplementary Table 1**. Whole-exome sequencing was undertaken using the SureSelect Human All Exon 50Mb kit (Agilent Technologies). Sequencing was performed with the SOLiD 4 system (Applied Biosystems).

Read mapping, variant calling and annotation. For the X-chromosome exome data, reads were aligned to reference genome hg19 by Burrows-Wheeler Aligner (BWA) 0.5.8 (ref. 28). BAM file manipulation and variant (single-nucleotide variant (SNV) and insertion and/or deletion (indel)) calling was performed with SAMtools 0.1.9 (ref. 29). The deletion in family A was detected by GMAP v3. 2011-03-28 (alignment)³⁰ and SAMtools 0.1.9 (variant calling). SeattleSeq Annotation 131 was applied to annotate all variants. Variants in dbSNP131 and the 1000 Genomes Project were filtered out for mutation screening. Whole-exome experiments, sequencing and preliminary analyses were undertaken at the Eastern Region Sequencing and Informatics Hub using the Genome Analysis Toolkit³¹.

Filtering strategy. In family A, variants shared by both boys were classified on the basis of their function; only exonic and splice-site variants were taken forward. We then filtered out all variants present in the local, in-house exome sequencing database, dbSNP, 1000 Genomes Project, LOVD and HapMap Project with an allele frequency of >1% in populations of European ancestry, which yielded no promising results. We therefore examined 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 these variants. For family B, we compared variants from the two brothers; only shared exonic and splice-site variants were taken forward to the filtering step. We filtered out all variants present in databases and the UK 10K genomes project and focused on previously unknown, 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 the manufacturer's protocol (primer sequences are listed in **Supplementary Table 4**). Products were purified with the QIAquick PCR purification kit (Qiagen) and mixed with 10 pmol of forward or reverse primer and sequenced with the Applied Biosystems 96-capillary 3730xl system.

Microarrays. We performed analysis on the cytogenetic whole-genome 2.7M array (Affymetrix) according to the manufacturer's protocol. In families C and D, this analysis identified the same haploblock of 23 Mb around the deletion (rs929590 to rs16979902), suggesting a common ancestral origin for these families, although no familial relationship is known for at least four generations.

Expression constructs. The epitope for the antibody to IGSF1 CTD encoded by the human Myc-IGSF1-HA construct (from P. Scheiffele) was modified (QuikChange, Stratagene) to match the mouse protein to facilitate detection with polyclonal antibody against IGSF1 CTD (P. Scheiffele). Mutations were introduced into the construct using QuikChange or Phusion (Thermo; to create the p.Ala713_Lys721del alteration) site-directed mutagenesis (primer sequences are listed in **Supplementary Table 5**). Constructs were verified by sequencing (McGill University and Genome Québec Innovation Centre).

Cell culture. For protein 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 the manufacturer's instructions. For immunofluorescence, HEK293 cells were plated in 24-well plates on glass coverslips with 50,000 cells per well and transfected with 100 ng of expression vector.

Protein blotting. Whole-cell extracts were prepared from transfected cells 24 h after transfection using RIPA buffer (150 mM NaCl, 50 mM sodium fluoride, 10 mM NaPO₄, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein lysates were deglycosylated with PNGaseF and EndoH (New England Biolabs), according to the manufacturer's instructions. Protein blots were performed as previously described³².

Cell immunofluorescence. Under non-permeabilizing conditions, antiserum to IGSF1 CTD (1:500 dilution in serum-free DMEM) was applied to cells for 2 h at 37 °C in 5% CO₂. Cells were transferred to room temperature, washed three times with serum-free DMEM and fixed with 4% paraformaldehyde (PFA). Cells were then washed three times with PBS and incubated in 5% BSA in PBS for 1 h. Secondary antibody (1:600 dilution in 5% BSA in PBS) was applied to cells for 1 h, and cells were washed three times with PBS and mounted using aqueous medium with DAPI. Under permeabilizing conditions, cells were fixed using 4% PFA, washed three times in PBS, incubated in 0.3% Triton X-100 in PBS (PBSX) and incubated in 5% BSA in PBSX for 1 h. Antiserum to IGSF1 CTD (1:500 dilution in 5% BSA in PBSX) was applied to cells for 2 h. Cells were washed three times with PBS. Secondary antibody (1:600 dilution in 5% BSA in PBSX) was applied for 1 h, and cells were washed three times with PBS and mounted under non-permeabilizing conditions.

Pituitary immunofluorescence. The morning when a vaginal plug was detected was considered E0.5. Pregnant mice at E18.5 were sacrificed, and embryo heads were fixed overnight in 4% PFA at 4 °C and embedded in paraffin, with sections sliced with 5 µm thickness. Sections were treated as previously described³³. 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 dilution, and (from Santa Cruz Biotechnology) TSH (sc7815) and LH (sc7824), 1:400 dilution, and GH (sc10365) and prolactin (sc7805) 1:200 dilution, in blocking buffer) overnight at 4 °C. Sections were washed three times with PBST, incubated in biotin-conjugated horse secondary antibody to goat (1:150 dilution in blocking buffer) for 1 h, washed three times with PBST, incubated in Streptavidin-Texas Red (1:400 dilution in blocking buffer) and Alexa 488-conjugated goat secondary antibody to rabbit (1:600 dilution) for 1 h, washed three times with PBST and mounted.

Cell surface biotinylation. Transfected cells were washed three times with PBS and incubated in 1 ml of 0.5 mg/ml EZ-link-sulfo-NHS-LC-biotin (Thermo) for 30 min at 4 °C, washed three times 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 (9 W for 10 s). Lysates were centrifuged, supernatant was collected and immunoprecipitation was carried out using EZview anti-HA affinity gel (E6779, Sigma) according to the manufacturer's instructions. Lysates were then processed for protein blotting. After blocking, membrane was incubated in 2.5% milk in TBST with two drops each of A and B reagents from the Vectastain kit (Vector) for 30 min. Membrane was washed three times with 10-min washes in TBST, and signal was visualized using the Western Lightning Plus-ECL kit (PerkinElmer).

Animals. *Igsf1*-deficient mice have been described previously¹⁷. 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 described³⁴ using riboprobes from human *IGSF1* cDNA clone 30387876 (IMAGE). Human embryonic material was provided by the Human Developmental Biology Resource, supported by Medical Research Council Grant G0700089 and Wellcome Trust Grant 082557.

RNA blotting. Human pituitary RNA was from the Netherlands Brain Bank (NBB; 02-055) and was obtained 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 S. Kimmins. Each RNA (13 µg) was blotted as previously described¹⁷, using a probe spanning exons 18–20 of mouse *Igsf1*.

Hormone concentration measurements. At diagnosis, serum FT4, TSH, T3 and prolactin concentrations were determined in the laboratories of the participating departments. At follow-up, samples were investigated in the Endocrine Laboratory at Erasmus Medical Center (inhibin B and AMH) and the Laboratory of Endocrinology and Radiochemistry at the Academic Medical Center (other measurements). Plasma LH and FSH concentrations were analyzed on a Roche E170; intra- and interassay variations were <5%. Plasma T3 concentration was measured by in-house radioimmunoassay (RIA); intra- and interassay variations were 6.3% and 7.8%, respectively. FT4, FT3, TSH, prolactin and GH concentrations were analyzed by fluoroimmunoassay using the Delfia 1232 Fluorometer (PerkinElmer); intra- and interassay variations, respectively, were 5.1% and 6.8% (FT4), 7.7% and 11.3% (FT3), 4.2% and 5.7% (TSH), 3.4% and 5.3% (prolactin) and 3.8% and 6.2% (GH). Plasma testosterone concentration was analyzed by in-house RIA; intra- and interassay variations were 9.2% and 10.8%, respectively. Insulin was analyzed by chemiluminescence using Immulite 2000 (Siemens); intra- and interassay variations were 3.7% and 5.1%, respectively. Serum inhibin B and AMH concentrations were estimated using enzyme immunometric methods (Beckman Coulter). For AMH concentration, the Gen II assay was used; intra- and interassay variations were published previously^{35,36}.

Mouse pituitary TSH and PRL content measurement was previously described³⁷ and used reagents supplied by A.L. Parlow (National Hormone and Peptide Program). In Chicago, serum total T4 and T3 levels were measured by coated-tube RIA (Siemens Medical Solutions Diagnostics) adapted for mouse. TSH concentration was measured as previously described³⁸. Free T4 levels were estimated from the ratio of total T4 to resin T4 uptake and expressed as the free T4 index (FT4I) as described³⁹.

Quantitative PCR. Mouse RNA was extracted from whole pituitaries and 3-mm hypothalamic blocks according to the following coordinates⁴⁰: anteroposterior-interaural 3.94 to 0.94 mm, dorsoventral-interaural 2.5 mm and below, and 1.44 mm lateral. RNA (2 µg) was extracted and reverse transcribed as previously described³². Quantitative PCR (qPCR) was performed

using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 0.4 pmol of each primer (**Supplementary Table 6**) on a Corbett Rotor-Gene 6200 HRM, using the manufacturer's protocol. Transcript levels were normalized to those of *Rpl19* (encoding ribosomal protein L19) and analyzed using the $2^{-\Delta\Delta CT}$ method^{41,42}.

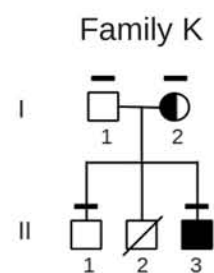
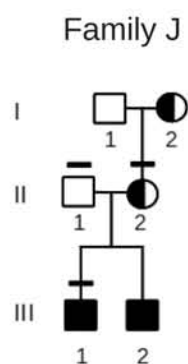
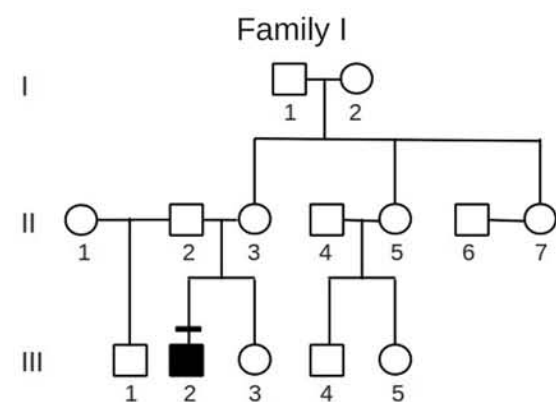
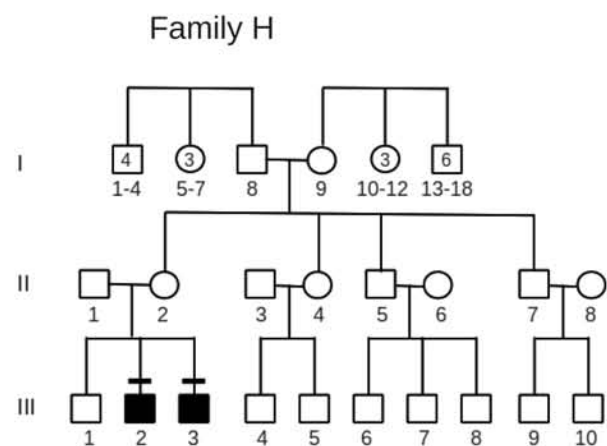
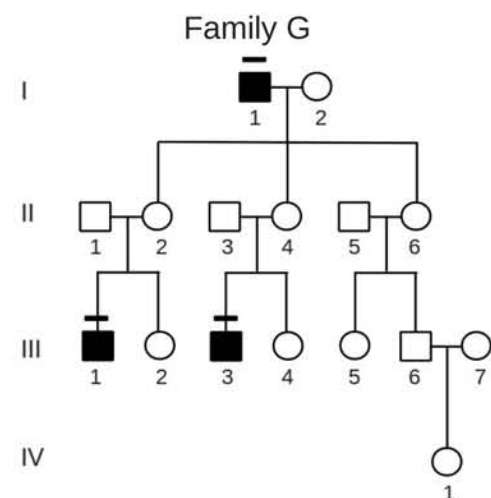
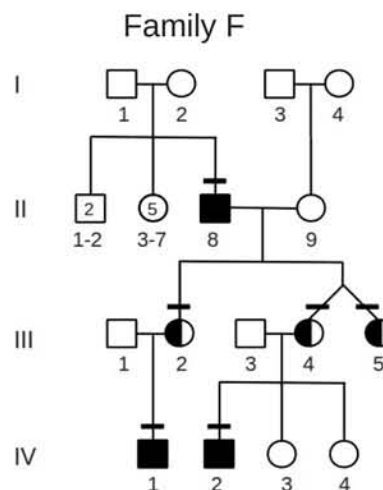
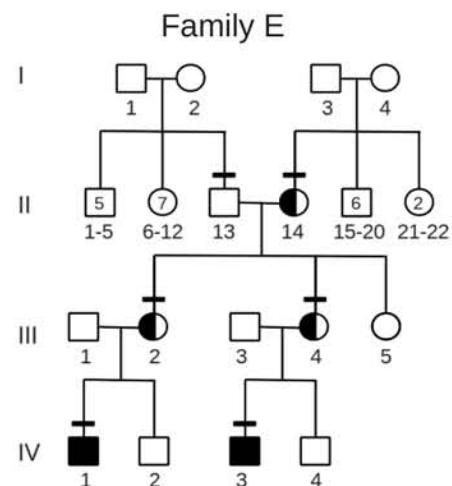
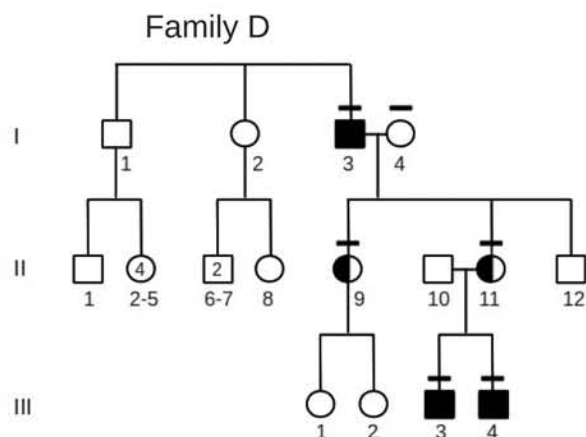
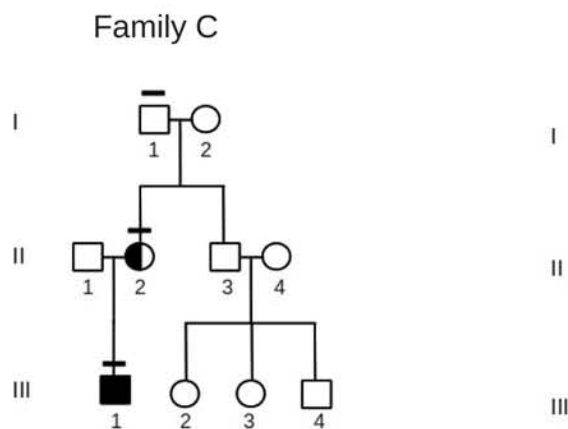
Statistics. In **Figure 4** and all supplementary figures, statistical significance was defined as $P < 0.05$ and was determined by two-tailed Student's *t* test.

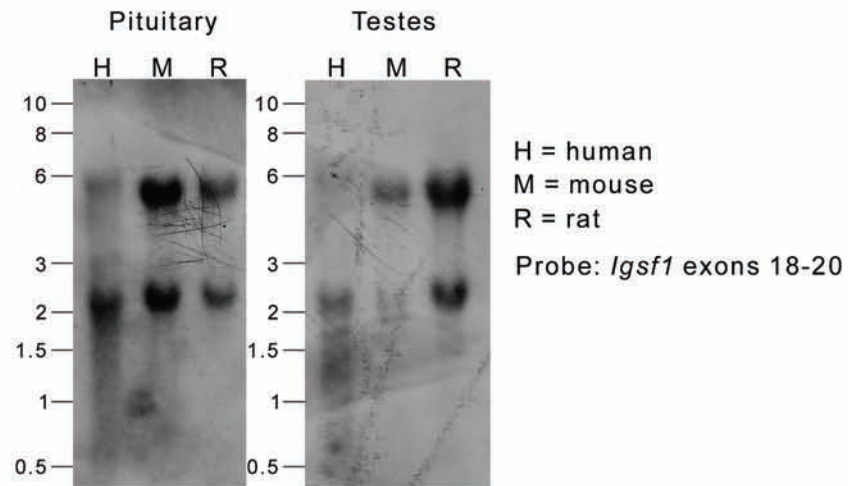
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Loss-of-function mutations in IGSF1 cause an X-linked syndrome of central hypothyroidism and testicular enlargement.

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

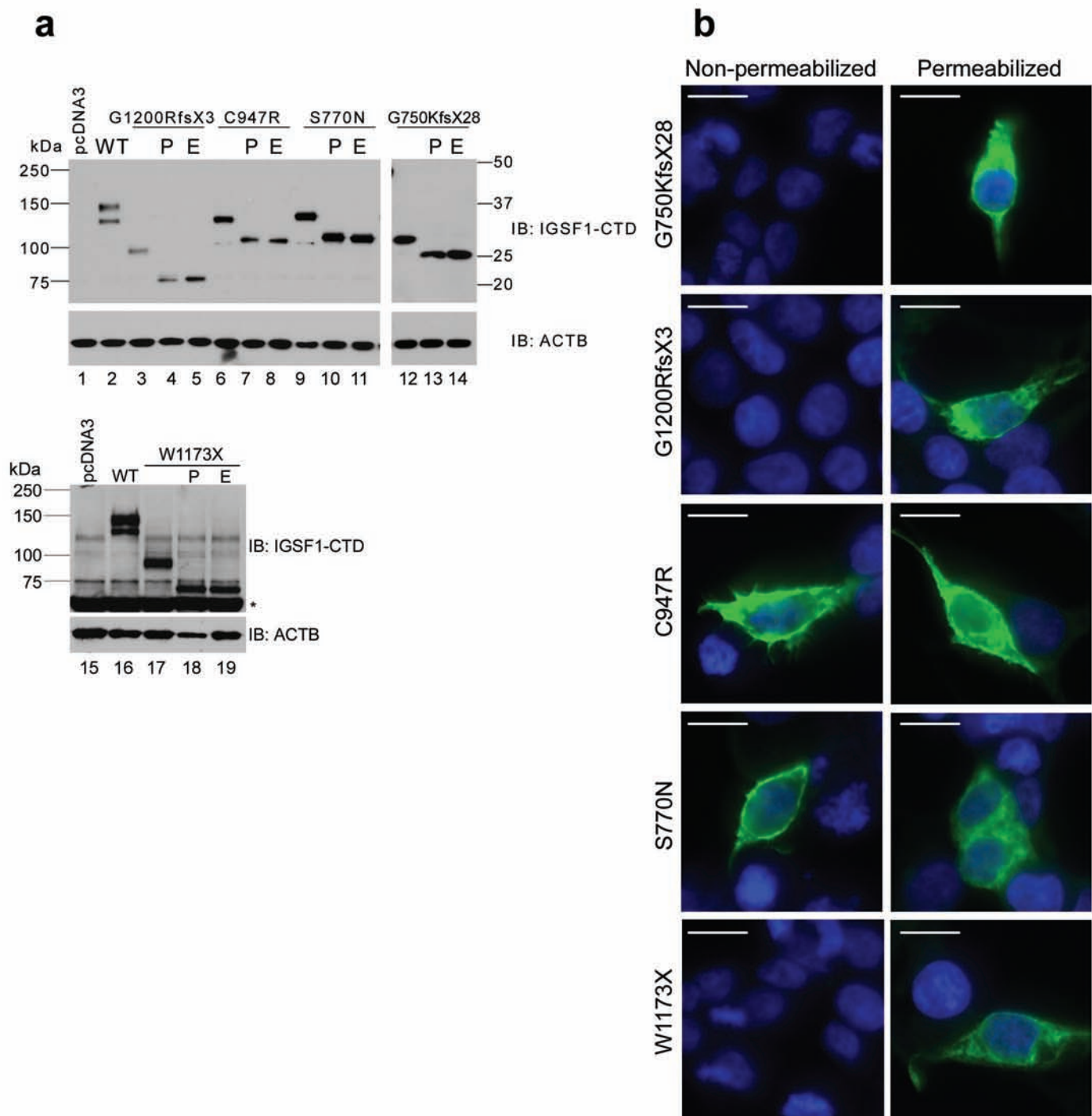
Supplementary information





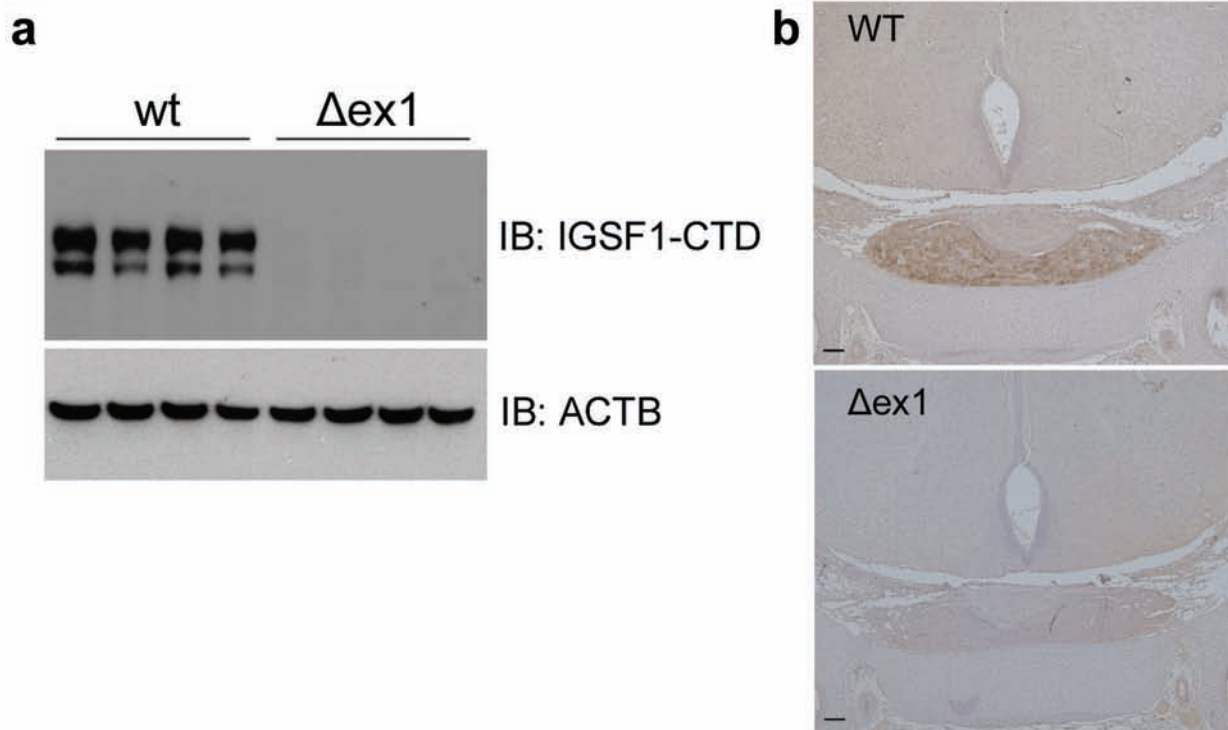
Northern blot comparing *IGSF1/lgsf1* expression

Northern blot comparing *IGSF1/lgsf1* expression in adult human (H), murine (M), and rat (R) pituitary and testes. Probe was directed against the 3' end of murine *lgsf1*.



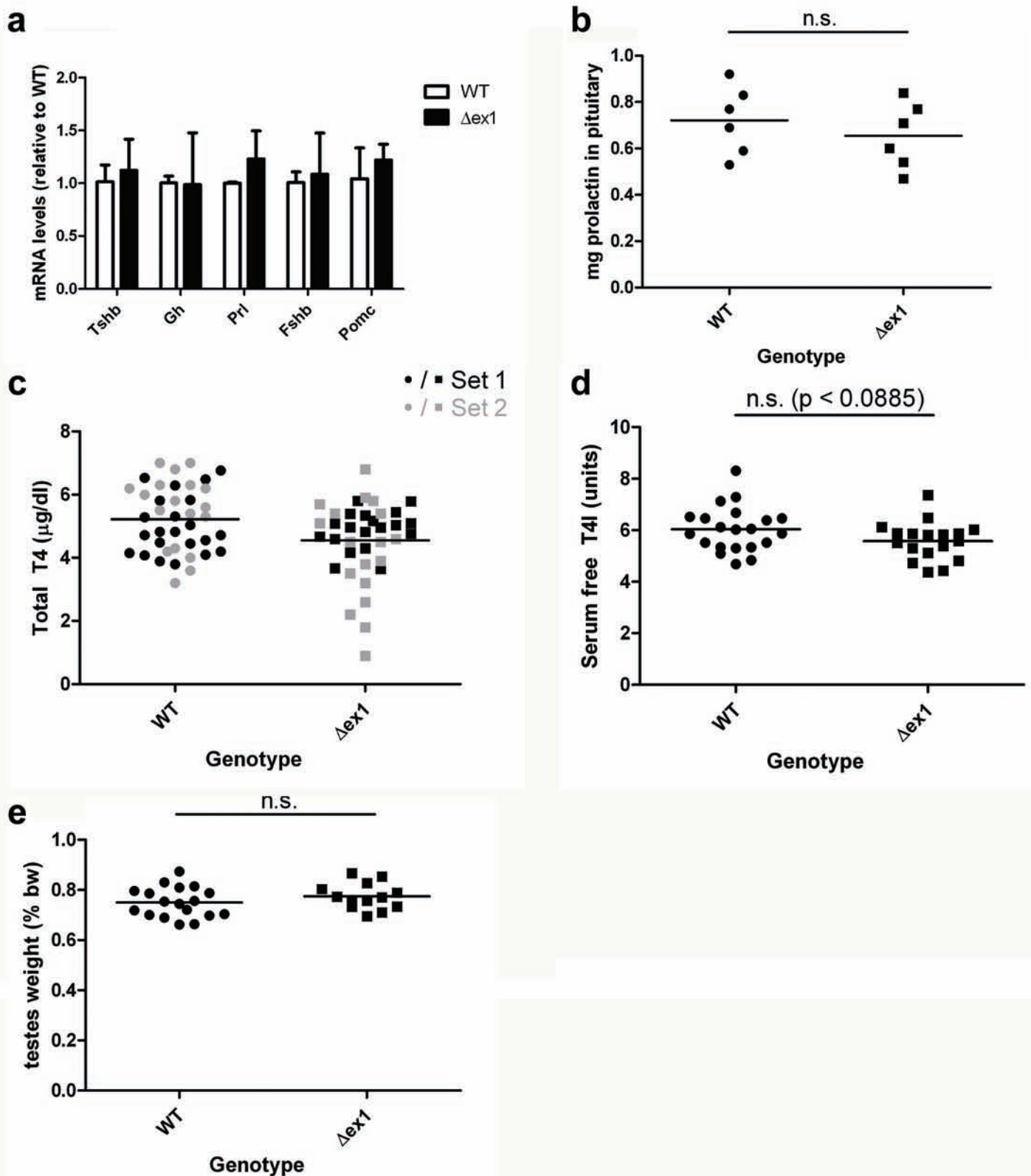
Expression of additional IGSF1 mutants

(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 α IGSF1-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 α IGSF1-CTD antibody under non-permeabilizing and permeabilizing conditions. Nuclei are stained with DAPI (blue). Scale bars, 10 μ m.



IGSF1-CTD expression in wild-type vs. *Igsf1*-deficient mice

(a) IGSF1-CTD protein is undetectable in pituitaries of *Igsf1* $\Delta ex1$ mice. Protein lysates from individual pituitaries of adult wild-type and *Igsf1* $\Delta ex1$ male mice were run on SDS-PAGE and subject to western blotting using IGSF1-CTD antiserum. **(b)** Immunohistochemistry for IGSF1 in WT and *Igsf1* $\Delta ex1$ male mice pituitary at embryonic day 18.5. Scale bars, 10 μm .



Hormone expression data and testes size in wild-type vs. *lgsf1*-deficient mice

(a) mRNA levels of anterior pituitary hormone encoding genes do not differ between adult wild-type and *lgsf1* $\Delta ex1$ 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 $\pm$ s.e.m., relative to wild-type for each hormone. (b) Pituitary prolactin content does not differ between adult male wild-type and *lgsf1* $\Delta ex1$ 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 *lgsf1* $\Delta ex1$ mice. (e) Testes weights (as a percentage of body weight) do not differ between adult male wild-type and *lgsf1* $\Delta ex1$ mice.

Supplementary Table 1. Overview of exome sequencing data

Patient	<i>A-III.11</i>	<i>A-III.7</i>	<i>B-III.7</i>	<i>B-III.8</i>
Total reads	15,039,708	13,712,350	83,312,890	83,457,089
Platform	GAI, Illumina	GAI, 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)		Δ FT4(%)	FT3 (pmol/L)		Δ FT3(%)
		0	peak	0	peak		0	peak	
<i>E-IV.1</i>	2 wk	3.0	8.4	8.4	9.8	16.7			
<i>C-III.1</i>	3 wk	2.3	7.8	9.1	9.6	5.5			
<i>A-III.11</i>	3 wk	2.6	10.4	8.3	9.4	13.3			
<i>B-II.11</i>	43.3 yr	1.7	7.6	7.2	8.0	11.1	4.5	5.1	13.3
<i>A-II.4</i>	52.4 yr	0.6	4.7	8.8	9.8	11.4	2.9	3.9	34.5
<i>F-II.8</i>	58.2 yr	2.3	8.5	8.5	10.2	20.0	3.9	6.0	53.8
<i>D-I.3</i>	62.8 yr	2.2	6.6	7.3	8.0	9.6	3.2	5.6	75.0
<i>B-I.4</i>	65.9 yr	1.7	4.5	9.1	9.9	8.8	4.0	4.8	20.0
<i>B-I-4³</i>	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	14.2¹	3.8	5.1	36.3²
SD		0.7	2.2	0.9	0.8	7.9	0.6	0.7	26.4

¹Reference 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	TCCTGATTGTTGGGATGTCTCC	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	CCAAGTGGAAGTGGAAGGA		
IGSF1_14_F	GGAACAATCCTGGGAGACAA	547	Exon 7
IGSF1_14_R_2	TTAAGGAGTGGGAGCAGGAA		
IGSF1_15_F_2	AAACCACCCTTGGA AAAACC	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	AGCAGCTTCAGCTCATACA		
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'-GGCAGCTGTAATTGCCTTGCTTTATCCTCCATTCTC-3'
c.2588C>T, p.(Ser863Phe)	5'-TATTATGACTTTTCTATCTGGTTTGAGCCCAGCGACC-3'	5'-GGTCGCTGGGCTCAAACCAGATAGAAAAGTCATAATA-3'
c.2839T>C, p.(Cys947Arg)	5'-GGACTCTGGGAAGTATAGCCGTATCTACTATGAGACAAC-3'	5'-GTTGTCTCATAGTAGATACGGCTATAGTTCCAGAGTCC-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:
<i>Tshb</i>	5'-gaacggtggaaataaccagga-3'	5'-agaaagactgcggcttggtgca-3'
<i>Trhr</i>	5'-ctcccaacataaccgacag-3'	5'-gcagagaaactgggctttga-3'
<i>Fshb</i>	5'-gtgcgggctactgctacact-3'	5'-caggcaatcttacggtctcg-3'
<i>Gh</i>	5'-acctcggaccgtgtctatga-3'	5'-gcagcccatagttttgagc-3'
<i>Prl</i>	5'-aagcagcttcttgaggagtt-3'	5'-tgttcgcaaagacaagatt-3'
<i>Pomc</i>	5'-ccaggaacagcagcagtg-3'	5'-acgttgggtacaccttcac-3'
<i>Trh</i>	5'- gaaagacctccagcgtgtg-3'	5'-tcttcggctcaacgtcttc-3'
<i>Rpl19</i>	5'-cgggaatccaagaggattga-3'	5'-ttcagcttggtgatgtgctc-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.