ISSN 2472-1972

From Consternation to Revelation: Discovery of a Role for IGSF1 in Pituitary Control of Thyroid Function

Daniel J. Bernard,[1,2] Emilie Brûlé,[2] Courtney L. Smith,[1] Sjoerd D. Joustra,[3] and Jan M. Wit[3]

[1]Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec H3G 1Y6, Canada; [2]Department of Anatomy and Cell Biology, McGill University, Montréal, Québec H3A 0C7, Canada; and [3]Department of Pediatrics, Leiden University Medical Center, 2333 ZA Leiden, the Netherlands

Immunoglobulin superfamily, member 1 (IGSF1) is a transmembrane glycoprotein highly expressed in the mammalian pituitary gland. Shortly after its discovery in 1998, the protein was proposed to function as a coreceptor for inhibins (and was even temporarily renamed inhibin binding protein). However, subsequent investigations, both *in vitro* and *in vivo*, failed to support a role for IGSF1 in inhibin action. Research on IGSF1 nearly ground to a halt until 2011, when next-generation sequencing identified mutations in the X-linked *IGSF1* gene in boys and men with congenital central hypothyroidism. IGSF1 was localized to thyrotrope cells, implicating the protein in pituitary control of the thyroid. Investigations in two *Igsf1* knockout mouse models converged to show that IGSF1 deficiency leads to reduced expression of the receptor for thyrotropin-releasing hormone (TRH) and impaired TRH stimulation of thyrotropin secretion, providing a candidate mechanism for the central hypothyroidism observed in patients. Nevertheless, the normal functions of IGSF1 in thyrotropes and other cells remain unresolved. Moreover, *IGSF1* mutations are also commonly associated with other clinical phenotypes, including prolactin and growth hormone dysregulation, and macroorchidism. How the loss of IGSF1 produces these characteristics is unknown. Although early studies of IGSF1 ran into roadblocks and blind alleys, armed with the results of detailed clinical investigations, powerful mouse models, and new reagents, the field is now poised to discover IGSF1's function in endocrine tissues, including the pituitary and testes.

Freeform/Key Words: central hypothyroidism, IGSF1, mutation, pituitary, TRH

1. A Brief History of IGSF1: The Inhibin Receptor That Wasn't

Immunoglobulin superfamily, member 1 (IGSF1) was born as an orphan. In 1998, two groups independently reported the cloning of large complementary DNAs coding for a novel member of the immunoglobulin superfamily [1, 2]. On the basis of the derived amino acid sequence, both groups predicted that the complementary DNAs, which they called IGSF1 and immunoglobulin-like domain containing 1, encoded a transmembrane glycoprotein with 12 extracellular immunoglobulin (Ig) loops, a single transmembrane domain, and a short cytoplasmic tail (Fig. 1). The protein's function was unknown at the time and remains so to this

Abbreviations: ALK, activin receptor-like kinase; ALK4, activin type IB receptor; ALK5, transforming growth factor β type I receptor; CTD, *C*-terminal domain; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; GH, growth hormone; HPT, hypothalamic-pituitary-thyroid; Ig, immunoglobulin; IGSF1, immunoglobulin superfamily, member 1; LoI/PTU, low-iodine diet, supplemented with propylthiouracil; mRNA, messenger RNA; NTD, *N*-terminal domain; T3, triiodothyronine; T4, thyroxine; TGFβ, transforming growth factor β; TGFBR3, betaglycan; TRH, thyrotropin-releasing hormone; TSH, thyrotropin.

Received 22 December 2017
Accepted 1 February 2018
First Published Online 6 February 2018

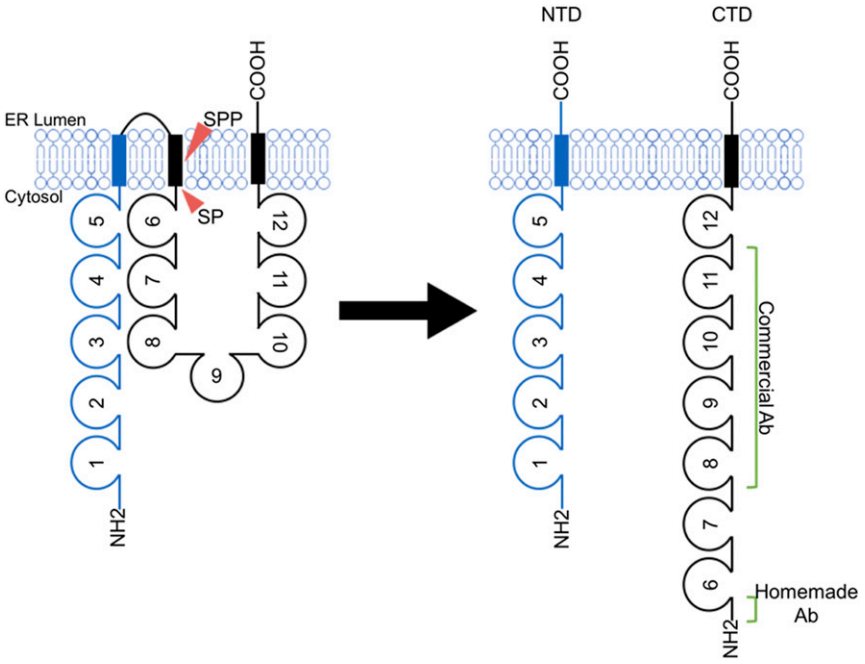

Figure 1. Schematic representation of IGSF1's topology before (left) and after (right) proteolytic cleavage. The 12 Ig loops are labeled, as are the cytosolic and ER luminal compartments. Approximate locations of cleavage by SP and SPP are marked with red arrowheads. Transmembrane domains are pictured as solid rectangles. The NTD and CTD are presented in blue and black, respectively. Note that the signal peptide at the *N*-terminus of the NTD is not pictured. The second transmembrane domain (*N*-terminal to Ig loop 6) serves as an internal signal peptide for the CTD. The approximate locations of the epitopes recognized by the two IGSF1 antibodies (Ab) described in the text are indicated in green. SP, signal peptidase; SPP, signal peptide peptidase.

day. However, research over the past 2 decades, and in particular over the past 5 years, has provided the necessary context for understanding IGSF1's role in cells.

In 2000, IGSF1 (then referred to as p120 or inhibin-binding protein) was "adopted" by the field of reproductive endocrinology when it was proposed to act as an inhibin coreceptor [3]. Inhibins are transforming growth factor β (TGFβ) superfamily ligands produced in the gonads, which act on pituitary gonadotrope cells to suppress follicle-stimulating hormone (FSH) synthesis and secretion [4]. Mechanisms of inhibin action were poorly understood at the time. In the context of a biochemical screen for inhibin receptors, a protein was purified from bovine pituitary membrane extracts by inhibin A affinity chromatography. *N*-terminal sequencing of the protein revealed a 17–amino acid peptide homologous to sequence between the fifth and sixth Ig loops of the newly characterized human IGSF1 (Fig. 1). The following year, on the basis of overexpression and promoter-reporter assays in heterologous cells, IGSF1 was further suggested to act specifically as an inhibin B coreceptor [5]. Contemporaneously, the TGFβ type III receptor, betaglycan (or TGFBR3), was also proposed to function as an inhibin coreceptor, but through a distinct mechanism [6]. Subsequent findings have largely supported a role for betaglycan, but not IGSF1, in mediating the actions of the inhibins. For example, both inhibin A and inhibin B can bind to betaglycan in *in vitro* heterologous binding assays, whereas neither protein binds IGSF1 under similar conditions [7–13].

In 2003, any potential role for IGSF1 in inhibin action was seemingly laid to rest when *Igsf1* knockout mice were reported to be fertile, with normal FSH levels [14]. Removal of a true inhibin receptor or coreceptor would be predicted to yield increases in FSH secretion and, in the case of females, enhanced ovarian folliculogenesis and fertility. Betaglycan's role as an inhibin coreceptor has not been established *in vivo* because *Tgfbr3* knockout mice die during embryonic development [15]. However, an antibody that associates with the part of betaglycan bound by

inhibins impaired inhibin A antagonism of FSH secretion in rat pituitary cells in culture [12], strongly suggesting a role for the protein in inhibin A action in gonadotropes.

With the emergence of betaglycan as the more likely inhibin coreceptor, research on IGSF1 slowed considerably and the protein regained its orphan status. However, in 2008, an unexpected feature of IGSF1 emerged with the reporting that the protein was cleaved cotranslationally into amino (*N*-) and carboxyl (*C*-) terminal domains of 5 and 7 Ig loops by signal peptidase or a related protease (Fig. 1) [16]. Moreover, only the *C*-terminal domain (CTD) appeared to traffic to the plasma membrane, with the *N*-terminal domain (NTD) remaining in the endoplasmic reticulum (ER). This discovery would prove important for at least two reasons. First, it explained why the *N*-terminal sequence of the purified bovine protein mapped to a segment between Ig loops 5 and 6 rather than at the *N*-terminus of the predicted full-length protein [3]: that is, the sequence corresponded to the *N*-terminus of the CTD following proteolytic cleavage. Second, it would later help to explain the remarkable clustering of mutations in the human *IGSF1* gene (Fig. 2), which were discovered starting 3 years later [17].

2. Discovery of IGSF1 as a Central Mediator of Thyroid Function

Between 2003 and 2012, few published studies referenced IGSF1. When mentioned, it was usually in the context of gene expression analyses in reproductive tissues or different tumors [18–23]. Unfortunately, none of these studies provided tangible insight into IGSF1 function in the pituitary gland or, indeed, any tissue or cell type. Then, fortune struck, not once, but twice, when pediatric endocrinologists in the Netherlands and the United Kingdom unleashed the power of next-generation sequencing on two families with unexplained central hypothyroidism [17].

Two male first cousins in the Netherlands who were diagnosed with idiopathic congenital central hypothyroidism were subjected to X chromosome sequencing, which revealed an in-frame 27-bp deletion in the *IGSF1* gene. This mutation was predicted to remove nine amino acids in the seventh Ig loop (see del27 bp in Fig. 2). Sequencing of DNA from other family members revealed that both cousins inherited the mutant allele from their mothers, who in turn inherited it from their father (*i.e.,* the probands' maternal grandfather). He was diagnosed with mild hypothyroidism, but only when evaluated in his 60s. Contemporaneously, whole exome sequencing revealed a nonsense mutation (Trp977*) in the *IGSF1* gene in two British brothers diagnosed with central hypothyroidism after referral for their prolonged neonatal jaundice. These boys inherited the mutation from their mother and maternal grandmother, both of whom were heterozygous for the mutant allele. The mutation prematurely truncated the IGSF1 protein in the 10th Ig loop. *In vitro* analyses revealed that both the Dutch and British mutations blocked or greatly attenuated plasma membrane trafficking of the IGSF1-CTD.

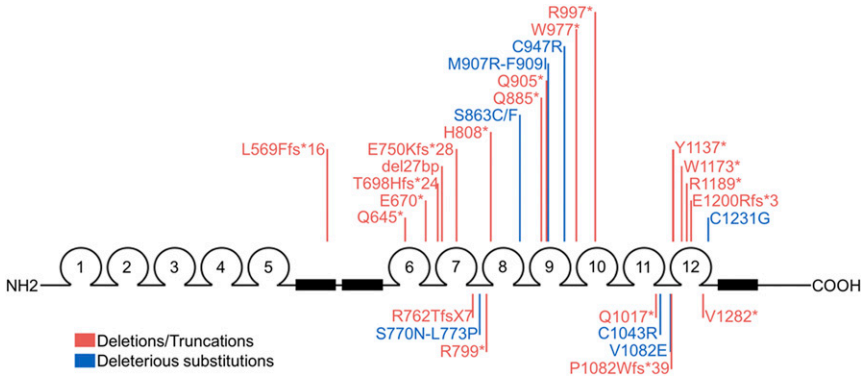

Figure 2. Approximate locations of pathogenic mutations in human IGSF1. Deletions and truncations are labeled in red. Deleterious missense mutations are in blue. Single-letter amino acid designations are used. fs, frame shift; *Stop codon.

These results suggested that loss-of-function mutations in the *IGSF1* gene might cause central hypothyroidism in these patients and perhaps others with idiopathic disease. Indeed, with a candidate gene in hand, eight additional *IGSF1* mutations were rapidly identified in nine other families [17]. Since the initial report on these families, at least 29 additional *IGSF1* mutations have been described in individuals and families from around the world (Fig. 2) [24–35]. These mutations include amino acid substitutions (missense), premature stop codons (nonsense), frame shifts (both insertions and deletions), and entire gene deletions. Remarkably, all intragenic mutations, with one exception (L569Ffs*16), map to the portion of the gene encoding the CTD. Most of the mutations prevent the CTD from trafficking from the ER to the plasma membrane, as assessed in heterologous cell models. These results, along with the cellular distribution of the NTD (in the ER) and CTD (at the plasma membrane), converge to suggest that the CTD is likely to be the functional part of the protein. It is also now clear that mutations in *IGSF1* represent the most common genetic cause of central hypothyroidism described to date [36, 37]. No genotype-phenotype relationships have been observed, including between missense mutations and entire gene deletions. Rather, the mutations identified up to this point all appear to cause near or complete loss of function.

Because *IGSF1* is X-linked, effects of mutations in the gene are most often observed in males. A few female heterozygous carriers have been clinically evaluated, but only a fraction show evidence of central hypothyroidism [27]. To our knowledge, no women with homozygous loss-of-function mutations in *IGSF1* have been described. Though central hypothyroidism is observed in all boys and men with pathogenic *IGSF1* mutations, carriers also display other common characteristics with variable prevalence, including hypoprolactinemia; growth hormone (GH) dysregulation, with deficiency in some children and increased or high-normal insulin-like growth factor-1 values in most adults; disharmonious pubertal development, with delayed rise of testosterone secretion in contrast to normal or even advanced start of testicular growth; and postpubertal macroorchidism (Table 1). In detailed pituitary hormone secretion analysis, men with IGSF1 deficiency exhibit loss of diurnal variation in thyrotropin (TSH) levels, a bimodal distribution of prolactin secretion (*i.e.,* severe deficiency or increased secretion), decreased luteinizing hormone pulse frequency, and increased FSH secretion [38]. Since the publication of the most comprehensive review of clinical features of IGSF1 deficiency [27], four additional case reports have been published [25, 26, 30, 35], which essentially confirm earlier findings. Novel observations included increased serum inhibin B and anti-Müllerian hormone in one patient [25] and hypercholesterolemia in another, which was resolved upon L-thyroxine treatment [35]. Finally, although memory and cognitive function appear to be normal in men with IGSF1 deficiency, whether treated with L-thyroxine or not, some individuals display or report attention deficits [39].

Table 1. Common Clinical Features in Individuals with *IGSF1* Mutations

Features	Patients Affected (%)
Males	
Central hypothyroidism	100
Prolactin deficiency	61
Small thyroid volume	74
Increased birth weight	26
Increased head circumference	20
Increased adult waist circumference	59
Childhood GH deficiency	16
Adult macroorchidism	88
Females	
Central hypothyroidism	18
Delayed menarche	31
Prolactin deficiency	22

Expression studies, particularly in rodents, have begun to provide some insight into how loss of IGSF1 may relate to the observed clinical phenotypes. For example, IGSF1 protein expression was initially reported in rat and mouse thyrotropes, lactotropes, and somatotropes, the pituitary cell lineages that make TSH, prolactin, and GH, respectively [17, 40]. As described, these are the hormones most often affected in IGSF1 deficiency. According to a more recent report, IGSF1 protein is expressed in rat thyrotropes and gonadotropes, but not lactotropes or somatotropes [25]. Importantly, the different studies used different antibodies (homemade vs commercial), which recognize different epitopes in the IGSF1-CTD (Fig. 1). Investigations with these two antibodies also indicated different patterns of IGSF1 expression in the testes within and between species [25]. Clearly, further investigation is needed to resolve the apparent inconsistencies between studies.

3. IGSF1 Regulates Pituitary Responsiveness to TRH

Central hypothyroidism derives from impairments in hypothalamic and/or pituitary function [41]. Within the hypothalamic-pituitary-thyroid (HPT) axis, thyrotropin-releasing hormone (TRH) is released from neurons in the paraventricular nucleus into the pituitary portal vasculature at the level of the median eminence. TRH travels to the anterior pituitary gland, where it binds to the TRH receptor (TRHR1 in rodents) on thyrotrope cells. TRH stimulates the secretion of TSH, which travels via systemic circulation to the thyroid gland. TSH binding to the TSH receptor stimulates synthesis of the thyroid hormones, thyroxine (T4) and, its active metabolite, triiodothyronine (T3). Thyroid hormones are secreted into systemic circulation and have pleiotropic effects throughout the body. At the level of the hypothalamus, they negatively feed back to suppress TRH synthesis and secretion. At the pituitary level, they inhibit expression of *Trhr1* and the two subunits that form TSH (*Tshb* and *Cga*). IGSF1 is expressed in the hypothalamus and pituitary, suggesting that loss-of-function mutations might affect the central control of the thyroid at both levels. However, *Igsf1* knockout mice have been instrumental in demonstrating that central hypothyroidism largely derives from a pituitary defect.

Thus far, two *Igsf1* knockout mouse models have been described. In the first, exon 1 (of the 20 exon gene; hereafter $Igsf1^{\Delta ex1}$ mice) was deleted by using conventional gene targeting in embryonic stem cells [14]. At the time these mice were produced, two *Igsf1* messenger RNA (mRNA) transcripts had been characterized in rat pituitaries [42]. One encoded the full-length (12 Ig loop) transmembrane protein described above (Fig. 1). The second was the product of alternative splicing and yielded a shorter transcript of the first five exons, plus sequence from the fifth intron. This transcript was predicted to encode a secreted protein containing the first two Ig loops of full-length IGSF1. Recent data, however, indicate that this isoform, which is also expressed in human pituitary, is retained in the ER, like the IGSF1-NTD (described above) [43]. Regardless, removal of the noncoding exon 1 eliminated detectable expression of both the full-length and truncated mRNA isoforms from pituitaries of $Igsf1^{\Delta ex1}$ mice [14]. Initial analyses of these mice revealed reduced pituitary and circulating TSH levels, as well as attenuated expression of pituitary *Trhr1* mRNA. Serum T3 and T4 levels varied between cohorts of mice, with one or the other hormone reduced in some experiments and neither reduced in others [17]. When thyroid hormone levels were reduced, the attenuation was relatively mild. Nonetheless, when the animals were treated with exogenous TRH, increases in serum TSH were significantly attenuated in $Igsf1^{\Delta ex1}$ relative to control mice [44]. Moreover, when the animals were challenged with a low-iodine diet, supplemented with propylthiouracil (LoI/PTU), which greatly reduces thyroid hormone production, the expected increases in serum TSH levels and pituitary expression of TSH subunits (*Tshb* and *Cga*) were greatly impaired in knockouts compared with controls [44]. At the level of the hypothalamus, *Trh* mRNA expression was modestly but significantly elevated in $Igsf1^{\Delta ex1}$ mice compared with controls, but both genotypes showed the appropriate suppression of *Trh* mRNA levels when challenged with exogenous T3 or T4 [44]. Collectively, these data suggested that the brains of $Igsf1^{\Delta ex1}$ mice were appropriately sensitive to thyroid hormones in

terms of *Trh* expression but that their pituitaries were impaired in their responses to TRH, perhaps because of decreases in *Trhr1* mRNA expression.

Recently, a second *Igsf1* knockout model was generated to make a strain with a mutation more akin to those described in many IGSF1-deficient patients and to remove the potential for compensation that might occur in the $Igsf1^{\Delta ex1}$ mice [44]. During the production of the latter, it was discovered that mice express at least two additional *Igsf1* mRNA isoforms in their pituitaries relative to what had been described in rats (and humans) [14, 42, 45]. One isoform, which is relatively low in abundance (isoform 3), was also eliminated by the removal of exon 1 and therefore was not considered problematic. In contrast, however, a fourth isoform (isoform 4), also of relatively low abundance, was retained in pituitaries of $Igsf1^{\Delta ex1}$ mice. This transcript, which appears to derive from an intronic promoter upstream of exon 10, was a potential concern because it encodes the entirety of the IGSF1-CTD [14, 16, 44]. Therefore, a strategy was used to disrupt expression of the IGSF1-CTD, whether it was derived from the full-length (isoform 1) or isoform 4 mRNA.

CRISPR-Cas9 was used to introduce mutations into the part of the *Igsf1*-gene encoding the 12th Ig loop. As shown in Fig. 2, at least eight mutations map to this part of human *IGSF1*. Mice carrying a 312-bp deletion (hereafter $Igsf1^{\Delta 312}$ mice) were characterized and shown to express a truncated form of IGSF1-CTD at greatly reduced levels, which failed to traffic to the plasma membrane. Most important, these mice showed phenotypes markedly similar to those of $Igsf1^{\Delta ex1}$ animals. Pituitary TSH content was reduced, as was expression of *Trhr1*, *Tshb*, and *Cga* mRNAs. In addition, their ability to secrete TSH in response to the LoI/PTU diet or TRH challenge was greatly impaired relative to that of controls [44]. Thus, at least in mice, loss of IGSF1 function clearly leads to impaired TRH action in pituitary thyrotrope cells.

It is not yet clear whether the same impairment underlies central hypothyroidism in humans with IGSF1 deficiency. Although we know that *IGSF1* mRNA is transcribed in developing and adult human pituitaries, no studies, to our knowledge, have determined whether IGSF1 protein is expressed in human thyrotropes (or other pituitary cell lineages). Nevertheless, some clinical data suggest that TRH action may in fact be impaired in humans with IGSF1 deficiency: that is, several patients challenged with exogenous TRH showed significant impairments in TSH release, particularly early in life [17, 25, 27].

4. Future Directions: Addressing the "Known Unknowns"

Though we have learned much about IGSF1, particularly over the past 5 years, we have clearly generated more questions than we have answered. In the following section, we pose some major avenues of investigation that should be pursued in the next few years.

A. What Is the Function of IGSF1 in Thyrotropes (and Other Cells)?

Loss of IGSF1 leads to impairments in *Trhr1* expression and TRH action, at least in mice. However, we still do not know the normal function(s) of IGSF1 or how the loss of the protein leads to impairments in TRH signaling. Our ignorance stems largely from the fact that IGSF1 lacks defined functional domains and its interactome (the proteins and small molecules with which it interacts) is poorly defined. Extracellular Ig loops are a common feature of many membrane proteins and are not, by themselves, predictive of particular functions or interactions, given the diverse roles that Ig loop–containing proteins play. IGSF1's intracellular domain is short and lacks defined functional motifs. It is rich in serine and threonine residues, suggesting that it may be a substrate for one or more kinases; however, it has not yet been established whether IGSF1 is a phosphoprotein.

The only published interaction partners for IGSF1 are itself and type I receptors in the TGFβ family, which are serine-threonine kinases [5]. In particular, IGSF1 was shown to interact with the activin type IB receptor (ACVR1B, also known as ALK4). Although it is tempting to speculate that the IGSF1 intracellular C-tail might be an ALK4 substrate, the two proteins interact in the absence of the ALK4 intracellular kinase domain. Regardless,

IGSF1 was originally suggested to impair activin A signaling, particularly in the presence of inhibin B, via its interaction with ALK4. A more recent report similarly showed that IGSF1 impaired activin A and a constitutively active form of ALK4 in a heterologous reporter assay [25]. However, in neither case was a clear mechanism of activin A or ALK4 antagonism articulated. For example, it is unclear whether IGSF1 impaired ligand-binding, ALK4 activity and/or receptor expression. Moreover, the effects of IGSF1 were always assessed in the context of overexpression in cell lines. Nevertheless, these data do raise the intriguing possibility that IGSF1 may somehow regulate TGFβ superfamily signaling.

Along these lines, a recent report suggested a candidate mechanism through which IGSF1 might regulate *Trhr1* expression [25]. In a heterologous cell system, overexpressed IGSF1 stimulated human *TRHR*-promoter–reporter activity. In the same cells, TGFβ1 modestly inhibited activity of the same reporter, and this effect was blocked by IGSF1 overexpression. In a rat somatolactotrope cell line (GH4C1), TGFβ1 similarly, but more robustly, inhibited endogenous *Trhr1* mRNA expression, and IGSF1 overexpression partially rescued (reversed) this inhibition. TGFβ1 signals predominantly through the type I receptor, TGFBR1 (ALK5). These data suggested that IGSF1 might inhibit TGFβ1 signaling via ALK5, akin to its proposed antagonism of activin A signaling via ALK4. Although interactions between IGSF1 and ALK5 have not been reported, to our knowledge, IGSF1 does appear to interact with several other type I receptors in the family, including activin receptor-like kinase (ALK)1, 2, 4, and 6 [5]. The interaction with ALK3 is relatively weak, and there are no published data on IGSF1-ALK7 interactions. However, an earlier report failed to show any effect of IGSF1 overexpression on TGFβ1-stimulated promoter-reporter activity in heterologous cells [5], although the cell lines (TSA vs HEK293FT) and reporters (3TP-luc vs CAGA-luc) used were different in the two studies.

Collectively, these observations suggest that IGSF1 may negatively regulate TGFβ signaling via its interaction with type I receptors in the family. If this is true and TGFβ1 is a bona fide negative regulator of *Trhr1* expression, we may be closer to establishing a mechanistic understanding of the cause of central hypothyroidism in individuals with IGSF1 deficiency. That is, under normal (wild-type) conditions, IGSF1 would function to attenuate TGFβ1 suppression of *Trhr1* expression. When IGSF1 is lost, TGFβ1 signaling would be enhanced, leading to increased suppression of *Trhr1* levels and impaired TRH signaling. This model should certainly be put to the test in the future by, for example, determining whether IGSF1 and ALK5 interact, examining the effects of loss of IGSF1 on TGFβ1 signaling in thyrotropes (thus far, all studies have involved IGSF1 overexpression), and elucidating a mechanism through which IGSF1 impairs ALK5 function. At the same time, we should not put all of our eggs in one basket and must continue to search for alternative IGSF1 functions. Newer proteomics methods should greatly facilitate the identification of novel IGSF1 interacting partners, in an unbiased manner, opening up entirely new lines of investigation.

B. How Does the Loss of IGSF1 Cause Other Common Phenotypes?

The defining characteristic of IGSF1 deficiency is central hypothyroidism (Table 1). However, hypoprolactinemia, GH dysregulation, and macroorchidism are also frequently observed in individuals with loss-of-function mutations in *IGSF1*. At present, we lack insight into the causes of these phenotypes. TRH can regulate prolactin and GH secretion [46]. It is therefore tempting to speculate that reductions in *Trhr1* expression in lactotropes and somatotropes might also explain alterations in prolactin and GH in some IGSF1-deficient individuals. Unlike the case with central hypothyroidism, it is not yet clear that *Igsf1* knockout mice will prove to be instructive in this context. Although both *Igsf1* knockout models are larger than wild-type mice, it has not yet been reported whether this is GH dependent and/or whether GH regulation is altered in these animals. Similarly, prolactin secretion has not been investigated in *Igsf1* knockout mice, although they do not appear to show any deficits in prolactin production in the pituitary gland (at the protein or mRNA level) [17]. Interestingly, TGFβ1 inhibited *Prl* mRNA expression in GH4C1 cells, as was described above for *Trhr1* expression

[25]. Therefore, enhanced TGFβ1 signaling in lactotropes could lead to reduced *Prl* expression, just as enhanced TGFβ1 signaling was proposed to inhibit *Trhr1* expression in thyrotropes in IGSF1 deficiency. However, IGSF1 overexpression did not alter TGFβ1 inhibition of *Prl* in these cells and the same researchers reporting these findings failed to detect IGSF1 protein expression in rat lactotropes and somatotropes (contrary to earlier findings) [25]. As a result, any alterations in prolactin and GH may not result from cell autonomous effects of IGSF1 loss of function. Going forward, it will be important to investigate both prolactin and GH secretion in *Igsf1* knockout models. If either hormone (or both hormones) is dysregulated, cell-specific knockout mice could be valuable in determining whether the phenotypes derive from loss of IGSF1 in thyrotropes, lactotropes, and/or somatotropes.

The mechanisms underlying macroorchidism are similarly unresolved. Two known endocrine causes of adult testicular enlargement are FSH excess and hypothyroidism during childhood or early puberty [47]. Because IGSF1 was first proposed to function as an inhibin receptor and IGSF1-deficient individuals are hypothyroid, either (or both) presents plausible explanations. In the case of FSH, however, the existing data fail to substantiate a role for IGSF1 in inhibin action. As indicated above, the protein does not bind to inhibin A or inhibin B [7]. In addition, *Igsf1* knockout mice also produce normal levels of FSH and do not exhibit macroorchidism [14, 44]. In most men with IGSF1 deficiency, FSH is not clearly elevated [27]. However, some individuals, from whom serial blood samples were collected, did show increased FSH secretion, although still within the normal range [38]. Also, in one case, where an individual with an entire *IGSF1* gene deletion was followed over many years, there was an apparent elevation in FSH levels at 14 days and 1 month after birth, which normalized by 5 months [25]. Reference ranges were not reported, however, so it is unclear to what extent FSH levels were increased. It is also unclear that this increase explained the testicular enlargement in this patient, as the events were only correlated (*i.e.,* they both occurred in the same individual, but years apart).

Although hypothyroidism could, in theory, explain testicular enlargement in IGSF1-deficient men, the extent of thyroid hormone deficiency is, in most cases, relatively mild. Moreover, in the more severe cases, where thyroid hormone replacement was implemented during neonatal development, macroorchidism still ensued. Therefore, unless these individuals have impairments in thyroid hormone transport, metabolism, or action in the testis, it is not clear that hypothyroidism explains their macroorchidism. Instead, we propose that loss of IGSF1 function in the testis, rather than endocrine effects, leads to macroorchidism. Although results vary between studies, IGSF1 mRNA and protein are expressed in the testes [1–3, 14, 17, 25, 40]. With a homemade antibody, the IGSF1 protein was detected in Sertoli cells and elongating spermatids of the adult rat testis [40]. The same antibody failed to detect IGSF1 protein in murine testes, either by immunohistochemistry or by western blotting (our unpublished data). A second report, through use of a commercial antibody, labeled IGSF1-immunoreactive protein in germ and Leydig cells of both murine and human testes [25]. In the future, it will be critical to further validate both antibodies to ensure that they crossreact specifically with IGSF1 in the testis (and other tissues). In addition, because *Igsf1* knockout mice do not exhibit macroorchidism and may not express IGSF1 protein in their testes, it may prove valuable to generate an *Igsf1*-deficient rat model for investigations into the mechanisms of testicular enlargement.

C. What Explains Phenotypic Heterogeneity in IGSF1-Deficient Humans and Mice?

Another intriguing aspect of IGSF1 deficiency is the heterogeneity in phenotypes within family members carrying the same mutation and in congenic knockout mouse strains. For example, in the Dutch index family, one 17-year-old male had isolated central hypothyroidism, whereas his 21-year-old cousin showed central hypothyroidism, prolactin deficiency, and transient growth hormone deficiency [28]. In both knockout mouse models, the mutations were created on or backcrossed onto the C57BL6 strain. Nonetheless, HPT axis function in knockout mice varies markedly both within and between litters: that is, under normal dietary and housing

conditions, some knockout mice exhibit very low levels of TSH, T3, and/or T4, whereas others secrete the hormones within the normal range and in a manner similar to that of their wild-type littermates. Because the mice are, in principle, genetically identical, the variation cannot easily be attributed to modifier genes, although this may be relevant in humans.

We propose that this variation may stem from IGSF1's role as modulatory rather than as an essential protein. As a result, some mice and humans with mutations in the *IGSF1/Igsf1* gene are able to compensate for its absence, at least under some conditions. For example, there were no apparent effects on circulating TSH, T3, or T4 in most $Igsf1^{\Delta 312}$ mice fed a normal diet. However, pituitary TSH protein content and expression of *Trhr1*, *Tshb*, and *Cga* mRNAs were reduced in these same animals [44]. Therefore, despite clear impairments in TSH synthesis by the pituitary, most of the mice were able to secrete sufficient TSH to maintain a euthyroid state. The mechanisms underlying this compensation are not yet clear and may vary between individuals or between physiological states. Nevertheless, when the HPT axis is challenged, the majority of knockout mice cannot fully compensate for the absence of IGSF1. For example, when rendered profoundly hypothyroid with a LoI/PTU diet, knockout mice were not capable of synthesizing or secreting TSH to the extent seen in wild-type animals. Specifically, they could not fully respond to the enhanced TRH drive on the pituitary. These observations suggest that IGSF1 may be most important when there is increased demand on the system, as might occur in response to different environmental stressors, including temperature, social cues, and nutrient availability. If this is true, differences in HPT axis function that we observe between individual people or mice with IGSF1 deficiency may reflect differences in acute or chronic demands on the system at the time of evaluation. Future investigations should attempt to identify extrinsic and intrinsic determinants of phenotypic variation both between and within individuals to test this idea.

In summary, studies of humans and mice with loss-of-function mutations in the *IGSF1/Igsf1* gene have demonstrated a role for IGSF1 in the central control of the thyroid, via regulation of TRH signaling in the pituitary gland. However, many questions remain to be addressed. In addition to areas of investigation outlined above, we will also need to solve other mysteries, such as the following: Are there IGSF1 orthologs in nonmammalian species? How did the IGSF1 gene evolve in mammals? Do the IGSF1-NTD and the two Ig loop isoform play functional roles in the ER or elsewhere in the cell? If not, why are their amino acid sequences so highly conserved? What is the role of IGSF1 in nonendocrine tissues, including the pancreas, choroid plexus, striated muscle, and fetal (but not adult) liver? Is IGSF1 expression in certain cancers functionally significant?

IGSF1 came onto the scene 20 years ago, yet we still know relatively little about this perplexing protein. With the discoveries made over the past few years, and a clear set of addressable questions, we are now well positioned to uncover what is hiding under the tip of a much larger iceberg.

Acknowledgments

Financial Support: This work was supported by operating grants from Canadian Institutes of Health Research (MOP-133557) and Natural Sciences and Engineering Research Council of Canada (NSERC; 2015-05178) to D.J.B. E.B. was supported by an MSc Fellowship from NSERC. C.L.S. was supported by a fellowship from Réseau Québécois en reproduction - Fonds de recherche du Québec - Nature et technologies.

Correspondence: Daniel J. Bernard, PhD, Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec H3G 1Y6, Canada. E-mail: daniel.bernard@mcgill.ca.

Disclosures: The authors have nothing to disclose.

References and Notes

1. Frattini A, Faranda S, Redolfi E, Allavena P, Vezzoni P. Identification and genomic organization of a gene coding for a new member of the cell adhesion molecule family mapping to Xq25. *Gene*. 1998;**214**(1-2):1–6.

2. Mazzarella R, Pengue G, Jones J, Jones C, Schlessinger D. Cloning and expression of an immunoglobulin superfamily gene (IGSF1) in Xq25. *Genomics*. 1998;**48**(2):157–162.
3. Chong H, Pangas SA, Bernard DJ, Wang E, Gitch J, Chen W, Draper LB, Cox ET, Woodruff TK. Structure and expression of a membrane component of the inhibin receptor system. *Endocrinology*. 2000;**141**(7):2600–2607.
4. Makanji Y, Zhu J, Mishra R, Holmquist C, Wong WP, Schwartz NB, Mayo KE, Woodruff TK. Inhibin at 90: from discovery to clinical application, a historical review. *Endocr Rev*. 2014;**35**(5):747–794.
5. Chapman SC, Woodruff TK. Modulation of activin signal transduction by inhibin B and inhibin-binding protein (INhBP). *Mol Endocrinol*. 2001;**15**(4):668–679.
6. Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM, Vale W. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature*. 2000;**404**(6776):411–414.
7. Chapman SC, Bernard DJ, Jelen J, Woodruff TK. Properties of inhibin binding to betaglycan, InhBP/p120 and the activin type II receptors. *Mol Cell Endocrinol*. 2002;**196**(1-2):79–93.
8. Makanji Y, Harrison CA, Stanton PG, Krishna R, Robertson DM. Inhibin A and B in vitro bioactivities are modified by their degree of glycosylation and their affinities to betaglycan. *Endocrinology*. 2007;**148**(5):2309–2316.
9. Makanji Y, Temple-Smith PD, Walton KL, Harrison CA, Robertson DM. Inhibin B is a more potent suppressor of rat follicle-stimulating hormone release than inhibin a in vitro and in vivo. *Endocrinology*. 2009;**150**(10):4784–4793.
10. Makanji Y, Walton KL, Wilce MC, Chan KL, Robertson DM, Harrison CA. Suppression of inhibin A biological activity by alterations in the binding site for betaglycan. *J Biol Chem*. 2008;**283**(24):16743–16751.
11. Wiater E, Harrison CA, Lewis KA, Gray PC, Vale WW. Identification of distinct inhibin and transforming growth factor beta-binding sites on betaglycan: functional separation of betaglycan co-receptor actions. *J Biol Chem*. 2006;**281**(25):17011–17022.
12. Wiater E, Lewis KA, Donaldson C, Vaughan J, Bilezikjian L, Vale W. Endogenous betaglycan is essential for high-potency inhibin antagonism in gonadotropes. *Mol Endocrinol*. 2009;**23**(7):1033–1042.
13. Farnworth PG, Harrison CA, Leembruggen P, Chan KL, Stanton PG, Ooi GT, Rahman NA, Huhtaniemi IT, Findlay JK, Robertson DM. Inhibin binding sites and proteins in pituitary, gonadal, adrenal and bone cells. *Mol Cell Endocrinol*. 2001;**180**(1-2):63–71.
14. Bernard DJ, Burns KH, Haupt B, Matzuk MM, Woodruff TK. Normal reproductive function in InhBP/p120-deficient mice. *Mol Cell Biol*. 2003;**23**(14):4882–4891.
15. Stenvers KL, Tursky ML, Harder KW, Kountouri N, Amatayakul-Chantler S, Grail D, Small C, Weinberg RA, Sizeland AM, Zhu HJ. Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Mol Cell Biol*. 2003;**23**(12):4371–4385.
16. Robakis T, Bak B, Lin SH, Bernard DJ, Scheiffele P. An internal signal sequence directs intramembrane proteolysis of a cellular immunoglobulin domain protein. *J Biol Chem*. 2008;**283**(52):36369–36376.
17. Sun Y, Bak B, Schoenmakers N, van Trotsenburg AS, Oostdijk W, Voshol P, Cambridge E, White JK, le Tissier P, Gharavy SN, Martinez-Barbera JP, Stokvis-Brantsma WH, Vulsma T, Kempers MJ, Persani L, Campi I, Bonomi M, Beck-Peccoz P, Zhu H, Davis TM, Hokken-Koelega AC, Del Blanco DG, Rangasami JJ, Ruivenkamp CA, Laros JF, Kriek M, Kant SG, Bosch CA, Biermasz NR, Appelman-Dijkstra NM, Corssmit EP, Hovens GC, Pereira AM, den Dunnen JT, Wade MG, Breuning MH, Hennekam RC, Chatterjee K, Dattani MT, Wit JM, Bernard DJ. Loss-of-function mutations in IGSF1 cause an X-linked syndrome of central hypothyroidism and testicular enlargement. *Nat Genet*. 2012;**44**(12):1375–1381.
18. Fafioffe A, Ethier JF, Fontaine J, JeanPierre E, Taragnat C, Dupont J. Activin and inhibin receptor gene expression in the ewe pituitary throughout the oestrous cycle. *J Endocrinol*. 2004;**182**(1):55–68.
19. Zhu R, Zhou X, Chen Y, Qiu C, Xu W, Shen Z. Aberrantly increased mRNA expression of betaglycan, an inhibin co-receptor in the ovarian tissues in women with polycystic ovary syndrome. *J Obstet Gynaecol Res*. 2010;**36**(1):138–146.
20. Fuller PJ, Zumpe ET, Chu S, Mamers P, Burger HG. Inhibin-activin receptor subunit gene expression in ovarian tumors. *J Clin Endocrinol Metab*. 2002;**87**(3):1395–1401.
21. Jarzab B, Wiench M, Fujarewicz K, Simek K, Jarzab M, Oczko-Wojciechowska M, Wloch J, Czarniecka A, Chmielik E, Lange D, Pawlaczek A, Szpak S, Gubala E, Swierniak A. Gene expression profile of papillary thyroid cancer: sources of variability and diagnostic implications. *Cancer Res*. 2005;**65**(4):1587–1597.

22. Patil MA, Chua MS, Pan KH, Lin R, Lih CJ, Cheung ST, Ho C, Li R, Fan ST, Cohen SN, Chen X, So S. An integrated data analysis approach to characterize genes highly expressed in hepatocellular carcinoma. *Oncogene*. 2005;**24**(23):3737–3747.
23. Younger ST, Corey DR. Transcriptional gene silencing in mammalian cells by miRNA mimics that target gene promoters. *Nucleic Acids Res*. 2011;**39**(13):5682–5691.
24. Asakura Y, Abe K, Muroya K, Hanakawa J, Oto Y, Narumi S, Hasegawa T, Adachi M. Combined growth hormone and thyroid-stimulating hormone deficiency in a japanese patient with a novel frameshift mutation in IGSF1. *Horm Res Paediatr*. 2015;**84**(5):349–354.
25. García M, Barrio R, García-Lavandeira M, Garcia-Rendueles AR, Escudero A, Díaz-Rodríguez E, Gorbenko Del Blanco D, Fernández A, de Rijke YB, Vallespín E, Nevado J, Lapunzina P, Matre V, Hinkle PM, Hokken-Koelega AC, de Miguel MP, Cameselle-Teijeiro JM, Nistal M, Alvarez CV, Moreno JC. The syndrome of central hypothyroidism and macroorchidism: IGSF1 controls TRHR and FSHB expression by differential modulation of pituitary TGFβ and Activin pathways. *Sci Rep*. 2017;**7**:42937.
26. Hughes JN, Aubert M, Heatlie J, Gardner A, Gecz J, Morgan T, Belsky J, Thomas PQ. Identification of an IGSF1-specific deletion in a five-generation pedigree with X-linked Central Hypothyroidism without macroorchidism. *Clin Endocrinol (Oxf)*. 2016;**85**(4):609–615.
27. Joustra SD, Heinen CA, Schoenmakers N, Bonomi M, Ballieux BE, Turgeon MO, Bernard DJ, Fliers E, van Trotsenburg AS, Losekoot M, Persani L, Wit JM, Biermasz NR, Pereira AM, Oostdijk W; IGSF1 Clinical Care Group. IGSF1 Deficiency: Lessons from an extensive case series and recommendations for clinical management. *J Clin Endocrinol Metab*. 2016;**101**(4):1627–1636.
28. Joustra SD, Schoenmakers N, Persani L, Campi I, Bonomi M, Radetti G, Beck-Peccoz P, Zhu H, Davis TM, Sun Y, Corssmit EP, Appelman-Dijkstra NM, Heinen CA, Pereira AM, Varewijck AJ, Janssen JA, Endert E, Hennekam RC, Lombardi MP, Mannens MM, Bak B, Bernard DJ, Breuning MH, Chatterjee K, Dattani MT, Oostdijk W, Biermasz NR, Wit JM, van Trotsenburg AS. The IGSF1 deficiency syndrome: characteristics of male and female patients. *J Clin Endocrinol Metab*. 2013;**98**(12):4942–4952.
29. Nakamura A, Bak B, Silander TL, Lam J, Hotsubo T, Yorifuji T, Ishizu K, Bernard DJ, Tajima T. Three novel IGSF1 mutations in four Japanese patients with X-linked congenital central hypothyroidism. *J Clin Endocrinol Metab*. 2013;**98**(10):E1682–E1691.
30. Nishigaki S, Hamazaki T, Fujita K, Morikawa S, Tajima T, Shintaku H. A Japanese family with central hypothyroidism caused by a novel IGSF1 mutation. *Thyroid*. 2016;**26**(12):1701–1705.
31. Reynaert N, Braat E, de Zegher F. Congenital nystagmus and central hypothyroidism. *Int J Pediatr Endocrinol* 2015;**2015**:7.
32. Tajima T, Nakamura A, Ishizu K. A novel mutation of IGSF1 in a Japanese patient of congenital central hypothyroidism without macroorchidism. *Endocr J*. 2013;**60**(2):245–249.
33. Tenenbaum-Rakover Y, Turgeon MO, London S, Hermanns P, Pohlenz J, Bernard DJ, Bercovich D. Familial central hypothyroidism caused by a novel IGSF1 gene mutation. *Thyroid*. 2016;**26**(12): 1693–1700.
34. Van Hulle S, Craen M, Callewaert B, Joustra S, Oostdijk W, Losekoot M, Wit JM, Turgeon MO, Bernard DJ, De Schepper J. Delayed adrenarche may be an additional feature of immunoglobulin super family member 1 deficiency syndrome. *J Clin Res Pediatr Endocrinol*. 2016;**8**(1):86–91.
35. Heinen CA, Zwaveling-Soonawala N, Fliers E, Turgeon M-O, Bernard DJ, van Trotsenburg ASP. A novel IGSF1 mutation in a boy with short stature and hypercholesterolemia: a case report. *J Endocr Soc*. 2017;**1**(6):731–736.
36. Joustra SD, van Trotsenburg AS, Sun Y, Losekoot M, Bernard DJ, Biermasz NR, Oostdijk W, Wit JM. IGSF1 deficiency syndrome: a newly uncovered endocrinopathy. *Rare Dis*. 2013;**1**(1):e24883.
37. Persani L, Bonomi M. The multiple genetic causes of central hypothyroidism. *Best Pract Res Clin Endocrinol Metab*. 2017;**31**(2):255–263.
38. Joustra SD, Roelfsema F, Endert E, Ballieux BE, van Trotsenburg AS, Fliers E, Corssmit EP, Bernard DJ, Oostdijk W, Wit JM, Pereira AM, Biermasz NR. Pituitary hormone secretion profiles in IGSF1 deficiency syndrome. *Neuroendocrinology*. 2016;**103**(3-4):408–416.
39. Joustra SD, Andela CD, Oostdijk W, van Trotsenburg AS, Fliers E, Wit JM, Pereira AM, Middelkoop HA, Biermasz NR. Mild deficits in attentional control in patients with the IGSF1 deficiency syndrome. *Clin Endocrinol (Oxf)*. 2016;**84**(6):896–903.
40. Joustra SD, Meijer OC, Heinen CA, Mol IM, Laghmani H, Sengers RM, Carreno G, van Trotsenburg AS, Biermasz NR, Bernard DJ, Wit JM, Oostdijk W, van Pelt AM, Hamer G, Wagenaar GT. Spatial and temporal expression of immunoglobulin superfamily member 1 in the rat. *J Endocrinol*. 2015;**226**(3): 181–191.
41. Beck-Peccoz P, Rodari G, Giavoli C, Lania A. Central hypothyroidism - a neglected thyroid disorder. *Nat Rev Endocrinol*. 2017;**13**(10):588–598.

42. Bernard DJ, Woodruff TK. Inhibin binding protein in rats: alternative transcripts and regulation in the pituitary across the estrous cycle. *Mol Endocrinol*. 2001;**15**(4):654–667.
43. Wang Y, Brûlé E, Silander T, Bak B, Joustra SD, Bernard DJ. The short mRNA isoform of the immunoglobulin superfamily, member 1 gene encodes an intracellular glycoprotein. *PLoS One*. 2017; **12**(7):e0180731.
44. Turgeon MO, Silander TL, Doycheva D, Liao XH, Rigden M, Ongaro L, Zhou X, Joustra SD, Wit JM, Wade MG, Heuer H, Refetoff S, Bernard DJ. trh action is impaired in pituitaries of male IGSF1-deficient mice. *Endocrinology*. 2017;**158**(4):815–830.
45. Tanaka S, Tatsumi K, Okubo K, Itoh K, Kawamoto S, Matsubara K, Amino N. Expression profile of active genes in the human pituitary gland. *J Mol Endocrinol*. 2002;**28**(1):33–44.
46. Kanasaki H, Oride A, Mijiddorj T, Kyo S. Role of thyrotropin-releasing hormone in prolactin-producing cell models. *Neuropeptides*. 2015;**54**:73–77.
47. De Sanctis V, Marsella M, Soliman A, Yassin M. Macroorchidism in childhood and adolescence: an update. *Pediatr Endocrinol Rev*. 2014;**11**(Suppl 2):263–273.