The hypothalamic-pituitary-thyroid axis is intact in male insulin receptor substrate 4 knockout mice

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Short title: Intact HPT axis in Irs4 knockout mice

Keywords: Pituitary, thyroid, hormone, hypothyroidism, mutation

Word count: 3250

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Abstract

Objective: Loss of function mutations in the insulin receptor substrate 4 (IRS4) gene cause a rare form of X-linked congenital central hypothyroidism in boys and men. Affected individuals show decreased thyroid-stimulation hormone (TSH) secretion. Members of the IRS family canonically act as scaffold proteins between tyrosine kinase receptors and downstream effectors. How loss of IRS4 affects TSH synthesis or secretion is unresolved. We therefore assessed IRS4’s role in the hypothalamic-pituitary-thyroid axis of Irs4 knockout mice. Methods: We generated two global Irs4 knockout mouse lines harboring either two or four base-pair deletions that result in frameshifts and loss of most of the IRS4 protein. Results: Under normal laboratory conditions, Irs4 knockout males did not exhibit impairments in pituitary expression of TSH subunit genes (Tshb or Cga) or in the thyrotropin-releasing hormone (TRH) receptor. Additionally, their serum thyroid hormone, T₃ (triiodothyronine) and T₄ (thyroxine), and hypothalamic Trh expression levels were normal. When Irs4 knockouts were rendered hypothyroid with a low-iodine diet supplemented with propylthiouracil (PTU) for 3 weeks, their serum TSH increased similarly to wild-type males. Conclusions: Overall, Irs4 knockout mice do not exhibit central hypothyroidism or otherwise appear to phenocopy IRS4 deficient patients. Compensation by another IRS protein may explain euthyroidism in these animals.
Introduction

Congenital central hypothyroidism (CCH) may affect as many as 1 in 13,000 people(1). In CCH, thyroid hormone levels are low to low-normal without the expected increases in thyrotropin (TSH), implicating defects in the brain and/or pituitary gland. TSH deficiency can occur in isolation or in the context of combined pituitary hormone deficiency(1, 2). Isolated CCH has thus far been associated with mutations in five genes in humans: TSHB, TRHR, IGSF1, TBL1X, and IRS4(2-8). TSHB encodes the beta subunit of TSH, TSHβ, and loss of function mutations in the gene prevent production of the dimeric hormone, leading to profound hypothyroidism(8, 9). Mutations in the other four genes generally lead to milder forms of the disease.

TSH secretion is regulated by thyrotropin-releasing hormone (TRH) from the paraventricular nucleus (PVN) of the hypothalamus, which acts on the TRH receptor (TRHR) on pituitary thyrotropes. Inactivating TRHR/Trhr mutations are associated with reduced TSH levels and/or bioactivity in humans and mice(7, 10, 11). Importantly, however, the hormone is still produced. This likely explains why hypothyroidism is milder in TRHR than in TSHB deficiency. These observations also demonstrate that TRH is not absolutely required for TSH synthesis or secretion. Indeed, Trh deficient mice show phenotypes similar to those of Trhr knockouts(12).

Mutations in the immunoglobulin superfamily member 1 gene (IGSF1) are the most common genetic cause of CCH(1, 5, 13, 14). IGSF1 is X-linked and most affected individuals are males. This is also the case with TBL1X and IRS4 mutations. In addition to CCH, IGSF1 deficiency is associated with prolactin deficiency, growth hormone dysregulation, and macroorchidism(5, 15). IGSF1 encodes a transmembrane protein of unknown function that is enriched in pituitary thyro trope, lactotrope, and somatotrope cells(5, 16, 17). TRH-stimulated TSH release is impaired
in IGSF1 deficient humans and mice. The latter also exhibit reductions in pituitary \textit{Tshb} and \textit{Trhr} expression\cite{5, 18, 19}, though how IGSF1 loss produces these effects has not yet been determined.

Transducin \textit{β-like 1X} (TBL1X) is a part of the thyroid hormone receptor/co-repressor complex\cite{20, 21}. \textit{TBL1X} mutations are associated with low thyroid hormone levels and hearing loss\cite{6, 22}. How \textit{TBL1X} mutations cause CCH has not yet been established; however, the TBL1X protein has been implicated in transcriptional regulation of \textit{TRH} and \textit{TSHB} in vitro\cite{23}.

Mutations in insulin receptor substrate 4 (\textit{IRS4}) were recently implicated in CCH \cite{3, 4} (Fig. 1A). Like IGSF1, IRS4 is highly expressed in the pituitary and hypothalamus\cite{3, 24, 25}. \textit{Irs4} expression is enriched in thyrotropes in murine pituitary \cite{26} and in select cell types of the murine PVN, including those producing TRH\cite{27}. IRS proteins, including IRS4, are best known for their roles in tyrosine kinase receptor signaling, particularly by insulin and insulin-like growth factor 1 (IGF1) receptors. However, it is unclear whether central hypothyroidism in these patients derives from impairments in insulin and/or IGF1 action. Affected individuals do not show alterations in insulin sensitivity or notable differences in height or weight compared to population-matched controls. As many of the effects of growth hormone are mediated via IGF1, these latter observations suggest that IGF1 signaling is intact. Insulin and IGF1 actions also appear to be relatively normal in \textit{Irs4} knockout mice\cite{25, 28}.

Hypothalamic-pituitary-thyroid (HPT) axis function was assessed in one \textit{Irs4} knockout mouse strain and no clear impairments in thyroid hormone production were observed\cite{3}. Female, but not male, knockouts had reduced pituitary \textit{Tshb} mRNA expression relative to wild-type animals, though their serum TSH was unaffected. Pituitary \textit{Trhr} expression was not assessed in these animals and sample sizes were small. Moreover, the animals’ responses to diet-induced hypothyroidism (e.g., chow low in iodine and supplemented with propylthiouracil) were not
examined. From our experience with Igsf1 knockout mice, this challenge may be needed to reveal robust impairments in TSH synthesis and secretion(18, 19). We therefore generated and characterized novel Irs4 knockout mouse strains to determine if or how IRS4 regulates TSH in vivo.

**Materials and Methods**

*Development of Irs4-deficient mice*

Mice were generated using CRISPR-Cas9 technology. A single guide RNA (gRNA) was designed to target the 5’ end of the single exon Irs4 gene (see Table 1). The gRNA (Integrated DNA Technologies Inc., Coralville, IA, USA) and Cas9 protein were injected into the cytoplasm of 0.5 dpc mouse zygotes (Strain 475, Charles River Laboratories, Senneville, QC, Canada) by the McGill Integrated Core for Animal Modelling at the Goodman Cancer Institute of McGill University. The injected zygotes were cultured overnight in EmbryoMax Advanced KSOM Embryo Medium (MR-101-D, MilliporeSigma) droplets under mineral oil in a 35-mm dish at 37°C in a 5% CO₂ incubator. Embryos were transferred to the oviducts of pseudopregnant CD1-Elite females (Strain 482, Charles River Laboratories). Live pups born from these females were genotyped by PCR amplification of genomic DNA extracted from tail biopsies followed by TA cloning (pGEM-T Easy, Promega Madison, WI) and sequencing (Génome Québec). A founder female had 2-base pair (bp) and 4-bp deletions in her two Irs4 alleles. This female was bred with a wild-type C57BL/6 male (Stain 027, Charles River Laboratories). Both deletions were germline transmissible, and we propagated them separately by backcrossing to C57BL/6 mice. We established lines with the 2 bp (Irs4<em1Djb> MGI:7413888; hereafter Irs4Δ2) and 4 bp
(Irs4<em2Djb> MGI:7413889; hereafter Irs4Δ) deletions. Females heterozygous for the
mutations (Irs4Δ2/+ or Irs4Δ4+) were crossed with wild-type males to generate control (Irs4<sup>+</sup>/y) and
hemizygous knockout males (Irs4Δ2/y or Irs4Δ4/y) for analysis. Mice were genotyped by high
resolution melt (HRM). Briefly, 50 ng of gDNA was used for HRM using EvaGreen (ABMMmix,
Diamed, Missisauga, ON, Canada) and primers listed in Table 1 on a Corbett Rotorgene 600
instrument (Corbett Life Science, Sydney, NSW, Australia). The reaction began with a hold step
at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing and extension
at 60°C for 10 sec. The HRM consisted of a 90 sec pre-melt, followed by a ramp from 70 to 95 ºC
in 0.1°C steps and held for 2 sec at each temperature. Analysis was performed using the Rotorgene
6000 software.

Animal housing

All mice were housed on a 12-hour lights on/12-hour lights off cycle and were given ad libitum
access to food and water. All animals were anesthetized with isoflurane and euthanized by
CO₂ asphyxiation. All animal work was conducted in accordance with federal and institutional
guidelines and with the approval of the Facility Animal Care Committee (Downtown Campus A)
at McGill University (protocol no. 5204).

Hypothyroid diet

To render mice hypothyroid, 8-week-old males were fed a low iodine (LoI) diet supplemented
with 0.15% propylthiouracil (PTU; TD.95125, Envigo) ad libitum for 3 weeks.

Blood collection and hormone assays
Blood was collected by submandibular venipuncture and coagulated at room temperature for 30 min, centrifuged for 10 min at 800xg, and serum collected. Serum TSH was measured with a MILLIPLEX assay (MPTMAG-49K, Millipore, detection range: 12.2 to 50,000 pg/mL) following manufacturer’s instructions. Serum T\textsubscript{3} and T\textsubscript{4} were measured with in-house radioimmunoassays(29). All samples from one experiment were measured within the same assay (intra-assay variations for T\textsubscript{3} and T\textsubscript{4} were 3.6% and 6.6%, respectively.

**Organ collection, RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)**

Pituitary glands and hypothalami were dissected from 8-week-old males on normal chow and from 11-week-old males following the LoI/PTU diet. Tissues were snap-frozen in liquid nitrogen and stored at −80°C. RNA was extracted from tissues using TRIzol Reagent following manufacturer’s protocol. Two-hundred ng total RNA was reverse transcribed as described previously described(30). The resulting cDNA was analyzed by qPCR using primers in Table 1. mRNA levels were determined using the 2\(^{-ΔΔCT}\) method, and gene expression normalized to ribosomal protein L19 (Rpl19).

**Expression plasmids**

Human myc-IRS4 expression plasmid (provided by Dr. Mizuno) (31) was used to produce murine myc-IRS4 expression vectors. Briefly, murine Irs4 open-reading frames were PCR amplified from genomic DNA of wild-type or Irs4\textsuperscript{Δ2/y} mice with the following nested primers: Outer - Fw: 5’-AAAACCACGGTGCATCACCATG-3’ and Rv 5-TCTCATTTCGGAGAGTGTTGTC-3’; and inner Fw-5’-GCTCAAGCTTCGGCGAGTTGCTCCTTCTCTGG-3’ and Rv: 5-GCCATCTAGATCACCCTGCTAGTCTCGTC-3’. Amplicons and the human IRS4-myc
plasmid were digested with \textit{XbaI} (NEB, R0145S) and \textit{HindIII}-HF (NEB, R3104S). After gel-purification from 1% agarose gels, the PCR amplicons (inserts) and vector backbone were ligated using T4-DNA ligase (NEB, M0202S). Plasmids were propagated in competent \textit{E. coli} and sequenced at GenomeQuebec (Montreal, QC).

\textit{Immunoblotting}

HEK293 cells (gift from Dr. Terry Hébert, McGill University) were seeded in a 6-well plate (300,000 cells/well) and cultured at 37°C/5% CO\textsubscript{2} in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum. The following day, cells were transfected with 2 µg per well of either pcDNA3.0, wild-type murine myc-IRS4, murine myc-IRS4\textsubscript{Δ2}, or human myc-IRS4 expression vectors using polyethylenimine. The next day, cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors (cOmplete™ Mini, EDTA-free Protease Inhibitor Cocktail, Roche Holding AG, 04693116001, Basel, Switzerland). Protein concentrations were measured using the Pierce BCA protein assay kit (23227, Thermo Fisher Scientific, Waltham, MA, USA).

Cell lysates were heated in Laemmli buffer (250 mM Tris pH 6.8, 10% SDS, 50% glycerol, 0.2% bromophenol blue, and 10% β-mercaptoethanol) at 70°C for 10 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% gels prepared using a 30% acrylamide/bis-acrylamide (29:1) solution in running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). Proteins were transferred to Protran nitrocellulose membranes (GE 10600001, MilliporeSigma) in Towbin buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol), blocked with 5% skim milk (w/v) in Tris-buffered saline [TBS; 150 mM NaCl, 10 mM Tris (pH 8.0)] containing 0.05% (v/v) Tween 20 (TBST) and incubated overnight at 4°C with agitation in anti-mouse myc antibody (1:1,000; M5546; RRID:AB_260581, Sigma-Aldrich) or anti-mouse tubulin
(1:1,000; ab7291; RRID:AB_2241126, Abcam) diluted in blocking buffer. The following day, membranes were washed in TBST and incubated in horseradish peroxidase-conjugated anti-mouse IgG (1:5,000 goat anti-mouse, 1706516; RRID:AB_11125547, Bio-Rad Laboratories, Hercules, CA, USA) in blocking buffer for 1 h at room temperature with agitation. Membranes were then washed in TBST, incubated in enhanced chemiluminescence substrate (NEL105001, PerkinElmer, Waltham, MA, USA), and bands visualized with an Amersham Imager 600 (GE Healthcare, Chicago, IL, USA).

**RNA-sequencing analysis**

We obtained murine and human pituitary single nucleus RNA sequencing datasets from the GEO data repository (GSE151961 and GSE178452, respectively; male data only). We analyzed adult male human hypothalamus single cell RNA sequencing data by integrating all datasets corresponding to hypothalamic dissections from the Human Brain Cell Atlas v1.0 hosted on CELLxGENE(32). Collection details were previously published(26, 32, 33). We applied standard preprocessing methods (cell quality filtering, doublet filtering, dimensionality reduction, and scaling) and subsequently integrated and analyzed the datasets using Scanpy 1.9.1(34, 35). For murine and human pituitary single nucleus RNA sequencing data, cell types were assigned as previously published, and Leiden clustering was performed using a resolution of 0.6 and 1.4, respectively(26, 33). Leiden clustering was performed using a resolution of 1.0 for the human hypothalamus single cell RNA sequencing data, and cell type was assigned by grouping cells by ‘cell_type’. Finally, we accessed the preprocessed, clustered, and annotated murine PVN single cell RNA sequencing atlas and restricted our analysis to male samples(36).
Statistical analysis

Pituitary and hypothalamic gene expression, body weight, and thyroid hormone concentrations were analyzed by two-tailed unpaired t tests with Welch’s correction. Serum TSH data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparisons test (MCT). Statistical analyses were performed using Prism 9, GraphPad software. P < 0.05 was considered statistically significant.

Results

Generation of Irs4 knockout mice

We generated Irs4 knockout mice using CRISPR-Cas9 and a guide RNA directed at the 5’ end of the coding sequence, in the part of the gene encoding the pleckstrin homology domain (Fig. 1A). We developed two strains from a single founder female with either a 2-bp or -4 bp deletion in her two Irs4 alleles. The mutations introduced frameshifts predicted to eliminate the majority of the IRS4 protein (Fig. 1B). In the Irs4Δ2 allele, base pairs 524-525 were deleted, leading to the loss of the IRS4 protein after Gln174 of the 1216 amino acid protein (96th amino acid of the pleckstrin homology domain, PHD; Fig. 1A-B). In the Irs4Δ4 allele, base pairs 525-528 were deleted, resulting in loss of the IRS4 protein after Asp175 (97th amino acid of the PHD). Using RT-qPCR with primers in this exon, but downstream of the deletions, we observed >40% reductions in Irs4 mRNA levels in pituitaries of the knockout strains (Fig. 1C and S1A). Thus, the locus was transcriptionally active in these mice, but the resulting mRNA levels were reduced. We were not able to measure the endogenous IRS4 protein in wild-type or mutant pituitaries (data not shown). However, we cloned the wild-type and mutant (Δ2) Irs4 open-reading frames into
mammalian expression vectors. When expressed in HEK293 cells, the mutant IRS4 protein was notably truncated and less abundant (lane 3, blue arrow) than wild-type murine (lane 2, black arrow) or human (lane 4) IRS4 (Fig. 1D).

**HPT axis function in euthyroid knockout mice**

In the 2-bp deletion model \( (Irs4^{Δ2/y}) \), serum triiodothyronine \( (T_3) \) and thyroxine \( (T_4) \) levels did not differ between knockout and wild-type male littermates (Fig. 2A-B). Note that we focused our analyses exclusively on males because \( Irs4 \) is X-linked, and no IRS4-deficient female patients have thus far been described. There were also no genotype differences in pituitary TSH subunit (\( Tshb \) and \( Cga \)), \( Trhr \), or growth hormone (\( Gh \)) expression (Fig. 2C-F) or in hypothalamic \( Trh \) expression (Fig. 2G). The mutation did not impact body weight (Fig. 2H).

**HPT axis function in hypothyroid knockout mice**

We next challenged adult \( Irs4^{Δ2/y} \) and wild-type males by placing them on a diet low in iodine and supplemented with propylthiouracil (LoI-PTU) for 3 weeks. Though not measured here, we previously reported (19) that this treatment renders \( T_3 \) and \( T_4 \) undetectable, leading to significant increases in TSH due to the loss of thyroid hormone negative feedback. We observed robust increases in serum TSH in both wild-type and \( Irs4^{Δ2/y} \) mice on the diet (Fig. 3A). However, TSH levels did not differ between genotypes (Fig. 3A). After three weeks on the LoI-PTU diet, there were also no genotype differences in pituitary \( Tshb \), \( Cga \), or \( Trhr \) mRNA levels (Fig. 3B-D). \( Irs4 \), but not \( Igf1 \) mRNA levels were lower in knockout mice (Fig. 3E-F). Hypothalamic \( Trh \) mRNA levels did not differ between wild-type and knockout mice (Fig. 3G).
We also challenged adult males in the second knockout strain, \( Irs4\Delta^{4/y} \), with the LoI-PTU diet and again did not observe differences between genotypes in pituitary gene expression (Fig. S1B-E), except for \( Irs4 \), which was reduced in \( Irs4\Delta^{4/y} \) mice (Fig. S1A). We did not observe changes in pituitary \( Irs1 \) or \( Irs2 \) mRNA levels (Fig. S1F-G). \( Irs3 \) was not reliably detectable (data not shown and Fig. 1C).

\( Irs4/IRS4 \) expression in pituitary and hypothalamus

Analyses of single nucleus RNA-seq data sets from male mice (Fig. 4A) and humans (Fig. 4B) revealed expression of \( IRS4/Irs4 \) in thyrotropes of both species(26, 33). In mice, \( Irs4 \) was more abundant than \( Irs2 \) in this cell type. The opposite was the case in humans. Analyses of single cell RNA-seq data of murine PVN revealed expression of both \( Irs4 \) and, to a lesser extent, \( Irs2 \) in \( Trh/Nfib \) expressing neurons (Fig. 4C). We were not able to find a comparable data set in humans (i.e., PVN specific). However, an analysis of human hypothalamic single cell RNA-seq revealed relatively high abundance of \( IRS2 \) in many cell types, with \( IRS4 \) at low to undetectable levels (Fig. 4D).

Discussion

Loss of \( IRS4 \) function in male mice does not recapitulate the central hypothyroidism observed in human males with \( IRS4 \) mutations. Our results are consistent with those of a previous report that similarly showed euthyroidism in another \( Irs4 \) knockout strain(3). We extended the analysis here to include mice challenged with a hypothyroid diet, as this can reveal phenotypes not apparent in mice under standard laboratory conditions(18, 19). Here, too, however, TSH synthesis and secretion were intact. In patients with \( IRS4 \) mutations, TSH release in response to exogenous TRH
challenge is often impaired(3, 4). We did not perform a similar challenge in our mice, though we doubt TRH-stimulated TSH release would have been negatively impacted, given the apparently normal TSH production and Trhr expression in these animals.

The discrepancy between humans and mice might reflect a difference in IRS4 function in the HPT axes of the two species. Alternatively, there may be functional redundancy in mice that does not occur in humans. For example, there is precedent for IRS2 and IRS4 playing redundant roles in murine neurons(25). Though, it is notable that IRS2 appears to be more abundant in human than murine thyrotropes, at least at the RNA level (Fig. 4A-B). Our analysis does not rule out species differences in IRS4 function in the brain. We did not, however, observe differences in hypothalamic Trh expression between genotypes in Irs4 knockout mice. While it is possible that loss of IRS4 function impairs TRH expression or secretion in humans, our analysis of publicly available RNA-seq data suggests that IRS4 expression is low in adult human hypothalamus. Moreover, the reduced TRH stimulated TSH secretion in IRS4 deficient patients indicates a pituitary defect in these individuals.

In addition to not treating Irs4 knockout mice with exogenous TRH, there are other shortcomings of our study. For example, we did not measure pulsatile TSH. It should be noted, however, that these are technically challenging experiments in mice and not fully justified considering the results we obtained under both euthyroid and hypothyroid conditions. We also did not challenge our mice with cold stress or with high fat diet. In the latter case, it is notable that IRS4 has been implicated in leptin signaling(37). Therefore, we cannot rule out phenotypes that might manifest under different conditions. Finally, and perhaps most significantly, we were not able to confirm the loss of the IRS4 protein in our models. We obtained two IRS4 antibodies [gift from Dr. Gustav Lienhard (38)]. While one detected the protein when over-expressed in
heterologous cells by western blot, neither worked in our hands with protein lysates from murine pituitary gland (data not shown). Nevertheless, in both strains, the 2 and 4 bp deletions generate frameshifts in the single exon *Irs4* gene, precluding production of most of the full-length protein. This was confirmed for the 2 bp deletion mutant when expressed in HEK293 cells. This analysis and the mRNA expression data in mice further suggest that any residual protein would be greatly reduced in abundance, likely as a result of nonsense mediated decay.

In summary, hemizygous mutations in *IRS4* cause central hypothyroidism in boys and men through presently uncharacterized mechanisms. Both our and previous *Irs4* mutant mouse strains appear to be euthyroid, suggesting species differences in the role of IRS4 in the central control of the thyroid. Concomitant disruption of additional IRS proteins, in pituitary thyrotropes and/or different hypothalamic nuclei, may be required to uncover IRS4’s role, if any, in the HPT axis in mice.

**Declaration of interests**

The authors have no conflicts of interest. Anita Boelen is on the editorial board of European Thyroid Journal. Anita was not involved in the review or editorial process for this paper, on which she is listed as an author.

**Funding**

This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR) to DJB.

**Author contributions**
EB and DJB conceived the study and wrote the paper. EB performed most experiments and analyzed the data. XZ contributed to the mouse work. YW performed the TSH assays. ERSB performed the bioinformatics analyses and generated corresponding figures. LO and ML analyzed hypothalamic *Trh* expression. LO cloned mutant and wild-type IRS4 expression vectors and performed the associated immunoblot experiment. AB performed the thyroid hormone assays. All authors reviewed the manuscript and approved submission.
References


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Figure legends

Figure 1: IRS4/Irs4 mutations in humans and mice. A) Schematic representation of IRS4 protein domain structure. Mutations identified in human IRS4 are shown, as is the location of the CRISPR-Cas9 introduced mutations in mice. PH, pleckstrin homology domain; PTB, phosphotyrosine binding domain; PI3K, PI3K binding motifs; Grb2, Grb2 binding site. B) CRISPR-Cas9 introduced 2 and 4 bp deletions in Irs4 in the mouse models analyzed here. WT, wild type; deleted bp in the Δ2 and Δ4 strains. The PAM sequence is marked in green. Amino acids are numbered. C) Pituitary Irs4 mRNA expression in euthyroid adult male WT and Irs4Δ2/y mice as determined by RT-qPCR. ****, p < 0.0001. D) Immunoblot (IB) of myc-tagged murine WT or Δ2 IRS4 and human IRS4. Black arrow indicates the location of the WT proteins. Blue arrow indicates the location of the Δ2 IRS4 protein. Tubulin was used as a loading control.

Figure 2: Irs4 knockout mice are euthyroid. Comparison of serum hormone levels and pituitary gene expression levels in adult male wild-type (Irs4+/y) and Irs4 knockout mice (Irs4Δ2/y). A) Serum T₃ and B) T₄ levels. Pituitary C) Tshb, D) Cga, E) Trhr, and F) Gh mRNA levels as measured by RT-qPCR. G) Hypothalamic Trh mRNA levels in a subset of the animals (hypothalami were not available for all animals in this experiment). H) Body weights. In all panels, bar heights show group averages. Individual data points are shown.

Figure 3: TSH production is normal in hypothyroid Irs4 knockout mice. Comparison of serum TSH levels and pituitary gene expression in adult male wild-type (Irs4+/y) and Irs4 knockout mice (Irs4Δ2/y) on a low iodine-PTU diet for three weeks. A) Serum TSH before (pre) and 3 weeks after (post) mice were transferred from normal chow to LoI-PTU diet. Note that in 5 animals per
genotype, TSH levels were below the limit of quantification (LOQ). These animals were assigned values of 153 pg/ml (the LOQ) for graphing and statistical purposes. ****, $p < 0.0001$, main effect of diet. Pituitary B) Tshb, C) Cga, D) Trhr, E) Irs4, and F) Igsf1 mRNA levels as measured by RT-qPCR. ****, $p < 0.0001$. G) Hypothalamic Trh mRNA levels in a subset of the animals (hypothalami were not available for all animals in this experiment). Bar heights show group averages. Individual data points are shown.

**Figure 4:** Expression of IRS/Irs subtypes in murine and human pituitary and brain. Dot plots showing expression of IRS subtypes in pituitary cell types in adult male A) mice and B) humans. Data were derived from snRNA-seq datasets(26, 33). Dot plots showing expression of IRS subtypes in C) PVN cell types in male mice and D) hypothalamic cell types in humans. Data were derived from snRNA-seq datasets(32, 36).
## Table 1: Oligonucleotides and primers

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gDNA, genomic DNA; HRM, high resolution melt;
A

CRISPR deletions

- p.(G215*)
- p.(R530Sfs*18)
- p.(G572Wfs*32)
- p.(K592Qfs*12)
- p.(C1054Yfs*12)

N  PH  PTB  PI3K  Grb2  C

B

WT  ...ACC|CAG|GAC|GAA|TAC|TTC...
Δ2  ...ACC|CAG|G--|GAA|TAC|TTC...
Δ4  ...ACC|CAG|GA-|--|--|TAC|TTC...

Thr  Gln  Asp  Glu  Tyr  Phe
173  178

C

**Figure 1**

D

IB: myc

IB: tubulin

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Figure 2
Figure 3

A) Serum TSH (ng/mL) in WT and Irs4Δ2/y mice before and after treatment.

B) Relative Tshb expression in WT and Irs4Δ2/y mice.

C) Relative Cga expression in WT and Irs4Δ2/y mice.

D) Relative Trhr expression in WT and Irs4Δ2/y mice.

E) Relative Irs4 expression in WT and Irs4Δ2/y mice.

F) Relative Igf1 expression in WT and Irs4Δ2/y mice.

G) Relative Trh expression in WT and Irs4Δ2/y mice.
Figure 4
Supplementary Figure S1: *Irs4* knockout mice are euthyroid. Comparison of pituitary gene expression in adult male wild-type (*Irs4*+/y) and *Irs4* knockout mice (*Irs4*Δ4/y). A) Pituitary *Irs4*, B) *Tshb*, C) *Cga*, D) *Trhr*, E) *Igsf1*, F) *Irs1*, and G) *Irs2* mRNA levels as measured by RT-qPCR. ****, \( p < 0.0001 \). In all panels, bar heights show group averages. Individual data points are shown.