A splice site variant in *MADD* affects hormone expression in pancreatic β cells and pituitary gonadotropes

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**Graphical abstract**
A splice site variant in MADD affects hormone expression in pancreatic β cells and pituitary gonadotropes

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MAPK activating death domain (MADD) is a multifunctional protein regulating small GTPases RAB3 and RAB27, MAPK signaling, and cell survival. Polymorphisms in the MADD locus are associated with glycemic traits, but patients with biallelic variants in MADD manifest a complex syndrome affecting nervous, endocrine, exocrine, and hematological systems. We identified a homozygous splice site variant in MADD in 2 siblings with developmental delay, diabetes, congenital hypogonadotropic hypogonadism, and growth hormone deficiency. This variant led to skipping of exon 30 and in-frame deletion of 36 amino acids. To elucidate how this mutation causes pleiotropic endocrine phenotypes, we generated relevant cellular models with deletion of MADD exon 30 (dex30). We observed reduced numbers of β cells, decreased insulin content, and increased proinsulin-to-insulin ratio in dex30 human embryonic stem cell–derived pancreatic islets. Concordantly, dex30 led to decreased insulin expression in human β cell line EndoC-βH1. Furthermore, dex30 resulted in decreased luteinizing hormone expression in mouse pituitary gonadotrope cell line LβT2 but did not affect ontogeny of stem cell–derived GnRH neurons. Protein-protein interactions of wild-type and dex30 MADD revealed changes affecting multiple signaling pathways, while the GDP/GTP exchange activity of dex30 MADD remained intact. Our results suggest MADD-specific processes regulate hormone expression in pancreatic β cells and pituitary gonadotropes.

Introduction

MAPK activating death domain (MADD) is a ubiquitously expressed protein (1) regulating activation of secretory small GTPases RAB3 and RAB27 (2–4). MADD has been implicated in exocytosis (4–10) and linking endocytic and secretory pathways (11). MADD regulates transportation of synaptic vesicles (10, 12) and storage granules (3, 4, 13), possibly by a mechanism involving localization of activated RAB3/RAB27 (3, 4, 13) and protein-protein interactions (12). MADD has an N-terminal DENN domain and C-terminal serine-rich and death domains. DENN domain is a common component of Rab GDP/GTP exchange factors (GEFs) (14), and it directly binds to target Rabs (12). Intact death domain is likewise important for the GEF activity of MADD (13, 15), as well as for protein-protein interactions (12, 16). Interaction of death domains of MADD and TNF receptor 1 (TNFR1) leads to activation of TNF-α–stimulated ERK1/2 phosphorylation and protection from apoptosis (16–19). Indeed, high expression of MADD appears to protect cells from apoptosis (17, 20–22), though this effect may be splice variant dependent (1, 20, 23, 24).

Polymorphisms in MADD locus are associated with fasting glucose and proinsulin levels in genome- and exome-wide studies (25–34). Conditional knockout of Madd in mouse β cells leads to hyperglycemia, glucose intolerance, and reduced and delayed glucose-induced insulin release (35), and rhythmic
alternative splicing of Madd transcript regulates glucose-stimulated insulin secretion in mouse β cells (36). However, biallelic MADD variants cause a complex phenotype ranging from mild developmental delay to severe multisystem disease affecting nervous, endocrine, exocrine, and hematological systems (37–39). The majority of pathogenic MADD variants affect the conserved protein domains, but there is no clear genotype-phenotype correlation. Most of the patients have hypopituitarism, with neonatal growth hormone deficiency (GHD) and low follicle-stimulating hormone (FSH) level being the most common findings (37–39). Patient-derived fibroblasts display decreased MADD expression, reduced TNF-α–stimulated ERK1/2 phosphorylation, increased susceptibility to cell death, and reduced endocytosis of EGF (37).

Here, we extend these observations by describing a homozygous exon 30–skipping variant in MADD leading to in-frame deletion of 36 amino acids close to the C-terminal death domain. Homozygous carriers of the exon 30–skipping variant had hyperglycemia, insulin resistance, congenital hypogonadotropic hypogonadism (CHH), hypopituitarism, and developmental delay. To model the endocrine disease, we deleted MADD exon 30 (dex30) from the genomic DNA of relevant cell types in vitro. We discovered reduced differentiation efficiency of stem cell–derived dex30 pancreatic progenitors and deficient insulin content and processing in dex30 pancreatic β cells. While dex30 did not affect the ontogeny of stem cell–derived gonadotropin-releasing hormone (GnRH) neurons, we observed reduced luteinizing hormone (LH) expression in dex30 gonadotropes. Surprisingly, dex30 did not cause crude secretory defects or reduced activation of RAB3A, suggesting that MADD may regulate hormone content via mechanisms independent of its GEF activity and that these functions are disrupted by dex30. Concordantly, dex30 led to changes in the protein-protein interactions of MADD.

Results

Clinical description of the patients

The consanguineous family with 2 affected siblings is depicted in Figure 1A. Clinical measurements are in Supplemental Data 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.167598DS1.

Patient 1. The index patient (individual V3 in Figure 1A) had a developmental delay, and at the age of 14 years, elevated level of glycated hemoglobin (HbA1c) was observed. The oral glucose tolerance test revealed elevated fasting plasma glucose and glucose intolerance. Insulin, C-peptide, and markers of β cell autoimmunity were within normal range, suggesting insulin resistance. The patient was prepubertal and had low estradiol and LH levels and minimal LH response to GnRH stimulation, suggesting gonadotropin deficiency. Her serum insulin-like growth factor 1 (IGF-1) was low for age, and low growth hormone (GH) response to arginine was suggestive of GHD. She was treated with metformin and estrogen but not with GH. At the age of 19 years her puberty was incomplete despite estrogen therapy, consistent with CHH. Her GH response to arginine stimulation remained low, confirming GHD.

Patient 2. Patient 2 (individual V2 in Figure 1A) had severe developmental delay and epilepsy. Elevated fasting plasma glucose and HbA1c were first observed at the age of 21 years. Patient 2 had prepubertal levels of testosterone and LH, and no physical signs of puberty, indicating absent puberty due to CHH. Patient 2’s IGF-1 level was low, suggesting GHD. At the age of 28 years, the patient developed polyuria due to hyperglycemia and was treated with metformin.

The family includes 2 other children, of which the firstborn (individual V1 in Figure 1A) died of asphyxia caused by strangulation of the umbilical cord during the delivery, and the youngest (individual V4 in Figure 1A) is developing normally at the age of 5 years.

A splice site variant in MADD leads to skipping of exon 30

To reveal the underlying mutation, we performed whole-genome sequencing (WGS) of the affected siblings and their parents at the Beijing Genomic Institute (BGI). WGS data had an average sequencing depth of 34.27 reads and coverage of at least 99.09% for each sample. Due to the consanguinity of the parents, a recessive homozygous mutation was considered the most likely cause of the phenotype. The WGS data were first filtered to include SNVs or insertions/deletions homozygous in both affected patients and heterozygous in their parents, then to include only variants in the coding regions or splice sites with a frequency of less than 1% in the 1000 Genomes database (40) (Figure 1B).
After the WGS data filtering, 14 SNVs (8 synonymous, 5 missense, and 1 splice site) were left (Figure 1B and Supplemental Table 1). According to predictions from the BGI, only a splice site variant in MADD had a high impact. This variant, c.4377+2T>G at National Center for Biotechnology (NCBI) Reference Sequence NM_003682.4, affects the canonical consensus splice donor site of exon 30 (Figure 1C) and has not been reported in the gnomAD, dbSNP, and Ensembl databases. Human Splicing Finder (41) predicted an alteration that most probably affects splicing, and this prediction was corroborated by SpliceAI/Pangolin.
and decreased mRNA levels of \( \text{NKX6-1} \) and \( \text{PDX1} \) at stages 3 and 4, \( \text{NKX2-2} \) at stages 3 and 4, and the wild-type cells at stages 3–5 (Supplemental Figure 4, D and E). However, between the \( \text{NGN3} \) cells, we verified robust expression of \( \text{MADD} \).

To elucidate the role of \( \beta \) cells, \( \text{MADD} \) regulates insulin expression in immortalized human pancreatic \( \beta \) cells

\( \text{MADD} \) and \( \text{MAFA} \) (Supplemental Figure 3A). We observed a significant reduction of insulin (INS) and pre\( \text{INS} \) mRNA levels in \( \text{siMADD} \)-treated cells compared with cells treated with scrambled siRNAs (siNT, Supplemental Figure 3, B and C). Silencing of \( \text{MADD} \) expression did not affect mRNA levels of \( \text{PDX1} \) and \( \text{MAFA} \) (Supplemental Figure 3, D and E), indicating that \( \text{MADD} \) regulates insulin expression independently from these transcription factors in EndoC-\( \beta \) H1 cells. Reduction of \( \text{INS} \) mRNA was reflected by decreased insulin content and glucose-stimulated insulin secretion in the \( \text{siMADD} \)-treated EndoC-\( \beta \) H1 cells (Supplemental Figure 3, F and G). However, the percentage secretion relative to the total insulin content was not affected, indicating that there were no crude defects in the secretion machinery (Supplemental Figure 3H).

In addition, the proinsulin/C-peptide ratio was comparable between \( \text{siMADD} \) and \( \text{siNT} \) cells (Supplemental Figure 3I). These results suggest that \( \text{MADD} \) regulates insulin expression in EndoC-\( \beta \) H1 cells.

\( \text{Dex30} \) leads to decreased insulin expression in immortalized human pancreatic \( \beta \) cells

To investigate the effect of skipping of \( \text{MADD} \) exon 30 on insulin expression in EndoC-\( \beta \) H1 cells, we directed CRISPR/Cas9-GFP by 2 guide RNAs that were designed to target the intronic sites around \( \text{MADD} \) exon 30 (Figure 2B). The genome-edited \( \text{dex30} \) cells were enriched by FACS and validated by PCR (Figure 2C). Again, we observed decreased pre\( \text{INS} \) and \( \text{INS} \) mRNA levels in \( \text{dex30} \) EndoC-\( \beta \) H1 cells (Supplemental Figure 3, J and K), while mRNA levels of \( \text{PDX1} \) and \( \text{MADD} \) remained unaltered (Supplemental Figure 3, L and M). Insulin content and basal insulin secretion into the culture media were significantly reduced in \( \text{dex30} \) EndoC-\( \beta \) H1 cells when normalized to total DNA (Figure 2, D and E). However, the basal and glucose-stimulated insulin secretion in \( \text{dex30} \) cells was comparable to wild-types when normalized to the total insulin content, suggesting that the secretion machinery remained functional (Supplemental Figure 3N). In addition, immunostaining suggested accumulation of proinsulin in some of the \( \text{dex30} \) EndoC-\( \beta \) H1 cells (Figure 2F).

In summary, our results indicate that \( \text{dex30} \) affects insulin expression in EndoC-\( \beta \) H1 cells.

\( \text{Dex30} \) in human embryonic stem cells reduces their differentiation efficiency into stem cell–derived pancreatic islets and affects insulin processing

To explore the effects of \( \text{dex30} \) on development of pancreatic \( \beta \) cells, we used the CRISPR/Cas9 strategy described for EndoC-\( \beta \) H1 cells in human embryonic stem cell (hESC) line H9 (49). Genome-edited cells were single-cell–sorted to generate clonal populations. We identified 2 homozygous \( \text{dex30} \) clones (Supplemental Figure 4A) that were differentiated into stem cell–derived pancreatic islets (SC-islets) alongside isogenic controls following a 7-stage differentiation protocol (Figure 3A) (50). The \( \text{dex30} \) cells differentiated into stage 1 definitive endoderm comparably to wild-types (Supplemental Figure 4B). At stage 4, we observed a reduced number of NKX6-1– pancreatic progenitors (Supplemental Figure 4C), indicating lower differentiation efficiency. The mRNA levels of \( \text{MADD} \) and \( \text{NNG3} \) were comparable between the \( \text{dex30} \) and the wild-type cells at stages 3–5 (Supplemental Figure 4, D and E). However, decreased mRNA levels of \( \text{PDX1} \) and \( \text{NKX2-2} \) at stages 3 and 4, \( \text{SOX9} \) at stages 4 and 5, and \( \text{NKX6-1} \) at...
stage 5 indicated that differentiation to pancreatic progenitors was less efficient in \textit{dex30} cultures, especially at stages 4 and 5 (Figure 3, B–E). This was reflected by lower number of C-PEP$^+$NKX6-1$^+$ \textit{dex30} \(\beta\) cells at stage 7 (Supplemental Figure 4F). The total number of endocrine cells in \textit{dex30} SC-islets was comparable to wild-types by flow cytometry and immunohistochemistry (Supplemental Figure 4, F–H), but \textit{dex30} SC-islets had significantly higher numbers of glucagon-positive \(\alpha\) cells concomitant with a trend of reduced insulin-positive \(\beta\) cells (Supplemental Figure 4, G and H). Concordantly, \(\text{INS}\) mRNA expression was reduced at stage 7 (Figure 3F).

During insulin biosynthesis, proinsulin is transported through the Golgi network to secretory granules, in which proprotein convertase 1/3 encoded by \(\text{PCSK1}\) and exopeptidase carboxypeptidase E/H encoded by \(\text{CPE}\) remove the C-peptide, leading to the formation of mature insulin (51). In humans \(\text{PCSK1}\) is specifically expressed in \(\beta\) cells, whereas proprotein convertase 2 encoded by \(\text{PCSK2}\) is specific to glucagon-producing \(\alpha\) cells (52). To study insulin processing in the \textit{dex30} SC-islets, we quantitated the mRNA expression of \(\text{PCSK1}, \text{PCSK2}, \text{and CPE}\) during stages 6–7. The mRNA level of \(\text{PCSK1}\) was significantly decreased, while the mRNA level of \(\text{PCSK2}\) was significantly increased in \textit{dex30} SC-islets at stage 7 (Figure 3, G and H), likely
Figure 3. Dex30 leads to reduced number of β cells, lowered insulin content, and impaired insulin processing in SC-islets. (A) Schematic of SC-islet differentiation protocol. (B) PDX1, (C) NKX2-2, (D) SOX9, and (E) NKX6-1 mRNA levels in WT and dex30 cultures at stages 3–5, relative to undifferentiated stem cells (n = 4–6 for WT, n = 8–11 for dex30). (F) INS, (G) PCSK1, and (H) PCSK2 mRNA levels in WT and dex30 cultures at stages 6–7, relative to undifferentiated stem cells (n = 6 for WT, n = 9 for dex30 at ST6, n = 10 for WT, and n = 13–14 for dex30 at stage 7). (I) Total insulin and (J) proinsulin content in WT and dex30 SC-islets at stage 7 (n = 7 for WT, n = 9 for dex30). (K) Proinsulin-to-insulin ratio in WT and dex30 SC-islets at stage 7 (n = 7 for WT, n = 9 for dex30). (L) Static insulin secretion from WT and dex30 SC-islets at stage 7, in 2.8 mM glucose (G2.8), 16.8 mM glucose (G16.8), 16.8 mM glucose + 50 mM Exendin 4 (Ex4), and 2.8 mM glucose with 30 mM KCl (KCL). (M) Dynamic insulin secretion in WT and dex30 SC-islets at stage 7. Conditions like in L (n = 7 for WT, n = 9 for dex30). (N) Area under curve quantification of dynamic insulin secretion in M. ***P < 0.001, **P < 0.01, and *P < 0.05 analyzed by Student’s t test (I–K and N) or multiple t tests (B–H and L).
reflecting the increased proportion of α cells. The mRNA level of CPE was not altered in dex30 SC-islets (Supplemental Figure 4J).

Concordant with the reduced insulin mRNA expression, insulin content was reduced in dex30 SC-islets when normalized to total DNA (Figure 3I) or to β cell DNA (Supplemental Figure 4M), suggesting that insulin content was lower in individual dex30 β cells. Interestingly, the proinsulin content was reduced to a lesser extent, leading to significantly increased proinsulin-to-insulin ratio in dex30 SC-islets (Figure 3, J and K), but immunohistochemistry revealed no clear changes in the proportions of proinsulin’, proinsulin’insulin’, and insulin’ cells (Supplemental Figure 4, K and L). These results suggest that insulin processing was compromised in dex30 β cells.

To assess the functionality of dex30 β cells, we analyzed insulin secretion in the SC-islets (Figure 3, L–N, and Supplemental Figure 4, N–P). We performed sequential incubations with low glucose (2.8 mM), high glucose (16.8 mM), high glucose with 50 nM glucagon like peptide-1 receptor agonist Ex4 (potentiates glucose-stimulated insulin secretion), and low glucose with 30 mM KCl (induces exocytosis of remaining insulin granules). In each tested condition, the dex30 SC-islets secreted significantly less insulin compared with wild-types when normalized to total DNA (Figure 3L). We studied the dynamics of insulin secretion using a perfusion assay with the same stimuli (Figure 3M). AUC quantifications verified the reduced insulin secretion in the dex30 SC-islets (Figure 3N). However, the relative responses, measured as the stimulation index (Supplemental Figure 4N) or insulin secretion normalized to total insulin content (Supplemental Figure 4, O and P), were not affected. These results indicate that the absolute insulin secretion in the dex30 SC-islets is reduced, but the secretory machinery remains intact. We did not observe increased apoptosis in dex30 SC-islets (Supplemental Figure 4, Q and R). In summary, the dex30 hESCs differentiated into SC-islets less efficiently than the wild-type cells, and their insulin content and secretion were markedly reduced, possibly reflecting defects in insulin processing as suggested by increased proinsulin-to-insulin ratio.

MADD is expressed in human and mouse hypothalamic neurons, but dex30 does not compromise hESCs’ potential to differentiate into GnRH-expressing neurons

Hypothalamic GnRH neurons regulate the onset of puberty by secreting GnRH and activating pituitary gonadotropes to release LH and FSH, which in turn regulate gonadal estrogen and testosterone production (53). The patients with MADD exon 30–skipping variant manifested absent puberty and low levels of LH, estrogen, and testosterone, which could originate from either hypothalamic or pituitary defects. To elucidate the hypothalamic component, we confirmed robust MADD mRNA expression by RT-PCR in a human hypothalamus cDNA library (Supplemental Figure 5A). To explore the coexpression of Madd and Gnrh1, we performed RNAscope mRNA in situ hybridization in mouse hypothalamus and observed ubiquitous expression of Madd, including in the Gnrh1-expressing cells (Figure 4A). A negative control demonstrating absence of Gnrh1 signal in a caudal section is shown in Supplemental Figure 5B, and negative and positive probe controls are shown in Supplemental Figure 5C. Concordantly, single-cell RNA-Seq data from mouse hypothalamus (54) revealed the highest expression of Madd in the cluster of GABAergic neurons (Supplemental Figure 5D), including GnRH neurons (55, 56). With relevance to the GHD in the proband, we observed coexpression of Madd and GH-releasing hormone (Ghrh) transcripts (Supplemental Figure 5E).

To explore the impact of deleting MADD exon 30 on the development of GnRH neurons, we differentiated the dex30 hESC clones into SC-derived GnRH neurons according to our established protocol (Figure 4B) (49, 55, 57, 58). The hESCs used in this study carry a GNRH1-tdTomato reporter gene allowing visualization of GNRH1-expressing cells (49). We detected tdTomato+ cells (Figure 4, C and D) and GnRH-immunopositive and neuron-specific class III β-tubulin–immunopositive (TuJ1-immunopositive) neurons in both wild-type and dex30 cultures on differentiation day 27 (Figure 4E), verifying that dex30 hESCs were able to differentiate to GnRH-expressing neurons. Additionally, dex30 cells secreted GnRH decapetide to the culture medium (Figure 4F). The variability in the differentiation efficiency is consistent with previous observations in wild-type cultures (57, 58). In summary, dex30 hESCs were able to differentiate to GnRH-secreting neurons.

MADD is expressed in pituitary hormone–producing cells, and dex30 pituitary gonadotropes display reduced LH expression while retaining their responsiveness to GnRH

We hypothesized that the skipping of MADD exon 30 may cause CHH by directly affecting the pituitary gonadotropes. We observed robust MADD mRNA expression in a human pituitary cDNA library by
RT-PCR (Supplemental Figure 5A) and verified MADD expression in a human gonadotrope cluster by using Single Nucleus Pituitary Atlas (59) (Supplemental Figure 5F). Furthermore, we detected robust Madd expression in mouse gonadotropes by RNAscope RNA in situ hybridization (Figure 5, A–C) and
and wild-type LβT2 cells. To investigate whether machinery remained functional in Lβ and stimulation indices (Supplemental Figure 6, F and G) were not affected, indicating that the secretory normalized to total protein (Figure 5I). However, the percentage LH secretion per LH content (Figure 5J) was significantly reduced in dex30 LβT2 cells compared with wild-types (Figure 5, F–H). To investigate was significantly reduced in dex30 and wild-type LβT2 cells (Supplemental Figure 6, C–E).

Interestingly, the expression of LH subunit β (Lhb) mRNA, LH content, and spontaneous LH secretion was significantly reduced in dex30 LβT2 cells compared with wild-types (Figure 5, F–H). To investigate stimulated LH secretion, we performed sequential incubations with no stimuli, 50 nM GnRH, and 60 mM KCl. Dex30 LβT2 cells secreted significantly less LH than the wild-types in all tested conditions when normalized to total protein (Figure 5J). However, the percentage LH secretion per LH content (Figure 5J) and stimulation indices (Supplemental Figure 6, F and G) were not affected, indicating that the secretory machinery remained functional in dex30 LβT2 cells. To investigate whether dex30 affects the responsiveness to GnRH stimulation, we primed dex30 and wild-type LβT2 cells with pulsatile GnRH stimulations as described earlier (61). In line with the previous observations (61), the subsequent increase of Lhb mRNA expression was approximately 2.4-fold, with no differences between the genotypes (Supplemental Figure 6H), suggesting that dex30 LβT2 cells are responsive to GnRH.

ERK1/2 phosphorylation is implicated in both basal and GnRH-stimulated Lhb expression (62). As MADD regulates ERK1/2 phosphorylation in LβT2 cells (16–18), we hypothesized that dex30 may affect ERK1/2 phosphorylation in LβT2 cells. We quantified total and phosphorylated ERK1/2 by Western blot in wild-type and dex30 LβT2 cells but did not detect significant differences in basal or GnRH-induced ERK1/2 phosphorylation (Supplemental Figure 6, I–K). These results suggest that dex30 leads to reduced Lhb expression independently of ERK1/2 phosphorylation and does not compromise GnRH-induced ERK1/2 signaling. As the death domain of MADD interacts with TNFR1 (16–18), we also assessed ERK1/2 phosphorylation in LβT2 cells after 15 minutes of 50 ng/mL TNF-α stimulation, but we observed a very low overall responsiveness to this stimulus in both wild-type and dex30 cells (data not shown).

Stability and activation of RAB3 proteins are not affected by dex30
MADD deficiency may reduce the stability of its target Rabs (4, 13). Therefore, we assessed the levels of RAB3A-D proteins by Western blot in dex30 and wild-type LβT2 cells but observed no differences (Supplemental Figure 7, A and B). To test if dex30 affects the GEF activity of MADD, we applied our CRISPR/Cas9 strategy in human embryonic kidney cell line HEK293 and generated 2 clones with homozygous dex30 (Supplemental Figure 7C). We transiently expressed human RAB3A in dex30 and wild-type HEK293 cells and quantified GTP-bound RAB3A by immunoprecipitation with conformation-specific antibody but observed no significant differences between dex30 cells and wild-types (Supplemental Figure 7, D and E).

Interactome of MADD reveals targets involved in multiple signaling pathways
To further elucidate the effects of dex30, we detected the stable and transient protein-protein interactions (PPIs) of wild-type and dex30 MADD in Flp-In T-REx 293 cells using affinity purification mass spectrometry (AP-MS) and proximity-based labeling mass spectrometry (BioID) assays (63). We detected in total 102 high-confidence interaction partners, of which 10 (9.8%) were detected by both methods (Figure 6 and Supplemental Table 3). Interactions between MADD and members of the 14-3-3 protein family and synaptotagmin 3 have been previously observed (64–68). Functionally, the largest group were the 17 interactors involved in signaling through GTPase activity, including 7 heterotrimeric G protein subunits and 10 regulators of Rho, Rab, Arf, and Rap subfamilies of small GTPases (Figure 6). In general, the interactors of MADD functioned in various signaling pathways, as they included 9 kinases, 7 serine/threonine-Protein phosphatase subunits, 2 STATs, and 15 DNA and/or RNA binding proteins (Figure 6 and Supplemental Table 3).

Gene Ontology (GO) analysis revealed that the most significantly overrepresented biological processes in the interactome of MADD were positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway (GO:1900740), signal transduction (GO:0007165), and cellular protein localization (GO:0034613) (Supplemental Table 4). Furthermore, Reactome and
Panther pathway analysis detected several overrepresented pathways, including signal transduction (R-HSA-162582), signaling by Rho GTPases (R-HSA-194315), EGF receptor signaling pathway (P00018), and FGF signaling pathway (P00021). Interestingly, diabetes-related pathways regulation of insulin secretion (R-HSA-422356), and integration of energy metabolism (R-HSA-163685), as well as puberty-related gonadotropin-releasing hormone receptor pathway (P06664), were enriched. Up to 50 enriched pathways are presented in Supplemental Table 5. Furthermore, we performed systematic PubMed searches and found that 35 of the interactors (34.3%) could be linked with diabetes/β cell function/insulin signaling.
and/or puberty/pituitary function and that 67 (65.7%) have been implicated in cellular functions previously linked with MADD (signaling through ERK1/2 or EGFR; exocytosis, endocytosis, or cell survival) (Figure 6 and Supplemental Table 6).

**Dex30 results in alterations in the interactome of MADD**

In total, we detected 36 proteins that interacted differently with dex30 and wild-type MADD (Figure 7 and Supplemental Table 7). Interestingly, 52.8% of these interactors could be linked with diabetes/β cell function/insulin signaling and/or puberty/pituitary function (Supplemental Table 7). Compared with wild-types, the relative abundances were significantly reduced for 17 interactors and increased for 19 interactors in protein complexes isolated from dex30 MADD-expressing cells, suggesting both loss-and gain-of-function effects. We detected the largest fold-change between wild-type and dex30 MADD for the ubiquitin-specific peptidase 9 X-linked, whose relative abundance in protein complexes isolated from dex30 MADD-expressing cells was approximately 24-fold higher compared with wild-type MADD-expressing cells. Notably, the AP-MS approach revealed that 6 members of 14-3-3 adaptor protein family (14-3-3β, 14-3-3ε, 14-3-3ζ, 14-3-3η, 14-3-3θ, and 14-3-3ζ encoded by YWHAE, YWHAQ, YWHAH, YWHAQ, and YWHAZ, respectively) interacted significantly less with dex30 than wild-type MADD (Figure 7). This finding was replicated by the BioID approach for 14-3-3β, 14-3-3γ, 14-3-3δ, and 14-3-3ζ. On the other hand, 2 heterotrimeric G protein inhibitory α subunits (Gi; GNAI1 and GNAI3) and 2 β subunits (GNB1 and GNB2) interacted more with dex30 compared with wild-type MADD. Both 14-3-3 proteins and subunits of heterotrimeric G proteins were highly represented in our pathway analysis (Supplemental Table 5), suggesting that dex30 causes changes in multiple signaling pathways.

**Discussion**

We identified an exon 30-skipping variant in MADD in 2 patients with developmental delay, diabetes, and pituitary hormone deficiency. In vitro disease-modeling experiments with dex30 cells recapitulated the diabetes and CHH in the probands and suggested that dex30 does not affect the GEF activity of MADD, but induces changes in the PPIs, likely affecting multiple signaling pathways.

The 2 patients with homozygous MADD exon 30-skipping variant were diagnosed with diabetes. Interestingly, polymorphisms at MADD locus have been previously associated with glycemic traits in genome-wide studies. The A allele at SNP rs7944584 and the G allele at SNP rs10501320 in MADD locus have been associated with higher fasting blood glucose levels (25, 27, 29) and rs7944584-A and 8 additional nonsynonymous MADD variants with type 2 diabetes (28). Results obtained with dex30 cells implicate multiple mechanisms that may contribute to the development of diabetes. First, dex30 caused reduced differentiation efficiency of stem cell-derived pancreatic progenitors at stages 4 and 5, leading to reduced number of β cells. MADD deficiency affects endocytosis of EGF (37), and the results of our PPI assays revealed that MADD interacts with 23 proteins implicated in EGFR signaling and that 52% of these interactions are altered by dex30. Given that EGFR signaling regulates pancreatic progenitor cell fate and organogenesis (69–71), and that EGF is one of the growth factors used in SC-islet differentiation during stages 3 and 4 (50), problems in EGFR signaling could explain the differentiation defect observed in dex30 SC-islets, and similar effects may occur in vivo.

Second, dex30 caused increased proinsulin-to-insulin ratio in SC-islets. Proinsulin levels have been associated with MADD locus in several genome- and exome-wide studies. A strikingly strong association \((P = 2.07 \times 10^{-71})\) was observed between MADD SNP rs7944584-A and higher fasting proinsulin levels (32). Further studies replicated this association (27, 29, 30) and identified additional SNPs rs10501320-G, rs10838687-T (29), rs1449626-A (34), and rs35233100-T (31) associated with higher fasting proinsulin levels. The increased proinsulin-to-insulin ratio observed in dex30 SC-islets is consistent with results of previous studies and implies that MADD regulates proinsulin processing and trafficking, though the exact mechanism remains unresolved.

Third, the index patient had diabetes with normal peripheral insulin levels, suggesting insulin resistance for which the β cells failed to compensate. Our PPI studies revealed that 33 of the proteins interacting with MADD have been implicated in diabetes, β cell function, and insulin signaling and that 52% of these interactions were altered by dex30, including with members of the 14-3-3 protein family and STAT1, which are implicated in peripheral insulin signaling and pathogenesis of diabetes in pancreas, muscle, and adipose tissue (72–74). Taken together, our results suggest that skipping of MADD exon 30
may lead to diabetes by affecting both β cell function and peripheral insulin signaling. To our knowledge, diabetes has not been previously reported in patients with biallelic variants in MADD (37–39). This discrepancy may be age related, as in the probands of the current study, hyperglycemia was first observed at the ages of 14 and 21 years, whereas most of the previously reported patients were considerably younger, with a median age of 1.6 years and 83% being below 8 years of age (37–39). Therefore, we recommend...
that the patients with deleterious MADD variants are followed up until early adulthood for the presence of hyperglycemia and diabetes.

Notably, we did not observe reduced RAB3A activation or reduced RAB3 protein stability in dex30 cells. RAB3 and RAB27 have been previously implicated in the exocytosis of pituitary hormones (75–77) and insulin (78–80), and it has been suggested that reduced activation of RAB3 and RAB27 may lead to problems in hormone secretion in patients with biallelic MADD variants (37). Rab3a- and Rab27a-deficient mice display reduced insulin secretion with normal or increased insulin content (79, 80), and conditional knockout of Madd in mouse β cells causes similar phenotype with disturbed exocytosis and accumulation of insulin granules (35). These results suggest that abolishment of Madd expression may affect insulin secretion by reducing activation of RAB3A/RAB27A. Instead, dex30 results in altered protein-protein interactions that may produce a variety of loss- and gain-of-function effects leading to problems in insulin production while secretory machinery remains functional.

The probands of the current study had CHH and GHD. This is consistent with the previously observed hypopituitarism in the patients with biallelic MADD variants. CHH has not been diagnosed in patients with biallelic MADD variants, possibly due to their young age (37–39). However, frequently observed small penis or micropenis and cryptorchidism suggest disorders of the hypothalamic-pituitary-gonadal axis (37, 38) (Supplemental Figure 2). Concordantly with the CHH in the probands, we observed reduced LH content and secretion in dex30 LβT2 gonadotropes. The relative GnRH responsiveness remained intact, suggesting
GnRH receptor–independent mechanism of LH deficiency. However, we cannot exclude a hypothalamic component contributing to the CHH of the patients with *MADD* exon 30–skipping variant. Haploinsufficiency of rabconnectin-3a, which is a putative scaffolding protein for MADD and RAB3 GTPase activating protein (81), caused a similar disorder with CHH, diabetes, and developmental delay (82). Functional studies implicated rabconnectin-3a in GnRH neuron specification and maturation (82, 83). Similarly, it is possible that skipping of *MADD* exon 30 may affect maturation of GnRH neurons in vivo, though the ontogeny of dex30 SC-derived GnRH neurons was not compromised.

Collectively, our results implicate *MADD* in the development and function of endocrine cells and suggest that it regulates multiple signaling pathways.

**Methods**

*Sex as a biological variable.* This study included 1 female patient and 1 male patient, and similar findings are reported for both sexes.

**WGS and data filtering.** Genomic DNA was extracted from the peripheral blood of the patients using standard methodology. The WGS was performed using Illumina HiSeq X Ten technology at the BGI (Shenzhen, China). The impact of the identified SNVs was further evaluated with the web tools SIFT (46), PolyPhen2 (47), Human Splicing Finder v3.0 (41), SpliceAI/Pangolin (42), NNSplice (43), HAL Splice Prediction (44), and Spliceator (45).

Exon 30 and the exon-intron borders were PCR-amplified from the genomic DNA with primers 5′-CTG-GATGAGGGGAATTCTTGGC-3′ and 5′-CGGTTCCTATGAGTTGGTGTTG-3′ with AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific). PCR products were purified with Illustra ExoProStar treatment (GE Healthcare, now Cytiva) and Sanger-sequenced at FIMM Genomics, University of Helsinki, Finland.

**RNA extraction and RT-PCR from patient’s blood.** RNA was extracted from fresh whole blood of the index patient and a healthy control with the QIAamp RNA Blood Mini Kit (QIAGEN). A total of 1 μg of RNA was converted to cDNA with SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The area covering *MADD* transcript (NM_003682.3) was PCR-amplified in 8 overlapping amplicons (Supplemental Table 8). PCR products were purified and Sanger-sequenced.

**Minigene assay.** See Supplemental Methods.

**RT-PCR in human cDNA libraries/cadaveric islet cDNA.** RT-PCRs were performed from 2 μL of Human Brain Hypothalamus Marathon-Ready cDNA (Takara Bio), human pituitary cDNA (Clontech), and cDNA from human primary islets (Nordic Network for Islet Transplantation, Uppsala University, Sweden) with AmpliTaq gold polymerase (Thermo Fisher Scientific). PCR products were purified with Illustra ExoProStar treatment (GE Healthcare, now Cytiva) and Sanger-sequenced.

**Assessing gene expression in EndoC-βH1 cells.** Total RNA was extracted using MACHEREY-NAGEL RNA isolation kit. cDNA was prepared using the Maxima first-stand cDNA synthesis kit (Thermo Fisher Scientific). RT-PCRs were performed from 2 μL of cDNA in 8 overlapping amplicons (Supplemental Table 1). PCR products were purified and Sanger-sequenced.

**Generating dex30 EndoC-βH1 cells.** *MADD* exon 30 was deleted by genome editing with CRISPR/Cas9. Two guide RNAs were designed with Benchling online tool (https://www.benchling.com). Guide sequences with protospacer adjacent motif sites marked in bold were: upstream from exon 30: 5′-CAGT-CATACATCCCTCTCAAGGG-3′ and downstream from exon 30: 5′-AAGGGCTATTGAGAGTCA-3′. Guide RNAs and Cas9 were electroporated to cells as a complexed ribonucleoprotein (RNP) using the Neon Transfection System kit (Invitrogen, Thermo Fisher Scientific). Briefly, 2 million cells were dissociated using TrypLE (Thermo Fisher Scientific), pelleted at 250 relative centrifugal force (RCF) for 5 minutes, and resuspended in R-buffer and mixed with the RNP complex containing fluorescent Alt-R CRISPR-Cas9 tracrRNA, ATTO 550 (Integrated DNA Technologies), then electroporated with 1,100 V, 30 ms, and 2 pulses. ATTO 550 cells were enriched using Sony SH800S Cell Sorter.

**Assessing gene expression in EndoC-βH1 cells.** Total RNA was extracted using MACHEREY-NAGEL RNA isolation kit. cDNA was prepared using the Maxima first-stand cDNA synthesis kit (Thermo Fisher Scientific). RT-PCRs were performed from 2 μL of cDNA in 8 overlapping amplicons (Supplemental Table 8). PCR products were purified and Sanger-sequenced.
Flow cytometry during SC-islet differentiation. Cells/SC-islets were dissociated with TrypLE for 3–10 minutes at 37°C and resuspended in 5% FBS-containing PBS. Stage 1 cells were stained with CXCR4 surface antibody for 20 minutes. Stage 4 cells and stage 7 SC-islets were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) for 20 minutes. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 30 minutes at RT in Perm/Wash buffer (BD Biosciences) + 5% FBS. FACS was performed with FACSCalibur cytometer (BD Biosciences); data were collected with CellQuest Pro v.4.0.2 (BD Biosciences) and analyzed with FlowJo v.10 (BD Biosciences). For antibodies, see Supplemental Table 10.
**Immunohistochemistry with SC-islets.** S7 SC-islets were fixed for 2 hours and embedded in paraffin. Sections of 5 μm were deparaffinized, and antigen retrieval was performed in 0.1 mmol/L citrate buffer in a pressure cooker (Biocare Medical). The slides were blocked with UV-block (Thermo Fisher Scientific) and incubated with primary antibodies (Supplemental Table 10) diluted in 0.1% Tween-20 overnight at 4°C and with secondary antibodies for 1 hour at RT. For detecting apoptosis, in situ Cell Death Detection Kit, Fluorescein (Roche), was used. The slides were imaged with Zeiss AxioImager using Apotome II with the same exposure and export setting for all slides of each immunostaining. Images were processed in Zen2 Blue Edition v.2 (ZEISS) and analyzed using CellProfiler v.4.0.

**Insulin secretion assays.** A total of 150 SC-islets were handpicked to 12-well plates and equilibrated in Krebs-Ringer buffer (KRB) with 2.8 mM glucose for 90 minutes, then subjected to sequential 30-minute incubations of 2.8 mM glucose, 16.8 mM glucose, 16.8 mM glucose + 50 μg/mL Ex4 (Tocris), and 2.8 mM glucose + 30 mM KCl in KRB. SC-islets were lysed in hypotonic conditions with sonication. Dynamic insulin secretion was assessed from another set of 150 SC-islets using a perfusion apparatus (Brandel Suprafusion SF-06) with a flow rate of 0.25 mL/min and sampling every 4 minutes. Insulin and proinsulin were quantified with ELISA kits (Mercodia), and DNA content was quantified using FluoReporter Blue dsDNA Kit (Invitrogen, Thermo Fisher Scientific).

**RNAscope mRNA in situ hybridization and immunohistochemistry in mouse hypothalamus and pituitary.** Brains from 10-week-old mice and pituitaries from 8-week-old mice (Charles River Laboratories, strain code 022) were fixed in 10% formalin (MilliporeSigma) overnight at RT. The tissue was paraffin-embedded following washes and dehydration through graded ethanol series. Samples were sectioned coronally at 4–5 μm. Double mRNA in situ hybridization was carried out with the RNAscope 2.5 HD Duplex Assay (ACD Bio). All probes used (Madd, Gnrh1, Lhb, Fshb, Ghrh, dapB, and Polr2A) were provided by the manufacturer. RNAscope-stained sections were scanned with a Nanozoomer-XR digital slide scanner (Hamamatsu). mRNA in situ hybridization combined with immunofluorescence was carried out using the RNAscope 2.5 HD Reagent Kit-RED assay (ACD Bio) and Madd probe with the following modification: Protease Plus was diluted 1:15 in PBS and incubated for 40 minutes at 40°C. After the Red detection step, slides were washed in PBS with 0.1% Triton X-100 (PBST) and incubated for 1 hour at RT in Blocking Buffer (0.15% glycine, 2 mg/mL BSA, 0.1% Triton X-100 in PBS) with 10% sheep serum. Primary antibodies (Supplemental Table 10) diluted in blocking buffer with 1% sheep serum were incubated at 4°C for 48 hours. Slides were washed in PBST and incubated with secondary antibodies for 1 hour at RT. Slides were washed before incubation with Hoechst (Invitrogen, Thermo Fisher Scientific) diluted 1:10,000 for 10 minutes at RT. Slides were washed and mounted with VectaMount (Vector Laboratories). Images were taken with a Leica TCS SP5 confocal microscope using an HCX Plan-Apochromat CS 20×/0.7 dry objective (Leica Microsystems).

**Single-cell RNA-Seq analysis.** Published data sets of mouse hypothalamus P45 (54) and of 7-week-old mouse pituitary (60) were reanalyzed following the respective published pipelines and interrogated for Madd expression using the Seurat package (54) in RStudio.

**Differentiation of SC-derived GnRH neurons.** hESCs were differentiated to GnRH-expressing neurons according to established protocol (57) with slight modifications. Briefly, differentiation was started (D0) in 90%-100% confluent cultures and carried out on Matrigel-coated plates in N2B27 basal medium. On days 0–9 cells were treated with 2 μM dorsomorphin (Selleckchem) and 10 μM SB431542 (MilliporeSigma). On day 10, cells were passaged with 200 U/mL collagenase IV and mechanical scraping, then replated at a dilution of 1:2 in N2B27 with 5 μM ROCK inhibitor. On days 11–19 cells were treated with 100 ng/mL FGF8 (PeproTech). On day 20 cells were passaged with PBS-EDTA and replated at a dilution of 1:8 in N2B27 + 100 ng/mL FGF8. On days 21–27 cells were treated with 20 μM DAPT (Selleckchem).

**Assessing tdTomato expression.** tdTomato signal was assessed from images acquired with ZOE fluorescent Cell Imager (Bio-Rad) on day 27 of differentiation. Red-colored cells from 6 images were counted manually using ImageJ (NIH).

**Immunocytochemistry with SC-derived GnRH neurons.** On day 20 of differentiation, cells were passaged with EDTA and seeded on Matrigel-coated glass coverslips. On day 27 cells were fixed with 4% paraformaldehyde for 15 minutes at RT and permeabilized in PBS containing 0.5% Triton X-100 (MilliporeSigma) for 7 minutes and blocked with BlockAid (Invitrogen, Thermo Fisher Scientific) for 30 minutes. Slides were incubated with primary antibodies (Supplemental Table 10) diluted in 0.1% Tween-PBS overnight at 4°C and with secondary antibodies for 1 hour at RT. Nuclei were counterstained with DAPI (MilliporeSigma). Slides were mounted...
with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific), and images were captured with Zeiss AxiosImager.Z1 upright epifluorescence microscope with 40×/NA 1.10 and 63×/NA 1.20 HC PL APO CS2 objectives (Biomedical Imaging Unit) and processed in Zen2 Blue Edition v.2 (Zeiss).

Assessing GnRH secretion. Day 23 and 25 cultures (on a 35 mm dish) were washed once with DMEM, and 1.5 mL of N2B27 + DAPT was added. After 48 hours media were collected, and GnRH was measured by a fluorescent enzyme immunoassay (Phoenix Pharmaceuticals Inc).

LβT2 cells. LβT2 cells (61) were purchased from MilliporeSigma and maintained in high-glucose DMEM (MilliporeSigma) with 10% FBS and penicillin-streptomycin. Cells were passaged twice a week with Trypsein-EDTA at the ratio of 1:2–1:4. Cell morphology images were captured with Leica DM IL LED Inverted Microscope using Leica HI PLAN I 10×/NA 0.22 PH 1 objective, and viable cells were counted with TC20 automated cell counter (Bio-Rad) in the presence of 0.2% trypan blue (Gibco, Thermo Fisher Scientific).

Generating LβT2 cells with Madd exon 30 deletion. LβT2 cells were genome-edited like hESCs with a few exceptions. Two different guide RNA pairs were used. Sequences for pair 1 were upstream from exon 30: 5′-GGAGTCTATGAAAGGGGCACTGG-3′, downstream from exon 30: 5′-AAACAAGGTGTGACCATAGCCAGG-3′, and for pair 2: upstream from exon 30: 5′-GGGGTGGGAGTCTATGAAGGGG-3′, downstream from exon 30: 5′-TACCATAAAATGGGTGCTTGGG-3′. LβT2 cells were harvested with Trypsin-EDTA and resuspended in cold PBS with 5% FBS. RNPs were mixed with 1 million LβT2 cells in 100 μL of R-buffer. Electroporation was performed with Neon Transfection system (Invitrogen, Thermo Fisher Scientific) with 2 pulses of 1,400 V for 25 ms. Cells were seeded on Matrigel-coated, 35 mm dishes with culture media with 30% FBS. After 48 hours cells were dissociated with Trypsin-EDTA and resuspended in FACS buffer (1% FBS, 5 μM EDTA, and 0.625 mM HEPES buffer from MilliporeSigma in HBSS from Life Technologies). ATTO 550+ cells were single-cell–sorted with BD Influx sorter into Matrigel-coated, 96-well plates with culture medium with 30% FBS. FBS concentration was reduced to 10% after 2 weeks. Clones that were 20%–80% confluent were screened for Madd exon 30 deletion by touchdown PCR with forward primer 5′-TGCCAGGCTTAACTC-3′ and reverse primer 5′-GCCCTAATCCTGCTTCCAG-3′ as described for hESCs. PCR products were Sanger-sequenced. Promising clones were expanded. Deletion of Madd exon 30 was confirmed by RT-PCR with forward primer: 5′-TCCATGTGGGGACCAGCTAGAG-3′ and reverse primer: 5′-CTCAGGCCAGGTTTGATGC-3′. PCR products were Sanger-sequenced.

Assessing gene expression in LβT2 cells. For assessing gene expression in the naive state, cells were collected during normal passaging. For assessing gene expression after GnRH training, 2 million LβT2 cells were seeded on Matrigel-coated 12-well plates. Pulsatile GnRH training was started the following day by stimulating cells with 50 nM GnRH (MilliporeSigma) for 15 minutes followed by 2 washes with DMEM and 75 minutes of rest without GnRH. Stimulation was repeated 4 times a day for 3 consequent days. On the fourth day cells were harvested with trypsin-EDTA. RNA was isolated with NucleoSpin RNA kit. A total of 1 μg of RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). qPCR reactions were performed with 25 ng of first-strand cDNA, HOT 5x FIREPol EvaGreen qPCR Mix Plus, and 0.5 μM forward and reverse primers (Supplemental Table 9) in a LightCycler 480 (Roche). Expression levels in dex30 cells relative to wild-types was calculated by ΔΔCt method, using TATA-box binding protein (Tbp) as a housekeeping gene.

Quantification of LH. A total of 3 million LβT2 cells were seeded on Matrigel-coated, 6-well plates and cultured until 70%–80% confluent. For assessing spontaneous LH secretion, cells were washed 2 times with culture media and supplied with 2 mL fresh medium. After 24 hours the media were collected. Cells were washed twice with ice-cold PBS and lysed by 10 minutes of incubation in ice-cold hypotonic lysis buffer (20 mM Tris-HCl at pH 7.6, 10 mM NaCl, 1 mM KCl, and 1.5 mM MgCl) supplemented with Protease inhibitor cocktail (Bio-Rad), followed by mechanical disruption by passing through a 29G needle 10 times. Lysates were centrifugated (12,000 g for 10 minutes at 4°C) and supernatant was collected. Total protein was quantitated by bicinechonic acid assay (BCA, Thermo Fisher Scientific). For stimulated LH secretion, 70%–80% confluent cells were washed twice with culture medium and incubated subsequently for 15 minutes with culture media, 50 nM GnRH (MilliporeSigma, L7134), and 60 mM KCl. Between stimuli cells were washed 2 times. Cells were lysed as described above. LH in the culture medium and lysates was quantitated by an immunofluorometric assay (85) at Reproductive Biology Unit, Turku Center for Disease Modeling, University of Turku, Finland.

Immunoblot analysis. A total of 1 million LβT2 cells were seeded on 12-well plates and cultured until 70%–80% confluent. Cells were washed twice with ice-cold PBS and lysed for 10 minutes in ice-cold
RIPA buffer (Thermo Fisher Scientific) supplemented with Halt Protease inhibitor cocktail, EDTA (Thermo Fisher Scientific), and Halt Phosphatase inhibitor cocktail when needed. Lysates were centrifuged at 12,000g for 10 minutes at 4°C and supernatant was collected. Total protein was quantitated by BCA. We added 4× Laemmli sample buffer (Bio-Rad) with β-mercaptoethanol (Bio-Rad), and lysates were boiled at 95°C for 5 minutes. Proteins were separated on SDS-PAGE (5 μg of total protein per slot) and transferred to a PVDF membrane. Membranes were blocked with 5% nonfat milk or BSA (used for phosphorylated proteins) in TBS-Tween followed by incubation with primary antibodies overnight at 4°C and with HRP-linked secondary antibodies at RT for 1 hour. Chemiluminescence signals were captured with ChemiDoc Touch imager (Bio-Rad). Band intensities were quantified with Image Lab v6.0 software (Bio-Rad).

**ERK1/2 phosphorylation.** A total of 1 million LβT2 cells were seeded on 12-well plates and cultured until 70% confluent. Cells were starved overnight in 0.1% FBS. The next day, cells were treated for 15 minutes with 50 nM GnRH (MilliporeSigma) or 50 ng/mL TNF-α (PeproTech) in starvation media or left untreated. Total and phosphorylated ERK1/2 were quantitated by immunoblotting as described above.

**Generating dex30 HEK293 cells.** HEK293 cells were purchased from American Type Culture Collection and maintained like LβT2 cells, except that a split ratio of 1:10 was used. The same guide RNA sequences were used as for EndoC-βH1 cells and hESCs. Linear DNA templates for expression of guide RNAs were prepared by PCR amplification (see Supplemental Methods). A total of 500,000 HEK293 cells were seeded on a 35 mm dish. The next day, 300 ng of guide RNA expression cassettes for each guide and 3 μg of a plasmid encoding wild-type Cas9, GFP, and puromycin resistance gene (a gift from Diego Balboa, Biomedical Stem Cell Centre, Helsinki, Finland; Addgene plasmid 89995) were transfected to cells with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific). After 2 days 2.5 μg/mL puromycin (MilliporeSigma) was added. After 2 days of selection, cells were harvested by trypsin-EDTA, diluted to a concentration of 10 cells/mL, and replated on a 96-well plate (1 cell/well). 70%–80% confluent clones were screened for the absence of MADD exon 30 as described for hESCs.

**RAB3A activation assay.** A total of 140,000 HEK293 cells/well were seeded in a 24-well plate. The next day, cells were transfected with 100 ng of pEGFP-C1A-RAB3A (see Supplemental Methods) and 400 ng of empty vector with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific). After 12 hours, the cells were subjected to RAB3A activation assay (NewEast Biosciences), based on pulldown of activated GTP-RAB3A with a conformation-specific antibody. Activated and total RAB3A were quantitated by immunoblot as described above.

**Generation of stable cell lines for PPI studies.** Constructs with MADD transcript ENST00000395336.7 with or without deletion of exon 30 in pcDNA3.1′ vector were generated at GenScript Biotech (the Netherlands). Entry clones compatible with the gateway system (Thermo Fisher Scientific) were generated according to the manufacturer’s instructions. MAC-tagged wild-type and dex30 MADD constructs were generated using Gateway LR Clonase II Enzyme Mix according to the manufacturer’s instructions (Thermo Fisher Scientific).

The Flp-In T-REx 293 cell line was purchased from Invitrogen, Thermo Fisher Scientific, and grown according to the manufacturer’s instructions. Cells were cotransfected with MAC-tagged MADD constructs and pOG44 vector (Invitrogen, Thermo Fisher Scientific) using the Fugene6 reagent (Promega) for 48 hours. Positive isogenic clones were selected with 100 μg/mL hygromycin for 2 weeks.

The stable cell line was cultured on 150 mm plates until 80% confluent. Expression of MAC-tagged MADD was induced by 1 μg/mL tetracycline for 24 hours. For BioID labeling, 50 μM of biotin was added simultaneously with tetracycline. Cells from 5 dishes were pooled and pelleted by centrifugation at 1,200g for 5 minutes at 4°C. Three replicate pellets were prepared for downstream analyses.

**Purification and mass spectrometry.** Cells with only tetracycline treatment were used for affinity purification. Cells treated with biotin and tetracycline were used for the BioID approach. Cells were lysed and processed as previously described (63). Purified protein complexes were processed and digested to peptides for mass spectrometry analysis by Orbitrap Elite hybrid mass spectrometer utilizing Xcalibur version 2.0.7 SP1 (Thermo Fisher Scientific), connected to an EASY-nLC II reverse-phase HPLC system through an electrospray ionization sprayer (Thermo Fisher Scientific). Mass spectrometry analysis was in a data-dependent acquisition mode using Fourier-transform mass spectrometry full scan (m/z 300–1,700) resolution of 60,000 and collision-induced dissociation scan of the top 20 most abundant ions.

**Data processing.** For protein identification, Thermo.RAW files were searched against selected human UniProtKB/SwissProt database with Sequest search engine. All reported data were based on high-confidence peptides assigned in Proteome Discoverer (Thermo Fisher Scientific) with a 5% FDR by Percolator.
Contaminant Repository for Affinity Purification (86) database and in-house GFP sample database were used as controls for identification of high-confidence interactions (87). Cytoscape 3.4.0 was used to construct the protein interaction network.

**Statistics.** Quantitative data were analyzed with GraphPad Prism 9 software and plotted as the mean ± standard error of the mean. Statistical significance was tested with Student’s *t* test or multiple *t* tests (all 2 tailed) with Holm-Šidák method for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

**Study approval.** This study has been approved by the Ethics Committee of Helsinki University Hospital, Helsinki, Finland, and carried out according to the Declaration of Helsinki. The guardians of the patients gave their written informed consent. Mouse studies were approved by King’s College London Research Ethics Committee, London, United Kingdom.

**Data availability.** The quantitative data presented in the figures are available in the Supporting Data Values file. Mass spectrometry data were deposited to MassIVE (https://massive.ucsd.edu/) with the web accession MSV000093584.

**Author contributions**

KP performed CRISPR editing and experimentation with hESC-derived GnRH neurons and LβT2 cells and RAB3A activation assays. JSV performed differentiations of SC-islets. PA conducted clinical investigations. XL and MV assayed PPIs. HI and VC performed experiments with EndoC-bH1 cells. AS and YK performed in situ hybridizations and single-cell RNA-Seq data analysis. YW performed part of the experiments with LβT2 cell clone 2. KV and API assisted in RAB3A activation assays, and KV was involved in data management. JK and JT performed the genetic analyses. KP and TR conceptualized the study and with SV, CLA, and TO designed the methodology. MV, SV, CLA, TO, and TR supervised the project. KP and JSV wrote the first manuscript draft. All the authors contributed to editing and accepted the final draft.

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