Regulation of cellular iron homeostasis is crucial as both iron excess and deficiency cause hematological and neurodegenerative diseases. Here we show that mice lacking iron-regulatory protein 2 (Irp2), a regulator of cellular iron homeostasis, develop diabetes. Irp2 post-transcriptionally regulates the iron-uptake protein transferrin receptor 1 (TfR1) and the iron-storage protein ferritin, and dysregulation of these proteins due to Irp2 loss causes functional iron deficiency in β cells. This impairs Fe–S cluster biosynthesis, reducing the function of Cdkal1, an Fe–S cluster enzyme that catalyzes methylthiolation of t6A37 in tRNAlysUUU to ms2t6A37. As a consequence, lysine codons in proinsulin are misread and proinsulin processing is impaired, reducing insulin content and secretion. Iron normalizes ms2t6A37 and proinsulin lysine incorporation, restoring insulin content and secretion in Irp2−/− β cells. These studies reveal a previously unidentified link between insulin processing and cellular iron deficiency that may have relevance to type 2 diabetes in humans.
Diabetes is characterized by high blood glucose levels due to the inability of pancreatic β cells to produce sufficient insulin to meet the needs of the body. Accumulating evidence reveals a role for iron in the pathogenesis of diabetes. Excess body iron stores are associated with an increased risk of type 2 diabetes (T2D) in patients with the genetic iron-overload disease hemochromatosis and in healthy individuals. Phlebotomy improves insulin sensitivity, insulin secretion, and glucose regulation in these individuals showing a causal role of iron in T2D. The mechanisms through which excess iron contributes to development of T2D are not yet fully understood but likely involves insulin resistance as well as impaired pancreatic β-cell function. Iron is important for normal glucose-stimulated insulin secretion; however, excess iron causes oxidative stress and increases apoptosis in β cells. While the consequences of excess iron in β-cell function and survival are established, the effects of iron deficiency on β-cell function and diabetes risk in humans are not yet fully understood.

Eukaryotic cells require iron for survival due to its presence in proteins involved in key cellular processes. Iron is used for the mitochondrial synthesis of Fe–S clusters and heme, which are cofactors for electron transport complexes and tricarboxylic acid (TCA) cycle enzymes and for the maturation of extra-mitochondrial Fe–S proteins involved in DNA metabolism, translation, and tRNA modification. Precise regulation of cellular iron content is crucial as excess iron generates reactive oxygen species (ROS) that damage DNA and proteins, while cellular iron deficiency causes mitochondrial dysfunction and cell cycle arrest. All organisms have thus developed mechanisms to sense, acquire, and store iron.

In vertebrates, cellular iron homeostasis is controlled post-transcriptionally by iron-regulatory protein 1 (Irp1, Aco1) and iron-regulatory protein 2 (Irp2, Ireb2). Irps bind to RNA stem-loops known as iron-responsive elements (IREs) in the 5′- or 3′- untranslated regions (UTRs) of mRNAs involved in iron uptake (transferrin receptor 1; TIR1, Tfrc) and sequestration (ferritin-H and -L subunits; Fth1, Ftl1), and regulate the stability or translation of these mRNAs. When cells are iron deficient, Irps bind IREs with high affinity, inhibiting the translation of ferritin mRNA, while stabilizing TIR1 mRNA. When cells are iron-sufficient, Irps bind with low affinity to IREs, increasing ferritin synthesis, and promoting TIR1 mRNA degradation. Iron regulates Irp1 and Irp2 by different post-transcriptional mechanisms: Irp1 assembles a Fe–S cluster and is converted to cytosolic aconitate lacking RNA-binding activity, while Irp2 is targeted for proteasomal degradation by the E3 ubiquitin ligase Fbxl5,13,14. By sensing cellular iron concentration, Irps regulate the amount of iron acquired by TIR1 and sequestered by ferritin, thus maintaining cellular iron within a narrow range to avoid adverse consequences of iron excess or depletion.

Our studies as well as others reported that Irp2−/− mice develop microcytic anemia caused by dysregulation of iron homeostasis and functional iron deficiency in erythroid precursor cells and in neurons, respectively. Here, we show that Irp2−/− mice develop diabetes as a consequence of functional iron deficiency in pancreatic β cells. We found that cellular iron deficiency impairs the function of Cdk5-regulatory subunit-associated protein 1-like 1 (Cdkal1), a radical S-adenosylmethionine (SAM) enzyme that contains 4Fe–4S clusters required for its methylthiotransferase activity. Cdkal1 catalyzes the methylthiolation of N9-threonylcarbamoyl adenosine 37 (tA37) in cytosolic tRNAAspUUU to generate ms2t6A37, which is required for the accurate translation of lysine codons in proinsulin. As a consequence of reduced Cdkal1 function in Irp2−/− β cells, lysine codons in proinsulin are misread and proinsulin processing is impaired, reducing insulin content and secretion. The ms2t6A37 modification in tRNAAspUUU is also relevant to humans as genome-wide association studies showed CDKAL1 as a strong T2D susceptibility gene. Our studies show a critical role for Irp2 in the regulation of β cell iron homeostasis and reveal a previously unrecognized role for iron in proinsulin processing and insulin secretion in these cells.

**Results**

Irp2−/− mice develop glucose intolerance. While performing neurological analysis of aged Irp2−/− mice, we found that plasma glucose concentrations were elevated in both random-fed and fasted 12-month-old male Irp2−/− mice compared with WT (random-fed: WT, 150 ± 13 mg/dl versus Irp2−/−, 211.2 ± 13.6 mg/dl glucose, n = 9–10, p < 0.01, and fasted: WT, 95.0 ± 5.3 mg/dl versus Irp2−/−, 118 ± 7.1 mg/dl glucose, n = 7–10, p < 0.05). Glucose concentration also showed a tendency to be elevated in random-fed 3-month-old male Irp2−/− mice, and significantly elevated in female Irp2−/− mice (Supplementary Table 1). The observed changes in glucose homeostasis in 3-month-old mice were not attributable to alterations in lipid homeostasis as plasma total cholesterol, triglycerides, nonesterified fatty acids, and electrolytes were similar in WT and Irp2−/− mice (Supplementary Table 1). Similar to aged Irp2−/− mice, male and female 3-month-old mice showed mild microcytic anemia characterized by reduced hemoglobin and hematocrit and decreased erythrocyte indices (Supplementary Table 2).

To assess whole-body glucose metabolism, intraperitoneal glucose tolerance tests (ipGTTs) were performed on overnight fasted male WT and Irp2−/− mice. After glucose injection, Irp2−/− mice at 5-months of age exhibited an increased peak glucose concentration and reduced glucose clearance compared with WT mice (Fig. 1a). Increased area under curve (AUC) values indicated that Irp2−/− mice had developed glucose intolerance already at age of 2.5-months without worsening over time in mice at ages 5, 12–16, and 18 months (Fig. 1b). At all ages, Irp2−/− mice showed significantly higher elevated fasting glucose levels compared with WT mice (Fig. 1c). WT and Irp2−/− mice had indistinguishable body weights at 3.5-months of age; however, starting around 7-months of age, body weight of Irp2−/− mice was significantly lower compared with WT mice, and Irp2−/− mice showed reduced fat mass and increased lean mass (Supplementary Fig. 1a–c). Irp2−/− female mice (10- and 20-weeks of age) also displayed significant but less pronounced glucose intolerance compared with males (Supplementary Fig. 2a, b), and therefore, subsequent studies were performed solely in male mice.

To determine whether impaired glucose clearance in Irp2−/− mice is due to insulin resistance, insulin tolerance tests were performed in 7-month-old WT and Irp2−/− mice. Comparable glucose disposal rates suggested glucose intolerance in Irp2−/− mice was not a consequence of insulin resistance (Fig. 1d). We next performed euglycemic-hyperinsulinemic clamps in overnight fasted 7-month-old mice in order to analyze whole-body and organ-specific insulin action in more detail. When exogenous insulin was administered to Irp2−/− mice, they displayed increased insulin sensitivity evident by higher glucose infusion rates compared with age- and body composition-matched WT mice (Fig. 1e). Comparable whole-body glucose turnover rates (Fig. 1f) in both genotypes suggested improved insulin sensitivity in Irp2−/− mice is not caused by peripheral alterations in insulin action, but rather a more pronounced suppression of hepatic glucose production by insulin (Fig. 1g). Taken together, these data suggest that glucose intolerance in Irp2−/− mice is primarily related to impaired β-cell function.
Insulin secretion is blunted in Irp2−/− mice. To determine whether diabetes in Irp2−/− mice is caused by insulin insufficiency, we measured plasma insulin levels after intraperitoneal glucose injection. In the fasted state (0 min), basal insulin levels in 7- and 18-month-old Irp2−/− mice were similar to their WT controls (Fig. 2a, b). Intraperitoneal glucose injection in 7-month-old Irp2−/− mice is followed by an increase in plasma insulin concentrations from baseline levels, but is blunted compared with WT mice, and was weaker in 18-month-old mice, suggesting an age-dependent effect (Fig. 2a, b). To measure pancreatic ß-cell sensitivity in response to elevations in plasma glucose, hyperglycemic clamps were carried out in overnight fasted 7-month-old WT and Irp2−/− mice. Irp2−/− mice showed fasting hyperglycemia (WT, 6.06 ± 0.23 versus Irp2−/−, 7.17 ± 0.39 mmol glucose/L, p = 0.02; Fig. 2c) and reduced basal plasma insulin concentrations (WT, 1.87 ± 0.773 versus 0.489 ± 0.105 ng/ml insulin, p < 0.05; Fig. 2d). Insulin secretion in response to hyperglycemia (~18 mM) is blunted in Irp2−/− mice (Fig. 2d). Calculation of the AUC during the first phase (0–15 min) and steady-state second phase (60–105 min) of the clamp showed that insulin secretion was reduced by 62% (p < 0.05) and 67% (p < 0.05), respectively, in Irp2−/− mice compared with WT mice (Fig. 2e). These results indicate that glucose intolerance in Irp2−/− mice is caused by impaired insulin secretion from ß cells.

Irp2 deficiency causes proinsulin accumulation in ß cells. To determine the basis for reduced insulin secretion in Irp2−/− mice, pancreatic insulin content was quantified in WT and Irp2−/− mice. The total pancreatic insulin was reduced in 2.5-, 7.5-, and 18-month-old Irp2−/− mice compared with age- and weight-matched WT mice (Fig. 3a). By contrast, pancreatic proinsulin content and the proinsulin-to-insulin (P/I) ratio significantly increased in 2.5-, 7.5-, and 18-month-old Irp2−/− mice compared with WT mice (Fig. 3b, c). The plasma P/I ratio also significantly increased in 7.5-month-old Irp2−/− mice compared with WT mice (Fig. 3d). Reduced insulin content in Irp2−/− mice was not due to changes in insulin transcription as transcript levels of the two insulin genes, Ins1 and Ins2, were similar to WT islets (Fig. 3e). Morphometric quantification of insulin-stained paraffin-embedded pancreatic sections showed that islet area and ß-cell mass in 2.5- and 7.5-month-old Irp2−/− mice were similar to age-matched WT mice, although islet area and ß-cell mass tended to be reduced in 18-month-old Irp2−/− mice (Fig. 3f, g; Supplementary Fig. 3a–d). Insulin, glucagon, and the glucose transporter Glut2 immunostaining displayed normal subcellular localization, showing islet morphology is normal in Irp2−/− islets (Supplementary Fig. 4a, b). These results suggest that proinsulin processing to mature insulin is impaired in Irp2−/− ß cells. We next assessed the secretory responses of WT and Irp2−/− islets under conditions of basal (2.5 mM) glucose and high (16.7 mM) glucose concentrations in a 1-h static assay. Consistent with pancreatic studies, insulin content decreased, and proinsulin content and the P/I ratio increased in Irp2−/− islets compared with WT islets (Fig. 3h–j). Insulin secretion was blunted in Irp2−/− islets under high glucose compared with WT islets (Fig. 3k), while proinsulin secretion increased in Irp2−/− islets under basal glucose compared with WT islets and further increased under high glucose (Fig. 3l). To account for reduced insulin content in Irp2−/− islets, insulin secretion was calculated as a percentage of total insulin content. Normalization of insulin secretion to total islet insulin content showed that Irp2−/− islets secreted similar amount of insulin compared with WT, suggesting that impaired insulin secretion results mostly from reduced insulin content (Fig. 3m). Normalization of proinsulin secretion to total proinsulin content showed that Irp2−/− islets secreted more of their total proinsulin compared with WT islets under high glucose, suggesting that proinsulin secretion is enhanced by Irp2 deficiency (Fig. 3n). Together, these data suggest that impaired glucose-stimulated insulin secretion in Irp2−/− islets is primarily caused by reduced insulin content, and that this defect is intrinsic to the ß cell.

Cellular iron homeostasis is dysregulated in Irp2−/− islets. Given that Irp2 controls cellular iron homeostasis by regulating
the expression of ferritin and TIR1, the levels of Fth1- and Ftl1-subunits, and TIR1 were assessed in WT and Irp2−/− islets. Western blot analysis showed no detectable Irp2 and no change in Irp1 levels in Irp2−/− islets (Fig. 4a). Fth1 levels were similar in WT and Irp2−/− islets, while Fth1 was not detected in WT islets, but notably increased in Irp2−/− islets (Fig. 4a). As expected, TIR1 protein and mRNA levels were lower in Irp2−/− islets compared with WT islets, consistent with destabilization of TIR1 mRNA caused by Irp2 loss (Fig. 4a, b). Ftl1 mRNA levels were similar in WT and Irp2−/− islets, but unexpectedly, Fth1 mRNA levels decreased in Irp2−/− islets, which may be a transcriptional response to compensate for increased Fth1 protein (Fig. 4b).

Double-immunofluorescence studies revealed prominent ferritin coexisting with insulin, and reduced TIR1 coexisting with insulin in Irp2−/− β cells compared with WT with TIR1 with agreement in western blot analysis (Fig. 4c, d). Consistent with reduced TIR1 levels, total iron content, as quantified by inductively coupled proton optical emission spectroscopy (ICP-OES), was reduced by 48% in Irp2−/− islets compared with WT islets, whereas the content of other metals (Cu, Mn, and Zn) was unchanged (Table 1). These data show that Irp2 deficiency in β cells reduces TIR1 levels and iron content, and increases ferritin levels and iron sequestration, causing functional cellular iron deficiency.

Iron normalizes insulin secretion in Irp2−/− mice. Because cellular iron homeostasis is dysregulated in Irp2−/− islets, we questioned whether insulin and proinsulin secretion can be normalized by iron supplementation in Irp2−/− islets. For these experiments, insulin and proinsulin secretion were first assessed in freshly isolated WT and Irp2−/− islets under basal glucose (2.5 mM). Islets were then cultured overnight in medium containing the iron chelator desferrioxamine (DFO) or iron (ferric ammonium citrate (FAC)) and assessed for insulin and proinsulin secretion under high glucose (16.7 mM). Consistent with islet studies in Fig. 3k, l, insulin secretion was blunted and proinsulin secretion increased in Irp2−/− islets compared with WT islets (Fig. 5a, b). DFO reduced insulin secretion and increased proinsulin secretion in WT islets compared with untreated WT islets (Fig. 5a, b), while FAC had no significant effect on insulin and proinsulin secretion in WT islets, but normalized insulin and proinsulin secretion in Irp2−/− islets to levels observed in untreated WT islets (Fig. 5a, b). Likewise, DFO reduced insulin content and increased proinsulin content in WT islets, while FAC normalized insulin content and partially normalized proinsulin content in Irp2−/− islets to levels observed in untreated WT islets (Fig. 5c, d). FAC also restored the proinsulin-to-insulin ratio in Irp2−/− islets to the untreated WT ratio (Fig. 5e). These results show that iron normalizes proinsulin and insulin secretion and content in Irp2−/− islets.

To determine whether iron supplementation normalizes insulin and proinsulin content in vivo, WT and Irp2−/− mice were intraperitoneally injected with iron dextran or PBS as a control for 5 days, after which mice were sacrificed, and pancreatic insulin and proinsulin content were quantified. Iron dextran increased pancreatic and liver iron content in WT and Irp2−/− mice compared with untreated mice (Fig. 5f, g). Iron dextran partially normalized insulin content and fully normalized proinsulin content in Irp2−/− mice to WT levels (Fig. 5h, i). The partial normalization of insulin content in Irp2−/− mice by iron may be due to reduced TIR1 expression in these islets (Fig. 4a). Of note, iron dextran reduced insulin content in WT
mice (Fig. 5h), suggesting that pancreatic iron overload may impair insulin production in normal mice. Collectively, these data indicate that maintenance of cellular iron homeostasis by Irp2 is critical for normal proinsulin processing and insulin secretion.

**Impaired insulin secretion in Irp2-depleted insulinoma cells.**

The role of Irp2 in insulin production and secretion was further studied in rat insulinoma INS-1 832/13 cells depleted of Irp2 by stable expression of a short-hairpin Irp2 RNA (shIrp2 RNA). These cells provided a tool for elucidating the mechanism...
Fig. 3 Irp2 deficiency leads to proinsulin accumulation in β cells. a Quantification of pancreatic insulin content, b proinsulin content, and c pancreatic proinsulin-to-insulin ratio (P/I) in 2.5-, 7.5-, and 18-month-old WT and Irp2−/− mice. d Plasma P/I ratio in 7.5-month-old WT and Irp2−/− mice. e qPCR shows no difference in Ins1 and Ins2 expression in WT and Irp2−/− islets from 10-month-old mice. Values are normalized to β-actin mRNA and are expressed as fold change relative to WT. f, g Quantification of islet area (f) and β-cell mass (g) in insulin-stained paraffin-embedded pancreatic sections from 2.5-, 7.5-, and 18-month-old WT and Irp2−/− mice. Mass was calculated by multiplying the fraction of insulin-positive β-cell area by pancreatic wet weight. h The total islet insulin content, i proinsulin content, and j islet P/I ratio measured in WT and Irp2−/− islets from 7.5-month-old mice. k, l Glucose-stimulated insulin (k) and proinsulin secretion (l) measured in islets under basal (2.5 mM) glucose and after stimulation with high (16.7 mM) glucose for 1 h and normalized to total islet protein. m, n Insulin (m) and proinsulin (n) secretion measured in islets in (k, l) normalized to total islet insulin or proinsulin content. Data are expressed as means ± s.e.m., unpaired two-tailed Student’s t test for a–j and a one-way ANOVA with Tukey’s multiple comparisons test for k–n, *p < 0.05, **p < 0.01, ***p < 0.001. The number of mice is indicated in parentheses. Source data are provided as a Source Data file.

Fig. 4 Cellular iron homeostasis is dysregulated in Irp2−/− islets. a Western blot analysis of Irp2, Irp1, TIR1, ferritin (Fth1 and Ftl1) in WT, and Irp2−/− islets from 8-month-old mice (n = 3 mice per genotype). β-Actin is a loading control. Note that murine Fth1 migrates slightly faster than Ftl1 on SDS-PAGE. b qPCR of Ftl1, Fth1, and TIR1 in WT and Irp2−/− islets from 8-month-old mice. Values are normalized to β-actin mRNA and expressed as relative fold change to WT islets (n = 6 mice per genotype). Data are expressed as means ± s.e.m., unpaired two-tailed Student’s t test, *p < 0.05, **p < 0.01. c, d Representative paraffin-embedded pancreatic sections from 8-month-old WT and Irp2−/− mice immunostained with antibodies to ferritin (green) and insulin (red) (c) or antibodies to TIR1 (green) and insulin (red) (d). The insulin antibody detects both insulin and proinsulin. Nuclei are stained with DAPI (blue). Scale bar: 50 µm. Source data are provided as a Source Data file.
underlying β-cell dysfunction caused by Irp2 deficiency. Western blot analysis confirmed reduced Irp2 expression in shIrp2 cells concomitant with the expected increase in ferritin and reduction in TIR1 levels compared with cells expressing empty vector (EV) (Fig. 6a). Treatment of cells with DFO stabilized Irp2, reduced ferritin and increased TIR1 levels, whereas FAC reduced Irp2, increased ferritin and reduced TIR1 levels in both EV and shIrp2 cells (Fig. 6a). Irp2 RNA-binding activity, as measured by an RNA-electrophoretic mobility shift assay (RNA-EMSA), decreased in shIrp2 cells concomitant with increased Irp1 RNA-binding activity, consistent with reduced total iron content in these cells (Fig. 6b, c). Consistent with Irp2−/− islets studies, shIrp2 cells displayed reduced insulin content, increased proinsulin content, as well as reduced glucose-stimulated insulin secretion and increased proinsulin secretion compared with EV cells (Fig. 6d–g). When the amount of secreted insulin was normalized to insulin content, shIrp2 cells secreted slightly less insulin compared with EV cells, suggesting that secretion may be somewhat impaired (Fig. 6b). No significant difference in proinsulin secretion was observed under high glucose in EV and shIrp2 cells after normalization to total proinsulin content (Fig. 6i). FAC supplementation restored insulin and proinsulin content, and glucose-stimulated insulin and proinsulin secretion to levels observed in EV cells (Fig. 6d–g). Together, these results show that shIrp2 cells recapitulate the abnormal proinsulin−/−phenotype observed in primary Irp2−/− islets.

### Table 1 Metal content in WT and Irp2−/− islets.

|       | WT (7) | Irp2−/− (6) |
|-------|--------|-------------|
| Cu    | 53.0 ± 8.8 | 48.8 ± 10.3 |
| Fe    | 475.4 ± 48.1 | 250.0 ± 46.7 |
| Mn    | 10.4 ± 1.23 | 10.2 ± 3.8 |
| Zn    | 779.9 ± 41.1 | 640.0 ± 61.3 |

Data are expressed as means (μg metal/islet) ± s.e.m., compared by unpaired two-tailed Students t test. *p < 0.001 relative to WT. Metal content was determined by ICP-OES. The number of mice used are indicated in parentheses. Age of mice, 10-month-old males. Source data are provided as a Source Data File.

### Irp2 deficiency impairs Fe−S cluster protein function.

Previous studies have shown that the activities of Fe−S clusters containing respiratory complexes I, II, and III, and aconitate are reduced in motor neurons of Irp2−/− mice18 and in hepatocytes of Irp1−/−; Irp2−/− mice.25 We therefore examined Fe−S cluster biosynthesis in Irp2-deficient INS-1 832/13 cells by measuring the activity of mitochondrial (m)- and cytosolic (c)-aconitases, and complex I, as well as complex IV, which lacks Fe−S clusters. For these studies, additional Irp2-deficient INS-1 832/13 cells lines were generated using CRISPR/Cas9-editing (sgIrp2.1 and sgIrp2.2), and Irp2 depletion and iron regulation were validated in these cell lines by western blot analysis (Fig. 7a). The total iron content was reduced in sgIrp2.1 and sgIrp2.2 cells compared with control parental INS-1 cells (Fig. 7b). M-aconitase and c-aconitase activity, and complex I activity were lower in sgIrp2.1 and sgIrp2.2 cells, and shIrp2 cells, respectively, compared with control cells, and FAC normalized activities of all proteins to levels observed in control cells (Fig. 7c–e). Reduced c-aconitase activity in sgIrp2.1 and sgIrp2.2 cells is consistent with increased Irp1 RNA-binding activity observed in shIrp2 cells (Figs. 7b, 6b). Complex IV activity was not significantly altered in shIrp2 cells (Fig. 7f). ATP production, as measured under basal glucose (5 mM) and after stimulation with 15 mM glucose, was blunted in sgIrp2.1 and sgIrp2.2 cells compared with control cells with FAC partially normalizing ATP production in sgIrp2.1 and sgIrp2.2 cells (Fig. 7g). Together, these data suggest that cellular iron deficiency caused by loss of Irp2 impairs Fe−S cluster biosynthesis, resulting in reduced mitochondrial and cytosolic Fe−S protein function and ATP production.

The UPR is activated in Irp2-depleted insulinoma cells. Accumulation of proinsulin in the endoplasmic reticulum (ER) lumen can lead to its misfolding or unfolding triggering ER stress and activating the unfolded protein response (UPR).26 Increased proinsulin levels in Irp2-deficient INS-1 cells suggested that the UPR might be activated in these cells. UPR activation was assessed using the UPR indicator markers, phosphorylated eukaryotic translation initiation factor 2α (eIF2α-P), a protein that attenuates translation, and Grp78/BiP, an ER chaperone that increases protein folding.27,28 For these experiments, eIF2α-P and Grp78/Bip levels were examined in control, sgIrp2.1, and sgIrp2.2 cells under basal glucose (5 mM) and after stimulation with 15 mM glucose to induce stress. eIF2α-P increased in sgIrp2.1 and sgIrp2.2 cells under basal and high glucose compared with control cells, while eIF2α-total levels were unchanged (Fig. 7h). Grp78/Bip was highly expressed in all cells and under all glucose conditions at similar levels (Fig. 7h). These data suggest eIF2α-P upregulation in Irp2-deficient INS-1 cells may provide a mechanism to attenuate proinsulin translation, ensuring cellular adaptation to iron deficiency.

### Loss of Irp2 impairs proinsulin translation.

The reduction in Fe−S cluster biosynthesis in Irp2-deficient INS-1 cells suggested the possibility that a Fe−S cluster protein related to proinsulin function might be impaired. After reviewing the literature, we focused on the radical SAM enzyme Cdkal1 that uses 4Fe−4S clusters to catalyze the methylation of t6A to ms2t6A on adenosine 37 in tRNA137,14,19,20 (Fig. 8a). This modification is critical for the accurate reading of lysine AAA and AAG codons in proinsulin, and its loss in β cells in Cdkal1 KO mice and in Cdkal1-deficient INS-1 cells results in reduced insulin content and secretion.20,29 Because many Fe−S proteins depend on their Fe−S clusters for stability, we examined Cdkal1 levels in sgIrp2.1 and sgIrp2.2 cells, and in Irp2−/− islets. Cdkal1 levels were modestly reduced in sgIrp2.1 and sgIrp2.2 cells, but were noticeably reduced with DFO compared with control cells (Fig. 8b). Notably, Cdkal1 levels were reduced in Irp2−/− islets compared with WT islets (Fig. 8c). Brambilla-Lasca et al.29 reported that (pro) chromogranin A (CgA), a protein that is co-sorted and co-processed with proinsulin in insulin secretory granules, was reduced in Cdkal1-deficient INS-1 cells. Similarly, we found reduced CgA levels in Irp2−/− islets compared with WT islets (Fig. 8c). Pcsk1, a prohormone convertase that processes proinsulin and CgA to their mature forms, was unchanged in Irp2−/− islets (Fig. 8c). These data show that Irp2 deficiency results in reduced levels of Cdkal1 as well as CgA in Irp2−/− islets.

Reduced Cdkal1 expression in Irp2−/− islets suggested that the ms2t6A modification may also be reduced. To test this premise, we used liquid chromatography−coupled mass spectrometry (LC-MS) to measure ms2t6A levels in control, sgIrp2.1, and sgIrp2.2 cells. Levels of ms2t6A were significantly reduced in sgIrp2.1 cells, and showed a tendency to be reduced in sgIrp2.2 cells compared with control INS-1 cells (Fig. 8d; Supplementary Fig. 5a). Iron supplementation restored ms2t6A levels in sgIrp2.1 and sgIrp2.2 cells to levels in control cells (Fig. 8d). No significant differences were observed in t6A levels in sgIrp2.1 and sgIrp2.2 cells compared with control cells (Fig. 8e; Supplementary Fig. 5b).

We next questioned the impact of reduced ms2t6A levels on proinsulin translation in Irp2-deficient INS-1 cells. Rat, mouse, and human proinsulin each contain a lysine residue at the
Pcsk1 cleavage site between the A-chain and the C-peptide, and lysine residues within the B-chain, and misreading of these lysine codons could impair proinsulin cleavage and/or folding. To examine proinsulin translation accuracy, control, sgIrp2.1, and sgIrp2.2 INS-1 cells were grown with or without supplemental iron, metabolically labeled with 3H-leucine and 14C-lysine, and the relative incorporation of 14C-lysine versus 3H-leucine in immunoprecipitated proinsulin was determined. The relative incorporation of 14C-lysine versus 3H-leucine in proinsulin in sgIrp2.1 and sgIrp2.2 cells was significantly reduced compared with control cells, and iron supplementation fully normalized proinsulin 14C-lysine incorporation in sgIrp2.1 cells and partially in sgIrp2.2 cells (Fig. 8f).

Together, these data show that reduced Cdkal1 function caused by Irp2 deficiency reduces the modification in tRNA^3CUC, leading to misreading of lysine codons in proinsulin, which impairs proinsulin processing and insulin production (Fig. 8g).
Discussion

Iron overload is a known risk factor in the development of T2D, but the physiological and cellular impact of iron deficiency in β-cell dysfunction and diabetes are not yet fully understood. In this report, we demonstrate that cellular iron deficiency as a consequence of Irp2 loss in β cells causes diabetes. We found that iron deficiency in β cells impairs Fe–S cluster biosynthesis that reduces Cdkal1 function and the ms²t₄As modification in tRNA⁷₄₅₆UUU. As a consequence, lysine codons in proinsulin are misread, proinsulin processing is impaired, and insulin content and secretion are reduced (Fig. 8g). Our work thus implicates iron deficiency as a potential mechanism for β-cell dysfunction in humans.

Single-nucleotide polymorphisms (SNPs) in the CDKAL1 gene locus are a strong risk factor for the development of T2D and are associated with impaired glucose metabolism and insulin secretion in humans. The importance of Cdkal1 in β-cell function arises from studies in Cdkal1 β-cell KO mice showing that the ms⁴t₄A modification in tRNA⁷₄₅₆UUU is critical for the accurate reading of lysine AAA and AAG codons, notably during conditions that increase protein synthesis. In this study, Bacillus subtilis wild-type and ygeV mutants (lack the ms⁴t₄A modification) were transformed with dual-luciferase reporter gene containing replacement of a lysine codon critical for luciferase activity with either AAA or AAG. Upon IPTG induction, luciferase activity was reduced in ygeV mutants expressing either the AAA or AAG compared with wild-type, consistent with misreading of lysine codons caused by loss of ygeV. In mammals, proinsulin synthesis constitutes ~30–50% of the total protein synthesis in the β cell, and the ms⁴t₄A modification in tRNA⁷₄₅₆UUU may be critical for efficient and accurate proinsulin translation. In agreement with studies in Cdkal1 β-cell KO mice, proinsulin accumulation in Irp2-deficient INS-1 cells is associated with UPR activation of PERK-dependent eIF2α phosphorylation, which is known to reduce global translation. Because β-cell mass was not reduced in 2.5- and 7.5-month-old Irp2–/– mice, this suggests that phosphorylated eIF2α-mediated translational attenuation may allow Irp2-deficient β cells to adapt to cellular iron deficiency, thereby providing protection against UPR activation of apoptotic signaling pathways.

Cdkal1 deficiency in INS-1 cells has been shown to be associated with reduced expression of proinsulin as well as other insulin secretory granule proteins, such as (pro) CgA and (pro) ICA512/IA-2, that are processed with proinsulin in insulin secretory granules. Consistent with this study, CgA expression was reduced in Irp2–/– islets compared with WT islets. It is therefore possible that Cdkal1 deficiency may affect the function of other insulin secretory granule proteins that could also contribute to insulin secretory defect observed in Irp2–/– mice.

Irp2 deficiency in β cells is associated with impaired mitochondrial function characterized by reduced complex I and m-aconitase activities, and reduced ATP production. Reduced activity of these proteins is likely due to impaired Fe–S cluster biosynthesis caused by iron deficiency in Irp2-deficient β cells. ATP is required for exocytosis of insulin secretory granules, and reduced ATP production in Irp2–/– β cells could affect glucose-stimulated insulin secretion. Our data, however, showed no major difference in insulin secretion in Irp2–/– islets or Irp2-deficient
INS-1 832/13 cells compared with controls when insulin secretion is normalized to insulin content, suggesting that reduced insulin secretion in Irp2−/− mice and Irp2-deficient cells is mostly caused by reduced insulin content. Of note, studies have reported Cdkal1 insulin secretion in β regulator of adipocyte differentiation in murine 3T3-L1 cells and control parental cells grown in medium with or without supplemental FAC and normalized to total cellular protein, e, f Complex I activity (εCO2/mv/min/mg protein) and complex IV activity (εCO2/mv/min/mg protein) in lysates from control EV and shIrp2 cells grown in medium with or without supplemental FAC and normalized to total cellular protein (n = 5 independent biological experiments). g ATP production in control INS-1, sgIrp2.1, and sgIrp2.2 cells grown in medium with or without supplemental FAC and assayed under basal (5 mM) glucose and after stimulation with 15 mM glucose for 1 h. ATP production was normalized to total cellular protein (n = 5 independent biological experiments). h Western blot analysis of elf2α-P, elf2α-total, and Grp78/BiP levels in sgIrp2.1 and sgIrp2.2 cells under basal glucose (5 mM) and after stimulation with 15 mM glucose. β-Actin is a loading control. Data are expressed as means ± s.e.m., unpaired two-tailed Student’s t test for b and f and one-way ANOVA with Tukey’s multiple comparisons test for c-e and g. *p < 0.05; **p < 0.01; ***p < 0.001. Source data are provided as a Source Data file.

A recent study using a distinct Irp2−/− mouse strain reported that 18-month-old male Irp2−/− mice displayed hyperglycemia, insulin resistance as determined by ITTs, and no change in early-phase glucose-stimulated insulin secretion, while we found that 18-month-old male Irp2−/− mice displayed reduced pancreatic insulin content and glucose-stimulated insulin secretion compared with WT mice. An explanation for this discrepancy is not clear, but may stem from differences in strain backgrounds or different assay conditions.

Iron deficiency and its role in anemia is well known, but the physiological and cellular effects of iron deficiency on non-erythropoietic cells and tissues are less clear. There is one case report of a patient with iron deficiency anemia and diabetes that was attributed to autoantibodies against the TIR1, suggesting the possibility that diabetes in this patient may be related to impaired proinsulin processing shown in our current study. Other studies in humans have shown that iron deficiency is associated with pulmonary arterial hypertension, cardiovascular, and neurodegenerative diseases. Studies in rats have revealed that chronic dietary iron deficiency causes mitochondrial dysfunction and remodeling of the pulmonary vasculature. Similarly, TIR1 deficiency in murine dopaminergic neurons causes mitochondrial dysfunction and neuronal degeneration. Recently, two patients with mutations in IREB2 have been identified that exhibit early onset and progressive neurological disease, and microcytic anemia; however, insulin...
Fig. 8 Irp2 deficiency reduces the ms²⁶A modification in tRNA¹⁰⁵UUU causing misreading of lysine codons in proinsulin. a Secondary structure and sequence of tRNA¹⁰⁵UUU with anticodon loop modifications 2-methylthio-N⁶-threonylcarbamoyl adenosine (ms²⁶A)⁶⁶, methoxycarbonylmethyl-2-thiouridine (mcm⁵s²U)⁶⁶ and pseudouridine-39 (ψ−39). The ms² group on ms²⁶A³⁷ is indicated by a dotted circle. Adapted from Vendeix, et al.⁶⁶. b Western blot analysis of Cdkal1 in control, sgIrp2.1, and sgIrp2.2 INS-1 cells grown in medium with or without DFO or FAC for 18 h. β-Actin is a loading control. Asterisk, nonspecific band. c Western blot analysis of Irp2, Cdkal1, Pcsk1, and CgA in islets isolated from 6-month old WT and Irp2−/− mice. Gapdh is a loading control for Irp2, Cdkal1 and Pcsk1 and β-actin is a loading control for CgA (n = 3 mice per genotype). d–e LC-MS analysis of ms²⁶A (d) and t⁶A (e) modifications in control, sgIrp2.1, and sgIrp2.2 INS-1 cells grown in medium with or without supplemental FAC (n ≥ 3 independent biological experiments). f Relative incorporation of 14C-lysine versus 3H-leucine in proinsulin immunoprecipitated from control, sgIrp2.1, and sgIrp2.2 INS-1 cells grown in medium with or without supplemental FAC (n = 3 independent biological experiments). Data in d, e are expressed as means ± s.e.m., unpaired two-tailed Student’s t test, *p < 0.05, **p < 0.01 relative to control INS-1 cells; data in f are expressed as means ± s.e.m., compared by one-way ANOVA with Tukey’s multiple comparison test, *p < 0.05, **p < 0.01. g Model of Irp2 regulation of proinsulin processing and insulin secretion in β cells. Source data are provided as a Source Data file.
sensitivity and secretion were not reported for these patients. We anticipate that our study will thus have relevance not only to β-cell function but also to other cell types and tissues that are functionally iron deficient. Our findings will also provide a foundation for further investigation of the role of iron deficiency in the pathogenesis of diabetes.

Methods

Animals. Mice with global deficiency Irp2−/− mice were generated by inserting a self-excision cassette containing a neomycin (Neo)’se linked to cre-recombinase (Cre) into exon 3 of the mouse Irp2+/- gene, as previously described. This cassette (pACN) contains the Cre gene (driven by the tester-specific angiotensin-converting enzyme (TACE promoter) linked to Neo’ (driven by the polymerase II promoter) and is flanked by loxp sites allowing for excision of Neo’, as it passes through the male germ line. Irp2−/− mice were generated on a C57BL/6J and 129/sv back- ground. Steady state homozygous with C57BL/6J for five generations, Irp2−/− littersm were obtained from intercrosses from Irp2+/− parents. Male and female mice from 2- to 18-months of age were used. Mice were kept in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah (Protocol Number: 18-12108). Mice were euthanized according to AVMA Guidelines for the Euthanasia of Animals. At the GMC, mice were maintained in IVC cages with free access to water and standard mouse chow containing 183 mg Fe/kg (Altromin no.1324, Lage, Germany). All experiments and housing of the animals in the GMC were performed according to the German Law on the Protection of Animals by the Government of Upper Bavaria (Regierung von Oberbayern).

Animal iron treatment. Iron overload in mice was achieved by daily intraper- tonal injections of 1 mg of iron dextran (Sigma-Aldrich, cat # D8517) or PBS as a control for 5 consecutive days. Mice were sacrificed, and pancreas and liver were harvested for iron content by ICP-MS, and pancreas for insulin and proinsulin measurements using ELISAs.

Preparations for glucose clamp experiments. To enable i.v. substrate infusion, a permanent silicone catheter was inserted into the left jugular vein under ketamine/xylazine anesthesia. For matching body fat and fat-free mass, mice were subjected to 1H-NMR analysis (MiniSpec, Bruker Optics Inc, Elltlingen, Germany) 6 days later. On the morning of the seventh postsurgical day, conscious mice were placed in restrainers (Opti-Lab, Munich, Germany) set on top of heating pads. The mice were fasted for 16–18 h but ad libitum water was given. Prior to surgery, the glucose infusion rate (GINF) from the rate of whole-body turnover was acutely raised and maintained at a physiological level until 120 min (10–17 h, and baseline blood glucose was determined from tail-vein blood (Ascencia Elite XL Glucometer, Bayer Corp and the Accu Check Aviva, Roche). Conscious animals were then challenged with an intraperitoneal (i.p.) injection of insulin (2 g/kg body weight, Sigma-Aldrich), and blood glucose levels were determined at 15, 30, 60, 90, and 120 min post injection. Animals were tested at 10–12 weeks, 20–22 weeks, and 74–78 weeks of age. For insulin tolerance tests, random-fed conscious animals (20–22 weeks and 50–64 weeks of age) were injected with human recombinant insulin (0.75 U/kg body weight, Novolin R, Novo Nordisk). Tail-vein blood samples were taken before and at 15, 30, 60, 90, 120, 130 and 180 min post injection.

Glucose and insulin tolerance tests. For glucose tolerance tests, mice were fasted for 16–17 h, and baseline blood glucose was determined from tail-vein blood (Ascencia Elite XL Glucometer, Bayer Corp and the Accu Check Aviva, Roche). Conscious animals were then challenged with an intraperitoneal (i.p.) injection of glucose (2 g/kg body weight, Sigma-Aldrich), and blood glucose levels were determined at 15, 30, 60, 90, and 120 min post injection. Animals were tested at 10–12 weeks, 20–22 weeks, and 74–78 weeks of age. For insulin tolerance tests, random-fed conscious animals (20–22 weeks and 50–64 weeks of age) were injected with human recombinant insulin (0.75 U/kg body weight, Novolin R, Novo Nordisk). Tail-vein blood samples were taken before and at 15, 30, 60, 90, 120, 130 and 180 min post injection.

Insulin and proinsulin measurements. For plasma insulin and proinsulin analyses, 100 µl of tail-vein blood was collected from overnight fasted mice for baseline insulin measurements, and 30 min post i.p. glucose injection (2 g/kg body weight). For pancreatic insulin and proinsulin content, freshly harvested pancreata were incubated in overnight in acid–ethanol mixture (1.5% HCl in 70% EtOH) at −20 °C. Tissue was homogenized and incubated overnight at −20 °C. Samples were centrifuged at 3000 × g for 15 min at 4 °C. Supernatants were neutralized with equal volume of 1 M Tris-HCl pH 7.5 and centrifuged at 15,000 × g for 10 min at 4 °C. Insulin and proinsulin were quantified in mouse pancreata, islets and plasma, and in INS-1 832/13 cells using a Mouse Insulin ELISA (ALPCO, cat# 80-INSMS-E01) and a Rat/Mouse Proinsulin ELISA (Merodia, cat# 10-1232-01), respectively, and adapted reagents from Olympus (Hamburg, Germany). Creatinine was measured using a kit from Biomed (Oberschleitheim, Germany) and NEFA (nonesterified fatty acids) using a kit of WAKO Chemicals (Neuss, Germany). Twenty-three different parameters were measured including various enzyme activities, and plasma concentrations of specific substrates and electrolyte. The routine applied clinical chemistry platform. The total iron-binding capacity (TIBC) was determined and transferrin saturation calculated in these animals. Data were statistically analyzed with the level of significance set at p<0.05 by ANOVA.

Glucose clamp and insulin secretion assays. For glucose clamp experiments, mice were fasted for 16–17 h, and baseline blood glucose was determined from tail-vein blood (Ascencia Elite XL Glucometer, Bayer Corp and the Accu Check Aviva, Roche). Concentric animals were then challenged with an intraperitoneal (i.p.) injection of glucose (2 g/kg body weight, Sigma-Aldrich), and blood glucose levels were determined at 15, 30, 60, 90, and 120 min post injection. Animals were tested at 10–12 weeks, 20–22 weeks, and 74–78 weeks of age. For insulin tolerance tests, random-fed conscious animals (20–22 weeks and 50–64 weeks of age) were injected with human recombinant insulin (0.75 U/kg body weight, Novolin R, Novo Nordisk). Tail-vein blood samples were taken before and at 15, 30, 60, 90, 120, 130 and 180 min post injection.

Euglycemic-hyperinsulimic clamp. Experiments were carried out after 16 h fasting, 30.5 ± 0.3 weeks old, male WT (n = 8) and Irp2−/− (n = 10) mice. By means of a primed-continuous insulin infusion (100 mU/kg min−1 within 3 min, then 3.5 mU/kg min−1), Humin-R, Eli Lilly, Indianapolis), insulin levels were acutely raised and maintained at a physiological level until 120 min (“steady state” conditions, pmol/L: 372 ± 8.66 Irp2−/− vs. 374 ± 6.4 in WT), as previously described. The resulting decline in plasma glucose concentration was counteracted by an i.v. glucose solution at 2% (110 mM) to maintain the glucose infusion rate. “Steady state” insulin infusion rate (GINF) equals whole-body glucose utilization, and is considered an index of an organ’s susceptibility to exogenous insulin. To assess whole-body glucose turnover, a continuous [3-3H]glucose infusion (0.1 µCi/min, Biotrein, Cologne, Germany) was administered during a 120 min basal tracer equilibration period and continued throughout the subsequent euglycemic-hyperinsulimic clamp. In order to allow estimation of insulin-stimulated glucose uptake in individual tissues, an iv. injection of a 2-deoxy-D-[3H]glucose bolus (10 µCi/30 g body weight, Biotrein, Cologne, Germany) was administered at “steady-state” min 75. The experiment was terminated by an i.v. ketamine/xylazine injection, and tissues (liver, M. gastrocnemius and quadriceps, epididymal adipose tissue, heart) were immediately dissected and freeze-clamped. Plasma [3-3H]glucose, H2O2 (measurement of water-borne glycolysis rate) and 2-14C[DG]CO2 concentration between clamp min 75 and 120 and in the final 10 min of the basal period were measured in Somogyi filtrates. The rate of whole-body glucose turnover was calculated as the ratio of [3-3H]glucose infusion rate (dpm/min) and plasma [3-3H]glucose-specific activity (dpm/min µmol) during “steady-state”. Hepatic [3-3H]glucose production (HGP) was deter- mined by subtracting the “steady-state” glucose infusion rate (GINF) from the rate of glucose whole-body turnover. Statistical analyses were made using a two-tailed Student’s t test, and for all hypotheses the significance level was p<0.05. All data are expressed as means ± s.e.m.
assays were performed for each mouse. Islets were then treated with 16.7 mM glucose for an additional 1 h at 37 °C. Islets were sonicated in 1 mL HBSS at setting 4 of a Branson Sonifier II (Branson Sonic Power Co.) and lysates used for insulin and proinsulin quantitation.

For iron and DFO experiments, freshly isolated islets were assessed for insulin and proinsulin secretion in KRB–HEPES buffer containing 2.5 mM glucose, as described above. Islets were then cultured overnight in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05% β-mercaptoethanol (β-ME), and 5% gentamicin along with DFO (50 μM) or FAC (50 μg/mL) at 37 °C with 5% CO2. Islets were washed and then incubated in KRB–HEPES buffer supplemented with 16.7 mM glucose for an additional 1 h at 37 °C. Supernatants were collected and islets sonicated as described above.

For glucose-stimulated insulin and proinsulin secretion assays in EV and shIrp2 expressing INS-1 832/13 cells. ~0.05 × 10^6 cells were plated onto a 24-well plate and grown to 100% confluence. The standard culture medium containing 11 mM glucose was changed to medium containing 5 mM glucose 18 h before performing secretion assay[51]. Cells were washed and pre-incubated for 2 h in HBSS (20 mM HEPES, pH 7.2 with 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 11.6 mM MgSO4, 2.5 mM CaCl2, 25.3 mM NaHCO3, and 0.2% BSA). Insulin and proinsulin secretion were measured by static incubation in 0.8 mL of HBSS containing 5 mM or 15 mM glucose for a 2 h-period. For all experiments, insulin and proinsulin secretion and content were assayed by the Mouse Insulin ELISA and the Rat-Mouse Proinsulin Plus Protein assay Reagent.

**Generation of stable Irp2-deficient insulinoma cell lines.** The rat insulinoma INS-1 832/13 cell line was kindly provided by Dr. Chris Newgard (Duke University). Cells were cultured in the RPMI-1640 medium supplemented with 10% fetal calf serum, 20 mM L-glutamine, 1 mM sodium pyruvate, 0.05% β-ME and 5.0 μg/mL puromycin, and grown at 37 °C with 5% CO2 as described[52]. For Irp2 depletion by short-hairpin RNA (shIrp2 RNA), INS-1 832/13 cells were stably transduced with empty vector (EV) (pENTL-OX3.7puro) or shIrp2 RNA (prientox3.7puro:IrP2-4) kindly provided by Dr. Othon Iliopoulos (Massachusetts General Hospital Cancer Center). The sequences for shIrp2 RNAs are: Irp2-forward: 5′-TGAGTTCTTGGGTGGGGTTGGGCG-3′; Irp2-reverse: 5′-TGCAAGAAAAAGATCTTGTGGGGGGTTGGAAGAGACCCCTTCCCACTTCTTTC-3′. shIrp2-2-forward: 5′-GGCTGGAAGAACAACGCGCC-3′; shIrp2-2-reverse: 5′-GGGAGGCGCACTACTACGGCG-3′; shIrp2-2-reverse: 5′-GGGAGGCGCACTACTACGGCG-3′; sh-Irp2-3-forward: 5′-GGCTCCTGGCTGTGAGGGCCAC-3′; sh-Irp2-3-reverse: 5′-GCCAACGATACCCCACTGCGG-3′. INS-1 832/13 cells were grown to 70–90% confluence in complete RPMI-1640 medium on 60-mm tissue culture plates. Cells were thoroughly washed with 1× Dulbecco’s phosphate-buffered saline (DPBS, Thermofisher cat# 14190250) and transfected with 20 μg of shIrp2-1 or shIrp2-2 plasmid constructs containing a CMV promoter using Lipofectamine 2000 (ThermoFisher cat# 15200-023; 11668027). Transfected cells were selected 48 h later with 5 μg/mL puromycin dihydrochloride. Puromycin resistant cells were assayed for loss of Irp2 by western blot analysis.

**Western blot analysis.** Isolated islets (100–150) were lysed in Triton Lysis Buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 mM DTT, and Halt Protease & Phosphatase Inhibitor Cocktail (Thermofisher, cat# 78464) using a micro-dounce. Lysates were cleared by centrifugation, and protein concentration was determined using Coomassie Plus Protein assay Reagent. Islets were lysed in bovine deoxycholate sodium (LDS) sample buffer (Invitrogen) containing 2.5% β-ME and analyzed by NuPAGE®T-12% Bis-Tris gel (Invitrogen) with MES SDS-running buffer. INS-1 832/13 cells expressing EV, shIrp2-1, or shIrp2-2 were treated with fenoldopam citrate (FAC) (50–50 μg/mL) or the iron chelator deferoxamine (50 μM) for 18 h, and lysed in Triton Lysis Buffer and cell lysates (15–30 μg) protein were analyzed by 4–12% Bis-Tris gels. Proteins were transferred to a Hybond®-ECL nitrocellulose membrane (Amersham) and probed with the following antibodies: chicken anti-Irp1 polyclonal antibody (1:10,000) (Dako, cat# A0564, 1:200) for 1 h at room temperature. Images were acquired using an Olympus FV1000 confocal microscope at the same time using identical camera settings.

**Quantitative real-time RT-PCR (qPCR).** The total RNA was extracted from isolated pancreatic tissues of 8–12-week-old WT and Irp2+/− mice using TRIzol reagent (Invitrogen). cDNA synthesis was performed with total RNA (0.5 μg) using SuperScript III First-Strand synthesis SuperMix for qPCR (Invitrogen). qPCR was performed on an ABI Prism 7900HT Sequence Detection System using TaqMan master mix and TaqMan Gene Expression Assays (ThermoFisher, cat# 20603835; 1:2000) or non-denaturing PAGE (acrylamide/methylene bisacrylamide ratio, 60:1), and the gel was exposed to a PhosphorImage scanner for analysis.

**Tissue metal content measurement.** Metal content was determined by digesting 20–30 mg of pancreas or liver or 100–200 isolated islets in 40% metal-free nitric acid at 95 °C. Samples were diluted in water and analyzed by PerkinElmer Optima 3100XL inductively coupled plasma optical emission spectrometer (ICP–OES) for Ag and Zn 7900 ICP (inductively coupled plasma mass spectrometer (ICP–MS)) for liver and pancreas. Metal concentrations were corrected for dilution and normalized to the total protein or number of islets. Iron content in shIrp2, sgIrp2.1, sgIrp2.2, and control INS-1 832/13 cell lines was determined by ICP-MS. Iron content was normalized to total protein.

**Immunohistochemistry and immunofluorescence.** pancreata were fixed with 4% buffered formalin immediately after dissection and embedded in paraffin. Pancreatic sections (3 μm) were rehydrated with xylene followed by decreasing concentrations of ethanol, and antigen retrieval was performed using Target Retrieval Solution (Dako, cat# S1699) at 90 °C for 30 min followed by cooling at room temperature for 20 min. Sections were incubated in blocking solution (5% donkey serum, 1% BSA) for 1 h at room temperature and then stained with guinea pig anti-insulin PAb (Dako, cat# A0564, 1:200) in PBS and incubated with Peroxidase AfinifPure Donkey anti-guinea pig IgG secondary antibody (Jackson ImmunoResearch, cat# C706-035-48, 1:1000) for 1 h and visualized by 3′,3-diaminobenzidine (DAB). For islet morphometric analyses, sections were taken at 120 μm intervals. Images (4×) spanning the entire pancreas were acquired using a Nikon Wildfield CCD/Spinning Disk microscope that automatically stitched images together to generate a compound image. Image J software was used to quantify islet area and the fraction of insulin-stained tissue area in four sections per pancreas for 2.5–7.5, and 18-month-old mice (n = 3–6 mice per genotype). Five cell clusters were considered to be an islet. β-cell mass was calculated by multiplying the fraction of insulin-stained tissue area by pancreas weight. Hematoxylin and eosin (H&E) staining of paraffin-embedded pancreatic sections was performed by the Huntsman Cancer Biorepository and Molecular Pathology Shared Resource (University of Utah).

For double-immunofluorescence studies, pancreatic sections were deparaffinized and antigen retrieval was performed as described above. Sections were incubated in TBST blocking solution (Tris-buffered saline containing 0.1% Tween) containing 1% fetal calf serum for 1 h at room temperature and then incubating with mouse anti-ferritin antibodies in TBST at 4 °C for 16 h: rabbit anti-ferritin PAb (UT106, 1:1000), mouse anti-TIRI MAb (H68.4, Thermofisher, cat# 13-6800, 1:250), rabbit anti-GLUT2 PAb (Millipore, cat# AB1822-2, 1:2000). Sections were washed in TBST, costained with guinea pig anti-insulin PAb (Dako, cat# A0564, 1:200) for 1 h, washed in PBS and incubated with secondary antibodies Alexa Fluor™ 488 goat anti-mouse (Thermofisher, cat# A-11007, 10 μg/ml) along with Alexa Fluor™ 488 goat anti-rabbit (Thermofisher, cat# A-11034, 10 μg/ml) or Alexa Fluor™ 488 goat anti-mouse (Thermofisher, cat# A-11001, 10 μg/ml) in TBST plus 1% BSA for 1 h at room temperature. Images were acquired using an Olympus FV1000 confocal microscope at the same time using identical camera settings.

**Enzyme activity and ATP measurements.** Complex I and IV activities were measured in INS-1 832/13 cell extracts using Complex I Enzyme Activity Dipstick Assay Kit (Abcam, cat# ab109720) and Complex IV Rotenone Enzyme Activity Assay Kit (Abcam, cat# ab109911) following the manufacturer’s protocols. Whole-
cell extracts (25 µg for Complex 1 assay and 30 µg for Complex IV assay) were used. Aconitase activity was measured in INS-1 832/13 cells enriched mitochondrial and cytosolic fractions (Mitochondria Isolation Kit for Cultured Cells, ThermoFisher, cat# 98974) using the Aconitase Enzyme Activity Microplate Assay Kit (Abcam, cat# 109712). Cell lysates (n = 4 independent biological experiments) were stored at –80 °C and then assayed together. For measurement of intracellular ATP, INS-1 832/13 cells were preincubated in HBBS (secretion buffer) with 2.5 mM glucose for 30 min at 37 °C. Cells were then incubated with 5 mM glucose or with 16.7 mM glucose for 1 h. ATP content was measured in triplicate samples using the ATP Determination Kit (Molecular Probes, cat# A22066) and normalized to total cellular protein. For enzyme activity and ATP assays, cells were treated with FAC (10 µg/mL) for 18 h before performing the assay.

**Mass spectrometric analysis of the ms24A modification.** All chemicals and reagents used were obtained at the highest purity available and were used without further purification unless stated. Benzonase, calf intestinal alkaline phosphatase, butylated hydroxytoluene, actoneitrile, and buffer salts were purchased from Sigma-Aldrich. Coformycin was obtained from the National Cancer Institute. Phosphodiesterase I was purchased from Worthington (cat# LS003926). Water Sigma-Aldrich. Coformycin was obtained from the National Cancer Institute. Parent 7 V, N2-gas temperature 250 °C, N2 gas electro-spray ionization (ESI-MS), fragmentor voltage 380 V, cell accelerator voltage 1800 V. Modiﬁcation 14C-lysine and 3H-leucine labeling mode. The MS was operated in positive ion mode with the following parameters:

\[
-\text{capillary} \ 1800 \ V; \ \text{MS acceleration} \ 14 \ \text{V/nsec} ; \\
-\text{ion source} \ \text{temperature} \ 327; \ \text{correction} \ \text{source} \ \text{voltage} \ 200 \ \text{Volts}.
\]

The mass of the parent ion was 123.15, and mass transitions of m/z 459 → 227, corresponding to the neutral loss of ribose. Data analysis: In each sample, the MS peak areas for the four canonical ribonucleosides (cytidine, uridine, guanosine, and adenosine) were integrated and summed. Peaks corresponding to the transitions for modiﬁed nucleosides were normalized to the summed peak areas of the canonical nucleosides to correct for the amount of injected RNA. The adjusted peak areas of the knockout and iron-supplemented samples were then calculated as a percentage of the INS-1 832/13 control sample.

**Metabolic 14C-lysin and 3H-leucine labeling.** Parental control, sgrp2.1, and sgrp2.2 INS-1 832/13 cells were grown to 90% confluence in medium with or without supplemented FAC (10 µg/mL) for 18 h. Cells were then incubated with KRB containing 0.1% BSA and 2.8 mM glucose for 30 min at 37 °C. The buffer was without supplemented FAC (10 µg/mL) for 18 h. Cells were then incubated with 2.8 mM glucose for 30 min at 37 °C. Cells were then incubated with 5 mM glucose or with 16.7 mM glucose for 1 h. ATP content was measured in triplicate samples using the ATP Determination Kit (Molecular Probes, cat# A22066) and normalized to total cellular protein. For enzyme activity and ATP assays, cells were treated with FAC (10 µg/mL) for 18 h before performing the assay.

**Statistical analysis.** All experiments were performed using at least three independent biological replicates that reflect different sources of material. Data are expressed as means ± standard error (s.e.m.). For two group comparison, an unpaired two-tailed Student’s t test was used. For multiple group comparisons, one-way ANOVA was used followed by Tukey’s multiple comparison test. For all hypotheses, the significance level was p < 0.05. Statistical analyses were performed using GraphPad Prism 7.0, 7.04 and 8.30, and Excel 2013.

**Data availability**

The source data underlying Figs. 1a–g, 2a–e, 3a–n, 4a,b,e, 5a–i, 6a, c–i, 7a–h, 8b–f, and Table 1 and unprocessed gel scans are provided as a Source Data file. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conceived the experiments and wrote the paper: E.A.L., M.C.F.S., C.P.A., S.N., K.B.Z., and P.C.D. Supervised the study at the GMSC: V.G., H.F., E.W., J.R. and M.H.A. Performed experiments: E.A.L., M.C.F.S., C.P.A., S.N., K.B.Z., S.R., M.B.S., B.R., J.R., W.M.C., M.R. and J.H. Analyzed the data: E.A.L., M.C.F.S., C.P.A., S.N., K.B.Z., and P.C.D. Writing, reviewing, and editing: E.A.L., M.C.F.S., S.N., M.R. and J.H. Acknowledgements
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