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Sel1L-Hrd1 ER-associated degradation maintains β-cell identity via TGFβ signaling

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Running title: ERAD maintains β-cell identity by suppressing TGFβ signaling

Keywords: Sel1L-Hrd1 ERAD, autophagy, single-cell RNA-Seq, β-cell identity, dedifferentiation, TGFβ signaling

Summary: Sel1L-Hrd1 ERAD maintains β-cell identity by suppressing TGFβ signaling, rather than cell death.
ABSTRACT

β-cell apoptosis and dedifferentiation are two hotly-debated mechanisms underlying β-cell loss in type 2 diabetes; however, the molecular drivers underlying such events remain largely unclear. Here, we performed a side-by-side comparison of mice carrying β-cell-specific deletion of endoplasmic reticulum (ER)-associated degradation (ERAD) and autophagy. We reported that while autophagy was necessary for β-cell survival, the highly conserved Sel1L-Hrd1 ERAD protein complex was required for the maintenance of β-cell maturation and identity. Using single cell RNA-sequencing, we demonstrated that Sel1L deficiency was not associated with β-cell loss, but rather loss of β-cell identity. Sel1L-Hrd1 ERAD controlled β-cell identity via TGFβ signaling, in part by mediating the degradation of TGFβ receptor 1 (TGFβRI). Inhibition of TGFβ signaling in Sel1L-deficient β-cells augmented the expression of β-cell maturation markers and increased the total insulin content. Our data revealed distinct pathogenic effects of two major proteolytic pathways in β-cells, providing a new framework for therapies targeting distinct mechanisms of protein quality control.
INTRODUCTION
Type 2 diabetes (T2D) is a heterogeneous multi-component disease, where disease initiation and progression are triggered and propelled by pancreatic β-cell dysfunction and death. Recent studies have suggested that β-cells may undergo dedifferentiation into progenitor-like cells (1), losing the expression of maturation markers such as MafA (2) and urocortin 3 (Ucn3) (3) while increasing the expression of endocrine progenitor cell markers, such as aldehyde dehydrogenase 1A3 (Aldh1a3) (4) and neurogenin 3 (Ngn3) (5). In both mouse diabetic models and humans with T2D, β-cell dedifferentiation may represent an early and reversible cause of β-cell loss (1, 4, 6, 7); however, its significance in disease pathogenesis remains under debate (4, 8-10). Recent studies have shown that disruption of TGFβ signaling, either genetically or pharmacologically, increases the expression of β-cell maturation markers (11, 12) and reverses β-cell dedifferentiation (3). These exciting findings have rekindled the hope for early intervention of β-cell loss in the treatment of diabetes; however, our understanding of the molecular events leading to β-cell dedifferentiation remains limited (8).

The ubiquitin-proteasome system and autophagy are the two major intracellular proteolytic pathways. Unlike autophagy, which mediates bulk protein degradation in different cellular compartments, ERAD is the principal mechanism that targets ER-resident proteins for degradation by the cytosolic ubiquitin-proteasome system (13, 14). The Sel1L-Hrd1 protein complex represents the most evolutionarily conserved ERAD machinery (15), in which a single-span ER-transmembrane protein Sel1L is an obligatory cofactor for the ER-resident E3 ligase Hrd1 (16-19). Using cell type-specific animal models, recent studies from several groups including ours have shown that mammalian Sel1L-Hrd1 ERAD mediates indispensable homeostatic processes such as immune cell development, systemic water balance, food intake, and energy metabolism in a largely substrate-specific manner (20-28). However, the physiological significance of ERAD remained largely unclear. Here we report a surprising finding linking Sel1L-Hrd1 ERAD to the maintenance of β-cell identity by suppressing TGFβ signaling, but not β-cell survival and proliferation.
RESULTS

ERAD expression in β-cells
Sel1L and Hrd1 (encoded by the Syvn1 gene) were ubiquitously expressed among different islet cell types, including α, β, δ and γ cells, as revealed by single cell RNA-Seq (scRNA-Seq) analysis of wildtype (WT) mouse islets (Figure S1A-B). Both Sel1L and Hrd1 proteins were detected in insulin-positive murine β-cells (Figure S1C-D). In human pancreas, Sel1L expression was lower in T2D islets than that of healthy islets (Figure 1A-1B, with patient information in Table S1).

Generation of β-cell specific Sel1L knockout (Sel1L\textsuperscript{Ins1}) mice
To elucidate the physiological role of Sel1L-Hrd1 ERAD in β-cells, we generated β cell-specific Sel1L knockout (Sel1L\textsuperscript{Ins1}) mice by crossing Sel1L floxed (Sel1L\textsuperscript{f/f}) mice (16) with Ins1-Cre knock-in mice (29). Sel1L protein level was largely abolished in β-cells of Sel1L\textsuperscript{Ins1} mice as was Hrd1 protein (Figure 1C-D), indicative of compromised Sel1L-Hrd1 ERAD function in Sel1L\textsuperscript{Ins1} β-cells. To assess the relative importance of ERAD in β-cells, we performed a side-by-side comparison of Sel1L\textsuperscript{Ins1} mice with β-cell specific autophagy-deficient mice (Atg7\textsuperscript{Ins1}), generated using the same breeding strategy. The known autophagy substrate p62 was highly elevated in Atg7\textsuperscript{Ins1} islets, as detected by Western blot (Figure 1C) and immunostaining (Figure 1E). In the studies below, age- and gender-matched Sel1L\textsuperscript{Ins1} and Atg7\textsuperscript{Ins1} mice were compared to their own Sel1L\textsuperscript{f/f} and Atg7\textsuperscript{f/f} littermates (collectively named as the WT cohort for simplicity, as there was no difference between them).

Progressive hyperglycemia and glucose intolerance of Sel1L\textsuperscript{Ins1} and Atg7\textsuperscript{Ins1} mice
While indistinguishable from their WT littermates in appearance (including body weight, Figure 2A), both male and female Sel1L\textsuperscript{Ins1} mice progressively developed hyperglycemia, post weaning (Figure 2B-C). In line with previous studies where the RIP-Cre line was used (30, 31), Atg7 deletion in β-cells also had no effect on body weight but both sexes of such animals developed progressive hyperglycemia starting at 8-9 weeks of age (Figure 2A-C). The onset of hyperglycemia in these mice was delayed by approximately one month in comparison to Sel1L\textsuperscript{Ins1} mice. Of note, both sexes of heterozygous Sel1L\textsuperscript{f/+};Ins1-Cre (Sel1L\textsuperscript{Ins1/+}) or Atg7\textsuperscript{Ins1/+} littermates, collectively termed “hets”, remained normoglycemic with age, similar to WT littermates (Figure 2B-C), thus excluding the possible effects of Sel1L, Atg7, or Ins1 haploinsufficiency in β-cell function in vivo.
In line with the trend of progressive hyperglycemia, Sel1L\textsuperscript{Ins1} mice developed glucose intolerance several weeks earlier than Atg7\textsuperscript{Ins1} mice (Figure S2A-B). By 10 weeks of age, both Sel1L\textsuperscript{Ins1} and Atg7\textsuperscript{Ins1} mice were glucose intolerant (Figure 2D and S2C-D) with reduced in vivo glucose-stimulated insulin secretion (Figure 2E) and lower fasting serum insulin levels (Figure 2F). Peripheral tissues such as liver, WAT and BAT all appeared indistinguishable from WT cohorts (Figure S3). Thus, Sel1L-Hrd1 ERAD is indispensable for β-cell function similar to autophagy; however, the onset of hyperglycemia and glucose intolerance in Sel1L\textsuperscript{Ins1} mice precedes those of Atg7\textsuperscript{Ins1} mice.

Sel1L-Hrd1 ERAD is dispensable for β-cell survival
To understand the mechanism underlying β-cell dysfunction in these animal models, we first evaluated islet histology in cohorts with mild hyperglycemia (~200-300 mg/dL glucose level). Morphometric analysis of islets revealed no significant changes in Sel1L\textsuperscript{Ins1} islets, in terms of morphology and β-cell mass, while Atg7\textsuperscript{Ins1} mice had increased β-cell mass (Figure 3A-B). However, cell proliferation and apoptosis as measured by Ki67\textsuperscript{+} and TUNEL\textsuperscript{+} β-cells, respectively, were comparable among the three cohorts (Figure 3C-D and S4A-B). Rather, unlike Sel1L\textsuperscript{Ins1} islets, Atg7\textsuperscript{Ins1} islet had a markedly expanded cell size with increased nucleus-to-nucleus distance (i.e. reduced nuclear density) in islets (Figure 3A and 3E), indicative of cytoplasmic swelling or cell hypertrophy rather than hyperplasia in the absence of autophagy. This surprising finding was further confirmed using flow cytometry measurements of the forward scatter (FSC), i.e. cell size (Figure 3F) and immunostaining with cell surface marker E-cadherin (Figure S4C). Moreover, when blood glucose reached severe hyperglycemia, Atg7\textsuperscript{Ins1} mice exhibited extensive vacuolization and β-cell loss (Figure 3G) as previously described (30, 31). By contrast, even under severe hyperglycemia, Sel1L\textsuperscript{Ins1} islets did not exhibit vacuolization or β-cell loss (Figure 3G).

Lack of cell death in Sel1L\textsuperscript{Ins1} islets prompted us to assess the activation of IRE1α branch of the UPR, a key regulator of β-cell survival and function (32-34). In line with our previous finding that the UPR sensor IRE1α is a substrate of Sel1L-Hrd1 ERAD (25), IRE1α protein accumulated by 3-4-fold in Sel1L\textsuperscript{Ins1} islets (Figure S5A); however, no significant activation of IRE1α was observed in Sel1L\textsuperscript{Ins1} islets as measured by levels of IRE1α phosphorylation using the phos-tag approach (35, 36) (Figure S5B) and Xbp-1 mRNA splicing (Figure S5C). The modest effect of Sel1L deletion on ER homeostasis was likely due to the induction of ER chaperones such as BiP and calnexin (Figure S5A), leading to cellular adaptation as previously reported (20, 21).
Recent in vitro studies using β-cell lines suggested that Sel1L-Hrd1 ERAD may be involved in proinsulin degradation and maturation (37, 38). Much to our surprise, in contrast to these studies, we did not observe any significant changes in proinsulin maturation in Sel1L<sup>inst</sup> islets as demonstrated by the pulse-chase labeling of primary islets to follow nascent proinsulin biogenesis (Figure S5D). Immunofluorescence co-labeling of proinsulin with the ER chaperone BiP further showed that proinsulin was able to mature beyond the ER (Figure S5E). Moreover, although insulin content in primary islets were lower, glucose stimulated insulin secretion in vitro was not defective in Sel1L<sup>inst</sup> islets (Figure S5F-G). After normalizing for insulin content, insulin secretion was even slightly elevated in Sel1L<sup>inst</sup> islets (Figure S5H). Hence, Sel1L effect in β-cells is uncoupled from cell survival, ER stress and insulin secretion.

**Downregulation of mature β-cell markers in Sel1L<sup>inst</sup> mice**

To explore how Sel1L deficiency caused β-cell dysfunction, we next performed non-biased genome-wide cDNA microarray of purified primary islets from 5-week-old mice. Unexpectedly, the endocrine progenitor cell marker Ngn3 was among the top upregulated genes, while β-cell-specific maturation markers such as MafA (39), Ucn3 (40), Glut2, Ins1 and Ins2 were among the top downregulated genes (Figure 4A-B), indicative of immature β-cells. By comparison, forbidden or disallowed genes known to prevent inappropriate insulin release, such as Hk1, Slc16a1, Ldha, Rest and Pdgfra (41) were unchanged in Sel1L<sup>inst</sup> islets (Figure 4B).

To further define the impact of Sel1L deficiency at the single cell level, we performed scRNA-Seq analysis of islets from 7-week-old littermates. Unbiased projection of the single cell transcriptome data identified 7 unique cell clusters, with β, α, δ and pancreatic polypeptide (γ) cells as the major clusters (Figure S6A-B). The percentage of β-cells were comparable (at approximately 62-64%) between Sel1L<sup>inst</sup> and WT islets (Table 1). Dramatically, the bulk of Sel1L<sup>inst</sup> β-cells clustered as a distinct population from WT β-cells, indicating significant changes in the transcriptional landscape in the absence of Sel1L (Figure 4C-D). Expression of mature β-cell markers such as Ins1, Ins2, Ucn3, and MafA were reduced in the Sel1L<sup>inst</sup> β-cell cluster, with concomitant increase in expression of dedifferentiation markers such as Aldh1a3 and the progenitor marker Ngn3 (Figure 4E).

We next confirmed the changes in β-cell identity in Sel1L<sup>inst</sup> islets using immunofluorescence staining and Western blotting. Insulin staining was reduced in Sel1L<sup>inst</sup> islets, with glucagon-
positive α- and somatostatin-positive δ-cells scattered within the core of adult $\text{Sel1L}^{\text{Inst}}$ islets, in contrast to their peripheral localization in WT islets (Figure 5A). Both MafA and Ucn3 were significantly reduced in $\text{Sel1L}^{\text{Inst}}$ β-cells compared to WT littermates (Figure 5B-C and S7). On the other hand, expression of Aldh1a3, a marker of endocrine progenitor cells and dedifferentiated β-cells in mice (6), was highly elevated in $\text{Sel1L}^{\text{Inst}}$ islets (Figure 5D-E). These changes were specific for $\text{Sel1L}^{\text{Inst}}$ islets and absent in $\text{Atg7}^{\text{Inst}}$ islets (Figure 5 and S7).

β-cell development is not affected in $\text{Sel1L}^{\text{Inst}}$ mice

We next asked whether $\text{Sel1L}$ deficiency triggered a β-cell developmental defect. At postnatal (P) day 14, blood glucose, serum insulin and total pancreatic insulin content in $\text{Sel1L}^{\text{Inst}}$ mice was comparable to WT littermates (Figure 6A-C). Moreover, the expression of insulin and glucagon in $\text{Sel1L}^{\text{Inst}}$ islets was robust at both P1 and P14 (Figure 6D-E). Similar to WT littermates, $\text{Sel1L}^{\text{Inst}}$ islets at P14 had normal islet architecture with α-cells at the periphery (Figure 6E). Furthermore, expression and localization of transcription factors MafA and Pdx1 were comparable between the cohorts at P14 (Figure 6F). Hence, β-cell development is unaffected by $\text{Sel1L}$ deficiency.

Elevated TGFβ signaling in $\text{Sel1L}^{\text{Inst}}$ β-cells

To further delineate the link between ERAD and β-cell dedifferentiation, we performed pathway analysis of significantly up- and down-regulated genes in both scRNA-Seq and cDNA profiling datasets. Consistent with the notion of resetting ER homeostasis or adaptation to Sel1L deficiency, genes associated with protein processing, folding and export in the ER were highly upregulated in $\text{Sel1L}^{\text{Inst}}$ β-cells (Figure 7A). By contrast, genes associated with regulation of insulin secretion and glucose metabolism were amongst the most downregulated (Figure 7B). Intriguingly, we noted a significant enrichment of “negative regulation of cell differentiation pathways” including TGFβ and WNT signaling pathways amongst the upregulated genes in scRNA-Seq (Figure 7A) and TGFβ signaling pathway in bulk cDNA microarray analyses (Figure S8A). Indeed, expression of TGFβ-activated genes such as $\text{Nedd9}$ and $\text{Smurf1}$ were increased while TGFβ-repressed genes such as $\text{Cited2}$ and $\text{Plat}$ were reduced in $\text{Sel1L}^{\text{Inst}}$ β-cells (Figure S8B).

TGFβ binding to its receptors triggers the phosphorylation and nuclear translocation of its downstream effectors Smad2/3 (42). Indeed, both phosphorylation and nuclear localization of Smad2/3 were elevated in $\text{Sel1L}^{\text{Inst}}$ islets (Figure 7C-F). These data strongly supported the
notion of increased TGFβ signaling in Sel1L<sup>Ins1</sup> islets. In comparison, Atg7<sup>Ins1</sup> islets did not show significant changes in Smad2/3 phosphorylation or nuclear translocation (Figure 7C-F), indicating that increased TGFβ signaling is specific to Sel1L deficiency in β-cells.

**TGFβ receptor 1 (TGFβRI) is an endogenous ERAD substrate in β-cells**

To explore the possible mechanism underlying increased TFGβ signaling in Sel1L-deficient β-cells, we measured the levels of TGFβRI in isolated islets and found that TFGβRI protein level and stability were increased in the absence of Sel1L (Figure 7G-H). In vitro, Hrd1 readily interacted with TGFβRI and also ubiquitinated TGFβRI in an E3 ligase activity-dependent manner (Figure S8C). Hence, Sel1L-Hrd1 ERAD may regulate TGFβ signaling in β-cells, at least in part via targeting TGFβRI for proteasomal degradation.

**TGFβ signaling links Sel1L to dedifferentiation**

To establish the causal relationship between altered TGFβ signaling and β-cell dedifferentiation in Sel1L<sup>Ins1</sup> islets, we next treated isolated Sel1L<sup>Ins1</sup> islets with TGFβRI-specific inhibitor (ALK5 inhibitor II, Alk5<sub>n</sub>). Inhibition of TGFβRI caused a significant reduction in the levels of phospho-Smad2/3 (Figure S9A) and nuclear exclusion of Smad2/3 proteins in Sel1L<sup>Ins1</sup> islets (Fig. 8A). Moreover, inhibition of TGFβRI significantly increased the gene expression of maturation markers such as MafA, Glut2 and Ucn3 in Sel1L<sup>Ins1</sup> islets (Figure 8B) and protein levels of MafA (Figure 8C and S9B). Indeed, a short-term treatment with the TGFβRI inhibitor increased insulin content in Sel1L<sup>Ins1</sup> islets (Figure 8D). Thus, Sel1L-Hrd1 ERAD regulates β-cell identity by suppressing TGFβ signaling in β-cells.
DISCUSSION

Cellular proteostasis has been implicated in the maintenance of β-cell function and the pathogenesis of diabetes (43). The current paradigm of the field states that disturbance of ER homeostasis is causally linked to proinsulin maturation defects and β-cell death (44). However, our data demonstrate that, unlike autophagy and UPR (32-34, 45, 46), ERAD is required for maintenance of β-cell identity while having no significant effect on cell survival. Hence, these principal quality-control mechanisms, ERAD, UPR and autophagy, play distinct roles in β-cells in T2D pathogenesis (Figure 8E). This difference is likely due to the distinct functions of ERAD vs. UPR and autophagy: unlike the latter two whose effects in β-cells are conspicuous, ERAD regulates signaling pathways and cellular function in a substrate-specific manner (28).

Loss of β-cell identity or dedifferentiation is an emerging concept in the pathogenesis of diabetes (8). It has been proposed that β-cells may undergo dedifferentiation as a result of persistent hyperglycemia (47). However, another recent study showed that lowering hyperglycemia in db/db model fails to reverse β-cell dedifferentiation (48). Here, our data show that Sel1L-Hrd1 ERAD plays a key role in the maintenance of β-cell identity by suppressing TGFβ signaling pathway. Inhibition of TGFβ signaling in Sel1L-deficient β-cells enhances the expression of β-cell maturation markers and insulin content. By direct contrast, despite being severely hyperglycemic, autophagy-deficient mice do not exhibit markers of dedifferentiation. Together, these data suggest that β-cell dedifferentiation in the absence of Sel1L-Hrd1 ERAD is causally linked to elevated TGFβ signaling, not merely as a result of an adaptive response to hyperglycemia.

Our data demonstrate that ER protein degradation by ERAD represents a primary mechanism in the maintenance of β-cell identity, rather than β-cell loss (which is more prominent with autophagy deficiency). Boosting the activity of SEL1L-HRD1 ERAD, in combination with modulating autophagy, may represent a superior strategy to protect β-cells in the treatment of T2D. We speculate that downregulation of SEL1L in human T2D islets may allow β-cells to undergo dedifferentiation. Future investigation into this question will inevitably advance this new exciting line of research.
MATERIALS AND METHODS

Mice. Sel1L\textsuperscript{ff} mice (16) and Atg7\textsuperscript{ff} (49) on the C57BL/6J background were crossed with B6(Cg)-\textit{Ins1}\textsuperscript{tm1.1(cre)ThorJ} (\textit{Ins1}\textsuperscript{Cre}, JAX 026801) (29) on the C57BL/6J background to generate β-cell-specific Sel1L (Sel1L\textsuperscript{Ins1}) and Atg7 (Atg7\textsuperscript{Ins1}) deficient mice with respective control littermates (Sel1L\textsuperscript{ff} and Atg7\textsuperscript{ff}). Atg7\textsuperscript{ff} mice were provided by Dr. Rajat Singh (Einstein College of Medicine) with the permission from Drs. Masaaki Komatsu and Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). \textit{Ins1}\textsuperscript{Cre} mice were provided by Dr. Scott Soleimanpour (University of Michigan). For some experiments, heterozygous Sel1L\textsuperscript{Ins1/+} and Atg7\textsuperscript{Ins1/+} mice were also generated as littermates for Sel1L\textsuperscript{Ins1} and Atg7\textsuperscript{Ins1} mice. In Figure 2, data from both Sel1L\textsuperscript{ff} and Atg7\textsuperscript{ff} control littermates were compiled as WT control littermates; and data from Sel1L\textsuperscript{Ins1/+} and Atg7\textsuperscript{Ins1/+} were compiled as the heterozygous control. All mice were housed in an ambient temperature room with 12 hr light cycle and fed a normal-chow diet (13% fat, 57% carbohydrate, and 30% protein, LabDiet 5LOD). Weekly measurements of body weight and glucose were performed at the same time of the day for consistency.

Power analysis of the animal size in metabolic studies. Based on sample size formula of the power analysis, \(N=8(CV)^2[1+(1-PC)^2]/(PC)^2\), to reach the error = 0.05, Power = 0.80, percentage change in means (PC) = 20%, co-efficient of variation (CV) = 10 ~ 15% (varies between the experiments), 4-6 mice per group are the minimal number of mice to obtain statistical significance. Together with our prior experience, we routinely used a total of 4-6 mice in each study to ensure adequate power. Mice in each group were randomly chosen based on the age, genotype and gender.

Human samples and quantification. Paraffin embedded pancreatic sections from cadaveric donors including non-diabetic controls and type 2 diabetes patients were obtained from the Human Islet Resource Center at the University of Pennsylvania. Donor information was presented in Table S1. The intensity of SEL1L fluorescence was quantified using Fiji software (NIH). In brief, we delimited the area of the islet using the plugin ‘ROI manager’. In each islet, the SEL1L signal was extracted from the green channel as integrated fluorescence intensity. We divided the integrated intensity by the area of the ROI to estimate the fluorescent intensity.
Generation of Sel1L-specific antibody. The cDNA sequence corresponding to the truncated human SEL1L (hSEL1L; amino acids 20 to 260) was sub-cloned into the pET28a(+) plasmid to allow for the expression of the recombinant His6-hSEL1L proteins in BL21(DE3) bacterial cells upon IPTG induction at 16°C for 16 hr. Recombinant His6-hSEL1L proteins were purified using Ni-NTA column chromatography followed by Superdex 200 size exclusion column chromatography. The polyclonal antibody was generated by immunizing rabbits with the recombinant hSEL1L proteins, and further affinity-purified using the same antigen coupled to cyanogen bromide-activated sepharose.

Glucose tolerance tests and in vivo glucose stimulated insulin secretion. Mice were fasted for 6 hr prior to the experiment. For intraperitoneal glucose tolerance test, blood was taken via tail nick. Basal blood glucose was sampled, and glucose administered intraperitoneally at a dose of 1.5 mg/kg body weight. Blood glucose was then measured at 15, 30, 60 and 120 min after glucose administration using OneTouch Ultra glucose strips. Serum was collected at the same time for insulin measurements using ultra-sensitive enzyme linked immunosorbent assay (Crystal Chem #90080) per the manufacturer’s instruction.

Pancreatic insulin content. Pancreata were isolated, weighed and placed into 2 ml of acid-ethanol solution [1.5% HCl in 75% (v/v) ethanol in water] and homogenized for 30 sec. The homogenate was rotated for 24 hr at 4°C for insulin extraction. After centrifugation at 3000 rpm for 30 min at 4°C, supernatant was diluted and insulin content was measured as above.

Hematoxylin and eosin staining (H&E) and morphometric analyses of islets. Pancreata were isolated, fixed in 10% neutral buffered formalin (VWR 95042-908) overnight at 4°C and processed by the University of Michigan Comprehensive Cancer Center for paraffin embedding, sectioning and H&E staining. Slides were imaged and analyzed using the Aperio Scanscope (Leica Biosystems). For β-cell mass, five randomly selected sections, at least 200 μm apart, per pancreas were used. Insulin stained pancreatic sections were imaged at 20x using Nikon A1 wide-field microscope at the University of Michigan Morphology and Image Analysis Core. Total islet area measured using thresholding feature by ImageJ was then divided by the total pancreas area and multiplied by the weight of the pancreas to obtain β-cell mass. To analyze islet area, islet size was measured manually based on their morphology using the Aperio scanscope and expressed as average islet area. For islet nuclei density, total number of nuclei were quantified in 150-200 islets from 5 mice of each genotypes. Islet nuclear density is
expressed as the number of nuclei per 1000 μm² area. All areas and cell quantification were processed with ImageJ software (NIH).

**Proliferation and TUNEL assay.** Paraffin embedded pancreas sections were co-stained with Ki67 (Abcam 15580; 1:100) and insulin (Biorad 5330-0104G, 1:100) as previously described (50). TUNEL assay was performed as per manufacturer’s protocol using the In-Situ Cell Death Detection Kit (Roche, 11684795910). Insulin co-staining was performed to identify β-cells. Images were acquired using Nikon A1 Confocal Microscope at the University of Michigan Morphology and Image Analysis Core from 45-80 islets per animal, which represented 1,500-3,000 β-cells per mice. β-cell proliferation and apoptosis was calculated as a percentage of Ki67 and TUNEL positive cells respectively per total insulin positive cells.

**Immunofluorescent staining.** Paraffin embedded pancreas sections were deparaffinized in xylene and rehydrated using graded ethanol series (100%, 90%, 70%) followed by rinse in distilled water. Antigen retrieval was performed by boiling the slides in microwave in either sodium citrate or EDTA. Sections were then incubated in a blocking solution (5% donkey serum, 0.3% TritonX-100 in PBS) for 1 hour at room temperature and with primary antibodies overnight at 4°C in a humidifying chamber. For MafA staining, cryosections were used. For staining with primary islets, purified islets were dissociated into single-cell suspension using trypsin for 3-5 min at 37°C and seeded in 8-well chamber (Nunc Lab-Tek II chamber slide; 12-565-8) for 24 hr prior to fixation. Cells were fixed in 4% PFA (EMS 15710) for 20 min, permeabilized in 0.3% PBST for 10 min followed by blocking in 5% donkey serum for 1 hour at room temperature. Following primary antibodies were used: Sel1L (1:200), p62 (Enzo BML-PW9860, 1:500), Insulin (Biorad 5330-0104G, 1:5000), Glucagon (Sigma G2654, 1:500), Somatostatin (Abcam ab30788, 1:200), Aldh1a3 (Novus Biologicals NBP2-15339, 1:100), MafA (Novus Biologicals NBP1-00121, 1:100), Ucn3 (Dr. Mark Huising, 1:1000), Pdx1 (Cell Signaling 5679, 1:100) Smad2/3 (Cell Signaling 8685; 1:1000), Proinsulin (DSHB GS-9A8; 1:100), E-Cadherin (BD 610181, 1:500), BiP (Abcam 21685, 1:500). Hrd1 antibody (1:200) was provided by Richard Wojcikiewicz (State University of New York Upstate Medical University, Syracuse, New York, USA). Next day, following three washes with PBST (0.03% Triton X-100 in PBS), slides were incubated with respective Alexa Fluor conjugated to secondary antibodies (Jackson ImmunoResearch; dilution 1:500) for 1 hour at room temperature followed by mounting with Vectashield mounting medium containing DAPI (Vector Laboratories, H-1500). Images were
captured using Nikon A1 Confocal Microscope at the University of Michigan Morphology and Image Analysis Core.

**Islet isolation and inhibitor treatments.** Pancreatic islets were isolated from mice as previously described (50). Briefly, mice were sacrificed by cervical dislocation and immediately processed for pancreatic perfusion. The pancreas was distended via the intraductal injection of 3 mL of Liberase and incubated at 37°C for 12 min in additional 1 mL of digestion solution. Liberase solution was prepared by dissolving 5 mg Liberase TL (Roche, 5401020001) in 20 ml of serum free RPMI (Fisher, 11875-085). Digestion was stopped by adding cold media containing 10% FBS. After gentle shaking for complete dissociation and brief centrifugation, the digested suspension was passed through a nylon mesh and islets were isolated by density gradient centrifugation on a Histopaque gradient (1.077 g/mL density; Sigma) for 20 min at 900 x g without brake. Islets were then collected from the interface, washed and hand-picked under a dissecting microscope. Isolated islets were recovered overnight in RPMI 1640 medium in a humidified incubator (95% air, 5% CO₂) at 37°C. For experiments with the TGFβRI inhibitor, islets from 5-6 week-old mice were cultured with Alk5 inhibitor II (10 µM; Cayman 14794) or vehicle (DMSO) for 24 hr.

**Flow cytometry.** Mouse islets were isolated and cultured with RPMI 1640 medium overnight, then dissociated with 0.5% Trypsin-EDTA. Cells were washed with PBS, fixed in 4% PFA at 4 °C for 15 min and permeabilized with a BD Cytofix/Cytoperm kit according to the manufacturer's protocol. Cells were stained with anti-glucagon antibody (Sigma K79bB10, 1:100) followed by Alexa Fluor-conjugated secondary antibody (Jackson ImmunoResearch, 1:200). Samples were analyzed using BD LSR cell analyzer at the Vision Research Core Facility at the University of Michigan Medical School. The glucagon-negative cell population was gated to analyze cell size. Data were analyzed using the CellQuest software (BD Biosciences) and Flowjo (Flowjo.com).

**Western blot.** Following an overnight recovery, islets were lysed in lysis buffer [50 mM Tris/Cl pH 7.4, 1 mM EDTA, 1x complete protease inhibitor (Sigma), 1x PhosSTOP (Sigma)] followed by brief sonication. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were heat-denatured at 65°C for 10 min in NuPAGE LDS sample buffer (Thermo Fisher Scientific), resolved by SDS-PAGE, and transferred to PVDF membranes (Bio-Rad). The membranes were incubated overnight at 4°C with antibodies prepared in 2% BSA (Sigma). The antibodies used are as follows: α-Tubulin (Santa Cruz sc-
Sel1L (Abcam ab78298; 1:1000), BiP (Abcam ab21685; 1:5000), Calnexin (Cell signaling 2679; 1:1000), Aldh1a3 (Novus Biologicals NBP2-15339; 1:1000), Hrd1 (Dr. Richard Wojcikiewicz, 1:300), Smad2/3 (Cell Signaling 8685; 1:1000), pSmad 2/3 (Cell Signaling 138D4 and 9520; 1:1000), p62 (Enzo BML-PW9860; 1:5000), TGFβRI (Abcam ab31013; 1:1000), Hsp90 (Abcam ab13492; 1:2000), Smad2/3 (Cell Signaling 8685; 1:1000), pSmad 2/3 (Cell Signaling 138D4 and 9520; 1:1000), p62 (Enzo BML-PW9860; 1:5000), TGFβRI (Abcam ab31013; 1:1000), Hsp90 (Abcam ab13492; 1:2000) and Insulin (Biorad 5330-0104G, 1:5000). Secondary antibodies were goat anti-rabbit IgG-HRP and anti-mouse IgG-HRP (1:5,000; BioRad). Phos-tag analysis of IRE1α phosphorylation was performed as previously described (35, 51).

**RNA extraction, microarray, cDNA synthesis and qPCR analysis.** Islets were collected from 5 week-old mice and recovered overnight prior to RNA extraction. RNA was extracted using RNeasy micro kit (Qiagen) including a column for elimination of genomic DNA as per manufacturer’s instructions. RNA concentration was determined using NanoDrop 2000 UV-Vis Spectrophotometer. The quality and concentration were determined using the RNA 6000 Nano kit on an Agilent 2100 bioanalyzer. The microarray was performed as previously described (25). RT-PCR for Xbp1 mRNA splicing and qPCR analysis were performed as previously described (52). All PCR data were normalized to the ribosomal L32 and Actin gene expression level. qPCR primer sequences are as follows:

**Actin** F: CCCGCAGTACAACCTTCT, R: CGTCATCCATGGCGAACT;  
**L32** F: GAGCAACAAGAAAACCAAGCA, R: TGCACAAGCCATCTACTCA;  
**MafA** F: ATCTGTACTGGATGCGGG, R: AGAGTGATGATGGTGCGAG;  
**Glut2** F: CTGCACCATCTTCATGTCGG, R: AATTGCAGACCCAGTTGCTG;  
**Ucn3** F: TGATGCCCACCTACTTCCTG, R: GGTGCGTTGTTGTTGCTCT;  
**Xbp1s** F: TTACGAGAAACTCATGGGC, R: GGGTCAAATGGCCAGAATGC.

**scRNA-Seq.** Islets were harvested from 7-week-old mice and recovered overnight. Cell suspension was prepared by trypsinization of islets in trypsin (Corning, 25-053-CI) diluted in Calcium-free PBS containing 1 mM EDTA for 15 min and immediately submitted for library preparation. A total of 26,061 pancreatic islet cells isolated from two *Sel1L*Δft and two *Sel1L*Δinst mice were processed using 10X Genomics CHROMIUM Single Cell 3' Solution at the sequencing core at the University of Michigan following the manufacturer’s guidelines. The libraries were sequenced using Illumina HiSeq 4000 platform. Sequencing raw reads were processed through demultiplexing, mapping and analysis by the pipeline in Cell Ranger v.3.0.0. A total of over 2 billion (2,352,307,194) reads with an average of 90,261 reads per cell were obtained. Approximately 78.8% of the sequence reads were confidently mapped to the mouse
transcriptome. Seurat package (version 2.3.4) was used to further analyze scRNA-seq data (53). After removing doublets and cells with low quality (high mitochondrial content or low sequencing depth), 18,612 cells that expressed more than 500 genes and 19,074 genes with transcripts detected in more than 3 cells were used for further analysis. Unique sequencing reads for each gene were normalized to total Unique Molecular Identifiers (UMIs) in each cell to obtain normalized UMI values. Unsupervised clustering was applied at a resolution of 0.2 using the top 17 dimensions of PCA. The cell cluster identification was based on the prior knowledge of marker genes. The t-SNE plots, violin plots, feature plots and heatmaps were generated by R 3.5.3 software.

**Statistical analysis.** Results are expressed as the mean ± SEM unless otherwise stated. Statistical analyses were performed in GraphPad Prism (GraphPad Software Inc.). Comparisons between two groups were made by unpaired two-tailed Student's t test. A one-way ANOVA followed by Bonferroni post-test was used to determine statistical significance for more than two groups with two factors. P value less than 0.05 was considered as statistically significant. All experiments were repeated at least twice, or performed with several independent biological samples, and representative data are shown.

**Study Approval.** All animal procedures were approved by and done in accordance with the IACUC at the University of Michigan Medical School (PRO00008989).

**Data Availability.** All microarray and RNA-seq data are available at NCBI GEO with accession numbers GSE143757 and GSE137785, respectively.
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AUTHOR CONTRIBUTION

N.S. designed and performed most of experiments; T.L. and J.D.L. performed scRNA-seq data analysis and discussion; X.L. and M.L. performed pulse chase of proinsulin; Y.J. made the initial crosses of Sel1L knockout mice and performed flow cytometry; R.B.R. performed some confocal microscopy experiments; M.T. performed the experiment with phostag gels; S.K. performed microarray analyses; C.L. and A.N. provided human pancreatic sections and performed immunostaining on one set of human samples; C.C.A.H. and C.H.A.T. generated SEL1L antibody; P.A. provided discussions and key reagents, and involved in the experimental design; L.Q. designed and supervised the project; L.Q. and N.S. wrote the manuscript. All other authors edited and approved the manuscript.
REFERENCES


Table 1. Percentage of cells in different clusters in WT and Sel1L<sup>Ins<sup>†</sup></sup> islets from single cell sequencing.

<table>
<thead>
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<th>#</th>
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Figure 1. SEL1L expression in human β-cells and generation of β-cell-specific Sel1L- and Atg7-deficient mice. (A-B) Representative immunofluorescence images of SEL1L (A) in human pancreas obtained from healthy and T2D donors (n=4 sample each) and quantified in (B, each dot represents an islet). (C) Western blot analyses in primary islets (n=2 mice for each genotype). Tubulin, a loading control. (D-E) Representative immunofluorescence images of Sel1L and insulin (D) and p62 and insulin (E) in pancreatic sections (n=2 mice for each genotype). Insets are shown in the lower panels. Values, mean ± SEM. **, p<0.01 by unpaired Student’s t-test.
Figure 2. Similar to Atg7 deficiency, β-cell-specific deletion of Sel1L leads to early-onset progressive hyperglycemia and glucose intolerance. (A) Growth curves of male and female mice (n=12 mice per group per gender per time point). (B-C) Weekly measurements of ad-libitum blood glucose in male (B) and female (C) (n=12 mice per group per time point). (D-E) Intraperitoneal glucose tolerance test in 10-week-old male mice showing glucose (D) and insulin (E) levels at indicated times (n=4-8 mice each group) with quantitation of area-under-curve shown on the right. (F) Fasting serum insulin levels in 10-week-old male mice (n=4-8 mice each group). Values, mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 by one-way ANOVA.
Figure 3. Unlike Atg7 deficiency, loss of Sel1L doesn’t lead to β-cell loss.
(A) Representative H&E images of pancreatic sections obtained from mice with mild hyperglycemia (n=5-6 for each genotypes). Age and blood glucose (BG) for the particular samples shown are indicated. (B) Quantitation of β-cell mass at 8-12 weeks of age (n=8-10 mice per group). Quantitation of (C) Ki67 and (D) TUNEL positive cells per insulin positive β cells (n=5-6 mice per group). (E) Quantitation of islet area (left, n=8-11 mice per group) and nuclear density (right, n=150-200 islets from 5 mice each) at 8-11 weeks of age. Each dot represents one mouse (left) or an islet (right). (F) Flow cytometric analysis of β-cell size as indicated by forward scatter (FSC) (n=4-6 mice per group). Quantitation shown below. (G) Representative H&E images of pancreatic sections obtained from mice with severe hyperglycemia (> 500mg/dl) (n=5-6 for each genotypes); *, vacuolization. Values, mean ± SEM. n.s., not significant; **, p<0.01; ***, p<0.001; ****, p<0.0001 by one-way ANOVA.
Figure 4. Non-biased sequencing analyses establish the importance of Sel1L-Hrd1 ERAD in β-cell identity.

(A-B) Results from cDNA microarray analysis of islets from 5-week-old mice (n=3 mice each group): (A) Volcano plot depicting transcriptomics data with dotted line marking p=0.05 on y-axis and fold change > 2 on x-axis. (B) Heat map showing log-fold change of mature β-cell markers and forbidden genes. (C-E) Results from scRNA-seq of islets from 7-week-old male mice (n = 2 mice each group): (C-D) Visualization of t-distributed stochastic neighbor embedding (tSNE) plots generated by unsupervised clustering analysis presented as merged (C) or individual (D) datasets. In D, β-cell population is highlighted. Each dot corresponding to a single cell. (E) Gene expression changes of representative β-cell markers associated with different processes.
Figure 5 Sel1L deficiency leads to downregulation of maturation markers and upregulation of immature markers in adult β cells.

(A) Representative immunofluorescence image showing major islet hormones (insulin, glucagon and somatostatin; DAPI in blue) in pancreatic sections of WT, Sel1L<sup>Ins1</sup> and Atg7<sup>Ins1</sup> mice (n=3 mice each). (B-D) Representative immunofluorescence image showing staining of (B) maturation marker MafA with quantitation in C (n=3 mice, each dot represents an islet), and (D) dedifferentiation marker Aldh1a3 in indicated genotypes. (E) Western blot analysis of Aldh1a3 in primary islets. Values, mean ± SEM. ****, p<0.0001 by one-way ANOVA.
Figure 6. *Sel1L* deficiency does not impact β-cell development. (A) Blood glucose, (B) serum insulin, and (C) total pancreatic insulin content in P14 pups. (D-E) Representative immunofluorescence images of insulin and glucagon in P1 (D) and P14 (E) (DAPI in blue, n=3 mice for each genotype). (F) Representative immunofluorescence images of β-cell transcription factors MafA and Pdx1 in P14 pups (DAPI in grey, n=3 mice for each genotype). Values, mean ± SEM. n.s., not significant by unpaired Student’s t-test.
Figure 7. Elevated TGF-β signaling in Sel1L-deficient islets. (A-B) GO analyses of the scRNA-Seq data showing significantly up- (A) and down- (B) regulated pathways in Sel1L<sup>ins<sup>1</sup></sup> vs. Sel1L<sup>f/f</sup> β-cells. (C) Western blot analysis of total and phosphorylated Smad2/3 in primary islets (with quantitation in D, n=3 independent repeats for Sel1L<sup>ins<sup>1</sup></sup> and one experiment for Atg7<sup>ins<sup>1</sup></sup>). (E) Representative confocal microscopic images of Smad2/3 in β-cells from primary islets (with quantitation in F, two independent repeats). (G) Western blot analysis of TGFβRI in isolated WT and Sel1L<sup>ins<sup>1</sup></sup> islets (with quantification on the right, n=4 mice, p<0.01 by unpaired Student’s t-test). (H) Cycloheximide (CHX) chase of TGFβRI protein in isolated islets following 4 hr of chase (with quantitation on the right, two independent repeats, data shown as normalized to basal 0 hr levels).
**Figure 8. TGFβ signaling links Sel1L to β-cell identity.**

(A-B) Representative immunofluorescence images of Smad2/3 and insulin (two independent repeats) (A) and qRT-PCR analysis of β-cell gene expression (B) in primary islets treated with vehicle- or 10 µM TGFβRI inhibitor (Alk5<sub>in</sub>) for 24 hr. qRT-PCR data normalized to WT vehicle controls, from n=3 biological replicates per genotype. (C) Representative confocal microscopic images of MafA in dispersed primary islets treated with vehicle (DMSO) or TGFβRI inhibitor Alk5<sub>in</sub> (two independent experiments, wider field of view shown in Figure S9B). ***, p<0.001; ****, p<0.0001 by one-way ANOVA. (D) Insulin content in primary islets following treatment with either DMSO or indicated dose of TGFβRI inhibitor for 24 hr (n=3). *, p<0.05 by two-way ANOVA. (E) Model for distinct effects of ERAD and autophagy in β-cell failure in T2D pathogenesis: while autophagy control cell survival, ERAD maintains β-cell identity by suppressing TGFβ signaling. Values, mean ± SEM.