TITLE: Sex differences in islet stress responses support female beta cell resilience

SHORT TITLE: Sex-specific stress responses promote resilience in female islets

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AUTHOR CONTRIBUTIONS

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ABSTRACT

Type 2 diabetes risk is ~40% higher in men than in pre-menopausal women. Despite evidence that sex differences in pancreatic β cells play a role in this differential diabetes risk, few studies have examined diabetes-associated changes to β cell function in each sex. Our single-cell analysis of human β cells revealed profound sex-specific changes to gene expression and function in type 2 diabetes. To gain deeper insight into sex differences in β cells, we generated a well-powered islet RNAseq dataset from 20-week-old male and female mice with equivalent insulin sensitivity. This unbiased analysis revealed differential enrichment of unfolded protein response pathway-associated genes, where female islets showed higher expression of genes linked with protein synthesis, folding, and processing. This differential expression was biologically significant, as female islets were more resilient to endoplasmic reticulum (ER) stress induction with thapsigargin. Specifically, female islets maintained better insulin secretion and showed a distinct transcriptional response under ER stress compared with males. Given the known links between ER stress and T2D pathogenesis, our findings suggest sex differences in β cells contribute to the differential T2D risk between men and women.

INTRODUCTION

Type 2 diabetes (T2D) is caused by failure to produce sufficient insulin to maintain glucose homeostasis, and to respond properly to the insulin that is made. Across many, but not all, population groups, men are at a higher risk of developing T2D than women (*1*–*4*). Some of the differential risk is explained by lifestyle and cultural factors (*4*–*6*); however, biological sex also plays a role, as the male-biased risk of developing diabetes-like phenotypes also exists in animals (*7*–*13*). One mechanism by which biological sex contributes to the differential T2D risk between men and women is via effects on insulin sensitivity. In humans, females show higher insulin sensitivity than males (*14*–*19*), a trend that exists in diverse species (*13*, *20*–*24*). Given the known association between loss of insulin sensitivity and T2D (*25*), this provides a simple explanation for the male-biased risk of T2D in rodents and humans. Yet, recent studies in humans show that sex differences in insulin production and/or secretion exist even when insulin sensitivity is equivalent between males and females (*26*). Despite a dominant role for β cell function in T2D pathogenesis (*27*, *28*), sex differences in β cell function have not been thoroughly explored.

Insight into potential sex differences in β cells has emerged from multiple studies. For example, large-scale surveys of gene expression in mice and humans illustrate that differences exist between the sexes in the pancreas (*29*–*31*), and also in β cells (*32*, *33*). While these data suggest differential β cell gene expression between the sexes, insulin sensitivity was not monitored in these studies. As a result, it remains unclear whether these differences simply reflect a male-female difference in peripheral insulin sensitivity. T2D-associated changes to β cell gene expression in each sex also remain unclear, as most efforts did not include biological sex as a variable in their analysis (*34*–*39*). Although one recent study found sexually dimorphic changes to β cell gene expression with aging (*40*), a sex-based analysis of single-cell RNA sequencing (scRNAseq) datasets with more donors without diabetes (ND) and with T2D is needed to extend our understanding of sex-specific changes to β cells in T2D.

Beyond gene expression, studies on insulin production provide additional insight into sex differences in β cells. Human female islets show higher glucose-stimulated insulin secretion than males in some (*41*), but not all (*40*) studies, with similar findings in rodents (*42*). These differences are likely shaped by gonadal hormones, as 17β-estradiol (E2) treatment in rodents increased insulin content and secretion, improved insulin sensitivity, and conferred protective effects against β cell apoptosis (*12*, *43*–*48*). Similar effects on β cell function were observed following E2 treatment of perfused pancreata and cultured islets (*12*, *44*, *46*–*53*), and protection against β cell apoptosis was also noted in humans (*46*, *47*, *54*, *55*). While this suggests significant male-female differences in β cell function, these studies did not fully explore the contribution of insulin sensitivity alongside β cell function. In addition, we lack a detailed understanding of changes to β cell function in T2D, as many large-scale studies on this topic do not analyze data according to sex (*56*, *57*). A detailed analysis of β

cell function under basal conditions and in T2D is therefore critical to determine whether sex differences in β cells contribute to the sex-biased risk of T2D in humans.

The overall goal of our study was to provide detailed knowledge of β cell gene expression and function in both males and females across multiple contexts to advance our understanding of sex differences in this important cell type. Collectively, our data suggest that sex differences in islet and β cell gene expression exist in healthy and T2D conditions, and contribute to sex differences in β cell resilience in these contexts. Because our data suggest these differences cannot be fully explained by differential peripheral insulin sensitivity between the sexes, biological sex should be an important variable in future studies on islet and β cell function.

RESULTS

Sex differences in β cell transcriptional and functional responses in ND and T2D human islets

To define sex-specific β cell-specific gene expression changes in T2D, we used a recently compiled meta-analysis of all publicly available scRNAseq datasets from male and female human islets (*58*). In line with prior reports (*40*), ND and T2D β cells displayed significant transcriptional differences. In β cells isolated from female T2D donors, mRNA levels of 127 genes were significantly different from ND female donors (77 downregulated, 50 upregulated in T2D) (Figure 1A-C). In β cells isolated from male T2D donors, 462 genes were differentially expressed compared with male ND donors (138 downregulated, 324 upregulated in T2D) (Figure 1A-C). Of the 660 genes that were differentially regulated in T2D, 71 were differentially regulated in both males and females (15 downregulated, 56 upregulated in T2D) (Figure 1A-C); however, we found that the fold change for these 71 shared genes was not the same between males and females (Figure 1-figure supplement 1A; Supplementary file 1). This suggests that for shared genes, the magnitude of the gene expression response to T2D was not shared between the sexes. Beyond shared genes, we observed that the majority of differentially expressed genes in T2D (589/660) were unique to each sex (Figure 1-figure supplement 1B, C; Supplementary file 1). Indeed, the most prominent gene expression changes in T2D occurred in only males or females (Figure 1-figure supplement 2A, B; Supplementary file 1). This suggests there are important sex differences in the β cell gene expression response in T2D.

To determine which biological pathways were altered in T2D in each sex, we performed pathway enrichment analysis. Genes that were upregulated in β cells isolated from T2D donors included genes involved in Golgi-ER transport and the unfolded protein response (UPR) pathways (Figure 1D-F; Supplementary file 1). While these biological pathways were significantly upregulated in T2D in both males and females, ~75% of the differentially regulated genes in these categories were unique to each sex (Table 1). Genes that were downregulated in T2D β cells revealed further differences between the sexes: biological pathways downregulated in β cells from female T2D donors included cellular responses to stress and to stimuli (Figure 1E; Supplementary file 1), whereas β cells from

male T2D donors showed downregulation of pathways associated with respiratory electron transport and translation initiation (Figure 1F; Supplementary file 1). Together, our analysis of scRNAseq data suggests that the β cell gene expression response in T2D was not fully shared between the sexes. Given this sex-specific β cell transcriptional response in T2D, we compared glucose-stimulated insulin secretion from ND and T2D human islets using data from the Human Pancreas Analysis Program (*59*). In ND donors, islets from males and females showed similar patterns of insulin secretion in response to various stimulatory media (Figure 1G, H). In T2D donors, we found that insulin secretion was impaired to a greater degree in islets from males than in females (Figure 1G-K). In male islets, insulin secretion was significantly lower following stimulation with both high glucose and IBMX (Figure 1G, I), which potentiates insulin secretion by increasing cAMP levels similar to the incretins (*60*). In contrast, islets isolated from female donors showed no significant defects in insulin secretion under identical treatment conditions (Figure 1K). Combined with our β cell gene expression data, these findings suggest that β cell transcriptional and functional responses in T2D are not shared between the sexes.

Sex differences in UPR-associated gene expression in mouse islets

In humans, β cell gene expression and functional responses in T2D may be affected by insulin sensitivity, disease processes, medication, and age (*4*, *6*). Because the contribution of these factors to the sex differences in β cell gene expression and functional responses in T2D was unclear (*58*), we generated a well-powered islet RNAseq dataset from 20-week-old male and female C57BL/6J mice to explore sex differences in islet gene expression and function in more detail in a more controlled model system. Importantly, insulin sensitivity was equivalent between males and females at this age (Figure 2-figure supplement 1A). Principal component analysis and unsupervised clustering clearly separated male and female islets on the basis of gene expression (Figure 2A; Figure 2-figure supplement 2A). Remarkably, 17.7% (3268/18938) of genes were differentially expressed between the sexes (1648 upregulated in females, 1620 upregulated in males), in line with estimates of sex-biased gene expression in other tissues (*61*, *62*). Overrepresentation and pathway enrichment analysis both identified UPR-associated pathways as a biological process that differed significantly between the sexes, where the majority of genes in this category were enriched in female islets (Figure 2B, C; Supplementary file 2). Specific categories that were enriched in females included genes associated with the gene ontology term “Cellular response to ER stress” (GO:0034976), which included many genes involved in regulating protein synthesis (Figure 2D). Indeed, female islets had significantly higher mRNA expression of most genes associated with protein synthesis compared with male islets (Figure 2D), and significantly higher levels of ribosomal protein genes (Figure 2E). Additional categories enriched in females included genes associated with protein folding, protein processing, and quality control (Figure 2D). Given that protein synthesis, processing and folding capacity are

intrinsically important for multiple islet cell types (*63*–*66*), including β cells (*67*, *68*), this suggests female islets may have a larger protein production and folding capacity than male islets.

Female islets are more resilient to endoplasmic reticulum stress in mice

The burden of insulin production causes endoplasmic reticulum (ER) stress in β cells (*69*–*71*). ER stress is associated with an attenuation of mRNA translation (*72*), and, if ER stress is prolonged, can lead to cell death (*73*–*75*). Given that female islets exhibited higher expression of genes associated with protein synthesis, processing, and folding than males, we examined global protein synthesis rates in male and female islets under basal conditions and under ER stress. We incubated islets with O-propargyl-puromycin (OPP), which is incorporated into newly-translated proteins and can be ligated to a fluorophore. This technique enabled us to monitor the accumulation of newly-synthesized islet proteins with single-cell resolution (Figure 3-figure supplement 1A). In basal culture conditions, male islet cells had significantly greater protein synthesis rates compared with female islet cells (Figure 3-figure supplement 1B). To investigate islet protein synthesis under ER stress in each sex, we treated islets with thapsigargin (Tg), a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that induces ER stress and the UPR by lowering ER calcium levels (*73*, *76*). At 2-hours post-Tg treatment, we found that protein synthesis was repressed in both male and female islet cells (Figure 3A, B; Figure 3-figure supplement 1C). At 24-hours post-Tg treatment, we found that protein synthesis was restored to basal levels in female islet cells, but not in male islet cells (Figure 3A, B; Figure 3-figure supplement 1C). This suggests that while protein synthesis repression associated with ER stress was transient in female islets, this phenotype persisted for longer in male islets. Because insulin biosynthesis accounts for approximately half the total protein production in β cells (*77*), one potential explanation for the sex-specific recovery from protein synthesis repression is a sex difference in transcriptional changes to insulin. To test this, we quantified GFP levels in β cells isolated from male and female mice with GFP knocked into the endogenous mouse *Ins2* locus (*Ins2*$^{GFP/WT}$) (*78*, *79*). While ER stress induced a significant reduction in *Ins2* gene activity, this response was equivalent between the sexes, suggesting *Ins2* transcriptional changes cannot fully explain the sex difference in protein synthesis repression under ER stress (Figure 3-figure supplement 2).

Given the prolonged protein synthesis repression in males following ER stress, we next quantified cell death, another ER stress-associated phenotype (*80*), in male and female islets. Using a kinetic cell death analysis, we observed no significant increase in apoptosis in female islet cells with 0.1 μM or 1.0 μM Tg treatment compared with controls after 84-hours (Figure 3C). In contrast, cell death was significantly increased at both the 0.1 μM and the 1.0 μM doses of Tg in male islet cells compared with vehicle-only controls (Figure 3D). While it is possible that female islets are resistant to Tg-induced cell death, we found a significant increase in apoptosis in female and male islet cells treated with 10 μM

Tg (Figure 3E, F), suggesting that female islets were simply more resilient to mild ER stress than male islets. To determine whether this increased ER stress resilience was caused by differential UPR signaling, we monitored levels of several protein markers of UPR activation including binding immunoglobulin protein (BiP), phosphorylated inositol-requiring enzyme 1 (pIRE1), phosphorylated eukaryotic initiation factor a (peIF2a), and C/EBP homologous protein (CHOP) (*81*, *82*) after treating male and female islets with 1 μM Tg for 24-hours. We found no sex difference in UPR protein markers between male and female islets without Tg treatment (Figure 3G-J; Figure 3-figure supplement 3A) and observed a significant increase in levels of pIRE1α and CHOP in islets from both sexes and BiP in female islets after a 24-hour Tg treatment (Figure 3G-J; Figure 3-figure supplement 3A). Lack of a sex difference in UPR protein markers suggests UPR activation was similar between male and female islets at 20 weeks of age. While this finding differs from other studies showing male-biased UPR activation (*10*), we reproduced the male-biased induction of ER stress in islets isolated from 60-week-old male and female mice (Figure 3K-M; Figure 3-figure supplement 3B), suggesting that age plays a role in the sex difference in UPR activation. Thus, despite equivalent UPR activation in male and female islets treated with Tg, significant sex differences exist in ER stress-associated protein synthesis repression and cell death.

Female islets retain greater β cell function during ER stress in mice

To determine whether sex differences in ER stress-associated protein synthesis repression and cell death affected islet insulin production, we examined glucose-stimulated insulin secretion in islets cultured under basal conditions and after ER stress induction (Figure 4A). In all conditions tested, high glucose significantly stimulated insulin secretion in both sexes (Figure 4B). To determine how Tg affects glucose-stimulated insulin secretion, we compared insulin secretion between low and high glucose at different timepoints post-Tg treatment. Female islets, in both low and high glucose, maintained robust insulin secretion following either short (0-hours) or longer (4-hours) Tg treatment (Figure 4-figure supplement 1A, B). In contrast, insulin secretion in high glucose was impaired in male islets after the 4-hour Tg treatment (Figure 4-figure supplement 1B). Given that insulin content measurements showed insulin content significantly increased during the 4-hour Tg treatment in female islets, but not male islets (Figure 4C), our data suggest female islets maintain higher insulin secretion during ER stress by augmenting islet insulin content. Similar results were obtained when we monitored proinsulin secretion in both male and female islets (Figure 4D); however, Tg treatment reduced islet proinsulin content to a greater degree in male islets compared with female islets (Figure 4E), suggesting a potential explanation for the reduced islet insulin secretion after Tg treatment. Together, these data suggest that female islets show improved β cell function under ER stress. To determine whether this trend persists in other contexts, we monitored glucose-stimulated insulin secretion, and glucose tolerance in mice at an age where we show insulin sensitivity was equivalent between the

sexes (Figure 4F-H; Figure 2-figure supplement 1). We found that despite higher fasting plasma insulin levels in males (Figure 4F), and similar glucose tolerance (Figure 4H), the magnitude of glucose-stimulated insulin secretion was greater in females (Figure 4G). Given that ER stress exists even in normal physiological conditions due to the burden of insulin production (*83*), this adds further support to a model in which female β cells maintain better insulin production than male β cells under ER stress.

Sex differences in the transcriptional and proteomic responses to ER stress in mouse islets

To gain insight into the differential ER stress-associated phenotypes in male and female islets, we investigated global transcriptional changes after 6- or 12-hour Tg treatments in each sex. Principal component analysis and unsupervised clustering showed that islets cluster by sex, treatment, and treatment time (Figure 5A; Figure 5-figure supplement 1A). The majority of the variance was explained by treatment (Figure 5B), and pathway enrichment analysis confirms the UPR as the top upregulated pathway in Tg-treated male and female islets at both 6- and 12-hours after treatment (Figure 5-figure supplement 2A, B; Supplementary file 3). While most of the UPR-associated genes differentially regulated by Tg treatment were shared between the sexes (6-hour: 29/36, 12-hour: 25/31), biological sex explained a large proportion of the variance in the gene expression response to ER stress, suggesting the transcriptional response to ER stress was not fully shared between the sexes. Indeed, after 6-hours Tg treatment, of genes that were differentially expressed between DMSO and Tg, 32.6% (2247/4655) were unique to one sex (881 to females, 1376 to males). After 12-hours Tg treatment, 29% (2259/7785) were unique to one sex (1017 to males, 1242 to females).

To describe the transcriptional response of each sex to Tg treatment in more detail, we used a two-way ANOVA to identify genes that were upregulated, downregulated, or unchanged in male and female islets between 6- and 12-hours post-Tg (Supplementary file 4). By performing pathway enrichment analysis, we were able to determine which processes were shared between the sexes, and which processes differed between the sexes, during Tg treatment. For example, we observed a significant increase in mRNA levels of genes corresponding to pathways such as cellular responses to stimuli, stress, and starvation in both male and female islets between 6- and 12-hour Tg treatments (Figure 5C; Supplementary file 4), suggesting Tg has similar effects on genes related to these pathways in both sexes. In contrast, there was a male-specific increase in mRNA levels of genes associated with translation during Tg treatment (Figure 5C; Supplementary file 4). In females, there was a decrease in mRNA levels of genes associated with β cell identity, such as *Pklr*, *Rfx6*, *Hnf4a*, *Slc2a2*, *Pdx1*, and *MafA* (Figure 5-figure supplement 3A), and in genes linked with regulation of gene expression in β cells (Figure 5C). Neither of these categories were altered between 6- and 12-hour Tg treatments in males (Figure 5C; Figure 5-figure supplement 3B). While this data suggests some

aspects of the gene expression response to ER stress were shared between the sexes, many genes were differentially regulated during Tg treatment in only one sex.

Beyond sex-specific transcriptional changes following Tg treatment, ER stress also had sex-specific effects on the islet proteome. Although the majority of proteins were downregulated by Tg treatment due to generalized repression of protein synthesis under ER stress (Figure 5D), we identified 47 proteins (35 downregulated, 12 upregulated in Tg) that were differentially expressed in female islets and 82 proteins (72 downregulated, 10 upregulated in Tg) that were differentially expressed after Tg treatment in male islets (Supplementary table 1). Proteins downregulated only in females include proteins associated with GO term 'endoplasmic reticulum to Golgi vesicle-mediated transport' (GO:0006888) (BCAP31, COG5, COG3, GOSR1), whereas proteins downregulated only in males include proteins associated with GO terms 'insulin secretion' (GO:0030073) (PTPRN2, CLTRN, PTPRN) and 'lysosome pathway' (KEGG) (NPC2, CTSZ, LAMP2, PSAP, CLTA). Importantly, only seven differentially expressed proteins were in common between the sexes (Figure 5D). This suggests that as with our phenotype and transcriptomic data, the proteomic response to Tg treatment was not shared between the sexes.

DISCUSSION

The goal of our studies was to comprehensively examine sex differences in islet and β cell gene expression and function in multiple contexts, including diabetes-relevant ER stress. In humans, we used a large scRNAseq dataset from ND and T2D donors to demonstrate significant male-female differences in the magnitude of gene expression changes, and in the genes that were differentially regulated, between ND and T2D donors. This suggests β cell gene expression changes in T2D are not fully shared between the sexes. Given that our analysis shows β cells from female T2D donors maintain better insulin production than β cells from male T2D donors, our findings suggest female β cells are more resilient than male β cells in the context of T2D. To gain further insight into this increased female β cell resilience, we explored sex differences in gene expression and β cell function in rodents. Our unbiased analysis of gene expression in islets from males and females with equivalent insulin sensitivity revealed sex differences in genes associated with the UPR. This differential gene expression was significant: female islets were more resilient to phenotypes associated with UPR activation than male islets, showed sex-specific transcriptional and proteomic responses to ER stress, and maintained better insulin secretion in this context. Collectively, these data suggest that in rodents, β cells from females are more resilient to ER stress. Considering the well-established links between ER stress and T2D (*81*, *84–86*), our data suggests a model in which female β cells maintain better function in T2D because they are more resilient to ER stress and UPR activation. While this model will be important to test in further detail in future studies, our findings highlight the importance of including

both sexes in islet and β cell studies to make accurate conclusions about β cell gene expression and function in both normal contexts and in disease.

With respect to gene expression, including both sexes in our analysis of β cell gene expression in human ND and T2D allowed us to uncover genes that were differentially regulated in T2D in each sex. Because many of these genes may have been missed if the scRNAseq data was not analyzed by sex, our findings advance our understanding of β cell changes in T2D by identifying additional genes that are differentially regulated in this context. This knowledge adds to a growing number of studies that identify sex differences in β cell gene expression during aging in humans (*40*), and in mice fed either a normal (*32*, *33*) or a high fat diet (*32*). Further, given that our RNAseq on islets from male and female mice with equivalent insulin sensitivity identifies genes and biological pathways that align with previous studies on sex differences in murine β cell gene expression (*32*, *33*), our data suggests that sex differences in islet and β cell gene expression cannot be explained solely by a male-female difference in peripheral insulin resistance. Instead, there is likely a basal sex difference in the β cell gene expression landscape that forms the foundation for sex-specific transcriptional responses to ER stress, and T2D. By generating large gene expression datasets from islet from male and female mice with equivalent peripheral insulin sensitivity and from islets subjected to pharmacological induction of ER stress, our studies provide a foundation of knowledge for future studies aimed at understanding sex differences in islet ER stress responses and β cell function following UPR activation. This will provide deeper mechanistic insight into the sex-specific phenotypic effects reported in animal models of β cell dysfunction (*8*–*12*, *87*–*90*) and the sex-biased risk of diseases such as T2D that are associated with β cell dysfunction (*13*, *40*, *91*, *92*).

Beyond gene expression, our sex-based analysis of mouse islets allowed us to uncover sex differences in ER stress-associated phenotypes (*e.g*. protein synthesis repression, cell death). While previous studies identify a sex difference in β cell loss in diabetic mouse models (*10*, *12*, *44*), and show that estrogen plays a protective role via ERα against ER stress to preserve β cell mass and prevent apoptosis in cell lines, mouse models, and human islets (*12*, *44*, *93*), we extend prior findings by showing that differences in ER stress-induced cell death were present in the context of equivalent insulin sensitivity between the sexes. This suggests sex differences in ER stress-associated phenotypes occur prior to male-female differences in peripheral insulin sensitivity. Indeed, islets isolated from males and females with equivalent sensitivity also show a sex difference in protein synthesis repression, a classical ER stress-associated phenotype (*80*). While estrogen affects insulin biosynthesis via ERα (*49*), future studies will need to determine whether estrogen contributes to the ability of female islets to restore protein synthesis to basal levels faster than male islets following ER stress. We currently lack this knowledge, as most studies on UPR-mediated recovery from protein translation repression use single- and mixed-sex animal groups, or cultured cells (*94*–*99*). It will also be important to determine whether the recovery of protein synthesis contributes to reduced cell death

in female islets following ER stress, as prior studies suggest the inability to recover from protein synthesis repression increases ER-stress induced apoptosis (*94*). Ultimately, a better understanding of sex differences in ER stress-associated phenotypes in β cells will provide a mechanistic explanation for the strongly male-biased onset of diabetes-like phenotypes in mouse models of β cell ER stress (*e.g*. Akita, KINGS, Munich mice) (*10*, *11*, *87*). Given the known relationship between ER stress, β cell death, and T2D, studies on the male-female difference in β cell ER stress-associated phenotypes may also advance our understanding of the male-biased risk of developing T2D in some population groups.

A further benefit of additional studies on the sex difference in β cell ER stress responses will be to identify mechanisms that help support β cell insulin production. In rodents, we found that female islets maintained high glucose-stimulated insulin secretion and increased insulin content following ER stress, whereas male islets showed significant repression of high glucose-stimulated insulin secretion under the same conditions. In humans, while β cells from T2D male and female donors have been shown to experience ER stress associated with β cell dysfunction (*100*), we found that changes to islet function in T2D were not the same between the sexes. Specifically, the magnitude of the reduction in insulin release by β cells from female donors with T2D was smaller than in β cells from male donors with T2D. Collectively, our data suggest that female β cells maintain enhanced insulin production and/or secretion in multiple contexts, where this increased β cell function cannot be solely attributed to a sex difference in peripheral insulin sensitivity. Clues into potential ways that female β cells maintain improved insulin production and secretion emerge from our examination of the transcriptional response to ER stress in each sex. While our data shows that Tg treatment induces gene expression changes characteristic of ER stress (*101*), we identified significant differences between male and female islets in the transcriptional response to ER stress over time. One notable finding was that a greater number of β cell identity genes were downregulated between 6- and 12-hour Tg treatments in females, but not in males. Because most studies on the relationship between β cell identity and function used a mixed-sex pool of islets and β cells (*69*, *102*, *103*), more studies will be needed to test whether a sex difference exists in changes to β cell identity during ER stress.

Overall, our work presents multiple lines of evidence, from rodents and humans, that sex differences exist in β cell gene expression and function. Importantly, these differences cannot solely be explained by male-female differences in insulin sensitivity. These findings represent a key step toward a more detailed understanding of how sex differences arise in phenotypes associated with β cell function, such as glucose homeostasis, and diseases linked with β cell dysfunction, such as T2D. Ultimately, a better understanding of changes to β cell gene expression and function in each sex will suggest effective ways to reverse disease-associated changes to this important cell type, improving equality in health outcomes.

MATERIALS AND METHODS

Animals. Mice were bred in-house or purchased from the Jackson Laboratory. Unless otherwise stated mouse islets were isolated from C57BL/6J mice aged 20-24 weeks. Animals were housed and studied in the UBC Modified Barrier Facility using protocols approved by the UBC Animal Care Committee and in accordance with international guidelines. Mice were housed in the temperature-controlled UBC Modified Barrier Facility on a 12-hour light/dark cycle with food and drinking water *ad libitum*. Mice were fed a regular chow diet (LabDiet #5053); 24.5% energy from protein, 13.1% energy from fat, and 62.4% energy from carbohydrates.

Islet Isolation, Culture, Dispersion and Treatment. Mouse islet isolations were performed by ductal collagenase injection followed by filtration and hand-picking, using modifications of the protocol described by Salvalaggio (*104*). Islets recovered overnight, in islet culture media (RPMI media with 11.1 mM D-glucose supplemented with 10% vol/vol fetal bovine serum (FBS) (Thermo: 12483020) and 1% vol/vol Penicillin-Streptomycin (P/S) (GIBCO: 15140-148)) at 37°C with 5% CO_2. After four washes with Minimal Essential Medium [L-glutamine, calcium and magnesium free] (Corning: 15-015 CV) islets were dispersed with 0.01% trypsin and resuspended in islet culture media. Cell seedings were done as per the experimental procedure (protein synthesis: 20,000 cells per well, live cell imaging: 5,000 cells per well). ER stress was induced by treating islets with the SERCA inhibitor, thapsigargin. For assays <24-hours, we used (11.1 mM D-glucose RPMI, 1% vol/vol P/S). For assays >24-hours we used (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS).

Analysis of protein synthesis. Dispersed islets were seeded into an optical 96-well plate (Perkin Elmer) at a density of approximately 20,000 cells per well islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS). 24-hours after seeding, treatments were applied in fresh islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S). After incubation, fresh culture media was applied (11.1 mM D-glucose RPMI, 1% vol/vol P/S), supplemented with 20 μM OPP (Invitrogen) and respective drug treatments. The assay was performed according to manufacturers instructions then cells were imaged at 10x with an ImageXpress^{MICRO} high-content imager and analyzed with MetaXpress (Molecular Devices) to quantify the integrated staining intensity of OPP-Alexa Fluor 594 in cells identified by NuclearMask Blue Stain.

Live cell imaging. Dispersed islets were seeded into 384-well plates (Perkin Elmer) at a density of approximately 5,000 cells per well and allowed to adhere for 48-hours in islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS). Cells were stained with Hoechst 33342 (Sigma-Aldrich) (0.05 μg/mL) and propidium iodide (Sigma-Aldrich) (0.5 μg/mL) for 1-hour in islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS) prior to the addition of treatments and imaging. 384-well plates were placed into environmentally controlled (37°C, 5% CO2)

ImageXpress^{MICRO} high content imaging system. To measure cell death, islet cells were imaged every 2-hours for 84-hours, and MetaXpress software was used to quantify cell death, defined as the number of Propidium Iodide-positive/Hoechst 33342-positive cells. To measure *Ins2* transcription, dispersed islets from *Ins2*$^{GFP/WT}$ mice aged 21-23 weeks were used. Islet cells were imaged every 30 minutes for 60-hours. MetaXpress analysis software and custom R scripts were used to perform single-cell tracking of *Ins2*$^{GFP/WT}$ β cells as previously described (*78*).

Western blot. After a 24-hour treatment with 1 μM Tg in islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS), mouse islets were sonicated in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 50 mM Tris (pH 7.4), 2 mM EGTA, 2 mM Na_3VO_4, and 2 mM NaF supplemented with complete mini protease inhibitor cocktail (Roche, Laval, QC)). Protein lysates were incubated in Laemmli loading buffer (Thermo, J61337AC) at 95°C for 5 minutes and resolved by SDS-PAGE. Proteins were then transferred to PVDF membranes (BioRad, CA) and probed with antibodies against HSPA5 (1:1000, Cat. #3183, Cell Signalling), eIF2α (1:1000, Cat. #2103, Cell Signalling), phospho-eIF2α (1:1000, Cat. #3398, Cell Signalling), IRE1α (1:1000, Cat. #3294, Cell Signalling), phospho-IRE1α (1:1000, Cat. #PA1-16927, Thermo Fisher Scientific), CHOP (1:1000, #ab11419, Abcam), β-actin (1:1000, NB600-501, Novus Biologicals). The signals were detected by secondary HRP-conjugated antibodies (Anti-mouse, Cat. #7076; Anti-rabbit, Cat. #7074; CST) and either Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) or Forte (Immobilon). Protein band intensities were quantified using Image Studio (LI-COR).

Islet Secretion and Content. Glucose-stimulated insulin/proinsulin production and secretion was assessed using size-matched islets (five islets per well in triplicates) seeded into 96-well V-bottom Tissue Culture Treated Microplates (Corning: #CLS3894). Islets were allowed to adhere for 48-hours in islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S, 10% vol/vol FBS). Islets were washed with Krebs-Ringer Buffer (KRB; 129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, 0.5% bovine serum albumin) containing 3 mM glucose then pre-incubated for 4-hours in 3 mM glucose KRB. 1 μM Tg was added to the 3 mM low glucose pre-incubation buffer 4-hours prior, 2-hours prior, or at the start of the low glucose incubation period. Islets were incubated in KRB with 3 mM glucose then 20 mM glucose for 45 minutes each. Supernatant was collected after each stimulation. Islet insulin and proinsulin content was extracted by freeze-thawing in 100 μL of acid ethanol, then the plates were shaken at 1200 rpm for 10 minutes at 4°C to lyse the islets. Insulin was measured by Rodent Insulin Chemiluminescent ELISA (ALPCO: 80-INSMR) and proinsulin by Rat/Mouse Proinsulin ELISA (Mercodia: 10-1232-01). Measurements were performed on a Spark plate reader (TECAN).

Blood collection and in vivo analysis of glucose homeostasis and insulin secretion. Mice were fasted for 6-hours prior to glucose and insulin tolerance tests. During glucose and insulin tolerance tests, tail blood was collected for blood glucose measurements using a glucometer (One Touch Ultra 2 Glucometer, Lifescan, Canada). For intraperitoneal (i.p.) glucose tolerance tests, the glucose dose was 2 g glucose/kg of body mass. For insulin tolerance tests, the insulin dose was 0.75U insulin/kg body mass. For measurements of *in vivo* glucose-stimulated insulin secretion, femoral blood was collected after i.p. injection of 2 g glucose/kg body mass. Blood samples were kept on ice during collection, centrifuged at 2000 rpm for 10 minutes at 4°C and stored as plasma at -20°C. Plasma samples were analysed for insulin using Rodent Insulin Chemiluminescent ELISA (ALPCO: 80-INSMR).

RNAseq. To assess basal transcriptional differences islets from male and female mice (n=9, 8) were snap frozen and stored at -80°C until RNA extraction. To assess Tg-induced transcriptional changes islets from each mouse were treated with DMSO or Tg for 6- or 12-hours in culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S) (8 groups, n=3-4 per group, each n represents pooled islet RNA from two mice). Islets were frozen at -80°C in 100 µL of RLT buffer (Qiagen) with beta mercaptoethanol (1%). RNA was isolated using RNeasy Mini Kit (Qiagen #74106) according to manufacturer's instructions. RNA sequencing was performed at the UBC Biomedical Research Centre Sequencing Core. Sample quality control was performed using the Agilent 2100 Bioanalyzer System (RNA Pico LabChip Kit). Qualifying samples were prepped following the standard protocol for the NEBNext Ultra II Stranded mRNA (New England Biolabs). Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp × 42bp reads. Demultiplexed read sequences were then aligned to the reference sequence (UCSC mm10) using STAR aligner (v 2.5.0b) (*105*). Gene differential expression was analyzed using DESeq2 R package (*106*). Pathway enrichment analysis were performed using Reactome (*107*). Over-representation analysis was performed using NetworkAnalyst3.0 (www.networkanalyst.ca) (*108*).

Proteomics. Islets were treated with DMSO or Tg for 6-hours in islet culture media (11.1 mM D-glucose RPMI, 1% vol/vol P/S) (4 groups, n=5-7 per group, each n represents pooled islets from two mice). Islet pellets were frozen at -80°C in 100 µL of SDS lysis buffer (4% SDS, 100 mM Tris, pH 8) and the proteins in each sample were precipitated using acetone. University of Victoria proteomics service performed non-targeted quantitative proteomic analysis using data-independent acquisition (DIA) with LC-MS/MS on an Orbitrap mass spectrometer. A mouse FASTA database was downloaded from Uniprot (http://uniprot.org). This file was used with the 6 gas phase fraction files from the analysis of the chromatogram library sample to create a mouse islet specific chromatogram library using the EncyclopeDIA (v 1.2.2) software package (Searle et al, 2018). This chromatogram library file was then

used to perform identification and quantitation of the proteins in the samples again using EncyclopeDIA with Overlapping DIA as the acquisition type, trypsin used as the enzyme, CID/HCD as the fragmentation, 10 ppm mass tolerances for the precursor, fragment, and library mass tolerances. The Percolator version used was 3.10. The precursor FDR rate was set to 1%. Protein abundances were log2 transformed, imputation was performed for missing values, then proteins were normalized to median sample intensities. Gene differential expression was analyzed using limma in Perseus (*109*).

Data from HPAP. To compare sex differences in dynamic insulin secretion, data acquired was from the Human Pancreas Analysis Program (HPAP-RRID:SCR_016202) Database (https://hpap.pmacs.upenn.edu), a Human Islet Research Network (RRID:SCR_014393) consortium (UC4-DK-112217, U01-DK-123594, UC4-DK-112232, and U01-DK-123716).

Statistical Analysis. Statistical analyses and data presentation were carried out using GraphPad Prism 9 (Graphpad Software, San Diego, CA, USA) or R (v 4.1.1) using a Student's *t*-test for parametric data and a Mann-Whitney test for non-parametric data. Statistical tests are indicated in the figure legends. For all statistical analyses, differences were considered significant if the p-value was less than 0.05. *: $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$. Data were presented as means ± SEM with individual data points from biological replicates.

Data Availability. Details of all statistical tests and *p*-values are provided in Supplementary file 5. All raw data generated in this study are available in Supplementary file 6. RNAseq data is available in Supplementary file 7 and Supplementary file 8.

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FIGURE LEGENDS

Figure 1 – Sex differences in human islet transcriptomic and functional responses in type 2 diabetes. scRNAseq data from male and female human β cells. (A-C) Venn diagrams compare the number of significantly differentially expressed genes between ND and T2D donors (*p*-adj<0.05). All differentially expressed genes (A), downregulated genes (B), upregulated genes (C) in T2D human β cells. For complete gene lists see Supplementary file 1. (D-F) Top 10 significantly enriched Reactome pathways (ND vs T2D) from non-sex-specific (D), female, or male (F) significantly differentially expressed genes (*p*-adj< 0.05). Gene ratio is calculated as *k/n*, where *k* is the number of genes identified in each Reactome pathway, and *n* is the number of genes from the submitted gene list participating in any Reactome pathway. For complete Reactome pathway lists see Supplementary file 1. (G-K) Human islet perifusion data from the Human Pancreas Analysis Program in ND and T2D donor islets in females (F, I) and males (G, H). 3 mM glucose (3 mM G); 16.7 mM glucose (16.7 mM

G); 0.1 mM isobutylmethylxanthine (0.1 mM IBMX); 30 mM potassium chloride (30 mM KCl); 4 mM amino acid mixture (4 mM AAM; mM: 0.44 alanine, 0.19 arginine, 0.038 aspartate, 0.094 citrulline, 0.12 glutamate, 0.30 glycine, 0.077 histidine, 0.094 isoleucine, 0.16 leucine, 0.37 lysine, 0.05 methionine, 0.70 ornithine, 0.08 phenylalanine, 0.35 proline, 0.57 serine, 0.27 threonine, 0.073 tryptophan, and 0.20 valine, 2 mM glutamine). (I-K) Quantification of area under the curve (AUC) is shown for the various stimulatory media in females (I), males (J) and donors with T2D (K). (I) In females, insulin secretion from ND islets was not significantly higher than T2D islets. (J) In males, insulin secretion from ND islets was significantly higher than T2D islets under 4 mM AAM +16.7 mM glucose (HG) + 0.1 mM IBMX stimulation (p=0.0442; unpaired Student's *t*-test). (K) Total insulin secretion was lower in T2D male islets than ND male islets (p=0.0503; unpaired Student's *t*-test). * indicates p<0.05, ** indicates p<0.01; ns indicates not significant; error bars indicate SEM.

Figure 2 – Sex-biased gene expression in mouse islet bulk RNAseq. (A) Principal component analysis (PCA) of RNAseq data from male and female mouse islets. (B) Over-representation analysis (ORA) of all significantly differentially expressed genes (*p*-adj < 0.01) from male and female mouse islets. Top 30 enriched KEGG pathways (large nodes; size = proportional to connections, darker red color = greater significance) and associated genes (small nodes; green = male enriched, yellow = female enriched). (C) Top significantly enriched Reactome pathways from the top 1000 significantly differentially expressed genes. (*p*-adj < 0.01) for males and females. Gene ratio is calculated as k/n, where k is the number of genes identified in each Reactome pathway, and n is the number of genes from the submitted gene list participating in any Reactome pathway. For complete Reactome pathway lists see Supplementary file 1. (D) All transcripts of differentially expressed genes under the gene ontology term "Cellular response to ER stress" (GO:0034976) and genes labeled by their role in transcription, translation, protein processing, protein folding, secretion and protein quality control. (E) All transcripts of differentially expressed ribosomal genes.

Figure 3 – Sex differences in mouse islet ER stress-associated phenotypes. (A) Protein synthesis was quantified in dispersed islet cells from 20-week-old male and female B6 mice after treatment with 1 μM Tg for 2- or 24-hours. In female islet cells, protein synthesis was significantly lower after a 2-hour Tg treatment compared to control (p=0.0152; paired Student's *t*-test) and significantly higher after a 24-hour Tg treatment compared to a 2-hour Tg treatment (p=0.0027; paired Student's *t*-test). In male islet cells, protein synthesis was significantly lower after a 2- and 24-hour Tg treatment compared to control (p=0.0289 [0-2 hour] and p=0.0485 [0-24 hour], respectively; paired Student's *t*-test). (B) In both male and female islet cells protein synthesis was repressed after 2-hours. By 24-hours, protein synthesis repression was resolved in female, but not male islet cells. (C-F) Quantification of propidium iodide (PI) cell death assay of dispersed islets from 20-week-old male and

female B6 mice treated with thapsigargin (0.1 μM, 1 μM or 10 μM Tg) or DMSO for 84-hours (n=6-8), compared to DMSO treatment in females (C, E) and males (D, F). In female islet cells, cell death was significantly higher in 10 μM Tg compared to control (p=0.0057; paired Student's *t*-test). In male islet cells, cell death was significantly higher in 0.1, 1.0 and 10 μM Tg compared to control (p=0.0313 [0.1 μM], p=0.0026 [1 μM] and p=0.0373 [10 μM], respectively; paired Student's *t*-test) (D). For C-F, %PI positive cells were normalized within each run to the DMSO control run average. (G-J) Levels of ER stress proteins were quantified in isolated islets from 20-week-old male and female B6 mice cultured in DMSO or 1 μM Tg for 24-hours. (G) BiP levels were significantly upregulated in female Tg vs DMSO (p=0.0011; paired Student's *t*-test) but not male Tg vs DMSO (p=0.1187; paired Student's *t*-test). (H) pIRE1α levels were significantly upregulated in female Tg vs DMSO (p=0.0001; paired Student's *t*-test) and in male Tg vs DMSO (p=0.0148; paired Student's *t*-test). (I) CHOP levels were significantly upregulated in female Tg vs DMSO (p=0.0333; paired Student's *t*-test) and in male Tg vs DMSO (p=0.0164; paired Student's *t*-test). (J) p-eIF2α levels were not significantly upregulated in either sex. (K-M) Levels of ER stress proteins were quantified in isolated islets from 60-week-old male and female B6 mice cultured in DMSO or 1 μM Tg for 24-hours. (K) BiP levels were significantly upregulated in male Tg vs DMSO (p=0.0048; paired Student's *t*-test) but not in female Tg vs DMSO (p=0.3319; paired Student's *t*-test). (L) p-IRE1α levels were not significantly upregulated in either sex (p=0.9257 [female] and p=0.8273 [male], respectively; paired Student's *t*-test). (M) p-eIF2α levels were not significantly upregulated in either sex (p=0.8451 [female] and p=0.3076 [male], respectively; paired Student's *t*-test). * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001; ns indicates not significant; error bars indicate SEM.

Figure 4 – Sex differences in *ex vivo* and *in vivo* insulin secretion. (A) Experimental workflow of static glucose-stimulated insulin secretion. (B) Insulin secretion at basal (3 mM; low glucose, LG) and stimulatory (20 mM; high glucose, HG) glucose. Female islet LG secretion was significantly higher compared with control after 2- and 4-hour Tg pre-treatments (p=0.0047 [2-hour] and p=0.0003 [4-hour], respectively; Mann Whitney test). Female islet HG secretion was significantly higher compared with control after 0- and 2-hour Tg pre-treatments (p=0.0012 [0-hour] and p=0.0061 [2-hour], respectively; Mann Whitney test). Male islet LG secretion was significantly higher compared to control after a 0-hour Tg pre-treatment (p=0.0371; Mann Whitney test). Male islet HG secretion was significantly lower compared with control after a 4-hour Tg pre-treatment (p=0.0012; Mann Whitney test). (C) Insulin content. Female islet insulin content was significantly higher compared with control after a 4-hour Tg pre-treatment (p=0.0269; Mann Whitney test). (D) Proinsulin secretion at basal (3 mM) and stimulatory (20 mM) glucose. Female islet HG secretion was significantly higher compared with control after 0- and 2-hour Tg pre-treatments (p=0.0075 [0-hour] and p=0.00437 [2-hour], respectively; Mann Whitney test). Male islet HG secretion was significantly lower compared with

control after a 4-hour Tg pre-treatment (p=0.0025; Mann Whitney test). (E) Proinsulin content. Female islet proinsulin content was significantly lower compared with control after a 2-hour Tg pre-treatment (p=0.0437; Mann Whitney test). Male islet proinsulin content was significantly lower compared with control after 2- and 4-hour Tg pre-treatments (p=0.0014 [2-hour] and p=0.0005 [4-hour], respectively; Mann Whitney test). (F-H) Physiology measurements after a 6-hour fast in 20-week-old male and female B6 mice. (F, G) Insulin levels from glucose-stimulated insulin secretion tests (F: nM, G: % basal insulin) following a single glucose injection (2 g glucose/kg body weight, i.p). Area under the curve (AUC) calculations (n=13 females, n=18 males). (F) Insulin levels were significantly higher in male mice at 0 minutes and 30 minutes post injection (p=0.0063 [0 minutes] and p=0.0009 [30 minutes], respectively; Student's *t*-test). AUC was significantly higher in males (p=0.0159; Student's *t*-test). (G) Insulin levels (% baseline). Glucose-stimulated insulin secretion was significantly higher in female mice 15 minutes post injection (p=0.0279; Student's *t*-test). (H) Glucose levels from glucose tolerance tests following a single glucose injection (2 g glucose/kg body weight). AUC calculations (n=11 females, n=11 males). For B-E, grey triangles indicate the concentration of insulin or proinsulin from five islets, black circles indicate the average values per mouse. For B, ## indicates p<0.01 and ### indicates p<0.001 for comparisons between treatments and DMSO in low glucose. For all other figures, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001; ns indicates not significant; error bars indicate SEM.

Figure 5 – Sex-specific transcriptomic and proteomic profiles following ER stress in mouse islets. (A) Principal component analysis (PCA) of RNAseq data from male and female mouse islets treated with DMSO or 1 μM Tg for 6- or 12-hours. (B) Spearman correlation depicting the variance for the first 5 principal components. (C) Top significantly enriched Reactome pathways from the top 1000 significantly differentially expressed genes (*p*-adj<0.01) for females and males that were upregulated or downregulated between 6-12 hours of Tg treatment. Gene ratio is calculated as k/n, where k is the number of genes identified in each Reactome pathway, and n is the number of genes from the submitted gene list participating in any Reactome pathway. (D) Protein abundance from proteomics data of female and male mouse islets treated with DMSO or 1 μM Tg for 6-hours. Top 45 differentially expressed proteins are shown (p< 0.05).

Table 1 – Human β cell pathway gene numbers. The number of genes corresponding to each T2D upregulated pathway in males, females or both sexes.

SUPPLEMENTAL FIGURE LEGENDS

Figure 1-figure supplement 1 – Sex-specific and non-sex-specific differentially expressed genes in T2D. scRNAseq data from male and female human β cells. (A-C) Top 60 significantly

differentially expressed genes (*p*-adj < 0.05). Non-sex-specific (A), female-specific (B), or male-specific (C). For complete gene lists see Supplementary file 1.

Figure 1-figure supplement 2 – Gene expression changes in T2D. scRNAseq data from male and female human β cells. (A, B) Top 60 differentially expressed genes (*p*-adj < 0.05) in females (A) and males (B). Sex-specific genes are indicated in red text. For complete gene lists see Supplementary file 1.

Figure 2-figure supplement 1 – Equivalent insulin sensitivity in male and female mice. (A) Insulin tolerance test (ITT). 20-week-old female and male B6 mice were fasted for 6 hours. Glucose levels (% baseline) from insulin tolerance tests (ITT) following a single insulin injection (0.75U insulin/kg body weight). AUC calculations (n=11 females, n=11 males). ns indicates not significant; error bars indicate SEM.

Figure 2-figure supplement 2 – Mouse islet gene expression clusters by sex. (A) Unsupervised hierarchical clustering of RNAseq data from female and male mouse islets. Sorting was based on all genes where the total count was >10 across all samples.

Figure 3-figure supplement 1 – ER stress-induced protein synthesis repression persists in male mouse islet cells. (A) Representative images of dispersed islets stained with nuclear mask and OPP labeled with Alexa Fluor 594. (B, C) Integrated staining intensity of Alexa Fluor 594 in nuclear mask positive islet cells in control media (B, FBS+) or after treatment with DMSO control or 1 μM Tg for 2- or 24-hours (C, FBS-). Protein synthesis is displayed on a per cell basis from data shown in Figure 3. n=4-5 mice, >1000 cells per group. Mean values are indicated under each group. (B) Protein synthesis was significantly higher in male islet cells than female islet cells in control media, 3.9% (p=0.027; unpaired Student's *t*-test). (C) In female islet cells, protein synthesis was significantly repressed from control-2 hour treatments (p=<0.0001; unpaired Student's *t*-test) and significantly increased from both control-24 hour treatments and 2-24 hour treatments (p=<0.0001; unpaired Student's *t*-test). In male islet cells, protein synthesis was significantly repressed from control-2 hour treatments (p=<0.0001; unpaired Student's *t*-test) and control-24 hour treatments (p=<0.0001; unpaired Student's *t*-test); however, was not significantly different between 2-24 hour treatments (p=0.07; unpaired Student's *t*-test). * indicates p<0.05, *** indicates p<0.001; ns indicates not significant; error bars indicate SEM.

Figure 3-figure supplement 2 – *Ins2* gene activity is repressed by ER stress induction. *Ins2* gene activity in β cells from 20-week-old male and female B6 mice treated with Tg (0.1 μM or 1 μM

Tg) or DMSO for 60 hours (n=6 mice per sex, > 1000 cells per group). (A, B) Average change in fluorescence intensity from all GFP expressing female (A) and male (B) β cells over time. Data was normalized to the first 2 hours to examine relative change in *Ins2* gene activity. (C, D) Density plot of *Ins2*$^{GFP/WT}$ β cell GFP fluorescence intensity, log transformed. Data is shown for each run for females (C) and males (D). (E-H) Average change in high (E, F) and low (G,H) GFP *Ins2*$^{GFP/WT}$ β cells fluorescence over time from females (E, G) and males (F, H). Data was normalized to the first two hours to examine relative change in *Ins2* gene activity.

Figure 3-figure supplement 3 – Representative western blot images of UPR protein markers. (A) Representative western blot images of 20-week Tg treated mouse islets [Figure 3G-I]. (B) Representative western blot images of 60-week Tg treated mouse islets [Figure 3K-M].

Figure 4-figure supplement 1 – Female mouse islets retain greater insulin secretion during ER stress. (A, B) Insulin secretion from data in Figure 4B shown as percent increase compared to control (DMSO) secretion in 3 mM glucose (A) and in 20 mM glucose (B). In low glucose, female islet insulin secretion was significantly higher than control in all conditions (p=0.0376 [0-hour], p=0.0219 [2-hour] and p=0.0004 [4-hour], respectively; Mann Whitney test). In low glucose, male islet insulin secretion was significantly higher than control after a 0-hour Tg pre-treatment (p=0.0253 [0-hour]; Mann Whitney test). In high glucose, female islet insulin secretion was significantly higher than control after a 0-hour and 2-hour Tg pre-treatment (p=0.0083 [0-hour] and p=0.0371 [2-hour], respectively; Mann Whitney test). In high glucose, male islet insulin secretion was significantly lower than control after a 4-hour Tg pre-treatment (p=0.0013 [4-hour]; Mann Whitney test). * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001; ns indicates not significant; error bars indicate SEM.

Figure 5-figure supplement 1 – Mouse islet gene expression clusters by sex, treatment and time. (A) Unsupervised hierarchical clustering of RNAseq data from female and male DMSO or Tg treated mouse islets. Sorting was based on all genes where the total count >10 across all samples.

Figure 5-figure supplement 2 – Female and Male mouse islets are enriched in similar pathways following 6- and 12-hour Tg treatments. (A, B) Most significantly enriched Reactome pathways from the top 1000 significantly differentially expressed genes. (*p*-adj < 0.01) for females and males between DMSO vs Tg after 6-hours (A) or 12-hours (B) of Tg treatment. Gene ratio is calculated as k/n, where k is the number of genes identified in each Reactome pathway, and n is the number of genes from the submitted gene list participating in any Reactome pathway.

Figure 5-figure supplement 3 – A greater number of β cell identity genes are downregulated between 6- and 12- hour Tg treatment times in female mouse islets. (A, B) Treatment:Time interaction plots of female islet (A) and male islet (B) β cell identity genes in Reactome pathway "Regulation of gene expression in β cells". The fold change (FC) for DMSO vs Tg was calculated for each sex and time point (Female 6-hour, Female 12-hour, Male 6-hour, Male 12-hour). The change in FC values (12-hour FC – 6-hour FC) were plotted according to *p*-adj values. In females, FC values between 6- and 12-hours are represented by orange and purple dots, respectively. In males, FC values at 6- and 12-hours are represented by green and blue dots, respectively. A solid black line connecting the dots indicates genes with a significant treatment:time interaction.

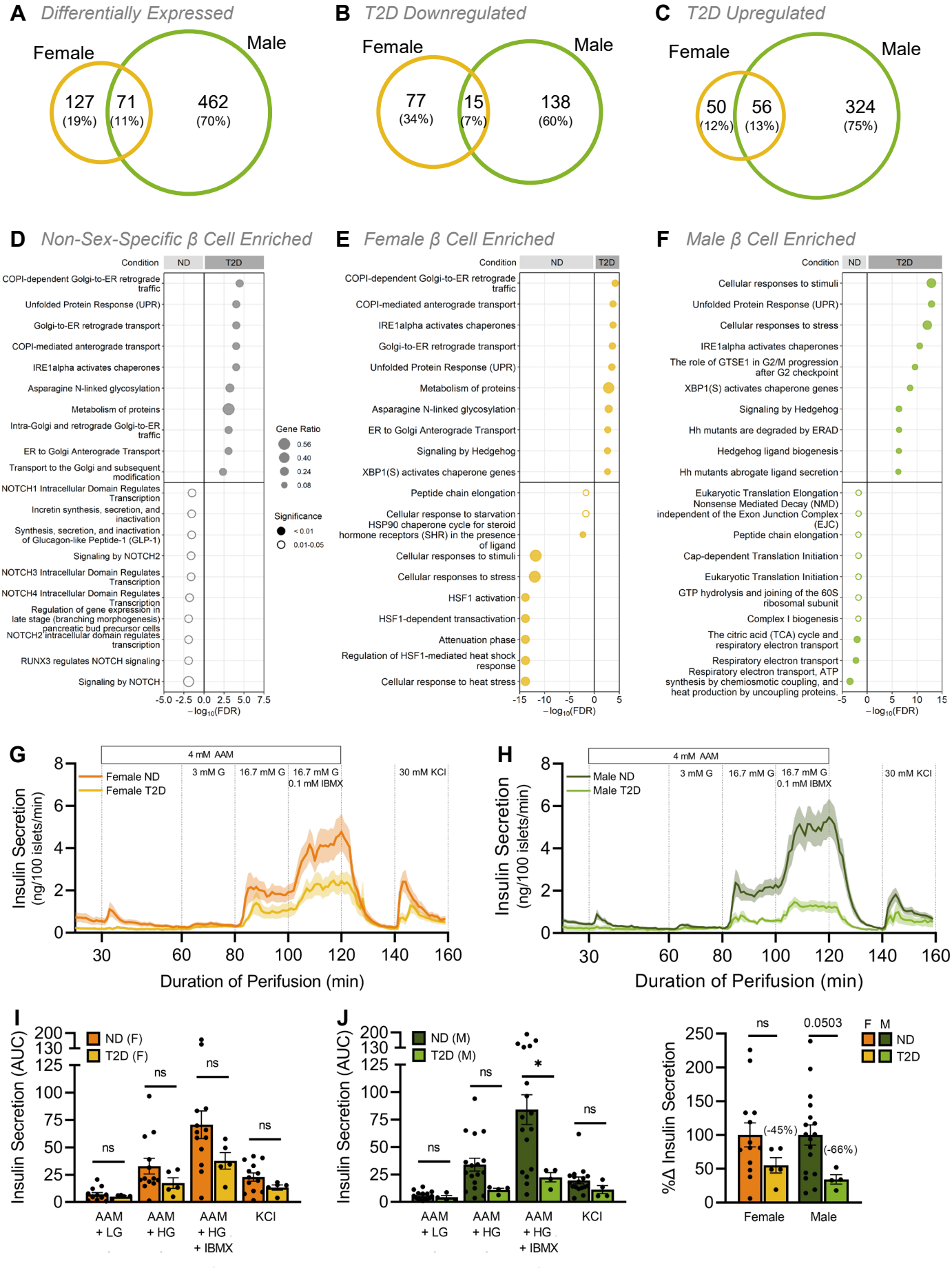

A Differentially Expressed
Female
Male
127 (19%)
71 (11%)
462 (70%)
B T2D Downregulated
Female
Male
77 (34%)
15 (7%)
138 (60%)
C T2D Upregulated
Female
Male
50 (12%)
56 (13%)
324 (75%)
D Non-Sex-Specific β Cell Enriched
Condition ND T2D
COPI-dependent Golgi-to-ER retrograde traffic
Unfolded Protein Response (UPR)
Golgi-to-ER retrograde transport
COPI-mediated anterograde transport
IRE1alpha activates chaperones
Asparagine N-linked glycosylation
Metabolism of proteins
Intra-Golgi and retrograde Golgi-to-ER traffic
ER to Golgi Anterograde Transport
Transport to the Golgi and subsequent modification
NOTCH1 Intracellular Domain Regulates Transcription
Incretin synthesis, secretion, and inactivation
Synthesis, secretion, and inactivation of Glucagon-like Peptide-1 (GLP-1)
Signaling by NOTCH2
NOTCH3 Intracellular Domain Regulates Transcription
NOTCH4 Intracellular Domain Regulates Transcription
Regulation of gene expression in late stage (branching morphogenesis) pancreatic bud precursor cells
NOTCH2 intracellular domain regulates transcription
RUNX3 regulates NOTCH signaling
Signaling by NOTCH
Gene Ratio 0.56 0.40 0.24 0.08
Significance < 0.01 0.01-0.05
-5.0 -2.5 0.0 2.5 5.0 7.5
−log10(FDR)
E Female β Cell Enriched
Condition ND T2D
COPI-dependent Golgi-to-ER retrograde traffic
COPI-mediated anterograde transport
IRE1alpha activates chaperones
Golgi-to-ER retrograde transport
Unfolded Protein Response (UPR)
Metabolism of proteins
Asparagine N-linked glycosylation
ER to Golgi Anterograde Transport
Signaling by Hedgehog
XBP1(S) activates chaperone genes
Peptide chain elongation
Cellular response to starvation
HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand
Cellular responses to stimuli
Cellular responses to stress
HSF1 activation
HSF1-dependent transactivation
Attenuation phase
Regulation of HSF1-mediated heat shock response
Cellular response to heat stress
-15 -10 -5 0 5
−log10(FDR)
F Male β Cell Enriched
Condition ND T2D
Cellular responses to stimuli
Unfolded Protein Response (UPR)
Cellular responses to stress
IRE1alpha activates chaperones
The role of GTSE1 in G2/M progression after G2 checkpoint
XBP1(S) activates chaperone genes
Signaling by Hedgehog
Hh mutants are degraded by ERAD
Hedgehog ligand biogenesis
Hh mutants abrogate ligand secretion
Eukaryotic Translation Elongation
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)
Peptide chain elongation
Cap-dependent Translation Initiation
Eukaryotic Translation Initiation
GTP hydrolysis and joining of the 60S ribosomal subunit
Complex I biogenesis
The citric acid (TCA) cycle and respiratory electron transport
Respiratory electron transport
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.
-5 0 5 10 15
−log10(FDR)
G
4 mM AAM
Female ND
Female T2D
3 mM G
16.7 mM G
16.7 mM G 0.1 mM IBMX
30 mM KCl
Insulin Secretion (ng/100 islets/min)
0 2 4 6 8
30 60 80 100 120 140 160
Duration of Perifusion (min)
H
4 mM AAM
Male ND
Male T2D
3 mM G
16.7 mM G
16.7 mM G 0.1 mM IBMX
30 mM KCl
Insulin Secretion (ng/100 islets/min)
0 2 4 6 8
30 60 80 100 120 140 160
Duration of Perifusion (min)
I
ND (F)
T2D (F)
Insulin Secretion (AUC)
200 130 125 100 75 50 25 0
ns
ns
ns
ns
AAM + LG
AAM + HG
AAM + HG + IBMX
KCl
J
ND (M)
T2D (M)
Insulin Secretion (AUC)
200 130 125 100 75 50 25 0
ns
ns
*
ns
AAM + LG
AAM + HG
AAM + HG + IBMX
KCl
%Δ Insulin Secretion
250 200 150 100 50 0
ns
0.0503
F M
ND
T2D
(-45%)
(-66%)
Female
Male

Figure 1

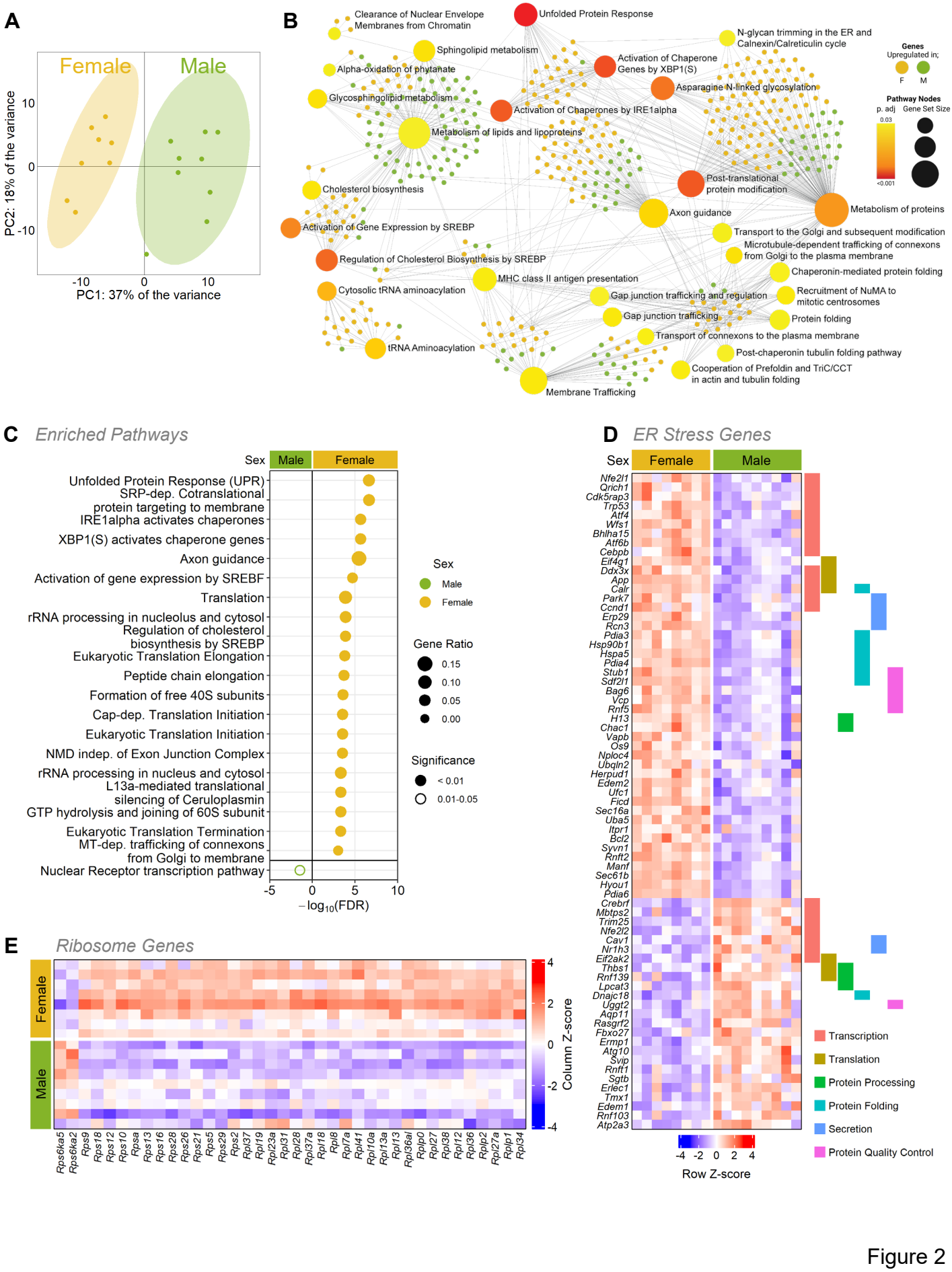

A
Female
Male
PC2: 18% of the variance
PC1: 37% of the variance
B
Clearance of Nuclear Envelope Membranes from Chromatin
Unfolded Protein Response
N-glycan trimming in the ER and Calnexin/Calreticulin cycle
Sphingolipid metabolism
Activation of Chaperone Genes by XBP1(S)
Alpha-oxidation of phytanate
Asparagine N-linked glycosylation
Glycosphingolipid metabolism
Activation of Chaperones by IRE1alpha
Metabolism of lipids and lipoproteins
Post-translational protein modification
Cholesterol biosynthesis
Axon guidance
Metabolism of proteins
Activation of Gene Expression by SREBP
Transport to the Golgi and subsequent modification
Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane
Regulation of Cholesterol Biosynthesis by SREBP
Chaperonin-mediated protein folding
MHC class II antigen presentation
Cytosolic tRNA aminoacylation
Recruitment of NuMA to mitotic centrosomes
Gap junction trafficking and regulation
Gap junction trafficking
Protein folding
tRNA Aminoacylation
Transport of connexons to the plasma membrane
Post-chaperonin tubulin folding pathway
Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding
Membrane Trafficking
Genes Upregulated in;
F M
Pathway Nodes
p. adj
Gene Set Size
C
Enriched Pathways
Sex
Male
Female
Unfolded Protein Response (UPR)
SRP-dep. Cotranslational protein targeting to membrane
IRE1alpha activates chaperones
XBP1(S) activates chaperone genes
Axon guidance
Activation of gene expression by SREBF
Translation
rRNA processing in nucleolus and cytosol
Regulation of cholesterol biosynthesis by SREBP
Eukaryotic Translation Elongation
Peptide chain elongation
Formation of free 40S subunits
Cap-dep. Translation Initiation
Eukaryotic Translation Initiation
NMD indep. of Exon Junction Complex
rRNA processing in nucleus and cytosol
L13a-mediated translational silencing of Ceruloplasmin
GTP hydrolysis and joining of 60S subunit
Eukaryotic Translation Termination
MT-dep. trafficking of connexons from Golgi to membrane
Nuclear Receptor transcription pathway
−log10(FDR)
Gene Ratio
0.15
0.10
0.05
0.00
Significance
< 0.01
0.01-0.05
D
ER Stress Genes
Sex
Female
Male
Nfe2l1
Qrich1
Cdk5rap3
Trp53
Atf4
Wfs1
Bhlha15
Atf6b
Cebpb
Eif4g1
Ddx3x
App
Calr
Park7
Ccnd1
Erp29
Rcn3
Pdia3
Hsp90b1
Hspa5
Pdia4
Stub1
Sdf2l1
Bag6
Vcp
Rnf5
H13
Chac1
Vapb
Os9
Nploc4
Ubqln2
Herpud1
Edem2
Ufc1
Ficd
Sec16a
Uba5
Itpr1
Bcl2
Syvn1
Rnft2
Manf
Sec61b
Hyou1
Pdia6
Crebrf
Mbtps2
Trim25
Nfe2l2
Cav1
Nr1h3
Eif2ak2
Thbs1
Rnf139
Lpcat3
Dnajc18
Uggt2
Aqp11
Rasgrf2
Fbxo27
Ermp1
Atg10
Svip
Rnft1
Sgtb
Erlec1
Tmx1
Edem1
Rnf103
Atp2a3
Row Z-score
Transcription
Translation
Protein Processing
Protein Folding
Secretion
Protein Quality Control
E
Ribosome Genes
Female
Male
Column Z-score
Rps6ka5
Rps6ka2
Rps9
Rps18
Rps12
Rps10
Rpsa
Rps13
Rps16
Rps28
Rps26
Rps21
Rps5
Rps29
Rps2
Rpl37
Rpl19
Rpl23a
Rpl31
Rpl28
Rpl37a
Rpl18
Rpl8
Rpl7a
Rpl41
Rpl10a
Rpl13a
Rpl13
Rpl36al
Rplp0
Rpl27
Rpl38
Rpl12
Rpl36
Rplp2
Rpl27a
Rplp1
Rpl34

Figure 2

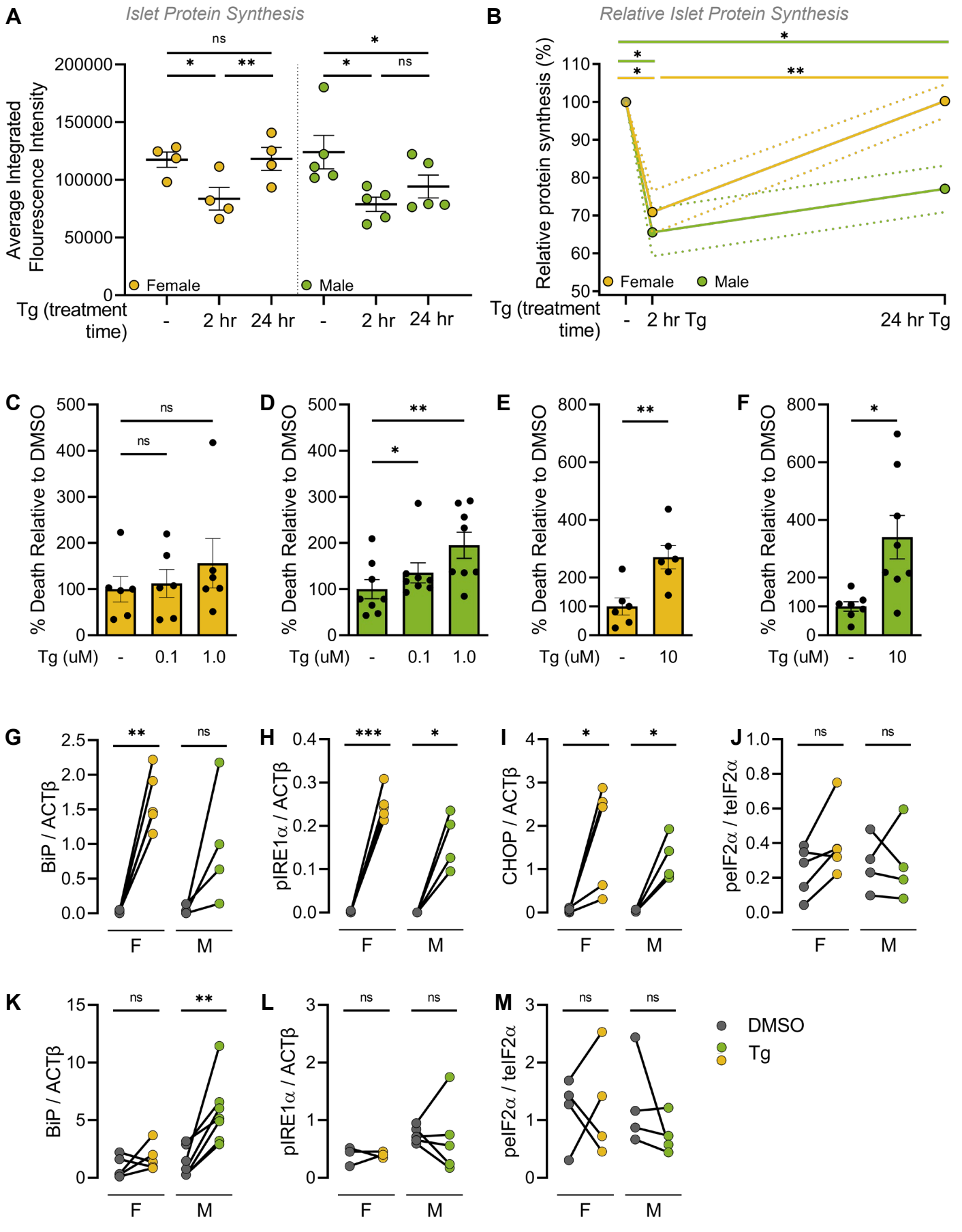

A
Islet Protein Synthesis
Average Integrated Flourescence Intensity
200000
150000
100000
50000
0
ns
*
**
*
*
ns
Female
Male
Tg (treatment time)
-
2 hr
24 hr
-
2 hr
24 hr
B
Relative Islet Protein Synthesis
Relative protein synthesis (%)
110
100
90
80
70
60
50
*
*
*
**
Female
Male
Tg (treatment time)
-
2 hr Tg
24 hr Tg
C
% Death Relative to DMSO
500
400
300
200
100
0
ns
ns
Tg (uM)
-
0.1
1.0
D
% Death Relative to DMSO
500
400
300
200
100
0
**
*
Tg (uM)
-
0.1
1.0
E
% Death Relative to DMSO
800
600
400
200
100
0
**
Tg (uM)
-
10
F
% Death Relative to DMSO
800
600
400
200
100
0
*
Tg (uM)
-
10
G
BiP / ACTβ
2.5
2.0
1.5
1.0
0.5
0.0
**
ns
F
M
H
pIRE1α / ACTβ
0.4
0.3
0.2
0.1
0.0
***
*
F
M
I
CHOP / ACTβ
4
3
2
1
0
*
*
F
M
J
peIF2α / teIF2α
1.0
0.8
0.6
0.4
0.2
0.0
ns
ns
F
M
K
BiP / ACTβ
15
10
5
0
ns
**
F
M
L
pIRE1α / ACTβ
3
2
1
0
ns
ns
F
M
M
peIF2α / teIF2α
3
2
1
0
ns
ns
F
M
DMSO
Tg

Figure 3

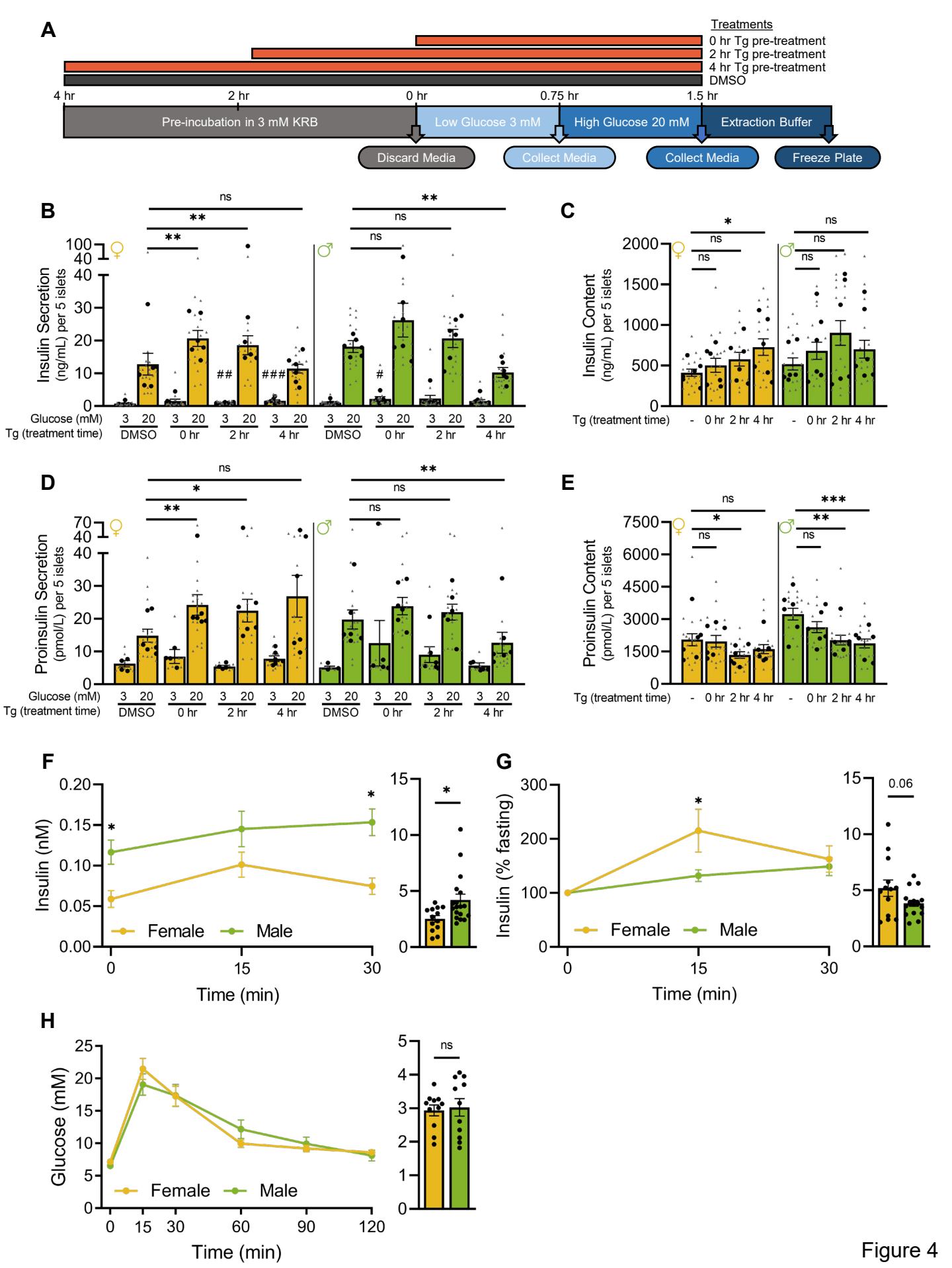

A
Treatments
0 hr Tg pre-treatment
2 hr Tg pre-treatment
4 hr Tg pre-treatment
DMSO
4 hr
2 hr
0 hr
0.75 hr
1.5 hr
Pre-incubation in 3 mM KRB
Low Glucose 3 mM
High Glucose 20 mM
Extraction Buffer
Discard Media
Collect Media
Collect Media
Freeze Plate
B
Insulin Secretion (ng/mL) per 5 islets
Glucose (mM)
Tg (treatment time)
DMSO
0 hr
2 hr
4 hr
C
Insulin Content (ng/mL) per 5 islets
Tg (treatment time)
D
Proinsulin Secretion (pmol/L) per 5 islets
E
Proinsulin Content (pmol/L) per 5 islets
F
Insulin (nM)
Female
Male
Time (min)
G
Insulin (% fasting)
0.06
H
Glucose (mM)
Female
Male
Time (min)

Figure 4

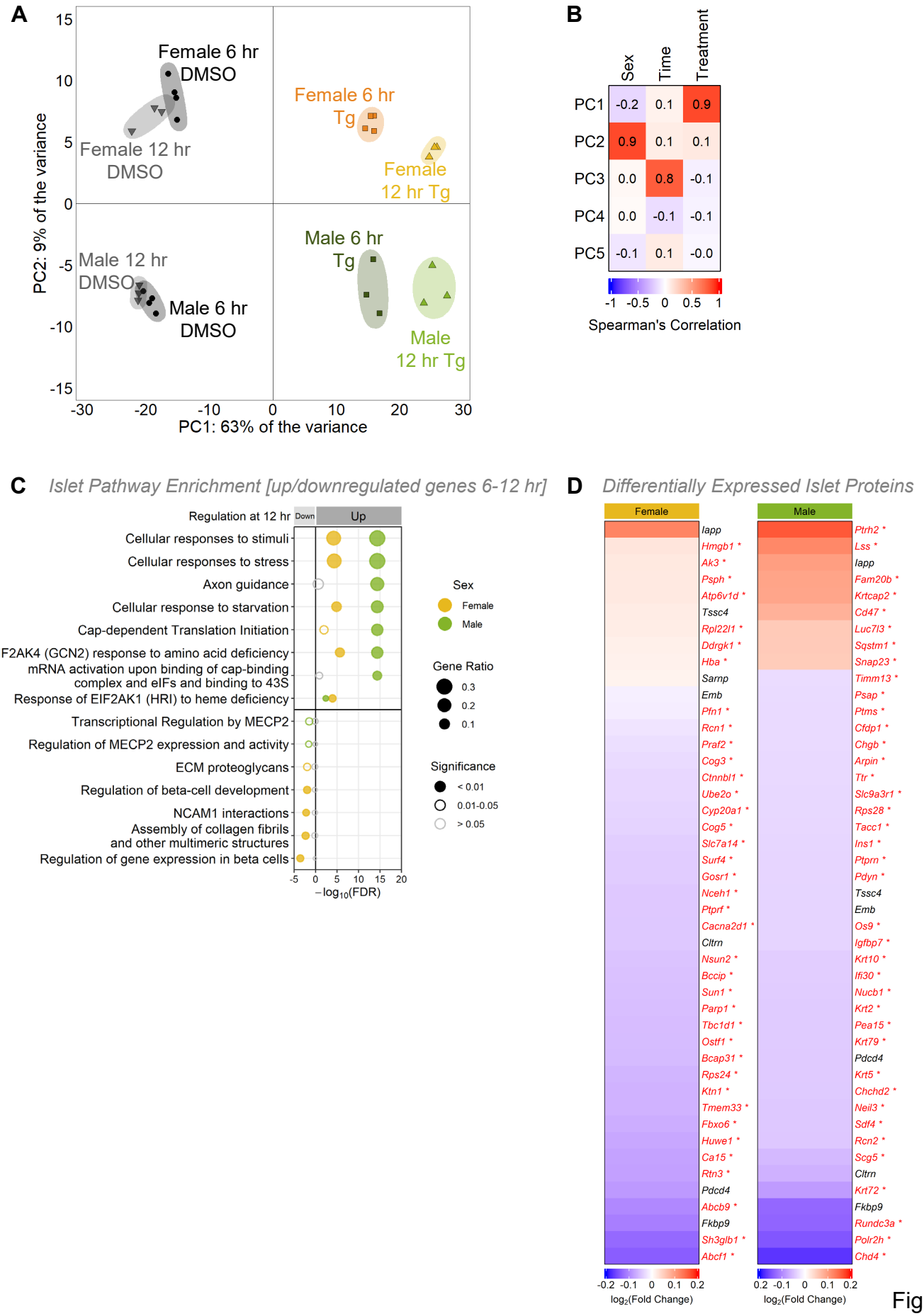

A
Female 6 hr DMSO
Female 12 hr DMSO
Female 6 hr Tg
Female 12 hr Tg
Male 12 hr DMSO
Male 6 hr DMSO
Male 6 hr Tg
Male 12 hr Tg
PC2: 9% of the variance
PC1: 63% of the variance
B
Sex
Time
Treatment
PC1 -0.2 0.1 0.9
PC2 0.9 0.1 0.1
PC3 0.0 0.8 -0.1
PC4 0.0 -0.1 -0.1
PC5 -0.1 0.1 -0.0
Spearman's Correlation
C
Islet Pathway Enrichment [up/downregulated genes 6-12 hr]
Regulation at 12 hr
Down
Up
Cellular responses to stimuli
Cellular responses to stress
Axon guidance
Cellular response to starvation
Cap-dependent Translation Initiation
EIF2AK4 (GCN2) response to amino acid deficiency
mRNA activation upon binding of cap-binding complex and eIFs and binding to 43S
Response of EIF2AK1 (HRI) to heme deficiency
Transcriptional Regulation by MECP2
Regulation of MECP2 expression and activity
ECM proteoglycans
Regulation of beta-cell development
NCAM1 interactions
Assembly of collagen fibrils and other multimeric structures
Regulation of gene expression in beta cells
−log10(FDR)
Sex
Female
Male
Gene Ratio
0.3
0.2
0.1
Significance
< 0.01
0.01-0.05
> 0.05
D
Differentially Expressed Islet Proteins
Female
Male
log2(Fold Change)
log2(Fold Change)

Figure 5

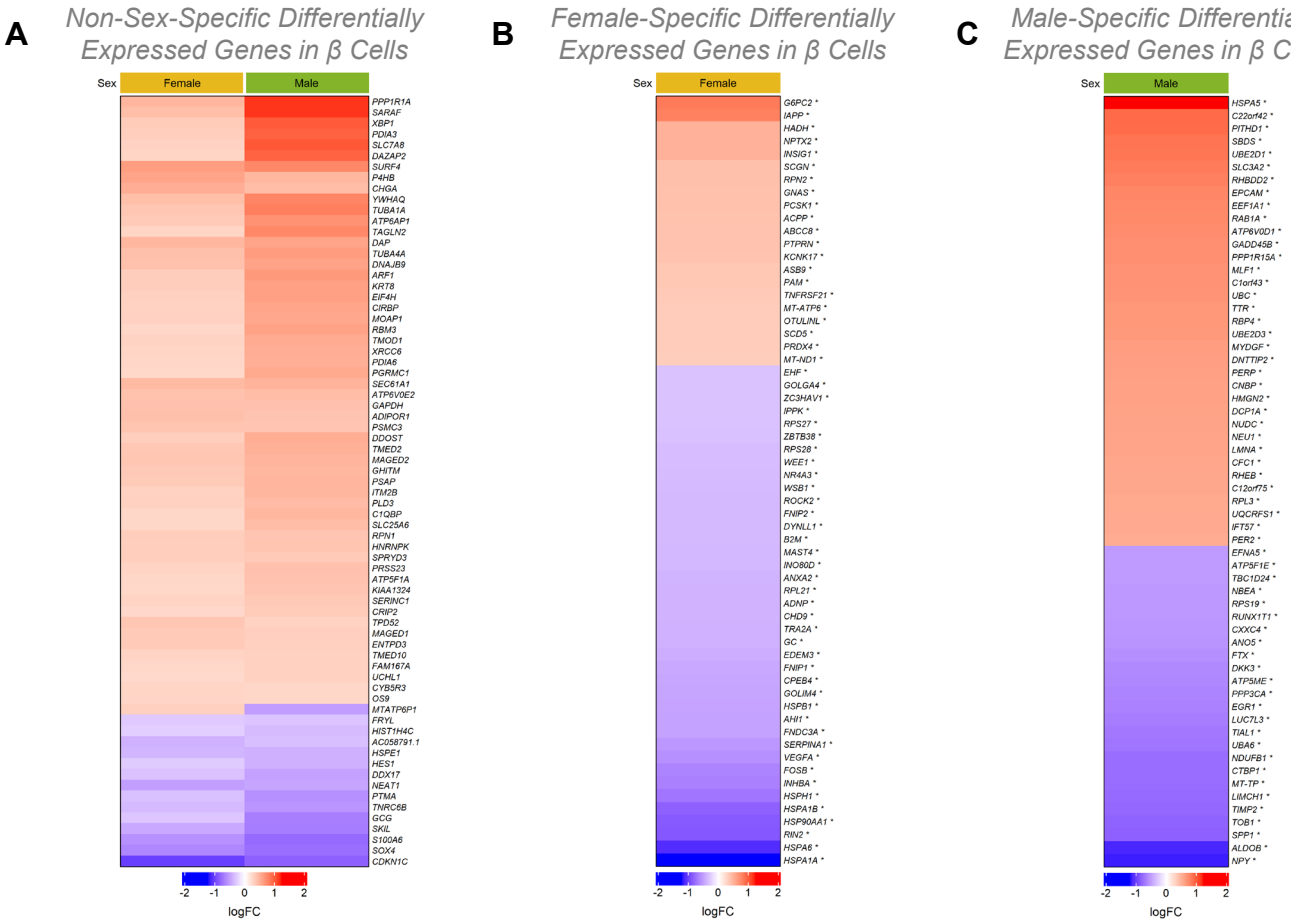
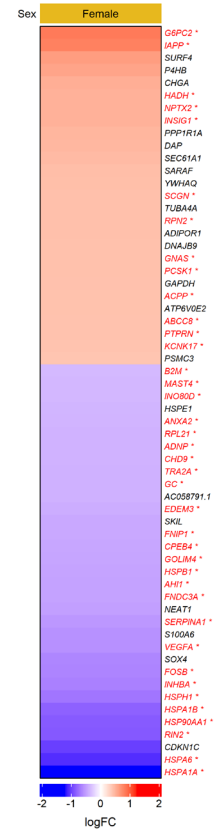


Figure 1-figure supplement 1

A

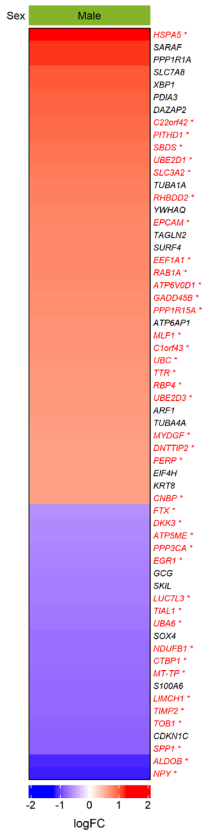
Top 60 Differentially Expressed Genes in Female β Cells



*Indicates genes unique to females

B

Top 60 Differentially Expressed Genes in Male β Cells



*Indicates genes unique to males

Figure 1-figure supplement 2

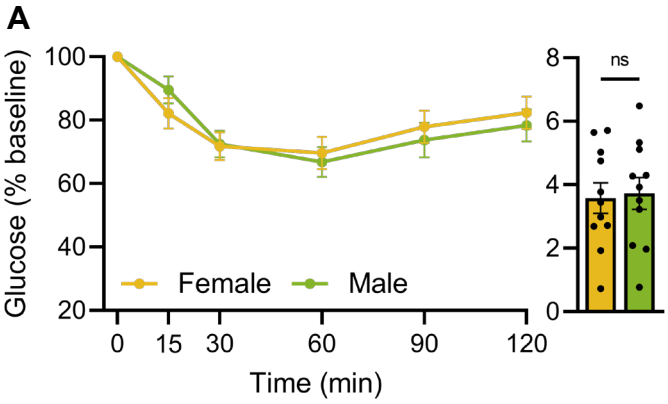


Figure 2-figure supplement 1

Figure 2-figure supplement 2

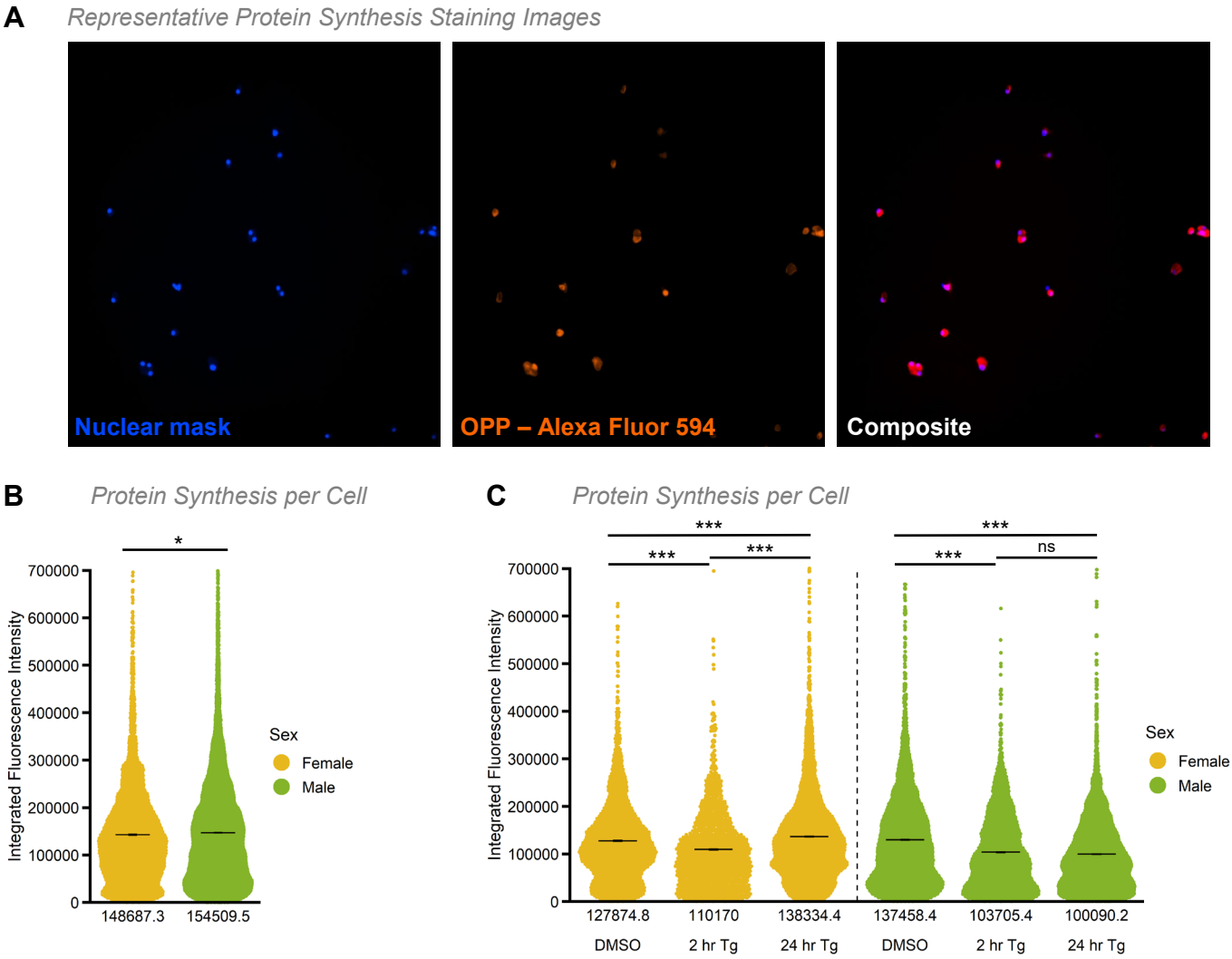


Figure 3-figure supplement 1

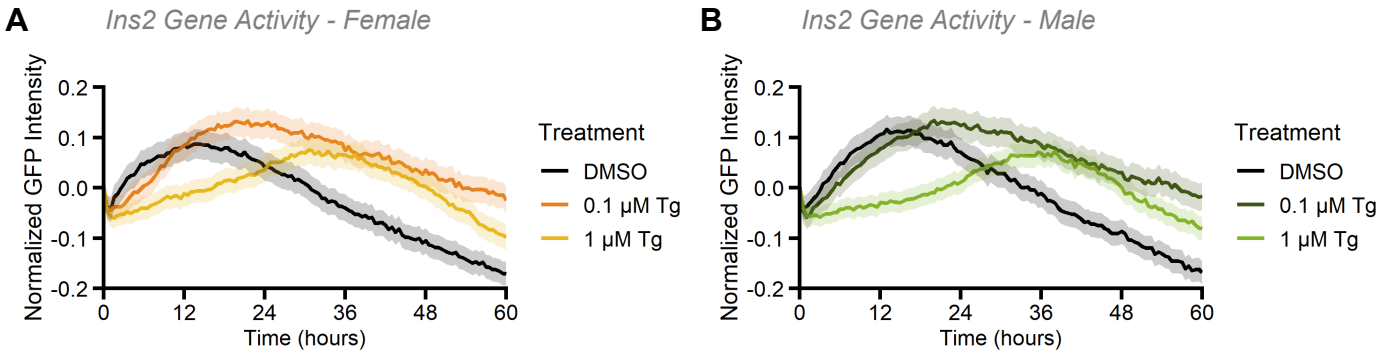


Figure 3-figure supplement 2

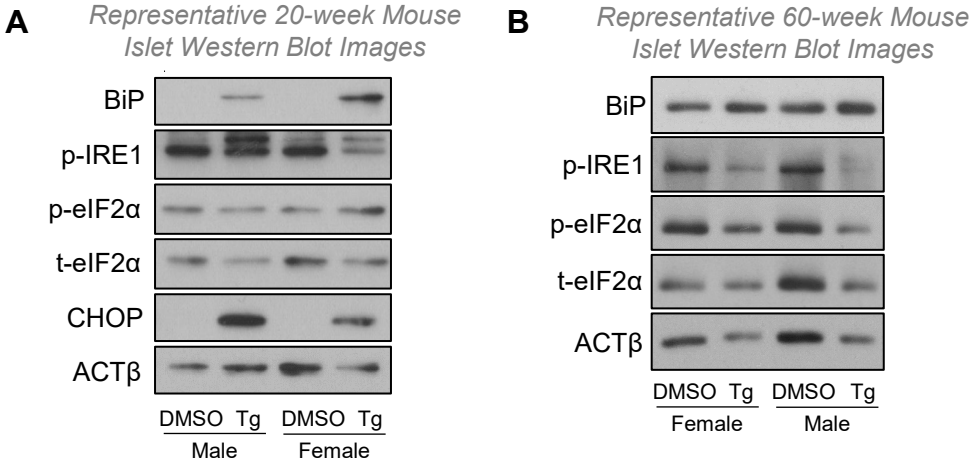


Figure 3-figure supplement 3

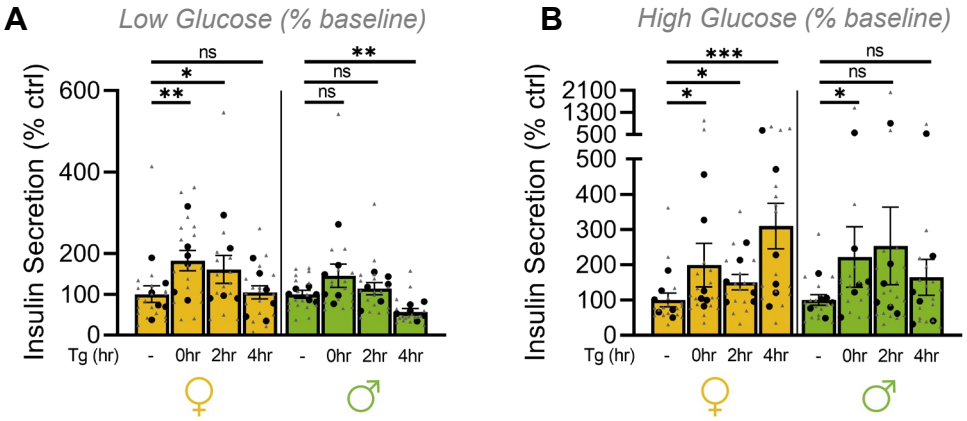


Figure 4-figure supplement 1

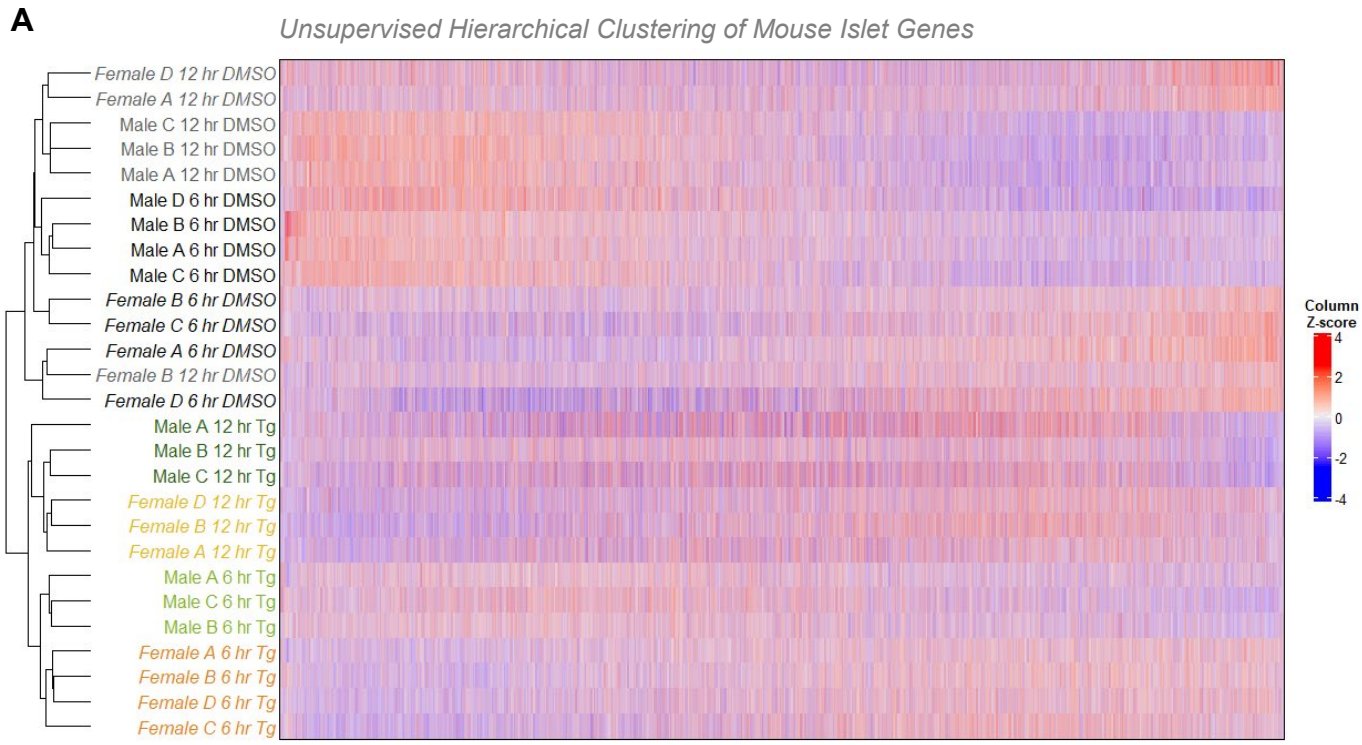
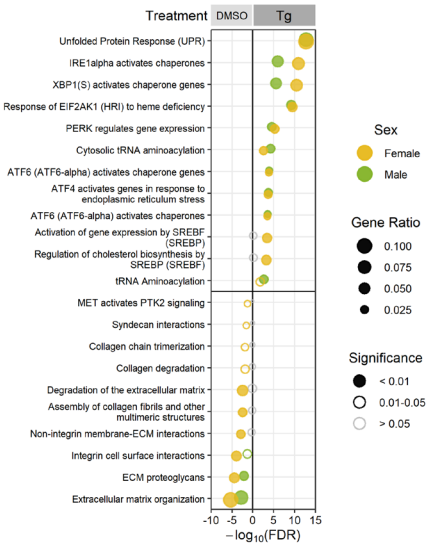


Figure 5-figure supplement 1

A

Islet Pathway Enrichment
[6 hr, DMSO vs Tg]



B

Islet Pathway Enrichment
[12 hr, DMSO vs Tg]

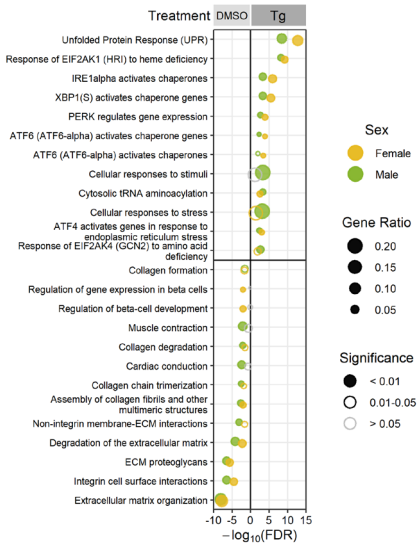


Figure 5-figure supplement 2

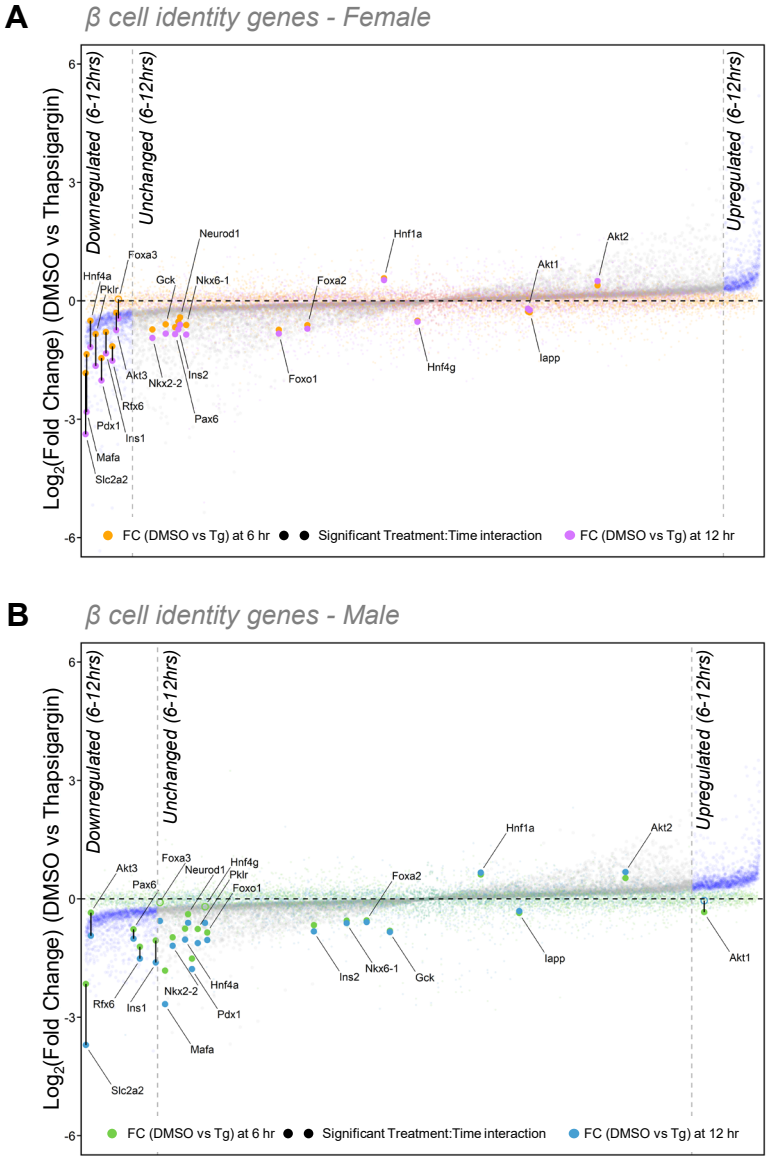


Figure 5-figure supplement 3

	Number of Pathway Genes		
Pathway Name	Unique Male	Common	Unique Female
Asparagine N-linked glycosylation	16	9	4
Cellular responses to stimuli	49	9	6
Cellular responses to stress	47	9	6
COPI-dependent Golgi-to-ER retrograde traffic	7	6	2
COPI-mediated anterograde transport	7	5	2
ER to Golgi Anterograde Transport	10	5	2
Golgi-to-ER retrograde transport	7	6	2
Hedgehog ligand biogenesis	12	4	1
Hh mutants abrogate ligand secretion	12	3	1
Hh mutants are degraded by ERAD	12	3	1
IRE1alpha activates chaperones	9	3	1
Metabolism of proteins	69	20	13
Signaling by Hedgehog	16	6	2
The role of GTSE1 in G2/M progression after G2 checkpoint	14	4	1
Unfolded Protein Response (UPR)	13	3	1
XBP1(S) activates chaperone genes	8	3	1