TITLE: The type 1 diabetes at-risk allele at rs3842753 increases beta cell *INS* mRNA expression and variance in a meta-analysis of single cell RNA sequencing data

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ABSTRACT

Type 1 diabetes is characterized by the autoimmune destruction of insulin secreting beta-cells. Genetic variations upstream at the insulin (*INS*) locus contribute to ~10% of type 1 diabetes heritable risk. Multiple studies showed an association between rs3842753 C/C genotype and type 1 diabetes susceptibility. Three small studies have reported an association between the rs3842753 C allele and increased whole pancreas *INS* expression. To date, no large-scale studies have looked at the effect of those genetic variations on insulin expression at the single cell level. We aligned all human islet single cell RNA sequencing datasets available to us in 2020 to the reference genome GRCh38.98 and genotyped rs3842753. We integrated 2315 beta-cells from 13 A/A donors, 23 A/C heterozygous donors, and 35 C/C at-risk donors. The donors included adults without diabetes and with type 2 diabetes. *INS* expression mean and variance were significantly higher in single β cells from females compared with males. Comparing across all β cells, we found that rs3842753 C containing cells (either homozygous or heterozygous) had the highest *INS* expression. We also found that β cells with the rs3842753 C allele had significantly higher ER stress marker gene expression compared to the A/A homozygous genotype. These findings support the emerging concept that inherited risk of type 1 diabetes may be associated with elevated insulin production at the mRNA level which may lead to β cell ER stress and fragility.

INTRODUCTION

Pancreatic β cells are the body's primary source of insulin, a hormone that promotes glucose uptake from the bloodstream into tissues (1). Type 1 diabetes constitutes ~10% of diabetes cases and is caused by the autoimmune destruction of a substantial proportion of β cells (1). Type 1 diabetes is highly heritable with a twin concordance between 30% and 70% (2). The HLA locus is the major genetic determinant of type 1 diabetes risk (OR = 0.02 to 11), but other genes play a significant role as well (3). Gene mapping and GWAS studies identified the human insulin (*INS*) locus as the 2nd most important contributor of type 1 diabetes genetic risk (OR = 2.38, ~10% of heritable risk) (4; 5). The region of the *INS* locus implicated in type 1 diabetes susceptibility has three genetic variants in near-perfect linkage disequilibrium with each other (4-6). Variants associated with increased type 1 diabetes risk are: a shortening of the repetitive region 400bp upstream of *INS* (class III *INS*-VNTR > class I *INS*-VNTR), a single nucleotide polymorphism (SNP) in the *INS* 5' intronic region (rs689 T>A), and a SNP in the *INS* 3'UTR (rs3842753 A>C) (4-8). Often, the SNP alleles are examined as surrogates for *INS*-VNTR length (6; 8-10). The rs3842753 C allele has been consistently correlated with increased type 1 diabetes susceptibility (5; 6; 8).

Despite genetic studies linking the rs3842753 C allele to increased type 1 diabetes susceptibility, there have only been three studies examining the effect of these genetic variants on *INS* expression in the pancreas (6; 9; 10). The studies found an allele specific association between rs3842753 C allele and increased *INS* expression in heterozygous adult and fetal whole pancreas tissue, but were confounded by a lack of cell type specificity. Moreover, the sample sizes were small (n = 3, 1, and 10) (6; 9; 10). These findings lend support to the β cell ER stress model of type 1 diabetes pathogenesis. It has been hypothesized that increased insulin demand could generate large proportions of misfolded or unfolded proinsulin, leading to ER stress (11). Under glucose stimulation (12) and even basal conditions (13), insulin production has been shown to be a significant source of ER stress. If the allele-specific increase in *INS* expression is translated into increased insulin production, there could be increased ER stress activated unfolded protein response leading to increased generation of unique antigen that present exclusively under specific conditions (neoautoantigens) (14). Some neoautoantigens translated from *INS* mRNA alternative reading frame have already been shown to stimulate T-cell proliferation and cytokine production, though the role of these neoautoantigens on type 1 diabetes autoimmunity remain unclear (15).

Recently, multiple studies have used single cell RNA sequencing (scRNAseq) in human pancreatic islets (16-23). Some studies have found distinct subpopulations of β cells, with

varying conclusions for differences in *INS* expression, indicating heterogeneity as a core feature of cellular biology (18; 23). Several studies have found transcriptomic differences in β cells from donors without diabetes compared to donors with type 2 diabetes (16-18). However, there was little overlap in differential gene expression changes between these two groups (24). This could be due to a combination of the complexity in type 2 diabetes etiology and limited sample size. Integrating all available single cell RNA sequencing (scRNAseq) data from human pancreatic islets into one dataset and conducting experiments with a greater sample size could resolve true differences.

In this study, we examined the effect of rs3842753 genotype, sex, and type 2 diabetes status on *INS* expression independently and in combination in single pancreatic β cells using an integrated dataset containing all available scRNAseq data.

METHODS

Data inclusion

We conducted all the scRNAseq alignments and initial dataset integration analysis using the Cedar Compute Canada cloud compute resource (www.computecanada.ca). All subsequent analyses were done in RStudio version 3.6.0. We included all published pancreatic islet scRNAseq datasets obtained using Smart-seq2 or Smart-seq methods for library preparation due to their higher sequencing depth for accurate SNP genotyping. We also included the Human Pancreas Analysis Program (HPAP) data available at the time of our study (22). We obtained the published datasets through the Gene Expression Omnibus (GEO) or the European Bioinformatics Institute (EMBL-EBI), and the HPAP datasets directly from investigators (Supplemental Table 1). All datasets metadata contain donor age, sex, and diabetes status. The read length ranges from 43bp to 100bp and median read depth per cell ranges from 0.75 to 4.4 million reads. In total, we used data from 71 donors, split into 48 donors without diabetes and 23 donors with type 2 diabetes.

Read alignment and genotyping

Single cell RNAseq reads were aligned to human reference genome GRCh38.98 using STAR version 2.7.1a (25) with gene read counts obtained using --quantMode GeneCounts. Read count files were aggregated into study specific read count matrices using a custom code. Each cell was genotyped at SNP rs3842753 (chr 11:2159830), using Samtools mpileup version 1.9 (26). Pancreatic β cells (indicated by total read count (DP4) at rs3842753 > 100) were used for donor genotype determination. Reference allele percent was calculated as

$\frac{(Reference_Forward_Read_Count+Reference_Reverse_Read_Count)}{DP4}$. The alternate allele percent was calculated as $\frac{(Alternate_Forward_Read_Count+Alternate_Reverse_Read_Count)}{DP4}$. Homozygosity was determined as reference or alternate allele percent > 80%, and heterozygosity otherwise. Donor genotype is determined as the majority of the individual's β cells' genotype. Less than 5% of individual β cells' genotype are non-concordant with donor genotype. Genotype non-concordant cells are included in downstream analysis and re-labeled with donor genotype.

Dataset filtering and cell type analysis

Initial filtering removed cells with abnormally high total RNA counts and genes expressed (i.e. doublet cells) and cells with low number of expressed genes and/or high mitochondrial gene percent (low viability), through visual inspection of distribution graphs for each dataset. The upper and lower limits were specific to each dataset to account for library preparation and sequencing protocol differences. The highest mitochondrial gene percentage was set to 25%.

Datasets were integrated into a single dataset in Seurat version 3.1 (27) using SCTransform (28) and clustered in UMAP space using default settings. Top ten differentially expressed genes in each cluster (compared to all other cells) was used to determine cluster cell type identify. As well, the location of pancreatic hormone genes (*GCG*, *INS*, *PPY* and *SST*) expression was used to determine Alpha (α), β, PP, and delta cell clusters, respectively. Enrichr (gene enrichment analysis) (29) was used when cluster identity could not be determined using previous methods.

Gene expression analysis

ER stress marker genes expression was normalized to all genes expressed in cell according to default Seurat settings. *INS* gene expression was normalized against select housekeeping genes rather than against all expressed genes in a cell due to the overwhelming amount of *INS* expression in pancreatic β cells (50% of all read counts) and to prevent self-normalization. Housekeeping genes for *INS* expression normalization were selected from a list of established human housekeeping gene *(ACTB, GAPDH, PGK1, PPIA, RPLP0, B2M, SDHA, TFRC, GUSB, HMBS, HPRT1, TBP*) for low inter-dataset variance and sufficient expression (relative to all genes in the cell). β cells *INS* read counts were normalized against the sum of select housekeeping genes read counts, scaled to a total of 10000, and natural log transformed. All cells with zero values for *INS* read counts or select house-keeping genes read counts were excluded from the downstream analysis.

Statistical analyses

Shapiro-Wilk test was used to test for normality. Fligner-Killeen or Bartlett's tests were used as a non-parametric or parametric test for difference in variance. Wilcox Rank Sum was used to test for differences in normalized *INS* expression between sexes or between disease statuses. Pairwise Kruskal-Wallis test was used to test for differences in normalized *INS* expression between genotypes, adjusting for multiple comparisons with Bonferroni correction. We did not study the effect of sex or diabetes status on normalized *INS* expression within genotype.

RESULTS

Dataset genotype summary

VNTR length cannot be determined directly with commonly used scRNAseq methods because reads are too short to span such repetitive regions. Therefore, for each cell, rs384275 genotype was determined as a surrogate for *INS*-VNTR length and rs689 genotype due to the near perfect linkage disequilibrium between these gene variants in Caucasian populations. Also, there were no read alignments to the rs689 region. Within donors without diabetes, 9 donors had rs384275 A/A genotype, 12 had rs384275 A/C genotype, and 27 had rs384275 C/C genotype. Within donors with type 2 diabetes, 4 donors had rs384275 A/A genotype, 11 had rs384275 A/C genotype, and 8 had rs384275 C/C genotype (Table 1).

Cell type clustering and identification

We performed cluster analysis and labeling to identify specific cell types since our dataset included a mixture of cells from pancreatic islets. We found 14 distinct clusters using 13622 pancreatic islet cells (Figure 1A). We classified clusters 0, 2, 4, 12 as α cell, clusters 1 and 8 as β cell, cluster 7 as PP cell, and cluster 10 as delta cells based on classical pancreatic hormone genes (*GCG, INS, PPY,* and *SST*) expression localization (Figure 1A-C, E). We classified cluster 11 as endothelial cells, cluster 3 as duct cells, and cluster 6 as acinar cells based on *PECAM1*, *SPP1*, and *PRSS1* expression localization (Figure 1A, B, D, E). We identified clusters 9 and 13 from Figure 1A as mesenchyme and CD14+ monocytes using Enrichr based on top ten conserved genes per cluster compared to all other cells (Figure 1B, E). We identified cluster 5 from Figure 1A as Beta-like cells due high *INS* expression compared to other clusters but lack of congregation in UMAP space. We only used the β cells cluster (2315 cells) for the *INS* expression analysis presented in the following section.

Effects of female sex and type 2 diabetes status on INS mRNA in single β cells

Sex and diabetes status have been shown to affect gene expression in pancreatic islets (16-18; 30). We examined the effect of sex and diabetes status on *INS* expression and we found that cells from females and from donors with type 2 diabetes have increased normalized *INS* expression variance (Figure 2A, C). Cells from female donors also had increased levels of normalized *INS* expression compared to males (Figure 2B). However, no significant difference in mean *INS* expression was observed between cells from diabetic and non-diabetic donors (Figure 2D). Changes in *INS* expression variance between sex and diabetes status provide additional evidence for heterogeneity in pancreatic β cell expression profile. As well, since *INS* expression level and variance changes between sex and diabetes status, it is important to account for these factors when studying the effect of rs3842753 genotype on *INS* expression.

rs3842753 C allele associates with increased INS expression in single β cells

Previous studies used allele specific expression to show a correlation between the at-risk type 1 diabetes rs3842753 C allele and increased pancreatic *INS* expression in a maximum of 10 heterozygous whole pancreas tissues (6; 9; 10). Here, we substantially increased the sample size (n = 71) and focused on β cell specific *INS* expression. To account for the overwhelming expression of *INS* in pancreatic β cells, and to avoid self-normalization to *INS*, we normalized *INS* expression to *B2M and RPLP0* expression, housekeeping genes we found to be both highly expressed and with a low inter-dataset variability in this dataset (Supplemental Figure 1). Donors with the rs3842753 A/C genotype had the highest level and variance of normalized *INS* expression, followed by C/C genotype, then A/A genotype (Figure 3 A, B). When examining the effect of rs3842753 genotype on *INS* expression within sex or diabetes status, the same trend of significantly increased variance of *INS* expression in donors with the heterozygous rs3842753 A/C genotype is present in cells from males and donors without diabetes (Figure 3 C, E). Additionally, cells from females and either diabetes status with the rs3842753 A/C genotype significantly expressed the highest level of *INS,* followed by C/C genotype, then A/A genotype (Figure 3 D, F). Together, these results indicate rs3842753 C allele affects *INS* expression, with the A/C genotype associated with both increased β cell *INS* expression variance and level.

rs3842753 C allele associates with increased ER stress markers in single β cells

Insulin production has been shown to sustain chronic baseline ER stress (13). Due to the increase in *INS* expression observed in cells with the at-risk rs3842753 C allele compared to the protective A allele, we were tested whether rs3842753 genotype also affected markers of ER stress. Indeed, the rs3842753 C allele was significantly associated with increased *ERN1* and

ATF6 expression (Figure 4A, B). These results indicate that rs3842753 C allele is associated with increased ER stress, possibly due to increased *INS* expression.

DISCUSSION

The linked genetic variants at the *INS* locus: Class I INS-VNTR, rs689 A allele, and rs3842753 C allele, are the main variants associated with increased type 1 diabetes susceptibility (5; 6; 8). Previously, only three small studies using whole pancreas tissues examined the effect of these variants on *INS* expression (6; 9; 10). The studies accounted for inter-donor differences by examining allele specific *INS* expression in heterozygous donors. Our study using 2315 single pancreatic β cells from 71 donors found that the heterozygous rs3842753 A/C genotype was associated with the highest *INS* expression, followed by C/C genotype, finally by the A/A genotype. This is consistent with the previous small studies using whole pancreas tissues that found that the C allele is associated with increased *INS* expression (6; 9; 10). Sex distribution for previous studies were not reported. Our study included 71 adult donors (48 donors without diabetes and 23 donors with type 2 diabetes), which is a substantial improvement from previous studies with a maximum of ten fetal pancreas tissues.

Our study represents the largest compilation of human islets single-cell RNA sequencing data to date and it offers several insights into the biology of *INS* gene expression. As with the individual studies, our work re-enforces the substantial variability in insulin gene expression between cells. We also found that the heterozygous rs3842753 A/C genotype was associated with the highest variance of *INS* expression followed by A/A genotype, then by the C/C genotype in single pancreatic β cells. The increase in normalized *INS* expression variance in cells with A/C genotype could be due to the expression profile being influenced by both the rs3842753 A allele and C allele. This difference in variance supports the established model of heterogeneity of pancreatic β cells and perhaps the rs3842753 genotype plays a role in heterogeneity of *INS* expression (18; 23; 31). Multiple other studies have found evidence of heterogeneity in β cells indicated by distinct subpopulations of β cells with varying *INS* expression (18; 23). In other work from our lab, we have recently found that these cells can fluctuate between these states of high and higher insulin gene expression (31). In the current study, we can, of course, not determine whether the cells with comparatively high or low *INS* expression was from dynamic or static activity due to the datasets we used.

Sex and diabetes status play an important role in insulin resistance and secretion. We found that females had more pancreatic *INS* expression than males. This is consistent with previous findings that males secrete less insulin after glucose stimulation than females, though insulin

secretion under basal conditions is less extensively studied (32). Interestingly, we found an increase in *INS* expression variance in cells from females and donors with type 2 diabetes compared to males and donors without diabetes, respectively. This is consistent with the hypothesis that female pancreatic β cells need to have more plasticity in gene expression to accommodate the physiological demands of the potential for pregnancy and T2D β cells could have more variance due to the range in disease state and its effects on cell health and function.

One perplexing observation from our study was that the highest *INS* mRNA levels were found in heterozygotes donors. While we can only speculate, it is possible that such an overdominance mode of *INS* expression could be through a transcription factor competitive binding mechanism of action, proposed for other systems (33). The rs3842753 C allele linked to shortening of *INS*-VNTR could increase accessibility of the chromatin by transcription factors at the *INS* loci and increase transcription of *INS*. In cells with the heterozygous genotype, transcription factor molecules could exceed binding site availability, limiting competition. Cells with the homozygous allele have increased number of *INS* transcription factor binding sites, which theoretically could induce competition amongst binding sites for transcription factors (at *INS* loci or other locus) thus lowering transcription efficiency and decreasing *INS* expression.

Our study has several limitations. This includes sample size limitation in the areas of age range, ethnic demographic, and sample size, which means that the results should be cautiously extrapolated and multiple subgroup analysis is challenging. Importantly, it was impossible to obtain sufficient data from pancreatic β cells from children without diabetes or with type 1 diabetes. Most cases of type 1 diabetes onset between 10 to 14 years of age (1). By studying the *INS* expression profile of pancreatic β cells from adults, we could be examining a different expression profile than the genetic risk factors that affect the onset of type 1 diabetes during childhood. The field of type 1 diabetes genetics in general is limited by an excessive focus on the Caucasian population (5; 6). The near perfect linkage for the three genetic variants *INS*-VNTR, rs689, and rs3842753 has only been extensively studied in Caucasian populations with the correlation factor r^2 unknown for other ethnicities (5). Our dataset only included ethnicity metadata for 31 out of 71 donors (29 of Caucasian descent). Thus, our assumption that genotyping for SNP rs3842753 as a surrogate for *INS*-VNTR length could not be formally validated. Finally, despite compiling all available data at the time of our work, our study is still limited by the relatively small sample number (n = 71) compared to GWAS studies, though it is a major improvement compared to the previous studies (n = 1, 3, 10) (9). Due to this limited sample size, we could not account for each of the large range of factors which could affect *INS*

expression including: age, BMI, dietary differences, and ethnicity (1). To address these limitations, we would need to increase the sample size, especially in younger age ranges.

We argue for an increase in studies using integrated scRNAseq datasets to take advantage of the massive amount of publicly available data. Our integrated dataset includes the most recent pancreatic islet scRNAseq data and will be available upon request. Diabetes status, sex, and age are included in the metadata allowing for investigations focusing on diabetes status specific transcriptome differences, sex specific transcriptome differences and age mediated transcriptome changes. Though Enge et al. (2017) have examined the transcriptional signatures of aging in human pancreas data with eight non-diabetic donors, it would be interesting to study the transcriptional signatures of aging between pancreatic islet cells from donors without diabetes compared to donors with type 2 diabetes (19).

Taken together, we confirmed the association between the rs3842753 C allele and increased *INS* expression at the single cell level using 2315 cells from 71 donors. These findings support the emerging concept that inherited risk of type 1 diabetes at SNP rs3842753 may be associated with elevated insulin production at the mRNA level and an increase in β cell ER stress. Insulin production-driven ER stress (13) could lead to errors in insulin mRNA translation, proinsulin protein processing and insulin peptide degradation, as well as other factors that could promote neoautoantigen generation and presentation, and the provocation of an autoimmune (Figure 4 C and D) (11)(34).

Tables:

Table 1: Donor number by sex and genotype for each dataset.

		Non-Diabetic				T2D			
Study		**A/A**	**A/C**	**C/C**	**Total**	**A/A**	**A/C**	**C/C**	**Total**
Xin et al., 2016 (16)	**Total Donor #**	3	4	5	12	1	3	2	6
	Male	3	1	3	7	0	2	1	3
	Female	0	3	2	5	1	1	1	3
Wang et al., 2016 (17)	**Total Donor #**	0	0	2	2	1	0	1	2
	Male	0	0	1	1	0	0	1	1
	Female	0	0	1	1	1	0	0	1
Segerstolpe et al., 2016 (18)	**Total Donor #**	3	0	3	6	1	2	1	4
	Male	3	0	2	5	0	1	1	2
	Female	0	0	1	1	1	1	0	2
Camunas-Soler et al., 2020 (21)	**Total Donor #**	1	4	13	18	1	4	2	7
	Male	1	2	6	9	0	2	1	3
	Female	0	2	7	9	1	2	1	4
Enge et al., 2017 (19)	**Total Donor #**	2	1	2	5	Not Applicable			
	Male	0	1	2	3				
	Female	2	0	0	2				
HPAP (22)	**Total Donor #**	0	3	2	5	0	2	2	4
	Male	0	1	1	2	0	0	1	1
	Female	0	2	1	3	0	2	1	3
Total Donor #		9	12	27	48	4	11	8	23
Male		7	5	15	27	0	5	5	10
Female		2	7	12	21	4	6	3	13

Genotype at SNP rs3842753.

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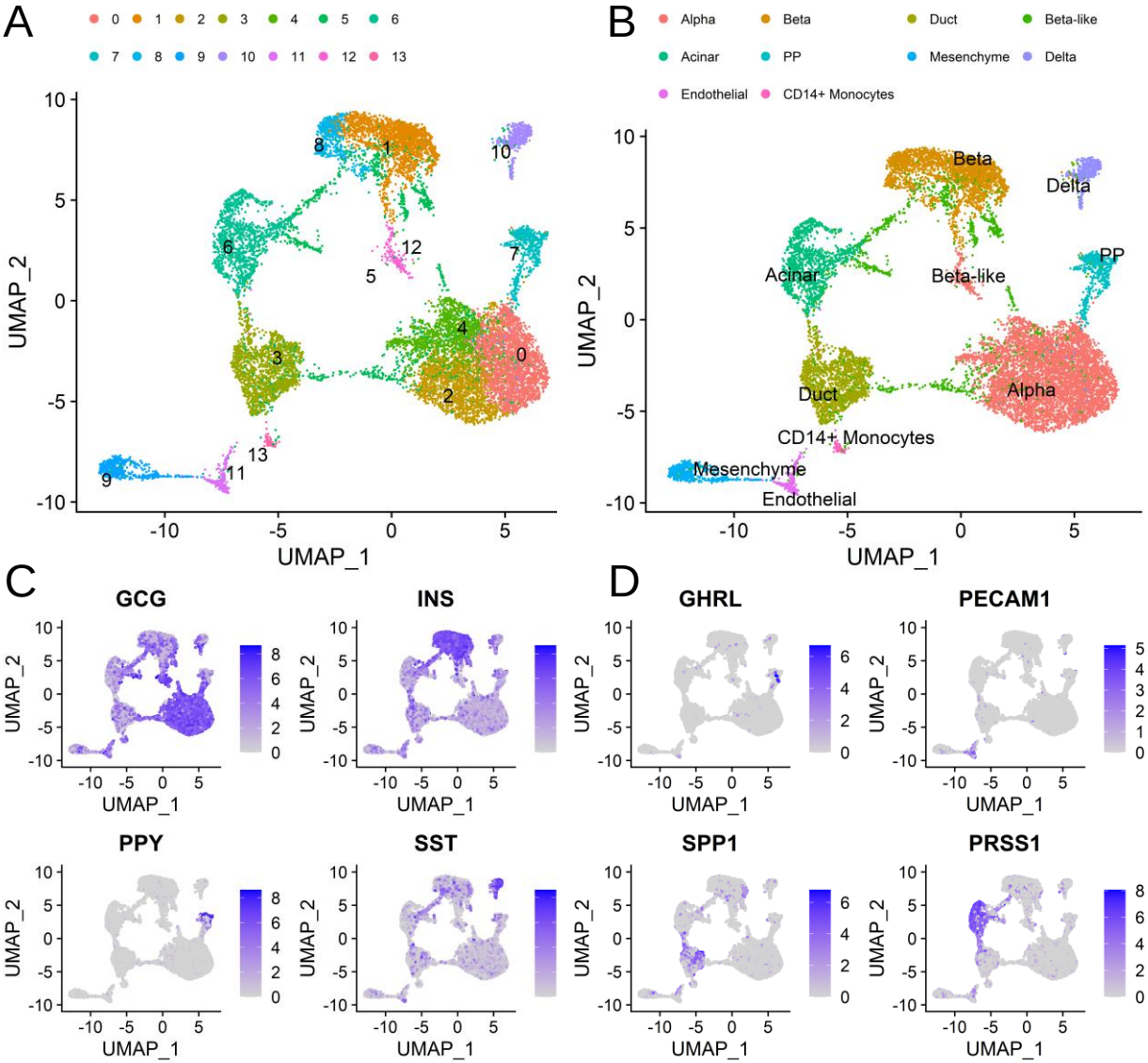
Figure Legends:

Figure 1: Cluster type clustering and identification of integrated dataset. UMAP projection of integrated dataset with clusters (A) and cell type identity separated by colour (B). UMAP projection of integrated dataset with gene expression localization for pancreatic hormonal genes (C) and other cell type (D) markers. Each point represents one cell. (E) Table with each identified cluster cell number.

Figure 2: Normalized *INS* expression in beta cells by sex and disease status. Distribution and mean normalized *INS* expression separated by sex (A, B), disease status (C, D). Red vertical lines represent median, blue vertical lines represent 1st and 3rd quartiles. Each point represents one cell. Bonferroni adjusted $p < 0.001$ = ***. ND = no diabetes, T2D = type 2 diabetes. Fligner-Killeen and Wilcox Rank Sum tests were used as non-parametric test for differences in variance and mean respectively.

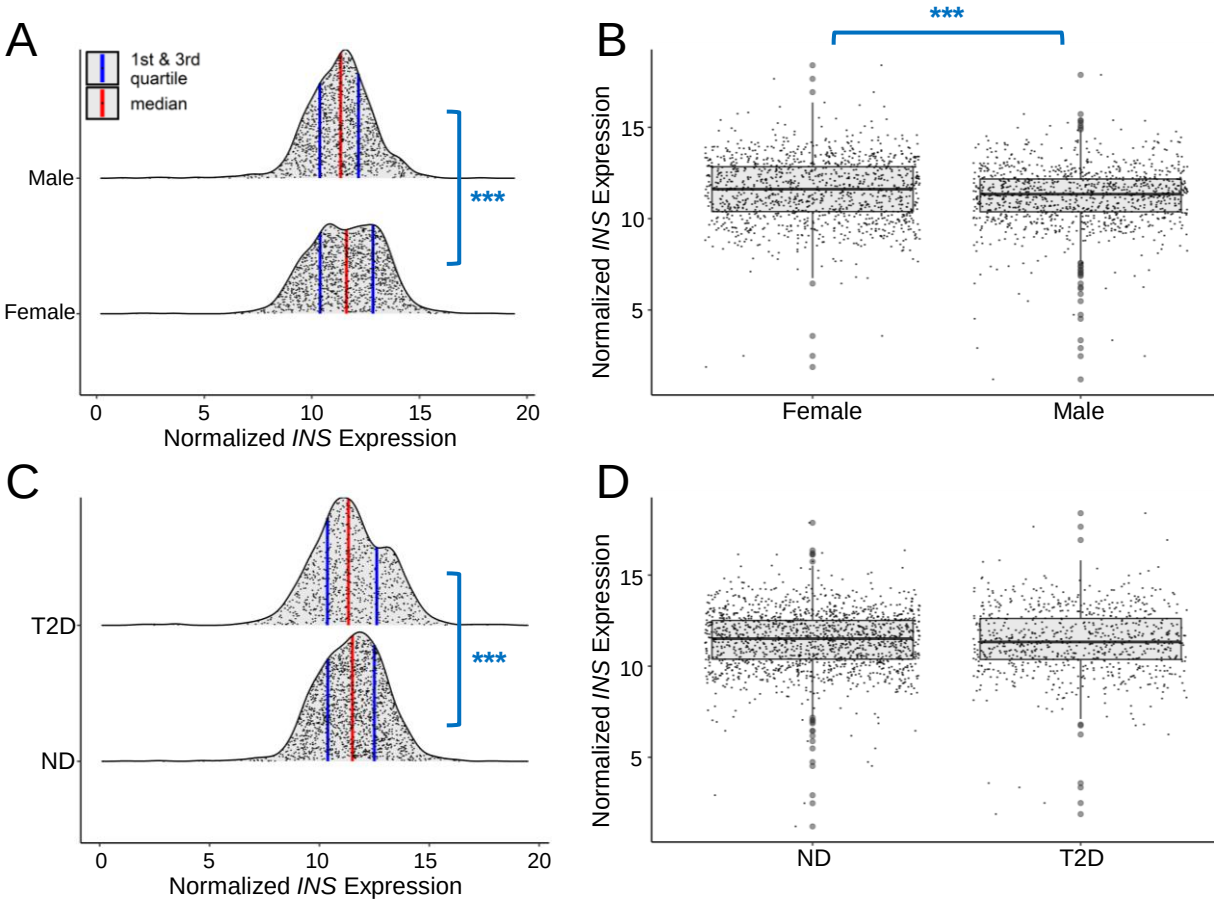
Figure 3: Normalized *INS* expression in β cells by genotype. Distribution and mean normalized *INS* expression separated by genotype (A, B), genotype and sex (C, D), and genotype and diabetes status (E, F). Red vertical lines represent median, blue vertical lines represent 1st and 3rd quartiles. Each point represents one cell. Bonferroni adjusted $P < 0.001$ = ***. F = female, M = male, ND = no diabetes, D = type 2 diabetes. Graphs are coloured by genotype. Fligner-Killeen and pairwise Kruskal-Wallis tests were used as non-parametric test for differences in variance and mean respectively.

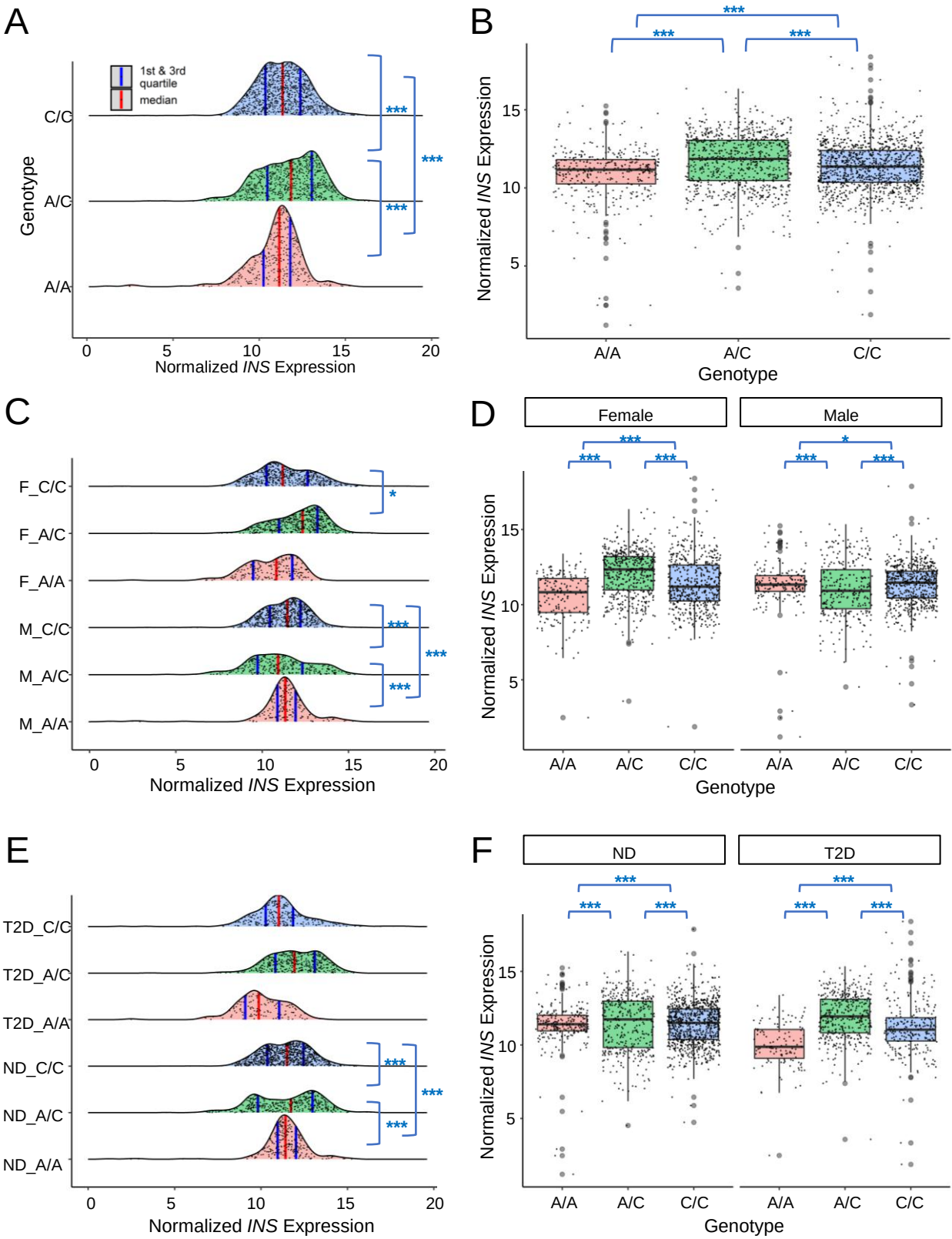
Figure 4: ER stress marked gene expression in β cells by genotype. Mean normalized INS expression of ERN1 (A) and ATF6 (B) separated by genotype. Bonferroni adjusted $P < 0.001$ =***. Graphs are coloured by genotype. Pairwise Kruskal-Wallis tests was used as non-parametric test for differences in mean. (C and D) Working model–rs3842753 associates with increased INS mRNA levels, presumably resulting in increased INS translation and increased misfolded INS protein, leading to increased ER stress.

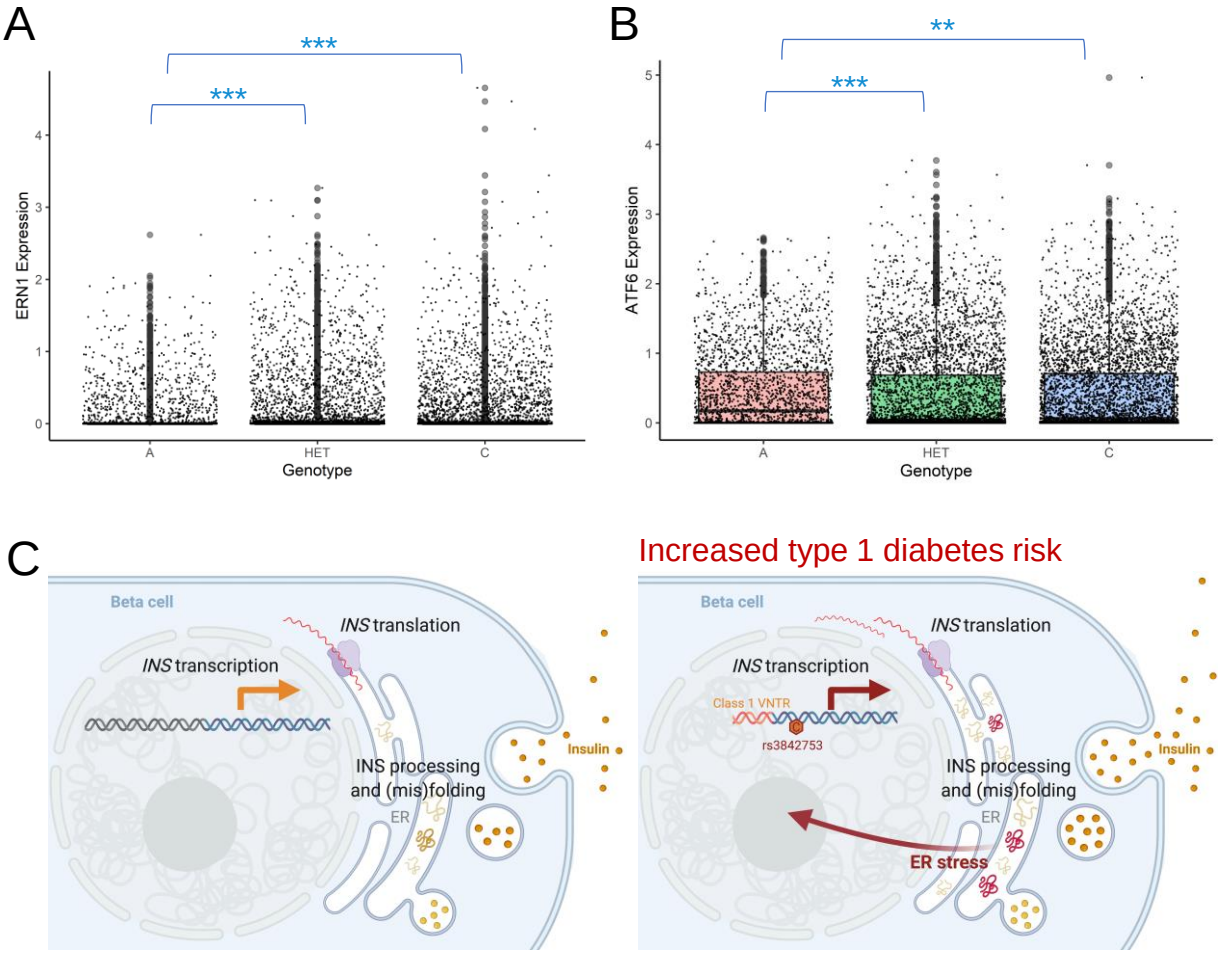


E

Alpha	Beta	Duct	Beta-like	Acinar	PP	Mesenchyme	Delta	Endothelial	CD14+ Monocyte
5519	2315	1502	1223	1188	586	531	451	226	81


A
1st & 3rd quartile
median
Male
Female
***
0
5
10
15
20
Normalized INS Expression
B
***
Normalized INS Expression
15
10
5
Female
Male
C
T2D
ND
***
0
5
10
15
20
Normalized INS Expression
D
Normalized INS Expression
15
10
5
ND
T2D


A
1st & 3rd quartile
median
C/C
A/C
A/A
Genotype
***
***
***
0
5
10
15
20
Normalized INS Expression
B
***
***
***
Normalized INS Expression
15
10
5
A/A
A/C
C/C
Genotype
C
F_C/C
F_A/C
F_A/A
M_C/C
M_A/C
M_A/A
*
***
***
***
0
5
10
15
20
Normalized INS Expression
D
Female
Male
***
***
***
*
***
***
Normalized INS Expression
15
10
5
A/A
A/C
C/C
A/A
A/C
C/C
Genotype
E
T2D_C/C
T2D_A/C
T2D_A/A
ND_C/C
ND_A/C
ND_A/A
***
***
***
0
5
10
15
20
Normalized INS Expression
F
ND
T2D
***
***
***
***
***
***
Normalized INS Expression
15
10
5
A/A
A/C
C/C
A/A
A/C
C/C
Genotype


A
***
***
ERN1 Expression
0
1
2
3
4
A
HET
C
Genotype
B
**
***
ATF6 Expression
0
1
2
3
4
5
A
HET
C
Genotype
C
Beta cell
INS translation
INS transcription
INS processing
and (mis)folding
ER
Insulin
Increased type 1 diabetes risk
Beta cell
INS translation
INS transcription
Class 1 VNTR
rs3842753
INS processing
and (mis)folding
ER
ER stress
Insulin