MAFA and MAFB regulate exocytosis-related genes in human β-cells

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Abstract

Aims: Reduced expression of exocytotic genes is associated with functional defects in insulin exocytosis contributing to impaired insulin secretion and type 2 diabetes (T2D) development. MAFA and MAFB transcription factors regulate β-cell physiology, and their gene expression is reduced in T2D β cells. We investigate if loss of MAFA and MAFB in human β cells contributes to T2D progression by regulating genes required for insulin exocytosis.

Methods: Three approaches were performed: (1) RNAseq analysis with the focus on exocytosis-related genes in MafA−/− mouse islets, (2) correlational analysis between MAFA, MAFB and exocytosis-related genes in human islets and (3) MAFA and MAFB silencing in human islets and EndoC-βH1 cells followed by functional in vitro studies.

Results: The expression of 30 exocytosis-related genes was significantly downregulated in MafA−/− mouse islets. In human islets, the expression of 29 exocytosis-related genes correlated positively with MAFA and MAFB. Eight exocytosis-related genes were downregulated in MafA−/− mouse islets and positively correlated with MAFA and MAFB in human islets. From this analysis, the expression of Rab3A, STXBP1, UNC13A, VAMP2, NAPA, NSF, STX1A and SYT7 was quantified after acute MAFA or MAFB silencing in EndoC-βH1 cells and human islets. MAFA and MAFB silencing resulted in impaired insulin secretion and reduced STX1A, SYT7 and STXBP1 (EndoC-βH1) and STX1A (human islets) mRNA expression. STX1A and STXBP1 protein expression was also impaired in islets from T2D donors which lack MAFA expression.
Conclusion: Our data indicate that STXBP1 and STX1A are important MAFA/B-regulated exocytosis genes which may contribute to insulin exocytosis defects observed in MAFA-deficient human T2D β cells.

KEYWORDS
diabetes, gene expression, insulin exocytosis, insulin secretion, MAFA, β cells

1 INTRODUCTION

In the last four decades, the worldwide prevalence of diabetes has quadruplicated reaching more than 420 million diabetic people in 2014 (https://www.who.int/news-room/fact-sheets/detail/diabetes). Although type 2 diabetes (T2D) development is usually accompanied by insulin resistance, this alone is insufficient to cause T2D. Instead, T2D is caused by a progressive decline in β-cell function and mass. Loss of β-cell function is caused by defects in glucose sensing/metabolism, insulin production and/or defects in the secretory machinery/exocytosis downstream of glucose metabolism.

Elevated glucose levels stimulate insulin secretion in a biphasic manner with an acute fast first phase (lasting 5-10 min) and a slow sustained second phase. The first-phase insulin secretion has a critical physiological role in the maintenance of post-meal glucose homeostasis. Glucose-intolerant subjects or patients in early stages of T2D have reduced or absent first-phase insulin secretion. First-phase insulin secretion relies on efficient exocytotic mechanisms related to insulin granule priming and membrane fusion. Therefore, reduced expression of genes required for insulin exocytosis may play an important role in the loss of first-phase insulin secretion in the early stages of T2D progression. In fact, global mRNA expression analysis of human islets from non-diabetic and T2D donors have demonstrated that reduced expression of exocytosis-related genes contributes to impaired insulin secretion and T2D pathogenesis. Moreover, a recent study has shown that human insulin secretion and exocytosis critically depend on the availability of membrane-docked granules and that T2D is associated with a strong reduction of insulin granule docking. Moreover, some genetic variants strongly linked to T2D have been associated with reduced insulin exocytosis in human β-cells. All these data support the idea that β-cell exocytosis is an important pathogenic mechanism of β-cell dysfunction contributing to T2D development.

Replenishing and maintaining the insulin exocytosis processes involve the transport of new insulin granules to the plasma membrane, exocytosis machinery assembly, granules docking to the plasma membrane, priming and insulin release. Insulin exocytosis is initiated within milliseconds and triggered by a glucose-stimulated rise of cytosolic Ca2+. Upon glucose stimulation, only insulin granules already docked to the plasma membrane are released, representing only 1% of the whole granule pool in an average mouse β-cell. Priming and fusion are processes involved in the cascade of events that precedes Ca2+-dependent exocytosis and insulin secretion. During the priming process, the SNARE complex assembles to prepare the granules for Ca2+-dependent fusion. The plasma membrane-associated proteins syntaxin 1A (STX1A) and synaptosomal-protein of 25 kDa (SNAP25) interact with vesicle-associated membrane protein (VAMP2), which gives rise to a tertiary SNARE complex that promotes fusion by pulling the vesicle membrane in close contact with the plasma membrane upon Ca2+ influx. Some isoforms of the family of synaptotagmins (SYTS) including SYT 1-3, 5-7 and 9-10 serve as Ca2+-sensing elements in regulating exocytosis. Apart from STX1A, SNAP25, VAMP2 and SYTS, the successful β-cell exocytosis involves several other proteins such as RIM1, RIM2, STXBP1 (or MUNC-18) and UNC-13 (or MUNC-13).

MAFA and MAFB are β-cell-enriched transcription factors required for β-cell differentiation/maturation and regulate the expression of several genes related to β-cell physiology including glucose sensing, insulin production and insulin exocytosis. Both MAFA and MAFB are expressed in mature human β-cells. MafA expression is restricted to maturing β-cells in mice and only detected from the juvenile period in humans, while the closely related MAFB transcription factor is present in developing and adult human β-cells. In fact, MAF mRNA expression levels are about six times higher in adult compared to foetal purified human β-cells, while MAFB expression is highest in developing human β-cells.

It has been established that MAFA expression is lost in human islets from subjects with T2D contributing to diabetic β-cell dysfunction. Recent studies have demonstrated that mutations in the MAFA gene are causing mature onset diabetes of the young further highlighting the importance of MAFA in human β-cell function. We and others have previously demonstrated that MAFA and MAFB regulate genes required for glucose-stimulated insulin secretion (GSIS) and are reduced in human T2D islets. However, the MAF-regulated genes required for insulin granule exocytosis in human β-cells have been poorly investigated. Identification of MAFA
and MAFB target genes required for insulin exocytosis provides an opportunity to better understand defects of β-cell function during T2D progression and provides potentially novel molecular targets to prevent and treat T2D.

2 | RESULTS

2.1 | MAFA and MAFB regulate the expression of exocytosis-related genes in mouse and human islets

RNAseq analysis of wild type and MafA total knock out (MafA\(^{-/-}\)) mouse islets was performed to determine how long-term loss of MafA affects the β-cell transcriptome. Sixty-two genes related to insulin exocytotic processes were significantly altered in pancreatic islets from MafA\(^{-/-}\) compared to control mice, with 30 genes being downregulated in the MafA\(^{-/-}\) (Figure 1A), suggesting that the transcriptional activator MafA regulates the expression of exocytosis-related genes in mouse β-cells.

In contrast to mouse β-cells, adult human β-cells co-express MAFA and the related MAFB transcription factor. To determine if MAF transcription factors also regulate exocytosis-related genes in human β-cells, an expression correlational analysis between MAFA, MAFB and exocytosis-related genes was performed in human islets (RNAseq). We found that the expression of 29 exocytosis-related genes correlated positively with both MAFA and MAFB (Table 1A), whereas 13 correlated only with MAFA (Table 1B) and 12 only with MAFB (Table 1C).

To determine if the transcriptional regulation of exocytosis-related genes is conserved between mouse and human β-cells, we investigated if genes downregulated in MafA\(^{-/-}\) islets were positively correlated with both MAFA and MAFB (Figure 1B), MAFA only (Figure 1C) or MAFB only (Figure 1D) in human islets. Eight genes were downregulated in MafA\(^{-/-}\) islets and positively correlated with MAFA and MAFB in human islets (STXBP1, UNC13A, SYT5, VAMP2, STX16, RAB3A, CAMK2N2 and ADRA2A; Figure 1B), whereas only one gene was downregulated in MafA\(^{-/-}\) islets and positively correlated with MAFA (NAPA; Figure 1C) or MAFB (NSF; Figure 1D). This suggests that a selected set of exocytosis-related genes are commonly regulated by MAF-transcription factors in mouse and human β-cells.
**TABLE 1** Expression of exocytosis-related genes correlated significantly with MAFA and/or MAFB in human islets

(A) Genes correlated positively with MAFA and MAFB (29 genes)

| Gene      | MAFA          | MAFB          |
|-----------|---------------|---------------|
|           | $\rho$ | $P$-value | $\rho$ | $P$-value |
| KCNJ11    | 0.86 | 2.55E−58 | 0.55 | 1.32E−16 |
| SNCB      | 0.83 | 2.04E−49 | 0.43 | 7.58E−10 |
| RAC3      | 0.81 | 2.11E−46 | 0.24 | 6.82E−04 |
| FOXA2     | 0.79 | 2.90E−42 | 0.33 | 3.78E−06 |
| PTPRN2    | 0.77 | 2.10E−38 | 0.58 | 3.30E−18 |
| **STX1A** | **0.74** | **9.80E−35** | **0.33** | **3.69E−06** |
| CAMK2B    | 0.74 | 1.33E−34 | 0.48 | 2.01E−12 |
| SNAP25-AS1| 0.74 | 9.35E−34 | 0.14 | 4.62E−02 |
| BSN       | 0.72 | 1.10E−31 | 0.46 | 1.45E−11 |
| CAMK2N2   | 0.71 | 3.41E−31 | 0.18 | 1.21E−02 |
| SYT5      | 0.65 | 9.06E−25 | 0.23 | 1.08E−03 |
| SYT3      | 0.65 | 1.90E−24 | 0.27 | 1.42E−04 |
| RAB3A     | 0.64 | 4.92E−23 | 0.72 | 3.23E−31 |
| **SYT7**  | **0.56** | **7.60E−17** | **0.70** | **6.01E−30** |
| GCK       | 0.53 | 1.90E−15 | 0.60 | 5.35E−20 |
| SYNGR4    | 0.50 | 2.59E−13 | 0.35 | 5.24E−07 |
| RIMBP2    | 0.49 | 9.32E−13 | 0.69 | 1.51E−28 |
| SIRT3     | 0.48 | 3.60E−12 | 0.34 | 1.63E−06 |
| ADRA2A    | 0.38 | 3.85E−08 | 0.19 | 7.94E−03 |
| **STXBP1**| **0.37** | **1.29E−07** | **0.78** | **1.22E−40** |
| CAMK2N1   | 0.33 | 2.59E−06 | 0.53 | 2.03E−15 |
| RPH3A     | 0.27 | 1.70E−04 | 0.48 | 1.67E−12 |
| CAMKV     | 0.24 | 9.95E−04 | 0.23 | 1.16E−03 |
| STX16     | 0.24 | 9.96E−04 | 0.34 | 2.06E−06 |
| UNC13A    | 0.21 | 3.12E−03 | 0.60 | 9.04E−20 |
| VAMP2     | 0.21 | 3.67E−03 | 0.41 | 3.53E−09 |
| RIMS2     | 0.20 | 5.23E−03 | 0.68 | 3.80E−27 |
| KCNB2     | 0.18 | 1.38E−02 | 0.70 | 5.83E−30 |
| STXBP5L   | 0.14 | 4.85E−02 | 0.61 | 3.62E−21 |

(B) Genes correlated positively with MAFA (13 genes)

| Gene      | MAFA          |
|-----------|---------------|
|           | $\rho$ | $P$-value |
| NAPA      | 0.83 | 1.97E−49 |
| **SYTL1** | **0.72** | **1.37E−31** |
| **STX10** | **0.65** | **1.63E−24** |
| **DOC2A** | **0.63** | **1.47E−22** |
| **SIRT7** | **0.58** | **6.65E−19** |
| **CAPS**  | **0.57** | **7.02E−18** |
| **SYT8**  | **0.51** | **4.93E−14** |
| **UNC13D**| **0.34** | **2.06E−06** |
2.2 \textbf{MAFA and MAFB regulate the expression of the exocytosis genes \textit{STX1A} and \textit{STXBP1} in human \beta \text{-cells}}

To determine whether MAFA and MAFB are regulating insulin secretion and expression of exocytosis-related genes in human \beta \text{-cells}, we silenced MAFA or MAFB in the human EndoC-\beta H1 cells and tested glucose-stimulated insulin secretion (GSIS), high K\textsuperscript{+}-stimulated insulin secretion (K-SIS) and gene and protein expression of exocytosis genes by qPCR and Western blot (Figure 2). Insulin secretion in response to high K\textsuperscript{+} stimulation bypasses the upstream mechanism of GSIS (the glucose metabolism-dependent block of K-ATP channel activity) relying mainly on the exocytosis machinery events. Thus, the K-SIS test is an indirect measure of the insulin exocytosis activity, since the depolarization by K\textsuperscript{+} act downstream of metabolism opening voltage-dependent Ca\textsuperscript{2+} channels necessary for influx of the Ca\textsuperscript{2+} that triggers exocytosis of insulin granules.

mRNA and protein expression of MAFA or MAFB was moderately reduced when specific MAFA or MAFB siRNA oligonucleotides were transfected in EndoC-\beta H1 cells respectively (Figure 2A,D,E). Interestingly, we observed that, MAFB silencing resulted in an increase of MAFA expression levels, suggesting compensatory upregulation (Figure 2A,E). Moderate reduction of MAFA or MAFB expression resulted in decreased GSIS and K-SIS (Figure 2B), although this effect was only significant in MAFA knockdown (KD) cells (Figure 2B). This may be because of compensatory upregulation of MAFA in MAFB KD cells (Figure 2A,D,E). The significant reduction of K-SIS supports the notion that MAF transcription factors are regulating the expression of genes participating in the exocytosis process in human \beta \text{-cells}.

To evaluate if the mRNA co-expression correlations observed in human islets were driven by MAFA and/ or MAFB transcriptional regulation on potential target genes, the expression level of these potential MAF-target genes was assessed in MAFA KD and MAFB KD EndoC-\beta H1 cells. Expression of genes identified in our bioinformatics analysis (\textit{RAB3A, STXBP1, UNC13A, VAMP2, NAPA, and NSF} (Figure 1B-D)) and two known critical exocytosis-related genes that positively

\begin{table}[h]
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\caption{(Continued)}
\begin{tabular}{|l|c|c|}
\hline
\textbf{Gene} & \textbf{MAFA} & \textbf{P-value} \\
& $\rho$ & \\
\hline
\textit{NCS1} & 0.32 & 5.72E−06 \\
\textit{STXBP2} & 0.30 & 2.24E−05 \\
\textit{VAMP1} & 0.24 & 8.06E−04 \\
\textit{RAC2} & 0.21 & 4.32E−03 \\
\textit{STX5} & 0.17 & 1.90E−02 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Genes correlated positively with MAFB (12 genes)}
\begin{tabular}{|l|c|c|}
\hline
\textbf{Gene} & \textbf{MAFB} & \textbf{P-value} \\
& $\rho$ & \\
\hline
\textit{EXOC8} & 0.487 & 9.23E−13 \\
\textit{RAB3B} & 0.463 & 1.59E−11 \\
\textit{STXBP5} & 0.441 & 1.69E−10 \\
\textit{SLC2A2} & 0.405 & 6.18E−09 \\
\textit{SYT9} & 0.382 & 4.96E−08 \\
\textit{NSF} & 0.371 & 1.22E−07 \\
\textit{CAPS2} & 0.378 & 2.75E−05 \\
\textit{LCA5} & 0.266 & 2.00E−04 \\
\textit{RACGAP1} & 0.259 & 3.00E−04 \\
\textit{UNC119B} & 0.244 & 6.87E−04 \\
\textit{CAMK4} & 0.178 & 1.39E−02 \\
\textit{STX2} & 0.176 & 1.47E−02 \\
\hline
\end{tabular}
\end{table}
**FIGURE 2** MAFA- and MAFB-regulated exocytosis-related genes in EndoC-βH1 cells. EndoC-βH1 cells were transfected with scramble or MAFA or MAFB or both siRNAs for 72 h. (A) MAFA and MAFB mRNA levels were quantified by qPCR. (B) Insulin secretion was stimulated for 1 h with SAB low glucose (1 mM), high glucose (20 mM) or low glucose + high K⁺ (35 mM) and insulin concentration in supernatant measured by ELISA. (C) mRNA expression level of selected exocytosis-related genes quantified by qPCR. (D) MAFA, MAFB, STX1A and STXBP1 proteins were identified by immunoblot and (E and F) protein levels quantified by densitometric analysis (normalized to total protein load). Graph bars represent mean ± SEM of n = 5 (A, C), 6 (B) and 3 (D-F) independent experiments. *P < .05, †P < .01 and ‡P < .001
correlated with MAFA and MAFB in human islets (STX1A and SYT7) was quantified by qPCR after acute silencing of MAFA or MAFB in human EndoC-βH1 cells. Synaptotagmin 7 (SYT7, \( P = .0004 \)) , Syntaxin Binding Protein 1 (STXBPI, \( P = .0052 \)) and Syntaxin 1A (STX1A, \( P = .0055 \)) mRNA levels were significantly downregulated in response to MAFA silencing, whereas the expression of SYT7 (\( P = .02 \)) and STX1A (\( P = .172 \)) was downregulated in response to MAFB silencing (Figure 2C). STXBPI protein levels were also reduced in response to MAFA KD (Figure 2F).

To assess if the reduction in gene expression of exocytosis-related genes observed in the human β-cell line was also preserved in primary human β-cells, MAFA and MAFB were silenced in human islets from three non-diabetic donors and mRNA expression of target genes was evaluated (Figure 3A,B). We observed a moderate reduction of MAFA (~50%) and MAFB (~40%) expression in siRNA-treated islets (Figure 3A). MAF transcription factor KD resulted in a significant downregulation of STX1A mRNA levels (Figure 3B, \( P = .0011 \)), consistent with the STX1A mRNA reduction observed in MAFA and MAFB KD EndoC-βH1 cells. MAFA and/or MAFB KD also reduced GSIS and K-SIS, albeit not significantly, most likely reflecting the heterogeneity of human donor islets, varying KD efficiency and compensatory upregulation of MAFA in MAFB KD (Figure 3C).

To evaluate the participation of potential human MAF target genes, STX1A, STXBPI and SYT7 in the insulin exocytosis process, we evaluated the correlation between STX1A, STXBPI and SYT7 mRNA levels with K-SIS and GSIS in human islets (Figure S1). A significant positive correlation between STX1A mRNA levels and K-SIS (Figure S1, \( P = .0036 \)) was observed while its association with GSIS was weaker (Figure S1, \( P = .059 \)). Our data indicate that STX1A and STXBPI are novel MAFA and MAFB target genes required for insulin exocytosis in primary human β-cells.

To evaluate if STX1A, STXBPI and SYT7 expression is affected by the long-term loss of MAFA as observed in islets from T2D donors, we performed immunohistochemical analysis for MAFA, STXBPI, STX1A and SYT7 in pancreatic sections from normoglycemic and T2D donors (Figure 4). Protein expression of MAFA, STXBPI and STX1A was reduced in T2D β-cells, whereas expression of SYT7 was not impaired (Figure 4). We observed also reduced STXBPI protein expression in pancreatic sections from MafA−/− animals (Figure S2) confirming our initial RNAseq analysis which showed reduced Stxbp1 mRNA levels in MafA-deficient islets. These results suggest that loss of MAFA expression in T2D islets contributes to impaired insulin exocytosis by reducing protein expression of exocytosis-related target genes.

### 2.3 Genetic variants in the MAFA gene correlate with MAFA, STXBPI and SYT7 expression in human islets

The expression and functional analysis of MAFA and exocytosis genes in human islets and β cells show that MAFA controls exocytosis gene expression and β-cell function. Expression quantitative trait loci (eQTL) analysis of 188 human islet\(^{29}\) was performed to see if there were any SNPs in the MAFA gene that are associated with islet expression, glucose clearance and type 2 diabetes. We identified a SNP that was associated with low expression of MAFA (Figure S3). Specifically, carriers of the alternate allele (A) for the MAFA SNP rs61731375 had significant reduction of MAFA transcript levels (Figure S3). Moreover, this SNP showed suggestive signals of association with type 2 diabetes.\(^{30}\) Next, we analysed if the presence of the same alternate allele affected the expression of STXBPI, STX1A and SYT7. STXBPI, STX1A and SYT7 expression was reduced in islets from donors carrying the alternate allele, however, only SYT7 showed a statistically significant reduction in non-diabetic donors (\( P = .025 \)). These results identify SNPs in the MAFA gene that are associated with type 2 diabetes and are correlated with lower expression of exocytosis genes indicating that reduced MAFA expression in pancreatic islets affects β cell function and insulin exocytosis.

### 3 DISCUSSION

In this study, we aimed to identify MAFA and MAFB regulated genes required for insulin exocytosis in human β-cells. We took advantage of transcriptomic analyses of MafA-deficient mouse islets (MafA\(^{−/−}\))\(^{31}\) and human islets from a large number of ND and T2D donors. MafA\(^{−/−}\) mouse islets are a good model to determine MAFA-regulated genes required for insulin exocytosis because of their reduced GSIS and K-SIS in vitro and reduced GSIS in vivo.\(^{32}\) Similarly, reduced depolarization-evoked exocytosis has been demonstrated in β-cells from MafA\(^{−/−}\) mice.\(^{33}\)

We identified exocytosis-related genes (30) with reduced expression in MafA\(^{−/−}\) islets and a positive correlation with MAFA and MAFB (29) or exclusively with MAFA (13) or MAFB (12) in human islets. Eight exocytosis-related genes had reduced expression in MafA\(^{−/−}\) islets and positive correlation with MAFA and MAFB in human islets. Among these genes, ADRA2A (alpha2A-adrenergic receptor), has previously been characterized as a MAFA target gene in mouse and human β cells\(^{34}\) and T2D risk alleles in the ADRA2A locus have been associated with increased ADRA2A expression,
FIGURE 3  MAFA- and MAFB-regulated exocytosis-related genes in human islets. Pancreatic islets from three non-diabetic donors were transfected with scramble or MAFA and MAFB siRNAs for 72 h. (A and B) mRNA expression level of (A) MAFA and MAFB and (B) of selected exocytosis-related genes were quantified by qPCR. Pancreatic islets from three non-diabetic donors were transfected with scramble or MAFA or MAFB or both siRNAs for 72 h. (C) Insulin secretion was stimulated for 1 h with SAB low glucose (2.8 mM), high glucose (16.7 mM) or low glucose+high K⁺ (35 mM) and insulin concentration in supernatant measured by ELISA. (D) MAFA, MAFB, STX1A and STXBP1 proteins were identified by immunoblot and (E and F) protein levels quantified by densitometric analysis (normalized to total protein load). Graph bars represent mean ± SEM of n = 3 independent experiments. *P < .05, †P < .01 and ‡P < .001
reduced insulin granule docking and depolarization-evoked insulin exocytosis in human β-cells.\textsuperscript{9,35} Thus, the identification of \textit{ADRA2A} illustrates that our bioinformatics analysis effectively detects MAF-regulated genes affecting insulin exocytosis in β-cells.

Our results show that acute silencing of \textit{MAFA} in human EndoC-βH1 cells and human islets reduced both GSIS and K-SIS. Similar results have been shown before in \textit{Mafa}-deficient mouse islets.\textsuperscript{27} The reduction of GSIS (−42\%) was stronger than the reduction of K-SIS (−25\%). These data are expected in a model where MAFA-regulated genes are involved in the two mechanisms mediating insulin secretion; the upstream glucose sensing and metabolism-dependent insulin secretion and, the K-ATP channel downstream cytosolic Ca\textsuperscript{2+}-mediated insulin exocytosis events. \textit{MAFB} silencing was less efficient and accompanied by a compensatory increase of \textit{MAFA} expression which resulted in a more modest reduction of insulin secretion. Similar to the compensatory \textit{MAFA} upregulation, during mouse pancreas development, there is a physiological sequential expression pattern between \textit{Mafb} and \textit{Mafa}, that is, \textit{Mafb} expression declines post-natally while \textit{Mafa} expression levels increase during differentiation/maturation processes of β-cells.\textsuperscript{18} In line with the idea that MAFA plays a more significant role in regulating genes necessary for insulin exocytosis in mature human β-cells, recently published data combining single-cell RNA sequencing and patch-clamp electrophysiology, show a significant correlation between total insulin exocytosis and \textit{MAFA} but not \textit{MAFB} mRNA levels in human β-cells.\textsuperscript{36}

\textit{STX1A}, \textit{SYT7} and \textit{STXBP1} mRNA levels were significantly reduced in \textit{Mafa} KD EndoC-βH1 cells while only \textit{SYT7} was significantly reduced in \textit{Mafb} KD EndoC-βH1 cells. Acute silencing of \textit{MAFA} and \textit{MAFB} in primary human islets resulted in the unique reduction of \textit{STX1A} mRNA levels, suggesting this is a robust MAFA and MAFB exocytosis target gene in primary human β-cells. \textit{STX1A} protein levels were not affected by short-term MAFA and/or MAFB KD, while MAFA-deficient pancreas from T2D donors showed reduced \textit{STX1A} protein expression. This may be explained by the reported long half-life of \textit{STX1A}.\textsuperscript{37} We also observed a reduction of \textit{STXBP1} protein levels in pancreatic sections from T2D donors and upon short-term knockdown of MAF transcription factors in EndoC-βH1 and human islets confirming that \textit{STXBP1} gene expression depends on MAF activity. In support of our findings, ChIPseq analysis showed the binding of MAFB to the upstream region of the \textit{STX1A} and \textit{STXBP1} genes in human islets.\textsuperscript{38} Our genetic analyses identified novel MAFA eQTLs and human islets from donors carrying two alternate alleles that had reduced MAFA, \textit{STX1A}, \textit{STXBP1} and \textit{SYT7} expression further supporting a critical role for MAFA in regulating these genes.

\textit{STX1A} and \textit{STXBP1} are essential proteins to form de novo clusters at nascent docking sites in the plasma membrane for insulin granule newcomers allowing them to fuse and release insulin.\textsuperscript{3} Quantitative measurements of several exocytosis proteins (\textit{STX1A}, \textit{SNAP25}, \textit{STXBP1}, \textit{MUNC13} and \textit{RAB3}) at the insulin granule release site have shown that docking coincides with rapid de novo formation of \textit{STX1A}/\textit{STXBP1} clusters at the nascent docking site. Thus, the findings that \textit{STXBP1} and \textit{STX1A} are novel MAF target genes in human β-cells highlight the importance of MAFA and MAFB.

\begin{figure}
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\includegraphics[width=\textwidth]{image.png}
\caption{MAFA, \textit{STX1A} and \textit{STXBP1} proteins are reduced in islet β-cells from T2D donors. Immunohistochemistry staining for (A) \textit{STXBP1} (green) and \textit{INSULIN} (red), (B) \textit{MAFA} (red) and \textit{INSULIN} (green), (C) \textit{STX1A} (green) and \textit{INSULIN} (red) and (D) \textit{SYT7} (green) and \textit{INSULIN} (red) in human pancreas sections from normoglycemic and type 2 diabetic. Nuclei are blue, scale bar is 20 µm.}
\end{figure}
expression in these cells for docking of insulin granules to the plasma membrane and consequently, for an efficient exocytosis process.

Interestingly, of all the exocytosis genes characterized so far, the three genes reduced in MAFA KD EndoC-βH1 cells; STX1A, SYT7 and STXBP1 have previously been highlighted by their association with different metabolic parameters in human islets. Thus, the expression levels of these three genes were negatively correlated with Hba1c and positively with GSIS. Another study found that the expression of these genes negatively correlated with Hba1c, whereas SYT7 mRNA levels positively correlated with stimulatory index (fold change of glucose-stimulated insulin secretion) and STX1A mRNA levels positively correlated with insulin granule docking in human β-cells.

Besides, STX1A protein levels are reduced in pancreatic islets from T2D donors. These data reinforce the important role of these MAFA and MAFB regulated genes for insulin exocytosis and glucose homeostasis in humans.

Our data indicate that STX1A and STXBP1 are novel MAFA and MAFB target genes critical for insulin granule docking and exocytosis in human β-cells and that eQTLs reducing MAFA expression levels are associated with T2D and contribute to β-cell dysfunction at least partially because of defective insulin granule docking and exocytosis processes.

### 4 | MATERIALS AND METHODS

#### 4.1 | Animals

MafA-deficient (MafA−/−) animals were generated by crossing MafA floxed (MafAfl/fl) with Sox2-Cre39 transgenic animals. Mice were maintained on a C57BL/6 background and housed in colony cages (three to five/cage) in a 12 h light/dark cycle with controlled humidity and temperature and free access to standard diet and water. All experimental procedures were approved by the Animal Welfare and Ethics committee in the Lund-Malmö region (Jordbruksverket; permit numbers: M 43-13, M 47-12 and M 385-12). All experimental procedures were carried out in accordance with approved Swedish national guidelines.

#### 4.2 | Mouse Islet isolation

Pancreatic islets were isolated from 6-month-old wild type and MafA−/− mice. Islets were collected for RNA extraction. For islet isolation, mice were euthanized by cervical dislocation, following V-incision on the lower abdomen to expose the pancreas. The pancreatic bile duct was identified and clamped. A small incision was made at the junction of the main pancreatic duct connecting with the duodenum, to inject a fresh mixture of collagenase P (20 U mL−1, Roche, Switzerland) dissolved in Hanks buffer (Sigma, USA) until the entire pancreas was filled with the solution. The pancreas was then digested at 37°C for 10-12 min, followed by tissue disruption through manual shaking and several washes with cold Hanks buffer. Thereafter, islets were hand-picked under an inverted bright-field microscope.

#### 4.3 | RNA extraction

Total RNA from 6-month-old MafAfl/fl and MafA−/− mice islets and from EndoC-βH1 cells was extracted using an adapted protocol combining trizol-chloroform and RNeasy Mini Kit. RNA quality was analysed with an Agilent 2100 bioanalyzer and samples with RIN (RNA integrity number) higher than seven were used for RNAseq and quantitative PCR. RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer, and concentrations were equalized for each round of complementary DNA (cDNA) synthesis.

#### 4.4 | cDNA synthesis and quantitative PCR

Reverse transcription was performed using SuperScript III reverse transcriptase (ThermoScientific) using 500-1000 ng total RNA for cDNA synthesis. Ten nanograms cDNA per well (in duplicates) was used for qPCR. Primer sequences for human genes are listed in Table 2. qPCR assays were performed using Fast SYBR Green Master Mix on a StepOnePlus Real-Time PCR System. Relative gene abundance was calculated using the ΔΔCt method with β-ACTIN and HPRT1 as housekeeping genes and expressed as FC to control.

#### 4.5 | RNA sequencing and data analysis

Islet RNA was processed for cDNA library preparation with TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat kit (Illumina) and sequenced using NextSeq 500/550 High Output Kit v2 (150 cycles) (Illumina). Quality assessment was made pre- and post-sample preparation on a 2100 Bioanalyzer (Agilent). Output reads were aligned to the mouse reference genome (GRM38.75) using STAR v.2.4.1. The dexseq_count python script was used by counting uniquely mapped reads in each exon. Gene and exon expression normalizations were then performed using the TMM
method, log-transformed and comparisons between groups were made using exactTest in edgeR. Data were analysed to yield Log-FC, FDR and P values.

### 4.6 MAFA-MAFB expression correlation analysis in human pancreatic islets

Human islets from 191 cadaver donors of European ancestry were provided by the Nordic Islet Transplantation Program Uppsala under full ethical clearance (Uppsala Regional Ethics Board, Pro00001754) and the donor families written informed consent and processed for RNA-sequencing (RNA-seq) for Gene Expression Omnibus (GEO) accession code GSE50398 and GSE108072. RNA extraction, quality control and sequencing were performed as described before. RNA samples were prepared using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequencing was performed on the Hi-Seq2000 platform. RNA-seq data were processed as previously described. Briefly, raw counts were normalized using trimmed mean of M-values and log2-transformed correlation coefficients. Spearman correlations (R) were calculated to assess the correlation of MAFA and MAFB expression with genes expressed in the human pancreatic islets using R language programming.

### 4.7 Cell culture

EndoC-βH1 cells were cultured as previously described in Matrigel/fibronectin-coated (100 mg mL⁻¹ and 2 mg mL⁻¹ respectively) (Sigma-Aldrich) vessels with DMEM containing 5.6 mmol L⁻¹ glucose, 2% BSA, 10 mmol L⁻¹ nicotinamide, 50 mmol L⁻¹ β-mercaptoethanol, 5.5 mg mL⁻¹ transferrin, 6.7 ng mL⁻¹ sodium selenite, 100 IU mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

### 4.8 siRNA Transfection in EndoC-βH1 cells and Human Islets

EndoC-βH1 cells were seeded (180,000 cells well⁻¹) in 48-well plates and 24 h later transfected with 50 nM of MAFA or MAFB and negative control siRNA#1 (NC) (ThermoScientific, codes: 4390771, 4392420 and 4390843 respectively) using Lipofectamine RNAiMAX (ThermoScientific). Seventy-two hours later GSIS or RNA was extraction performed.

MAFA and MAFB double knockdown of human islets from non-diabetic donors (n = 3, Table 3) was performed by MAFA and MAFB siRNA (30 nM) transfection using Lipofectamine RNAiMAX has previously described. The islets were obtained through the EXODIAB network via the Nordic Network for Islet Transplantation and cultured as previously described. All procedures were approved by Swedish authorities.

### TABLE 2 Oligonucleotide primer sequences for qPCR analysis of human genes

| Gene name (GenBank accession no.) | Forward sequence | Reverse sequence | Size (bp) |
|-----------------------------------|------------------|------------------|-----------|
| MAFA (NM_201589.4)                | CTTCAGCAGAGAGGGAGTCA | TGTTACAGGTCGCGCTCTTT | 195 |
| MAFB (NM_005461.5)                | GAACGAGAAGACGACGCTCAT | CGAGTTTCTCAGACTGACCT | 104 |
| RAB3A (NM_002866.5)               | ACCATCACCAGCCCATACTA | TCAAAGAATCGAAACAAG | 233 |
| SYT7 (NM_004200.3)                | CTCAAAGGAGTACGACTCGG | TCCAGGACAGGATGATCCTTG | 247 |
| STXB1P (NM_003165.3)              | GCTGACCCGCGAGCTCGG | TGAGATGTTGAGTATCTTT | 214 |
| UNC13A (NM_001050421.2)           | GAGAGCTCCAGTTCACGT | AGAGATTGCGAGCAGCGT | 220 |
| VAMP2 (NM_014232.2)               | GTGGATGATGTCGGTGAGCA | GCGGAAATCACTCCCAAG | 197 |
| STX1A (NM_00115903.2)             | CATTCCAGGAGCAGACTATGT | TGGAGATGTTGAGTATCTTT | 185 |
| NAPA (NM_003827.4)                | AGTCTCCAGGAGTCCAGG | GTTTCAGCAGCAGCTCAG | 241 |
| NSF (NM_006178.4)                 | CCCGAGTGTGGTGAAGAC | ACTGTTAGGAAACACCC | 180 |

### TABLE 3 Characteristics of non-diabetic human islets donors

| Islet preparation | Donor 1 | Donor 2 | Donor 3 |
|-------------------|---------|---------|---------|
| Donor identifier  | 227     | 229     | 239     |
| Islet purity (%)  | 80      | 55      | 86      |
| Donor age (y)     | 76      | 41      | 67      |
| Donor sex (M/F)   | M       | M       | F       |
| Donor BMI (kg/m²) | 22.7    | 24.5    | 29.5    |
| Donor HbA1c       | 5.4     | 5.6     | 5.8     |
4.9 Western blot analysis

EndoC-βH1 cells (700,000 cells/condition) and human islets from ND donors (500-1,000 islets/condition) were lysed using RIPA buffer with protease inhibitor. Whole-cell extracts (15-20 µg) were separated in Tris-glycine sodium dodecyl sulphate (SDS) gels and transferred onto polyvinylidene difluoride (PVDF) membrane. The following primary antibodies were used: MAFB (1:1000; Bethyl Laboratories), MAFA (1:500; Novus Biologicals); STX1A (1:500; Synaptic Systems) and STXBPI (1:1000; Synaptic Systems). Band density for target proteins was normalized against total protein load and analysed using Image Lab software.

4.10 Insulin secretion of EndoC-βH1 cells

EndoC-βH1 cells were starved in the DMEM medium with low glucose (2.8 mM) overnight (18 h). On the day of assay, the cells were washed two times with PBS and starved for 2 h with 0.5 mL well⁻¹ of low glucose (1 mM) secretion assay buffer (SAB) (1.16 mmol L⁻¹ MgSO₄, 4.7 mmol L⁻¹ KCl, 1.2 mmol L⁻¹ KH₂PO₄, 114 mmol L⁻¹ NaCl, 2.5 mmol L⁻¹ CaCl₂, 25.5 mmol L⁻¹ NaHCO₃, 20 mmol L⁻¹ HEPES and 0.2% BSA, pH 7.4). Then, the cells were stimulated for 1 h with 0.25 ml well⁻¹ of SAB buffer with low glucose (1 mM), high glucose (20 mM) or low glucose and high KCl (1 mM + 35 mM respectively). Secreted insulin levels were measured using mouse insulin ELISA (Mercodia).

4.11 Insulin secretion of human islets

After transfection or treatment, human pancreatic islets (10 islets/well (24-WP), four to six replicates/condition) were starved for 1 h in SAB buffer, LG (1 mM) and stimulated for 1 h with SAB buffer LG (2.8 mM) or HG (16.7 mM). Total insulin content was measured by extracting total islets protein with RIPA buffer. Insulin concentration was measured from supernatants (accumulated during 1 h of stimulation) and from islets lysates (10 islets/well) by ELISA and insulin secretion expressed as the percentage of insulin secreted from the total insulin content.

4.12 Immunohistochemistry

Immunohistochemical analysis was performed as described previously. Briefly, Pancreata from six months old wild-type and MafA mutant mice as well as human pancreas biopsies from normoglycemic and T2D donors were fixed in 4% paraformaldehyde and embedded in paraffin and 6-µm sections were mounted on glass slides. The primary antibodies used were: Insulin (1:1,000; Dako), MAFA (1:100; Bethyl Laboratories), STX1A (1:100; Synaptic Systems) and STXBPI (1:250; Synaptic Systems). Secondary antibodies used were: Cy2-, Cy3- and Cy5-conjugated α-guinea pig, α-mouse, α-goat and α-rabbit (1:500; Jackson ImmunoResearch). Nuclear staining was performed using DAPI (1:6,000; Invitrogen).

4.13 MAFA eQTL analysis

Gene expression from 188 donors was processed as described previously. Briefly, post-processing of expression data was performed by alignment to reference genome build 37, and gene counts were computed using feature counts. Counts were further normalized for sequencing depth and rank-based inverse normal transformation was applied. Linear models were applied to assess the association between gene expression and genotypes, with age, sex, BMI, purity and days of culture as covariates. For eQTL analysis, rs61731375 was the lead signal for association with MAFA expression and was assessed for association with exocytosis genes.

4.14 Statistical analysis

Data are shown as mean ± SEM of at least three independent experiments. Student’s t test or one-way ANOVA were used for single or multiple comparisons respectively. Statistical significance was set at P < .05. PRISM 8.0 (GRAPHPAD) was used for statistical analysis and graph generation.

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