Calcineurin/NFATc2 and PI3K/AKT signaling maintains β-cell identity and function during metabolic and inflammatory stress

Highlights
Acute metabolic and inflammatory stress activates Ca²⁺/CN/NFATc2 signaling in β-cells

NFATc2 induces differentiation genes and suppresses disallowed genes in β-cells

Chronic stress hyperstimulates [Ca²⁺], and delinks NFAT transcriptional activity

Loss or exhaustion of Ca²⁺/CN/NFATc2 signaling results in β-cell dedifferentiation
Calcineurin/NFATc2 and PI3K/AKT signaling maintains β-cell identity and function during metabolic and inflammatory stress

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SUMMARY

Pancreatic islets respond to metabolic and inflammatory stress by producing hormones and other factors that induce adaptive cellular and systemic responses. Here we show that intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and ROS signals generated by high glucose and cytokine-induced ER stress activate calcineurin (CN)/NFATc2 and PI3K/AKT to maintain β-cell identity and function. This was attributed in part by direct induction of the endocrine differentiation gene RFX6 and suppression of several β-cell “disallowed” genes, including MCT1. CN/NFATc2 targeted p300 and HDAC1 to RFX6 and MCT1 promoters to induce and suppress gene transcription, respectively. In contrast, prolonged exposure to stress, hyperstimulated [Ca\(^{2+}\)]\(_i\), or perturbation of CN/NFATc2 resulted in downregulation of RFX6 and induction of MCT1. These findings reveal that CN/NFATc2 and PI3K/AKT maintain β-cell function during acute stress, but β-cells dedifferentiate to a dysfunctional state upon loss or exhaustion of Ca\(^{2+}\)/CN/NFATc2 signaling. They further demonstrate the utility of targeting CN/NFATc2 to restore β-cell function.

INTRODUCTION

Glucose is a central metabolic fuel for sustaining cellular energy requirements and physiological processes. Pancreatic islet cells freely uptake and metabolize glucose to sense its concentration in the blood and release peptide hormones to maintain glucose homeostasis. Specifically, islet β cells produce and release insulin when blood glucose concentrations are high, stimulating glucose disposal by its uptake and utilization. When glucose concentrations remain high, metabolic and inflammatory stress ensues. Under these conditions, β cells must adapt by reducing metabolic load or be subjected to exhaustion, apoptosis, and cell death. This may be accomplished in part by altering expression of genes required for β-cell sensitivity and responsiveness to glucose (Jonas et al., 2009; Mezza et al., 2016; Swisa et al., 2017; Hu et al., 2020). Thus, β cells can avoid overstimulation and exhaustion detrimental to survival by becoming less “β-like.”

Mounting evidence suggests that the loss of β-cell function in stressed islets not only arises from β-cell death, but also results from changes in expression of genes that define β-cell identity and state of differentiation (Talchai et al., 2012; Spijker et al., 2015; Cinti et al., 2016). Metabolic and inflammatory stress can induce transdifferentiation of islets to other nonendocrine cell types or dedifferentiation to a nonfunctional progenitor state. This altered cell identity has been described in both type 1 and type 2 diabetes models, and several studies have shown that trans- and dedifferentiated (hereon referred to as dedifferentiated) islet precursor cells can be reconverted to a β-cell phenotype (Jonas et al., 1999; Brereton et al., 2014; Wang et al., 2014).

Several clusters of genes have been identified and tracked during β-cell dedifferentiation. Among these are downregulated transcription factors required for β-cell differentiation and upregulated “disallowed” genes that are normally selectively silenced in β cells to maintain identity and function (Thorel et al., 2010; Spijker et al., 2013). Many of these genes are differentially regulated under diabetic conditions that promote metabolic, oxidative, and ER stress in β cells (Lombardi et al., 2012; Guo et al., 2013; Swisa et al., 2017; Kitakaze et al., 2018). These cellular processes contribute to mechanisms that elevate intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), produce reactive oxygen species (ROS), and elicit the unfolded protein response, which provide signals for adaptation of β cells to cell stress and metabolic demand. However,
prolonged or unresolved metabolic stress results in sustained or overstimulated release of intracellular Ca²⁺ and ROS levels that can suppress genes required for β-cell function and induce disallowed genes to drive dedifferentiation (Guo et al., 2013; Stancill et al., 2017; Swisa et al., 2017). For example, sustained metabolic and oxidative stress reduces expression and activity of β-cell transcription factors PDX1, FOXO1, PAX6, NKX6.1, and MAFA (Guo et al., 2013; Dai et al., 2016; Swisa et al., 2017). In parallel, diabetic conditions can induce disallowed genes in β cells including SLC16A1 (MCT1), OAT, ITIH5, and CXCL12 (Lemaire et al., 2016).

Both high glucose and cytokines are major contributors to ER and oxidative stress signals via increased mitochondrial metabolism, secretory protein requirements, and production of ROS. However, cell signaling mechanisms regarding cellular processes that regulate genes required for maintenance of β-cell identity and function are largely unknown. In this study, we identified β-cell differentiation and disallowed genes that are acutely regulated by high glucose and cytokines and examined contributions of oxidative and ER stress signaling components to regulate their expression. We demonstrate requirements of ER-derived Ca²⁺ and ROS signaling components for induction and suppression of genes, namely RFX6 and MCT1 that contribute to β-cell identity and function. We show that both Ca²⁺-dependent calcineurin (CN)/nuclear factor of activated T cells 2 (NFATc2) and ROS-mediated phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) signaling pathways are required to maintain β cells in a differentiated and functional state during stress.

Moreover, we elucidate mechanisms whereby Ca²⁺/CN/NFATc2 signaling mediates recruitment of p300/histone deacetylase 1 (HDAC1) to RFX6 and MCT1 gene promoters during acute and sustained exposure to high glucose and IL-1β to differentially regulate gene expression. We further delineate requirements of ROS-mediated PI3K/AKT signaling for activation of NFATc2-p300/HDAC1 complexes on the RFX6 and MCT1 gene promoters. Loss of signaling components of either pathway results in stress-induced β-cell dedifferentiation. The mechanism of chronic effects of glucose and IL-1β to induce dedifferentiation was attributed in part to sustained elevated [Ca²⁺], and depletion of [Ca²⁺], stores to impair CN/NFATc2 signaling. Conditional β-cell-specific knockout (βKO) of NFATc2 in mouse islets mimicked effects of dedifferentiation and induction of disallowed genes during stress.

The small molecule differentiation inducer, isoxazole-9 (ISX9), could restore glucose-stimulated insulin secretion (GSIS) in β cells with prolonged exposure to high glucose and IL-1β. This translated to improved β-cell function in transplanted islets. Effects of ISX9 were attributed in part to preventing overstimulation of intracellular Ca²⁺ release and maintaining CN/NFATc2 signaling in β cells. ISX9 could not restore RFX6 gene expression in NFATc2βKO mice, indicating a direct requirement of CN/NFATc2 signaling for the therapeutic effect to sustain β-cell differentiation. These studies provide insight into signaling mechanisms of differential expression of differentiation and disallowed genes during β-cell adaptation in metabolic and inflammatory stress. The results also demonstrate that these converging pathways can be targeted to sustain β-cell differentiation for potential therapeutic interventions for diabetes.

RESULTS
High glucose and IL-1β regulate genes that control cytokines, oxidative and ER stress, and β-cell differentiation in human islets

To identify genes expressed in islets in response to metabolic and inflammatory stress, we performed total RNA-Seq on isolated human islets exposed to IL-1β in the presence of high glucose. Results revealed 3651 genes (padj <0.05) differentially regulated within 2 h of cytokine treatment (Figure S1). Gene ontology analysis of upregulated transcripts indicated enrichment of genes associated with cytokine signaling and proinflammatory responses (Table S1A). Multiple cytokines and chemokines were induced, and several key regulators of oxidative and ER stress responses were upregulated (Tables S1B and S1C). The results revealed acute induction of proinflammatory transcripts and upregulation of genes involved in oxidative and ER stress in human islets in response to IL-1β and high glucose.

Upregulation of oxidative and ER stress genes correlated with downregulation of transcription factors essential for islet cell differentiation and function, including PDX1, NEUROD1, MAFA, MAFB, NKX6.1, NKX2.2, ISL1, PAX4, PAX6, MNX1, FOX O1, SIX2, GATA6, ONECUT1, and ONECUT2 (Figure 1A). This paralleled the induction of several β-cell disallowed genes, including SLC16A1(MCT1), PDGFRA, GDA, MGLL, TNS1, ACOT7, NDRG4, ZDHHC9, RARRES2, ZFP36L1, and ARAP2. Moreover, regulator of G protein
signaling 16 (RGS16), a marker shown to be temporally expressed in developing islets and reexpressed in β cells of diabetic and metabolically stressed adult mice (Villasenor et al., 2010), was also upregulated. However, expression of pancreatic endoderm progenitor PTF1A and islet progenitor NEUROG3 transcripts remained undetected, and early upstream regulator of islet cell differentiation RFX6 remained unchanged.

Figure 1. Identification of genes and transcription factors that regulate β-cell differentiation and disallowed genes in human islets exposed to metabolic and inflammatory stress
(A) Relative abundance of RNA-Seq differentially expressed mRNA transcripts determined by DESeq2 average normalized count values for β-cell differentiation and disallowed genes in human islets exposed to 16.7 mM glucose (G16.7) and IL-1β.
(B and C) Enrichr enrichment analysis visualization grid, network, and bar graph and (C) table of results ranked by combined score of odds ratio and p value (p < 0.05) as determined by TRANSFAC and JASPAR software analyses of position weight matrices of transcription factors for gene targets identified by RNA-Seq (padj < 0.05). The grid represents enriched terms that cluster with highest significance. Network nodes represent enriched terms and links represent gene content similarity among enriched terms. Brighter colors represent higher significance.

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Top FK506 downregulated gene integrated pathways (Enrichr/NIH BioPlanet)

A

Gene expression regulation in endocrine-committed (NEUROG3+) progenitor cells
Gene expression regulation in pancreatic beta cells
Pancreatic beta-cell development regulation
Depression of pain sensation by the transcriptional regulator DREAM
Sonic Hedgehog (Shh) pathway
DAIPP-32 events
Maturity onset diabetes of the young

Index | Name | p-value | Odds Ratio | Combined score
--- | --- | --- | --- | ---
1 | Gene expression regulation in endocrine-committed (NEUROG3+) progenitor cells | 1.60E-03 | 51.61 | 332.13
2 | Gene expression regulation in pancreatic beta cells | 1.10E-04 | 19.49 | 177.73
3 | Pancreatic beta-cell development regulation | 5.11E-05 | 14.49 | 143.18
4 | Repression of pain sensation by the transcriptional regulator DREAM | 8.51E-04 | 19.42 | 137.28
5 | Sonic Hedgehog (Shh) pathway | 1.04E-03 | 17.93 | 123.16
6 | DAIPP-32 events | 2.72E-04 | 14.85 | 121.88
7 | Maturity onset diabetes of the young | 2.72E-04 | 14.85 | 121.88

D
Top β-cell development and functional genes affected by FK506

| Gene | P-value |
|---|---|
| RFX6 | 5.68E-18 |
| PAX6 | 1.57E-09 |
| IAPP | 3.77E-07 |
| NKX2-2 | 2.51E-05 |
| INS1 | 3.33E-05 |
| SLC2A2 | 1.52E-04 |
| IRX2 | 1.43E-02 |

E

β-cell Differentiation Genes

β-cell Disallowed Genes

F

FK506

NFATC2βKO

Human islets

Mouse islets

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under these conditions (not shown). Expression of insulin, glucagon, and somatostatin were also unchanged (not shown). The results indicate that islets undergo global suppression of genes that maintain β-cell identity and function in response to high glucose and IL-1β but remain resistant to complete reversion to an endocrine cell progenitor state during acute stress.

NFATc2-dependent genes are enriched in human islets exposed to high glucose and IL-1β
To identify transcription factors that may be regulating genes in response to IL-1β and high glucose, we performed TRANSFAC and JASPAR position weight matrices analysis of top upregulated genes identified by RNA-Seq. The results indicated enrichment of gene promoter targets of transcription factors, including NFATC2, IRF1, RELA, SND1, TBX5, HIVEP1, NFkB1, STAT1, RELB, and NR4A2 (Figures 1B and 1C). We and others have previously shown that CN and its downstream target NFAT have both adaptive and functional roles in β cells during metabolic and inflammatory stress (Lawrence et al., 2005, 2011, 2015; Heit et al., 2006; Keller et al., 2016; Yoshimatsu et al., 2017; Zhao et al., 2019). Indeed, clinical use of the CN-inhibitor tacrolimus (FK506) as an immunosuppressant during organ transplantation results in loss of β-cell function and manifestation of post-transplant diabetes (Armitage et al., 1992; Odocha et al., 1993). Thus, we hypothesized that NFATc2 might be a key transcription factor in targeting genes that regulate β-cell adaptation to metabolic and inflammatory stress.

CN/NFATc2-dependent genes maintain β-cell identity and function
To identify CN/NFAT-dependent gene targets that may be required for β-cell adaptation to metabolic and inflammatory stress, we analyzed RNA-Seq gene sets of human islets exposed to high glucose and IL-1β in the presence of FK506. NIH BioPlanet integrated pathway analyses of gene changes in stressed islets pretreated with FK506 revealed downregulation of clusters of genes involved in processes of islet cell differentiation, development, and function (Figures 2A–2C). Genes among top clusters downregulated by exposure of human islets exposed to FK506 included RFX6, INSM1, NXX2-2, PAX6, IRX2, SLC2A2 (GLUT-2), and IAPP (Figure 2C). The results indicate that blockade of CN/NFAT signaling results in suppression of genes required for β-cell differentiation and function.

The downstream CN target NFATc2 scored highest among enriched regulators of top induced genes in human islets exposed to high glucose and IL-1β (Figure 1B). Thus, we used a conditional transgenic β-cell-specific NFATc2 knockout (NFATc2 KO) mouse model to analyze requirements of NFATc2 to regulate genes in response to metabolic and inflammatory stress. Transcriptome analysis of NFATc2 KO islets exposed to high glucose and IL-1β revealed downregulation of genes known to promote β-cell differentiation and upregulation of β-cell disallowed genes relative to WT control islets. Blockade of differentiation factors RFX6 and INSM1 overlapped with effects observed in human islets exposed to FK506, indicating that CN/NFAT-dependent regulation of islet cell differentiation factors are conserved in mouse and man (Figures 2E and 2F). Moreover, β-cell disallowed genes SLC16A1 (MCT1), ZFP36L1, ARAP2, ITIH6, and TST were induced in both NFATc2 KO mouse and FK506-treated human islets (Figures 2D–2F). Collectively, these results suggest that CN/NFATc2 signaling is required for expression of genes that maintain β cells in a differentiated state, and its inactivation allows genome-wide transcriptional changes that promote β-cell dedifferentiation when exposed to metabolic and inflammatory stress.

Regulation of RFX6 and MCT1 gene expression is dependent upon duration of exposure to high glucose and IL-1β
Transcriptome analyses of human and transgenic mouse islets indicated that RFX6 expression is affected by inhibition of CN or deletion of NFATc2 in β cells. RFX6 is a key factor required for β-cell differentiation and
To determine effects of high glucose and IL-1β on RFX6 gene expression, we performed time-course analysis of RFX6 gene transcription in human and mouse islets and MIN6 β-cell line. Results confirmed elevated or sustained expression after 6 h exposure to high glucose and IL-1β (Figure 3B). Notably, MIN6 had much lower overall expression of Rfx6 which could be stimulated to much higher folds compared to islets. This could be attributed in part to long-term culture of MIN6 in high glucose. Although MIN6 lose β-cell characteristics under these conditions, they do retain glucose-stimulated insulin secretion. Moreover, both β and α cells produce RFX6 which could mask stimulatory effects in islets. Thus, increased RFX6+ β cells concurred with decreased RFX6+ α cells in response to high glucose and IL-1β, which could minimize the overall net expression observed in islets. The induction in RFX6 gene expression was lost by 24 h, and transcription was suppressed by 48 h MIN6 transfected with an Rfx6 promoter reporter showed increased reporter activity within 6 h, indicating regulation occurring at the gene level (Figure 3C). Inhibition of CN by FK506 and co-transfection of MIN6 with a dominant-negative NFAT plasmid indicated a CN/NFAT signaling requirement for induction of the Rfx6 gene promoter in response to metabolic and inflammatory stress.

To examine contributions of high glucose and IL-1β on Rfx6 gene expression in MIN6 β cells, we compared effects of high glucose and IL-1β alone to basal conditions for up to 48 h exposure. MIN6 cells exposed to high glucose showed up to 4-fold increase in Rfx6 gene expression within 6 h (Figures 3B and 3D). IL-1β alone showed similar induction (3.3-fold) of Rfx6 in β cells within 2 h (Figure 3D). Combined high glucose and IL-1β provided an enhanced effect on Rfx6 expression (9.1-fold) within 2 h. In all cases, Rfx6 expression was suppressed more than 50% compared to basal 5.5 mM glucose time-matched controls within 24 h. These results indicate dual mechanisms by which Rfx6 is acutely upregulated by high glucose and IL-1β and subsequently suppressed after prolonged exposure.

Transcriptome analyses also revealed several β-cell disallowed genes including MCT1 were induced by CN inhibition or NFATc2 knockout. As MCT1 is a clinically relevant β-cell-disallowed gene related to exercise-induced hyperinsulinism, we also tracked its expression in response to metabolic and inflammatory stress. Mct1 gene expression was suppressed up to 35% within 2–6 h of treatment with high glucose and IL-1β compared to 5.5 mM glucose time-matched controls (Figure 3E). Mct1 was induced 3.8-fold to 16.6-fold within 12–24 h and its expression was sustained (16.2-fold) for up to 48 h in MIN6 cells exposed to high glucose and IL-1β. The data indicate that high glucose and IL-1β acutely enhance gene transcription of differentiation factor Rfx6 while Mct1 is suppressed. In contrast, prolonged exposure of β cells to high glucose and IL-1β results in suppression of Rfx6 and induction of Mct1. Taken together, these data suggest that there are dual mechanisms by which Rfx6 and Mct1 genes are inversely regulated during acute and prolonged exposure to metabolic and inflammatory stress.

Inverse regulation of RFX6 and MCT1 requires CN and PI3K evoked by ER stress

Sirolimus or Rapamycin (RAPA) is another immunosuppressant often used in transplantation maintenance therapy that like FK506 binds to immunophilins with calcineurin but instead inhibits mTOR activity, which is
also associated with β-cell toxicity. Moreover, PI3K/AKT-mTOR signaling has been shown to have a role in regulating β-cell mass function (Soleimanpour et al., 2010; Demozay et al., 2011). Thus, we sought to investigate whether PI3K/AKT-mTOR had a role in stress-induced regulation of β-cell differentiation/dedifferentiation and determine its upstream signaling components. To determine cell signaling requirements contributing to the regulation of RFX6 and MCT1 gene expression in β cells, MIN6 cells were pretreated with FK506, the mTOR inhibitor rapamycin (RAPA), and the PI3 kinase inhibitor wortmannin (WM) before exposure to high glucose and IL-1β. FK506 and WM but not RAPA blocked upregulation of Rfx6 (70.7 and 88.2%, respectively) at 2 h, indicating requirements of both CN and PI3K (Figure 3F). FK506 prevented suppression of Rfx6 at 24 h while WM further inhibited its expression. FK506 also prevented suppression of Mct1 at 2 h while WM inhibited its expression (Figure 3G). Mct1 induction at 24 h was blocked by both FK506 and WM. Collectively, the data suggest there are inverse mechanisms by which Rfx6 and Mct1 are regulated by CN and PI3K signaling during acute and prolonged exposure to high glucose and IL-1β.

As metabolic stress and inflammatory stress are known to induce ER stress in β cells, we examined the effects of the ER stress inducer, thapsigargin (Tg), on Rfx6 and Mct1 gene expression in MIN6 cells. ER stress induction by Tg in MIN6 at basal glucose mimicked induction and suppression of Rfx6 and Mct1 with kinetics similar to those observed with high glucose and IL-1β. Moreover, requirements of CN and PI3K were similar for Tg-regulated Rfx6 and Mct1 gene expression as observed for high glucose and IL-1β (Figures 3J–3K). These data suggest that CN and PI3K signaling inversely regulates Rfx6 and Mct1 gene expression in response to high glucose and IL-1β by mechanisms evoked by ER stress. CHOP indicator of ER stress was similarly expressed in MIN6 treated by either Tg or IL-1β, further suggesting that these signaling mechanisms are mediated by ER stress (Figure 3N). Effects on gene expression for both Rfx6 and Mct1 closely paralleled those observed in gene promoter activity in MIN6 cells, indicating that the stress-induced transcriptional changes occur at the level of the gene promoter (Figures 3F–3I and 3J–3M).

NFATc2 associates with RFX6 and MCT1 gene promoters in response to high glucose and IL-1β and is dependent on CN

To evaluate effects of CN signaling on the association of NFATc2 with Rfx6 and Mct1 genes, we performed NFATc2 chromatin immunoprecipitation (ChIP) in MIN6 cells after 2 h and prolonged 24 h exposure to high glucose and IL-1β. ChIP analysis showed enrichment of NFATc2 on 5' flanking proximal gene promoters of both Rfx6 and Mct1 genes within 2 h. Enrichment of NFATc2 on promoters of both genes was significantly reduced (p < 0.005) in the presence of FK506. Similar effects of NFATc2 association and inhibition by FK506 were also observed at 24 h. However, NFATc2 enrichment on Rfx6 and Mct1 promoters at 24 h was largely reduced (81.6 and 55.1%, respectively) compared to 2 h (Figures 3O and 3P). These data indicate that acute stimulation of NFATc2 direct association with the Rfx6 and Mct1 promoters by high glucose and IL-1β requires CN. They also show that CN-dependent stimulation of NFATc2 enrichment upon promoters significantly declines by 24 h.

CN/NFATc2 and PI3K regulate enrichment of p300 and HDAC1 on RFX6 and MCT1 promoters

Previous studies have demonstrated capabilities of NFAT to recruit histone-acetylating p300 and histone-deacetylating (HDAC) enzymes to gene promoters to regulate transcription (García-Rodríguez and Rao, 1998; Avots et al., 1999). Thus, we sought to determine if NFATc2 influenced the association of p300 and HDAC with the Rfx6 and Mct1 promoters. NFATc2 ChIP analysis revealed rapid accumulation of NFATc2 on both Rfx6 and Mct1 promoters that peaked within 10 min and was sustained for up to 60 min of exposure to high glucose and IL-1β (Figures 4A and 4D). FK506 but not RAPA or WM inhibited NFATc2 promoter accumulation, indicating the selective requirement of CN. NFATc2 ChIP and subsequent re-ChIP of p300 showed increased and sustained co-occupancy of p300 with NFATc2 on the Rfx6 promoter (Figure 4B). Both FK506 and WM inhibited accumulation of p300, indicating requirements for both CN and PI3K signaling. Accumulation of p300 corresponded with a concomitant sustained decrease in HDAC1 on the Rfx6 promoter, which was also prevented by FK506 and WM (Figure 4C).

In contrast, NFATc2 ChIP-p300 re-ChIP showed a transient (5-15 min) enrichment of p300 on the Mct1 promoter followed by inhibition and sustained (up to 60 min) reduction of p300 occupancy during exposure to high glucose and IL-1β (Figure 4E). Sustained reduction in p300 corresponded with increased accumulation of HDAC1 on the Mct1 promoter and acute suppression of Mct1 gene expression (Figure 4F). WM inhibited p300 enrichment while promoting HDAC1 occupancy on both Rfx6 and Mct1 promoters. In all cases, FK506
prevented changes in occupancy of either p300 or HDAC1. Collectively, these data indicate that NFATc2-mediated enrichment and displacement of p300 and HDAC1 on *Rfx6* and *Mct1* promoters is dependent on both CN and PI3K signaling. Although CN is required for changes in NFATc2-p300 and NFATc2-HDAC1 co-occupancy, sustained accumulation or displacement of p300 and HDAC1 requires PI3K signaling.

**Figure 4.** Cell-signaling requirements for NFATc2 co-occupation with p300 and HDAC1 on *RFX6* and *MCT1* gene promoters

(A–L) Effects of 16.7 mM glucose (G16.7) and IL-1β in the presence of (A–F) FK506, RAPA, and WM and (G–L) Nif, Dant, and NAC on association of NFATc2 (A, D, G, and J), p300 (B, E, H, and I), and HDAC1 (C, F, I, and L) on *RFX6* and *MCT1* gene promoters by ChIP-Re-ChIP assay in MIN6. Data shown are representative of results from at least three independent experiments (n = 3) using replicate assays. Asterisks denote statistical significance (#p < 0.05, ##p < 0.01, ###p < 0.001 as compared to control, *p < 0.05, **p < 0.01, ***p < 0.001 as compared to no drug) in mean values based on the two-tailed Student t test.
Overall, sustained promoter co-occupancy by NFATc2-p300 correlated with acute gene upregulation, whereas increased co-occupancy by NFATc2-HDAC1 correlated with acute gene downregulation.

NFATc2 and p300/HDAC1 promoter co-occupancy requires intracellular Ca²⁺ and redox signaling

The current data suggest that high glucose and IL-1β regulate Rfx6 and Mct1 gene expression by mechanisms involving ER stress. As ER stress responses can be propagated via perturbation in intracellular Ca²⁺ and redox signaling, we evaluated effects of inhibitors of extracellular Ca²⁺ influx (nifedipine), intracellular Ca²⁺ release (dantrolene), and redox transitions (N-acetyl cysteine or NAC) on NFATc2, p300, and HDAC1 promoter occupancy in response to high glucose and IL-1β. Blockade of [Ca²⁺], from extracellular or intracellular compartments prevented association of NFATc2 with Rfx6 and Mct1 promoters (Figures 4Ga and 4J). However, NAC did not significantly affect sustained elevation of NFATc2 promoter occupancy, indicating that stimulation of NFATc2 association is primarily dependent on intracellular Ca²⁺. In contrast, ChIP-re-ChIP analysis showed that NAC suppressed sustained enrichment of NFATc2-p300 and enhanced accumulation of NFATc2-HDAC1 on the Rfx6 and Mct1 promoters (Figures 4G–4L). The data suggest that redox perturbation or oxidative stress promotes p300 displacement of HDAC1 during acute exposure to high glucose and IL-1β. In each case, inhibition of intracellular Ca²⁺ signaling prevented accumulation of either NFATc2-p300 or NFATc2-HDAC1.

K⁺ depolarization and hydrogen peroxide (H₂O₂) induce NFATc2 and p300/HDAC1 promoter co-occupancy

To identify potential contributions of intracellular Ca²⁺ and redox perturbation on NFAT-mediated p300/HDAC1 promoter association, we tested effects of (1) Ca²⁺ elevation by K⁺-induced depolarization and (2) ROS H₂O₂ on MIN6 cells. K⁺ depolarization but not H₂O₂ transiently induced (5-15 min) NFATc2 enrichment with Rfx6 and Mct1 gene promoters (Figures 5A and 5D). However, combined treatment with K⁺ depolarization and H₂O₂ resulted in sustained (up to 60 min) accumulation of NFATc2. NFATc2 enrichment correlated with co-occupancy of p300 and displacement of HDAC1 on the Rfx6 promoter (Figures 5B and 5C). This contrasted with effects of K⁺ depolarization and H₂O₂ to enhance NFATc2-HDAC1 enrichment and displace NFATc2-p300 on the Mct1 promoter (Figures 5E and 5F). These results suggest that intracellular Ca²⁺ signaling directly contributes to NFAT-mediated accumulation of p300/HDAC1, whereas ROS signaling contributes to sustained NFATc2-p300 and NFATc2-HDAC1 complexes on Rfx6 and Mct1 gene promoters.

ROS directly induces PI3K/AKT signaling

The current data suggest that intracellular Ca²⁺ directly promotes CN/NFATc2 signaling and that both ROS and PI3K signaling is required for stabilization of NFATc2-p300/HDAC1 complexes. However, upstream mechanisms by which PI3K is activated and downstream effects remain unclear. Although results with RAPA indicate mTOR is not required for promoter regulation during cytokine or ER stress, we hypothesized that ROS might contribute to activation of PI3K and its downstream effector AKT. Immunoblot analysis showed increased phosphorylation of AKT in response to high glucose and IL-1β. WM but not FK506 prevented AKT phosphorylation, indicating a selective requirement of PI3K to activate AKT (Figure 5G). H₂O₂ mimicked PI3K-dependent activation of AKT and was prevented in the presence of NAC (Figures 5H and 5I). These results suggest that ROS directly contributes to induction of PI3K and downstream activation of AKT under conditions of oxidative stress.

ISX9 prevents overstimulation of intracellular Ca²⁺ stores and maintains NFATc2-mediated RFX6 gene expression in β cells

ISX9 was shown to improve β-cell function by modulating [Ca²⁺], in β cells and promoting expression of β-cell transcription factors (Pujol et al., 2018). We hypothesized that Ca²⁺-dependent effects of ISX9 to maintain differentiation of β cells might be conferred by Ca²⁺/CN/NFAT-mediated regulation of RFX6. Evaluation of [Ca²⁺], in MIN6 cells showed a significant elevation of basal [Ca²⁺], and hyperstimulation of depolarization-induced [Ca²⁺], after 24 h exposure to high glucose and IL-1β (Figures 6A and 6B). Pretreatment of MIN6 with ISX9 prevented elevations in basal [Ca²⁺], and hyperstimulation of [Ca²⁺], during extended exposure of β cells to high glucose and IL-1β and prevented. Indeed, ISX9 maintained basal [Ca²⁺], levels similar to controls under all conditions tested (Figure S2).
To determine if ISX9 could influence NFAT-mediated gene transcription, we analyzed changes in NFAT-Luc promoter reporter activity in MIN6 cells exposed to high glucose and IL-1β. Promoter reporter analysis showed increased NFAT activity in MIN6 acutely exposed up to 2 h to high glucose and IL-1β, which was lost and suppressed within 24 h of extended exposure (Figure 6C). Pretreatment of MIN6 with ISX9 maintained and prevented loss of NFAT activity during both acute and extended exposure to high glucose and IL-1β. Moreover, NFAT-mediated promoter activity correlated with the association of NFATc2 with the Rfx6 gene promoter in which NFATc2 was highly enriched in response to high glucose and IL-1β and lost after

Figure 5. Signal-selective enrichment of NFAT-p300 and NFAT-HDAC1 on the RFX6 and MCT1 gene promoters (A–I). Effects of 16.7 mM glucose (G16.7) and IL-1β, K⁺-induced depolarization and H₂O₂ on association of NFATc2 (A and D), p300 (B and E), and HDAC1 (C and F) on RFX6 and MCT1 gene promoters by ChIP-Re-ChIP assay in MIN6. Effect of (G) FK506 and WM on G16.7 and IL-1β–induced activation of AKT by phosphorylated-AKT (pAKT) immunoblot. Effect of (H) WM and (I) NAC on H₂O₂–induced activation of AKT. Data shown are representative of results from at least three independent experiments (n = 3) using replicate assays. Blots were performed in duplicates (n = 2) or are representative of at least three independent experiments performed in MIN6 (n = 3). Asterisks denote statistical significance (#p < 0.05, ##p < 0.01, ###p < 0.001 as compared to control, *p < 0.05, **p < 0.01, ***p < 0.001 as compared to no drug) in mean values based on the two-tailed Student t test.
The results suggest that conditions that promote metabolic and inflammatory stress or directly promote islet cell dedifferentiation to an immature or dysfunctional state, which can be prevented or restored by ISX9 unless CN/NFATc2 signaling is disrupted. In MIN6 cells cultured in high glucose and cytokines, which could be partially restored by ISX9 (Figure 6E). In contrast, ISX9 could not rescue loss of Rfx6 gene expression in NFATc2KO islets. Moreover, ISX9 could improve GSIS stimulation indices in mouse islets unless NFATc2 was ablated in β cells (Figure 6G). These results indicate that CN/NFATc2 signaling is required for effects of ISX9 to induce or recover RFX6 expression to maintain β-cell differentiation and function during metabolic and inflammatory stress.

NFATc2KO results in dysregulated insulin secretion in islets exposed to high glucose and IL-1β

To examine consequences of disrupting NFATc2 on β-cell function during stress, we evaluated GSIS in NFATc2KO mouse islets after long-term exposure to high glucose and IL-1β. NFATc2KO islets cultured in basal glucose conditions showed an overall reduction (48.9%) in GSIS compared to WT controls (Figure 7A). In contrast, NFATc2KO islets exposed to high glucose and IL-1β for 24 h exhibited spontaneous insulin release at both low (3 mM) and high (16.7 mM) glucose conditions. Similar results were observed in MIN6 β cells which also released insulin at low glucose concentrations (Figure 7B). Effects of stress on GSIS in MIN6 could be prevented by ISX9 (Figure 7C). Collectively, the data suggest that loss of CN/NFAT signaling because of metabolic and inflammatory stress results in β-cell dedifferentiation to an immature or dysfunctional state, which can be prevented or restored by ISX9 unless CN/NFATc2 signaling is disrupted.

It was previously shown that unsilenced MCT1 or its overexpression in islets can allow pyruvate flux into β cells to inappropriately stimulate insulin secretion (Ishihara et al., 1999; Schuit et al., 2012). Indeed, patients with promoter-activating mutations in the MCT1 gene exhibit hyperinsulinemia during anaerobic exercise or exogenous pyruvate load. Thus, we hypothesized that induction of MCT1 in β cells by metabolic and inflammatory stress or NFATc2 gene ablation might also have effects on pyruvate-stimulated insulin secretion (PSIS). NFATc2KO islets showed a reduced capacity of PSIS (Figure 7D). However, NFATc2KO islets with 24 h exposure to high glucose and IL-1β exhibited spontaneous secretion of insulin at both low (3 mM) and high (20 mM) glucose conditions. Similarly, MIN6 cells exposed to FK506 showed impaired PSIS under basal glucose culture conditions (Figure 7E). However, MIN6 cells cultured in high glucose and IL-1β for 24 h resulted in spontaneous insulin release at both low (3 mM) glucose and high (20 mM) pyruvate conditions. Effects of high glucose and IL-1β could be prevented by ISX9 (Figures 7C and 7F). Taken together, these data indicate that CN/NFATc2 signaling is essential for maintaining β-cell differentiation,
and its inhibition results in dysregulated GSIS. Moreover, CN/NFATc2 signaling GSIS and PSIS can be preserved or restored by ISX9 during metabolic and inflammatory stress.

ISX9 improves graft function in diabetic nude mice transplanted with human islets

Islets undergo multiple modes of stress during transplant procedures including ischemia, hypoxia, and mechanical and physical stress during islet isolation, resulting in release of cytokines and induction of a highly inflammatory state known to be detrimental to islet mass and function. We hypothesized that the

Figure 7. Requirement of CN/NFATc2 signaling to preserve β-cell function during metabolic and inflammatory stress

(A–H) Effect of long-term (24 h) 16.7 mM glucose (G16.7) and IL-1β exposure with (A and D) NFATc2KO mouse islets and FK506-treated MIN6 (B, C, E, and F) on (A–C) G (glucose)-stimulated insulin secretion (GSIS) and (D–F) pyruvate-stimulated insulin secretion (PSIS). (C and F) Effect of ISX9 on GSIS and PSIS after 24 h exposure to G16.7 and IL-1β. Effect of ISX9 on marginal doses of human islets transplanted to nude mouse recipients on (G) blood glucose up to 30 days posttransplant and (H) intraperitoneal glucose tolerance test performed 30 days posttransplant in recipients of pre-graft and post-graft removal by nephrectomy (PN). Data shown are representative of results from three to five mice (n = 3-5) per treatment group experiments and assays with three replicates. Asterisks denote statistical significance (#p < 0.05, ##p < 0.01, ###p < 0.001 as compared to control, *p < 0.05, **p < 0.01, ***p < 0.001 as compared to no drug) in mean values based on the two-tailed Student t test.
inflammatory state imposed on islets during transplantation procedures may contribute to loss of β-cell function by promoting dedifferentiation. Thus, we tested if ISX9 could protect or improve β-cell function and reverse diabetes in recipients with suboptimal or “marginal” doses of islets. Human islets pretreated with ISX9 ex vivo and transplanted into diabetic nude mouse recipients achieved and maintained normoglycemic blood glucose for 30 days posttransplant, whereas control islets were unable to maintain normoglycemia (Figure 7G). An intraperitoneal glucose tolerance test performed in mice after 30 days confirmed improved graft function within 120 min in islets treated with ISX9 compared to marginal islets with no treatment (Figure 7H). Removal of grafts by nephrectomy resulted in reversion to a diabetic state, supporting the finding that normalized blood glucose profiles were a result of direct effects of improved islet graft function by ISX9.

DISCUSSION

CN/NFAT has been implicated in multiple roles in regulating β-cell development, proliferation, and function. We previously showed that CN/NFAT can regulate insulin and isletokine gene expression in pancreatic β-cells which can be modulated by glucose and proinflammatory cytokines (Lawrence et al., 2001, 2008, 2009, 2011, 2015; Lawrence et al., 2002; Yoshimatsu et al., 2017). Other groups have shown that CN is required for β-cell proliferation and survival, which was linked to an IRS2-driven PI3K-AKT signaling pathway and type 2 diabetes (Heit et al., 2006; Soleimanpour et al., 2010; Demozay et al., 2011; Keller et al., 2016). Exogenous expression of constitutively active NFATc1 could restore β-cell proliferation and mass in β-cell specific CN knockout mice (Heit et al., 2006). Moreover, CN was shown to influence genes that promote β-cell proliferation and maturation in neonatal mice, and ablation of CN in islet cell progenitors results in diabetes by the time of weaning (Goodyer et al., 2012). Thus, we hypothesized that CN/NFAT signaling plays a role in β-cell adaptation to metabolic and inflammatory stress, which is often imposed upon islets during high metabolic demand or under diabetic conditions.

The overall goal of the current study was to identify upstream cell signaling mechanisms and downstream molecular targets of CN/NFAT by which β-cells adapt to metabolic and inflammatory stress. Specifically, we investigated CN/NFAT-dependent signaling events that regulate genes which control the state of differentiation and dedifferentiation in β-cells under metabolic and inflammatory stress. Initial screening and analyses of RNA-Seq transcripts revealed a global loss of β-cell differentiation factors and induction of β-cell disallowed genes highly influenced by NFATc2. This prompted us to further investigate mechanistic cell signaling aspects of NFATc2 KO in islets under metabolic and inflammatory stress. This is the first study to our knowledge describing signaling requirements of NFAT in a conditional β-cell-specific transgenic knockout model.

We performed transcriptome analyses to determine overlapping FKS06-sensitive and NFATc2-dependent gene targets that were conserved between human and mouse islets, respectively. As RFX6 is the earliest determinant of islet progenitor cell lineage among conserved NFATc2-dependent genes identified in our study, we primarily focused on signaling aspects of CN/NFAT to regulate its expression in β-cells. We chose MCT1 for further study of regulation of a conserved NFATc2-dependent β-cell disallowed gene because its silencing allows for unique metabolic utilization of glucose required for β-cell GSIS. Loss of expression of RFX6 and/or gain of function of MCT1 in β-cells resulted in loss of function or inappropriate release of insulin from islets. Notably, we showed that the ratio of RFX6 expressing β-cells to α-cells increases during acute stress. This suggests that stress may have an opposing role to regulate RFX6 in alpha cells with respect to beta cells to influence differentiation and cell type specification. Further studies will be required to address this hypothesis.

The results indicated that β-cells induce RFX6 to promote differentiation and maintain function during acute metabolic and inflammatory stress. However, upon prolonged exposure, RFX6 and downstream islet cell differentiation factors are lost but β-cells remain viable in an immature precursor state. This suggests that β-cells dedifferentiate to protect themselves from nutrient exhaustion or stress overload. Ablation of NFATc2 or blockade of its upstream signaling components calcium and calcineurin prevents upregulation of RFX6 and allows activation of MCT1, resulting in the loss of GSIS. Collectively, these results highlight the importance of CN/NFATc2 in regulating genes that determine the state of β-cell differentiation to maintain or modulate β-cell function.
The study further delineates signaling mechanisms involved in the biological events arising from ER stress to propagate metabolic and inflammatory responses. We define integration of signaling components of Ca\(^{2+}\)-modulated CN/NFATc2 and ROS-dependent PI3K/AKT signals propagated via ER stress to regulate genes contributing to β-cell differentiation and identity. We provide evidence to show that RFX6 and MCT1 genes can be inversely regulated by these signaling mechanisms. Mechanistically, CN/NFATc2 recruited p300/HATs to gene promoters while PI3K/AKT enhanced p300 activity to provide an overall sustained effect on transcription. Further mechanistic analysis indicated that ROS/redox signaling was required for activation of PI3K/AKT and promoter enrichment of p300. Moreover, NFATc2-p300 displacement of HDAC1 could be mimicked by either direct stimulation of ROS signaling by H\(_2\)O\(_2\) or increasing [Ca\(^{2+}\)], by KCl-induced cell depolarization. However, both ROS and intracellular Ca\(^{2+}\) signals were required for sustained NFATc2-p300 enrichment on the RFX6 promoter, but alone were not sufficient for sustained displacement of NFATc2-HDAC1 from the MCT1 promoter. These observations suggest that there is yet another unknown signaling factor contributing to recruitment or stabilization of HDAC1 on the gene promoter. Taken together, the results show that although Ca\(^{2+}\)-dependent CN/NFATc2 signaling is capable of recruiting both p300 and HDAC1 to the gene promoter, ROS-dependent PI3K/AKT signaling is required for sustaining or increasing p300 occupancy and displacing HDAC1. Given the known counterregulatory properties of these enzymes to modify acetylation histones, we propose that stress-induced Ca\(^{2+}\)/CN/NFATc2 and ROS/PI3K/AKT signals might be upregulating and/or suppressing genes by controlling access of transcription factors to the DNA-chromatin complex of targeted gene promoters. These transcription factors could potentially provide an additional level of regulation and contribute to the observed selectivity of inverse ratios of p300/HDAC1 occupancy upon RFX6 and MCT1 gene promoters.

In contrast to acute adaptive responses of β cells to metabolic and inflammatory stress, prolonged exposure resulted in loss of NFATc2 signaling and reversal of NFAT-mediated effects on RFX6 and MCT1 gene promoters and respective gene expression. Loss of NFATc2 signaling because of prolonged exposure of β cells to high glucose and IL-1β was attributed at least in part to exhausted release of Ca\(^{2+}\) from intracellular stores. Islets lacking NFATc2 in the β cells had significantly downregulated insulin production but maintained GSIS. However, NFATc2-KO islets exposed to high glucose and IL-1β showed dysregulated secretion, similar to immature β cells which secrete insulin under low glucose conditions. As transplantation procedures induce inflammatory and immune responses, these results might provide some insight to the high incidence of post transplant diabetes observed in patients exposed to CN inhibitors after solid organ transplantation. Collectively, the data suggest that NFATc2 is required to maintain RFX6-dependent differentiation and functional maturity of β cells under metabolic and inflammatory stress conditions. However, upon overstimulation or exhaustion of intracellular Ca\(^{2+}\) signaling, NFATc2 activity is lost, and β cells undergo dedifferentiation characterized by suppression of RFX6 and downstream β-cell differentiation genes, induction of β-cell disallowed genes, and dysregulated GSIS.

Effects of prolonged high glucose and IL-1β could be mimicked by Tg, indicating that dysregulated Ca\(^{2+}\) and redox signals are propagated by perturbation of ER calcium stores and ER stress. ISX9 was able to prevent dedifferentiation of β cells and spare CN/NFAT activity. This was attributed in part to the prevention of overstimulation and dysregulation of intracellular Ca\(^{2+}\) signaling in β cells exposed to high glucose and IL-1β. These findings support previous studies showing effects of ISX9 to induce calbindin-D28K via NFATc1/NFATc2 in β cells to buffer excessive [Ca\(^{2+}\)], and prevent apoptosis (Pujol et al., 2018). Moreover, ISX9 has been shown to protect islets exposed to high glucose and fatty acids by altering its metabolome (Kalwat et al., 2016). We suggest that dedifferentiation to turn off metabolic circuitry required for GSIS to become less “β-like” may be a protective mechanism for cell survival in response to inducers of ER stress. ISX9 prevents exhaustive Ca\(^{2+}\) and redox signaling components of ER stress so that β cells may maintain metabolic sensitivity.

Finally, it was previously shown that ISX9 can induce genes that enhance insulin gene transcription and improve GSIS in islet cultures (Dioum et al., 2011; Kalwat et al., 2016). Furthermore, the use of ISX9 to improve β-cell function indicated that stress-induced dedifferentiation could be reversed in a β-cell regeneration mouse model. We show in our study that ISX9 prevents overstimulation or exhaustion of Ca\(^{2+}\) signaling to sustain CN/NFATc2-mediated β-cell differentiation and preserve both GSIS and PSIS in islets during metabolic and inflammatory stress. We further demonstrate that pretreating human islets with ISX9 ex vivo can improve graft function in a nude mouse transplant model. These results indicate that the Ca\(^{2+}\)/CN/NFATc2 signaling axis can be potentially targeted for effects to maintain or restore islet cell function under diabetic or proinflammatory conditions.
The findings provide insight to understanding signaling mechanisms for the loss of β-cell function under diabetic conditions or during transplantation. The calcineurin inhibitor tacrolimus (FK506) routinely used as the primary immunosuppressant in organ transplant is associated with high risk of posttransplant diabetes. Both FK506 and high glucose have been primarily attributed to β-cell toxicity. Our studies indicate that β cells can also dedifferentiate (rather than die) to a precursor state when exposed to high glucose or FK506. Importantly, the observations of reversible β-cell dedifferentiation open possibilities for therapeutic interventions to re-differentiate β cells under diabetic conditions or during islet transplantation.

Limitations of the study
Owing to limitations in availability of donor pancreases for research isolation and purification of primary beta cells from human islets, most of the mechanistic and validation experimentation was performed in MIN6 β cells and/or whole intact mouse and human islets. Thus, it should be noted that some of the observations may be either mouse-specific or effects related to culturing of cell lines. As noted, the MIN6 cells expressed significantly lower amounts of RFX6 compared to mouse and human islets. This could be attributed to prior extended exposure of MIN6 β cells to high glucose culturing conditions which would be predicted to lower RFX6 based on results observed in islets. Moreover, differences in expression of RFX6 in MIN6 and islets could be a result of contributions of non-β cell subtypes present in whole intact islets.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Mouse studies
  - Generation of NFATc2βKO mice
  - Isolated pancreatic islets
  - Cell and tissue culture and treatment
- METHOD DETAILS
  - Flow cytometry
  - Immunoblotting
  - ChIP assay
  - Real-time QPCR
  - DNA transfection and promoter assays
  - Calcium assay
  - Insulin release studies
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS

C.M.D. designed experimentation, acquired and analyzed data, and contributed to manuscript production. S.V. analyzed data and edited the manuscript. J.M. edited the manuscript. Y.L. analyzed the data. C.J.R. generated NFATc2fl/fl mice. B.N. guided experimentation. M.C.L. designed experimentation, acquired and analyzed the data, contributed to manuscript production, guided experimentation, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. We worked to ensure ethnic or other types of diversity in the recruitment of human subjects. We worked to ensure sex balance in the selection of non-human subjects. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| Anti-NFATc2 Antibody (G1-D10) | Santa Cruz Biotechnology | Cat#sc-7295X; RRID:AB_628013 |
| Anti-p300 Antibody (F-4) | Santa Cruz Biotechnology | Cat#sc-48343X; RRID:AB_628075 |
| Anti-Histone Deacetylase 1 (HDAC1) Antibody (10E2) | Santa Cruz Biotechnology | Cat#sc-81598X; RRID:AB_2118083 |
| Phosphorylated-AKT (Ser473) | Cell Signaling Technologies | Cat#9271; RRID:AB_329825 |
| Akt (pan) (C67E7) | Cell Signaling Technologies | Cat#4691; RRID:AB_915783 |
| β-actin (13E5) Rabbit mAb | Cell Signaling Technologies | Cat#4970; RRID:AB_2223172 |
| CHOP (L63F7) Mouse mAb | Cell Signaling Technologies | Cat#2895; RRID:AB_2089254 |
| Human RFX6 Antibody | R&D Systems | Cat#MAB7780; RRID:AB_2910122 |
| Alexa Fluor 647 Mouse Anti-Insulin | BD Biosciences | Cat#565689; RRID:2739331 |
| BV421 Mouse Anti-Glucagon | BD Biosciences | Cat#565891; RRID:AB_2739385 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| (Z)-4-hydroxytamoxifen | Millipore Sigma | Cat#H7904 |
| Collagenase IV | Sigma | Cat#C9407 |
| Thapsigargin | Santa Cruz | Cat#sc-24017 |
| Isoxazole-9 | R&D Systems | Cat#4439 |
| IL-1β | Biologend | Cat#579402 |
| Tacrolimus (FK506) | Enzo | Cat#ALX-380-008-M001 |
| N-Acetyl-L-Cysteine | Sigma | Cat#A7250 |
| Rapamycin | MedChemExpress | Cat#HY-10219 |
| Wortmannin | Sigma | Cat#W1628 |
| Hydrogen Peroxide | Sigma | Cat#216763 |
| Streptozotocin | Millipore Sigma | Cat#S0130 |
| Fluo-4 dye | ThermoFisher | Cat#F14201 |
| **Critical commercial assays** |        |            |
| Alexa Fluor 488 Protein Labeling Kit | ThermoFisher | Cat#A10235 |
| Mouse Ultrasensitive Insulin ELISA | ALPCO | Cat#80-INSMSU-E01 |
| Pierce BCA Protein Assay Kit | ThermoFisher | Cat#23225 |
| RNeasy Mini Kit | Qiagen | Cat#74104 |
| Lipofectamine 3000 Transfection Reagent | ThermoFisher | Cat#L3000001 |
| Secrete-Pair Dual Luminescence Assay Kit | GeneCopoeia | Cat#LF031 |
| Dual Luciferase Assay Kit | Promega | Cat#E1910 |
| High Capacity Reverse Transcription Kit | ThermoFisher | Cat#4368813 |
| **Deposited data** |        |            |
| Raw data | This paper; Mendeley | Mendeley Data: https://doi.org/10.17632/4jb5cb3w5b.1 |
| RNA-seq data | This paper | GEO Series Accession Number GSE197710 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE197710) |

(Continued on next page)
**REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER**
--- | --- | ---
Experimental models: Cell lines  
MIN6 | AddexBio | Cat#C0018008, RRID:CVCL_0431

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| NFATc2/OKO | In house | |
| Human Islets | IIDP | https://iidp.coh.org |

**Oligonucleotides**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| NFATc2-5'arm-2 | IDT | 5' TGAGAAGGCACCGGCACCGCTGG 3' |
| NFATc2-3'arm-2 | IDT | 5' CTCCTACTGAACTGCTTTGAAGCTGG 3' |
| LAR3 primer | IDT | 5' CACCGGTCTTCTGTAGCTCC 3' |
| INS1-cre/ERT primer 22475 | IDT | 5' CAGCCAAATTTGGGTGTACCGG 3' |
| INS1-cre/ERT primer 22608 | IDT | 5' CGACGGTATCGATAAGCTTGA 3' |
| INS1-cre/ERT primer OIMR1544 | IDT | 5' CAGTGGGGCTCAGCATT 3' |
| INS1-cre/ERT primer oIMR3580 | IDT | 5' TCACCGACATTCTGCCCTTG 3' |
| Mouse RFX6 promoter | IDT | 5' CCTGAGAATTGATGGGGAAA and 5'-GTGAACAGGTACCGGAA from -329 to -99 of the 5'-flanking |
| Mouse MCT1 promoter | IDT | 5'-TGACCAGCGCTTGCTCTAAAC and 5'-TGAATGGACGGACACTTGTA from -625 to -411 of the 5'-flanking |
| Mm00624115_m1 (Rfx6) | ThermoFisher | Cat#4331182 |
| Mm01306379_m1 (Slc16a1) | ThermoFisher | Cat#4331182 |

**Recombinant DNA**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Plasmid: pGL3-NFAT-Luc | Cliffstone and Crabtree, 1992 | Addgene plasmid #17870; RRID:Addgene_17870 |
| Plasmid: pRL-SV40 | Promega | Cat#E2231 |
| Slc16a1 Promoter Reporter Clone | Genecopoeia | MPRM40340-PG04 |
| Rfx6 Promoter Reporter Clone | Genecopoeia | MPRM33989-PG04 |

**Software and algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GraphPad Prism Version 9.0 | GraphPad | https://www.graphpad.com |
| ImageJ | Schneider et al., 2012 | https://imagej.nih.gov/ij/ |
| FlowJo version 10.7.2 | BD Biosciences | https://flowjo.com |

**RESOURCES AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Michael Lawrence (Michael.Lawrence@BSWHealth.org).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Original data have been deposited at Mendeley Data Repository and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. This paper does not report original code. Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse studies

Animals were housed and managed at animal facilities at Baylor University at Waco, Texas and the Dallas Veterans Affairs Medical Center. All animal breeding and experimental protocols were approved by the Institutional Animal Care and Use Committee at both institutions prior to the study. Mice used in studies were 7-9 weeks old including both male and female.

Generation of NFATc2βKO mice

Pancreatic β-cell–specific NFATc2βKO mice were generated by cross-breeding NFATc2fl/fl (gifted to us by Dr. Christopher J. Rhodes) and INS1Cre/ERT2 mice (JAX). Briefly, NFATc2fl/fl and INS1Cre/ERT2 were crossed to produce NFATc2fl/wt: INS1Cre/ERT2 (F1) offspring. F1 mice were then back-crossed with NFATc2fl/fl mice to generate NFATc2fl/fl: INS1Cre/ERT2 (F2) offspring. F2 mice along with WT control mice were injected intraperitoneally with 2 mg tamoxifen in corn oil at 24 h intervals for five consecutive days to produce NFATc2βKO mice.

Genotyping quantitative polymerase chain reaction (QPCR) assays were performed using the following primers: NFATc2-5’arm-2: TGAGAAGGCCAACGGGCAGCCCTGG; NFATc2-3’arm-2: CTCTAAGACAGAATGGGGTGTGG; LAR3 primer: CAACGGGTTCTTCTGTTAGTCC. INS1-cre/ERT primers: 22475: CAGGCAATTTTGGTGTACGG; 22608: CGACGGTATCGATAAGCTTGA; OIMR1544: CACGTGGGCTCCAGCATT; oIMR3580: TCACCACTTATTCTGCCCCGG. Experimental assays were performed after a 7-day recovery period following the final injection. In vitro ablation of NFATc2 in isolated NFATc2βKO mouse islets was performed by 48-h exposure to 100 nM 4-hydroxytamoxifen followed by an additional 48-h recovery period.

Isolated pancreatic islets

Human islets from multiple donors were isolated from research-grade pancreases at Baylor University Medical Center by the Baylor Scott & White Islet Cell Research team and also provided by the Integrated Islet Distribution Program at City of Hope (Table S2). Mouse islets were isolated from C57BL/6 mice from Jackson Laboratories (JAX) and NFATc2βKO transgenic mice. Briefly, pancreases were extracted from mice after collagenase perfusion and digested for 10 min at 37°C. Islets were separated from digested pancreas preparations by centrifugation on a 1.077 and 1.100 g/mL Ficoll gradient solution. The separated islet tissue layer was collected and washed with Hanks’ balanced salt solution. Intact islets were hand-selected from the purified prep by pipette and cultured overnight at 5.5 mM glucose RPMI, 5% CO2, and 37°C.

Cell and tissue culture and treatment

Isolated islets and MIN6 β cells (passages 25-45) were cultured in RPMI 1640 supplemented with 11 mM glucose, 10% fetal bovine serum, 10 mM HEPES, 10 mM sodium pyruvate, and 0.1 mg/mL penicillin-streptomycin at 37°C in 5% CO2 humidified air. Cell treatments included 1 μM Thapsigargin (Tg), 50 μM ISX9, 50 ng/mL IL-1β, 100 nM Tacrolimus (FKS06), 3 mM N-acetylcysteine (NAC), 100 nM Rapamycin (RAPA), 3 nM Wortmannin (WM), and 200 μM H2O2.

Islet transplantation. Athymic nude mice (Harlan Laboratories) recipients (n = 5 per group) were injected with streptozotocin to induce diabetes as determined by blood glucose exceeding 350 mg/dL for two subsequent readings prior to transplantation. Human islets (1500 IEQ) treated with or without ISX9 for 48 h post-isolation were transplanted into the renal subcapsular space. Nonfasting blood glucose levels were measured with Accu-Check Aviva (Roche Diagnostics, Indianapolis, IN) three times a week for 60 days post-transplantation. Normoglycemia was defined as two consecutive blood glucose levels <200 mg/dL. Intraperitoneal glucose tolerance tests were performed on all mice 30 days after transplantation (pre- and post-nephrectomy) after fasting for 12 h after an intraperitoneal injection of 2 g/kg glucose solution. Blood glucose levels were measured 0, 15, 30, 60, and 120 min post-injection.

METHOD DETAILS

Flow cytometry

The fluorescent dye-labeled monoclonal antibodies against human intracellular molecule included anti-INS, anti-GCG, and anti-RFX6. Cells were fixed and permeabilized using BD Cytofix/Cytoperm kit. Fluorescence minus one controls for each antibody were utilized to establish gates correctly and to determine the frequency of positively stained cells. The cells were analyzed by a BD Canto II (BD Biosciences) and FlowJo software.
Immunoblotting
In brief, 2000-5000 IEQ islets and MIN6 cells were washed and lysed in 500 μL radioimmunoprecipitation (RIPA) assay buffer with complete protease inhibitor cocktail. MIN6 cells were vortexed and pelleted by centrifugation. Islets were vortexed for 1 min, sonicated for 2 min in 30-s on/off cycles, and pelleted by centrifugation. Supernatant protein concentration was quantified by bicinchoninic acid (BCA) assay. Protein lysates were resolved on a 4%-20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane by a semi-dry transfer method. Primary antibodies included phosphorylated AKT and total AKT (Cell Signaling Technologies) and run on an iBlot system.

ChIP assay
MIN6 cells were fixed and chromatin DNA-protein was cross-linked with 1% formaldehyde for 10 min, then quenched with 125 mM glycine for 5 min, and finally washed with phosphate-buffered saline. Fixed cells were sonicated with a Bioruptor 200 (Diagenode) and lysed in a RIPA assay buffer containing protease inhibitors. DNA–protein complexes were immunoprecipitated with indicated antibodies or IgG isotype controls and washed. Cross-links were reversed at 65°C for 4 h in Tris buffer containing 5 M NaCl and 0.5 M EDTA. DNA was extracted by phenol/CHCl3 and precipitated with ethanol followed by proteinase K and RNase A treatment. Precipitated DNA and 1% control inputs were analyzed by QPCR. For Re-ChIP samples, eluates were diluted 10-fold to reduce SDS concentration, and multiple samples were pooled and scaled up 18-fold to provide sufficient material for a second round of immunoprecipitations with a second pull-down antibody. Cross-links were reversed with 0.2 M NaCl at 65°C for 5 h, and the DNA was purified by phenol-chloroform extraction. Promoter DNA precipitate was measured by QPCR and normalized to IgG and input DNA control samples.

Real-time QPCR
Total RNA was isolated with the Qiagen RNeasy Mini Kit. Equal amounts of cDNA were synthesized using a High Capacity Reverse Transcription kit (ThermoFisher). TaqMan primers were mixed with TaqMan Universal Master Mix II, with uracil N-glycosylase (ThermoFisher). 185 was amplified as an internal control. QPCR was performed using the Bio-Rad CFX connect system with TaqMan Primer Assays to detect 185 and target genes (ThermoFisher). Primer pair sequences used to detect input and immunoprecipitated DNA sequences for ChIP assay were 5’-CCTGAGAATTGATGGGGAAA and 5’-GTGAACAGGTACCGCACAGA from -329 to -99 of the 5’-flanking RFX6 promoter and 5’-TGACCGGCTTGTCCTTAAAC and 5’-TGAATGACGGACGGACACTGGTA from -625 to -411 of the 5’-flanking mouse MCT1 promoter.

DNA transfection and promoter assays
NFAT-dependent promoter-reporter (pGL3-NFAT-Luc, Addgene plasmid #17870), Gluc-ON reporters for RFX6 (MPRM33989-PG04), and MCT1 (MPRM40340-PG04) promoters were obtained from GeneCopoeia. For NFAT-Luc assays, MIN6 cells were co-transfected with pGL3-NFAT-Luc and normalizing vector pRL-SV40 (Promega). Transfection of MIN6 cells was performed using Lipofectamine™ 3000 method. Cells were grown to 50%-60% confluence in 11 mM glucose and co-transfected with pGL3-NFAT-Luc. Transfection was performed in six-well plates. A total of 3 μg/μL DNA was mixed in P3000 Reagent and then incubated in Lipofectamine™ 3000 Reagent for 15 min at room temperature. DNA-lipid complex was added drop-wise to the cell culture for a final concentration of 2 μg DNA. Cells were incubated at 37°C and 5% CO2. After 24 h, the medium was replaced with 2 mL fresh medium and incubated for another 24 to 48 h. Conditioned medium was collected and assayed for alkaline phosphatase activity using the Secreted Pair Dual Luminescence Assay Kit (GeneCopoeia) to normalize for RFX6 and MCT1 promoter-reporter transfection. Cells were then lysed in passive lysis buffer, and Firefly and Renilla luminescence were measured with a dual luciferase assay kit (Promega). Luminescence was measured using the Cytation5 Cell Imaging Multi-Mode Reader (BioTek).

Calcium assay
MIN6 cells were seeded at 100,000 cells/well in a 96-well flat, clear bottom, black-walled plate and treatments were applied at staggered time points to end at times to perform all calcium measurements simultaneously. On the day of the assay, cells were incubated in low glucose (1.1 mM) HEPES-buffered Krebs-Ringer bicarbonate solution (KRBH) for 30 min, and then probenecid was loaded in high glucose.
(16.7 mM) KRBH for 15 min. Buffer containing Fluo-4 (ThermoFisher) dye was added and cells incubated for 1 h at 37°C. Buffer was replaced and 60 mM KCl was auto-injected and read by the Cytation5 Cell Imaging Multi-Mode Reader (BioTek).

Insulin release studies
Islet cells were size-matched and seeded 10/well in 24-well plates. Cells were allowed to rest in culture medium during treatments including control RPMI media at 5.5 mM glucose or stimulation treatments of 16.7 mM glucose RPMI + 50 ng/mL IL-1β. Media was replaced with 300 μL of KRBH containing bovine serum albumin (BSA) and 1 mM glucose. Cells were incubated for 1 h at 37°C and then the buffer was replaced with 300 μL low glucose (3 mM) and incubated for another hour. After 1 h, the buffer was saved for testing and then replaced with high glucose (16.7 mM) buffer. Separate pyruvate stimulation of insulin was set up similarly. Buffer was replaced with 300 μL low glucose for 1 h and replaced by low glucose +20 mM sodium pyruvate for an hour. After incubations, the supernatant was removed and stored at −20°C for analysis by ALPCO Insulin enzyme-linked immunosorbent assay. Stimulation indexes were calculated as the ratio between insulin secreted at high glucose versus low glucose.

QUANTIFICATION AND STATISTICAL ANALYSIS
Graph Pad (version 9.0) was used to create graph 6A. Enrichr was used to create graphs 1B and 2A-C. Flow cytometry data were analyzed using FlowJo software, version 10.7.2. Statistical details of experiments can be found in the figure legends. All data are presented as mean ± standard error of the mean. p value <0.05 were considered statistically significant.