Regulation of STAT1 signalling in human pancreatic β-cells by the lysine deacetylase, HDAC6; a new therapeutic opportunity in type 1 diabetes?

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Abstract

Type 1 diabetes arises from the selective destruction of pancreatic β-cells by autoimmune mechanisms and intracellular pathways driven by Janus (JAK)-kinase mediated STAT isoforms (especially STAT1 & STAT2) are implicated as mediators of β-cell demise. Despite this, the molecular mechanisms that regulate JAK-STAT signalling in β-cells during the autoimmune attack remain only partially disclosed and the factors acting to antagonise pro-inflammatory STAT1 signalling are uncertain. We have recently implicated Signal Regulatory Protein (SIRP)-α in promoting β-cell viability in the face of ongoing islet autoimmunity and now reveal that this protein controls the availability of a cytosolic lysine deacetylase, HDAC6, whose activity regulates the phosphorylation and activation of STAT1. We provide evidence that STAT1 serves as a substrate for HDAC6 in β-cells and that sequestration of HDAC6 by SIRPα in response to anti-inflammatory cytokines (such as interleukin-13) leads to increased STAT1 acetylation. This then impairs the ability of STAT1 to promote gene transcription in response to pro-inflammatory cytokines including interferon-gamma (IFNγ). We further find that SIRPα is lost from the β-cells of subjects with recent-onset type 1 diabetes under conditions when HDAC6 is retained and STAT1 levels are increased. On this basis, we report a previously unrecognised role for cytokine-induced regulation of STAT1 acetylation in the control of β-cell viability and propose that targeted inhibition of HDAC6 activity may represent a novel therapeutic modality to promote β-cell viability in the face of active islet autoimmunity.

Article Highlights

- Signal regulatory protein-alpha (SIRPα) is present in human islet cells but its levels decline in β-cells in type 1 diabetes.
- Decreases in SIRPα expression are associated with a reduction in the viability of cultured β-cells.

- Immunoprecipitation of β-cell SIRPα reveals a direct interaction with the lysine deacetylase, HDAC6.
- Sequestration of HDAC6 by SIRPα results in increased acetylation, reduced phosphorylation and impaired activation of STAT1 during exposure of β-cells to IFNγ.
- Pharmacological targeting of HDAC6 might yield improvements in β-cell viability during progression to type 1 diabetes.

Introduction

The molecular events that lead to selective β-cell loss in type 1 diabetes (T1D) involve a diversity of immune mechanisms, environmental factors and genetic predisposition (1-4). These culminate in the development of autoimmunity against key islet antigens and ultimately lead to β-cell loss mediated by immune cells (notably CD8+ cytotoxic T-cells) which infiltrate pancreatic islets (5-7). Although direct immune cell-endocrine cell contact may contribute to the attack, the majority of infiltrating CD8+ T-cells are found at the periphery of islets implying that cytokine secretion and remote activation of pro-apoptotic pathways are also likely to be important (5-8). Interferons are among the most critical cytokines which impinge on β-cells to impair their function and viability in type 1 diabetes (9-14). These agents interact with specific cell surface receptors to initiate signalling events which lead to the activation of specific Signal Transducer and Activators of Transcription (STAT) molecules, especially STAT1 and STAT2 (12). These, in turn, drive gene transcriptional changes which result in the hyperexpression of canonical HLA class I proteins (HLA-A,B,C) on β-cells to exacerbate CD8+ T-cell cytotoxicity; as well as increases in various pro-apoptotic proteins which enhance the vulnerability of the cells to cytotoxic insults (14-21).

In the face of this increasingly intense immune-mediated assault, it has become clear that β-cells do not simply adopt the stance of silent bystanders; rather, they mount various anti-inflammatory responses designed to repel and restrict the cytotoxic onslaught (3; 13). These include the upregulation of non-classical HLA class I isoforms (e.g. HLA-E, F, G) and PDL-1 which may attenuate cell-mediated toxicity (10; 17; 20). They can also respond by the engagement of anti-inflammatory signalling pathways activated by additional cytokines that may be present in the islet milieu, such as IL-4 and IL-13 (22-25). These actively promote β-cell viability by regulation of an alternative STAT isoform, STAT6, whose transcriptional activity opposes the actions of STAT1 and STAT2 (23; 24). However, the activity of this

STAT6-dependent mechanism may be down-regulated in type 1 diabetes and the final outcome for any given β-cell will then reflect the net balance of the competing activities of the various pro- vs anti-apoptotic pathways (22).

We are studying the molecular events that derive from anti-inflammatory cytokine signalling in β-cells and have identified a pathway involving the STAT6-mediated upregulation of signal regulatory protein-α (SIRPα) as an important means by which the pro-inflammatory actions of STAT1 are attenuated (22). SIRPα is primarily considered to play an important role in the recognition of "self" during immune regulation but we, and others, have found that it is also involved in a more direct attenuation of cytotoxicity in β-cells (24; 26; 27). In the present work, we provide evidence that SIRPα recruits a cytosolic lysine deacetylase enzyme, HDAC6, as a principal component of this mechanism. We show that STAT1 is a substrate for HDAC6 in β-cells and that failure to deacetylate STAT1 impairs its ability to promote gene transcription in response to interferons. We propose that targeting of HDAC6 may offer a novel therapeutic route to promote β-cell viability in the face of developing islet autoimmunity.

Research Design & Methods

Imaging studies

Samples of formalin-fixed paraffin-embedded (FFPE) pancreas from three subjects with recent-onset type 1 diabetes and three controls, matched for age and gender, were selected from the Exeter Archival Diabetes Biobank (www.pancreatlas.com; (ESM Table 1)) and used with full ethical approval (West of Scotland REC:15/W/0258). Immunoperoxidase staining was used for detection of single antigens (28) while fluorescently labelled antisera were used for detection of multiple antigens. Labelled sections were scanned using a Vectra® Polaris™ (VP) slide scanner (Akoya Biosciences). Autofluorescence was subtracted using InFORM software and immunopositivity analysed using the Indica HALO image analysis platform. Statistical analysis was conducted in Rstudio version R4.3.1.

Culture of EndoC-βH1 cells and human islets

Human EndoC-βH1 β-cells derived from tissues of undefined gender (29) were obtained from Prof Rapheal Scharfmann and cultured on ECM and fibronectin at an initial confluency of 5 x 10^4 cells/cm^2. Cells were free from Mycoplasma as judged by routine testing with MycoAlert (Lonza, Basel, Switzerland).

Isolated human islets from organ donors without diabetes were kindly provided from the Oxford Centre for islet transplantation and were cultured in DMEM medium (Lonza, Basel, Switzerland) supplemented with 5% FBS, L-glutamine (2mM) and antibiotics.

RNA interference in EndoC-βH1 cells

Small interference RNAs (siRNA) from the Silencer Select range (ThermoFisher) were exploited for targeted knockdown of transcripts encoding human SIRPα and HDAC6. Controls were transfected with scrambled siRNA. Cells were transfected with 10 nmol of each siRNA using Optimem medium (ThermoFisher) and lipofectamine RNAi Max transfection reagent (Invitrogen, Boston, MA, USA). Following transfection, cells were cultured for 48 h prior to exposure to test reagents (22).

Luciferase reporter assays

EndoC-βH1 cells plated at 2 x 10^5 cells/well in 24 well plates were transfected with siRNA and the Cignal Reporter Assay kit (Qiagen, USA) containing a Gamma Associated Sequence (GAS) promoter, in parallel with either SIRPα or HDAC6 24 h. After transfection, cells were treated with IFNγ for a further 16 h and luciferase activity was then measured using a Dual Luciferase Reporter Assay system (Promega). Empty vector constructs were used as controls.

Western blotting and immunoprecipitation

EndoC-βH1 cells or human islets were harvested, lysed and proteins electrophoresed then transferred to PVDF membranes for Western blotting as described (24). Immunoreactive proteins were detected using CDP-star chemiluminescent substrate (Merck) and Licor C-digit. In the case of fluorescently labelled secondary antibodies, detection was achieved with the Licor Odyssey system (Licor, Cambridge, UK). Quantitative Western blot analysis was performed using the Licor's Image Studio version 5.2, after normalisation of expression to either α-tubulin or GAPDH.

Co-immunoprecipitation experiments were performed with cell extracts obtained by lysis in 50 mmol/L Tris (pH 8) buffer containing 150 mmol/L NaCl; 1 mmol/L EDTA, 1 mM EGTA and 1% (v/v) Triton-X100 supplemented with protease and phosphatase inhibitors. Lysates were incubated with relevant primary antibodies overnight at 4^0C. Protein G Sepharose beads were added for 4 h at 4^0C and elution of immunoprecipitated proteins was achieved with LDS sample buffer containing 10% (v/v) β-mercaptoethanol at 70°C for 10 min. Immunoprecipitates were probed for target antigens using Western blotting. Where SIRPα was detected after immunoprecipitation, pulldown was achieved with an antibody from Sinobiological and blots were probed with a second antibody from Cell Signalling (ESM Table 2).

Mass spectrometric analysis of immunoprecipitated proteins

Mass spectrometry analysis was outsourced to the Proteomics Facility, University of Bristol, UK, and performed as described in ESM Methods (30).

Cell viability assays

Cell viability was assessed using propidium iodide (Merck). Each experimental condition was performed with at least six technical replicates and a minimum of three biological repeats (31).

Statistics

Data were analysed using either Graphpad Prism version 9.4.1 or RStudio version R4.3.1 and results expressed as mean values ± SEM unless otherwise specified. Sex of subjects was not considered as a separate variable. Statistical significance between mean values was assessed using either an unpaired Student's t test or ANOVA (with post hoc Tukey's test).

Data and Resource Availability

All data are available from the corresponding authors on request

Results

SIRPα is expressed in human β-cells but is lost in T1D

In previous work, we provided firm evidence that SIRPα is increased in expression when human β-cells are exposed to IL-13 (22). However, it has been argued that SIRPα is not an abundant constituent of the human β-cell proteome under control conditions since a recent study failed to detect it by Western blotting or immunohistochemistry in human islet cells (32). To address this discrepancy, we re-examined SIRPα expression in human pancreas sections from control subjects using two different antisera, including that employed in the recent negative report (32). Immunohistochemical staining revealed a strongly positive signal in islets immunostained with either of two different anti-SIRPα reagents (obtained from Abcam and Origene respectively (Figure 1A)). Co-staining with anti-insulin confirmed a β-cell localisation for SIRPα (Figure 1B). We also verified that SIRPα can be immunoprecipitated from isolated human islets (Figure 1C) and is present in the human β-cell line EndoC-βH1 cells by Western blotting (22) and mass spectrometry (see below)

SIRPα expression is not restricted solely to β-cells since it was also co-localised with anti-glucagon staining in α-cells (Figure 2A). Quantification revealed that SIRPα is more highly expressed by human β- than α-cells in healthy donor pancreas (Figure 2B). However, in

subjects with recent-onset type 1 diabetes, a significant decline in SIRPα intensity was noted in both α- and β-cells (Figure 2C) relative to controls. Targeted knock down of SIRPα in EndoC-βH1 cells was associated with a significant increase in apoptosis (ESM Figure 1) corroborating our previous conclusions in rodent β-cells (22).

SIRPα knockdown increases the activity of STAT1 in EndoC-βH1 cells

To explore this phenomenon further, experiments were performed to investigate the status of the STAT1 signalling pathway under conditions of SIRPα knockdown in EndoC-βH1 cells. Knockdown of SIRPα with siRNA typically led to a ~70% reduction in protein intensity on Western blots (not shown). Measurement of transcriptional activity using a luciferase reporter construct under the control of a STAT1-responsive GAS promoter revealed a significant increase in activity when SIRPα levels were attenuated (ESM Figure 2). Conversely, over-expression of SIRPα led to a marked reduction in IFNγ-induced reporter activity (ESM Figure 2). These data imply that the presence of SIRPα acts to limit the extent of STAT1 activation both under control conditions and in response to IFNγ.

Sequestration of STAT1 by SIRPα in EndoC βH1 and human islets

Having discovered that depletion of SIRPα leads to increased STAT1 activity whereas overexpression attenuates the increase in STAT1 activity caused by IFNγ, we next considered the possibility that a key role for SIRPα might be to sequester STAT1 as a means to minimise its availability for activation. Accordingly, SIRPα was immunoprecipitated and the products subjected to mass spectrometric analysis (LCMS). LCMS analysis revealed that SIRPα does, indeed, interact with STAT1 but it was also clear that peptides arising from fragmentation of this transcription factor did not form the major components of the interaction complex (ESM Table 3). Unexpectedly, the most abundant peptides were derived from a cytosolic lysine

deacetylase, HDAC6 since the Sequest HT sequence score for identified peptides was 74.9 for HDAC6 but only 6.8 for STAT1 (ESM Table 3). We were able to confirm by Western blotting that both HDAC6 and STAT1 are found in the immunoprecipitates of lysates prepared from EndoC-βH1 β cells (Figure 3 A-C) or human islets in the absence or in the presence of IL-13 (Figure 3 D-F). Indeed, the extent of complex formation was enhanced by exposure to IL-13.

Inhibition of HDAC6 reduces β cell death induced by pro-inflammatory cytokines

Given the unexpected finding that HDAC6 appears to be a primary target for sequestration by SIRPα, and that loss of SIRPα leads to enhanced STAT1 activity, we considered the possibility that STAT1 might be under the control of HDAC6 in β-cells. This hypothesis was tested initially with a selective small molecule inhibitor of HDAC6, BRD9757 (33), which binds directly to the catalytic domain of the enzyme and is highly selective for HDAC6 relative to other HDAC isoforms.

The actions of BRD9757 were verified by studying the extent of tubulin acetylation in EndoC-βH1 cells since this protein is known to be subject to control by acetylation and to serve as a substrate for HDAC6 in other cells (33; 34). EndoC-βH1 cells were treated with BRD9757 overnight at increasing concentrations, and then exposed to IFNγ for 30 min (Figure 4A). Western blot analysis (Figure 4A; confirmed by densitometry (ESM Figure 4)) revealed a dose-dependent increase in acetylated tubulin under these conditions, consistent with HDAC6 inhibition. Interestingly, parallel studies in which the cells were exposed to IL-13 in the absence of the HDAC6 inhibitor also revealed a very early (30 min) increase in the acetylation of tubulin which persisted for up to 24 h, implying that IL-13 may directly promote a rapid loss of HDAC6 activity to enhance protein acetylation (Figure 4B).

Having confirmed that BRD9757 acts to inhibit HDAC6, EndoC-βH1 cells were treated with 1 μM BRD9757 overnight and the medium replaced to include a cocktail of pro-inflammatory cytokines for 48 h before assessment of the extent of cell death. Treatment of cells with BRD9757 alone did not alter their viability but the pro-inflammatory cytokines induced a significant increase in β cell death (Figure 4C). Strikingly, the HDAC6 inhibitor attenuated significantly the detrimental effects of pro-inflammatory cytokines. BRD9757 was similarly protective to rat INS-1E cells exposed to a pro-inflammatory cytokine cocktail (ESM Figure 3). Importantly, targeted knockdown of HDAC6 using siRNA also yielded significant cytoprotection in cytokine exposed EndoC-βH1 cells (Figures 4D).

HDAC6 inhibitor reduces the activity of STAT1

To evaluate the effects of HDAC6 on STAT1 activity more directly, reporter assays were performed in EndoC-βH1 cells and it was confirmed that either knockdown of HDAC6 or exposure to BRD9757 significantly reduced IFNγ-induced STAT1 reporter gene activity (Figure 5A,B). BRD9757 also caused a marked reduction in IFNγ-induced phosphorylation at residue Y701 (Figure 5C) implying that variations in the activity of HDAC6 may influence the extent of STAT1 phosphorylation and thereby, its activity, in β-cells. To ensure that this response was not simply due to the use of a reporter construct to measure STAT1 activity, the induction of the downstream IFNγ–induced gene, *MX1*, was assessed and found to be attenuated dramatically by knockdown of HDAC6 (Figure 5D). The longer-term induction of STAT1 characteristically seen under such conditions (12) was also attenuated (Figure 5E).

Finally, experiments were undertaken to confirm, more directly, that STAT1 is subject to acetylation in β-cells and that this response is influenced by the activity of HDAC6. Cells were treated overnight with BRD9757 followed by IFNγ treatment for 30 mins. STAT1 was then

immunoprecipitated and the products run on Western blots prior to probing with an antibody directed against acetylated lysine residues. STAT1 was clearly immunodetected under control conditions suggesting that it is acetylated and the extent of this acetylation was reduced markedly following exposure to IFNγ stimulation, consistent with enhanced HDAC6 activity under these conditions (Figure 6). In support of this, the IFNγ-induced reduction in STAT1 acetylation was attenuated in the presence of BRD9757. To verify that acetylation of STAT1 is associated with an attenuation of its phosphorylation, samples were probed, in parallel, for acetylated or tyrosine-phosphorylated STAT1 after exposure of cells to IFNγ in the absence or presence of BRD9757 (ESM Figure 5). The results confirmed the prediction that enhanced acetylation of STAT1 led to a reduction in IFNγ-induced phosphorylation.

Since the data imply that the activity of STAT1 is regulated by HDAC6 and that deacetylation is associated with enhanced STAT1 signalling, we examined HDAC6 levels in the islets of subjects with type 1 diabetes. Total STAT1 levels are increased under such conditions (19) and we now find that this is associated with the retention of HDAC6 (Figure 7). Given that β-cell SIRPα expression is reduced in type 1 diabetes (Figure 1) and that SIRPα recruits and inhibits HDAC6; relief of this inhibition is likely to contribute to the deacetylation of STAT1. This, in turn, will promote its full activation when interferon levels are increased.

Discussion

In earlier work, we established that the cytoprotective actions of anti-inflammatory cytokines (IL-13 and IL-4) in pancreatic β-cells involve the upregulation of an immunoregulatory protein, SIRPα (22; 24). Gene expression array analysis indicated a large-scale proportional up-regulation of SIRPα in response to IL-13 in β-cells but the apparent extent of this increase is magnified because of the low basal expression of the gene. Given that gene expression profiles often provide only a poorly accurate estimate of protein levels (and noting that a recent study

failed to detect SIRPα in human islet cells (32)), we considered it imperative to verify its expression in human pancreatic islets at the protein level. This goal was achieved using two independent antisera which allowed the ready detection of SIRPα in islet endocrine cells of the human pancreas using immunohistochemical approaches. Secondly, by employing lysates of freshly isolated human islets we were able to selectively immunoprecipitate SIRPα with one target antiserum and to detect it by Western blotting of the immunoprecipitate with a second, directed against a different epitope. Thirdly, mass spectrometric analysis of the SIRPα immunoprecipitate recovered from human EndoC-βH1 cells lysates provided direct and independent chemical confirmation that the protein is present. When coupled with our consistent ability to detect immunoreactive SIRPα in EndoC-βH1 cells, these studies provide firm evidence that, although transcriptomic data imply relatively low basal expression of the SIRPα gene in islet cells, the protein product is present. Moreover, as shown previously using isolated human islets, its levels are increased markedly by exposure to IL-13 (22).

Against this background, and in the light of our previous demonstration that targeted knockdown of SIRPα leads to a net loss of β-cell viability, these data imply that SIRPα forms a component of a system regulating the viability of these cells (22). Building on this, we now demonstrate that SIRPα protein levels are diminished in the residual β-cells of subjects with recent-onset type 1 diabetes (Figure 1) suggesting that this loss may contribute to the increased susceptibility of these cells to the pro-apoptotic actions of inflammatory mediators during the development of type 1 diabetes. When coupled with our previous evidence that STAT6 levels are also depleted from β-cells in type 1 diabetes and that STAT6 activation drives SIRPα expression (22), a decrease in STAT6 levels may provide an explanation for the decline in SIRPα.

To evaluate the validity of this conclusion and to understand the functional role of SIRPα in β cells, we monitored the activity of a STAT1-responsive reporter construct in transfected

EndoC-βH1 cells and noted that selective knockdown of SIRPα leads to an increase in STAT1 reporter activity while overexpression of SIRPα causes an attenuation of IFNγ-induced STAT1 activity. This implies that one function of SIRPα is to restrain STAT1 signalling in β-cells. Thus, these results suggest that absolute SIRPα protein levels are titrated in β-cells to modulate signalling pathways regulating cell viability.

To gain a more complete understanding of the molecular basis of these actions, we considered the possibility that SIRPα might act as a sink to sequester STAT1 to prevent its activation. Credence was lent to this possibility by the results of immunoprecipitation experiments which revealed that SIRPα can interact directly with STAT1 in EndoC-βH1-cells and mass-spectrometric analysis provided unequivocal verification of the presence of STAT1 in the SIRPα immunoprecipitates. However, application of this technology revealed that a previously unanticipated protein ligand, HDAC6, was also present in the SIRPα immunoprecipitates. Indeed, HDAC6 appeared to be the more abundant binding partner for endogenous SIRPα in terms of peptide sequences identified in the mass spectrometry. Strikingly, we also obtained evidence that HDAC6 was recruited to the SIRPα complex in greatest amounts when the cells were exposed to IL-13. Thus, we are drawn to the conclusion that HDAC6, rather than STAT1, may be a key binding partner for SIRPα in pancreatic β-cells; especially in cells exposed to IL-13.

Under the conditions of our experiments, the sequestration of HDAC6 by SIRPα in cells exposed to IL-13, occurred more rapidly than would be expected if gene expression changes were involved. Thus, we postulate the existence of two, distinct, actions of IL-13 in β-cells which are likely to contribute to its cytoprotective actions. The first occurs rapidly and involves the early sequestration of HDAC6 by SIRPα. This is followed, over a longer time course, by an increase in total SIRPα levels mediated by a STAT6-responsive increase in gene

transcription. Both actions contribute to the attenuation of pro-inflammatory signalling mediated via STAT1.

Since STAT1 and HDAC6 can each be found in a complex with SIRPα in β-cells, this raises the possibility that SIRPα might act as a signalling hub to coordinate the interactions of HDAC6 and STAT1 in these cells. However, given the apparently different stoichiometries of HDAC6 and STAT1 when present in complex with SIRPα, we consider this improbable. Rather, we propose that the sequestration of HDAC6 by SIRPα is the more significant mechanistic interaction and that attendant changes in the ability of HDAC6 to deacetylate target proteins at relevant lysine residues is likely to be the critical factor. Thus, we envisage that, in response to IL-13, SIRPα recruits HDAC6 to the plasma membrane and thereby limits its interaction with target substrates present in the cytoplasm. This model is illustrated diagrammatically in Figure 8. A primarily cytoplasmic localisation for the principal HDAC6 substrates is consistent with the known subcellular distribution of HDAC6 because, despite its moniker as a "histone"-deacetylase, the enzyme is understood to be mainly cytosolic in distribution and to control the extent of acetylation of cytoplasmic proteins (35). In support of this, we found that selective inhibition of HDAC6 using the inhibitor BRD9757, led to a marked rise in tubulin acetylation and that addition of IL-13 also caused a dramatic, early, increase in tubulin acetylation in EndoC-βH1 cells. This is consistent with the induction of a rapid sequestration of cytoplasmic HDAC6 following exposure of cells to IL-13.

Strikingly, we observed a second effect of BRD9757 in that, in parallel with its action to promote tubulin acetylation in β-cells, it reduced the extent of tyrosine phosphorylation of STAT1 in cells exposed to IFNγ. This raises the possibility that STAT1 may also be a bona fide endogenous target for the activity of HDAC6 and implies that the degree of acetylation might influence its propensity for phosphorylation in response to IFNγ. STAT1 is reported to be subject to acetylation in certain other cells and we have verified this in β-cells by Western

blotting of STAT1 immunoprecipitates with an antiserum directed against anti-acetylated lysine residues (36; 37). Indeed, we show that STAT1 acetylation is lost rapidly upon exposure of cells to IFNγ and that, under conditions when STAT1 acetylation is enhanced by inhibition of HDAC6, STAT1 phosphorylation in response to IFNγ is decreased (ESM Figure 5). We hypothesise, therefore, that dynamic regulation of acetylation (by the opposing actions of protein acetylases and HDAC6) may impose a specific level of regulation on the STAT1 pathway whereby the extent of acetylation of STAT1 dictates the potential for IFNγ-induced transcriptional activity (Figure 8). This mechanism has not been reported previously in β-cells but has been suggested from studies in other cell types (37; 38). Consistent with this proposal, we show that BRD9757 reduced significantly the induction of a STAT1 reporter luciferase construct in EndoC-βH1 cells and that a similar effect was evident in cells depleted of HDAC6 expression by siRNA-induced knockdown. This leads to the conclusion that an adequate availability of catalytically active HDAC6 is required to sustain STAT1-mediated gene expression in β-cells. This notion was further verified by our demonstration that induction of the mRNA encoding MX1, a key STAT1-responsive transcript in β-cells, was dramatically inhibited following HDAC6 knockdown in IFNγ-treated cells. More strikingly still, we observed that the capacity of a combination of pro-inflammatory cytokines to cause the loss of β-cell viability was also compromised upon addition of BRD9757.

When considered in parallel with our evidence that SIRPα is lost from the islets of people with recent onset type 1 diabetes and that this correlates with a rise in STAT1 levels and retention of HDAC6, we propose that the activation of STAT1 is favoured under these conditions (Figure 8). This is likely to contribute to β-cell loss under the proinflammatory conditions prevailing in islets in type 1 diabetes and implies that targeting of HDAC6 with selective inhibitors may offer a novel therapeutic approach to slow β-cell loss. Since HDAC6 inhibitors are already in

development for use in other clinical conditions, consideration might be given to repurposing of such agents in type 1 diabetes, either alone or in parallel with JAK inhibitors which act upstream in the interferon regulated pathway (39; 40). It is noteworthy that recent clinical trials have reported the successful use of JAK inhibitors in type 1 diabetes and this raises the intriguing possibility that combination therapies involving HDAC inhibitors might prove even more efficacious (41; 42).

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Professor Noel Morgan and Dr Mark Russell are guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Contributions

KAL, SJR, NGM and MAR designed the research. KAL collected and analysed the majority of data. CL performed immunostaining of human pancreas tissue and analysed these data. NGM and KAL wrote the first draft of the manuscript. NGM, KAL, CL, SJR and MAR edited each subsequent draft, and approved the completed manuscript. NGM and MAR take responsibility for the accuracy and integrity of data and its presentation.

Conflicts of Interest

No potential conflicts of interest relevant to this paper were reported.

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Figure 1: SIRPα is expressed in human pancreatic islet cells.

(**A,B**) Representative images are from pancreas sections of individuals without diabetes. FFPE pancreas sections were immunostained to detect SIRPα using antibodies from two different suppliers (Abcam and Origene). The sections were stained using either immunoperoxidase **(A;** Abcam right panel; Origene – left panel**)** or immunofluorescences **(B)** protocols. In the immunofluorescence studies (**B**) cell nuclei were stained with DAPI (dark blue) and insulin was immunostained in cyan. Scale bar = 50 μm, SIRPα (green-Origene, red-Abcam), and insulin (light blue). (**C**) Western blot analysis was performed on protein lysates obtained from isolated human islets after immunoprecipitation with anti-SIRPα. The arrow and red box indicate the migration of the immunoreactive band corresponding to SIRPα. H and L are used to denote the migration of IgG Heavy and Light chains respectively.

Figure 2: SIRPα expression is depleted in pancreatic β cells from people with T1D.

(A) Representative immunofluorescence micrographs were imaged after immunostaining of human pancreas sections from a control individual (upper panels) or a subject with recent-onset type 1 diabetes (lower panels). Sections were incubated with antisera raised against SIRPα (green), glucagon (red) or insulin (cyan) while nuclei were stained with DAPI (blue). The arrows in the lower panel indicate a β-cell lacking SIRPα expression (cyan arrows) and an α-cell with retained SIRPα (red arrows). **(B & C)** Quantification of the mean fluorescence intensity (MFI) of SIRPα immunostaining in human β- and α- cells in sections of human pancreas stained as in (**A**). Labelling of SIRPα was quantified in both β and α cells in subjects without diabetes or those with type 1 diabetes. Data are representative of 3 control subjects and 3 with type 1 diabetes. ****$p<0.0001$

Figure 3: SIRPα interacts with STAT1 and HDAC6 in EndoC-βH1 cells and human islets. SIRPα was immunoprecipitated from lysates of EndoC-βH1 cells and the extracts analysed by mass spectrometry. Proteins were identified by peptide sequence analysis. **(A, B, C)** The identity of HDAC6 and STAT1 as partner proteins interacting with immunoprecipitated SIRPα in lysates of EndoC-βH1 cells was confirmed by Western blotting with antisera raised against SIRPα itself (**A**) HDAC6 (**B**) or STAT1 (**C**) respectively. (**D-F**) Alternatively, isolated human islets were cultured for 24 h after arrival in the laboratory then treated with either vehicle or IL-13 (20 ng/ml) for 60 min. SIRPα was immunoprecipitated after islet cell lysis and binding partners were identified by Western blotting of the immunoprecipitates with antisera raised against SIRPα itself (**D**) HDAC6 € or STAT1 (**F**) respectively. In all cases the specificity of labelling was verified by incubation of samples with non-immune serum (IgG) as shown. The arrows and red boxes indicate the migration of the immunoreactive band corresponding to the relevant protein. Blots are representative of 3 independent experiments.

Figure 4: Inhibition of, or knockdown of, HDAC6 protects EndoC-βH1 cells from proinflammatory cytokine-induced toxicity.

(A) EndoC-βH1 cells were treated with increasing concentrations (0-10 μM) of BRD9757 overnight followed by 30 min IFNγ (20 ng/ml) stimulation. Lysates were extracted and Western

blots were performed and probed with antisera against acetylated-tubulin with GAPDH as a loading control. Densitometric analysis of these blots is shown in ESM Figure 4. **(B)** EndoC-βH1 cells were treated with 20 ng/mL IL-13 for up to 48 h and then lysed before Western blotting to detect either acetylated-tubulin or total tubulin (as a loading control) with specific antisera. Blots are representative of 3 independent experiments. **(C, D)** The viability of EndoC-βH1 cells was examined following inhibition of HDAC6 activity in two ways; (**C**) cells were either incubated with 1 μM of the selective HDAC6 inhibitor BRD9757 overnight, or (**D**) HDAC6 was knocked down by treatment of EndoC-βH1 cells with siRNA for 48 h. Control cells received either **(C**) vehicle or (**D**) scrambled siRNA. After this time, the cells were exposed for a further 48 h to either vehicle or a cocktail of proinflammatory cytokines treatment (IL1β, TNFα, IFNγ and IL6; each at 20 ng/ml) prior to measurement of cell viability by flow cytometry after propidium iodide staining. 48 h was established as a suitable time point to achieve cytokine-induced loss of viability in preliminary studies. Data are from 12 replicates across 3 independent experiments, $^{**}p<0.01$, $^{****}p<0.0001$,

Figure 5: STAT1 activity is attenuated by inhibition of, or knockdown of, HDAC6**.**

(A,B) EndoC-βH1 β cells were transfected with siRNA to knockdown HDAC6 or with BRD9757 (1 μM). Cells were transfected with a STAT1-responsive luciferase reporter and exposed to IFNγ (20 ng/ml) for 8 h prior to measurement of luciferase activity (n=12). $^{****}p<0.0001$. **(C)** EndoC-βH1 were treated with increasing concentrations of BRD9757 overnight followed by IFNγ (20 ng/ml for 30 min). Lysates were extracted and Western blots performed with antisera against total STAT1 and phospho-STAT1 (pSTAT1). α-Tubulin was also probed as an additional loading control. Image is representative of 3 independent experiments. **(D,E)** Expression of HDAC6 was reduced in EndoC-βH1 cells by transfection

with siRNA for 48 h and the cells were then exposed to IFNγ (20 ng/ml) overnight. Control cells received scrambled siRNA and vehicle. RNA was extracted and the expression of either *MX1* (**D**) or *STAT1* (**E**) examined by RT-qPCR. n=6 *p<0.05, ***p<0.001, ****p<0.0001.

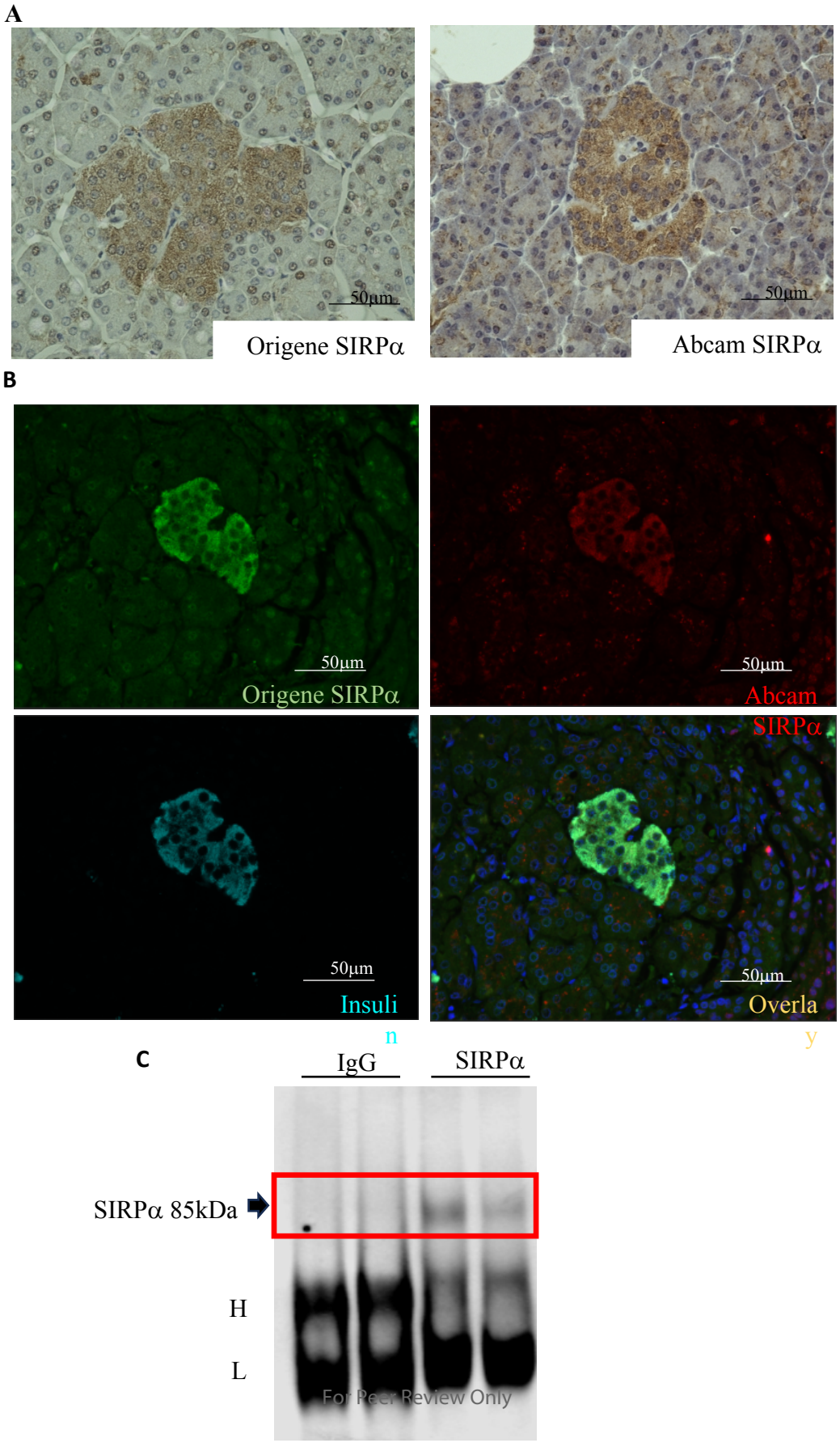
Figure 6: STAT1 is acetylated in an HDAC6-dependent manner in EndoC-βH1 cells. EndoC-βH1 cells were treated with the HDAC6 inhibitor (BRD9757 (1 μM) for 24h then exposed to either vehicle or IFNγ (20 ng/ml) for 30 min. The cells were lysed and proteins immunoprecipitated with anti-STAT1 antibody. Immunoprecipitates were probed by Western blotting for **(A)** STAT1 and **(B)** acetylated-lysine. The red boxes indicate the migration of the immunoreactive bands corresponding to the relevant proteins. (C) Densitometric analysis was performed on the blots to allow statistical evaluation. n=3, *p<0.05; **p<0.01

Figure 7. HDAC6 is expressed in human islet cells in control subjects and is retained in type 1 diabetes. Representative micrographs of pancreatic islets from 3 individuals without (ND; upper panels; i-iii)) and 3 with type 1 diabetes (T1D; lower panels; iv-vi)) stained with anti-HDAC6 and detected immunohistochemically. Whole slide scans were imaged at x40. Scale bars = 100 μm.

Figure 8. Model illustrating the regulation of STAT1 signalling by HDAC6 in β-cells**.** (**Left panel)** Under conditions when HDAC6 is available within the cytosol of β-cells, STAT1 acetylation is minimised such that signalling via the IFNγ receptor leads to JAK kinase activation and thence to phosphorylation and transcriptional activation of STAT1. By contrast (**right panel**), elevation of SIRPα (e.g in response to IL-13) leads to increased sequestration of HDAC6 and, as a result, to enhanced STAT1 acetylation. Increased acetylation is accompanied

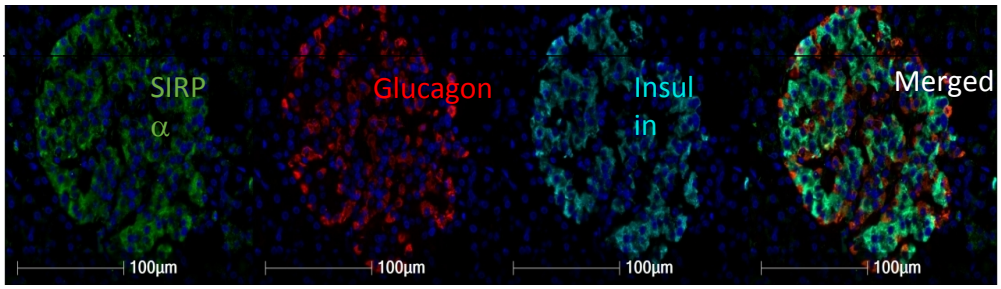
by a concomitant reduction in the propensity for phosphorylation of STAT1 by JAK kinases culminating in attenuation of its transcriptional activity.

Figure 1

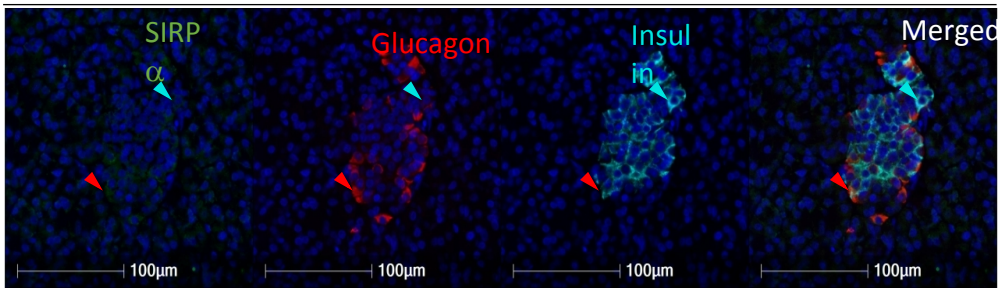


A

No Diabetes



Type 1 diabetes



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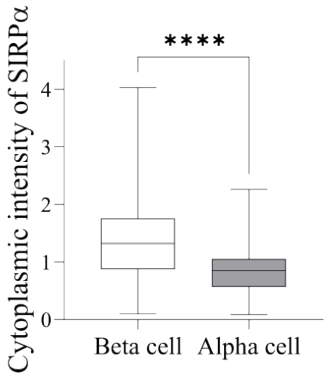

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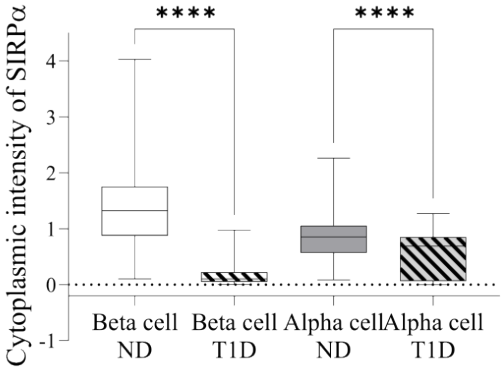


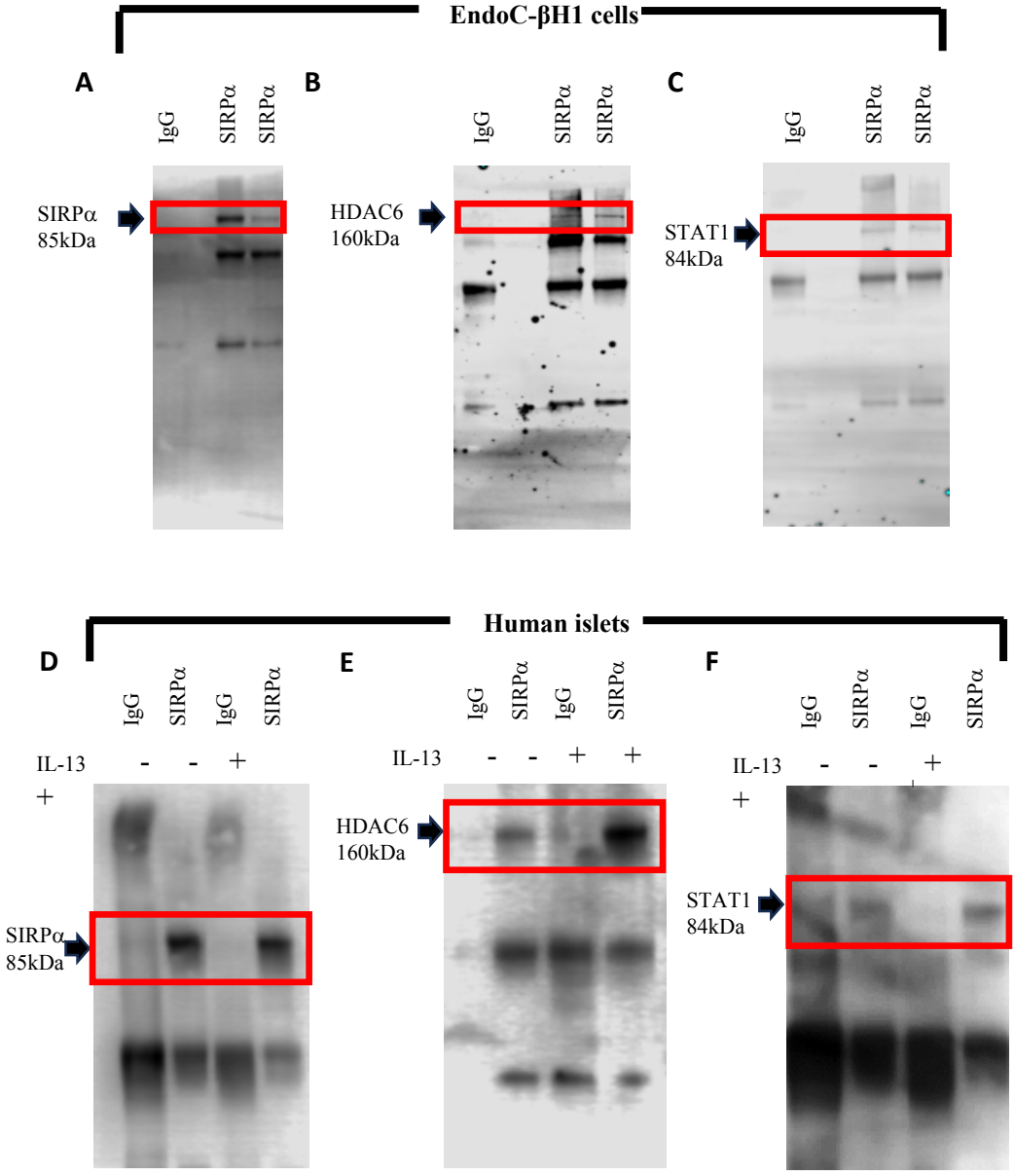
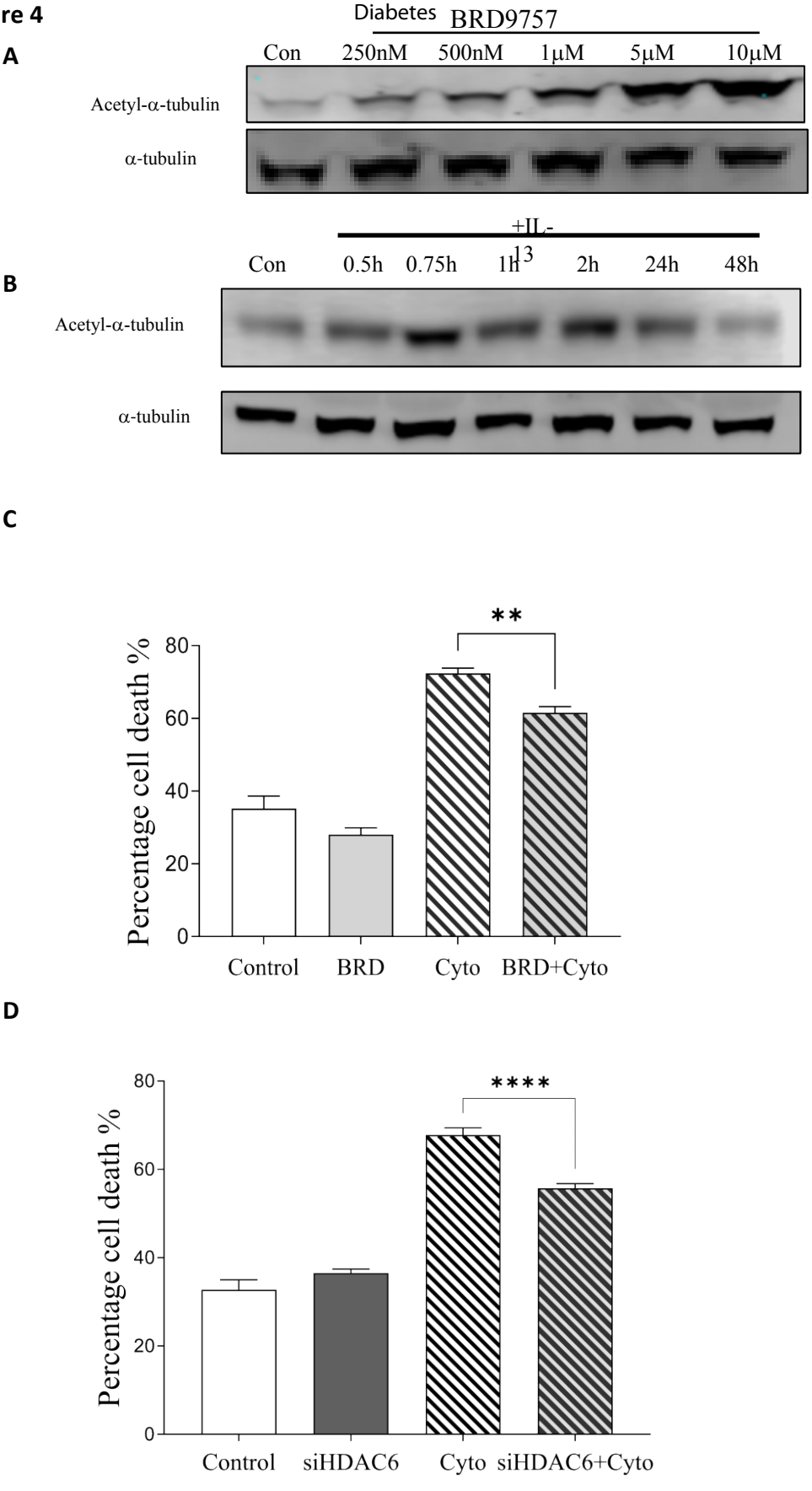


Figure 4



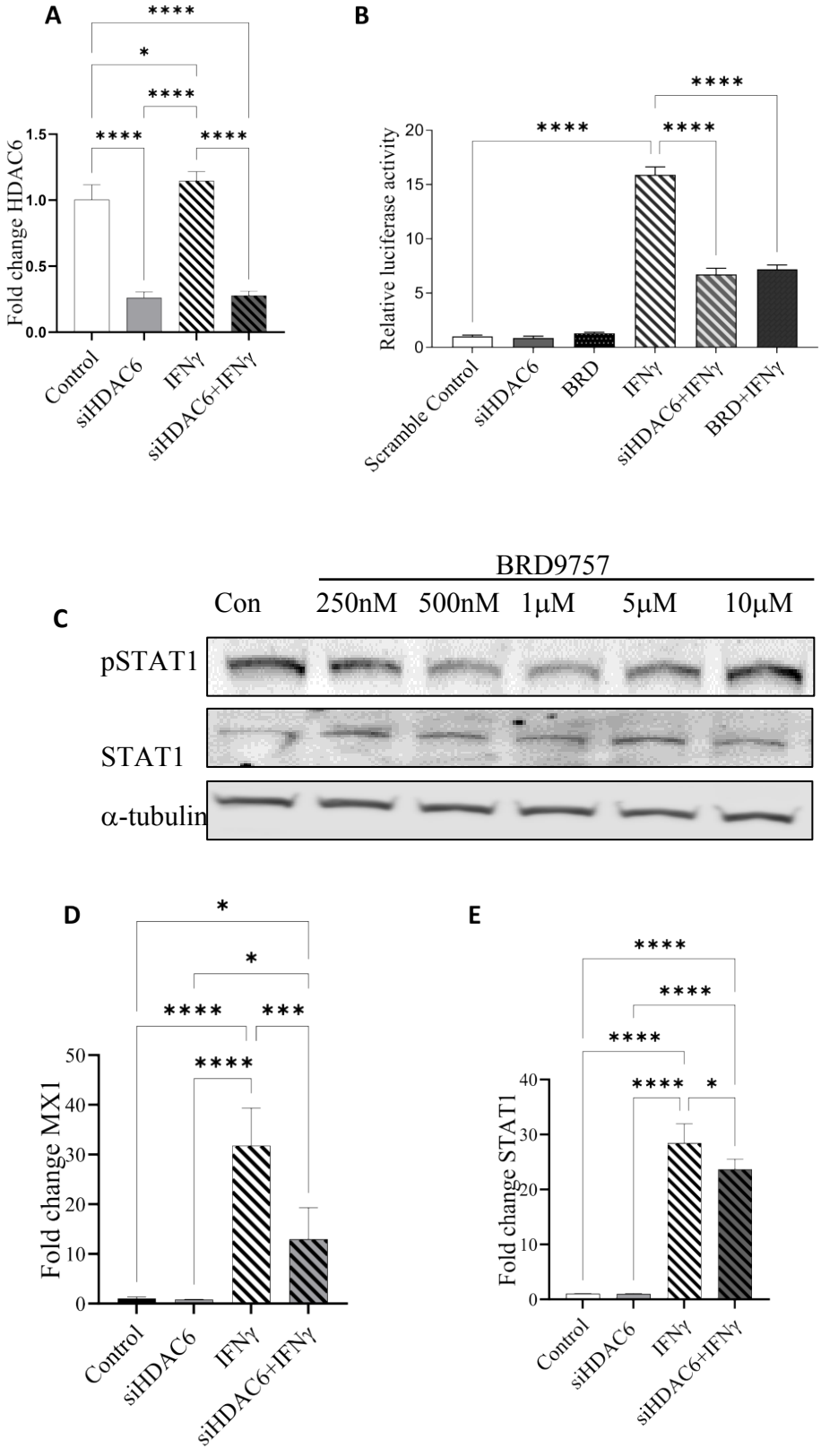
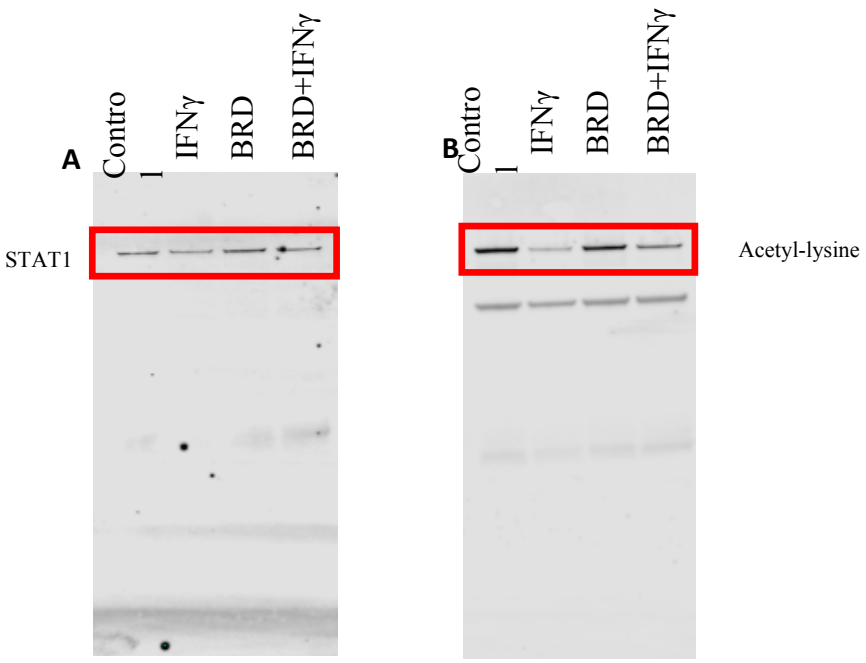
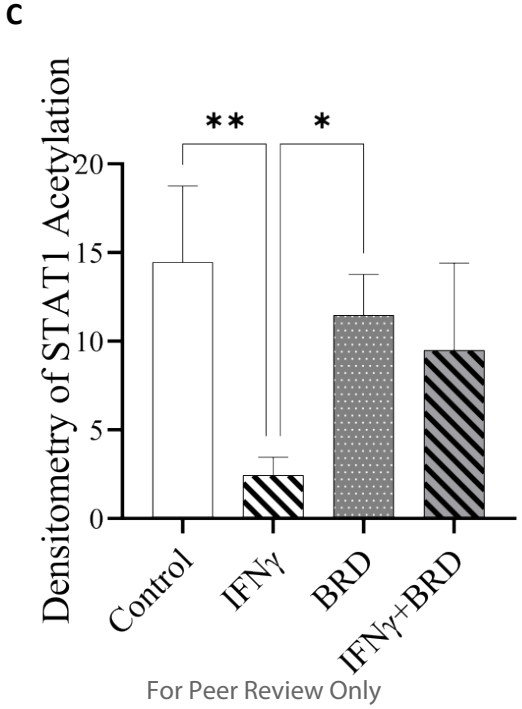
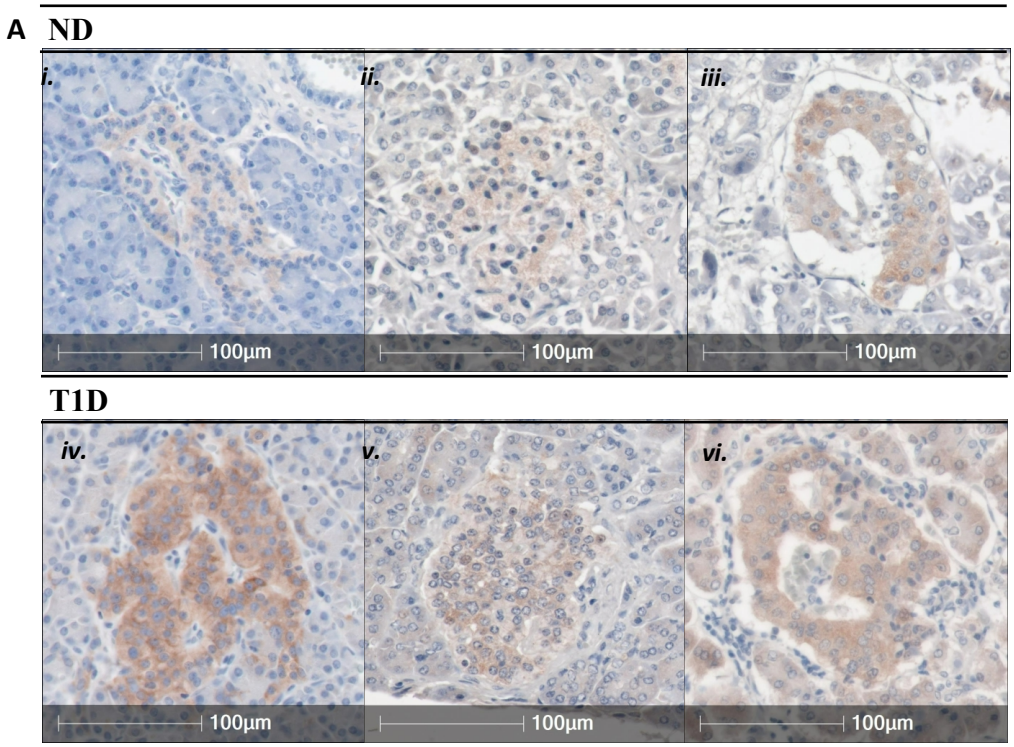
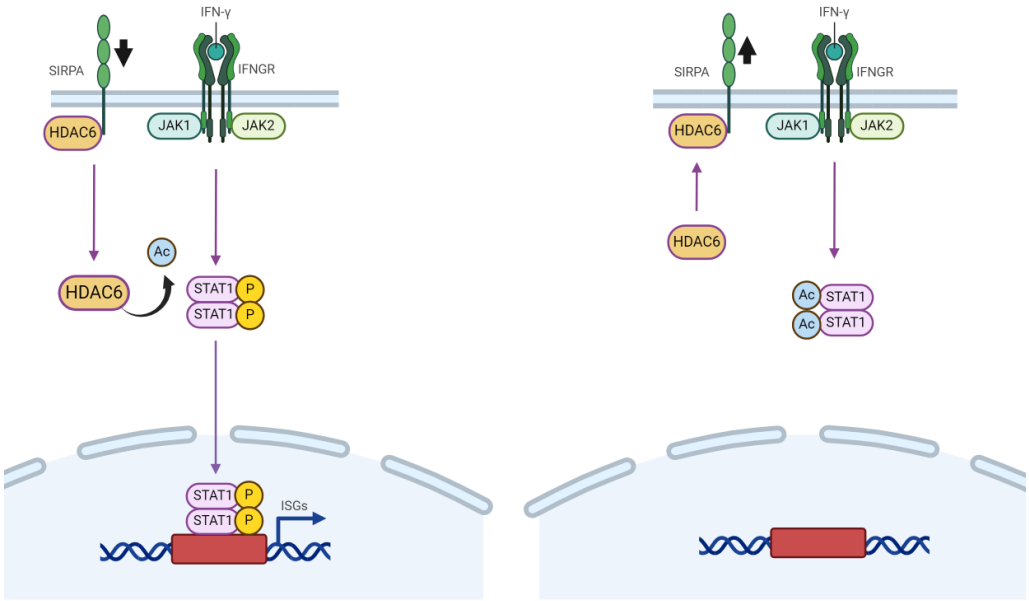


Figure 6









ESM Table 1: Details of pancreas samples from the Exeter Archival Diabetes Biobank (EADB) used for immunohistochemical analysis.

Case ID	Group	Source	Donor Status	Sex	Age (years)
242 89	No diabetes control	EADB	Autopsy	M	3
146 66	No diabetes control	EADB	Autopsy	F	18
21 89	No diabetes control	EADB	Autopsy	F	4
E560	Type 1 diabetes	EADB	Organ Donor	F	42
E385	Type 1 diabetes	EADB	Autopsy	F	19
SC41	Type 1 diabetes	EADB	Autopsy	F	4
SC19	Type 1 diabetes	EADB	Autopsy	F	1.75

ESM Table 2: Antibody details and conditions of use.

Antibody	Species raised	Dilution	Catalogue number	Company
Anti-SIRPA	Rabbit	1:1000	13379S	Cell signalling
Anti-SIRPA	Rabbit	1:500	ab191419	Abcam
Anti SIRPA	Mouse	1:250	TA807751S	Origene
Anti-SIRPA	Rabbit	1-100	11612-RP02	Sinobiological
Anti-pSTAT1	Rabbit	1:1000	9167	Cell signaling
Anti-STAT1	Mouse	1:1000	9176	Cell Signaling
Anti-Acetyl Lysine	Mouse	1:1000	623401	Biolegend
Anti-Acetyl tubulin	Rabbit	1:1000	5335	Cell signaling
Anti-STAT1	Rabbit	1:1000	9172	Cell signaling
Anti-tubulin	Mouse	1:10000	11224-1-AP	Proteintech
Anti-HDAC6	Rabbit	1:1000	ab133493	Abcam
Anti-mouse 680	Goat	1:5000	SA5-10154	Invitrogen
Anti-Rabbit 800	Goat	1:5000	SA5-10036	Invitrogen
Anti-mouse-AP	Goat	1:25000	A3562	Sigma-Aldrich
Anti-Rabbit-AP	Goat	1:25000	A3687	Sigma-Aldrich
Anti-insulin	Goat	1:360	A0564	Dako
Anti-glucagon	Rabbit	1:2000	ab92517	Abcam

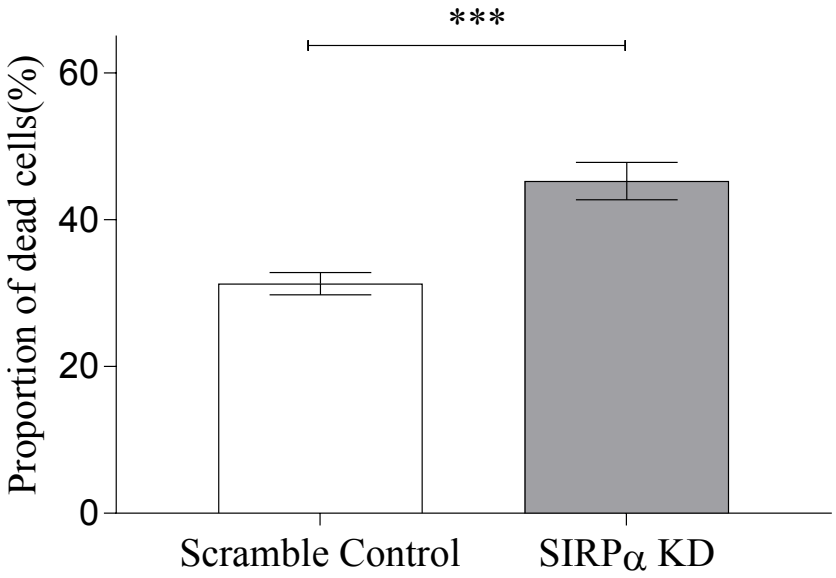
ESM Table. 3: Results mean values of differentially bound proteins to SIRPα relative to IgG controls by LC-MS

Protein	Coverage	Peptides	PSM	Unique Peptides	Area:F2	Score Sequest HT
SIRPA	73.51190476	39	164.5	26	6650346423	426.99
HDAC6	15.0617284	13	29.5	13	492822060.1	74.89
SIRPB1	13.5678392	8.5	30	1	1000926.461	76.04
VCP	12.71712159	7.5	8	7.5	15071702.97	17.27
GPRASP2	12.17183771	7	8	7	64604190.29	16.41
PTPRN2	5.024630542	3	3	2.5	6190975.507	5.84
STAT1	3.329119258	2	2	2	4662181.746	4.42
NEDD4L	4.307692308	3	3	3	3915384.052	6.99

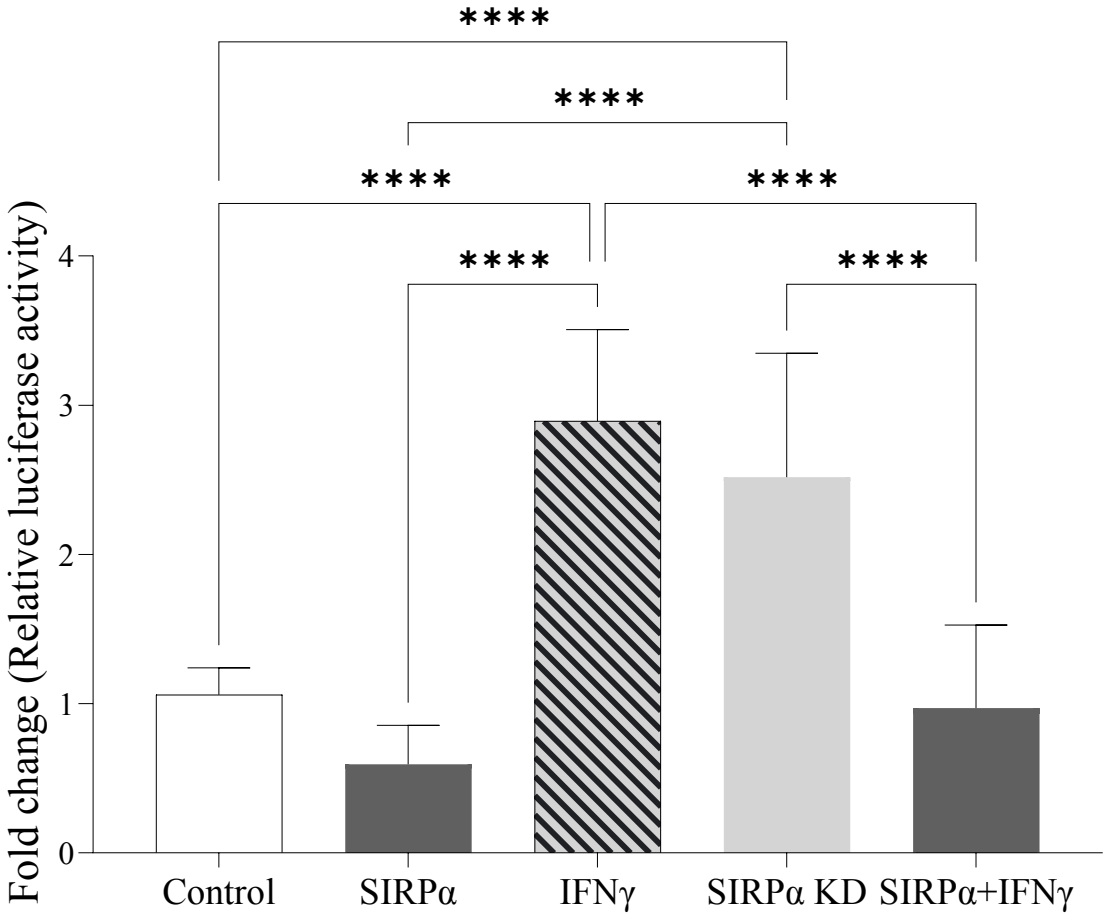
ESM Methods:

Mass spectrometry analysis was performed by the Proteomics Facility, University of Bristol, UK according to methods described previously (30). Brielfy, after co-immunoprecipitation of EndoC-βH1 cell lysates with anti-SIRPα serum or control IgG, samples were transferred to Bristol Proteomics Facility who separated, digested and fractionated the proteins using a Dionex Ultimate 3000 nano-HPLC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). The peptides were purified using Acclaim PepMap C18 columns (Thermo Fisher) and analysed using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) operated in data-dependent acquisition mode. Data were quantified using Proteome Discoverer software v2.1 (Thermo Scientific) and searched against the UniProt Human database (downloaded January 2022; 178486 sequences) using the SEQUEST HT algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.995 Da), acetylation of the protein N-terminus (+42.011 Da) and methionine loss plus acetylation of the protein N-terminus (-89.03 Da) as variable modifications and carbamidomethylation of cysteine (+57.021 Da) as a fixed modification. Searches were performed with full tryptic digestion and a maximum of 2 missed cleavages were allowed. The reverse database search option was enabled and all data were filtered to satisfy a false discovery rate (FDR) of 5%.

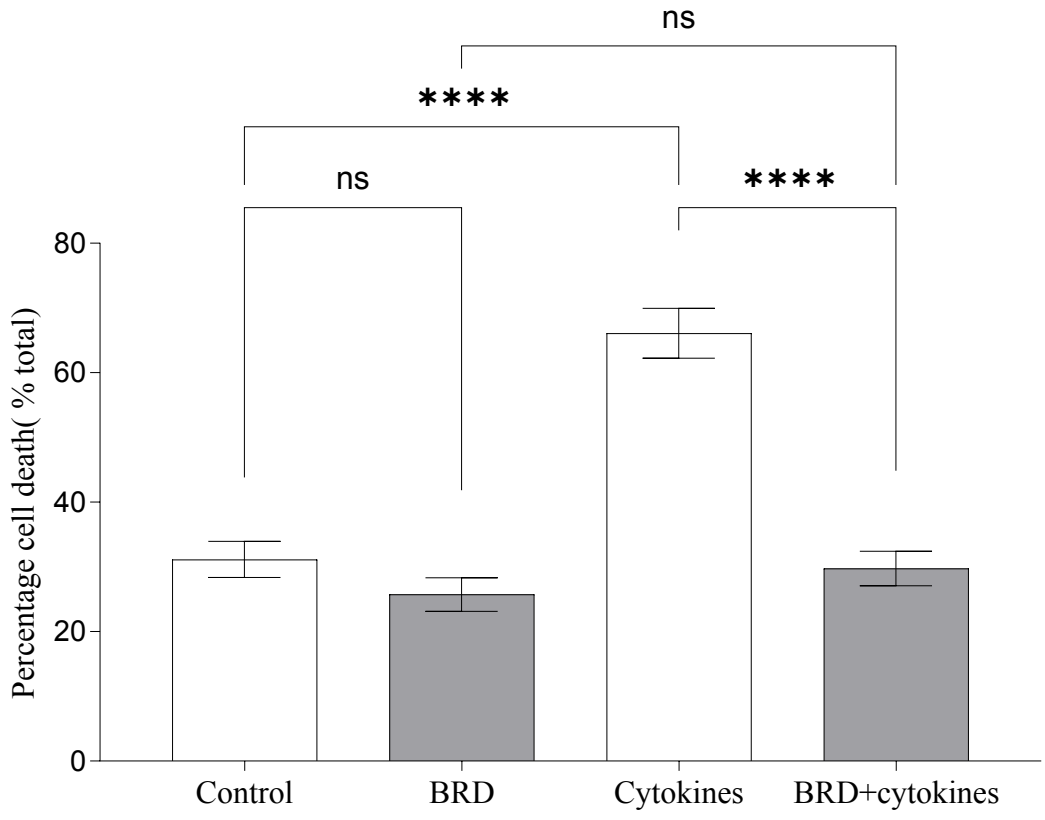
A



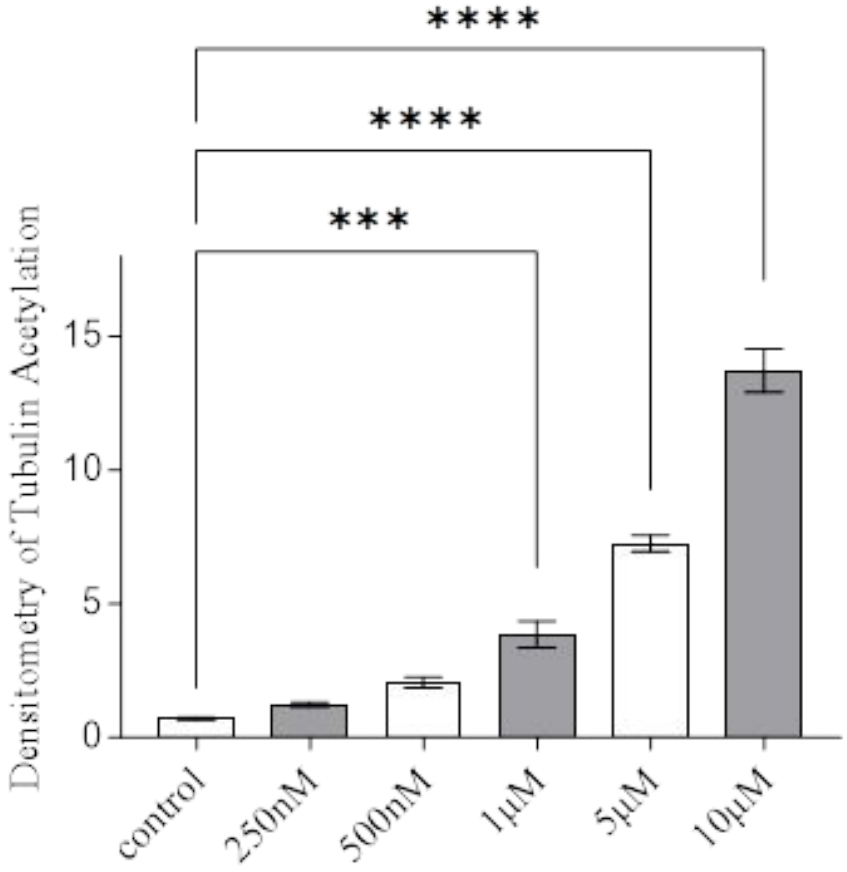
ESM Figure 1. Targeted knockdown of SIRPα with siRNA leads to a net loss of viability in EndoC-βH1 cells over a period of 96h of culture. ***p<0.001



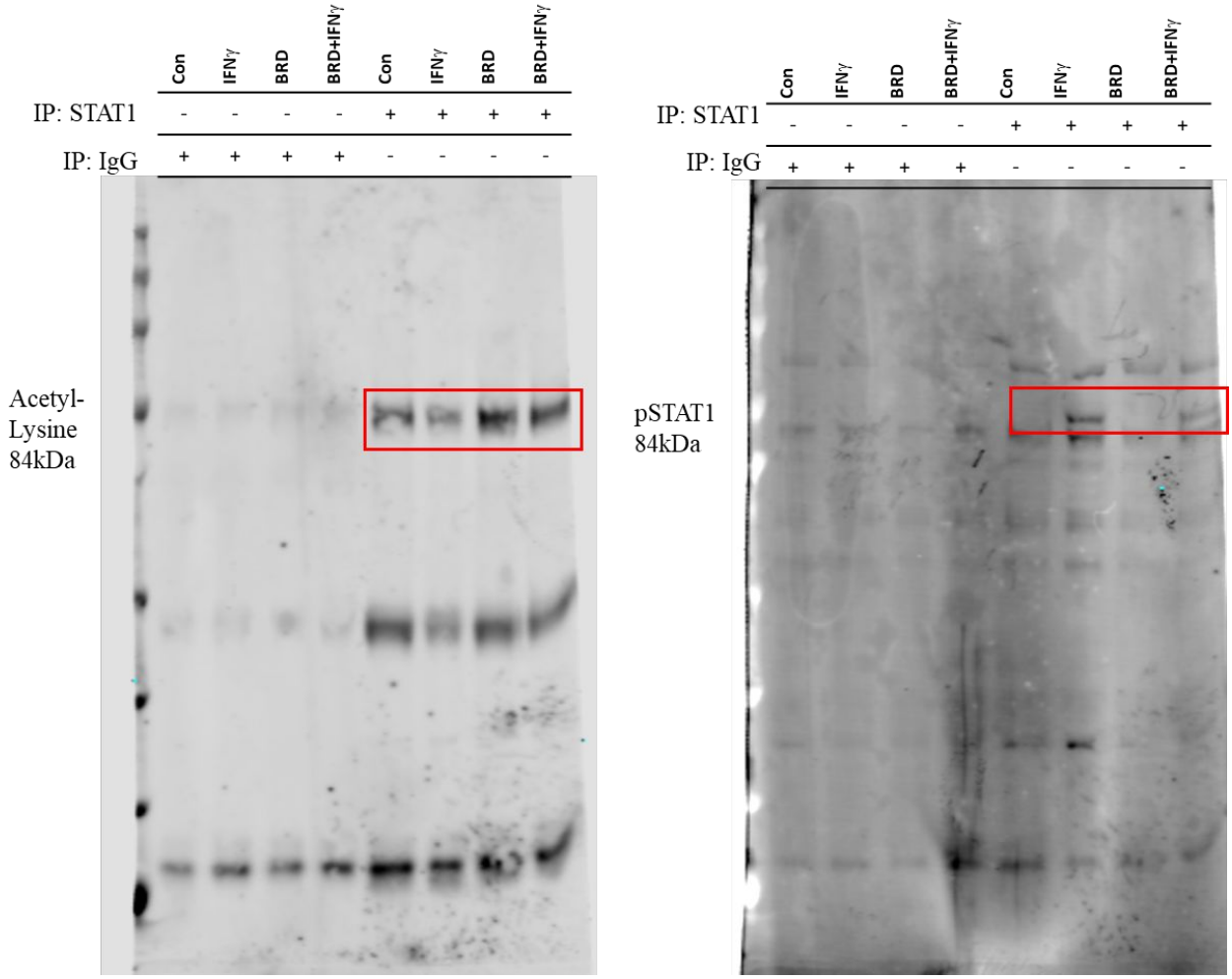
ESM Fig. 2: EndoC-βH1 β cells were transfected with a SIRPα expression plasmid (dark grey bars) or with siRNA to knockdown SIRPα (light grey) and a GAS-luciferase reporter construct. Cells were then treated with vehicle (control) or IFNγ (20 ng/ml) for 8 h, as shown, prior to measurement of luciferase activity. ****p<0.0001.



ESM Fig 3. The selective HDAC6 inhibitor, BRD9757 (10 μM) attenuates the cytotoxic actions of pro-inflammatory cytokines in rodent INS-1E cells. ****p<0.0001.



ESM Fig. 4: EndoC-βH1 were stimulated with increasing concentration of BRD9757 for 18 h followed by protein extraction and western blotting to detect acetylated tubulin by densitometry. ***p<0.001; ****p<0.0001



ESM Fig. 5: STAT1 was immunoprecipitated from human EndoC-βH1 cells after exposure to IFNγ and/or BRD9757 and blots probed for acetylated-lysine (left panel) and pSTAT1 (right panel).