Original Article

MOLECULAR METABOLISM

Deletion of histone deacetylase 3 in adult beta cells improves glucose tolerance via increased insulin secretion

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ABSTRACT

Objective: Histone deacetylases are epigenetic regulators known to control gene transcription in various tissues. A member of this family, histone deacetylase 3 (HDAC3), has been shown to regulate metabolic genes. Cell culture studies with HDAC-specific inhibitors and siRNA suggest that HDAC3 plays a role in pancreatic β-cell function, but a recent genetic study in mice has been contradictory. Here we address the functional role of HDAC3 in β-cells of adult mice.

Methods: An HDAC3 β-cell specific knockout was generated in adult *MIP-Cre*ERT transgenic mice using the Cre-loxP system. Induction of HDAC3 deletion was initiated at 8 weeks of age with administration of tamoxifen in corn oil (2 mg/day for 5 days). Mice were assayed for glucose tolerance, glucose-stimulated insulin secretion, and islet function 2 weeks after induction of the knockout. Transcriptional functions of HDAC3 were assessed by ChIP-seq as well as RNA-seq comparing control and β-cell knockout islets.

Results: HDAC3 β-cell specific knockout (HDAC3βKO) did not increase total pancreatic insulin content or β-cell mass. However, HDAC3βKO mice demonstrated markedly improved glucose tolerance. This improved glucose metabolism coincided with increased basal and glucose-stimulated insulin secretion *in vivo* as well as in isolated islets. Cistromic and transcriptomic analyses of pancreatic islets revealed that HDAC3 regulates multiple genes that contribute to glucose-stimulated insulin secretion.

Conclusions: HDAC3 plays an important role in regulating insulin secretion *in vivo*, and therapeutic intervention may improve glucose homeostasis.

Keywords HDAC3; Glucose tolerance; Insulin secretion

1. INTRODUCTION

Glucose homeostasis is a tightly controlled process that is critical for normal physiology, which is maintained by pancreatic endocrine cells secreting peptide hormones and peripheral tissues responding accordingly. Specifically, the role of the β-cell in the pancreas is to secrete insulin in response to glucose, but dysfunction can lead to diabetes mellitus. Type 1 diabetes mellitus is of growing clinical significance in the population and is hallmarked by autoimmune recognition of pancreatic β-cells [1]. This proinflammatory environment created by the immune system leads to impaired function and death of the insulin producing β-cells of the pancreas, whereas type 2 diabetes mellitus is characterized by insulin resistance and failure of the insulin secreting β-cells, classically associated with obesity and low-grade inflammation [2,3]. Growing evidence suggests that changes in transcription, regulated by epigenetic changes may play a larger role in the pathogenesis of both these diseases than previously appreciated [4].

Histone deacetylase 3 (HDAC3) is a member of the class I HDACs. It functions as part of multi-protein complexes that deacetylate histone tails, thereby modifying chromatin structure and resulting in gene repression. HDAC3 has been shown to form stable complexes *in vivo* with, and be activated by, the nuclear receptor corepressor (NCoR1) and the silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) [5,6]. Class I HDACs are ubiquitously expressed and have been implicated in regulation of metabolic gene signatures [7]. In the past several years, multiple studies of siRNA knockdown and pharmacological inhibition of HDAC3 have suggested a role for HDAC3 in β-cells, with loss of HDAC3 function protecting β-cells from cytokine-induced apoptosis and helping to maintain proper glucose-stimulated insulin secretion [8–12]. Furthermore, an HDAC3-specific inhibitor was reported to improve glucose homeostasis and insulin secretion in a diabetic rat model [11]. To determine the physiologic role of HDAC3 in β-cells, we applied mouse genetics to conditionally ablate HDAC3 *in vivo*. Here we demonstrate that the deletion of HDAC3 in β-

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cells of adult male mice improves glucose tolerance by increasing insulin secretion.

2. METHODS

2.1. Animal studies

The *MIP-Cre*ERT and HDAC3$^{f/f}$ lines have previously been described [13,14]. Mice were maintained on a C57BL/6 background and normal chow unless otherwise noted. Analyses were restricted to male mice. Tamoxifen (Sigma T5648) was dissolved in corn oil at 20 mg/mL and administrated at 2 mg/day via gavage for five days. Animals were assayed 2 weeks after tamoxifen induction. Intraperitoneal glucose tolerance tests (IPGTTs) were performed as previously described [15]. Glucose-stimulated insulin secretion (GSIS) assays were performed by administering mice a bolus of glucose (3 g/kg) following a 16 h fast. Plasma was separated using heparinized tubes, and insulin and C-peptide were measured using ELISA kits (Crystal Chem #90080 and #90050, respectively). Total pancreatic insulin and glucagon content were determined by radioimmunoassay (RIA) in which acid–ethanol extractions were performed on whole pancreata (EMD Millipore). All the animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania in accordance with the guidelines of the US National Institutes of Health.

2.2. Immunohistochemistry and immunofluorescence

Pancreata were dissected, weighed, fixed in 10% formalin for 16 h at 4°C, washed with PBS, and embedded in paraffin. Tissue sections were stained as previously described using HDAC3 (H-99 sc-11417), Insulin (ab7842), and Glucagon (N-17 sc-7780) antibodies [15]. Insulin immunohistochemistry (IHC) was performed as previously described [15]. Insulin signal was detected with Vectastain Elite ABC kit (standard; Vector, PK6100) and DAB Peroxidase Substrate Kit (Vector, SK4100). To quantify β-cell mass, sections were digitally scanned using an Aperio ScanScope CS2 and analyzed using ImageScope as previously described [16].

2.3. Islet isolation and static incubations

Islets were isolated using the standard collagenase (EC 3.4.24.3 Serva, 17449) digestion protocol as previously described [17]. For static incubations, islets were cultured for 3 days and then transferred to KREBS buffer. An equal number of islets were glucose starved for 30 min and then glucose-stimulated for 40 min. Supernatants were collected and insulin measurements performed by RIA.

2.4. RNA analysis

RNA was immediately extracted from isolated mouse islets two weeks after tamoxifen induction, and quantitative reverse transcription-PCR (RT-PCR) was performed as described [18], using primers as

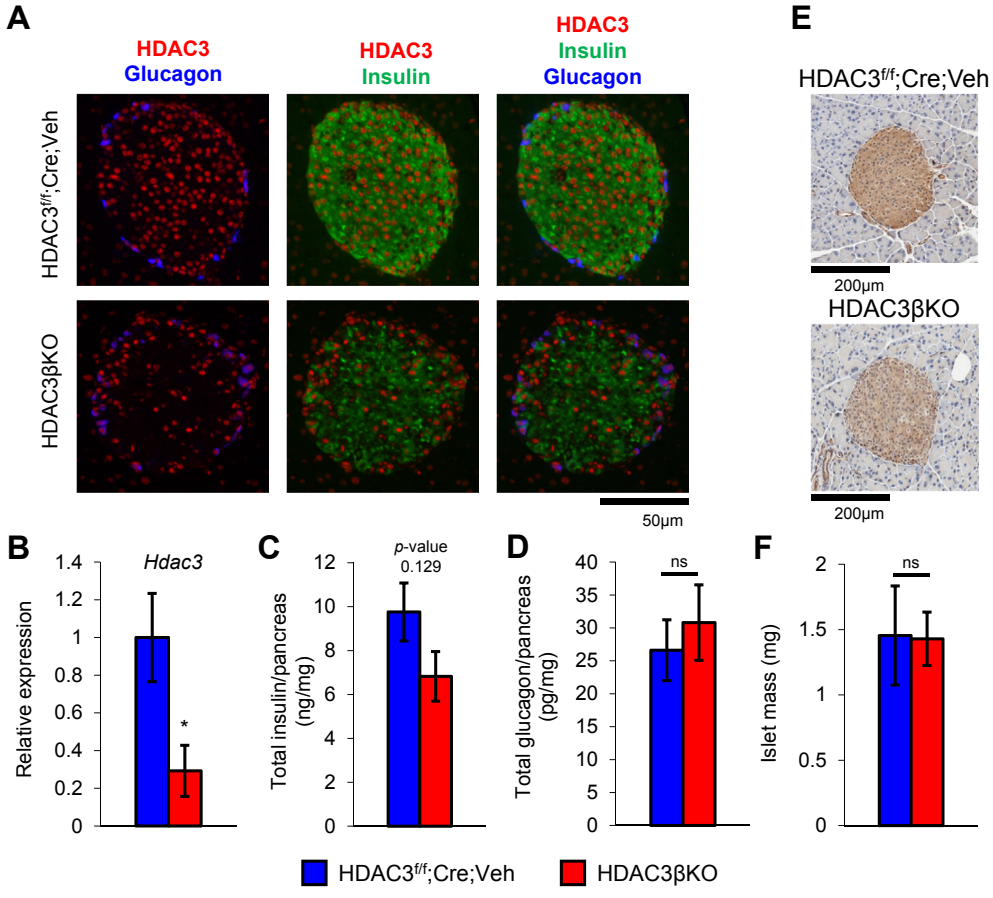

Figure 1: HDAC3 β-cell KO does not increase insulin content or β-cell mass. (**A**) Co-immunofluorescence for HDAC3, Insulin, and Glucagon (20×). (**B**) Quantitative RT-PCR of freshly isolated islets (n = 5). (**C, D**) Total pancreatic insulin and glucagon content normalized to pancreatic weight (n = 4–6). (**E**) Insulin immunohistochemistry (IHC) staining (20×). (**F**) β-Cell mass quantified from insulin IHC staining (n = 4). All error bars, s.e.m. (t-test, *$p < 0.001$).

indicated and normalized to *Actb* (Supplemental Table). RNA-seq libraries were generated using the Tru-seq kit (Illumina). Raw reads were aligned to mm9 reference genome using Tophat version 2.1.0 and the parameters recommended by the original author [19]; gene level quantification was performed by HTSeq using default parameters [20], and differential expression analysis was performed using DESeq2 according to original authors' instructions [21]. RNA-seq datasets have been deposited at GEO.

2.5. ChIP-seq

Isolated mouse islets were washed with PBS, fixed with 1% formaldehyde at room temperature for 15 min, quenched with 125 mM glycine for 5 min, and washed with PBS. Fixed islets were probe sonicated at 10 W and 15 W for 10 s on and 10 s off, twice. Sonicated islets were lysed in RIPA buffer containing protease inhibitors and PSMF. ChIP was performed using 10 μg HDAC3 antibody (ab7030) and protein A agarose. Cross links were reversed at 65°C overnight and proteinase K digested, followed by phenol/chloroform isolation. Libraries were prepared and sequenced as previously described [22]. Briefly, sequencing reads of biological replicates were aligned to the mm9 genome using Bowtie v0.12.7 [23]. Duplicate reads were removed, and replicates were pooled using HOMER v4.7 [24]. Genome-browser tracks were generated, and peaks were called using HOMER with default parameters and genomic DNA as input. Peaks from HDAC3$^{f/f}$;Cre;Veh and HDAC3βKO experiments were pooled, and an average profile was generated using HOMER. Additional analysis was limited to peaks in HDAC3$^{f/f}$;Cre;Veh greater than 1 read per million (RPM) and more than 4 fold over HDAC3βKO. Distribution of peaks in the genome was found using HOMER. BEDTools v2.26 was used to find peaks within 100 kb of gene transcriptional start site (TSS) [25], and gene ontology analysis was performed on said peaks using GREAT v3.0 [26]. STRING analysis [27] was performed on transcription factors identified in *de novo* motif analysis of HDAC3 peaks and expressed in RNA-seq with greater than 1 normalized read count. Transcription factors with known interactions with HDAC3 are presented using Cytoscape v3.3.0. ChIP-seq datasets have been deposited at GEO.

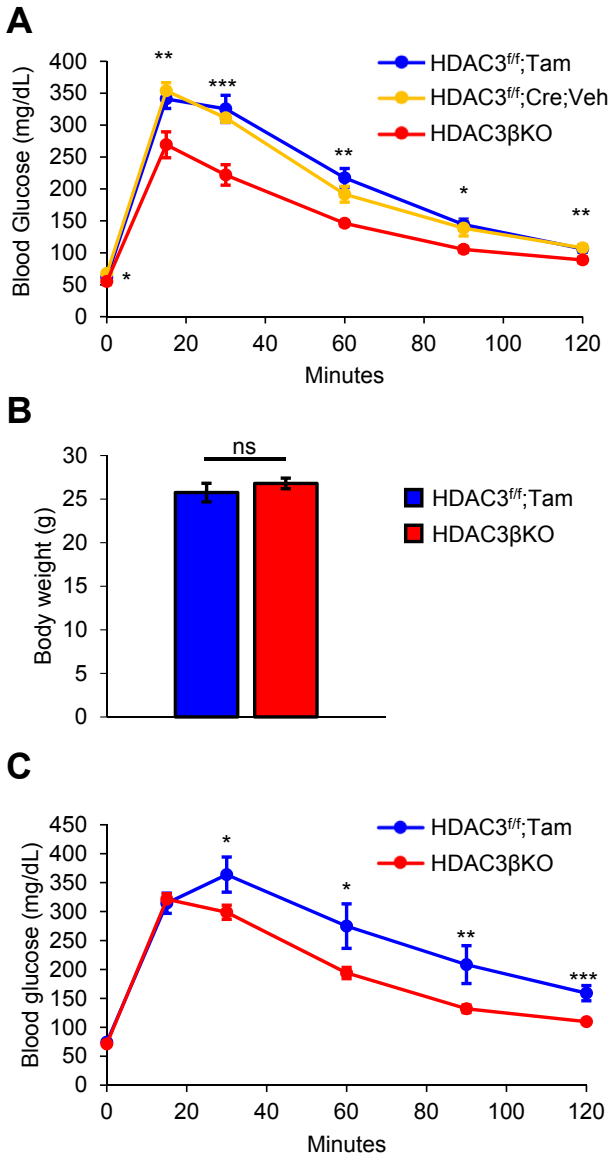

Figure 2: Deletion of HDAC3 in β-cells of adult mice markedly improves glucose tolerance. (**A**) Intraperitoneal glucose tolerance test (IPGTT) of mice on normal chow (NC) at 10 weeks of age, 2 weeks after tamoxifen administration, 2 g/kg glucose (n = 5, t-test compares HDAC3$^{f/f}$;Cre;Veh and HDAC3βKO). (**B**) Weight of mice on NC 6 weeks after tamoxifen induction of KO. (**C**) IPGTT 6 weeks after KO induction (n = 6–11). All error bars, s.e.m. (t-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

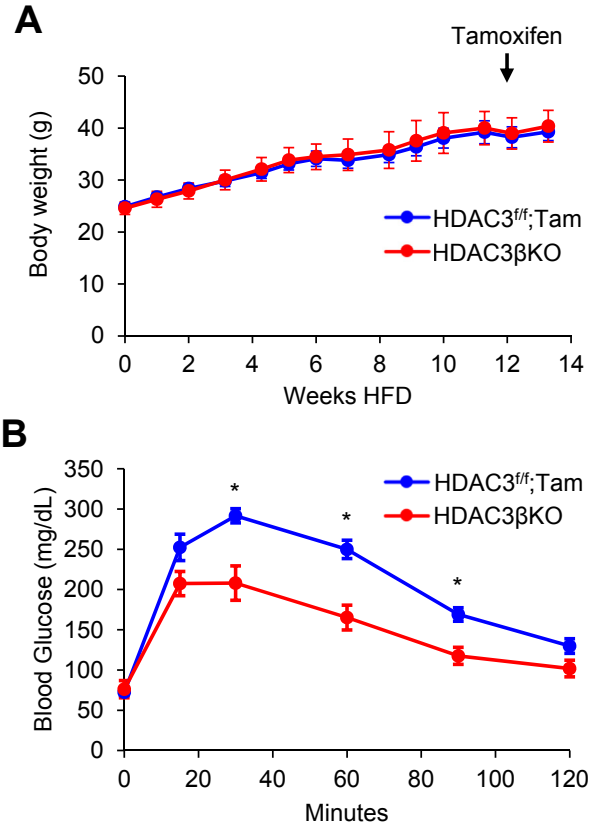

Figure 3: HDAC3 β-cell KO improves glucose tolerance in the setting of diet-induced obesity. (**A**) Body weights of mice on high fat diet (HFD). (**B**) IPGTT in mice after 14 weeks high fat diet and 2 weeks after tamoxifen administration, 1 g/kg glucose (n = 5). All error bars, s.e.m. (t-test, $*p < 0.01$).

3. RESULTS

3.1. Deletion of HDAC3 in β-cells does not significantly alter insulin content or β-cell mass

To generate β-cell specific deletion of HDAC3 in C57BL/6 mice, HDAC3$^{f/f}$ mice were crossed with mice expressing tamoxifen-inducible Cre recombinase under control of the mouse insulin 1 gene promoter (*MIP-Cre*ERT) [13,14]. This Cre line has previously been shown to be β-cell specific without recombination detected in the brain, unlike other putatively β-cell specific deletion models such as *RIP*-Cre [28]. The HDAC3 β-cell knockout (HDAC3βKO) was induced by 5 days of tamoxifen administration (2 mg/day) to 8 week old mice, and immunofluorescence studies confirmed the knockout of HDAC3 in insulin-positive islet cells when compared to HDAC3$^{f/f}$ mice expressing the *MIP-Cre*ERT transgene administered corn oil vehicle (HDAC3$^{f/f}$;Cre;-Veh) (Figure 1A). The remaining islet HDAC3 signal was likely due to non-beta cells in the islet in addition to any β-cells in which the knockout was incomplete. The β-cell deletion of HDAC3 was supported by quantitative RT-PCR for *Hdac3* transcript in freshly isolated islets (Figure 1B). There were no significant differences in the total pancreatic insulin (Figure 1C) or glucagon content (Figure 1D) in the HDAC3βKO mice. Islet architecture, assessed by insulin immunohistochemistry staining (Figure 1E), and β-cell mass (Figure 1F) were not appreciably altered in the HDAC3βKO mice.

3.2. HDAC3 ablation in β-cells of adult mice markedly improves glucose tolerance

To characterize the physiological consequences of the HDAC3βKO on β-cell function, we determined their glucose tolerance. Notably, there was a marked improvement in glucose tolerance in HDAC3βKO mice during glucose challenge, as well as a modest fasting hypoglycemia. (Figure 2A). The improvement in glucose tolerance was observed when comparing HDAC3βKO animals to HDAC3$^{f/f}$ mice administered tamoxifen (HDAC3$^{f/f}$;Tam) or to HDAC3$^{f/f}$ mice expressing the *MIP-Cre*ERT administered corn oil vehicle (HDAC3$^{f/f}$;Cre;Veh). This result is consistent with a recent finding that the *MIP-Cre*ERT transgene does not impair or enhance glucose homeostasis despite expressing human growth hormone [16]. The HDAC3βKO did not significantly alter body weight relative to control 6 weeks after induction of the knockout (Figure 2B). In addition, the improved glucose phenotype remains present over time, as mice assayed 6 weeks after knockout induction have significantly increased glucose tolerance (Figure 2C).

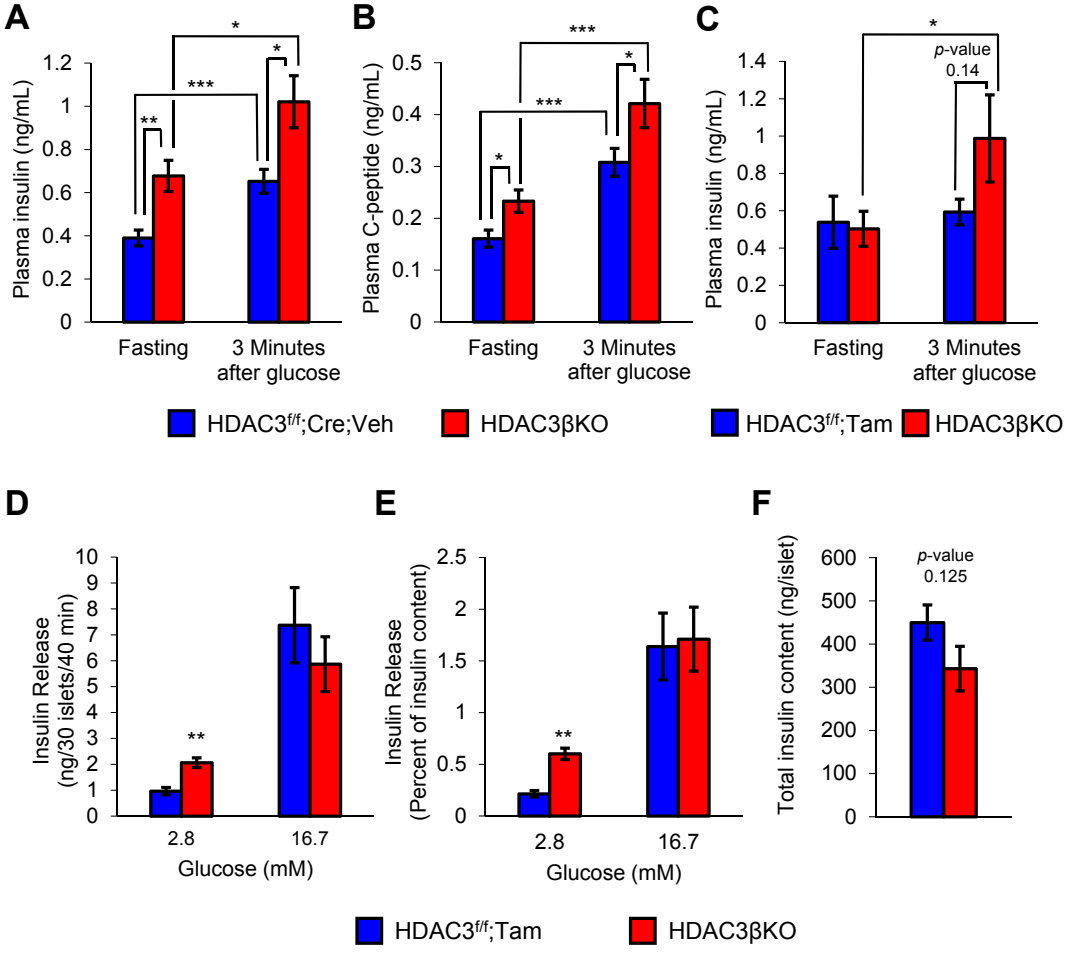

Figure 4: HDAC3 β-cell KO mice have increased insulin secretion. (**A, B**) Plasma insulin and C-peptide of mice on normal chow after a 16 h fast, and 3 min after intraperitoneal glucose injection (3 g/kg) (n = 14). (**C**) Plasma insulin in mice on HFD for 15 weeks after a 16 h fast, and 3 min after intraperitoneal glucose injection (3 g/kg) (n = 5). (**D–F**) Glucose-stimulated insulin secretion (GSIS) *ex vivo* from isolated islets and corresponding total insulin content (n = 5–9). All error bars, s.e.m. (t-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

3.3. Deletion of HDAC3 in β-cells of obese mice markedly improves glucose tolerance

To determine if the loss of β-cell HDAC3 improves glucose tolerance in a model of obesity with associated insulin resistance, mice were placed on high fat diet starting at 6 weeks of age. Tamoxifen was administered at 18 weeks of age, and glucose tolerance was assessed at 20 weeks of age. The knockout of HDAC3 in β-cells of obese mice did not alter body weight (Figure 3A) but did result in significant improvement in glucose tolerance (Figure 3B). These data show that acute deletion of HDAC3 has beneficial effects on glucose metabolism in the setting of diet-induced obesity.

3.4. Improved glucose tolerance is a due to increased insulin secretion

To investigate the physiological basis of improved glucose tolerance in HDAC3βKO mice, we next explored the effect of HDAC3 β-cell specific knockout on insulin secretion. It has been shown previously that pharmacological HDAC3 inhibition or knockdown in cell culture can maintain glucose-stimulated insulin secretion in the presence of cytokines [8,10,12]. Of note, HDAC3βKO mice on normal chow displayed significantly increased circulating insulin after a 16 h fast, as well as 3 min after a glucose challenge (Figure 4A). This finding was corroborated by increased plasma C-peptide in the fasted and glucose-stimulated conditions (Figure 4B). Further, in the setting of diet-induced obesity, glucose-stimulated insulin secretion was increased in HDAC3βKO mice (Figure 4C). These data suggest that the loss of HDAC3 in β-cells led to increased insulin secretion and was responsible for the improved glucose tolerance. To determine if the increased insulin secretion was a cell-autonomous effect of β-cell depletion of HDAC3, glucose-stimulated insulin secretion was examined *ex vivo* in isolated islets from mice on normal chow. Indeed, HDAC3βKO islets secreted more insulin at lower glucose concentrations than control islets, whether normalized to the number of islets (Figure 4D) or to total insulin content (Figure 4E), which was not significantly altered by the loss of HDAC3 (Figure 4F). The enhanced insulin secretion at low glucose concentrations *ex vivo* is consistent with the increased basal insulin secretion observed during fasting of HDAC3βKO mice, whereas the plateau of insulin secretion at high glucose for 40 min may not be directly comparable to the *in vivo* GSIS measured 3 min after glucose challenge.

3.5. HDAC3 cistrome in isolated islets

To further investigate the means by which HDAC3 influences insulin secretion *in vivo*, we sought to determine the HDAC3 cistrome in isolated islets. To achieve this goal, islets from HDAC3$^{f/f}$;Cre;Veh and HDAC3βKO were isolated and pooled for chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq). There was robust enrichment for HDAC3 in the HDAC3$^{f/f}$;Cre;Veh control islets compared to HDAC3βKO (Figure 5A). We identified 9972 peaks in the HDAC3$^{f/f}$;Cre;Veh control with greater than 1 read per million (RPM) and 4 fold over input library; 8975 of these peaks had greater than 4 fold enrichment over the HDAC3βKO cistrome. Consistent with HDAC3 cistromes from other tissues, the majority of genomic binding was intronic or intergenic, with only a minority of binding sites at promoters or transcriptional start sites (Figure 5B) [29]. Using the genomic regions enrichment of annotations tool (GREAT), HDAC3 binding was found to be enriched at a variety of biological processes critical for β-cell function, including endocrine development and regulation of insulin secretion (Figure 5C). These data suggest that HDAC3 could be a critical transcriptional regulator of genes involved in β-cell function and maintaining glucose homeostasis.

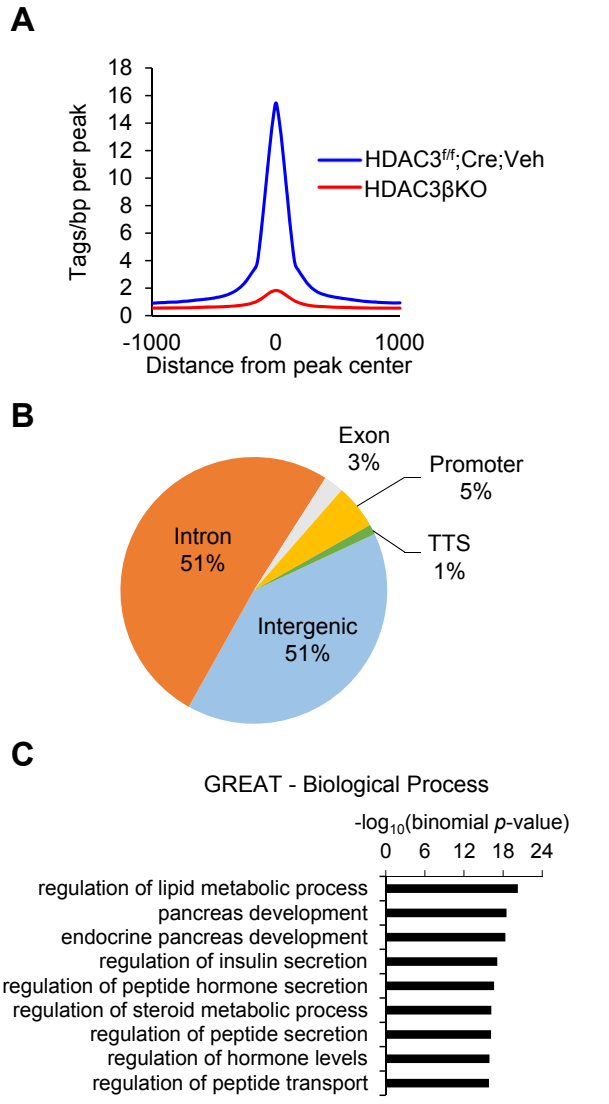

Figure 5: HDAC3 cistrome in isolated islets. (**A**) Average profile of HDAC3 binding in HDAC3$^{f/f}$;Cre;Veh and HDAC3βKO. (**B**) Distribution of HDAC3 genomic binding relative to known genes. (**C**) GREAT analysis of HDAC3$^{f/f}$;Cre;Veh specific peaks filtered against HDAC3βKO.

3.6. Transcriptome of HDAC3 β-cell KO reveals potential pathways contributing to increased insulin secretion

To better understand the functional role of HDAC3 in the β-cell, we performed RNA-seq on islets from HDAC3βKO mice in two separate cohorts of mice, one relative to HDAC3$^{f/f}$;Tam controls that lack the *MIP-Cre*ERT transgene, and the other compared to the HDAC3$^{f/f}$;Cre;Veh mice. Since the two control models displayed indistinguishable glucose homeostasis, we reasoned that genes significantly regulated in both experiments would shed light on the mechanisms by which HDAC3 influences insulin secretion. This was particularly relevant given the recent report that *Tph1* is dramatically induced in the *MIP-Cre*ERT transgenic mouse, leading to increased islet serotonin levels [16], which have been correlated with increased β-cell mass in pregnancy [30]. Using both control groups, transcriptomic analysis revealed 264 and 222 significantly up- and down-regulated genes in the HDAC3βKO islets (Figure 6A). Gene ontology analysis of the up-regulated genes revealed amino acid activation, vesicle transport,

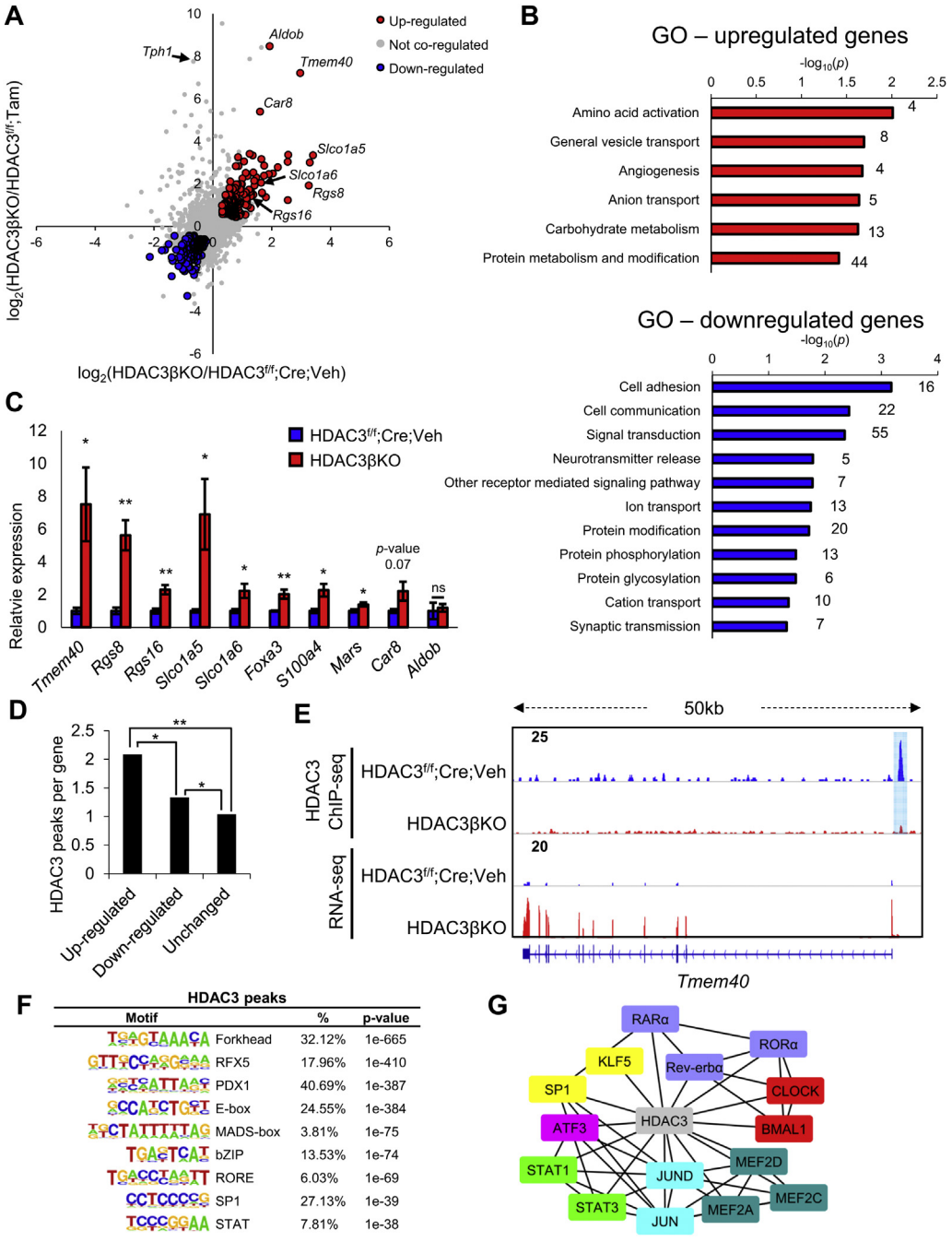

Figure 6: RNA-seq analysis of HDAC3βKO compared to HDAC3$^{f/f}$;Cre;Veh and HDAC3$^{f/f}$;Tam mice. (**A**) Scatter plot of $\log_2$ fold change from two separate RNA-seq experiments. Red dots are significant (FDR < 0.1) in both experiments and up-regulated 1.5 fold or greater in either HDAC3βKO. Blue dots are significant (FDR < 0.1) in both experiments and down-regulated 1.5 fold or greater in either HDAC3βKO. (**B**) Gene ontology (GO) biological process enrichment results using 264 upregulated genes and 222 downregulated genes. Only significantly enriched ($p < 0.05$) terms are shown. For each term, the numbers of the genes that contribute to the enrichment is labeled. (**C**) Quantitative RT-PCR confirming significant upregulation of transcripts from RNA-seq in the HDAC3βKO (n = 5) (All error bars, s.e.m., t-test, $*p < 0.05$, $**p < 0.01$). (**D**) HDAC3 peak frequency within 100 kb of gene TSS from RNA-seq analysis (Mann–Whitney–Wilcoxon test, $*p < 0.05$, $**p < 0.0001$). (**E**) Genome-browser view of ChIP-seq and RNA-seq tracks of the *Tmem40* gene. The major HDAC3 peak is highlighted in light blue. Scale is reads per ten million. (**F**) *De novo* motif analysis at HDAC3 binding sites genome-wide using HOMER. Motifs with *p*-value $<$ 1e−150 or known interactions are shown. (**G**) STRING analysis of transcription factors identified by *de novo* motif analysis of HDAC3 peaks. Transcription factor families are denoted by different colors.

anion transport, and carbohydrate metabolism as significantly enriched processes (Figure 6B). Quantitative RT-PCR confirmed RNA-seq results for many of the up-regulated transcripts in the HDAC3βKO (Figure 6C). Intersection of the islet transcriptomes and HDAC3 cistrome revealed that HDAC3 bound more prevalently near genes that are subsequently up-regulated in the β-cell KO (Figure 6D). The concordance of HDAC3 genomic binding with derepressed transcription in the HDAC3βKO islet is exemplified at the *Tmem40* locus (Figure 6E). Genome-wide *de novo* analysis of DNA sequences enriched under HDAC3 binding peaks revealed Forkhead, RFX5, PDX1, E-box, MADS-box, bZIP, nuclear

receptor, SP1, and STAT motifs (Figure 6F). Intersection of these motifs with of transcription factors expressed in the islet transcriptome revealed several signal-responsive factors with the potential to function with HDAC3, including circadian proteins Rev-erbα, ROR, and CLOCK (Figure 6G), which may reflect the known role of HDAC3 in the circadian clock in other tissues [29].

4. DISCUSSION

Our work demonstrates that HDAC3 in beta cells regulates insulin secretion and glucose metabolism. HDAC3 is a critical epigenetic modifying enzyme and regulator of metabolic gene signatures. Previous studies suggested a role for HDAC3 in mediating cytokine-induced apoptosis in cultured β-cell models and in a diabetic rat model, but the present study is the first to demonstrate that adult mice lacking HDAC3 in their β-cells display markedly increased glucose clearance and tolerance. The present studies, determining the physiological consequence of acute HDAC3 ablation in adult mouse beta cells, suggest that HDAC3 plays an important role in modulating insulin secretion in normal conditions as well as in the setting of diet-induced obesity. While these conclusions are consistent with the beneficial result of HDAC3-specific inhibition in a diabetic rat model [11], they are contrary to those of a recent report where HDAC3 ablation was generated using *RIP*-Cre [31]. A major concern about the *RIP*-Cre model is that it has been shown to produce off-target tissue recombination, particularly in the brain, where HDAC3 has been shown to have important effects on behavior [32–34]. Also, the *RIP*-Cre model is not inducible; therefore, the effects in β-cells may emphasize the role of HDAC3 in development rather than in the mature adult pancreas. Indeed, our cistromic results suggest that HDAC3 genomic binding is enriched near genes involved in endocrine development, and two genes upregulated in the HDAC3βKO, *Rgs8* and *Rgs16*, have been shown to be dynamically regulated in the developing pancreas [35]. Nevertheless, the *MIP-Cre*ERT model that we have used to delete HDAC3 avoids the problem of brain expression and clearly demonstrates an inhibitory role of HDAC3 on insulin secretion from adult beta cells *ex vivo* and *in vivo*.
We have identified a number of genes controlled by HDAC3 in β-cells with known or plausible roles in glucose-stimulated insulin secretion. Among these are the organic anion transporters *Slco1a5* and *Slco1a6*. Intriguingly, the *Slco1a6* gene locus has been identified as an important quantitative trait locus (QTL) in β-cells, regulating gene transcription by altering transport of bile acids in islets [36]. Notably, bile acids have been shown to potentiate glucose-stimulated insulin secretion [37]. In addition to these transporters, *Rgs16* was shown to be regulated, which has been shown to increase glucose-stimulated insulin secretion [38]. Furthermore, *S100a4* and its paralog *S100a6* are identified as up-regulated in the HDAC3βKO RNA-seq analysis. These genes are defined to be calcium binding proteins, and *S100a6*, also known as Calcyclin, has been shown to potentiate Ca^{2+} stimulated insulin release [39].
In silico analysis of the HDAC3 binding sites in islets suggested a potential role of several signal-responsive transcription factors including STAT and bZIP family members, as well as components of the circadian clock. Although highly speculative, the latter is intriguing given the known role of the molecular clock in controlling beta cell functions [40,41]. Interestingly, we did not detect an increase in insulin gene transcription in HDAC3βKO islets (data not shown). Although this is consistent with the physiologic data presented here, it is worth noting that a previous study using the pan-HDAC inhibitor Trichostain A (TSA) increased insulin gene transcription [42]. However, this drug inhibits all class 1 HDACs and thus the present results suggest that HDACs and/or other targets are responsible for the effects of TSA in beta cells.
In summary, we have described the physiological role of HDAC3 in adult mouse β-cells and identified several target genes that may in combination act to increase glucose-stimulated insulin secretion *in vivo*. Thus, HDAC3 is a critical regulator of gene transcription in β-cells and, as a druggable enzyme that modulates the epigenome, a potential therapeutic target to improve glucose homeostasis, especially in the setting of diet-induced obesity and metabolic stress.

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CONFLICTS OF INTEREST

MAL is a member of the Scientific Advisory Boards of KDAC, Eli Lilly and Company, and Pfizer, Inc.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.11.007.

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