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Original Research

The Deubiquitinating Enzyme Otub2 Modulates Pancreatic Beta-Cells Function and Survival

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Abstract

Background: We have previously demonstrated that ovarian tumor (Otu) domain-containing ubiquitin aldehyde-binding protein 2 (Otub2), a deubiquitinating enzyme, exerts anti-apoptotic effects in primary human islets. The present study aims to further elucidate the molecular mechanisms underlying the role of Otub2 as a regulator of insulin secretion and β-cell function. **Methods**: Otub2 overexpression or silencing was employed to study its effects on cultured MIN6 cells and dispersed human islets. To evaluate its *in vivo* effects, Otub2 knockout (KO) mice were employed, as well as a pancreata-specific Otub2 overexpression model. RNA sequencing was performed on pancreatic tissue from Otub2-KO and control mice to study its effects on gene expression patterns. Co-immunoprecipitation followed by mass spectrometry identified Otub2-interacting proteins. **Results**: Overexpression of Otub2 inhibited NF-κB activity and enhanced glucose-stimulated insulin secretion (GSIS) in cultured MIN6 cells and primary human islets. Otub2 KO mice exhibited impaired glucose tolerance and upregulation of NF-κB target genes. Conversely, selective *in vivo* overexpression of Otub2 in pancreata of C57BL wild-type mice resulted in significantly lower (~30%) blood glucose levels, post glucose injection, compared to control mice. Transcriptomic analysis of KO pancreata revealed downregulation of K^+ transporter-related genes and upregulation of oxidative phosphorylation genes, consistent with defective insulin secretion. Mass spectrometry identified the voltage-gated potassium channel subunit Kv9.3 as a major Otub2 binding partner, along with paternally expressed 3 (Peg3) and calcium/calmodulin dependent protein kinase II delta (Camk2d) proteins known to promote NF-κB signaling and β-cell apoptosis. **Conclusions**: Otub2 is a critical regulator of β-cell function, acting through modulation of NF-κB signaling and K^+ channel-associated complexes. By deubiquitinating components such as Peg3 and Camk2d, Otub2 may protect β-cells from cytokine-induced apoptosis and sustain insulin secretory capacity. These findings position Otub2 as a potential therapeutic target for preserving β-cell function in diabetes.

Keywords: Otub2; pancreatic beta cells; NF-κB; apoptosis; potassium channels

1. Introduction

Type-1 and Type-2 diabetes mellitus involve selective and progressive loss of pancreatic β-cells that might eventually require life-long insulin replacement therapy [1,2]. Transplantation of islets and β-cells regeneration are two approaches for β-cells replenishment [3]. Currently, islet transplantation is a potential treatment that brings about insulin independence, improves quality of life, and increases beta-score [4–6]. However, a large proportion (up to 70%) of the grafts are lost during the initial few days following islet infusion. This is mainly attributed to inflammatory reactions, generation of reactive oxygen species (ROS), and cytokine secretion [5,7]. For example, oxygen tension in the liver, the preferred site for islet transplantation, is well below that of the pancreas. Further, the low expression level of major cellular antioxidant enzymes in islets contributes to less-than-optimal islet survival. Autoimmune and alloimmune responses and the diabetogenic action of immunosuppressive drugs also contribute to islet deterioration [5]. Several strategies have been employed to improve graft survival, such as heparinization of the islets' medium prior to transplantation and peri-transplant insulin therapy. These approaches may improve β-cell function and reduce immediate β-cell apoptosis, but cannot prevent loss of β-cell function over time, with most patients returning to insulin-dependence after five years [4,8].

To improve β-cells' survival following transplantation, we have previously developed and performed high throughput screens (HTS) of ~730 pre-selected siRNAs in search for genes that affect survival of isolated human pancreatic islets treated with pro-inflammatory cytokines [9,10]. These studies identified a number of novel genes, for example, ovarian tumor (Otu) domain-containing ubiquitin aldehyde-binding protein 2 (Otub2) [9], Nedd4 family

interacting protein 1 (Ndfip1) [11], and transmembrane 7 superfamily member 3 (TM7SF3) [12], whose roles in promoting survival of cytokine-affected human β-cells had not been previously examined.

Otub2, a family member of cysteine proteases having a deubiquitinase activity [13], was a highly significant 'hit' in the above screens. Silencing of Otub2 expression increased caspase-3/7 activity in primary human islets treated with a mixture of cytokines (TNF-α, IL-1β, IFN-γ), inhibited insulin secretion, and increased NF-κB activity [9]. These findings suggest that Otub2 may function as a novel promoter of viability and insulin secretion in β-cells treated with cytokines. At the molecular level, Otub2 was demonstrated to have a preference for cleaving K63–linked ubiquitin [14], unlike its homologue Otub1, which has high specificity for K48-linked chains [15]. In doing so, Otub2 could hamper NF-κB activation by modifying scaffold elements. In this study, we demonstrate the beneficial effects of Otub2 *in vivo* and show its action as a pro-survival protein for β-cells that protects them from apoptotic death and maintains their functionality. These findings suggest that Otub2 might be a potential candidate for improved survival of transplanted β-cells.

2. Materials and Methods

2.1 Cells

MIN6 murine pancreatic β-cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 11 mM glucose, 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 5 µM β-mercaptoethanol. 100 U/mL penicillin and 100 µg/mL streptomycin were added to non-transfected MIN6 cells, while 400 µg/mL geneticin (G418, InvivoGen, Toulouse, France) was supplemented to stably transfected MIN6 cells. Cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. MIN6 cells were used within 20 passages and were routinely tested for mycoplasma contamination. No additional authentication was performed, as the identity of this β-cell line was confirmed by its characteristic morphology and glucose-stimulated insulin secretion response.

2.2 Plasmids

pEGFP-c1 plasmid (Addgene, https://www.addgene.org/) harboring G418 resistance was used for creating stable MIN6 cell lines that overexpress Otub2 (mouse isoform 2) with a Flag tag (instead of EGFP). The pEGFP-c1 vector, in which the Flag tag was cloned instead of the EGFP sequence (pFlag), served as a control. pEGFP-c1 plasmids which contained the original EGFP sequence (p-EGFP-c1) and EGFP-Otub2 sequence (pEGFP-Otub2) were used for transient transfection of MIN6 cells. To create these constructs, the Otub2 cDNA was amplified by PCR using the 5′ primer (5′-CAGTCCGGAGACACTATG AGTGAAACATCTTTCAACC-3′); and the 3′ primer (5′-CGCGGATCCGCGGTAGTCAGTGTTTCTCGGCTGC-3′). The PCR product was digested using BspE1 and BamHI restriction enzymes and ligated into the pEGFP-c1 plasmid. An additional construct was generated using the following primers containing Flag tag: 5′ primer (5′-CTAGCATGGACTACAA AGACGATGACGACAAGT-3′) and 3′ primer (5′-CCGGACTTGTCGTCATCGTCTTTGTAGTCCATG-3′). The primers were hybridized and ligated into pEGFP-c1 digested with NheI and BspEI restriction enzymes. The EGFP cDNA was then excised from the plasmid and replaced by a Flag tag, creating the pFlag-Otub2 construct. pFlag plasmid was generated after digestion of pFlag-Otub2 construct with BspE1 and BamHI restriction enzymes (excising Otub2 out of the construct) and used as a control.

2.3 Antibodies

Polyclonal anti-Flag and anti-GAPDH antibodies were from Sigma (St. Louis, MO, USA). Polyclonal anti-Otub2 antibodies were from Novus Biologicals (Centennial, CO, USA). Polyclonal anti-GFP antibodies were from Abcam (Waltham, MA, USA). Alexa488 conjugated goat anti-mouse secondary antibodies were from Life Technologies (Waltham, MA, USA).

2.4 Cytokines

The cytokine mixture referred to as '1x-cytomix' consisted of 3 nM TNF-α (Prospec-Tany Technogene, Rehovot, Israel), 3 nM IFN-γ (Prospec-Tany Technogene, Rehovot, Israel), and 1.5 nM IL-1β (MD Biosciences, Ness Ziona, Israel). Their biological activities were 10 units/ng (TNF-α, IFN-γ) and 200 units/ng (IL-1β).

2.5 Dispersion and Culture of Human Islets

Isolated human islets (~80% purity confirmed by dithizone staining) were provided by the European Consortium for Islets Transplantation (Islet for Basic Research program) through a Juvenile Diabetes Research Foundation Award 31-2008-413. Isolated human islets were grown in CMRL 1066 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin, and 40 µg/mL gentamycin, and were cultured at 37 °C in a 5% CO_2 humidified atmosphere. Islets media was replaced every other day. Intact human islets were dispersed by incubation at 37 °C (4 minutes) with 1 mg/mL Trypsin/EDTA by pipetting the cells, and by passing them twice through a 21G needle. Trypsinized islets were washed and resuspended with CMRL 1066 medium supplemented with 10% FBS. Cells were used within 48 hours following dispersion. Human islets studies received Ethics approval from the Bioethics and Embryonic Stem Cell Research Oversight Committee (ESCRO) of the Weizmann Institute of Science.

2.6 siRNA Transfection

MIN6 cells or human islets were seeded in 96 well plates (30,000 cells/well or 1000 dispersed human islets cells/well) in 100 µL medium (DMEM for MIN6 cells and CMRL for human islets), and immediately transfected with sequences of non-targeting siRNA or Otub2 siRNA (to a final concentration of 25 nM) using DarmaFECT-4 transfection reagent (Dharmacon, Waterbeach, UK) for MIN6 cells and Darma-FECT-1 transfection reagent (Dharmacon) for human islets, according to manufacturer's instructions.

2.7 Transient DNA Transfection

MIN6 cells or human islets were seeded (500,000 cells/well in 6-well plates for MIN6 cells, and 1000 islets/well in 96-well plates for human islets) and 24 hours post-seeding, the cells or human islets were transfected with pFlag or pFlag-Otub2 vectors (3 µg/well for a 6-well plate and 0.2 µg/well for a 96-well plate) using the transfection reagent Lipofectamine 2000 (Thermo-Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

2.8 Generation of MIN6 Cells That Stably Over-Express Otub2

MIN6 cells were seeded in 12-well plates and 24 hours post-seeding, cells were transfected using jetPEI transfection reagent (Polyplus Transfection, Illkirch, France), with pEGFP-c1 vectors (1 µg/well) harboring G418 resistance to calibrate for the cells' G418 resistance. pEGFP-c1 vector in which Flag tag was cloned instead of EGFP sequence (pFlag) served as control; and pEGFP-c1 with Flag-Otub2 cDNA clone without EGFP sequence (pFlag-Otub2) was utilized to overexpress Otub2. Non-transfected cells served as a control and 48 hours after transfection media were replaced with media containing elevated concentrations of G418. After the calibration process was complete and the proper concentration of G418 required for selection was established, MIN6 cells were seeded in 6-well plates (150,000 cells/well) to create stable cell lines, 24 hours post-seeding, cells were transfected with pEGFP-c1 constructs (3 µg/well), and 48 hours thereafter, the media were replaced with media containing 400 µg/mL G418 for the selection process. After completion of selection, the media were replaced with media containing 200 µg/mL G418 for maintenance.

2.9 Caspase 3/7 Activity Assay

MIN6 cells were seeded in 96-well plates (30,000 cells/well) and transfected with siRNA and 48 hours later, cells were treated with a cytokine mixture, referred to as 1x-cytomix. For stably transfected cell lines, cells were treated with 1x-cytomix 24 hours post-seeding and 24 hours later caspase 3/7 activity was examined using Sensolyte homogeneous RH110 caspase 3/7 assay kit (AnaSpec, Fremont, CA, USA). Human islets were dispersed and seeded in 96-well plates (1000 islets/well). Islets were transfected with p-Flag or pFlag-Otub2 constructs that stably express Flag-Otub2. The rest of the experiment was similar to the assay in MIN6 cells as described above.

2.10 NF-κB Activity Assay

NF-κB activity was measured using the Ready-To-Glow secreted luciferase assay kit (Clontech, San Jose, CA, USA). Stably transfected MIN6 cells were seeded in 96-well plates (30,000 cells/well) and 24 hours post-seeding, cells were transfected with a secreted luciferase plasmid (250 ng/well), coupled to the NF-κB enhancer elements. Constitutively secreted luciferase plasmid, transfected into MIN6 cells, served as a control. After 24 hours, cells were treated with 1x-cytomix for 4 hours to induce NF-κB activity and 50 µL of the media of the cells was harvested and transferred to black 96-well plates containing a luciferase assay mix (5 µL/well). Luminescence was read using an Infinite 2000 PRO reader (Tecan, Männedorf, Switzerland).

2.11 Glucose Stimulated Insulin Secretion (GSIS)

Stably transfected MIN6 cells were seeded in 96-well plates (30,000 cells/well). After 48 hours, cells were incubated for 1 hour in Krebs-Ringer bicarbonate HEPES buffer (KRBH), containing 124 mM NaCl, 5.6 mM KCl, 2.5 mM $CaCl_2$, and 20 mM HEPES, pH 7.4, at 37 °C, followed by incubation for 1 hour in KRBH with 0 mM or 20 mM glucose. Insulin concentration in the culture medium was determined using insulin detecting HTRF kit (Cisbio, Codolet, France) according to the manufacturer's instructions.

2.12 RNA Analysis

Following treatment in 24-well plates, cells were harvested, and total RNA was extracted using the PerfectPure RNA kit (5-prime, Montreal, Canada). RNA was quantified using nano-drop. cDNA was generated by the cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA), following the manufacturer's instructions. qRT-PCR was carried out using an ABI-Prism 7300 instrument (Applied Biosystems, Waltham, MA, USA), utilizing SYBR Green PCR mix (Invitrogen, Carlsbad, CA, USA) and specific primers (100 nM final concentration). Expression levels of Actin or Hypoxanthine Phosphoribosyltransferase 1 (*HPRT*) were used to normalize mRNA levels. Primers used are given herein (see Table 1).

2.13 RNA Sequencing

RNA was extracted from the pancreas and liver of mice using the PerfectPure RNA kit (5-prime, Montreal, Canada) following the manufacturer's instructions. An Agilent 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA) was used to assess RNA quality. RNA-seq libraries were generated by the MARS-seq protocol [16]. Libraries were sequenced by the Novaseq 6000

Table 1. qRT-PCR primers (5′ to 3′).

Gene	Origin	Forward primer	Reverse primer
iNOS	Mouse	GCCCTGCTTTGTGCGAAGTG	AGCCCTTTGTGCTGGGAGTC
MCP-1	Mouse	AGGTGTCCCAAAGAAGCTGTA	ATGTCTGGACCCATTCCTTCT
IL-1β	Mouse	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
IP-10	Mouse	ATGACGGGCCAGTGAGAATG	TCAACACGTGGGCAGGATAG
Otub2	Mouse	AACCGAGCTGACTTCTTCCG	CGTCGACGTACTCTACCTGC
Glut2	Mouse	TTTTCAGCCAAGGACCCCGT	GCCCAAGGAAGTCCGCAATG
Nkx6.1	Mouse	AACACACCAGACCCACGTTCT	ATCCCCAGAGAATAGGCCAAG
MafA	Mouse	CAAGGAGGAGGTCATCCGAC	TCTCCAGAATGTGCCGCTG
HPRT	Mouse	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT
Actin	Mouse	GGCCAACCGTGAAAAGATGA	CACAGCCTGGATGGCTACGT

iNOS, nitric oxide synthase 2; MCP-1, C-C motif chemokine ligand 2; IP-10, C-X-C motif chemokine ligand 10; Otub2, ovarian tumor (Otu) domain-containing ubiquitin aldehyde-binding protein; Glut2, glucose transporter 2; Nkx6.1, NK6 homeobox 1; MafA, MAF BZIP transcription factor A; HPRT, hypoxanthine phosphoribosyltransferase 1.

(Illumina, San Diego, CA, USA), using SP mode 100 cycles kit (Illumina, San Diego, CA, USA). Alignment of sequences to the genome and count matrix determination were performed by the UTAP pipeline (Weizmann Institute, Rehovot, Israel). Normalization of libraries, low count genes filtration, and calculation of differentially expressed genes were performed using the edgeR and Limma packages in R (https://www.r-project.org/). Gene Ontology (GO) and MsigDB pathways enrichment analysis were performed using the Camera method from the Limma package in R. The enrichment results are given in *p*-values, with –log10(*p*-value) >1.3 considered to be significant. A functional protein association network was built using STRING (https://string-db.org/).

2.14 Immunoprecipitation and Mass Spectrometry

Protein A-agarose beads were washed with 0.1 M Tris-HCl (pH 8.0, 4 °C), followed by incubation with Flag or GFP antibodies in 0.1 M Tris-HCl (pH 8.0) for 4 h at 4 °C. Supernatants (centrifuged at 20,000 ×g 15 min 4 °C) of cell extracts in extraction buffer (~0.8–1.2 mg of protein), were incubated at 4 °C overnight with the immobilized antibodies. Immunocomplexes were washed three times with extraction buffer and then incubated for 30 min at 4 °C with PBS containing Flag or GFP peptide in order to elute Otub2 and the proteins that formed a complex with it. Immunocomplexes were mixed with Laemmli sample buffer and resolved by 10% SDS-PAGE. The complexes were subjected to mass spectrometry analysis as previously described [17].

2.15 Western Blot Analysis

Cells were seeded and treated as indicated. Treated cells were washed three times with PBS and were harvested in lysis buffer (25 mM Tris-HCl [pH 7.4], 10 mM sodium orthovanadate, 10 mM pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulphonyl fluoride). Lysates were centrifuged (20,000 ×g, 15 min, 4 °C). Samples (40–150 µg) were mixed with 5× Laemmli buffer, boiled, and resolved by 10–12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane for Western blotting with the indicated antibodies: Polyclonal anti-Flag (Sigma, F7425, diluted 1:1000), Polyclonal anti-GAPDH (Sigma, ABS16, diluted 1:1000).

2.16 Generation of Otub2 Knockout Mice

All *in vivo* experiments were carried out in accordance with relevant guidelines and regulations. Experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science (application number 23980116-3). Mice were housed under standard light/dark conditions and were given access to food and water ad libitum. The study is reported in accordance with the ARRIVE guidelines.

The mouse strain C57BL/6NTac-Otub2$^{tm1a(EUCOMM)Wtsi}$/WtsiH was ordered from the Wellcome Trust Sanger Institute, London, UK, as part of the EUCOMM Mutant Mouse Project. One heterozygous male and two heterozygous female mice on a C57BL/6NTac background were ordered from the European Mouse Mutant Archive (EMMA) mouse repository. EUCOMM vector contains 5′ and 3′ homology arms, facilitating homologous recombination, and a targeting cassette that disrupts gene function, flanked by flipase recognition target (FRT) recombination sites to allow removal by Flp recombinase. Further, exons 3/4 of Otub2 were flanked by a pair of loxP recombination sites, inducing a frame shift upon removal of the exons and leading to complete gene inactivation. For genotyping, mouse tail genomic DNA preparations were extracted using REDExract-NAmp tissue PCR kit (Sigma), followed by amplification reactions performed with oligonucleotide pairs (see Table 2) specific for the foreign or wild-type sequences, to amplify ~200–900 bp fragments.

Table 2. Genotyping primers (5′ to 3′).

	Forward	Reverse	Product size
Wild-type 1	GATGGTCAGCCTTGTTAGCA	CCGTTCAGTCAGGTCCCTAG	940 bp
Wild-type 2	GATGGTCAGCCTTGTTAGCA	CTTGAGGGAACAGGGCATGT	970 bp
Tm1a allele 1	GAGGACAGCTTGGGAGAGAT	CCACAACGGGTTCTTCTGTT	635 bp
Tm1a allele 2	AGGCGCATAACGATACCACGAT	CCACAACGGGTTCTTCTGTT	204 bp

2.17 Pancreas Removal From Mice

Otub2 knock-out and wild-type (WT) mice were sacrificed by cervical dislocation. An incision was made from the upper abdomen downwards to expose the liver and intestines. The duodenum was exposed by shifting the intestines to the right, and the pancreas was isolated by pulling the intestines carefully from the duodenum downwards. Spleen was then removed, and the pancreas was detached from the large intestine to complete its isolation.

2.18 Construction and Administration of Adeno Associated Viral Vectors (AAVs)

Adeno-associated viral vectors (AAVs) that can stably express Flag or Flag-Otub2 were generated by inserting the Flag or Flag-Otub2 inserts into the pAAV-RIP-VEFG-WPRE vector, after eliminating the VEGF coding sequence, as described [18,19]. The pAAV-RIP-Flag (for control purposes) and pAAV-RIP-Flag-Otub2 vectors were used for production of the AAV vectors. To ensure selective expression of Otub2 in pancreatic β-cells, the rat insulin promoter (RIP) was used to drive expression of Otub2. WPRE (WHP Posttranscriptional Regulatory Element) was used to enhance expression. Following vector generation, transgene expression was evaluated first *in vitro* in cultured cells. Then the DNA was amplified, and single-stranded AAV-Otub2 vectors were produced. Single-stranded AAV8 was used throughout. Administration of the AAV-8 viral vectors into the pancreatic duct of live mice was carried out as described [20]. For vector administration, mice were anesthetized with ketamine (100 mg/kg body weight, working concentration 25 mg/mL) and xylazine (10 mg/kg body weight, working concentration 2.5 mg/mL), administered intraperitoneally prior to surgery.

2.19 Glucose Tolerance Test

Mice were fasted overnight with water access ad libitum. Mice were intraperitoneally injected with glucose (1.8 g/kg body weight). Blood samples were taken at timed intervals (0–120 minutes) from a tail vein, and Glucose levels were monitored using MediSense Optium Blood Glucose test strips (Abbott Laboratories, Abbott Park, IL, USA). Glucose levels were plotted versus the time points, and the area under the curve (AUC) was calculated using the computeAUC function from the PharmacoGx package in R.

2.20 Immunofluorescence of Pancreatic Sections

For immunofluorescence, pancreata were fixed for 24 h in 4% paraformaldehyde and then transferred to 70% ethanol until embedding in paraffin by a standard protocol (by using automated tissue processing) as follows: pancreata were first dehydrated by immersing them sequentially for 45 minutes each in a series of ethanol-water mixtures (70%; 95% ($\times$3) and 100% ($\times$2; 30 minutes)) followed by immersion with ethanol-xylene mixture (1:1; for 45 minutes) and xylene ($\times$2; 1 hour). The tissues were then embedded in paraffin at 60 °C (3 times for 1 hour). Cross sections of paraffin blocks were incubated in xylene ($\times$2) and ethanol (95–70%). Antigen retrieval was performed by boiling in 0.01 M sodium citrate (pH 6.0; 12.5 minutes in microwave). Sections were then blocked (with 0.5% Triton) and incubated overnight at 4 °C with anti-Otub2 antibodies (Novus Biologicals, OTI11B3, diluted 1:1000). Anti-Otub2 was detected with fluorescent-tagged secondary antibodies (conjugated to Alexa488 dye). Sections were later washed with PBS and DAPI for nucleus staining.

2.21 Statistics

Statistical analysis was performed in R. Differences between experimental conditions were determined by a two-tailed Student's *t* test, unless otherwise mentioned in the figure legends.

3. Results

3.1 Overexpression of Otub2 Decreases NF-κB Activity

To characterize the effects of Otub2 on β-cells, a MIN6 cell line that stably overexpresses Flag-tagged Otub2 was generated. Over-expression of Otub2 was validated by qRT-PCR (for mRNA levels, Fig. 1A) and by Western blotting (for protein levels, **Supplementary Fig. 1A,B**). Fluorescence microscopy indicated that there are no morphological changes between control Flag-expressing cells and Flag-Otub2 expressing cells (**Supplementary Fig. 1C**).

To examine the effects of Otub2 on NF-κB activity, a luciferase-reporting system was employed. Min6 cells were treated with 'cytomix', a mixture of cytokines (TNF-α, IL-1β, IFN-γ) that significantly increases the expression of NF-κB target genes [21] and promotes β-cell apoptosis [21,22]. Indeed, cytomix treatment significantly increased the level of NF-κB promoter activity by ~1.8-fold, while stable overexpression of Otub2 resulted in ~78% de-

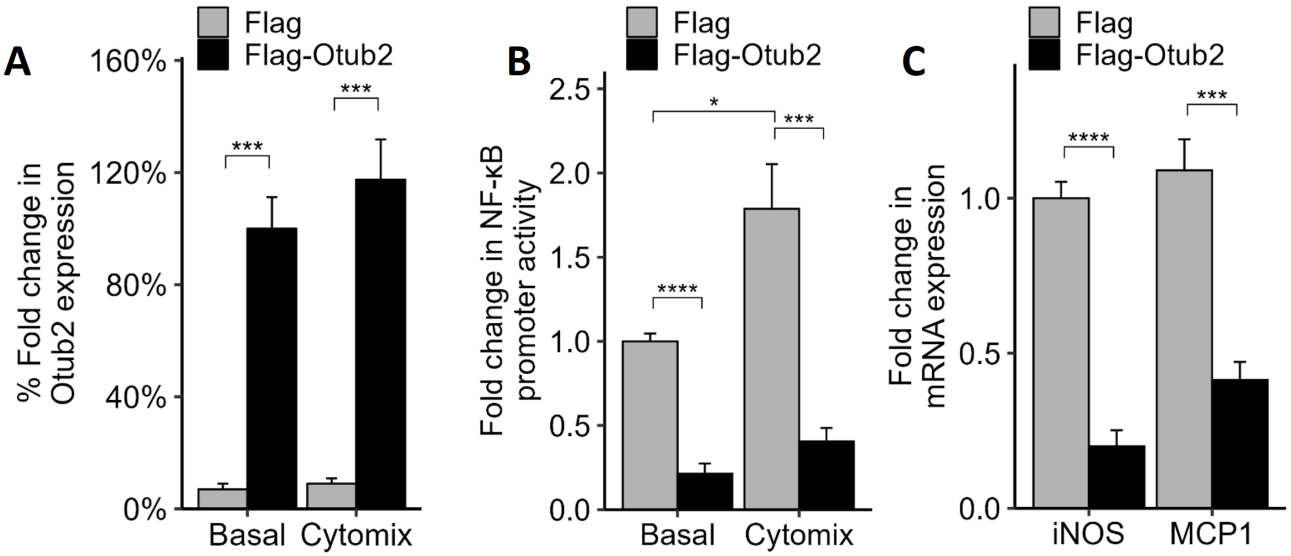

Fig. 1. Effects of OTUB2 overexpression on NF-κB activity in MIN6 cells. (A) MIN6 cells were transfected with pEGFP-c1 plasmid constructs: pFlag and pFlag-Otub2 vectors. The stable cell line was subjected to qRT-PCR analysis following treatment with 1x-cytomix for 4 h. Non-treated cells served as a control. mRNA levels were normalized to Actin. (B) MIN6 cells overexpressing Flag (control) or Flag-OTUB2 were transfected with pNF-κB-MetLuc2 or with pMetLuc2 (control). 24 h following transfection, cells were treated with or without 1x-cytomix for 4 h. NF-κB activity was determined. Data presented were normalized to control vector transfection values. (C) MIN6 Flag or Flag-OTUB2 over-expressing cells were treated with 1x-cytomix for 4 h and then harvested. Total mRNA was extracted, and qRT-PCR was conducted. mRNA levels of NF-κB target genes were normalized to Actin. Data are means $\pm$ SEM of four replicates in each of four experiments (B) and of duplicates in each of three experiments (C). Asterisks indicate the p-values as follows: *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ vs. control. Flag stable cells.

crease in NF-κB promoter activity, both in control and cytomix-treated cells (Fig. 1B). Overexpression of Otub2 in cytokine-treated cells also reduced by 60% and 80%, respectively, the mRNA levels of the NF-κB target genes *MCP1* and *iNOS* (Fig. 1C). These results complement our previous findings that Otub2 knock-down increased NF-κB activation that significantly increased the expression of NF-κB target genes [21].

3.2 Over-Expression of OTUB2 Decreases Caspase 3/7 Activity

The reduced NF-κB activity in cells overexpressing Otub2 may induce anti-apoptotic effects. To test this possibility, we examined Caspase 3/7 activity in Otub2 over-expressing or Otub2-depleted MIN6 cells. As expected, basal Caspase 3/7 activity was significantly decreased (by ~40%) in Otub2-overexpressing cells compared to the control Flag-overexpressing cells (Fig. 2A). In addition, the ~5-fold increase in caspase activity observed upon cytokine treatment in control (Flag over-expressing) cells, was also significantly reduced (~65%) in Flag-Otub2 overexpressing cells (Fig. 2A). Conversely, and consistent with our previous observations [9], silencing of Otub2 in MIN6 cells using siRNAs, significantly increased caspase 3/7 activity, under basal conditions as well as following treatment with cytokines (Fig. 2B).

Even stronger effects were observed in dispersed human islets. An approximate 5-fold decrease in caspase 3/7 activity was observed in human islets that were transfected with the Flag-Otub2 construct, when compared with Flag-transfected islets (Fig. 2C). Similar results were observed upon treatment of dispersed human islets with IFN-γ (Fig. 2C). Conversely, opposite effects were observed in dispersed human islets in which Otub2 has been silenced by siRNA. These islets demonstrated ~2.4-fold increase in caspase 3/7 activity compared to control, mock-transfected cells (Fig. 2D). These results indicate that overexpression of Otub2 decreases caspase 3/7 activity and, therefore, confers a pro-survival effect on human pancreatic β-cells.

3.3 Over-Expression of Otub2 Affects β-Cell Function

To determine whether overexpression of Otub2 affects β-cell function, glucose-stimulated insulin secretion (GSIS) was studied in MIN6 cells. Basal insulin secretion was ~2.5 higher in Flag-Otub2 expressing cells, when compared to controls (Fig. 3A). Glucose challenge (20 mM) increased insulin secretion 2.5-fold in control cells and 2.2-fold in Otub2 expressing cells, resulting in an approximately 2.2-fold higher GSIS in Otub2 expressing cells. These results favor our hypothesis that Otub2 promotes β-cell function, in addition to or independent of its inhibitory effects on NF-κB activity. Next, we assessed the influence

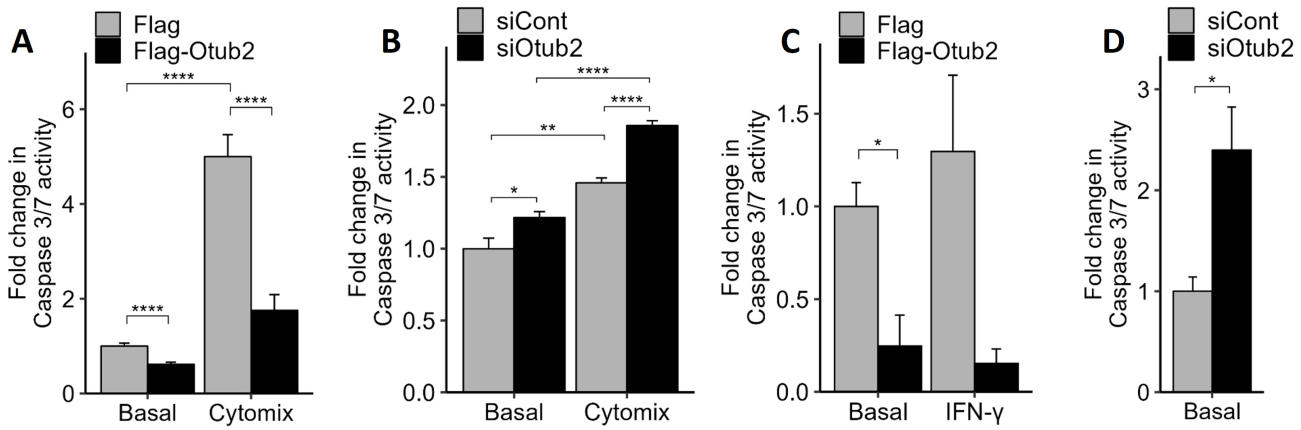

Fig. 2. Effects of Otub2 on caspase 3/7 activity. (A) Control, Flag over-expressing MIN6 cells or Flag-Otub2 over-expressing cells were treated with 1x-cytomix for 24 h (or remained untreated). Apoptosis was assayed by caspase-3/7 activity measurements. (B) MIN6 cells, transfected with the indicated siRNAs, remained untreated or were treated with 1x-cytomix for 24 h. Apoptosis was assayed by caspase-3/7 activity measurements. Control-siRNA transfected cells served as control. (C,D) Dispersed human islets transiently transfected for 48 h with pFlag (control) or pFlag-Otub2 constructs, remained untreated or were treated with IFN-γ (C) or transfected with the indicated siRNAs (D), were assayed for apoptosis by caspase-3/7 activity. Data represent means $\pm$ SEM of five replicates in each of three experiments (A), 4–5 replicas (B), or four replicas of human islets (C,D). In all panels, asterisks indicate the p-values as follows: *$p < 0.05$, **$p < 0.01$, **** $p < 0.0001$ vs. control cells.

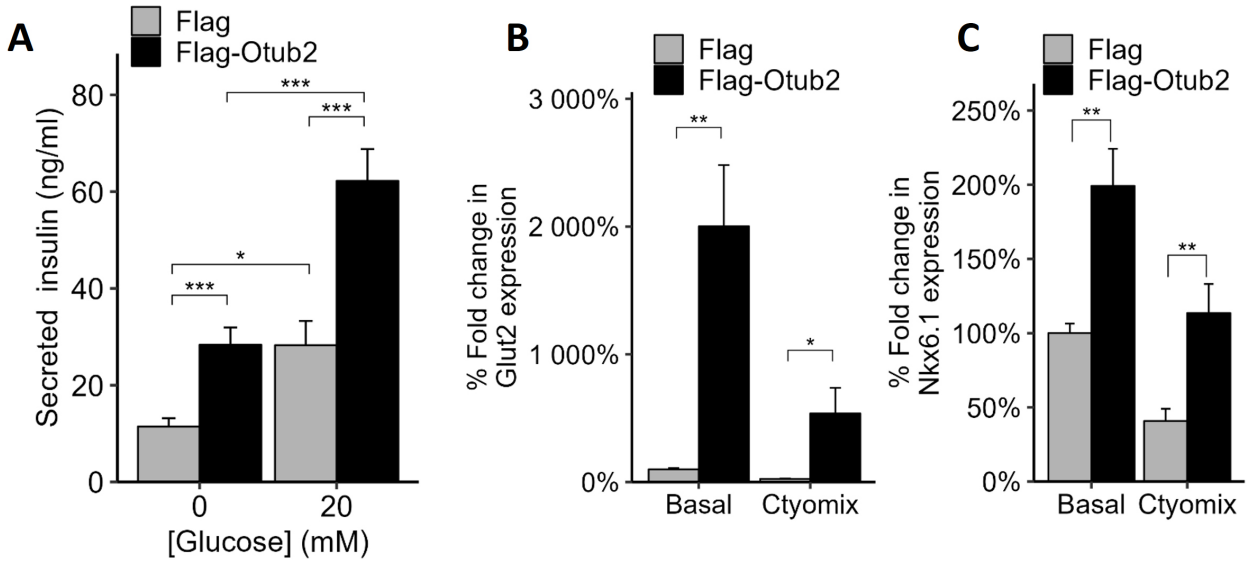

Fig. 3. Effects of OTUB2 stable over-expression on β-cell function. (A) MIN6 cells over-expressing Flag or Flag-Otub2 were incubated for 1 h in Krebs-Ringer bicarbonate HEPES buffer (KRBH) at 37 °C, followed by an additional 1 h incubation in 0 mM or 20 mM glucose. Insulin secretion was then determined using insulin detecting Homogeneous Time-Resolved Fluorescence (HTRF) kit according to the manufacturer's instructions. (B,C) MIN6 cells overexpressing Flag or Flag-Otub2 were treated with 1x-cytomix for 4 h and harvested. Non-treated cells served as the control. Total mRNA was extracted. Quantitative detection of *GLUT2* (B) and *NKX6.1* (C) was carried out by qRT-PCR. mRNA levels were normalized to Actin. Data are means $\pm$ SEM of four replicates in each of four experiments (A), or two replicates in each of three experiments (B,C). In all panels, asterisks indicate the p-values as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

of Otub2 on glucose transporter 2 (Glut2), the glucose transporter mediating glucose uptake in β-cells [23]. As shown in Fig. 3B, overexpression of Otub2 dramatically increased the mRNA levels of Glut2 by approximately 20-fold. A similar increase was found in cells treated with cytokines, which by themselves drastically inhibited Glut2 expression, as previously described [24].

Table 3. Otub2 binding proteins (selected hits from Mass-Spec).

Protein	Gene	Ratio OTUB2/FLAG	Ratio OTUB2/GFP	Function
OTUB2	*Otub2*	145.09	179.16	
PEG3	*Peg3*	Inf	Inf	Acts synergistically with TRAF2 through activation of NF-κB
KCC2D	*Camk2d*	Inf	133.39	Calcium/calmodulin-dependent kinase
KCNS3	*Kcns3*	Inf	29.11	Voltage-gated potassium channel subunit Kv9.3
SSRD	*Ssr4*	Inf	8.37	Binds calcium to the ER membrane and regulates the retention of ER resident proteins
SSRG	*Ssr3*	2.53	3.35	Binds calcium to the ER membrane and regulates the retention of ER resident proteins
AT1A1	*Atp1a1*	Inf	3.62	Sodium/potassium-transporting ATPase
ATPG	*Atp5c1*	Inf	2.25	Mitochondrial membrane ATP synthase
AT2A3	*Atp2a3*	3.09	4.56	Catalyzes the hydrolysis of ATP coupled with the transport of calcium

PEG3, paternally expressed gene 3; TRAF2, TNF receptor associated factor 2; KCC2D, calcium/calmodulin dependent protein kinase II delta; KCNS3, potassium voltage-gated channel modifier subfamily S member 3; SSRD, signal sequence receptor subunit 4; ER, endoplasmic reticulum; SSRG, signal sequence receptor subunit 3; AT1A1, ATPase Na^+/K^+ transporting subunit alpha 1; ATPG, ATP synthase F1 subunit gamma; AT2A3, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 3.

Another gene of interest is NK6 homeobox 1 (Nkx6.1), a known transcription factor required for β-cell development, as well as glucose sensing and insulin secretion [25]. As shown in Fig. 3C, overexpression of Otub2 significantly increased the mRNA levels of Nkx6.1 by approximately 2- and 2.8-fold under basal or cytomix treatments, respectively. Collectively, these findings suggest that overexpression of Otub2 is involved in preserving β-cells functionality, mainly in terms of insulin secretion.

3.4 Otub2 Binding Partners Regulate β-Cell Function

To identify proteins that interact with Otub2, immunoprecipitations (IPs) with Otub2 antibodies were carried out using MIN6 cells transiently overexpressing Otub2 (and appropriate controls), followed by mass spectrometry analysis. Several proteins were enriched at least 100-fold in immunoprecipitates derived from Flag-Otub2 overexpressing cells (Table 3, **Supplementary Table 1**) compared to controls. A similar trend was observed when GFP-Otub-2 Min6 cells were used. Top hits were Peg3 and Camk2d, which enhance the NF-κB pathway and β-cell death [26,27]. We assume that Otub2 deubiquitinates protein complexes containing Peg3 and Camk2d, and thereby might inhibit propagation of the NF-κB cascade that promotes β-cell apoptosis. The third protein, kv9.3, is a potassium channel responsible for repolarization of β-cell membranes after insulin release [28]. Potentially, Otub2 can deubiquitinate and inhibit this channel and thereby prolong insulin release and improve β-cell function.

3.5 Otub2 Affects β-Cell Functionality In Vivo

The effects of Otub2 on β-cell functionality *in vivo* were evaluated next. To this end, we employed a mouse model that harbors either heterozygous ($Otub2^{+/-}$ (or homozygous) $Otub2^{-/-}$(whole-body deletion of the Otub2 gene. Pancreata were isolated from wild-type (WT, $Otub2^{+/+}$) and heterozygous Otub2 knockout mice (het, $Otub2^{+/-}$), and the expression of several NF-κB target genes was evaluated. As shown in Fig. 4A, the expression levels of Otub2 itself were decreased by approximately 2-fold in $Otub2^{+/-}$ mice compared to $Otub2^{+/+}$ mice, confirming the expected partial down-regulation of the Otub2 gene expression in the $Otub2^{+/-}$ animals. Conversely, there was a marked increase in the expression of several NF-κB targets in $Otub2^{+/-}$ mice, including IP-10, MCP-1 and IL-1β, whose expression levels increased by approximately 5-, 6- and 2-fold, respectively (Fig. 4A). These results suggest an increased inflammatory state of the pancreas upon partial Otub2 depletion, supporting the physiological role of Otub2 as a negative regulator of NF-κB activity in pancreatic islets *in vivo*. Immunofluorescence staining of pancreatic sections from those mice confirmed the expected decrease in Otub2 protein levels in the $Otub2^{+/-}$ and $Otub2^{-/-}$ mice (Fig. 4B).

Next, glucose tolerance tests (GTT) were performed in $Otub2^{+/+}$, $Otub2^{+/-}$, and $Otub2^{-/-}$ mice. As shown in Fig. 4C, basal glucose levels of $Otub2^{+/+}$, $Otub2^{+/-}$, and $Otub2^{-/-}$ male and female mice were approximately similar. However, following glucose injection, blood glucose levels, at all-time points, were significantly higher in $Otub2^{+/-}$ and $Otub2^{-/-}$ male mice, when compared to $Otub2^{+/+}$ animals (Fig. 4C). Accordingly, the area under the curve (AUC) was ~40% greater in $Otub2^{+/-}$ and $Otub2^{-/-}$ male mice compared to WT controls (Fig. 4D). The effects of Otub2 deletion on the female mice were a bit more complex. There were no differences in GTT between $Otub2^{+/+}$ and $Otub2^{-/-}$ female mice, but the $Otub2^{+/-}$ animals exhibited a slight, yet significant higher GTT response (Fig. 4C) that was also evident by a modest, yet significant higher (approximately 20%) AUC (Fig. 4D). These

results suggest a positive role for Otub2 in the improvement of islet functionality under physiological conditions in an *in vivo* setting, with possible compensatory pathways in KO female mice.

3.6 Effects of Otub2 Over-Expression In Vivo on Glucose Tolerance of Mice

To complement and validate the above findings that were based upon Otub2 KO mouse models, we used a second, independent model in which Otub2 was selectively overexpressed in the pancreas of C57BL wild-type mice. To introduce the Otub2 gene into the pancreas, we made use of adeno-associated viral constructs (AAVs) that are considered a favorable delivery system to express exogenous proteins due to their low immunogenicity and excellent safety profile [29]. C57BL wild-type mice were subjected to pancreatic intraductal administration of AAV8 constructs containing Flag (control) or Flag-Otub2 sequences (**Supplementary Fig. 2A**). As shown in **Supplementary Fig. 2B**, islets derived from mice, subjected to intraductal injection of AAV8-Flag-Otub2, showed a strong fluorescent signal, while a much weaker signal was observed in mice injected with AAV8-Flag viral control vector. We further validated these findings by extracting RNA from the pancreases and performing qRT-PCR (**Supplementary Fig. 2C**). These results verified the efficacy of increasing Flag-Otub2 expression in pancreases, by intra-ductal AAV8 injection.

Glucose-tolerance tests in these mice revealed that basal glucose levels in AAV8-Flag-Otub2 mice were almost identical to the AAV8-Flag control animals, however, blood glucose levels of Flag-Otub2 mice were significantly lower (approximately 30%) at 15–45 minutes post glucose injection (Fig. 5A). Accordingly, the AUC decreased by 20% in AAV8-Flag-Otub2 injected mice compared with control animals (Fig. 5B). These findings further support our conclusion that Otub2 exerts favorable functional effects on β-cells *in vivo*.

3.7 Deletion of Otub2 Affects Pancreatic Gene Expression Patterns

To reveal the genes whose expression is affected upon Otub2 deletion, RNA was extracted from the pancreases of Otub2$^{-/-}$, Otub2$^{-/+}$, and Otub2$^{+/+}$ (WT) mice, and RNAseq analysis was performed. The expression levels of Otub2 were indeed below detection in most Otub2$^{-/-}$ and Otub2$^{+/-}$ pancreases (**Supplementary Fig. 3A**). Both heterozygous and homozygous pancreases showed significantly different patterns of gene expression compared to WT pancreases, as shown by the volcano plots (**Supplementary Fig. 3B**). Of note, more than 20 genes were significantly up- or down regulated, both in heterozygous and homozygous mice when compared to wild-type animals (**Supplemental Table 2**). Enrichment analysis of curated signatures employing the MsigDB database and the Camera method [30] revealed several gene-sets that were significantly upregulated (**Supplementary Fig. 3C**). Most significant were the MOOTHA_VOXPHOS gene families [31], involved in oxidative phosphorylation. Other relevant families were WANG_NFKB_TARGETS, which includes NF-κB target genes [32]; as well as REACTOME_DIABETES_TARGETS [33], and ALCALA_APOPTOSIS [34]; all gene-sets expected to be upregulated upon depletion of Otub2.

To gain a deeper insight into the gene networks affected by Otub-2 depletion, pathway enrichment analysis was carried out using the GO database. Such analysis revealed that potassium ion transport was significantly affected in Otub-2$^{-/-}$ and Otub-2$^{-/+}$ mice (Fig. 6A,B), with several genes related to this pathway being down-regulated in OTUB2 KO animals (Fig. 6B). One is Ank2 that encodes the ankyrin-2 (AnkB) protein, which is essential for localization and membrane stabilization of potassium channels. Other relevant genes were (potassium voltage-gated channel subfamily C member 3) KCNC3, encoding a voltage-dependent potassium channel involved in insulin secretion [35], and Cacna1a, encoding the α1A pore-forming subunit of the neuronal channel P/Q, which is a member of the voltage-gated calcium channels superfamily.

Conversely, genes related to oxidative phosphorylation were mostly upregulated in heterozygous and homozygous Otub2 KO mice (Fig. 6A,C), in accordance with the increased enrichment of the MOOTHA_VOXPHOS gene-set (**Supplementary Fig. 3C,D**). Independently, STRING analysis revealed a cluster of oxidative phosphorylation-related genes among those genes that were significantly upregulated in pancreata of Otub-2$^{-/+}$ mice (Fig. 6D).

Given that β-cells are highly susceptible to oxidative stress [36,37], the increase in oxidative phosphorylation might be associated with β-cells dysfunction and apoptosis observed upon Otub2 depletion. Of note, the ATP-dependent potassium channels, discussed above, are specific targets of oxidative damage [38]. Indeed, the upregulated genes in Otub2 KO pancreases, related to oxidative phosphorylation, were almost exclusively encoding for components of the electron transport chain (ETC) complexes in the mitochondria (Fig. 6C). Conversely, the highest upregulated gene in this pathway, Cox4i1, was previously shown to be upregulated in type 2 diabetic mice [39]. This suggests that oxidative stress is a potential trigger of the impaired β-cells function observed in the Otub2 KO mice.

The alterations in the transcriptomic landscape of pancreata depleted of Otub2 were quite unique, as largely different alterations in transcriptomic landscapes were observed in livers of heterozygous and homozygous Otub2 KO mice when compared to livers of WT animals (**Supplementary Fig. 4A,B**). Still, pathway enrichment analysis revealed that oxidative phosphorylation genes, as well as components of the ETC complexes in the mitochon-

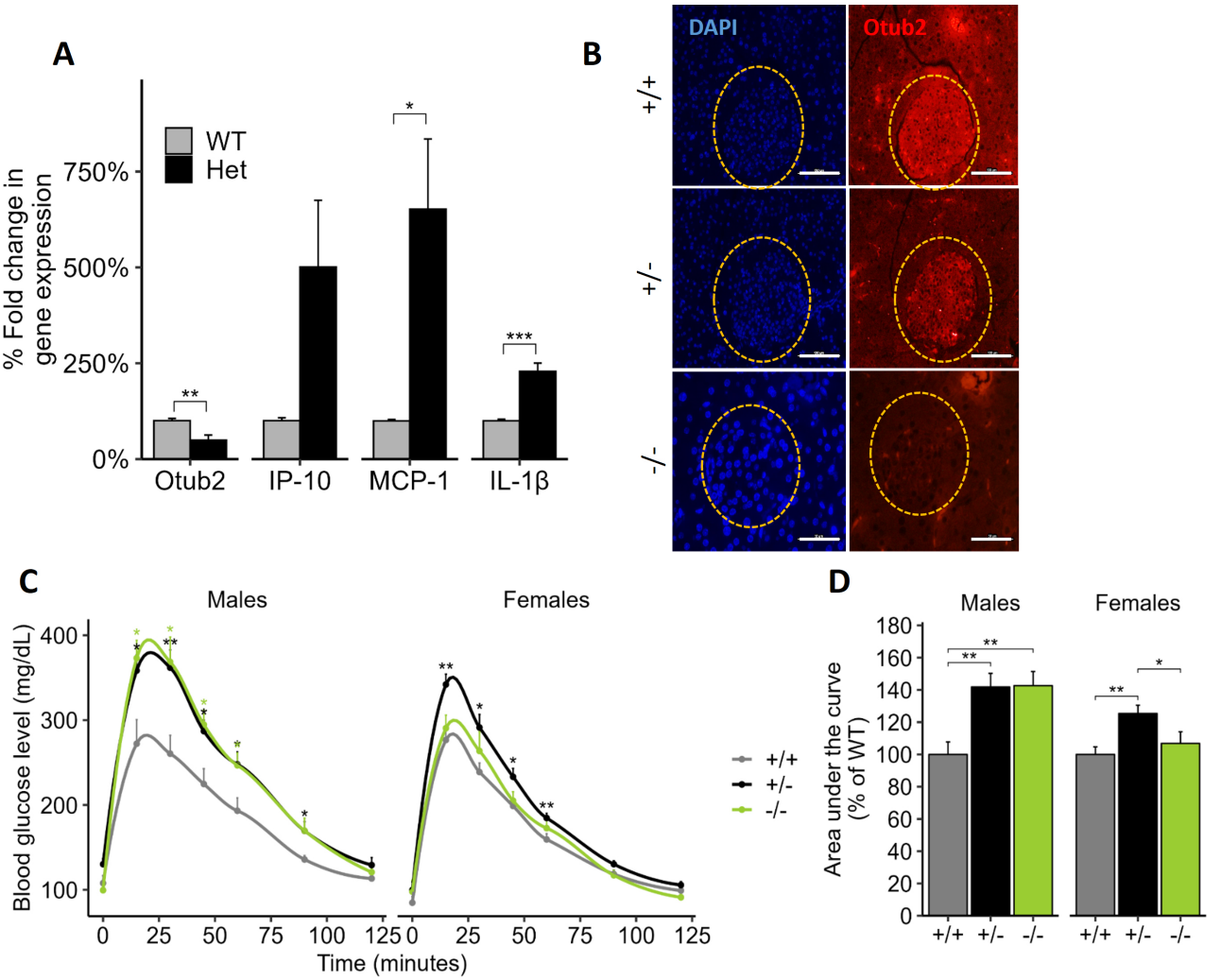

Fig. 4. Effects of OTUB2 knockdown on pancreatic NF-κB activity *in vivo*. Pancreata from Otub2$^{+/+}$ (wild-type (WT)) and Otub2$^{+/-}$ (het) female and male mice were excised. (A) Total mRNA was extracted, and qRT-PCR was conducted. mRNA levels of NF-κB target genes were normalized to Actin. Data is Otub2$^{+/+}$ n = 5; Otub2$^{+/-}$ n = 5 (2 males and 3 females per group). Data represent means ± SEM of three experiments. Asterisks indicate the *p*-values: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. Otub2$^{+/+}$ mice. (B) Pancreas sections were stained for Otub2 and DAPI. Scale bar = 100 µm. (C,D) Otub2 knock-out mice are glucose intolerant. Male and female mice at 8 weeks of age were subjected to an intraperitoneal (i.p.) glucose tolerance test (GTT; 1.8 g D-glucose per kg body weight) after overnight fasting. Blood samples were taken at the indicated time points (0'–120') and glucose levels were determined by a glucometer (C). A graph representing the area under the curve of the GTT is indicated as (D). Data represent means ± SEM of six experiments. Mice numbers: Males: Otub2$^{+/+}$ n = 7; Otub2$^{+/-}$ n = 12; Otub2$^{-/-}$ n = 8. Females: Otub2$^{+/+}$ n = 8; Otub2$^{+/-}$ n = 10; Otub2$^{-/-}$ n = 8. Asterisks indicate the *p*-values: *$p < 0.05$, **$p < 0.01$.

dria, were enriched in livers as well (**Supplementary Fig. 4C**). Although these hepatic genes were not upregulated to the same extent as they did in the pancreas (Fig. 6C, **Supplementary Fig. 3D**), our results suggest that knockdown of Otub2 ubiquitously upregulates genes involved in oxidative phosphorylation and mitochondrial ETC.

4. Discussion

We have previously shown that down-regulation of Otub2 by siRNAs in MIN6 cells and human islets increased caspase-3/7 activity, reduced GSIS, and elevated expression of NF-κB target genes, both under basal levels and following cytokine treatment [9]. This implicates Otub2 as a potential physiological regulator of β-cell survival. In the present study, we employed animal models to strengthen these findings and reveal new aspects of Otub2 action as an inhibitor of NF-κB signaling and as a pro-survival protein of human β-cells that promotes β-cell functionality *in vivo*.

Several lines of evidence support this conclusion. First, we showed that overexpression of Otub2 decreases NF-κB activity and expression of its target genes MCP-1 and iNOS. The increased expression of these genes was

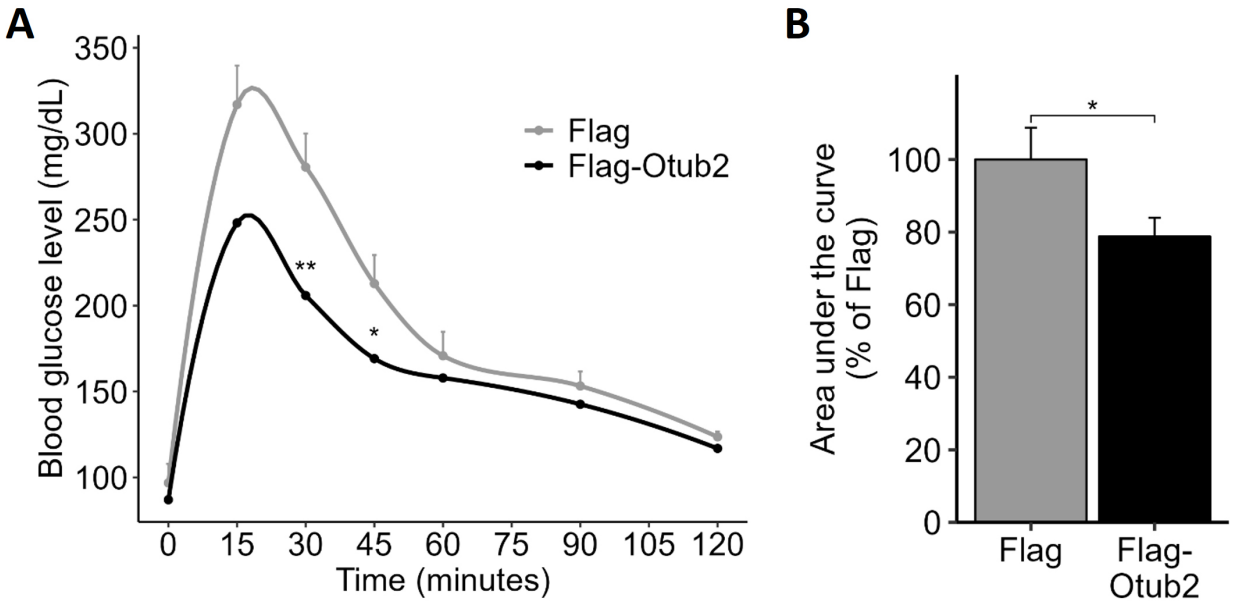

Fig. 5. Effects of intra-ductal injection of AAV8-Flag-Otub2 vector on glucose tolerance. (A) C57BL WT, 10 weeks old, male mice, were anesthetized and subjected to retrograde pancreatic intraductal AAV administration. After two weeks of recovery and overnight fasting, mice were subjected to an intraperitoneal (i.p.) glucose tolerance test (GTT; 1.8 g D-glucose per kg body weight). Blood samples were taken at the indicated time points (0–120 minutes), and glucose levels were determined by a Glucometer. (B) Area under the curve of the GTT. Data represent means ± SEM of five experiments. *T*-test was used to measure the *p*-values: **p*-value < 0.05, ***p*-value $<$ 0.01 of AAV8-Flag mice compared to AAV8-Flag-Otub2 mice. Mice numbers: n = 5 AAV8-Flag mice; n = 7 AAV8-Flag-Otub2 mice.

also observed in pancreatic sections derived from Otub2-knockout mice, thus highlighting the physiological relevance of these findings. Given that NF-κB signaling promotes apoptosis of pancreatic β-cells [40,41], it was of no surprise that overexpression of Otub2 inhibited cytokine production and Caspase 3/7 activity in β-cell lines and human pancreatic islets, while inhibition of Otub2 expression exerted opposite effects.

At the molecular level, earlier work has shown that the level of ubiquitination of TRAF6, which is involved in cytokine-induced NF-κB activation, is elevated in MIN6 cells upon Otub2 silencing [9,42]. Additional studies showed that Otub2 is a negative regulator of type I IFN-γ induction through deubiquitination of TRAF3 and TRAF6 [43]. Thus, our working hypothesis predicts that K63-deubiquitination of TRAF6, induced by Otub2, inhibits cytokine-induced K48-ubiquitination and degradation of IkB. This inhibits propagation of NF-κB signaling and the expression of NF-κB target genes such as MCP-1, IP-10, IL-1β, and iNOS [44,45], which contribute to β-cell demise.

The physiological relevance of Otub2 as a regulator of β-cell function was established using animal models. We showed that partial or complete knockdown of the Otub2 gene, mainly in male mice, significantly impairs their glucose tolerance. Interestingly, while depletion of Otub2 in heterozygous mice resulted in impaired GTT, in homozygous mice the effect was evident only in male mice, suggesting a yet unknown, gender-specific compensatory effect that affects only female homozygous knockout animals.

Several mechanisms could account for the impaired glucose tolerance in Otub2$^{+/-}$ and Otub2$^{-/-}$ animals. Most likely, these effects could be attributed to the inhibitory effects of Otub2 on NF-κB activity as discussed above. We found that Otub2 forms complexes with Peg3 and Camk2d, both enhancers of the NF-κB pathway and β-cells death [26,27]. Peg3, a regulator of TNF response, acts synergistically with TRAF2 to activate the NF-κB signaling pathway [26]. Hence, deubiquitination of Peg3 by Otub2 might inhibit NF-κB signaling in response to cytokines. Another key player in this process might be the calcium/calmodulin-dependent kinase (CaMKIID) that forms complexes with Otub2. CaMKIID phosphorylates Bcl-10 during activation of the NF-κB pathway. Ubiquitination of phospho-Bc1-10 promotes its interaction with Malt-1, which binds TRAF6 [46], another Otub2 target [9]. Hence, complex formation between Otub2 and CaMKIID might facilitate Bcl-10-CaMKIID interaction and prevent its ubiquitination by TRAF6 and termination of NF-κB signaling. The specific effect of Otub2 on its binding partners and the downstream result of this binding on β-cells function requires further studies.

Yet, Otub2 seems to exert additional effects that might be only partially related to NF-κB signaling. Overexpression of Otub2 significantly increases expression of Glut2,

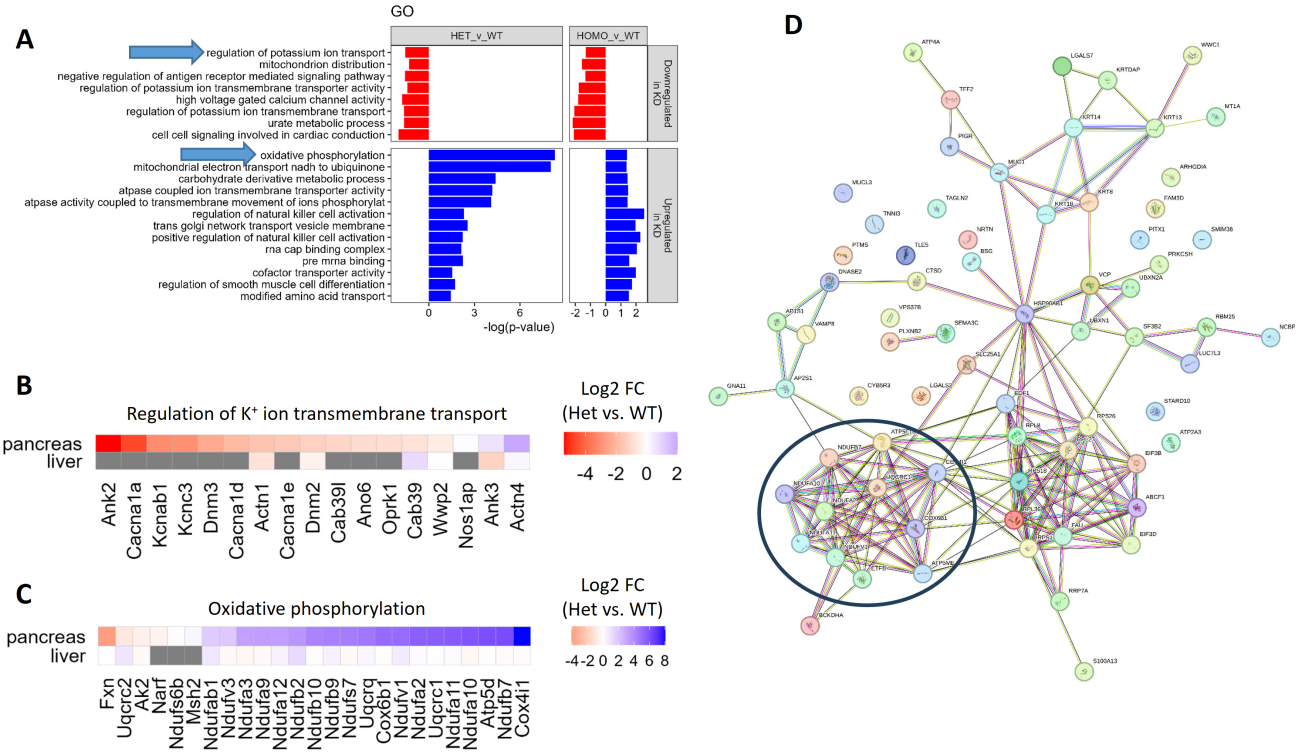

Fig. 6. RNA-seq analysis of pancreas from OTUB2 KO mice. Pancreases and livers were extracted from OTUB2$^{+/+}$ (WT, n = 3), OTUB2$^{+/-}$ (Heterozygotes, n = 5) and OTUB2$^{-/-}$ (Homozygote, n = 3) mice. RNA was extracted, and RNA-seq was performed to determine differential gene expression between the homozygotes/heterozygotes and the WT mice. (A) Geneset enrichment of gene ontology (GO) terms, performed by the CAMERA method. Arrows indicate interesting GO terms. Analysis is based on gene expression in the pancreas. (B,C) Heatmaps representing the fold change (log2) in gene expression between heterozygotes and WT mice in the pancreases and livers. Genes related to potassium ion transport (B) and oxidative phosphorylation (C) are taken from the respective GO terms. (D) STRING analysis of genes significantly upregulated in Otub2$^{+/-}$ vs. Otub2$^{+/+}$ pancreases. The black circle depicts a cluster of genes associated with oxidative phosphorylation.

the major glucose transporter of β-cells, which is essential for insulin secretion stimulated by glucose [23]. Further, overexpression of Otub2 upregulates the expression of Nkx6.1, a transcription factor essential for maintaining the functional and molecular traits of mature β-cells (e.g., insulin biosynthesis and insulin secretion [47]).

The beneficial effects of Otub2 under physiological conditions were further illustrated when its overexpression in beta cells was studied. We found that overexpression of Otub2 promotes insulin secretion in cultured β cell lines, but even more relevant, we found that mice subjected to intra-ductal injection of AAV8-Flag-Otub2 vectors showed significantly lower (~30%) blood glucose levels, when compared with control animals, following glucose injection. Accordingly, the AUC decreased by 20% in the AAV8-Flag-Otub2 injected mice compared to the controls. These findings further support our hypothesis that Otub2 exerts favorable functional effects on β-cells *in vivo*.

A broader perspective concerning the physiological functions of Otub2 was gained by analyzing alterations in the transcriptomic landscape of pancreata depleted of Otub2. While the use of whole-body depletion is a limitation of this study, it is obvious that the effects of Otub2 on the transcriptomic landscape seem to be specific, as different gene sets are affected when we compare liver to pancreata in wild type vs. Otub2 knockout animals.

Concerning the pancreas, the analysis highlighted a significant reduction in the expression of genes that regulate potassium ion channel transport, which directly influences insulin secretion [48]. Of interest, one of these family members, Kv9.3, which is expressed in human pancreatic islets [28], forms complexes with Otub2. This channel is involved in repolarization of excitable cells; blocking the activity of this delayed-rectifier potassium channel is expected to increase intracellular free calcium and promote GSIS [28]. Potassium channels need to be closed for proper insulin secretion, in a process initially mediated by ATP [48]. Therefore, activating mutations in potassium channels may induce adult and neonatal diabetes [49]. Indeed, impaired expression of the potassium channel regulator AnkB, observed in Otub2 KO mice, impairs insulin secretion and induces diabetes [50]. Similarly, downregulation of the dy-

namin genes Dynamin 2 (DNM2) and Dynamin 3 (DNM3) [51], observed in Otub2 KO mice, attenuates internalization of potassium channels on one hand, while inhibiting exocytosis of insulin granules [52,53]. Hence, maintenance of higher concentrations of potassium channels at the plasma membrane of Otub2 KO mice might impair glucose-stimulated insulin secretion, which is the characteristic feature of these animals. This model, linking Otub2 to GSIS via regulation of potassium channels, still merits further experiments for its establishment.

In line with this hypothesis, knockdown of Otub2 promotes transcription of oxidative phosphorylation pathways, with the NDUF (NADH:ubiquinone oxidoreductase subunits) gene-family members being the targets. These proteins are components of complex I in the mitochondrial electron transport chain [54]. Hence, increased ATP production in the KO animals might be a compensatory mechanism utilized by these mice to foster closure of the highly abundant potassium channels and thus promote insulin secretion. Yet, overexpression of NDUF gene-family members, particularly when it disrupts the balance of mitochondrial complex 1 activity, can induce oxidative stress, which is detrimental for β-cell function and survival [25]. It is well established that β-cells are sensitive to oxidative stress due to their low antioxidative capacity [36]. Hence, overexpression or mutation of the NDUF gene-family members, such as NDUFA11, might disrupt complex I formation, impairing mitochondrial structure and increasing ROS levels [55]. Furthermore, it has been previously shown that Otub2 silencing in ovarian cancer stabilizes sorting nexin 29 pseudogene 2 (SNX29P2), which inhibits hypoxia-inducible factor-1 alpha (HIF-1α) from von Hippel-Lindau tumor suppressor-mediated degradation. HIF-1α induces the transcription of Carbonic anhydrase 9, promoting glycolysis and progression of ovarian cancer [56]. Hence, reprogramming and activation of ATP-generating systems might be a common outcome of Otub2 silencing. Yet, Otub2 silencing might exert opposite effects in some model systems. For example, Otub2 depletion reduces glucose consumption, lactate production, and cellular ATP production in colorectal cancer cells [57]. Similarly, decreased expression of MOOTHA_VOXPHOS genes contributes to the development of type-2 diabetes in human muscles [31]. Hence, the effects of Otub2 on oxidative phosphorylation and ATP production might well be tissue-specific. The wide differences in gene expression profiles between the liver and pancreas of Otub2 KO mice support this assumption.

5. Conclusion

Our findings highlight the role of Otub2 as an anti-apoptotic regulator of β-cell survival that preserves their mass and functionality. Otub2 inhibits NF-κB signaling and cytokine production, downregulates membrane potassium channels, promotes insulin secretion, and reduces oxidative stress. In doing so, Otub2 may protect β-cells from cytokine-induced apoptosis and sustain insulin secretory capacity. This raises the possibility of developing novel therapeutic strategies that target Otub2 and increase its expression for the benefit of diabetic patients.

Disclosure

The paper is listed as, "The Deubiquitinating Enzyme Otub2 Modulates Pancreatic Beta-Cells Function and Survival" as a preprint on bioRxiv at: https://www.biorxiv.org/content/10.1101/2024.09.30.615641v1.

Availability of Data and Materials

The RNAseq generated during the current study is available in the Gene Expression Omnibus database under accession number GSE285973, and can be viewed in: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE285973. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

MO, RI, SB-H, EC and YV performed the studies and analyzed the data. SS, SL, FB, YV, and YZ contributed to the design of the experiments and drafting the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All *in-vivo* experiments were carried out in accordance with ARRIVE guidelines. Experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science (application number 23980116-3). Human islets studies received Ethics approval from the Bioethics and Embryonic Stem Cell Research Oversight Committee (ESCRO) of the Weizmann Institute of Science (date: 21 May 2015). The study was conducted in accordance with the principles of the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL44406.

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