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Abstract

Proinsulin is a misfolding-prone protein and its efficient breakdown is critical, when β-cells are confronted with high insulin biosynthetic demands, to prevent endoplasmic reticulum stress, a key trigger of secretory dysfunction and, if uncompensated, apoptosis. Proinsulin degradation is thought to be performed by the constitutively expressed standard proteasome, while the roles of other proteasomes are unknown. We recently demonstrated that deficiency of the proinsulin chaperone GRP94 causes impaired proinsulin-handling and defective insulin-secretion associated with a compensated endoplasmic reticulum stress-response. Taking advantage of this model of restricted folding-capacity, we investigated the role of different proteasomes in proinsulin degradation, reasoning that insulin secretory dynamics require an inducible protein degradation-system.

We show that expression of only one enzymatically active proteasome subunit, namely the inducible β5i-subunit, was increased in GRP94 CRISPR/Cas9 KO cells. Additionally, the level of β5i containing intermediate proteasomes was significantly increased in these cells, as was β5i-related chymotrypsin-like activity. Moreover, proinsulin levels were restored in GRP94 KO upon β5i siRNA-mediated knock down. Finally, the fraction of β-cells expressing β5i subunit is increased in human islets from type 2 diabetes patients. We conclude that β5i is an inducible proteasome subunit dedicated to the degradation of mishandled proinsulin.

1. Introduction

The insulin secretory dynamics and associated transients in proinsulin biosynthesis endanger the pancreatic β–cells for the detrimental consequences of protein misfolding. Even in normal conditions 20 % of proinsulin is misfolded, implicating that β–cells critically depend upon a highly flexible protein degradation system to avoid endoplasmic reticulum (ER) stress, cellular dysfunction and death (24). Misfolded proinsulin is disposed from the ER through the Endoplasmic-Reticulum-Associated protein Degradation (ERAD) pathway with final proteolysis performed by the proteasome (10). There are four major types of proteasomes that differ in composition of β subunits forming the enzymatically active inner rings of the 20S proteasome core: the constitutively expressed standard proteasome (composed of β1, β2, β5; s-proteasome), the immunoproteasome (β1i, β2i, β5i), the intermediate proteasome (containing a combination of standard and inducible β subunits; int-proteasome) and the thymus-specific proteasome (containing β5t subunit) (17). Inducible subunits are constitutively expressed not only in immune cells but also in the pancreatic islets, as reported recently by us and the Human Protein Atlas database (11, 26). Expression of the inducible subunits in β-cells is further upregulated by cytokines e.g. INFγ/β and IL-1β (6, 11, 15) during viral infections (16), and their constitutive expression has also been reported in e.g. heart and liver where they form int-proteasomes constituting 1-50% of total proteasomes of a cell (4, 8, 21). However, the role of non-standard proteasomes in proinsulin degradation has not been investigated. Most studies of proteasome biology employ broad-spectrum proteasome inhibitors such as MG132 and lactacystin (9, 13) that cannot clarify the roles of individual proteasome subunits in protein degradation. Moreover, β-cell investigative models have focused on the fate of *mutated* e.g. AKITA proinsulin and not on misfolded *wild-type* proinsulin, as it has been assumed that the relevant degradation mechanisms are similar if not identical (9).

We recently showed that Glucose Regulated Protein 94 (GRP94), an ER resident chaperone, is critical for proinsulin handling. Deficient GRP94 activity results in substantial loss of intracellular proinsulin and a reduction in insulin secretion (7). As the loss of proinsulin in GRP94 knockout (KO) cells occurs at the level of ER (7), we hypothesized that mishandled proinsulin becomes an ERAD and proteasome substrate.

We therefore examined the activity and composition of proteasome(s) in GRP94 KO clonal cell lines derived from β-cell line Ins1-E and showed that these cells specifically overexpress the inducible β5i proteasome subunit that is incorporated into enzymatically active proteasomes. Furthermore, siRNA-based reduction of β5i expression increased intracellular levels of proinsulin. Finally, we demonstrated that the β5i subunit is expressed in an increased fraction of β-cells from type 2 diabetes patients.

2. Methods

Cell culture Ins1-E GRP94 KO cells were generated as described in (7). The cells were grown in RPMI-1640 (Life Technologies, Naerum, Denmark) supplemented with 10 % fetal bovine serum (FBS), 1 % Penicillin/Streptomycin (P/S), 10 mM N-2-hydroxyehtylpiperazine-N-2-ethane sulfonic acid (HEPES**),** 50 µM β-mercaptoethanol and 1 mM sodium pyruvate, on fibronectin coated plasticware. The GRP94 inhibitor PU-WS13 (GRP94i) was described in (19) and used as in (7).

Western Blotting Cells were lysed and protein concentration calculated as in (7). Samples were run on Nu-Page 4-12 % bis-tris gels (Thermo Fisher Scientific, Hvidovre, Denmark), transferred to PVDF membranes before an overnight incubation with primary antibodies (Abcam: β5i ab183506, β2i ab3329, β1i ab243556; Sigma: β1 HPA029635, β2 HPA026322, β5 SAB210895, Thermo Fisher scientific: GRP94 MA3-016, Santa Cruz biotech: Tubulin T6074; Cell Signaling:

Insulin 8138S). Blots were developed using chemiluminescence and captured using the Azure®Saphire Biomolecular Imager. Quantification of Western blots was by ImageJ software (v.1.52a, (22)).

Proteasome activity assay GRP94 KO and control cells were plated in 96-well plates, treated with 50 nM ONX-0914 (Selleck Chemicals, Rungsted, Denmark, IC50:~10 nM for β5i, (18)) or vehicle-containing control medium for 2 h prior to experiments. As previously described (15) chymotrypsin-, trypsin- and caspase-like activities were measured through luminescent assay using Proteasome-GloTM Assay (Promega, Nacka, Sweden). Statistical analysis was done on the average of each biological triplicate each point represented by a technical replicate.

Proteasome Mass-spectrometry analysis Cells were grown to 90 % confluence, washed with Hank's Balanced Salt Solution (HBSS) before incubation with culture media supplemented with 0.1 % formaldehyde for cross-linking for 15 minutes. Next, 125 mM glycine was added for 10 minutes at 37°C to quench the formaldehyde. The cells were then washed with HBSS, centrifuged and pellets stored at -80°C. Immuno-purification of the proteasomes from the *in vivo* cross-linked lysates, liquid chromatography mass spectrometry (LC-MS/MS) analysis, protein identification, validation and quantifications were performed as previously described (2, 4). Briefly, proteasomes were purified by incubating the lysates with CNBr sepharose beads (GE Healthcare) covalently bound to the antibody specific for the α2 subunit of the proteasome (MCP21), using 150 million cells per 50 mg of grafted beads. Two additional cycles of purification were conducted, re-incubating the collected supernatant with antibody-grafted beads. All fractions were pooled and LC-MS/MS analysis performed.

Single-Cell RNA Sequencing of Pancreatic Islets Pancreatic islet cell subpopulations were analyzed for *β5i, β1i* and *β2i* expression with human islet single-cell sequencing data (23). FastQ

files were downloaded from ArrayExpress (accession: E-MTAB-5061). Data was analyzed with bcbio nextgen (https://github.com/chapmanb/bcbio-nextgen), using the hisat2 algorithm (12) to align reads to human genome version hg38 and the Salmon algorithm (20) for quantitation of gene counts.

β5i siRNA-mediated knockdown GRP94 KO cells were grown in 6-well plates to 80 % confluence and transfected with β5i-directed SMART-POOL siRNA (Dharmacon®, Cambridge, Great Britain), using the Lipofectamine 3000 transfection kit according to manufacturer's protocol (Thermo Fisher Scientific, Hvidovre, Denmark). Supernatants were collected for measurements of proinsulin and insulin by ELISAs (Cat. #10-1232-01 and 10-1250-01, Mercodia, Uppsala, Sweden) according to manufacturer's protocols. Cell viability assay was performed using the staining reagent AlamarBlue (Life Technologies, Naerum, Denmark) added to the cell culture for 4h, incubated at 37^{o}C. The resulting fluorescence was read at 570 nm and 600 nm (reference) on a plate reader. Thapsigargin was used at the dose of 0.1 μM for 24 h.

Real-time Quantitative PCR RNA was isolated using the NucleoSpin® RNA kit (Macherey-Nagel, Bethlehem, USA), followed by cDNA synthesis (iScripttm cDNA Synthesis Kit BioRad, Copenhagen, Denmark) and quantification using SYBR® Green master mix (Life Technologies Naerum, Denmark). The primers used: PSMB8 (F: CCAGGAAAGGAAGGTTCAGAT, R: ATCTCGATCACCTTGTTCAC); ins-1 (F: GGGGAACGTGGTTTCTTCTAC, R: CCAGTTGGTAGAGGGAGCAG); ins-2 (F: CAGCACCTTTGTGGTTCTCA, R: CACCTCCAGTGCCAAGGT); HPRT1 (F: GCAGACTTTGCTTTCCTT, R: CCGCTGTCTTTTAGGCTT); β-actin (F: CACCCGCGAGTACAACCTTC, R: CCCATACCCACCATCACACC); PPIA (F: AGCACTGGGGAGAAAGGATT, R: GATGCCAGGACCTGTATGCT). The best reference genes for normalization were determined as

147 in (1), and normalization was carried out using the 2^(-ΔCT) method to provide the relative gene
148 expression levels.

149 **Donor human islets** were received at 90 % purity from the European Consortium for Islet
150 Transplantation (ECIT). Donor information: female, 56 y.o., BMI 19.4, cause of death was cerebral
151 bleeding, blood group A+, HLA (A:B) 1,29 : 18,57, HLA (DR) 11,11, cold ischemia time 4 h, time
152 for islets isolation to shipment 16 h, estimated purity 80 %. After a 24 h pre-incubation in RPMI
153 medium supplemented with 10 % FBS, 1 % P/S and 5.6 mM glucose, 500 islets per experimental
154 condition were transferred to RPMI medium supplemented with 1 % HS and 24 h later experiments
155 were performed.

156 **Statistical analysis** Quantified data of Western blots and proteasome assays were assessed by
157 Bonferroni corrected two-tailed Student's t-test. GraphPad Prism (v. 7.04, La Jolla, CA, USA) was
158 used for all statistical analyses and data presentation. Data is presented as means ± SD with p-
159 values of ≤0.05 considered significant.

160 **3. Results**

161 **Increased expression and activity of β5i in GRP94 KO cells**

162 To investigate the expression of enzymatically active proteasome β subunits, we analyzed protein
163 contents of GRP94 KO and control clonal cell lines. We found all standard and inducible β subunits
164 expressed to varying degrees in the tested cells (Fig. 1) but only the β5i subunit was expressed
165 significantly more in GRP94 KO cells compared to controls (Fig. 1A-B). β5i mRNA was
166 upregulated in a similar fashion in GRP94 KO cells (Fig. 1C).

167 Next, we measured levels of overall substrate-specific proteasome activities. GRP94 KO cells
168 showed increased chymotrypsin-like activity (characteristic for β5, β5i, β1i subunits (25)) compared
169 to the control group while the other substrate-specific activities remained unchanged (Fig. 1F).

Treatment of cells for 2 h with 50 nM of ONX-0914, a β5i specific inhibitor, reduced chymotrypsin-like activity in the GRP94 KO to the levels observed in control cells treated with ONX-0914, indicating that increase in this type of activity is β5i dependent (Fig. 1F).

Increased incorporation of β5i subunit into active proteasomes in GRP94 KO cells

Next, we investigated the cellular composition of proteasomes through immunoprecipitation (IP) of the 20S α2 proteasome subunit followed by LC-MS/MS analysis. We identified the presence of two types of proteasomes: the s-proteasome and int-proteasome, containing β1, β2, β5 and β1, β2, β5i subunits respectively, with no other detectable inducible subunits incorporated (Fig. 2A). The s-proteasome was the dominant form present in control (83.2 %) and GRP94 KO cells (79.9 %). Int-proteasome represented 16.8% of proteasomes in control group while its presence was increased to 20.1% in GRP94 KO cells. Furthermore, we identified a significant increase in the 11S regulatory particles (PA28αβ and PA200), known to be part of the immunoproteasomes (3), in active proteasomes of GRP94 KO cells (Fig. 2B).

Inhibition of β5i activity increases proinsulin levels in GRP94 KO cells

As β5i was the only proteasome subunit upregulated in GRP94 KO cells and incorporated into active proteasomes to a higher degree, we evaluated the possible role of the β5i subunit in proinsulin degradation in GRP94-deficient cells. β5i was knocked down with three individual siRNAs (Fig. 2C). Concurrently, levels of intracellular proinsulin and mature insulin increased as compared to siRNA control treated cells (Fig. 2D) with no apparent upregulation in *Ins1-2* genes transcription (Fig. 2E). The observed increase was accompanied by higher amount of constitutively secreted proinsulin but not insulin (experiment was performed in 2 mM glucose containing media; Fig. 2F). Finally, β5i KD induced partial loss of cell viability (Fig. 2G).

β5i expression is increased upon glucose stimulation and GRP94 ATP-ase inhibition

In order to mimic increased insulin demand conditions and increased proinsulin production, Ins1-E cells and human islets were cultured in the presence of 20 mM glucose. As expected, intracellular proinsulin levels increased accompanied by the smaller increase in insulin levels, presumably because mature insulin is secreted at 20 mM glucose concentration. After 24 and 48 h we observed 10-25 % increase in the β5i expression (Fig. 3A, n=3 and 3B, n=1), induced further by the addition of 5-20 μM of GRP94i (Fig. 3C; n=1). Off note, proinsulin and insulin levels were diminished after treatment with GRP94i, as reported previously (7). At this point, human islets results should be taken with a reservation as they represent only a single experiment with a single islet donor.

β5i expression is increased in pancreatic islet cells in type 2 diabetes

To investigate whether inducible β subunits are overexpressed in islets of type 2 diabetes (T2D) patients, we analyzed RNA-sequencing data of single-cells dispersed from pancreatic islets (23). We found that 20 % more of α-, and 40 % more of β- and δ-cells from T2D patients tested positive for expression of the β5i subunit compared to non-diabetic controls (Fig. 4A). Similarly, 10 % more of all islet cell-types were β1i-positive in T2D compared to control cases (Fig. 4B) with no significant changes observed for β2i (Fig. 4C). When β5i- (and β2i-) positive cells were analyzed for expression levels of β subunits, we found no significant changes (Fig 4D) between control and T2D cases. The β1i subunit exhibited lower expression in T2D cases but significance was reached only in α-cells (Fig. 4E).

Next, we analyzed the inter-dependence between GRP94 and β5i expression and found statistically significant correlation only in β-cells but the correlation was weak (R2=0.2) indicating that only a fraction of β-cells exhibited the inter-dependence in expression of these genes (Fig. 4G-I).

4. Discussion

Here, we report that the expression of the inducible proteasome subunit β5i was significantly higher in GRP94 KO cells, a β-cell model of restricted ER folding capacity and proinsulin mishandling, and in islets of T2D patients. Moreover, we have shown that proteasomes containing the β5i subunit are engaged in proinsulin degradation.

Misfolded proinsulin has been thought to be degraded by the s-proteasome but the experimental procedures employed have not been able to distinguish proteasome subtypes involved in the process (9, 10, 13). Low levels of intracellular proinsulin, accompanied by unaffected preproinsulin transcription in GRP94 KO (7) indicate enhanced proinsulin degradation. We expected a broad upregulation of expression of proteasome subunits to counteract a higher load of mishandled proinsulin. Unexpectedly, we show that limiting folding capacity (GRP94 KO) induces upregulation of only β5i expression (Fig. 1A). The increase in β5i expression was accompanied by an increase in chymotrypsin-like activity in GRP94 KO that was reduced as a result of the treatment with a β5i selective small-molecule inhibitor, ONX-0194 (Fig. 1F). The particular position of the β5i in the inducible proteasomal response in β-cells was confirmed by mass spectrometry showing increased presence of β5i-containing int-proteasomes in GRP94 KO cells (Fig. 2A). We did not detect incorporation of other inducible β subunits despite their demonstrated expression in control and GRP94 KO cells (Fig. 1). Finally, we showed that β5i can be upregulated in response to prolonged exposure to high glucose concentration and ATP-ase inhibition of GRP94 activity that mimics the limited β-cell folding capacity (Fig. 3, note that human islets experiments represent n=1 and thus should be viewed with caution).

The increased presence of the β5i subunit containing int-proteasomes likely signifies the evolution of a positive and inducible adaptive response dedicated to proinsulin degradation under conditions of biosynthetic stress. Conversely, we showed that β5i KD results in an increase in intracellular proinsulin and insulin (Fig. 2C and D) and loss of cell viability (Fig. 2G).

What signaling processes could be responsible for the changes in subunits expression? Our experiments were performed with Ins-1E cells *in vitro* and therefore the upregulation of the expression of β5i had to occur in non-stimulated conditions with cells employing internal mechanisms to upregulate it. Theoretically, constitutive activation of NACHT, LRR and PYD domains-containing protein 3 (NLRP3)-inflammasome may be involved. Classically, this activation requires signal-1 that increases intracellular pro-interleukin (IL)-1β concentration in response to binding of ligands to toll-like receptors (TLR) and signal-2 that triggers inflammasome-dependent activation of caspase-1 that in turn processes pro-IL-1β into the mature biologically active cytokine. Some substances can provide both signal-1 and 2 (27) and these include ER stress-inducing drugs such as thapsigargin (14). We have previously reported that GRP94 KO cells mount a compensated ER stress, with a limited PERK response (7) that would lead to increased inflammasome activity through TXNIP (as in (5)), and production of IL-1β. We have recently showed that externally provided non-toxic concentrations of IL-1β induce expression of β5i and β1i in Ins-1E cells and mouse and human islets (11). How that result relates to those reported here, with overexpression of only β5i, remains to be clarified.

T2D patients exhibited increased fractions of α-, β- and δ-cells expressing β5i mRNA (Fig.4A) and weak but significant correlation between increased GRP94 and β5i mRNAs in T2D. As the demand for insulin biosynthesis increases during the progression of T2D, β-cells respond by increasing proinsulin synthesis and upregulating folding machinery. Despite that compensation, T2D patients display increased proinsulin misfolding. The observed β5i upregulation is therefore likely a positive adaptive mechanism that through the increased degradation of mishandled proinsulin contributes to β-cell homeostasis.

The role of specific proteasomes in proinsulin degradation remains to be uncovered in detail and our results do not preclude involvement of s-proteasomes in this process. However, the perspective

that in a compromised ER folding environment in islets of T2D patients, β-cells specifically overexpress and incorporate the β5i proteasome subunit into inducible int-proteasomes that contributes to proinsulin degradation warrants further investigation into its significance for human cases.

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6. Disclosures

The authors declare that there are no conflicts of interests except for AW who is an employee of AstraZeneca and has shares in the company.

7. Author Contributions

MSK, SEB, DV, THB, CP, PAKA, JBA, CKN, KK developed the protocols, conducted experiments, the statistical analysis, constructed figure, wrote and reviewed the manuscript. TD developed GRP94 KO clones and participated in writing the manuscript. Bioinformatics analysis of single-cell RNA-sequencing data was conducted by AW and BT. M-PB and DZ performed and analyzed mass spectrometry and wrote the manuscript. SJR and NGM performed human islet

related experiments and participated in critical analysis of the data. TMP developed the protocols, participated in data analysis and writing the manuscript. MTM is the study guarantor and was its initiator; developed the protocols, performed the statistical analysis and constructed figures and tables as well as wrote the manuscript.

REFERENCES

1. **Andersen CL, Jensen JL, and Orntoft TF**. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research* 64: 5245-5250, 2004.
2. **Bertrand Fabre TL, David Bouyssié, Thomas Menneteau, Bernard Monsarrat, Odile Burlet-Schiltz, Marie-Pierre Bousquet-Dubouch**. Comparison of label-free quantification methods for the determination of protein complexes subunits stoichiometry. *EuPA Open Proteomics* 4: 82-86, 2014.
3. **Fabre B, Lambour T, Garrigues L, Amalric F, Vigneron N, Menneteau T, Stella A, Monsarrat B, Van den Eynde B, Burlet-Schiltz O, and Bousquet-Dubouch MP**. Deciphering preferential interactions within supramolecular protein complexes: the proteasome case. *Mol Syst Biol* 11: 771, 2015.
4. **Fabre B, Lambour T, Garrigues L, Ducoux-Petit M, Amalric F, Monsarrat B, Burlet-Schiltz O, and Bousquet-Dubouch MP**. Label-free quantitative proteomics reveals the dynamics of proteasome complexes composition and stoichiometry in a wide range of human cell lines. *Journal of proteome research* 13: 3027-3037, 2014.
5. **Fan P, Tyagi AK, Agboke FA, Mathur R, Pokharel N, and Jordan VC**. Modulation of nuclear factor-kappa B activation by the endoplasmic reticulum stress sensor PERK to mediate estrogen-induced apoptosis in breast cancer cells. *Cell Death Discov* 4: 15, 2018.
6. **Freudenburg W, Gautam M, Chakraborty P, James J, Richards J, Salvatori AS, Baldwin A, Schriewer J, Buller RM, Corbett JA, and Skowyra D**. Reduction in ATP levels triggers immunoproteasome activation by the 11S (PA28) regulator during early antiviral response mediated by IFNbeta in mouse pancreatic beta-cells. *PloS one* 8: e52408, 2013.
7. **Ghiasi SM, Dahlby T, Hede Andersen C, Haataja L, Petersen S, Omar-Hmeadi M, Yang M, Pihl C, Bresson SE, Khilji MS, Klindt K, Cheta O, Perone MJ, Tyrberg B, Prats C, Barg S, Tengholm A, Arvan P, Mandrup-Poulsen T, and Marzec MT**. Endoplasmic Reticulum Chaperone Glucose-Regulated Protein 94 Is Essential for Proinsulin Handling. *Diabetes* 68: 747-760, 2019.
8. **Guillaume B, Chapiro J, Stroobant V, Colau D, Van Holle B, Parvizi G, Bousquet-Dubouch MP, Theate I, Parmentier N, and Van den Eynde BJ**. Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. *Proc Natl Acad Sci U S A* 107: 18599-18604, 2010.
9. **Hoelen H, Zaldumbide A, van Leeuwen WF, Torfs EC, Engelse MA, Hassan C, Lebbink RJ, de Koning EJ, Resssing ME, de Ru AH, van Veelen PA, Hoeben RC, Roep BO, and Wiertz EJ**. Proteasomal Degradation of Proinsulin Requires Derlin-2, HRD1 and p97. *PLoS One* 10: e0128206, 2015.
10. **Hu Y, Gao Y, Zhang M, Deng KY, Singh R, Tian Q, Gong Y, Pan Z, Liu Q, Boisclair YR, and Long Q**. Endoplasmic Reticulum-Associated Degradation (ERAD) Has a Critical Role in Supporting Glucose-Stimulated Insulin Secretion in Pancreatic beta-Cells. *Diabetes* 68: 733-746, 2019.
11. **Khilji MS, Verstappen D, Dahlby T, Burstein Prause MC, Pihl C, Bresson SE, Bryde TH, Keller Andersen PA, Klindt K, Zivkovic D, Bousquet-Dubouch MP, Tyrberg B, Mandrup-Poulsen T, and Marzec MT**. The intermediate proteasome is constitutively expressed in pancreatic beta cells and upregulated by stimulatory, low concentrations of interleukin 1 beta. *PloS one* 15: e0222432, 2020.

12. **Kim D, Langmead B, and Salzberg SL**. HISAT: a fast spliced aligner with low memory requirements. *Nature methods* 12: 357-360, 2015.
13. **Kitiphongspattana K, Mathews CE, Leiter EH, and Gaskins HR**. Proteasome inhibition alters glucose-stimulated (pro)insulin secretion and turnover in pancreatic {beta}-cells. *J Biol Chem* 280: 15727-15734, 2005.
14. **Lerner AG, Upton JP, Praveen PV, Ghosh R, Nakagawa Y, Igbaria A, Shen S, Nguyen V, Backes BJ, Heiman M, Heintz N, Greengard P, Hui S, Tang Q, Trusina A, Oakes SA, and Papa FR**. IRE1alpha induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. *Cell Metab* 16: 250-264, 2012.
15. **Lundh M, Bugliani M, Dahlby T, Chou DH, Wagner B, Ghiasi SM, De Tata V, Chen Z, Lund MN, Davies MJ, Marchetti P, and Mandrup-Poulsen T**. The immunoproteasome is induced by cytokines and regulates apoptosis in human islets. *The Journal of endocrinology* 233: 369-379, 2017.
16. **McCarthy MK, and Weinberg JB**. The immunoproteasome and viral infection: a complex regulator of inflammation. *Front Microbiol* 6: 21, 2015.
17. **Morozov AV, and Karpov VL**. Biological consequences of structural and functional proteasome diversity. *Heliyon* 4: e00894, 2018.
18. **Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, Lauer C, Sylvain C, Ring ER, Shields J, Jiang J, Shwonek P, Parlati F, Demo SD, Bennett MK, Kirk CJ, and Groettrup M**. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 15: 781-787, 2009.
19. **Patel PD, Yan P, Seidler PM, Patel HJ, Sun W, Yang C, Que NS, Taldone T, Finotti P, Stephani RA, Gewirth DT, and Chiosis G**. Paralog-selective Hsp90 inhibitors define tumor-specific regulation of HER2. *Nature chemical biology* 9: 677-684, 2013.
20. **Patro R, Duggal G, Love MI, Irizarry RA, and Kingsford C**. Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods* 14: 417-419, 2017.
21. **Pelletier S, Schuurman KG, Berkers CR, Ovaa H, Heck AJ, and Raijmakers R**. Quantifying cross-tissue diversity in proteasome complexes by mass spectrometry. *Mol Biosyst* 6: 1450-1453, 2010.
22. **Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, and Eliceiri KW**. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* 18: 529, 2017.
23. **Segerstolpe A, Palasantza A, Eliasson P, Andersson EM, Andreasson AC, Sun X, Picelli S, Sabirsh A, Clausen M, Bjursell MK, Smith DM, Kasper M, Ammala C, and Sandberg R**. Single-Cell Transcriptome Profiling of Human Pancreatic Islets in Health and Type 2 Diabetes. *Cell Metab* 24: 593-607, 2016.
24. **Sun J, Cui J, He Q, Chen Z, Arvan P, and Liu M**. Proinsulin misfolding and endoplasmic reticulum stress during the development and progression of diabetes. *Molecular aspects of medicine* 42: 105-118, 2015.
25. **Tanaka K**. The proteasome: overview of structure and functions. *Proc Jpn Acad Ser B Phys Biol Sci* 85: 12-36, 2009.
26. **Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, and Ponten F**. Proteomics. Tissue-based map of the human proteome. *Science* 347: 1260419, 2015.

27. **Wali JA, Gurzov EN, Fynch S, Elkerbout L, Kay TW, Masters SL, and Thomas HE**. Activation of the NLRP3 inflammasome complex is not required for stress-induced death of pancreatic islets. *PLoS One* 9: e113128, 2014.

Figure 1. Expression and activity of proteasome β subunits.

SDS-PAGE and Western blot analysis with quantification of relative levels of expression of β inducible **(A-B)** and **(D-E)** standard proteasome subunits in Ins-1E derived GRP94 KO and control clonal cells (n=3). **C.** mRNA levels of *β5i* gene were analyzed by quantitative reverse transcription-PCR (qRT-PCR) in control and GRP94 KO cells (n=6, individual clones data shown). **F.** Proteolytic-specific activities exhibited by proteasome subunits treated with β5i subunit specific inhibitor ONX-0914 (2 h, 50 nM). Proteasome activity was evaluated in cultured cells and the data is shown as luminescence per cell. Statistical analysis performed by Bonferroni corrected unpaired two-tailed Student's t-test. The data are presented as means±SD.

Figure 2. Proteasome activity and structure in GRP94 KO clonal cells and an effect of β5i KD on proinsulin/insulin contents.

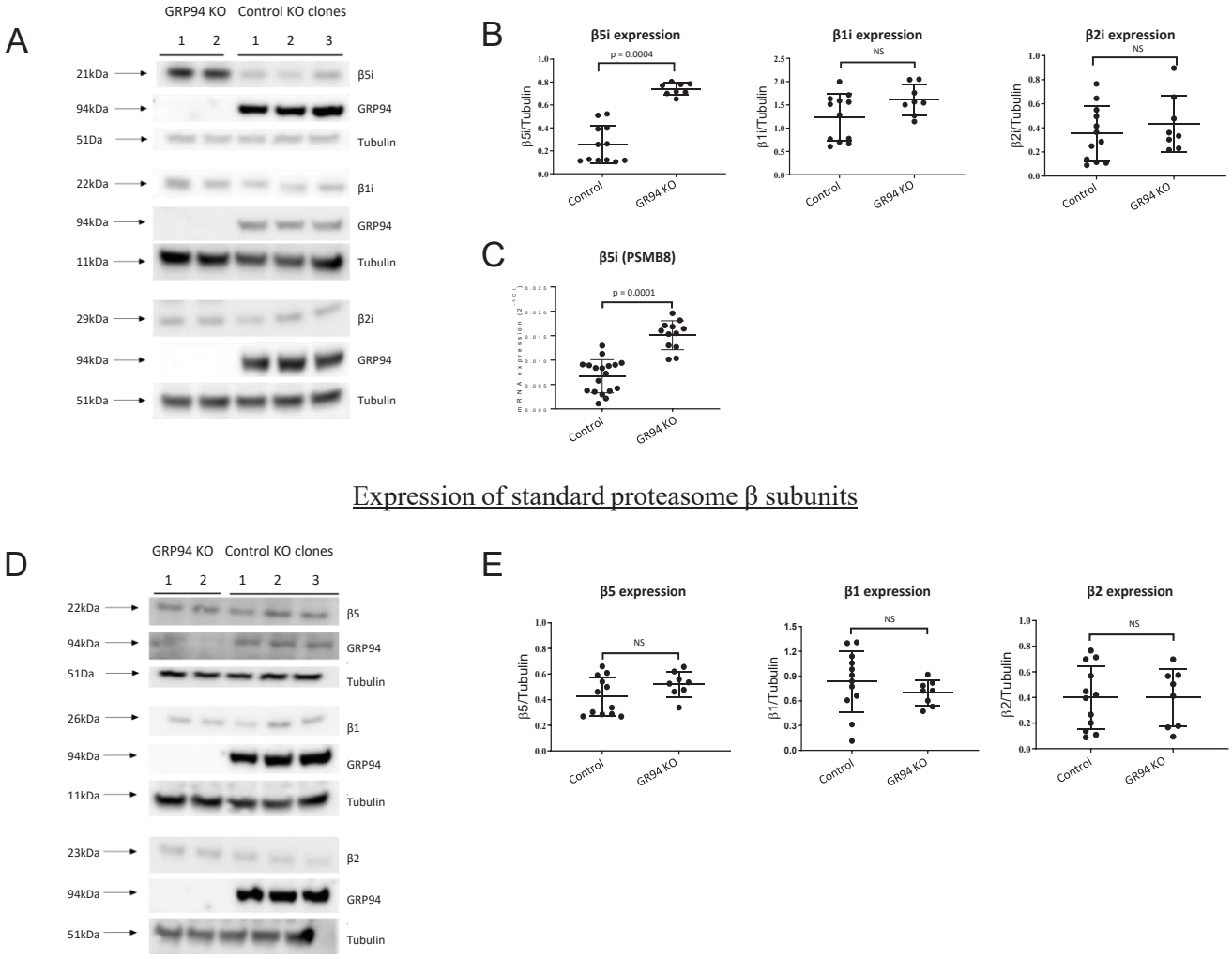
A-B Proteasomes were precipitated with anti-MCP21 from $4x10^8$ cells and analyzed by LC-MS/MS. The absolute quantities of each of the β subunits and regulatory particles measured by the LC-MS/MS method were computed to calculate the stoichiometry of 20S proteasome subtypes and 20S proteasome-associated regulators, n=4. Statistical analysis performed by Bonferroni corrected unpaired two-tailed Student's t-test. **C-G** siRNA KD of β5i in GRP94 KO cells was analyzed 72 h post siRNA delivery via: **C.** SDS-PAGE and Western blot analysis of proinsulin and insulin expression; **D.** quantification of WB data from six independent experiments; data depicted as percentage of control; **E.** mRNA levels of *β5i* and *Ins-1-2* genes were analyzed by qRT-PCR in control and GRP94 KO cells (n=4); **F.** evaluation by ELISA assays of proinsulin and insulin secretion after additional 2 h culture in 2 mM glucose containg medium (n=3); **G.** Cell viability evaluated with AlamarBlue reagent. Thapsigargin was used at the dose of 0.1 μM for 24 h (n=4). Statistical analysis performed by Bonferroni corrected paired two-tailed Student's t-test. The data is presented as means±SD.

Figure 3. β5i expression is increased upon glucose stimulation and GRP94 ATP-ase inhibition.

A-C Ins1-E cells and human islets from deceased donor were cultured in depicted glucose conditions for 24-48 h and their protein content analyzed by SDS-PAGE and Western blotting (3A: n=3 and 3B: n=1). **C.** Human islets were cultured for 24 h in the presence of 20 mM glucose and 20 μM of GRP94i and their content analyzed (n=1).

Figure 4. Proteasome inducible subunits expression in islets of control and T2D patients. A-C Single cell RNA sequencing analysis of *β5i* (A), *β1i* (B) and *β2i* (C) gene expression in human pancreatic islets α-, β- and δ-cells from healthy individuals (n=6) and T2D patients (n=4). **D-F** Expression levels of inducible subunits were quantified as expression values (CPM, log2) per individual cell. Data is presented as means±SD. **G-I** Pair-wise expression correlation between *GRP94* (*HSP90B1*) and inducible subunit *β5i* (*PSMB8*) expression in α-, β- and δ-cells from healthy and T2D patients (Pearson's correlation coefficients are shown; blue: healthy, green: T2D). Statistical analysis was performed using ANOVA with Bonferroni correction for multiple comparisons of T2D versus healthy donors.

Fig. 1


Expression of inducible proteasome β subunits
A
GRP94 KO
Control KO clones
1 2 1 2 3
21kDa
β5i
94kDa
GRP94
51Da
Tubulin
22kDa
β1i
94kDa
GRP94
11kDa
Tubulin
29kDa
β2i
94kDa
GRP94
51kDa
Tubulin
B
β5i expression
p = 0.0004
β5i/Tubulin
Control
GR94 KO
β1i expression
NS
β1i/Tubulin
Control
GR94 KO
β2i expression
NS
β2i/Tubulin
Control
GR94 KO
C
β5i (PSMB8)
p = 0.0001
Control
GR94 KO
Expression of standard proteasome β subunits
D
GRP94 KO
Control KO clones
1 2 1 2 3
22kDa
β5
94kDa
GRP94
51Da
Tubulin
26kDa
β1
94kDa
GRP94
11kDa
Tubulin
23kDa
β2
94kDa
GRP94
51kDa
Tubulin
E
β5 expression
NS
β5/Tubulin
Control
GR94 KO
β1 expression
NS
β1/Tubulin
Control
GR94 KO
β2 expression
NS
β2/Tubulin
Control
GR94 KO

F

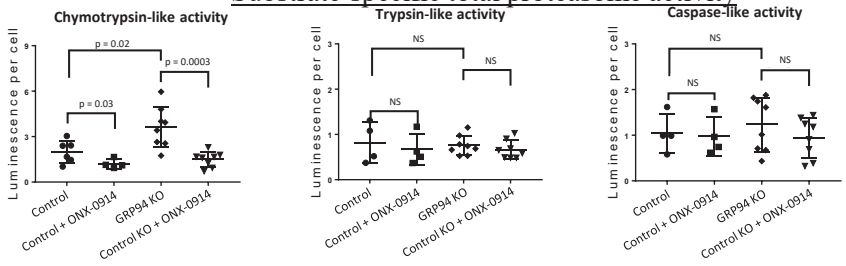
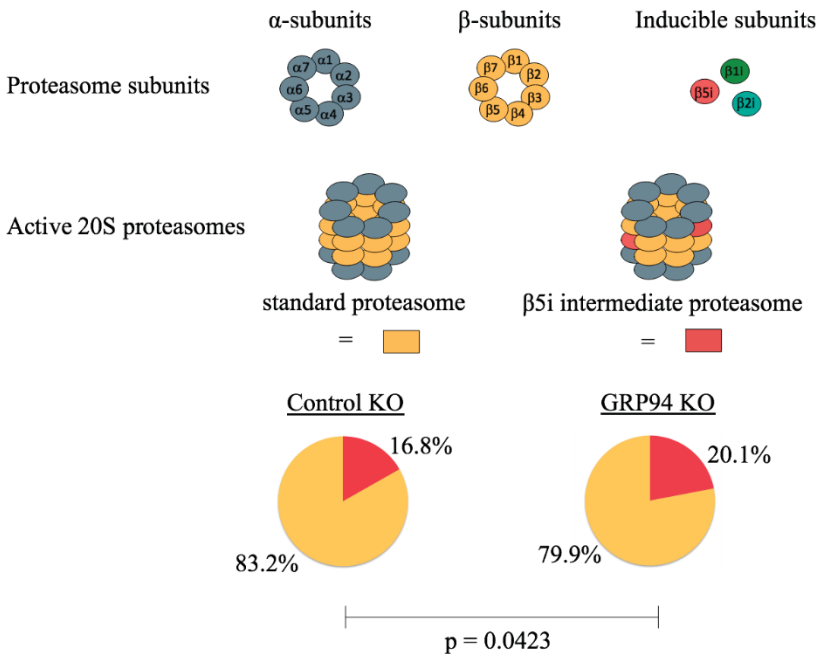

Substrate-specific total proteasome activity
Chymotrypsin-like activity
p = 0.02
p = 0.0003
p = 0.03
Luminescence per cell
Control
Control + ONX-0914
GRP94 KO
Control KO + ONX-0914
Trypsin-like activity
NS
NS
NS
Luminescence per cell
Control
Control + ONX-0914
GRP94 KO
Control KO + ONX-0914
Caspase-like activity
NS
NS
NS
Luminescence per cell
Control
Control + ONX-0914
GRP94 KO
Control KO + ONX-0914

Fig. 2

A

Proteasome types detection by mass spectrometry

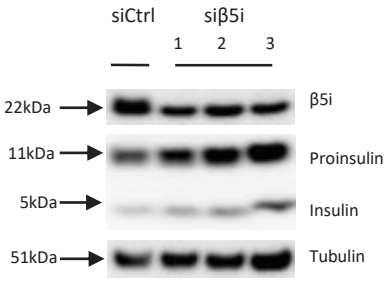


B

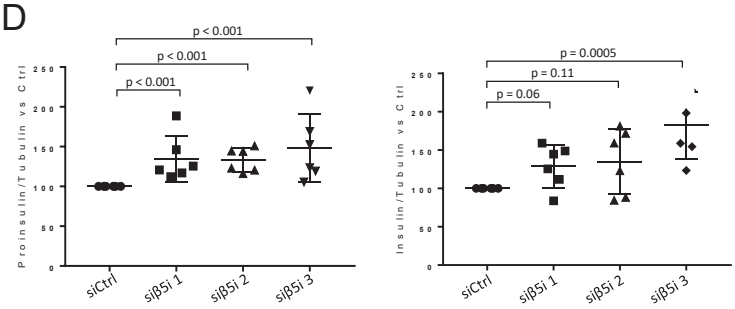
Regulatory particle	GRP94 KO control	GRP94 KO	*p* value
19S	86.13	70.15	0.174
PA28αβ (11S)	1.93	3.4	0.045
PA28γ (11S)	1.53	1.5	0.614
PA200 (11S)	0.76	1.25	0.019

siRNA-based knockdown of β5i subunit- protein expression

C

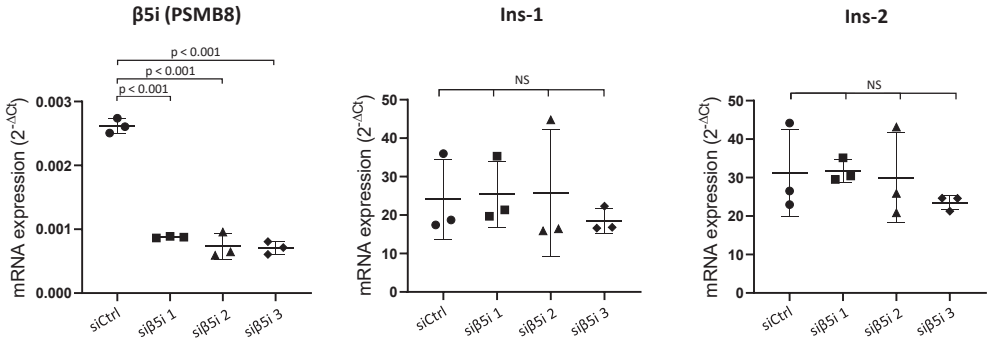


D



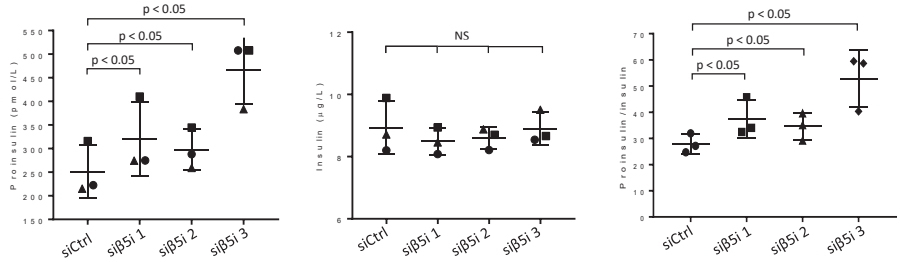
siRNA-based knockdown of β5i subunit – mRNA expression

E



F

siRNA-based knockdown of β5i subunit – secretion



G

siRNA-based knockdown of β5i subunit – cell viability

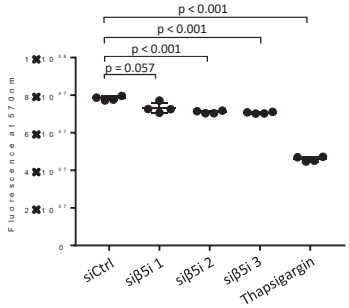


Fig. 3

β5i expression upon glucose stimulation and/or GRP94 ATP-ase inhibition

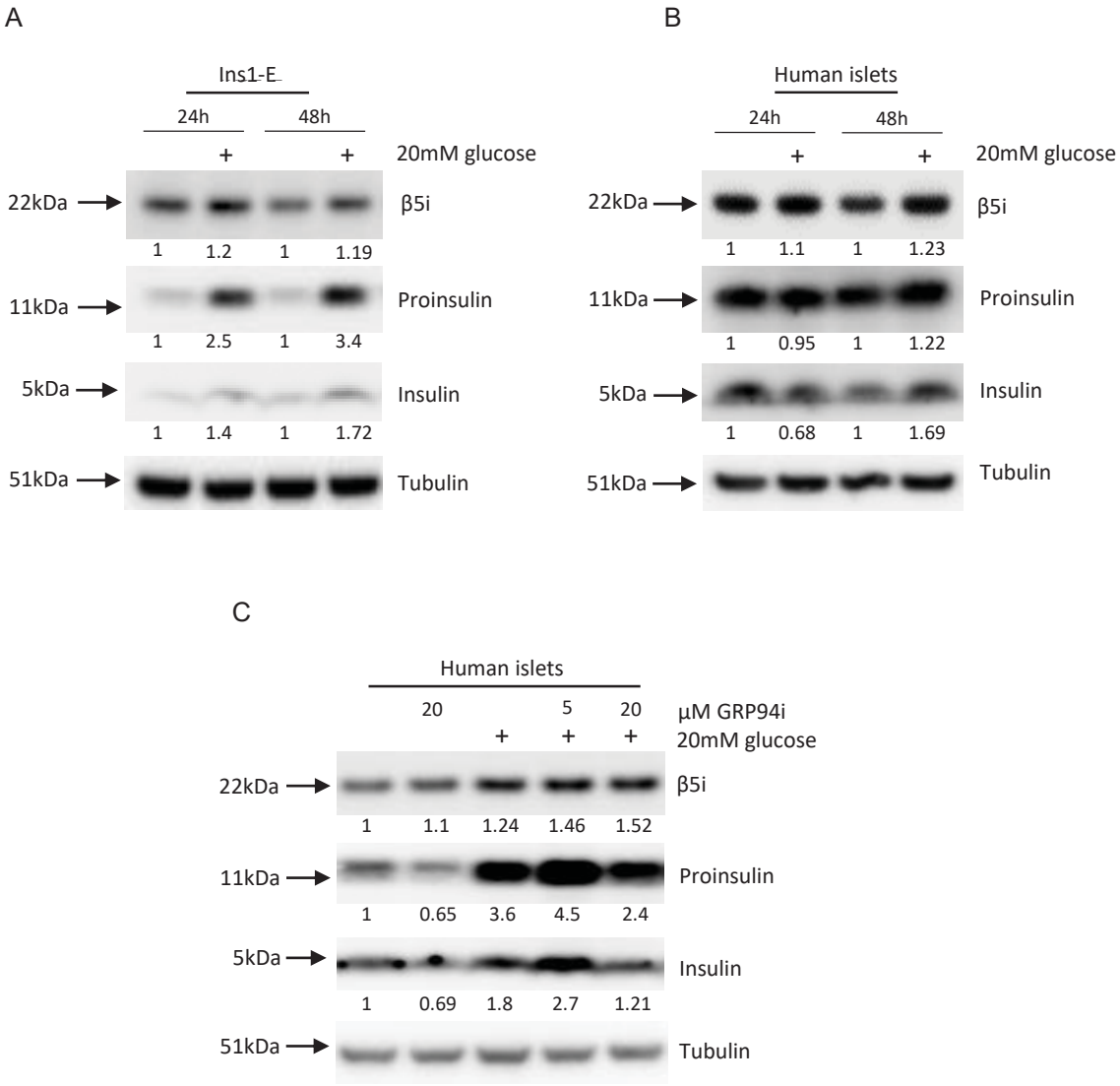


Fig. 4 mRNA sequencing of single cells from human islets