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## Metabolomic analyses reveal profound differences in glycolytic and tricarboxylic acid cycle metabolism in glucose-responsive and -unresponsive clonal $\beta$ -cell lines

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Running Head: Metabolic regulation in  $\beta$ -cells

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**Insulin secretion from pancreatic  $\beta$ -cells is controlled by complex metabolic and energetic changes provoked by exposure to metabolic fuels. Perturbations in these processes lead to impaired insulin secretion, the ultimate cause of Type 2 Diabetes. To increase our understanding of stimulus-secretion coupling and metabolic processes potentially involved in the pathogenesis of Type 2 Diabetes, a comprehensive investigation of the metabolic response in the glucose-responsive INS-1 832/13 and glucose-unresponsive INS-1 832/2  $\beta$ -cell lines was performed. For this metabolomics analysis, we used gas chromatography/mass spectrometry combined with multivariate statistics. We found that perturbed secretion in the 832/2 line was characterized by disturbed coupling of glycolytic and TCA-cycle metabolism. The importance of this metabolic coupling was reinforced by our observation that insulin secretion partially could be reinstated by stimulation of the cells with mitochondrial fuels which bypass glycolytic metabolism. Furthermore, metabolic and functional profiling of additional  $\beta$ -cell lines (INS-1, INS-1 832/1) confirmed the important role of coupled glycolytic and TCA-cycle metabolism in stimulus-secretion coupling. Dependence of the unresponsive clones on glycolytic metabolism was paralleled by increased stabilization of hypoxia-induced factor 1 $\alpha$  (HIF-1 $\alpha$ ). The relevance of a similar perturbation for human Type 2 Diabetes was suggested by increased expression of HIF-1 $\alpha$  target genes in islets from Type 2 Diabetes patients.**

## INTRODUCTION

Failure of the pancreatic  $\beta$ -cells to release insulin appropriately is a major pathogenetic abnormality in Type 2 Diabetes. Insulin secretion from  $\beta$ -cells is controlled by two different mechanisms: the triggering ( $K_{ATP}$ -dependent) and the amplifying ( $K_{ATP}$ -independent) pathways [1]. While the triggering pathway has been thoroughly characterized [2, 3], the mechanisms and signals underlying the amplifying pathway remain largely unknown [4, 5]. Yet, several metabolites, mainly originating from mitochondrial metabolism, have been implicated as potential coupling factors in the amplifying pathway of glucose-stimulated insulin secretion (GSIS). These include long-chain acyl-CoAs [6], glutamate [7], and NADPH [8, 9].

In the present study, the metabolome of two clonal  $\beta$ -cell lines, the glucose-responsive 832/13 and the glucose-unresponsive 832/2 line, both derived from the INS-1 rat insulinoma cell line [10], were examined. This enabled us to investigate possible differences in metabolic regulation that underlie perturbed function of the 832/2 cells. Expression of several glycolytic and tricarboxylic acid (TCA) cycle enzymes has previously been found to differ between these cell lines [11]. Most strikingly, the 832/2 cells were found to express lactate dehydrogenase A (LDH-A) and release lactate. Hence, the glucose-unresponsive 832/2 line relied heavily on glycolytic metabolism, while the robust insulin secretor, the 832/13 line, exhibited strong glucose concentration-dependent coupling of cytosolic and mitochondrial fuel metabolism. Moreover, it has previously been shown that 832/13 cells are characterized by an active anaplerotic pathway, which is less pronounced in 832/2 cells [12]. Nevertheless, a detailed analysis of the impact of these metabolic differences on the levels of individual metabolites and how they are controlled has not yet been performed.

To date, the bulk of investigations on the coupling of  $\beta$ -cell metabolism to insulin secretion has been performed in a univariate fashion. One or a few metabolites or metabolic enzymes have been studied at a time. Due to the complexity of the metabolic networks, univariate investigations are likely to be insufficient. An unbiased characterization of metabolism is therefore warranted. Thus, in the present study, gas chromatography/mass spectrometry (GC/MS)-based metabolomics [13-15] combined with multivariate statistical analyses [16] were applied to investigate the metabolome. These metabolomic analyses generated hypotheses, which we aimed to verify with functional studies and analysis of human islets.

A prominent finding was the lacking response of levels of late glycolytic and TCA cycle intermediates in the unresponsive 832/2 cell line despite an increase in extracellular glucose. The perturbed coupling of cytosolic and mitochondrial metabolism could partially be circumvented by specifically stimulating mitochondrial metabolism. This manoeuvre reinstated fuel-stimulated mitochondrial respiration and insulin secretion. Interestingly, we found that hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), a transcription factor regulating the balance between glycolytic and mitochondrial metabolism, was stabilized in the glucose-unresponsive cells.

## EXPERIMENTAL

### Metabolomics

Clonal  $\beta$ -cell lines INS-1 832/2 and INS-1 832/13 were stimulated with either 2.8 or 16.7 mM glucose for 1 h followed by an unbiased analysis of the metabolome using GC/MS. The generated data were normalized and evaluated using multivariate statistics to highlight alterations in metabolite levels unique to or shared between the two cell lines. To validate the finding in the 832/2 and 832/13 clones, a metabolite profiling of the glucose responsive INS-1 and the glucose unresponsive INS-1 832/1 clones was undertaken. Details of the metabolomics protocols are found in the Supplemental information.

### Insulin secretion

INS-1, 832/1, 832/2 and 832/13 clones were cultured in 24-well culture plates to reach 90-95 % confluency, whereafter insulin was assayed as previously described in detail [10]. Insulin secretion was measured after incubation for 1 h in 2.8 mM glucose, 16.7 mM glucose or 10 mM leucine and 10 mM glutamine.

### Oxygen consumption

The oxygen consumption rate (OCR) was measured in the 832/2 and 832/13 clones using the Extracellular flux (XF) analyzer XF24 (Seahorse Bioscience, Billerica, MA), as previously described in detail [11]. Following a pre-incubation at 2.8 mM glucose for 2 h, the OCR was assayed at 2.8 mM glucose subsequent to a transition to 16.7 mM glucose or 2.8 mM glucose together with 10 mM leucine and 10 mM glutamine. OCRs were first normalized by protein content and then by the average basal OCR at 2.8 mM glucose. An area under the curve analysis was performed for fuel-stimulation conditions.

### Glucose uptake

The cells were prepared as for analysis of insulin secretion [10]. After removing the secretion assay buffer (SAB), 500  $\mu$ l of SAB containing 3-O-methyl-D-[3H(N)]glucose (specific activity, 80.2 Ci/mmol; PerkinElmer Life Sciences) and 3-O-methyl-D-glucose to a final concentration of 2.8 or 16.7 mM 3-O-methyl-D-glucose, respectively, were added to each well. Cells were incubated for 10 min at 37 °C. Next, 250  $\mu$ l of lysis buffer containing 200 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.4) and 1% SDS were added. Subsequently, 150  $\mu$ l of lysate were transferred into scintillation vials. Protein content was determined by the bicinchoninic acid (BCA) method. 3-O-methyl-D-[3H(N)]glucose content was measured by liquid scintillation spectrometry [17].

### Western blotting

Whole cells were solubilized in homogenization buffer (100 mM HEPES, 9 M urea, 1% Triton X-100, 2 mM EDTA, pH 7.2) mixed 1:100 (v/v) with protease inhibitor cocktail (Sigma, St. Louis, MO). Protein content was determined by the BCA method. Samples were mixed 1:10 (v/v) with loading buffer (100 mM HEPES, 10% SDS, 10% dithiothreitol, 20% glycerol, pH 7.2). 40  $\mu$ g protein were run on an 8% SDS-PAGE gel, and subsequently blotted onto PVDF membranes. HIF-1 $\alpha$  protein was detected with a primary mouse anti-HIF-1 $\alpha$  monoclonal antibody (Abcam, Cambridge, UK) in dilution 1:500 (v/v).  $\beta$ -Tubulin was detected with a primary rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in dilution of 1:400 (v/v). Horse-radish peroxidase-coupled goat-anti-rabbit IgG (1:8000, v/v), goat-anti-mouse IgG (1:6000, v/v), and donkey-anti-goat IgG (1:8000, v/v) (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies. Blots were developed with enhanced chemiluminescence (ECL) and detection was by Amersham Hyperfilm ECL (Amersham Biosciences, Uppsala, SE). Films were scanned using the Bio-Rad GS-800 Calibrated Densitometer (Bio-Rad, Sundbyberg, SE). Measurements obtained for HIF-1 $\alpha$  were corrected for protein loading with  $\beta$ -tubulin. For loading control blots were stripped with 2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7.

### Human islets

Human pancreatic islets from 55 non-diabetic and 9 T2D deceased donors were obtained from the Human Tissue Laboratory at Lund University Diabetes Centre. The 29 male and 26 female non-diabetic donors were aged  $56.7 \pm 9.8$  years, had a BMI of  $25.9 \pm 3.6$  kg/m<sup>2</sup> and HbA<sub>1c</sub> of  $5.7 \pm 0.8$ . The 5 male and 4 female T2D donors were aged  $57.0 \pm 13.1$  years, had a BMI of  $28.5 \pm 4.7$  kg/m<sup>2</sup> and HbA<sub>1c</sub> of  $7.3 \pm 1.2$ . Islets were prepared by collagenase digestion and density gradient purification. After isolation, islets were cultured free floating in CMRL 1066 culture medium (ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10 mmol/l HEPES, 2 mmol/l l-glutamine, 50  $\mu$ g/ml gentamicin, 0.25  $\mu$ g/ml Fungizone (GIBCO BRL, Gaithersburg, MD, USA), 20  $\mu$ g/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/l nicotinamide at 37°C (5% CO<sub>2</sub>) prior to RNA and DNA preparation. The donor before death or her/his relatives upon admission to Intensive Care Unit (ICU) had given their consent to donate organs and the local ethics committees approved the protocols.

Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany). The microarrays were performed following the Affymetrix standard protocol. Briefly, 200 ng total RNA were processed following the GeneChip® Expression 3'-Amplification Reagents One-cycle cDNA synthesis kit instructions (Affymetrix Inc, Santa Clara, CA, US) to produce double-stranded cDNA. This was used as a template to generate biotin-targeted cRNA following

manufacturer's specifications. 15  $\mu$ g of the biotin labeled cRNA were fragmented to strands between 35 and 200 bases in length, 10  $\mu$ g of which were hybridized onto the GeneChip® Human Gene 1.0 ST whole transcript based assay overnight in the GeneChip® Hybridization oven 6400 using standard procedures. The arrays were washed and stained in a GeneChip® Fluidics Station 450. Scanning was carried out with the GeneChip® Scanner 3000 and image analysis was performed using GeneChip® Operating Software. The array data were summarized and normalized with Robust Multi-array Analysis (RMA) method using the software "Expression Console" (Affymetrix).

### Statistical analysis

All data are shown as mean  $\pm$  standard deviation or standard error of the mean for indicated number of experiments. A Mann-Whitney U-test was used to compare data from human islets. Unless stated otherwise, Student's t-test was used when comparing two groups. The Kruskal-Wallis test in combination with Mann-Whitney followed by Bonferroni's test *post hoc* was applied for the analysis of HIF-1 $\alpha$  protein expression.

## RESULTS

### Metabolomics

Multivariate statistical calculations were performed to identify which metabolites out of the 164 detected were uniquely regulated by the glucose stimulation in the INS-1 832/2 and the INS-1 832/13 clones (see Supplemental information). These analyses revealed a unique regulation of the late glycolytic and TCA-cycle intermediates in the 832/13 clone (Figure 1A and B). Whereas glucose-6-phosphate was found to exhibit a glucose-stimulated increase in both cell lines, downstream intermediates 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate were found to increase exclusively in the glucose responsive 832/13 clone. This lack of response in the 832/2 clone was reflected also by the levels of the TCA-cycle intermediates; levels of all measured TCA-cycle intermediates increased in the 832/13 clone whereas they were unaltered in the 832/2 clone (Figure 1B). The fold of the glucose-stimulated increase in lactate was similar in the two clones (Figure 1A), although the levels were significantly higher in the un-responsive clone (see Supplementary Figures S4 and S8). The level of the pentose phosphate shunt intermediate ribose-5-phosphate rose more in the glucose responsive 832/13 clone.

### Oxygen consumption

Given the lack of rise in TCA-cycle intermediates in the 832/2 clone in response to glucose, we were interested to find out whether this had any further functional implications. To this end, we chose to assess oxygen consumption, which reflects overall mitochondrial activity. Based on an area under the curve analysis, glucose stimulation caused a sustained  $1.84 \pm 0.26$ -fold ( $n=7$ ) increase in OCR in the 832/13-clone, whereas no change in OCR was observed upon glucose stimulation of the 832/2-clone (Figure 2A,C). However, leucine and glutamine increased the OCR in both clones; OCR was significantly increased  $1.81 \pm 0.18$ -fold ( $n=6$ ) and  $1.26 \pm 0.07$ -fold ( $n=6$ ) in 832/13 and 832/2 cells, respectively (Figure 2B,C), compared with respiration at 2.8 mM glucose alone.

### Insulin secretion

The lack of a glucose-stimulated response in levels of TCA-cycle intermediates in the glucose-unresponsive 832/2 clone is likely to constrain insulin secretion via both the triggering pathway, which is dependent on the mitochondrial ATP production, and the amplifying pathway, which has been suggested to be coupled to TCA-cycle metabolism. Basal insulin secretion at 2.8 mM glucose was  $15.9 \pm 1.8$  ( $n=8$ ) and  $8.39 \pm 1.96$  ( $n=6$ ) ng/mg/h for 832/13 and 832/2 cells, respectively. Indeed, while stimulation with 16.7 mM glucose yielded a  $12.6 \pm 1.3$ -fold ( $n=8$ ) increase of insulin secretion in the glucose-responsive 832/13 cell, no rise in GSIS was observed in the glucose-unresponsive 832/2 line (Figure 2D). Leucine and glutamine, on the other hand, significantly stimulated insulin secretion in 832/2 cells ( $2.88 \pm 0.22$ -fold ( $n=6$ ) increase vs. basal), whereas the same stimulus yielded a  $14.1 \pm 5.5$ -fold ( $n=6$ ) increase in the glucose-responsive 832/13 line (Figure 2D).

### Metabolite profiling of additional clonal $\beta$ -cell lines

The results generated from the study of the glucose responsive 832/13-clone and the glucose unresponsive 832/2 clone suggested that insufficient coupling of glycolytic and TCA-cycle metabolism may be a factor underlying perturbed GSIS. To investigate whether this phenotype is unique for the glucose unresponsiveness of the 832/2 clone or a more general phenomenon in  $\beta$ -cells, two additional  $\beta$ -cell lines were investigated. To this end, the glucose-responsive INS-1 line and the glucose-unresponsive INS-1 832/1 sub-clone were examined. A targeted metabolite profiling revealed a significantly lower fold-change of glucose-stimulated alterations in glycolytic and TCA-cycle metabolite levels in the unresponsive clone (Figure 3), reminiscent of the metabolic phenotype of the 832/2 clone (Figure 1A-B).

### Functional characterization of additional clonal $\beta$ -cell lines

Next, the functional consequences of the reduced response in the glycolytic and TCA-cycle intermediates upon glucose stimulation were assessed. Glucose was found to stimulate insulin secretion  $6.6 \pm 2.6$  fold ( $n=3$ ) in the INS-1 parental clone, whereas insulin secretion from the 832/1 sub-clone was not stimulated by the hexose ( $1.2 \pm 0.3$  fold,  $n=3$ ). To investigate whether stimulus secretion coupling was intact and the observed lack of response in the 832/1 clone was due to insufficient coupling of glycolytic and TCA-cycle metabolism, insulin secretion in response to leucine and glutamine was also assessed. Indeed, leucine and glutamine provoked a significant ( $p < 0.05$ )  $7.8 \pm 2.4$  fold and a  $3.1 \pm 1.0$  fold increase in insulin secretion in the INS-1 and the 832/1-clone, respectively.

### Glucose uptake

To investigate whether differences in glucose uptake could account for the observed metabolic differences between the glucose-responsive and -unresponsive  $\beta$ -cell clones, uptake of the non-metabolizable analogue of D-glucose, 3-O-methyl-D-glucose, was assessed. No significant differences between the four clones could be observed (see Supplementary Figure S9), indicating that differences in glucose uptake alone can not explain the perturbed metabolic response in the glucose unresponsive clones.

### Stabilization of HIF-1 $\alpha$

The metabolomics data suggested that the lacking response of the 832/2-clone to glucose stimulation may be caused by impaired aerobic glycolysis concurrent with lactate production and a perturbation in the coupling between cytosolic and TCA-cycle metabolism. This together with the fact that the cell-lines are tumour derived [10] suggested involvement of HIF-1 $\alpha$ . Stabilization of this protein may account for a switch of cellular metabolism to dependence on glycolysis, the so called Warburg-effect [18]. To explore this possibility, we determined the presence of HIF-1 $\alpha$  by Western blotting. Indeed, while HIF-1 $\alpha$  was present in all INS-1 clones, its protein levels were e.g.  $227 \pm 30$  % ( $p=0.006$ ) higher in the glucose-unresponsive 832/2 clone, in comparison to the glucose-responsive 832/13 clone (Figure 4A-B).

It has recently been shown that a low level of  $\alpha$ -ketoglutarate in the presence of a high level of succinate stabilizes HIF-1 $\alpha$  [19, 20]. To assess whether such a mechanism is operating in the clonal cell lines, we plotted the HIF-1 $\alpha$  protein levels against the ratios of  $\alpha$ -ketoglutarate and succinate (fold responses of the metabolite levels to glucose stimulation were employed; Figure 4C). Indeed, we observed that the lower the ratios of  $\alpha$ -ketoglutarate to succinate, the higher the protein level of HIF-1 $\alpha$ . Next, we examined whether the change in HIF-1 $\alpha$  protein levels coincided with metabolic changes. To this end, we plotted HIF-1 $\alpha$  protein levels against the ratios of citrate to pyruvate (Figure 4D). The rationale for this analysis is that this ratio may reflect coupling of glycolysis and TCA-cycle activities. A low ratio would imply a low level of TCA-cycle activity, suggesting that coupling of glycolysis and TCA-cycle is perturbed. The plot clearly shows that high HIF-1 $\alpha$  protein levels coincided with a low ratio of citrate to pyruvate in the clonal  $\beta$ -cell-lines. This translated into impaired GSIS, as shown in figure 4E.

### Expression of HIF-1 $\alpha$ target genes in human islets

Stabilization of HIF-1 $\alpha$  can establish a pseudo-hypoxic metabolic phenotype under normoxic conditions. This involves primarily increased expression of glycolytic enzymes. Thus, to investigate whether our findings in the clonal cell-lines are of relevance for the pathogenesis of Type 2 Diabetes in humans, we first analyzed expression of HIF-1 $\alpha$  in human islets. Although HIF-1 $\alpha$  was expressed at the mRNA-level, we did not observe any differences between human islets from healthy controls and patients with Type 2 Diabetes (data not shown). This is in agreement with the fact that signalling through HIF-1 $\alpha$  requires stabilisation on the protein level, a phenomenon which occurs independent of changes in mRNA-level of HIF-1 $\alpha$  [21]. Instead, to ascertain whether regulation by HIF-1 $\alpha$  occurs in human islets, we examined expression of HIF-1 $\alpha$  target genes in human islets from healthy controls and patients with Type 2 Diabetes. Indeed, we found increased mRNA levels for hexokinase 2, lactate dehydrogenase, and phosphofructokinase-2/fructose-2,6-bisphosphatase-3. Unexpectedly, there were minor, but significant, decreases in pyruvate dehydrogenase kinase-1 (~13%) and enolase (~22%) mRNA levels.

### DISCUSSION

The complexity of  $\beta$ -cell metabolism prompts novel analytical approaches that allow analyses of more than one or a few metabolites at time. In the present study, metabolomics allowed the simultaneous analysis of 164 putative metabolites, out of which 44 could be identified. Analyses of four clonal  $\beta$ -cell-lines with variable glucose responsiveness and hence stimulated insulin secretion pinpointed a fundamental disruption of metabolism in the glucose-unresponsive clones. The disruption appeared to be at the level of coupling glycolysis to TCA-cycle metabolism. As metabolism moved down the path of glycolysis, the weaker the glucose-stimulated response in metabolite levels became. In fact, even lactate, a metabolite not thought to be compatible with robust  $\beta$ -cell function, was increasingly produced in glucose-unresponsive cell-lines [11].

To explore whether this observed metabolic perturbation impacted mitochondrial activity, we analyzed OCR in presence of either glucose, which requires glycolytic metabolism, or a combination of leucine and glutamine, which directly activates TCA-cycle metabolism. In agreement with the metabolomics data, glucose was ineffective to stimulate oxygen consumption in glucose-unresponsive cells with the observed block of coupling between glycolysis and TCA-cycle metabolism. In contrast, fuels that were directly metabolized in the TCA-cycle increased oxygen consumption also in glucose-unresponsive cells, albeit not to the same extent as in glucose-responsive cells. This suggests that TCA-cycle metabolism and oxidative phosphorylation in the glucose-unresponsive cell-lines have adapted to a metabolic situation where provision of fuels from glycolysis is constrained. In support of this notion, we have previously shown that respiratory complex activities and protein expression of key subunits of the complexes are decreased in 832/2 versus 832/13 cells [11]. Nevertheless, when mitochondrial fuels are provided, a metabolic response from mitochondria can still be provoked. Accordingly, fuels metabolized directly in the TCA-cycle were able to stimulate insulin secretion also in the clones totally lacking a secretory response to glucose.

At this point, the question remained how the adaptive transition from coupled glycolytic and TCA-cycle metabolism to an increasing dependence on glycolysis was regulated. As we previously observed [11], metabolism in the 832/2 line exhibits Warburg-like features. Moreover, the metabolite profiling revealed that TCA-cycle intermediates, particularly succinate, that can inhibit proline hydroxylases when coinciding with low levels of  $\alpha$ -ketoglutarate, were increasingly abundant in cells with low glucose responsiveness. Proline hydroxylases hydroxylate HIF-1 $\alpha$ , priming it for proteolytic degradation. The inhibition of this process is an important component of the Warburg transition establishing a pseudohypoxic normoxic condition. HIF-1 $\alpha$  serves a transcription factor, enhancing expression of genes in the glycolytic pathway. Hereby energy homeostasis is maintained under hypoxic conditions, a situation commonly found in tumour-derived cells even under normoxic conditions. Indeed, the cell lines examined in this study are of tumour origin, originally derived from a transplantable rat insulinoma [22]. Accordingly, we found that HIF-1 $\alpha$  protein was present in all the clonal cell lines, being most abundant in the cell lines with the lowest level of glucose responsiveness. In support of this observation, we found that increased levels of HIF-1 $\alpha$  protein coincided with a perturbed coupling of glycolysis and TCA-cycle metabolism as evident from the citrate to pyruvate

ratio. Moreover, higher HIF-1 $\alpha$  protein levels corresponded to impaired GSIS. Collectively, these observations suggest that HIF-1 $\alpha$  is a key regulator of the balance between glycolytic and TCA-cycle metabolism in  $\beta$ -cells. In this capacity, HIF-1 $\alpha$  could serve as a regulator of  $\beta$ -cell stimulus-secretion coupling. However, while our experiments have established a relation between HIF-1 $\alpha$  and glucose responsiveness, it remains to be shown that this is a casual effect. Supporting a casual relationship are recent findings showing that genetic targeting of the von Hippel-Lindau factor in mouse  $\beta$ -cells stabilizes HIF-1 $\alpha$  resulting in impaired glucose tolerance and perturbed insulin secretion [23]. Yet another study shows that HIF-1 $\alpha$  present at low levels in mouse  $\beta$ -cells under normoxic conditions is required for proper  $\beta$ -cell function [24]. This circumstance could explain why we also found HIF-1 $\alpha$  protein in the glucose-responsive cell-lines, albeit at very low levels.

The relevance of our findings for the pathogenesis of Type 2 Diabetes is supported by studies of animal models for the disease. For instance, lactate production has been shown to be increased in the Goto-Kakizaki (GK) rat [25]. Moreover, it has previously been shown that leucine-stimulated glutamate metabolism is intact in the GK rat and that the combination of these amino acids stimulates insulin secretion [26]; the latter is reminiscent of what we observed in the glucose-unresponsive cell lines. In fact, although patients with Type 2 Diabetes exhibit severely perturbed GSIS, glutamine-leucine-stimulated insulin secretion is largely preserved [27]. Substantiating our findings on HIF-1 $\alpha$ , it has previously been reported that the protein is expressed in human islets [24]. Using our repository of human islets, we could confirm this finding. However, there was no difference between patients with Type 2 Diabetes and healthy controls. This was no surprise given that HIF-1 $\alpha$  mRNA levels are not thought to contribute to its regulatory function. Instead, this is controlled by its stabilization at the protein level. Therefore, we explored expression of HIF-1 $\alpha$  target genes in human islets. Indeed, we found that some of the known targets of this transcription factor, e.g. hexokinase 2, lactate dehydrogenase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 were up-regulated in islets from patients with Type 2 Diabetes. While these data are compelling, there should be call for caution. The human islets used in our studies, as well as in any other studies, have been cultured for different lengths of time. In this situation, the circulation in the islets has been compromised. This implies that hypoxia may prevail in these islets, particularly in the core of the islets. Clearly, such hypoxia could stabilize HIF-1 $\alpha$ . This notwithstanding, we did observe a difference between healthy controls and patients with Type 2 Diabetes.

In conclusion, we have used metabolomics analysis to identify a profound perturbation in stimulus-secretion coupling in clonal  $\beta$ -cells. The metabolite profile suggested to us that HIF-1 $\alpha$  could play a role in the observed transition of metabolism to dependence on glycolysis. The functional studies as well as observations in human islets suggest that an improper alignment of glycolysis with TCA-cycle metabolism may underlie perturbed GSIS. As such it may play a role in the pathogenesis of human Type 2 Diabetes. Further work is required to establish a casual relationship of these phenomena.

#### **AUTHOR CONTRIBUTION**

Peter Spégel performed metabolomics analyses and wrote the first draft of the manuscript. Siri Malmgren performed insulin secretion and oxygen consumption measurements. Vladimir Sharoyko performed glucose uptake measurement, provided feedback on results and the manuscript and, assisted in metabolite extraction. Thomas Koeck determined HIF-1 $\alpha$  stabilization, performed oxygen consumption measurements, and provided feedback on results and the manuscript. Philip Newsholme provided feedback on results and the manuscript throughout the project. Hindrik Mulder conceived and directed the project and finalized the paper.

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## FIGURE LEGENDS

### Figure 1 Glucose-stimulated fold changes in metabolite levels.

Fold changes of metabolite levels after glucose stimulation were calculated by dividing the peak areas for the metabolite levels at 16.7 and 2.8 mM glucose derived from the glucose-unresponsive INS-1 832/2 and the glucose-responsive INS-1 832/13 lines, respectively. (A) Glycolytic and related metabolites. (B) TCA-cycle and related intermediates. The data are represented as mean±SEM. Statistical comparisons were made by t-test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001). Abbreviations: Rib5P - ribose-5-phosphate; Glu6P1 - glucose-6-phosphate peak 1; Glu6P2 - glucose-6-phosphate peak 2; GlyA3P - 3-phosphoglycerate; GlyA2P - 2-phosphoglycerate; PEP - phosphoenol pyruvate; Pyr - pyruvate; Lac - lactate; Cit - citrate; Aco - aconitate; AKGA - α-ketoglutarate; Succ - succinate; Fum - fumarate; Mal - malate.

### Figure 2 Respiration and insulin secretion in glucose-responsive 832/13 and glucose-unresponsive 832/2 cells.

Oxygen consumption rate (OCR) in INS-1 832/13 (closed triangles) and 832/2 (open triangles) cells after addition of metabolic fuels. (A) 16.7 mM glucose or (B) 10 mM leucine + 10 mM glutamine. (C) Fuel-stimulated OCR during the stimulation period normalized to protein content and basal OCR at 2.8 mM glucose; calculated based on an area under curve (AUC) analysis [28]. (D) Fold-change of insulin secretion in INS-1 832/13 and INS-1 832/2 β-cells at 16.7 mM glucose and 10 mM of leucine + 10 mM of glutamine relative 2.8 mM glucose. Data are presented as means ±SEM (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

**Figure 3 Targeted metabolite profiling of the glucose –responsive INS-1 parental clone and the glucose-unresponsive INS-1 832/1 sub-clone.**

A targeted metabolite profiling was performed to investigate whether the observed metabolic perturbation was present also in other clonal  $\beta$ -cell lines. Fold-changes were calculated and compared as described in legend to Figure 1; (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001). Abbreviations as in Figure 1.

**Figure 4 Stabilization and functional relations of HIF-1 $\alpha$  with metabolic and functional parameters in clonal  $\beta$ -cell lines.**

HIF-1 $\alpha$  stabilisation was examined by Western-blotting as described in Experimental Procedures.  $n=3-6$ ; data were compared by Kruskal-Wallis test in combination with Mann-Whitney test followed by Bonferroni's test *post hoc*. **(A)** Western blot data. **(B)** Quantified levels of HIF-1 $\alpha$ . **(C)** Stabilization of HIF-1 $\alpha$  in relation to the ratio of  $\alpha$ -ketoglutarate (AKGA) to succinate (Succ) (calculated as the ratio of their fold-changes to stimulatory glucose). **(D)** Stabilization of HIF-1 $\alpha$  in relation to fold-changes of citrate (Cit) to pyruvate (Pyr) (see C). **(E)** Relationship between the fold-change of GSIS and HIF-1 $\alpha$  stabilization.

**Figure 5 Islet mRNA expression from patients with Type 2 Diabetes and healthy controls.**

Expression of islet mRNA, derived from microarray analysis, of hexokinase 2 (HK2), enolase (ENO2), phosphofructokinase-2/fructose-2,6-bisphosphatase-3 (PFKFB3), pyruvate dehydrogenase kinase-1 (PDK1), and lactate dehydrogenase (LDHA). Data are given as means  $\pm$  standard deviation and were compared with a Mann-Whitney U-test, (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001).

**Figure 6 Cartoon of the suggested involvement of HIF-1 $\alpha$  in  $\beta$ -cell stimulus-secretion coupling.**

The cartoons show the proposed different modes of metabolic regulation in the glucose-responsive and -unresponsive clonal  $\beta$ -cell lines involving hypoxia-induced factor 1 $\alpha$  (HIF-1 $\alpha$ ).  $\alpha$ -ketoglutarate – AKGA; hypoxia response element – HRE; lactate dehydrogenase – LDH; mitochondrion – M; nucleus – N; proline hydroxylases – PHD; pyruvate dehydrogenase – PDH; PDH kinase 1 – PDK1; oxidative phosphorylation – OXPHOS; succinate – suc; tricarboxylic acid cycle – TCA; von Hippel-Lindau factor – VHL

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Figure 1(A)

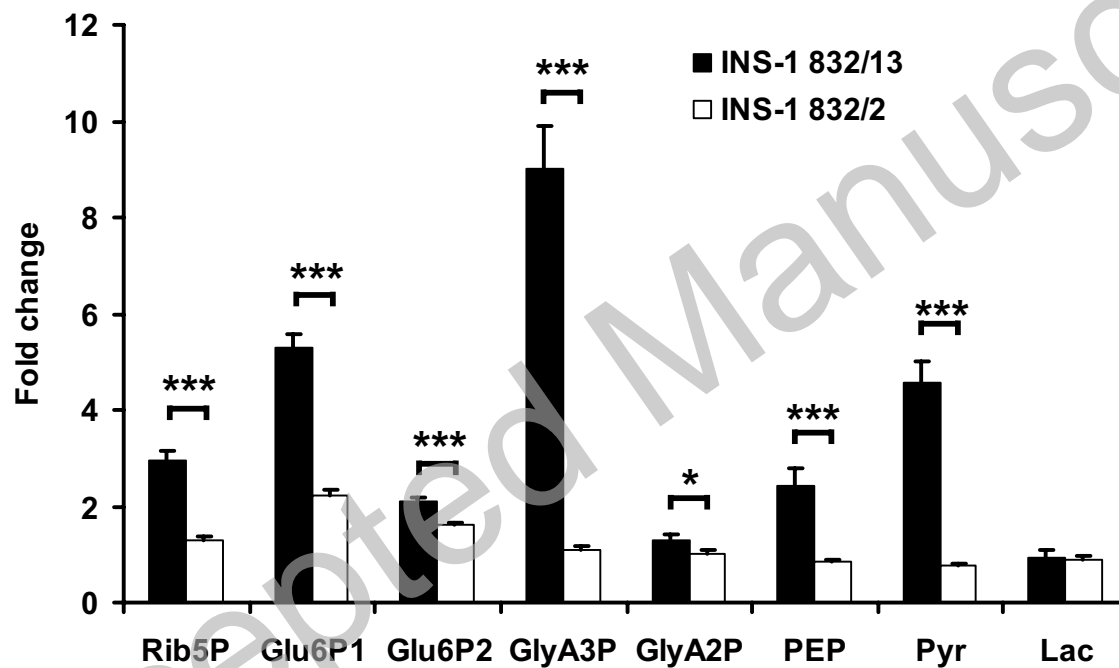
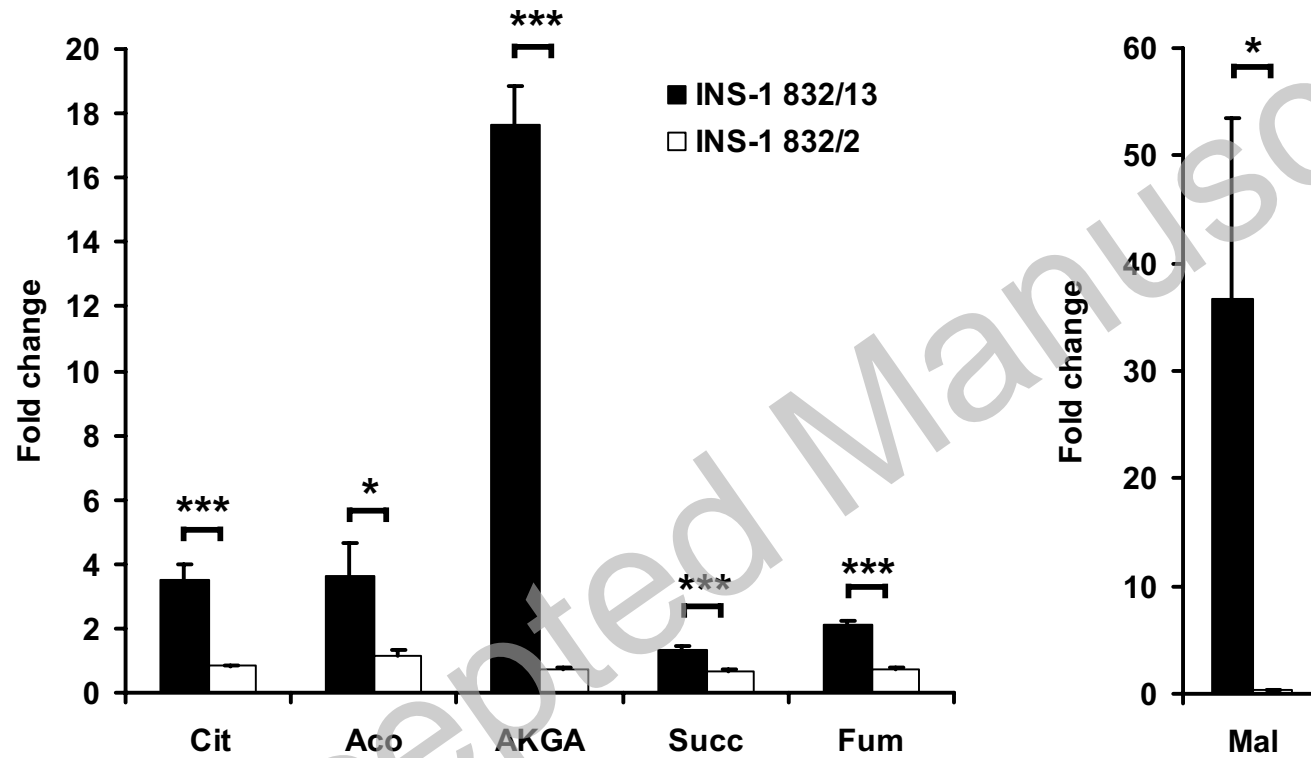


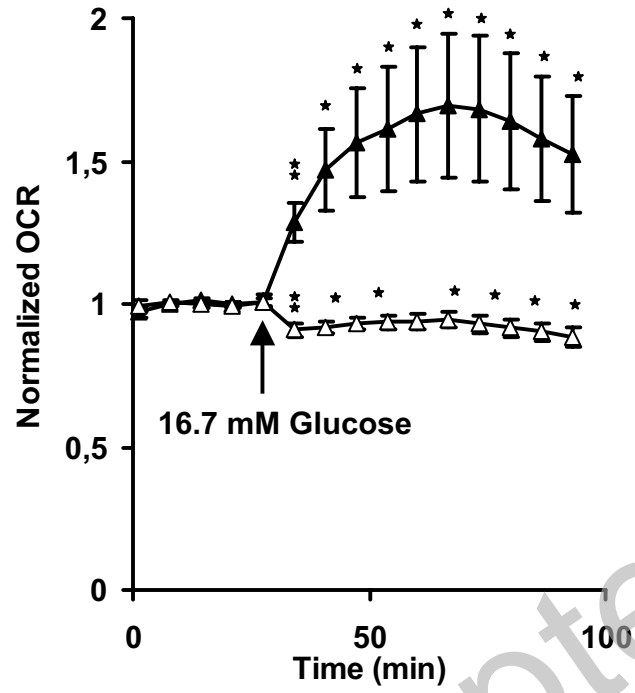
Figure 1(B)



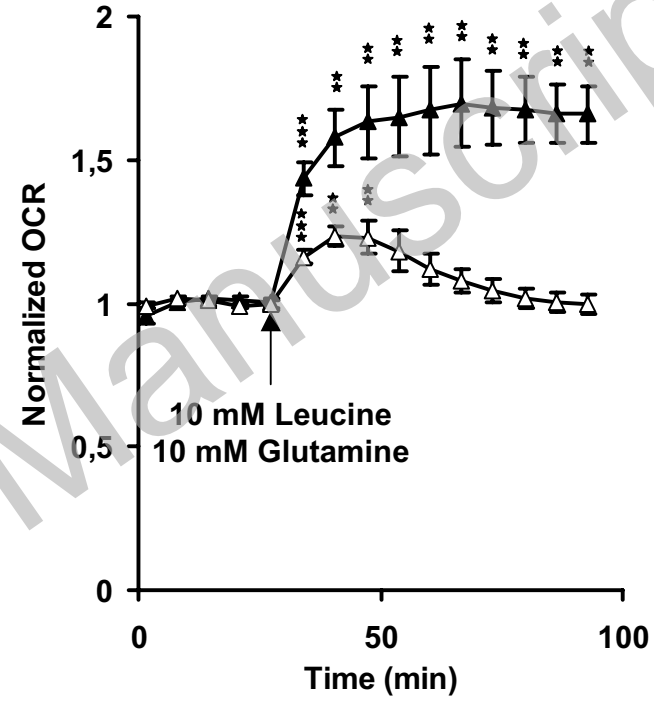
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**Figure 2**

(A)                      ▲— INS-1 832/13  
                               △— INS-1 832/2



(B)



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Figure 2 (C)

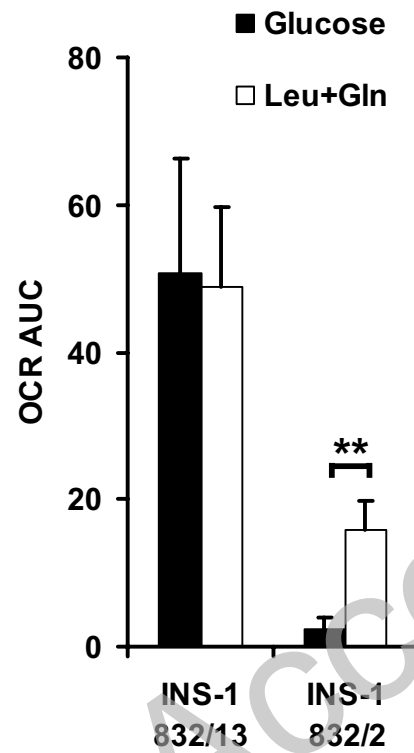


Figure 2 (D)

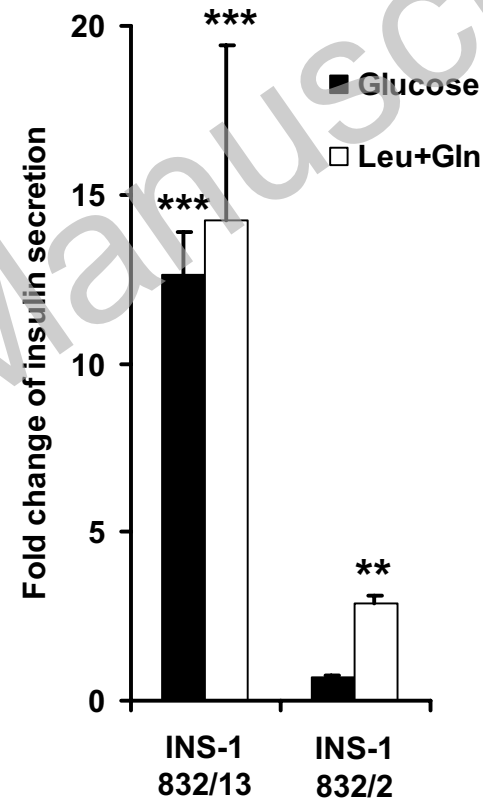
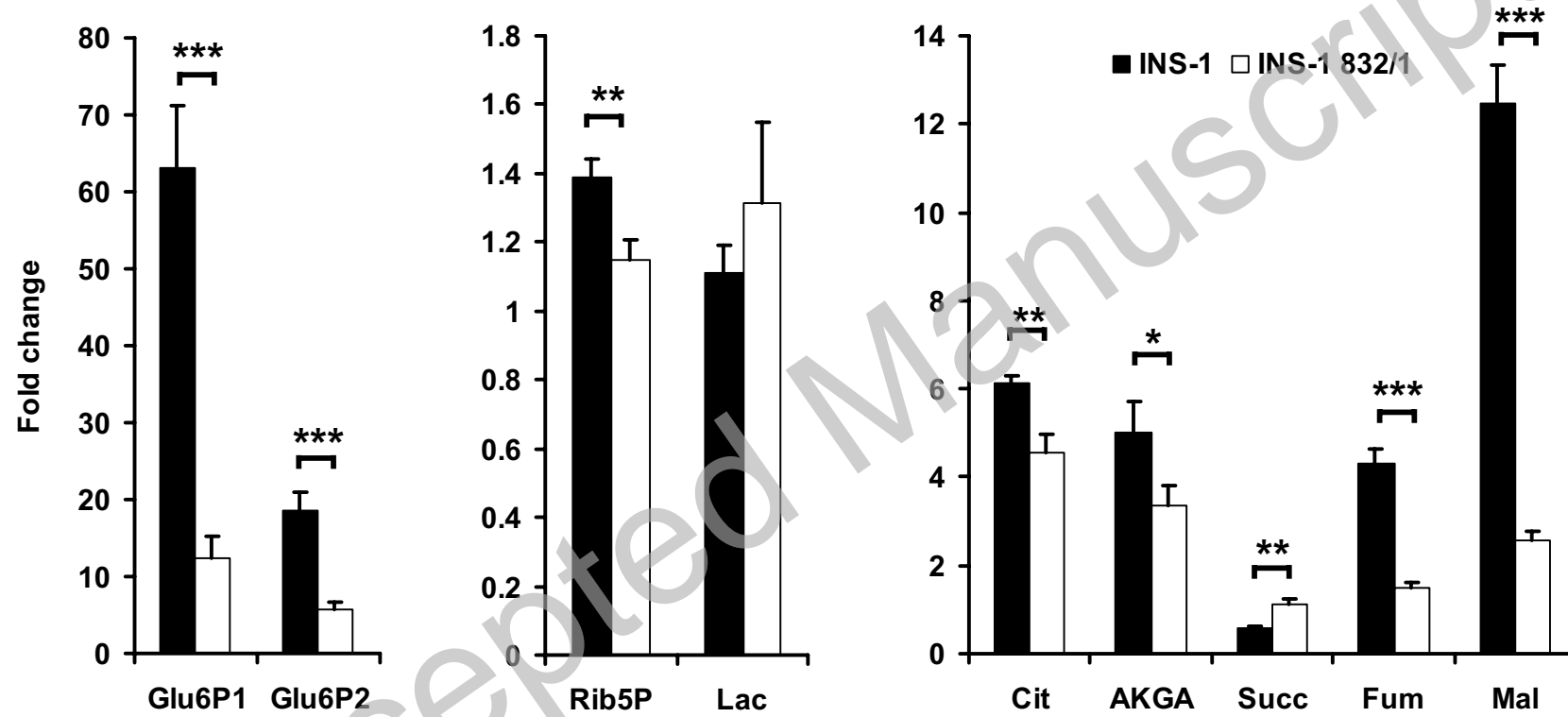


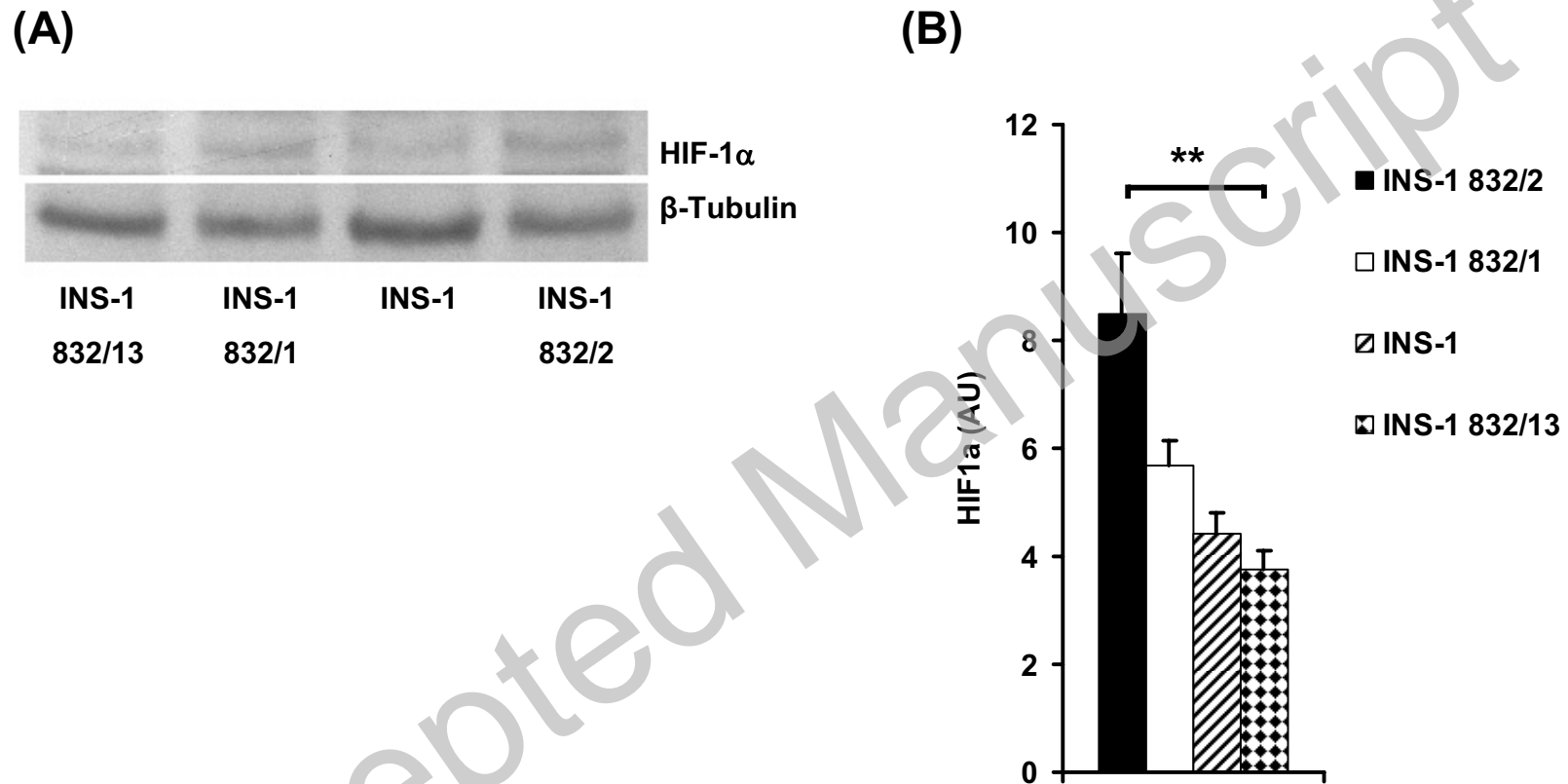
Figure 3



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**Figure 4**



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Figure 4

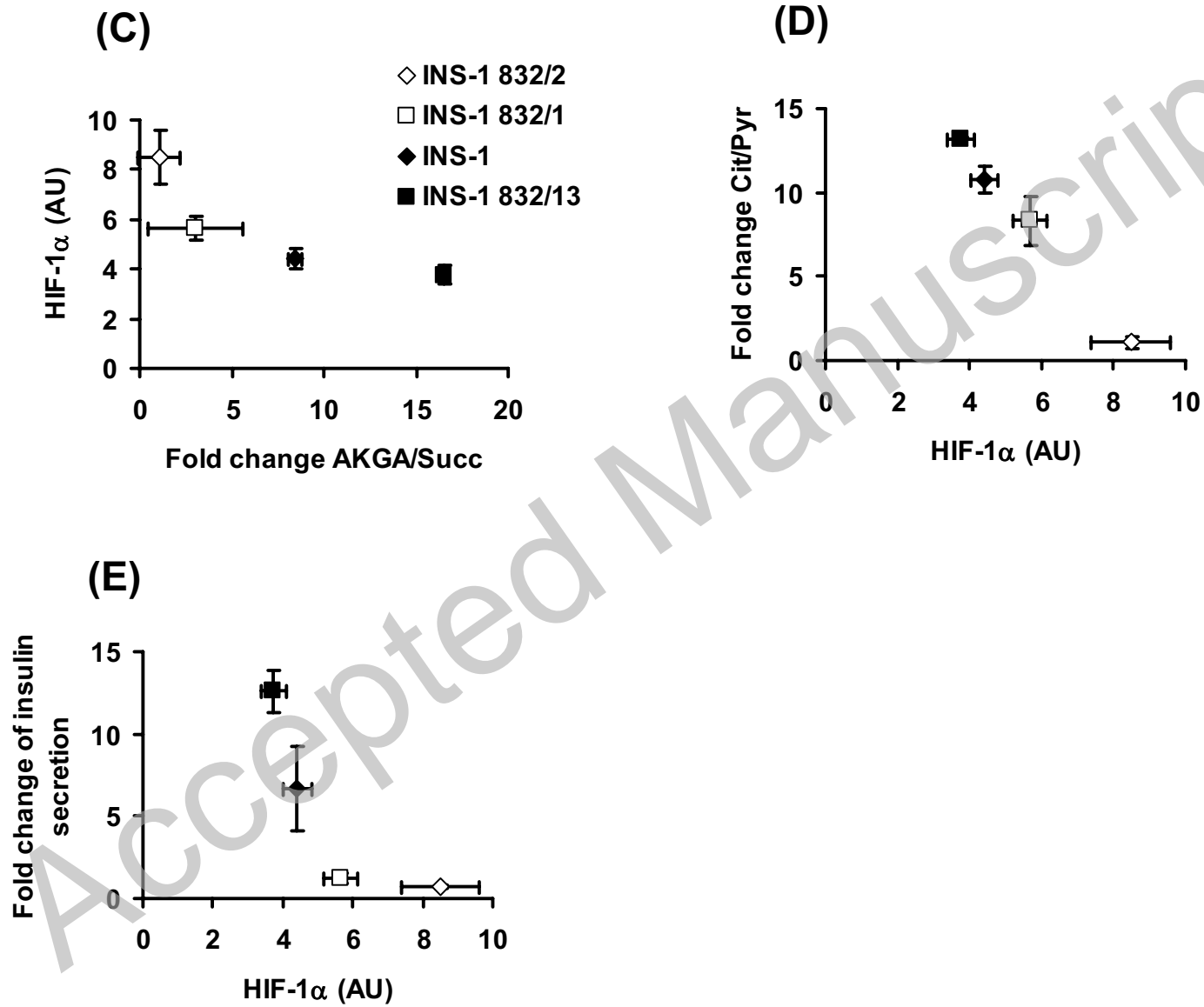
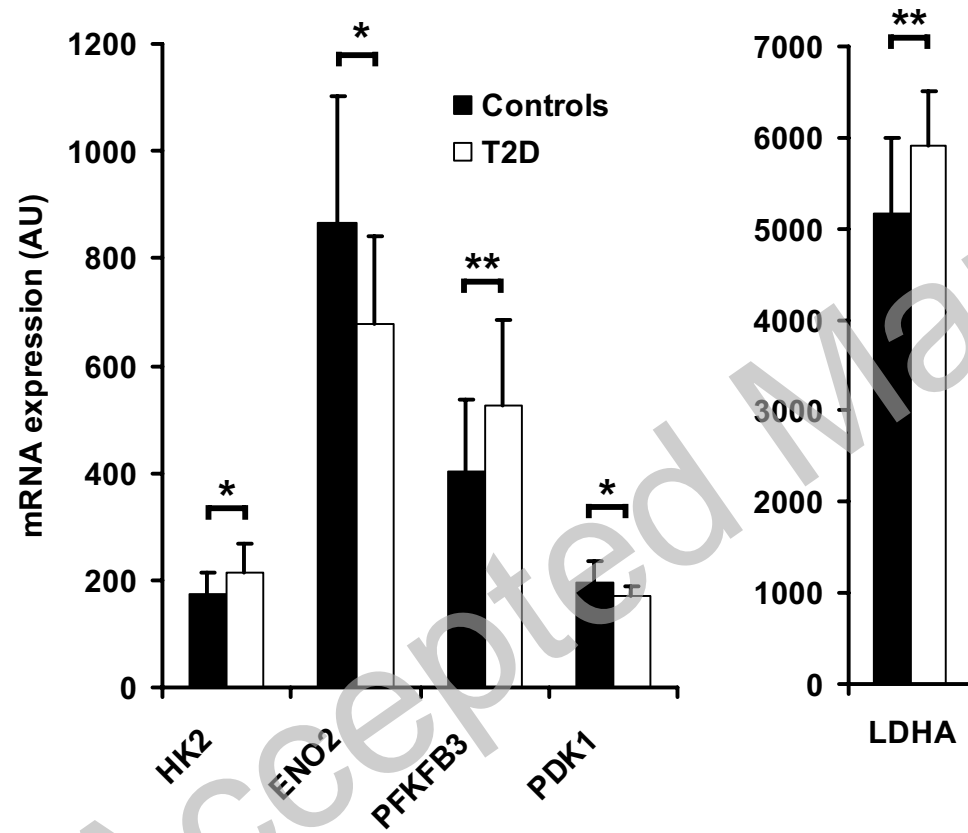
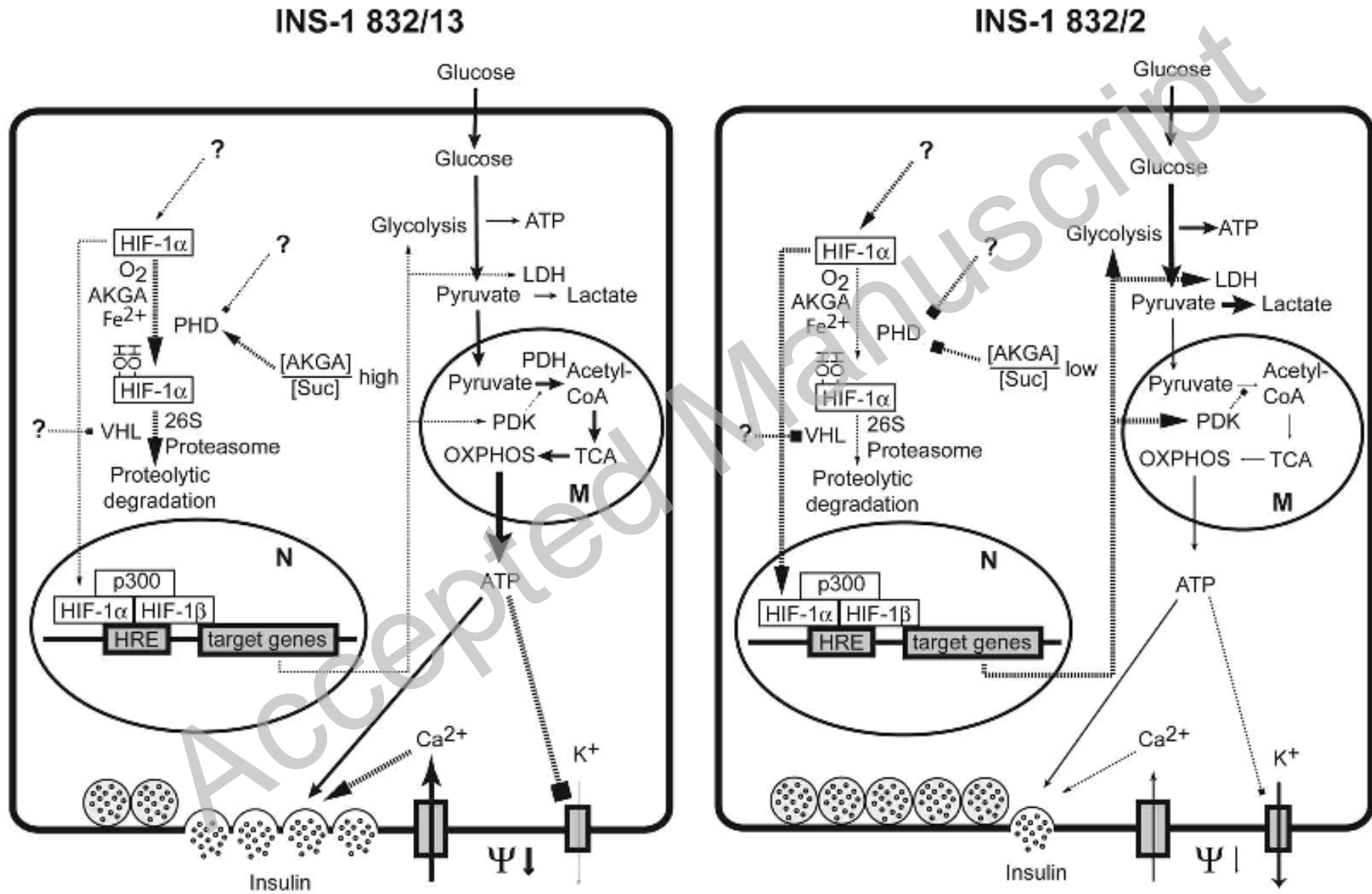


Figure 5



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Figure 6



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