**SUPPLEMENTAL DATA**

**Amendments to the original Article for Diabetes**

**Glucose-stimulated insulin secretion fundamentally requires H2O2 signaling by NADPH oxidase 4**

**Lydie Plecitá-Hlavatá1, Martin Jabůrek1, Blanka Holendová1, Jan Tauber1, Vojtěch Pavluch1, Zuzana Berková2, Monika Cahová2, Katrin Schroeder3, Ralf P. Brandes3, Detlef Siemen4 and Petr Ježek1,\***

**1**Department of Mitochondrial Physiology, No.75, Institute of Physiology of the Czech Academy of Sciences, Prague, 14220, Czech Republic

**2**Institute of Clinical & Experimental Medicine, Prague, 14021, Czech Republic

**3**Institut für Kardiovaskuläre Physiologie, Goethe-Universität, Theodor-Stern Kai 7, Frankfurt, 60590, Germany

**4**Klinik für Neurologie, Universität Magdeburg, Leipziger Str.44, Magdeburg, 39120, Germany

**\*Corresponding author: *jezek@biomed.cas.cz***

Part I Verification of silencing and overexpression in INS-1E cells

Part II Cytosolic NADPH increases simultaneously with cytosolic H2O2 release upon GSIS

Part III Verification of -cell-specific NOX4 knockout (NOX4KO) mice

Part IV Possible causes of attenuated responses to glibenclamide for NOX4KO mice

Part V 3-point sampling of individual mice

Part VI Detailed documentation of the IGT phenotype of NOX4KO and NOX4KO mice

Part VII Mice weight and normal pancreatic islet morphology in NOX4KO mice

Part VIII H2O2 signaling targeting KATP is not a significant part of fatty acid-stimulated insulin secretion

Part IX Original data for 14C-glucose uptake into the glycogen of diaphragm and lipids of epidydimal adipose tissue

Part X **Appendix** – absolute P values for ANOVA using Holm-Sidak tests

**Part I** Verification of silencing and overexpression in INS-1E cells

To verify silencing procedures for NOX4 (Fig.S1A), we employed both qRT-PCR (according Ref.: Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 2001, 29(9):e45) as well as Western blotting. Verification using Western blots for NOX4 siRNA see Fig.1C,D.

**Fig. S1A. Verification of NOX4 silencing in INS-1Ecells Fig. S1B. Verification of SUR1 silencing in INS-1E**

– using qRT-PCR; data ±SDs from four transfections. **cells** – Western blots; data ±SDs from two exp.

**SUR1.tif**

**Silencing of sulfonylurea receptor SUR1** – verification for results of Fig.1A, Fig.6E,F.

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**Silencing of glucose-6-phosphate dehydrogenase** – verification for results of Fig.1E

**Fig. S1C. Verification of G6PDH silencing in INS-1Ecells** – via its activity was performed using a MAK015-1KT kit (Sigma Aldrich) based on G6PDH reaction quantifying NADPH; data ±SDs from three transfections are shown.

**Overexpression of catalase** – verification for results of Fig.1F

**Fig. S1D. Verification of catalase overexpression by Western blot** –

Typical Western blots indicates catalase overexpression 24 and 48 hours after transfection. *Bars*: Fold-expression normalized Coomassie blue staining.

**Silencing of branched-chain ketoacid dehydrogenase BCKDH** – verification for results of Fig.8B,C,D.

**Fig. S1E. Verification of BCKDH silencing** – using qRT-PCR; data ±SDs from three transfections are shown.

**Part II**  Cytosolic NADPH increases simultaneously with cytosolic H2O2 release upon GSIS

NOX4 is the only constitutively expressed NADPH oxidase directly producing H2O2 [18]. Our data demonstrated that NOX4 does so upon GSIS in INS-1E cells (see Fig.2) and PIs (see Fig.4I–K). The observed H2O2 increase was mitochondria-independent, since the superoxide release into the mitochondrial matrix diminished upon glucose addition (Fig.2I), due to the export of NADPH (Fig.S2A,B) resulting from activated redox shuttles, supplying NADPH also for NOX4. Note that GSIS as such was unaffected by the matrix antioxidant SkQ1 (Fig.8K).

The cell cytosolic ROSrelease rates *J*c were estimated from slopes of DCF time-lapsed monitoring (such as in Fig.2A, Fig.4I,J). To identify species as H2O2 we also monitored cytosolic ROSrelease rates using the strictly H2O2-selective probe HyPer-C (Fig.2F), indicating a similar extent in cytosolic H2O2 increase upon GSIS as the third probe used, *i.e*. HSP-FRET monitoring ROS on the basis of Forster resonance energy transfer (FRET) (Fig.2G). The pattern was similar to the pattern when only the normalized fluorescence intensities at a given time point (F) were compared (Fig.2C,D,E,H).

10 M DPI, non-specifically inhibiting NOX enzymes, largely prevented the glucose-stimulated ROS increase (Fig.2C), similarly to the inhibition of oxidative phosphorylation and hence also an overall metabolism via a feedback provided with a Complex III inhibitor stigmatellin (0.5 M, Fig.2C); or similarly to blockage of the pentose phosphate pathway (PPP) with 1 mM 6-aminonicotinamide (“6-AN”), or 40 M oxythiamine (Fig.2H). Note that the PPP blockage prevented also NADPH elevation as demonstrated in Fig.S2. All these data together with results of G6PDH silencing partially abolishing GSIS (Fig. 1E) support a mechanism based upon elevation of cytosolic NADPH due to acceleration of G6PDH reaction followed by the increased NOX4 activity fed by the resulting NADPH.

The independent evaluation was performed by estimations of cytosolic ratios between bound NADPH (NADPHB) and bound NADH (NADHB) from fluorescence life time confocal microscopy imaging (FLIM) of INS-1E cells according to Duchen and colleagues (Blacker *et al*., see Ref. in Fig.S2 legend). These ratios are derived using an empirical formula from the respective autofluorescence time decay. Again, a profound elevation is indicated for NADPH (being in a bound state to the cytosolic enzymes).

NADPH for supplement.tifA B

**Fig. S2. Changes in cytosolic NADH and NADPH during GSIS in INS-1E cells**

(**A**) **Total cell NADPH levels** in INS-1E cells after addition of glucose, assayed by a specific enzyme reaction kit (BioVision, Milpitas, CA) normalized to an average prior to GSIS, at 10 min (*gray bars*) or 1 hour (*dashed gray bars*) after glucose addition; *n* = 5.

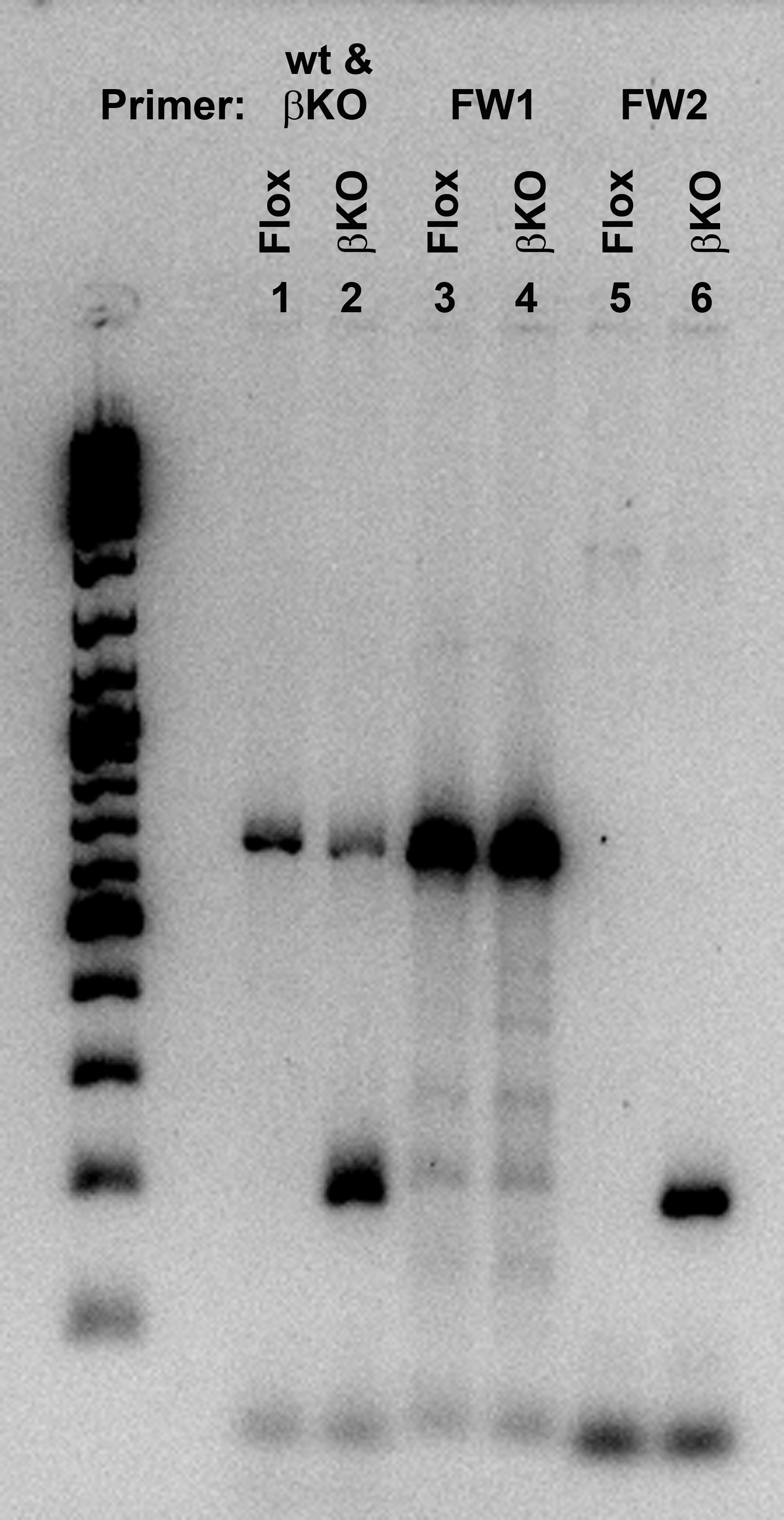
(**B**) Bound NADPH/NADH in the cytosol or nucleus as derived from fluorescence lifetime imaging *In situ* NAD(P)H autofluorescence was quantified using a double-channel fluorescence lifetime imaging attachment (Becker&Hickl, Berlin, Germany) to a Leica TSC-SP8 confocal microscope (Leica Microsystems, Mannheim, Germany) [*Blacker TS, Mann ZF, Gale JE, Ziegler M, Bain AJ, Szabadkai G, and Duchen MR. Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. Nat Commun 5: 3936, 2014.*].

ANOVA Tukey test **K**: \*\*\* P < 0.001; \*\* P< 0.05; \* P< 0.1; Holm-Sidak test see Appendix in this Supplement on-line.

**Part III** Verification of -cell-specific NOX4 knockout (NOX4KO) mice

**A) Verification based on PCR of genomic DNA from mouse pancreatic islets**

Verification has been performed as described in Experimental Design and Methods. In DNA from PIs of the control NOX4*Flox/Flox* mice, no KO band was detected. In DNA from PIs of NOX4KO mice, both bands representing the control and the “KO” alleles were detected (Fig.S3A). Consequently, we verified the presence of β-cells having the NOX4 deletion in PIs of our NOX4KO mice.



**Fig. S3A. Verification of -cell-specific NOX4 knockout (NOX4KO) – PCR of genomic DNA from mouse pancreatic islets**

Genomic DNA of isolated pancreatic islets (PIs) from control NOX4*Flox/Flox* mice (“**Flox**”, *odd lanes*) and NOX4KO mice (“**KO**”, *even lanes*) visualized on an agarose gel, using both control andKO primers (“**wt & KO**”, lanes 1 and 2), sole NOX4FW1 primer with the reverse primer to detect only control allele (“**FW1**”, lanes 3 and 4), and sole NOX4FW2 primer with the reverse primer to detect -cell specific NOX4 deletion (“**FW2**”, lanes 5 and 6).

**B) Estimation of *Cre* activity in mouse pancreatic islets isolated from β-cell-specific NOX4 knockout mice**

The constructed mouse strain (B6.Cg-Nox4<tm1Ams>Tg(Ins2-cre)25Mgn) mice were intercrossed with the reporter mouse strain B6.129(Cg)-Gt(ROSA)26Sor<tm4(ACTB-tdTomato,-EGFP)Luo expressing two-color *Cre*-reporter allele. Prior to Cre recombination, cells show tdTomato fluorescence while Cre recombinase expressing cells replace red fluorescence for EGFP. The resulting mouse population was used to verify the expression of the *Cre* allele necessary for deletion of the NOX4 specifically in pancreatic β-cells.

Fig CRE for Supplement.tifThe resulting images show unequivocal co-localization of the GFP-expressing cells (Cre-positive) with Cy5- (*i.e.* insulin-) positive cells (Fig.S3B).

**Fig. S3B. Visualization of the mouse pancreatic islets obtained from the progeny of the cross-bred reporter (EGFP-tdTomato) and β-cell- specific NOX4 knockout (NOX4KO) – A)** Merged image; **B)** detail of the merged image; **C)** Cy5-positive (*i.e*. insulin-positive cells; *pink*);  **D)** tdTomato positive (*i.e*. *Cre*-negative cells; red); **E**) EGFP positive (*i.e*. *Cre*-positive cells; *light green*); **F)** DAPI stained nuclei (*blue*).

**Method:**

Mice were killed after bleeding with cervical dislocation and the pancreases were pulled out and processed using standard fixation in 3.8–4% formalin in PBS for 24 hours, followed by dehydration and transfer to paraffin with graded alcohol series and xylene soaking (ASP6025, Leica). Finally samples were embedded into paraffin blocks (EG1150 H+C, Leica). Sections 2.5–3 μm thick were prepared on a rotary microtome (RM2255, Leica) and mounted on Superfrost PlusTM glass slides (ThermoFisher Scientific). The samples were subsequently IHC-stained using a Ventana Discovery Ultra machine as follows. Slides were rehydrated and treated using pH 6 antigen-retrieval buffer for antigen unmasking. Specific labeling of cell population was performed using *anti*-Insulin antibody (Abcam, ab181547, dilution 1:500) with *anti*-rabbit Cy5-secondary antibody (Abcam, ab6564, dilution 1:800). Mounted slides were then scanned using Axio Scan.Z1 slide scanner (Carl Zeiss) at 10**×** magnification and 0.22 μm/pixel resolution. Fluorescence of Cy5, EGFP, tdTomato and DAPI was detected. The images were analyzed using ZEN 2.3 lite software.

**Part IV** Possible causes of attenuated responses to glibenclamide for NOX4KO mice

NOX4KO mice exhibited roughly half responses to glibenclamide when compared to NOX4KO mice (Fig.3E,F; Fig.4E,F), indicating certain inhibition or impairment of the KATP-dependent pathways of insulin secretion. Such inhibition is only manifested for the KATP-dependent pathways (cf.Fig.7E,F for OIC), since the predominantly GPR40-dependent pathway of fatty acid-induced insulin secretion (FASIS) was kept intact (Fig.4G,H). Concerns could be raised due to the original construction of our NOX4KO mouse strain based on the Cre recombinase, beginning crossing of NOX4Flox/Flox mice with RIP2-Cre (*Ins2*-*cre*) mice. This is because the RIP2-Cre mouse strain was shown recently to induce serotonin/pregnancy-related phenotypic changes suppressing GSIS as a possible consequence of induction of ectopic serotonin induced by increased expression of human growth hormone minigene (hGH), which is a part of a construct to enhance transgene (*Cre*) expression. We have quantified transcript of tryptophan hydroxylase (encoded by the *Tph1* gene), a major enzyme producing serotonin (5-hydroxytryptamine; cf. Kim *et al*., Nat.Med. 2010; 16, 804-808). Depending on the “housekeeping gene” used for normalization, there was 30–50% of *Tph1* transcript in our own-constructed NOX4KO mouse strain relatively to RIP2-Cre mice (Fig.S4A). Interestingly, the expression of hGH didn´t affect GSIS on a cellular level, performed by expression of the identical construct in INS1E cells (Fig.S4B). The hGH minigene expression decreased the first phase but only slightly the second phase of GSIS in perifused islets isolated from RIP2-Cre mice (Fig.S4C), and in the same extent also responses to glibenclamide. Even if the hGH minigene expression was reported to suppress GSIS on a systemic level in RIP2-Cre mice, other authors demonstrated that after backcrossing of RIP2-Cre mice into the C57Bl6/J background, this side effect disappeared (Fex *et al*. Journal of Endocrinology 2007; 194, 551–555).

We must emphasize that when isolated islets from NOX4Flox/Flox mice are compared to those from RIP2-Cre mice, a partial inhibition of GSIS is recognized (Fig.S4C). In contrast the islets isolated from RIP2-Cre mice have less suppressed the first phase of GSIS when compared to NOX4KO islets (Fig.4B), whereas the second phase in RIP2-Cre islets was inhibited only marginally (Fig.S4C). Consequently, the additional supression of GSIS in the NOX4KO islets should be ascribed to further inhibition due to the ablated NOX4. Such NOX4-dependence is independently supported by observations of the H2O2 rescue (Fig.4N) and rescue due to the lentiviral overexpression (Fig.4L).

**A B**

**Fig. S4. Ability to produce serotonin and effects of expression of human growth hormone minigene in RIP2-Cre islets** –

**A) Tryptophan hydroxylase (*Tph1*) transcript levels** in islets of NOX4KO mice compared to RIP2-Cre mice can be related to the ability to produce serotonin; data normalized to housekeeping genes -glucuronidase (vs GUSB) and peptidylprolyl isomerase A (vs PPIA);  **B) GSIS unaffected** by hGH minigene expression in INS-1Ecells; **C) Affected GSIS in RIP2-Cre islets** (*purple*)**; E) Comparison of RIP2-Cre *vs*. NOX4KO** **islets** (*red* data with omitted SDs from Fig.4B) and islets from control NOX4Flox/Flox mice (*black*) – experiments were performed as described in

Research Design and Methods.

**C D**



**Part V**  3-point sampling of individual mice and detailed documentation of the IGT phenotype of NOX4KO and NOX4KO mice

Three points DMB+CTRL.tifTo ascertain that peaks are real within the ensemble of all collected points of the first 10–15 min for the fast insulin release in mice, three time-point sampling was used for selected mice within each experiment. In order to show that our sampling is able to distinguish various amounts of released insulin, we compare the net GSIS (*black points* in Fig.S5) with a stimulation by an agonist of the glucagon-like peptide receptor (GLP1R) 6,7-dichloro-2-methylsulfonyl-3-N-tert-butylaminoquinoxaline (DMB) at simultaneous administration of glucose (*white points* in Fig.S5).

**Figure S5. 3-point sampling from individual NOX4*Flox/Flox* mice**

Insulin released to the blood circulationwas sampled at three time points of each individual mouse from eye plexus blood after i.p. injection of glucose bolus 1 mg *per* g body weight (**A**, **B** *black points*) or the same glucose bolus together with 1.74 mg.kg-1 body weight of DMB (~100 nmol DMB *per* mice) (*white points*). Mice were 14 hr-fasted. experiment.

**Part VI** Detailed documentation of the IGT phenotype of NOX4KO and NOX4KO mice

Both NOX4KO mice (Fig.S6A,C) and NOX4O mice (Fig.S6B,D) were inspected for long time courses of insulin release (Fig.S6A,B) and glycemia (Fig.S6C,D) to demonstrate that despite the profoundly decreased insulin release response on the initial glucose dose, glycemia always returned to the basal state. This delay is a typical symptom of the impaired glucose tolerance. In contrast, a diabetic state is recognized when the second phase of increased glycemia lasts permanently, Experiments were performed as those described in Fig.3A–D.

**240min ALL.tif**

Figure S6. 4-hr time courses for NOX4KO and NOX4KO mice

(**A**,**B**) **Insulin released to the blood** **circulation** and (**C**,**D**) **glycemia surveyed up to 240 min**, both probed in eye plexus after i.p. injection of glucose bolus (1 mg *per* g body weight) to 14 hr-fasted wt (backcrossed) or NOX4KO mice (**A**,**C**); and NOX4*Flox/Flox* or NOX4KO mice (**B**,**D**). Each mouse was used for two to three time points and different littermates are indicated by the different symbols. Delayed return of glycemia indicates the IGT, in contrast to a non-existing diabetic state (when the second increased glycemia would last permanently). Experiments were performed as those described for Fig.3A–D. Student’s T-test (each time point contained at least 2–8 estimates): \*\*\* P < 0.001; \*\* P< 0.05; \* P< 0.1; ns non-significant.

**Part VII** Mice weight and normal pancreatic islet morphology in NOX4KO mice

cutaroundNEW BodyWeight.tifThe two months old male NOX4KO mice had similar weight as backcrossed wt mice, whereas female NOX4KO mice weighted even a little bit more (Fig.S7). In contrast, both two months old male and female NOX4KO mice had significantly lower weight as compared to age-matched control NOX4*Flox/Flox* mice (Fig.S7). The only other apparent phenotype of aging for NOX4KO or NOX4O mice was delayed gray fur appearance *vs*. controls, which turned gray more intensively.

**Figure S7.** Body weight of 8 week old mice as indicated. Student’s T-tests: \*\*\* P< 0.001; \*\* P< 0.05.

We also intended to describe in more details pancreatic islet morphology of our novel NOX4KO mice strain. The islet morphology was normal (Fig.S8A,B).

**Figure S8. Non-diabetic phenotype – normal pancreatic islet morphology of NOX4KO mice**

**A,B**) **Sections of pancreas from NOX4Flox/Flox mice**,(**A**) **NOX4O mice** (**B**): glucagon (*brown*) and insulin immunostaining (*pink*). Cell nuclei: *blue*. Normal typical -cell mantle is observed in pancreatic sections of all mice tested, encompassing the rich mass of -cells and accounting for ~90% of islet section area. The average number of -cells in optimum PI sections (defined as being larger than 105 m2) was 160 and 145 for NOX4KO and NOX4*Flox/Flox* mice, respectively. The average size of -cell sections (among 40 to 70 islets) was insignificantly different for NOX4KO and NOX4*Flox/Flox* mice, being 121 ± 27 m2 and 119 ± 31 m2, respectively, and 30 ± 8 m2 and 30 ± 14 m2, respectively was for -cell sections.

**Part VIII** H2O2 signaling targeting KATP is not a significant part of fatty acid-stimulated insulin secretion

We have reported previously that palmitic acid-augmented insulin secretion *via* G-protein-coupled receptor GPR40 is stimulated by phospholipase iPLA2-cleaved fatty acids. Under these conditions, mitochondrial -oxidation provides the H2O2 burst necessary for iPLA2 activation. Now, we demonstrate that such palmitic acid-stimulated insulin release is not significantly suppressed in NOX4-silenced INS-1E cells (Fig.S8). Likewise, the KATP closure in the nontransgenic or NOX4-silenced cells was not a part of such a stimulating mechanism.

**Figure S9 Negative controls for essential contribution of NOX4 to the first phase of GSIS – Fatty acid-stimulated insulin secretion (FASIS) is NOX4- and KATP-independent**

C

Insulin release stimulated by 20 M palmitic acid (“Palm“) in NOX4-silenced cells (*filled* *red and open dark red squares* – see Legend) *vs*. ntg controls (*black and gray triangles*), assayed without glucose (*gray triangles, dark red squares*) or with 25 mM glucose (“Glc“) (*black or purple, triangles, red squares*). Typical time courses are displayed among the three evaluated.

**Part IX** Original data for 14C-glucose uptake into the glycogen of diaphragm and lipids of epidydimal adipose tissue

Orange+Red.tifOriginal data are shown for 14C-glucose uptake into the glycogen of diaphragm (Fig.S10A,B) and lipids of epidydimal adipose tissue (Fig.S10C).

**Figure S10. Accessment of peripheral insuilin resistance** using 14C-glucose incorporation stimulated with insuilin into the glycogen of diaphragm and lipids of epidydimal adipose. tissue4-hr time courses for NOX4KO and NOX4KO mice

 (**A**,**B**) **Data for incorporation into lipids of epidydimal adipose tissue**: each individual tissue sample of each mouse (*N*=7) was assayed without and with insulin. *Gray thick bars*: indicate averages, while gray thin bars +/- SDs. **C)** **Data for incorporation into diaphragm**: when diaphragms from 7 mice were pooled to achieve better resolution of obtained values. SDs were obtained from repeated 14C counting.

The tissues were incubated in the absence or presence of 250 U/ml insulin for two hours in Krebs-Ringer bicarbonate buffer with 5 mmol/l glucose, 0.1 Ci (U-14C)-glucose per ml (UVVR, Řež, Czech Republic) and 2% bovine serum albumin, under 95% O2 and 5% CO2 at 37oC in sealed vials while shaking. Neutral lipids were extracted to chlorophorm / methanol (2:1, vol. to vol.) and the radioactivity was counted by scintillation counting. 14C-glucose accumulation into glycogen in diaphragm was determined after diaphragm digestion by boiling in 30% KOH and precipitation in 96% ethanol.

Part X

**Appendix** – absolute P values for ANOVA using Holm-Sidak tests and other statistics

**Figure 1 B** ***Insulin secretion rates averaged during 60 min, INS-1E cells silenced for NOX4***

Sample: Bar.No. : P by Holm-Sidak test: ordered: Tukey test:

11mM Glc 1 1vs2 1.11E-16 1vs2 1.109E-16 P<0.001

11mM siNOX 2 1vs4 1.79E-15 1vs3 1.06E-11 P<0.001

3mM Glc 3 1vs3 1.06E-11 1vs4 1.793E-15 P<0.001

3mM Glc siNOX 4 3vs4 0.0000216 2vs3 0.0000272 P<0.001

3vs2 0.0000272 2vs4 0.434 0.856

2vs4 0.434 3vs4 0.0000216 P<0.001

**Figure 1 C** ***NOX activity, INS-1E cells silenced for NOX4***

Sample: Bar.No. : P by Holm-Sidak test: ordered: Tukey test:

11mM Glc 1 1vs4 1.658E-019 1vs2 5.536E-019 P<0.001

11mM siNOX 2 1vs2 5.536E-019 1vs3 1.603E-012 P<0.001

3mM Glc 3 1vs3 1.603E-012 1vs4 1.658E-019 P<0.001

3mM Glc siNOX 4 3vs4 0.000000000860 2vs3 0.000000162 P<0.001

3vs2 0.000000162 2vs4 0.0101 P<0.047

2vs4 0.0101 3vs4 0.00000000086 P<0.001

**Figure 2 B** ***Cytosolic ROS by DCF fluorescence rates, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered:

ntg 11 mM to 25 mM 1 2vs1 3.40E-12 1vs2 3.40E-12

si NOX4 11 mM to 25 mM 2 2vs3 3.40E-12 1vs3 1

ntg 3 mM to 25 mM 3 1vs4 3.525E-11 1vs4 3.525E-11

si NOX4 3 mM to 25 mM 4 3vs4 3.525E-11 2vs3 3.40E-12

4vs2 0.0183 2vs4 0.0183

1vs3 1 3vs4 3.525E-11

**Figure 2 C** ***Cytosolic ROS by DCF fluorescence intensity, INS-1E cells***

D=DPI, S=Stigmatellin Sample No.: P by Holm-Sidak test: ordered:

11mM Glc 1 1vs2 8.06E-09 1vs2 8.06E-09

11mM Glc to 25 mM 2 2vs4 7.12E-05 1vs3 0.053500

D+11mM Glc to 25 mM 3 2vs5 6.23E-04 1vs4 0.172

S+11mM Glc to 25 mM 4 2vs3 0.00123 1vs5 0.040700

S+D+11mM Glc to 25 mM 5 5vs1 0.0407 2vs3 0.001230

3vs1 0.0535 2vs4 0.000071

4vs1 0.172 2vs5 0.000623

5vs4 0.538 3vs4 0.554

3vs4 0.554 3vs5 0.996

3vs5 0.996 4vs5 0.538

Sample No.: P by Holm-Sidak test: ordered:

3mM Glc 1 1vs2 0.00000003 1vs2 3.00E-08

3mM Glc to 25 mM 2 2vs5 0.000129 1vs3 0.104

D+3mM Glc to 25 mM 3 2vs3 0.00032 1vs4 0.067200

S+3mM Glc to 25 mM 4 2vs4 0.000598 1vs5 0.096300

D+S+ 5 4vs1 0.0672 2vs3 0.000320

5vs1 0.0963 2vs4 0.000598

3vs1 0.104 2vs5 0.000129

5vs4 0.803 3vs4 0.851

3vs4 0.851 3vs5 0.957

3vs5 0.957 4vs5 0.803

**Figure 2 D** ***Cytosolic ROS by HSP-FRET fluorescence intensity, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered:

11mM Glc 1 1vs4 0.00000019 1vs2 0.04300

11mM Glc to 25 mM 2 3vs4 0.0000403 1vs3 0.0442

3mM Glc 3 2vs.4 0.00072 1vs4 0.00000019

3mM Glc to 25 mM 4 2vs1 0.04300 2vs3 0.695

3vs1 0.0442 2vs4 0.00072

2vs3 0.695 3vs4 0.0000403

**Figure 2 F** ***Cytosolic ROS by cHYPer fluorescence rates, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered:

11mM Glc 1 1vs4 0.00000369 1vs2 0.00017

11mM Glc to 25 mM 2 3vs4 0.0000612 1vs3 0.153

3mM Glc 3 2vs1 0.00017 1vs4 0.00000369

3mM Glc to 25 mM 4 2vs3 0.00386 2vs3 0.00386

4vs2 0.0621 2vs4 0.0621

1vs3 0.153 3vs4 0.0000612

**Figure 2 G** ***Cytosolic ROS by HSP-FRET fluorescence rates, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered: Tukey test:

11mM Glc 1 1vs4 0.00001640 1vs2 0.00104 0.005

11mM Glc to 25 mM 2 3vs4 0.000726 1vs3 0.169 0.498

3mM Glc 3 2vs1 0.00104 1vs4 0.00001640 P<0.001

3mM Glc to 25 mM 4 2vs3 0.03600 2vs3 0.03600 0.144

4vs2 0.0794 2vs4 0.0794 0.281

1vs3 0.169 3vs4 0.000726 0.004

**Figure 2 H** ***Cytosolic ROS by HSP-FRET intensity, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test, ordered: Tukey test:

11mM Glc to 25 mM 1 1vs2 0.00000688 P<0.001

6AN+11mM Glc to 25 mM 2 1vs3 0.000167 P<0.001

Oxythia+11mM Glc to 25 mM 3 2vs3 0.108 0.233

3mM Glc to 25 mM 1 1vs2 0.00164 0.004

6AN+3mM Glc to 25 mM 2 1vs3 0.0054 0.014

Oxythia+3mM Glc to 25 mM 3 2vs3 0.56 0.824

**Figure 2 I** ***Mitochondrial matrix ROS by MitoSOX fluorescence rates, INS-1E cells ntg vs. siRNA NOX4***

Sample: Sample No.: P by Holm-Sidak test: ordered: Tukey test:

11mM Glc 1 1vs2 4.44E-12 1vs2 4.44E-12 <0.001

11mM Glc to 25 mM 2 3vs2 1.94E-11 1vs3 1 1

siNOX4 11mM Glc 3 1vs4 6.32E-11 1vs4 6.32E-11 <0.001

siNOX4 Glc to 25mM 4 3vs4 1.58E-10 2vs3 1.94E-11 <0.001

2vs4 0.289 2vs4 0.289 0.639

1vs3 1 3vs4 1.58E-10 <0.001

3mM Glc 1 3vs2 0.00000699 1vs2 0.00002060 <0.001

3mM Glc to 25 mM 2 1vs2 0.00002060 1vs3 0.438 0.857

siNOX4 3mM Glc 3 3vs4 0.00002270 1vs4 0.000111 <0.001

siNOX4 3mM 25 mM 4 1vs4 0.000111 2vs3 0.00000699 <0.001

3vs1 0.438 2vs4 0.606 0.952

2vs4 0.606 3vs4 0.00002270 <0.001

**Figure 3 A** ***Insulin secretion time course during 60 min, NOX4KO vs. backcrossed mice***

Sample: P by T test: Mann-Whitney Ran Sum Test:

10 min P<0.001

15 min P=0.071

**Figure 3 B** ***Insulin secretion time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

5 min P=0.003

10 min P<0.001

15 min P<0.001

20 min P<0.001

60 min P=0.792

0 min P=0.558

**Figure 3 C** ***Glycemia during 60 min, NOX4KO vs. backcrossed mice***

Sample: P by T test:

5 min P=0.026

10 min P=0.257

15 min P=0.358

20 min P=0.005

60 min P=0.138

0 min P=0.683

**Figure 3 D** ***Glycemia during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

5 min P=0.243

10 min P=0.002

15 min P<0.001

20 min P<0.001

60 min P<0.001

**Figure 3 E** ***Insulin = response to glibenclamide : secretion time course during 60 min, NOX4KO vs. backcrossed mice***

Sample: P by T test:

5 min P=0.205

10 min P=0.178

15 min P=0.098

20 min P=0.744

60 min P=0.124

0 min P=0.280

**Figure 3 F** ***Insulin = response to glibenclamide : secretion time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

5 min P=0.02

10 min P=0.003

15 min P<0.017

20 min P=0.002

60 min P=0.730

0 min P=0.965

**Figure 3 G** ***Glycemia during response to glibenclamide : time course during 60 min, NOX4KO vs. backcrossed mice***

Sample: P by T test:

5 min P=0.488

10 min P=0.478

15 min P=0.327

20 min P=0.01

60 min P=0.183

0 min P=0.062

**Figure 3 H** ***Glycemia during response to glibenclamide : time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

5 min P=0.109

10 min P=0.005

15 min P<0.001

20 min P=0.001

60 min P<0.001

0 min P=0.827

**Figure 3 I** ***Insulin = response to glucose: secretion time course during 60 min, NOX2KO vs. NOX2-backcrossed mice***

Sample: P by T test:

5 min P=0.870

10 min P=0.910

15 min P=0.636

20 min P=0.483

60 min P=0.107

0 min P=0.325

**Figure 3 J** ***Glycemia during response to glucose : time course during 60 min, NOX2KO vs. backcrossed mice***

Sample: P by T test:

5 min P=0.845

10 min P=0.714

15 min P=0.940

20 min P=0.443

60 min P=0.221

0 min P=0.679

**Figure 3 K** ***Insulin RESISTANCE Diaphragm of NOX4KO and NOX4KO mice vs. respective controls***

Sample: Bar.No. : P by Holm-Sidak test: ordered: Tukey test:

backcrossed 1 1vs4 2.48E-10 1vs2 0.000000231 P<0.001

NOXKO = null 2 3vs4 2.48E-10 1vs3 1.00E+00 1.00E+00

Flox/Flox 3 1vs2 2.31E-07 1vs4 2.48E-10 P<0.001

NOX4KO 4 3vs2 0.000000231 2vs3 0.000000231 P<0.001

2vs4 0.0000705 2vs4 0.0000705 P<0.001

1vs3 1.00E+00 3vs4 2.48E-10 P<0.001

**Figure 3 L** ***Insulin RESISTANCE WAT of NOX4KO and NOX4KO mice vs. respective controls***

Sample: Bar.No. : P by Holm-Sidak test: ordered: Tukey test:

backcrossed 1 1vs2 3.31E-05 1vs2 0.0000331 P<0.001

NOX KO = null 2 3vs.2 3.31E-05 1vs3 1.00E+00 1.00E+00

NOX4 Flox/Flox 3 3 1vs4 3.64E-03 1vs4 0.00364 0.019

NOX4KO 4 3vs.4 0.00364 2vs3 0.0000331 P<0.001

2vs4 0.14 2vs4 1.40E-01 0.445

1vs3 1.00E+00 3vs4 0.00364 0.019

**Figure 4 A** ***Perifused Islets: Insulin secretion time course during 60 min, NOX4KO vs. backcrossed mice***

Sample: P by T test:

0 min P=0.879

2 min P=0.056

4 min P<0.001

6 min P=0.006

8 min P=0.011

10 min P=0.047

12 min P=0.145

14 min P=0.188

16 min P=0.591

18 min P=0.619

20 min P=0.063

25 min P=0.012

30 min P=0.166

35 min P=0.362

40 min P=0.012

45 min P=0.086

50 min P=0.310

55 min P=0.193

60 min P=0.610

**Figure 4 B** ***Perifused Islets: Insulin secretion time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

4 min P<0.001

6 min P<0.001

8 min P<0.001

10 min P=0.003

12 min P=0.009

14 min P=0.009

16 min P=0.296

30 min P=0.021

35 min P=0.002

40 min P=0.002

45 min P<0.001

50 min P<0.001

55 min P<0.001

60 min P<0.001

**Figure 4 G** ***Perifused Islets in the presence of fatty acids: Insulin secretion time course during 60 min, NOX4KO vs.backcrossed mice***

Sample: P by T test:

0 min P=0.008

2 min P=0.678

4 min P<0.837

6 min P=0.089

8 min P=0.005

10 min P=0.008

12 min P=0.113

14 min P=0.891

16 min P=0.813

18 min P=0.418

20 min P=0.034

25 min P=0.604

30 min P=0.411

35 min P=0.226

40 min P=0.260

45 min P=0.097

50 min P=0.026

55 min P=0.116

60 min P=0.083

**Figure 4 H** ***Perifused Islets in the presence of fatty acids: Insulin secretion time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

4 min P=0.001

6 min P<0.001

8 min P=0.004

10 min P=0.353

12 min P=0.002

14 min P=0.01

16 min P=0.008

18 min P<0.001

20 min P=0.014

22 min P=0.004

25 min P=0.379

30 min P=0.319

35 min P=0.973

40 min P=0.015

45 min P=0.104

50 min P=0.318

55 min P=0.332

60 min P=0.356

**Figure 4 K** ***Cytosolic ROS*** ***in islets by fluorescence DCF rate – data from NOX4 KO vs. backcrossed and NOX4KO vs. NOX4Flox/Flox mice***

Sample: Sample No.: P by Holm-Sidak test: ordered:

PI from wt = baccrossed 1 2vs1 3.396E-12 1vs2 3.396E-12

PI from NOX4 KO 2 2vs3 3.396E-12 1vs3 1

PI from NOX4Flox/Flox 3 1vs4 3.525E-11 1vs4 3.525E-11

PI from NOX4KO 4 3vs4 3.525E-11 2vs3 3.396E-12

4vs2 0.0183 2vs4 0.0183

1vs3 1 3vs4 3.525E-11

**Figure 4 L** ***Insulin secretion at 6 min NOX4KO pancreatic islets - Rescue by nox4expression***

Sample: Bar.No. : P by Holm-Sidak test: ordered: Tukey test:

GFP 1 1vs2 0.00160 1vs2 0.00160 0.007

NOXbetaKO 2 1vs4 0.222 1vs3 0.04420 0.158

NOX4+/+ 3 1vs3 0.04420 1vs4 0.222 0.574

NOX4beta KO plus nox4 4 3vs4 0.321 2vs3 0.000107 P<0.001

3vs.2 0.000107 2vs4 0.000324 0.002

2vs.4 0.000324 3vs4 0.321 0.722

**Figure 5 F** ***NPo from patch clamp siRNA NOX4 vs ntg, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered: Tukey test:

ntg 11 mM 1 2vs3 3.09E-07 1vs2 0.0914 0.311

si NOX4 11 mM 2 1vs3 6.44E-06 1vs3 6.44E-06 <0.001

ntg 11 mM to 25 mM 3 4vs3 0.00028 1vs4 0.0697 0.25

si NOX4 to 25 mM 4 2vs4 0.00179 2vs3 3.09E-07 <0.001

1vs4 0.0697 2vs4 0.00179 0.009

2vs1 0.0914 3vs4 0.00028 0.002

**Figure 6 B** ***RSIS siRNA NOX4 vs. ntg, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered: Tukey test:

H2O2 1 3vs2 0.00006220 1vs2 0.000548 0.003

siRNA NOX4 H2O2 2 2vs1 0.000548 1vs3 0.4 0.827

H2O2 +Glc 3 3vs.4 0.00099 1vs4 0.00798 0.037

Si NOX4 H2O2+Glc 4 4vs1 0.00798 2vs3 0.00006220 0.001

4vs2 0.286 2vs4 0.286 0.698

1vs3 0.4 3vs4 0.00099 0.005

siSUR glc 5 P<0.001

**Figure 6 C** ***GSIS, RSIS & agents INS-1E cells siRNA NOX4 vs ntg***

Sample No. vs. CTRL P by Holm-Sidak method

Diazoxide 2 3.925E-14

Cromakalim 3 5.489E-15

Nimodipine 4 2.564E-14

H2O2+Nimodipine 5 6.597E-14

Oligo 6 3.097E-14

Glc+oligp 7 1.695E-14

H2O2+Oligo 8 0.179

siNOX4 Nimodipine 2 0.000000361

siNOX4 Oligo 3 0.000000802

siNOX4 Glc+Oligo 4 0.0000219

**Figure 6 D** ***Thalium flux glybenclamide sens., INS-1E cells siRNA NOX4 vs ntg***

Sample No. vs. CTRL P by Holm-Sidak method

***ntg cells:***

11mM Glc to 25 mM 2 1.28E-13

H2O2 3 3.00E-13

2x H2O2 4 5.17E-14

H2O2+Oligo 7 7.49E-16

Oligo 8 8.31E-11

Diazoxide 11mM Glc to 25 mM 2 1.72E-08

Diazoxide H2O2 3 0.705

Diazoxide No add 5 0.999

***siRNA NOX4 cells:***

11mM Glc to 25 mM 2 0.000896

H2O2 3 6.45E-10

2x H2O2 4 6.25E-13

H2O2+Oligo 7 2.25E-13

Oligo 8 0.000896

DIAZOxide H2O2 3 0.00000118

DIAZOxide no add 5 0.0298

Catalase overexpres. 9 0.094

**Figure 6 E** ***Thalium assay SUR1 silencing, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test, ordered: Tukey test:

ntg H2O2 2 0.00004160 P<0.001

siSUR glc 3 1.93E-08 P<0.001

siSUR H2O2 4 0.00000484 P<0.001

**Figure 6 G** ***Thalium flux, glybenclamide sens., INS-1E cells, siRNA NOX4 vs. ntg cells***

Sample: Sample No.: vs. CTRL P by Holm-Sidak test, ordered:

11mM Glc to 25 mM 2 5.234E-13

OIC 3 9.57E-13

Palmitic acid 4 1

si NOX4 11 mM to 25 mM 5 0.000197

siNOX4 OIC 6 6.94E-12

si NOX4 Palmitic acid 7 1

Between two selected samples: P by Holm-Sidak test, ordered:

11mM Glc to 25 mM 1 1vs2 0.000002

si NOX4 11 mM to 25 mM 2 1vs3 0.204

siNOX4 OIC 3 2vs3 0.0000115

**Figure 6 F** ***Plasmamembrane potential assay - siRNA NOX4 silencing, INS-1E cells***

Sample: Sample No.: vs. CTRL P by Holm-Sidak test, ordered: Tukey test:

11mM Glc to 25 mM 2 0.00000033 <0.001

H2O2 after Glc 3 0.00000153 <0.001

H2O2 before Glc 4 0.00000189 <0.001

Catalase overexpression 5 0.00000030 <0.001

**Figure 7 C** ***Insulin secretion stimulated by OIC, INS-1E cells, siRNA NOX4 vs. ntg cells***

Sample: Sample No.: vs. CTRL P by Holm-Sidak test, ordered:

OIC 1 = CTRL

OIC +SkQ1 2 6.07E-14

OIC +AOA 3 0.241

siBCKDH KIC 4 4.426E-13

siBCKDH KIC +SkQ1 5 3.64E-13

siBCKDH no KIC 6 2.848E-14

siRNA NOX4 KIC 7 0.00029

siRNA NOX4 KIC +SkQ1 8 1.377E-12

**Figure 7 D** ***Mitochondrial ROS by MitoSOX Red assay, i.e. superoxide stimulated by OIC, INS-1E cells, siRNA NOX4 vs. ntg cells***

Sample: Sample No.: vs. CTRL P by Holm-Sidak test, ordered:

OIC 2 = CTRL

OIC +SkQ1 3 6.07E-14

OIC +AOA 4 0.241

siBCKDH OIC 5 4.426E-13

siBCKDH OIC +SkQ1 6 3.64E-13

siBCKDH no OIC 7 2.848E-14

P by Holm-Sidak test

1vs2 2.09E-08

1vs3 0.003

1vs4 1.19E-08

1vs5 0.537

1vs6 0.537

1vs7 0.53

2vs3 0.00002

2vs4 0.836

2vs5 3.93E-09

2vs6 3.93E-09

2vs7 1.20E-07

3vs4 9.74E-06

3vs5 0.00045

3vs6 0.00045

3vs7 0.017

4vs5 2.28E-09

4vs6 2.28E-09

4vs7 6.72E-08

5vs6 1

5vs7 0.217

6vs7 0.217

**Figure 7 E** ***OIC-induced insulin secretion time course during 60 min, NOX4 KO vs. backcrossed mice***

Sample: P by T test:

5 min P=0.394

10 min P=0.586

15 min P=0.229

20 min P=0.02

60 min P=0.167

0 min P=0.009

**Figure 7 F** ***OIC-induced insulin secretion time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

5 min P<0.001

10 min P<0.001

15 min P=0.011

20 min P=0.025

60 min P=0.076

0 min P=0.07

**Figure 7 G** ***OIC-induced glycemia time course during 60 min, NOX4 KO vs. backcrossed mice***

Sample: P by T test: **all nonsignificant**

5 min P=0.394

5 min P=0.700

10 min P=0.599

15 min P=0.308

20 min P=0.219

60 min P=0.766

0 min P=0.409

**Figure 7 H** ***OIC-induced glycemia time course during 60 min, NOX4KO vs. NOX4Flox/Flox mice***

Sample: P by T test:

5 min P=0.327

10 min P=0.02

15 min p<0.001

20 min P=0.01

60 min P=0.005

0 min P=0.134

**Figure S3 A** ***Total cell NADPH assay, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered: Tukey test:

11mM Glc 1 2vs4 2.49E-10 1vs2 0.000171 0.002

11mM Glc to 25 mM 2 2vs5 1.90E-08 1vs3 0.343 0.868

1 hr 11mM Glc to 25 mM 3 3vs4 1.30E-07 1vs4 2.73E-07 <0.001

6AN+11mM Glc to 25 mM 4 1vs4 0.000000273 1vs5 0.00012 0.001

Oxythia+11mM Glc to 25mM 5 3vs5 0.0000331 2vs3 0.00363 0.027

1vs5 0.00012 2vs4 2.49E-10 <0.001

2vs1 0.000171 2vs5 1.90E-08 <0.001

2vs3 0.00363 3vs4 1.30E-07 <0.001

5vs4 0.0241 3vs5 0.0000331 <0.001

3vs1 0.343 4vs5 0.0241 0.149

3mM Glc 1 2vs4 0.00000105 1vs2 0.0000666 <0.001

3mM Glc to 25 mM 2 2vs5 0.00000990 1vs3 0.00144 0.011

1 hr 3mM Glc to 25 mM 3 3vs4 0.00001820 1vs4 0.0734 0.355

6AN+3mM Glc to 25 mM 4 1vs2 0.0000666 1vs5 0.408 0.913

Oxythia+3mM Glc to 25 mM 5 3vs5 0.0002 2vs3 0.202 0.683

3vs1 0.00144 2vs4 0.00000105 <0.001

1vs4 0.0734 2vs5 0.00000990 <0.001

2vs3 0.202 3vs4 0.00001820 <0.001

4vs5 0.309 3vs5 0.0002 0.002

1vs5 0.408 4vs5 0.309 0.832

**Figure S3 B** ***FLIM derived NADPH/NADH bound, INS-1E cells***

Sample: Sample No.: P by Holm-Sidak test: ordered:

cytosol 11mM Glc 1 2vs3 0.00000028 1vs2 0.0059

cytosol 11mM Glc to 25 mM 2 2vs4 0.00000785 1vs3 0.00008

nucleus 11mM Glc 3 3vs1 0.00008 1vs4 0.00464

nucleus11mM Glc to 25 mM 4 4vs1 0.00464 2vs3 0.00000028

2vs1 0.0059 2vs4 0.00000785

4vs3 0.0669 3vs4 0.0669