

1 Supplemental Materials

2 Supplemental Methods

3 Pancreatic islet isolation and culture

4 Human pancreatic islets were cultured in Medium 199 with Earle's salts (Sigma-Aldrich Inc.)
5 containing 5 mmol/l glucose and supplemented with 10% v/v heat-inactivated fetal bovine serum
6 (FBS), 1% v/v penicillin and streptomycin, 50 µg/ml gentamicin (all from ThermoFisher
7 Scientific), and 0.25 µg/ml amphotericin (Aurogene s.r.l., Rome, Italy).

8 Mouse pancreatic islets were cultured in RPMI 1640 medium containing 11 mmol/l glucose and
9 supplemented with 10% v/v heat-inactivated FBS and 1% v/v penicillin and streptomycin.

10 INS-1E cells culture

11 Rat insulin-secreting INS-1E cells were cultured in RPMI 1640 medium containing 11 mmol/l
12 glucose and supplemented with 10% v/v heat-inactivated FBS, 1% v/v penicillin and streptomycin,
13 1% v/v non-essential amino acids (ThermoFisher Scientific), 10 mmol/l HEPES pH 7.4, 1 mmol/l
14 pyruvic acid, and 50 µmol/l beta-mercaptoethanol (all from Sigma-Aldrich) in a 5% CO₂
15 atmosphere at 37 °C.

16 Pancreatic islets and INS-1E cells treatments

17 Palmitate was prepared by dissolving the powder in 0.1 mol/l NaOH at 70 °C to obtain a 5 mmol/l
18 palmitate solution, then complexing it with 10% FA-free BSA (FA to BSA molar ratio of 3.3:1).
19 Oleate was prepared by complexing a solution of 0.5 mol/l oleate in ethanol with 10% FA-free BSA
20 at 37 °C.

21 In INS-1E cells, the insulin concentration was 9 nmol/l (\pm 1 nmol/l, n=3) in the KRBH buffer (after
22 the wash out and prior to stimulation with 10 nmol/l insulin), and 71 nmol/l (\pm 9 nmol/l, n=3) in the
23 culture medium (after stimulation with BSA as control for palmitate stimulation and prior to
24 stimulation with 100 nmol/l insulin). Therefore, the doses of insulin used for the execution of the
25 experiments can be considered consistent with the insulin physiological levels in INS-1E cells.

26 To achieve p66^{Shc} knockdown, INS-1E cells were seeded in 6-well dishes with 2 ml of complete
27 medium until a confluence of 70%, then transfected with 100 nmol/l p66^{Shc} siRNA using 2.5 µl/well
28 Lipofectamine® RNAiMAX Reagent and 0.5 ml/well Opti-MEM® medium (ThermoFisher
29 Scientific) for 48 h. During the last 24 h, the cells were incubated with palmitate or BSA as control,
30 then stimulated with insulin. Control cells were treated with Lipofectamine only.

31 To achieve p66^{Shc} hyperexpression, INS-1E cells were grown in 6-well dishes until they reached
32 60% confluency. The cells were infected with 1 µl of the adenovirus constructs in 400 µl of
33 medium and incubated at 37 °C and 5% CO₂ for 90 min, after which 1600 µl of the medium was
34 added. After 24 hours, the cells were treated according to the different experimental conditions.
35 Where indicated, INS-1E cells were transfected with an adenoviral p66^{Shc} construct harboring a
36 Ser³⁶ to Ala³⁶ mutation. An empty adenovirus vector was used as control (mock). Viral titers used

37 were: 5.2×10^7 PFU/ml for Ad/mock, 2.5×10^5 PFU/ml for Ad/p66^{Shc}, and 1.2×10^5 PFU/ml for
38 Ad/p66^{Shc} (Ala³⁶).

39 Where indicated, INS-1E cells were treated with 30 μ mol/l SP600125 for 2 h or 30 μ mol/ pifithrin-
40 alpha for 1 h (both Sigma-Aldrich) prior to stimulation with palmitate/BSA to achieve the inhibition
41 of JNK or p53 protein activity, respectively.

42 Immunoblotting

43 Equal amounts of proteins were resolved by electrophoresis on 6% or 10% w/v sodium dodecyl
44 sulfate polyacrylamide gels and transferred onto polyvinylidene difluoride or nitrocellulose
45 membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories). Proteins were
46 visualized by a chemiluminescence reaction, and images were captured using a Versadoc or a
47 ChemiDoc Imaging System, and quantified by densitometric analysis using Quantity One image
48 analysis software or the Image Lab Software 6.1, respectively (all from Bio-Rad Laboratories).

49 Quantitative Real-Time PCR

50 Pancreatic islets RNA was prepared using 1 ml of TRIzol reagent (ThermoFisher Scientific) for
51 each pellet. Islets were homogenized using IKA T10 basic homogenizer (Sigma-Aldrich) for 1 min,
52 200 μ l chloroform (Merck KGaA, Darmstadt, Germany) was added to each sample, and the tubes
53 were centrifuged at $1200 \times g$ for 15 min at 4 °C. The upper aqueous phase was collected.

54 To obtain RNA from INS-1E cells, they were lysed in RLT buffer, and the lysate was homogenized
55 by using QIAshredder columns (Qiagen).

56 Total RNA from pancreatic islets or beta-cells lysates was purified using the RNeasy Mini Kit
57 (Qiagen); genomic DNA contamination was eliminated by DNase digestion (Qiagen). RNA
58 concentrations were determined by Qubit Fluorometric Quantification (ThermoFisher Scientific).
59 cDNA synthesis was performed on 500 ng total RNA using the iScript Reverse Transcription
60 Supermix for RT-qPCR (Bio-Rad Laboratories). mRNA reverse transcription was performed using
61 an Eppendorf Thermal Cycler (Eppendorf, AG, Hamburg, Germany). Real-time PCR reactions
62 were performed using a 2X ready-to-use master mix (iTaQ Universal SYBR Green Supermix
63 purchased by Bio-Rad Laboratories) in 96-well hard-shell PCR plates covered with optically clear
64 Microseal 'B' PCR plate sealing film (all from Bio-Rad Laboratories). Real-time PCR was
65 performed in a Cfx Connect Real-Time System (Bio-Rad Laboratories) under the following
66 conditions: 95 °C for 3 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Relative RNA levels
67 were determined by analyzing changes in SYBR green fluorescence during PCR using the $2^{-\Delta\Delta C_t}$
68 method. p66^{Shc} mRNA levels were normalized using 18S mRNA as the reference gene. No template
69 controls were included for each analysed gene.

70 Reactive oxygen species (ROS) production measurements

71 Cells were stimulated with 1 mmol/l N-Acetyl-L-cysteine (NAC, Sigma-Aldrich) for 2 h, then
72 cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h or 100 μ mol/l H₂O₂
73 for 1 h. Intracellular ROS were detected through the evaluation of dihydroethidium (DHE)
74 oxidation. INS-1E cells were seeded on glass coverslips, and incubated with 15 μ mol/l DHE
75 (ThermoFisher Scientific) in the dark at 37 °C for 15 min. The ROS-dependent oxidation of the

76 fluorescent probe (excitation 488 nm/emission 585 nm) was measured by acquiring fluorescent
77 images on a Nikon ECLIPSE Ti-S fluorescence microscope (Nikon, Minato, Tokyo, Japan).

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79 **Supplemental Table 1.** Characteristics of antibodies used for immunoblotting.

Specificity	Antibody	Dilution used
Phospho-AKT (Ser ⁴⁷³)	Cell Signaling Technology Inc., #9271	1:1000
Total AKT	Cell Signaling Technology Inc., #4691	1:1000
Beta-actin	Santa Cruz Biotechnology, sc-47778	1:1000
Phospho-IRS 1 (Ser ³⁰⁷)	Cell Signaling Technology Inc., #2381	1:500
Total IRS-1	Millipore, #06-248	1:500
Phospho-p70 S6K (Thr ³⁸⁹)	Cell Signaling Technology Inc., #9205	1:1000
Total Shc	Merck Millipore, #06-203	1:1000
Phospho-p66 ^{Shc} (Ser ³⁶)	Invitrogen, #44828M	1:500
Phospho-c-Jun (Ser ⁶³) II	Cell Signaling Technology Inc., #9261	1:1000
Acetyl p53 (Lys ³⁸²)	Cell Signaling Technology Inc., #2525	1:500

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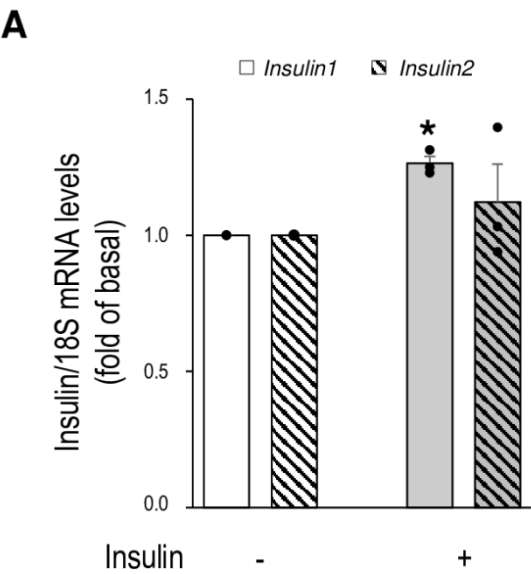
81 **Supplemental Table 2.** Primers used for quantitative real-time PCR analysis.

Gene	Species	Forward primer sequence	Reverse primer sequence
<i>RNA18S5</i>	Homo sapiens	5'-CGAACGTCTGCCCTATCAACTT-3'	5'-ACCCGTGGTCACCATGGTA-3'
<i>p66^{Shc}</i>	Homo sapiens	5'-CCCCAAGCCCAAGTACAA-3'	5'-GACCCAGAAGCCCCTTCCT-3'
<i>RNA18S</i>	Rattus Norvegicus	5'-TGATTAAGTCCCTGCCCTTTGT-3'	5'-GATCCGAGGGCCTCACTAAA-3'
<i>INS1</i>	Rattus Norvegicus	5'-CTGCCCAGGCTTTTGTCAA-3'	5'-TCCCCACACACCAGGTACAGA-3'
<i>INS2</i>	Rattus Norvegicus	5'-GCAAGCAGGTCATTGTTCCA-3'	5'-GGTGCTGTTTGACAAAAGCC-3'

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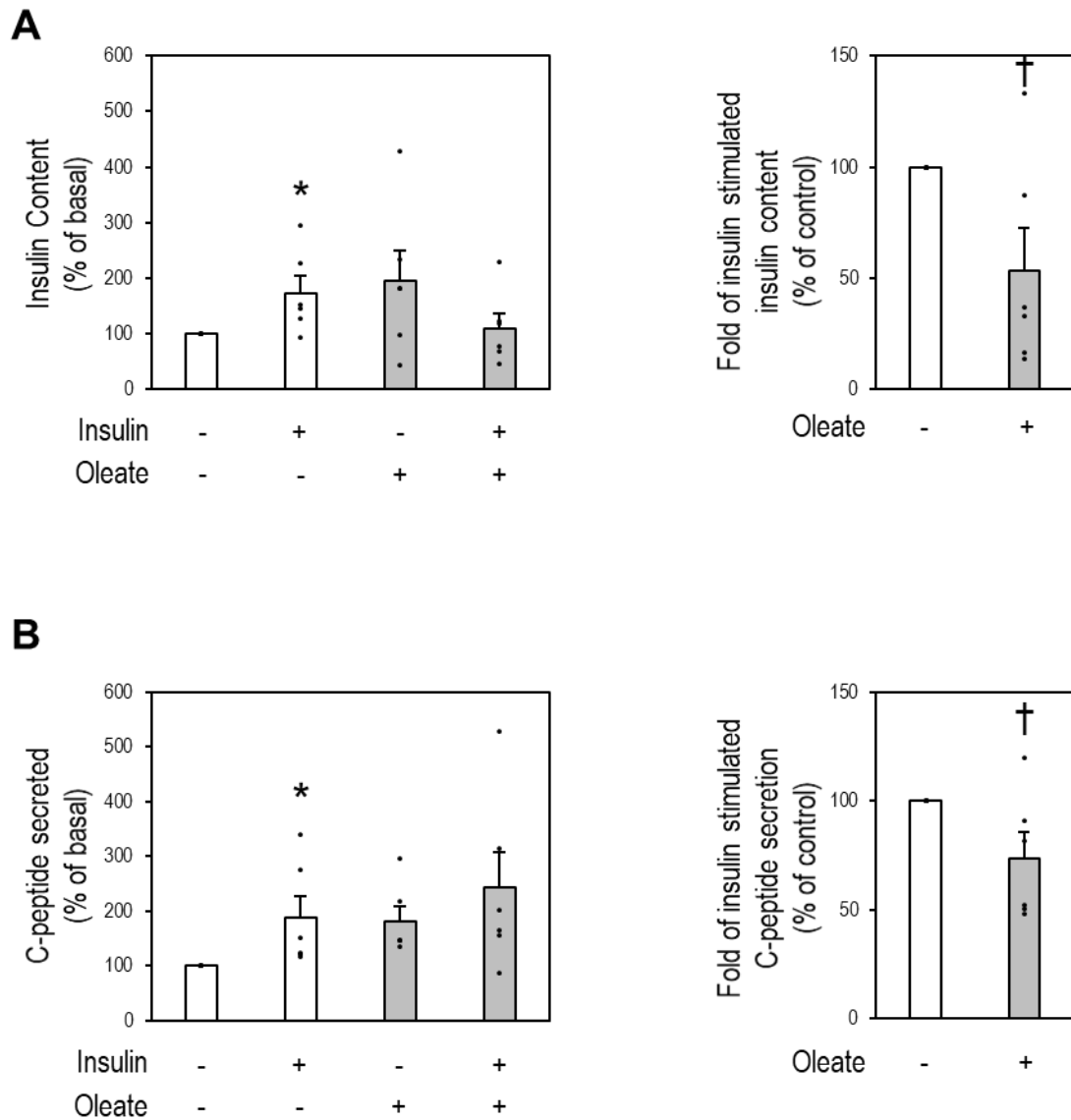
83 **Supplemental Figures**

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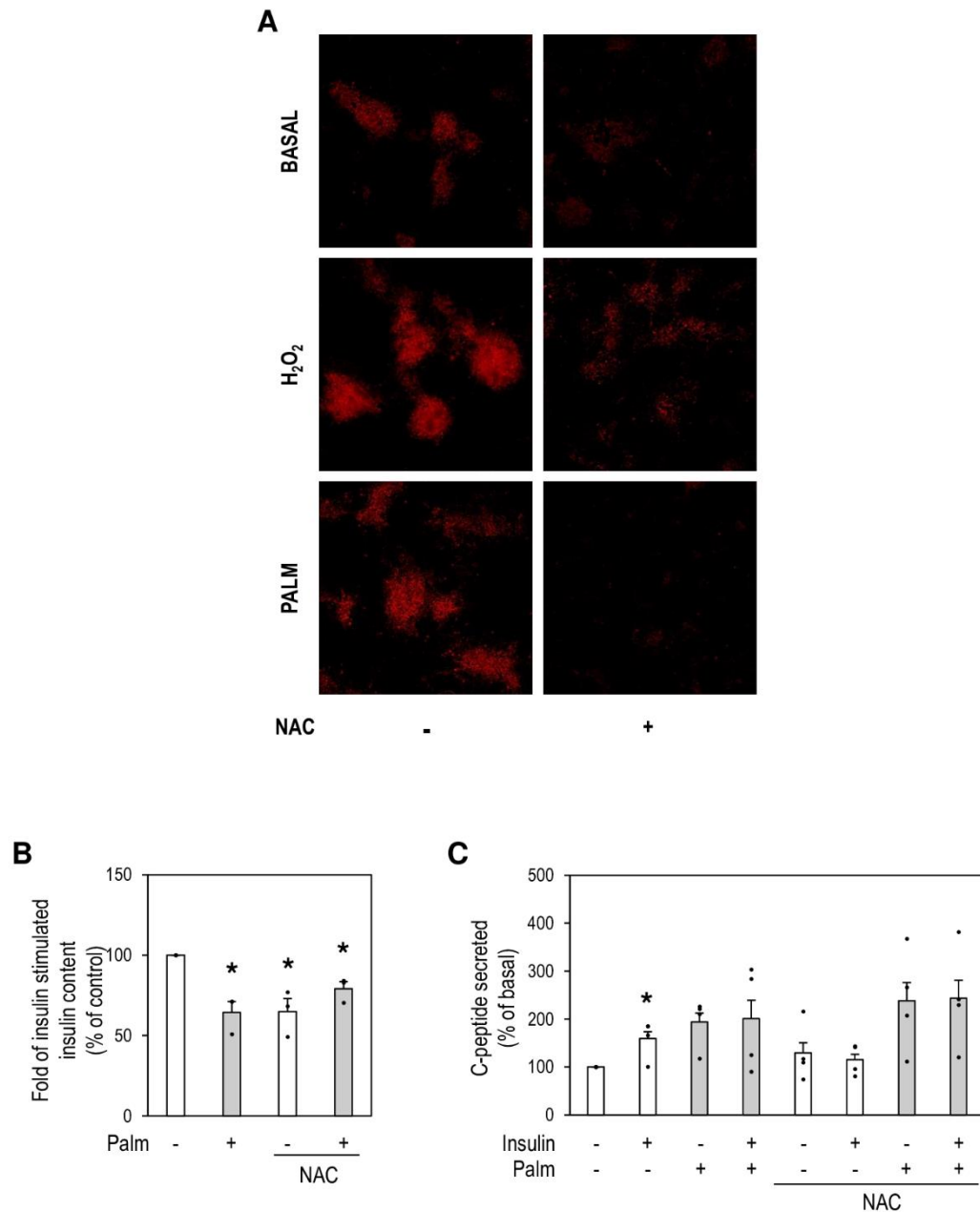
86 **Supplemental Figure 1.** Insulin increases *Insulin 1* gene expression levels in INS-1E cells. **A:** Cells
87 were incubated in KRBH buffer for 100 min, then stimulated with 10 nM insulin in fresh KRBH
88 buffer for 40 min. *Insulin 1* and *Insulin 2* gene expression was evaluated by quantitative RT-PCR
89 analysis normalized to 18S gene expression (n = 3 independent experiments). *p < 0.05 vs control.
90 Data are expressed as the mean ± SEM.



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93 **Supplemental Figure 2.** Effects of oleate on insulin-induced insulin content and C-peptide release
 94 in INS-1E cells. **A, B:** Cells were cultured in the presence of 0.5 mmol/l oleate (grey bars) or BSA
 95 (white bars), as a control, for 24 h, followed by incubation in KRBH buffer for 100 min, and
 96 stimulation with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. **A:** Insulin content was
 97 measured by enzyme-linked immunosorbent assay (ELISA), normalized to protein concentration,
 98 and expressed as a percentage of untreated control (n = 6 independent experiments); the fold-
 99 increase of insulin-stimulated insulin content over control (not treated with oleate) is also shown.
 100 **B:** Secreted C-peptide levels were measured by ELISA, normalized against total protein
 101 concentration, and expressed as a percentage of the untreated control (n = 6 independent
 102 experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not
 103 treated with oleate) is also shown. *p < 0.05 vs control without insulin; †p < 0.05 vs control
 104 without oleate. Data are expressed as the mean ± SEM.



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Supplemental Figure 3. Effects of reactive oxygen species (ROS) on insulin activity in INS-1E cells.

A: The treatment with NAC reduces dihydroethidium (DHE) fluorescence (red) under basal condition and after stimulation with H₂O₂ or palmitate in INS-1E cells. **B, C:** INS-1E cells were stimulated with 1 mmol/L NAC for 2 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h prior to be incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40 minutes. **B:** Insulin content was measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (n = 3 independent experiments). **C:** Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration, and expressed as a percentage of the untreated control (n = 4 independent experiments). *p < 0.05 vs control; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs control without NAC. Data are expressed as the mean ± SEM. Palm, palmitate; NAC, N-Acetyl-L-cysteine; H₂O₂, hydrogen peroxide.