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**The p66<sup>Shc</sup> protein mediates insulin resistance and secretory dysfunction in pancreatic beta-cells under lipotoxic conditions**

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Complete List of Authors:	<p>Biondi, Giuseppina; University of Bari, Department of Emergency and Organ Transplantation  Marrano, Nicola; University of Bari, Department of Emergency and Organ Transplantation  Dipaola, Lucia; University of Bari, Department of Emergency and Organ Transplantation  Borrelli, Anna; University of Bari, Department of Emergency and Organ Transplantation  Rella, Martina; University of Bari, Department of Emergency and Organ Transplantation  D’Oria, Rossella; University of Bari, Department of Emergency and Organ Transplantation  Genchi, Valentina A.; University of Bari, Department of Emergency and Organ Transplantation  Caccioppoli, Cristina; University of Bari, Department of Emergency and Organ Transplantation  Porreca, Immacolata; Genetic Research Centre “Gaetano Salvatore” (IRGS), Biogem  Cignarelli, Angelo; University of Bari, Department of Emergency and Organ Transplantation  Perrini, Sebastio; University of Bari, Department of Emergency and Organ Transplantation  Marchetti, Piero; University of Pisa, Department of Clinical and Experimental Medicine  Vincenti, Leonardo; University Hospital Polyclinic, Division of General Surgery  Laviola, Luigi; University of Bari, Department of Emergency and Organ Transplantation  Giorgino, Francesco; University of Bari, Department of Emergency and Organ Transplantation  Natalicchio, Annalisa; University of Bari, Department of Emergency and Organ Transplantation</p>

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**Title page****Full title:**

The p66<sup>Shc</sup> protein mediates insulin resistance and secretory dysfunction in pancreatic beta-cells  
under lipotoxic conditions

**Short running title:**

p66<sup>Shc</sup> in beta-cell insulin resistance

**Authors:**

Giuseppina Biondi<sup>1</sup>, Nicola Marrano<sup>1</sup>, Lucia Dipaola<sup>1</sup>, Anna Borrelli<sup>1</sup>, [Martina Rella<sup>1</sup>](#), Rossella  
D'Oria<sup>1</sup>, Valentina A. Genchi<sup>1</sup>, Cristina Caccioppoli<sup>1</sup>, Immacolata Porreca<sup>2</sup>, Angelo Cignarelli<sup>1</sup>,  
Sebastio Perrini<sup>1</sup>, Piero Marchetti<sup>3</sup>, Leonardo Vincenti<sup>4</sup>, Luigi Laviola<sup>1</sup>, Francesco Giorgino<sup>1</sup>,  
Annalisa Natalicchio<sup>1</sup>

**Affiliation:**

<sup>1</sup>Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy.

<sup>2</sup>Genetic Research Centre "Gaetano Salvatore" (IRGS), Biogem, Ariano Irpino (AV), Italy.

<sup>3</sup>Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

<sup>4</sup> University Hospital Polyclinic, Division of General Surgery, Bari, Italy.

**Corresponding author:**

Francesco Giorgino, M.D., Ph.D.; Section of Internal Medicine, Endocrinology, Andrology and  
Metabolic Diseases; Department of Emergency and Organ Transplantation; University of Bari Aldo  
Moro; ORCID iD: 0000-0001-7372-2678; Piazza Giulio Cesare, 11, I-70124 Bari, Italy;  
Phone +39 080.5593522 080.5478689 080.5478152; E-mail: [francesco.giorgino@uniba.it](mailto:francesco.giorgino@uniba.it).

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23 **Abstract**

24 We evaluated the role of the p66<sup>Shc</sup> redox adaptor protein in pancreatic beta-cell insulin resistance  
25 that develops under lipotoxic conditions and with excess body fat. Prolonged exposure to palmitate  
26 in vitro or the presence of overweight/obesity augmented p66<sup>Shc</sup> expression levels and caused an  
27 impaired ability of exogenous insulin to increase cellular insulin content and secreted C-peptide  
28 levels in INS-1E cells and human and murine islets. ~~When compared with pancreatic islets from~~  
29 ~~lean subjects, islets from overweight/obese subjects showed increased p66<sup>Shc</sup> mRNA levels and an~~  
30 ~~impaired insulin effect to increase insulin content and secreted C-peptide.~~ In pancreatic beta INS-1E  
31 cells, p66<sup>Shc</sup> knockdown resulted in enhanced insulin-induced augmentation of insulin content and  
32 C-peptide secretion and prevented the ability of palmitate to impair these effects of insulin.  
33 Conversely, p66<sup>Shc</sup> overexpression impaired insulin-induced augmentation of insulin content and C-  
34 peptide secretion both in the absence and presence of palmitate. Under lipotoxic condition, the  
35 effects of p66<sup>Shc</sup> ~~appear to require~~ are mediated by p53-induced increase in p66<sup>Shc</sup> protein levels and  
36 JNK-induced p66<sup>Shc</sup> phosphorylation at Ser<sup>36</sup> and appear to involve the phosphorylation of the  
37 ribosomal protein S6 kinase at Thr<sup>389</sup> and of insulin receptor substrate-1 at Ser<sup>307</sup>, resulting in the  
38 inhibition of insulin-stimulated protein kinase b phosphorylation at Ser<sup>473</sup>. Thus, the p66<sup>Shc</sup> protein  
39 mediates the impaired beta-cell function and insulin resistance induced by saturated fatty acids and  
40 excess body fat.

## 41 **Introduction**

42 The loss of pancreatic beta-cell functional mass is a necessary and early event during the  
43 development of type 2 diabetes, as well as a potential target for the treatment and potential cure of  
44 type 2 diabetes (1). Insulin-secreting beta-cells are targeted by insulin itself, which acts in an  
45 autocrine manner to promote beta-cell viability and function (reviewed in 2). Constitutively  
46 secreted insulin is necessary to maintain beta-cell glucose sensitivity (3), and alterations in insulin  
47 receptor and insulin receptor substrate-1 (IRS-1) result in secretory dysfunction and glucose  
48 intolerance (reviewed in 2). However, the physiological relevance of autocrine insulin activity  
49 remains somewhat controversial due to the different insulin doses and glucose levels used to assess  
50 beta-cell secretory function (2, 4).

51 Several metabolic ‘stressors’ can result in impaired insulin activity or ‘insulin resistance’ in  
52 pancreatic beta-cells, as shown in other insulin-targeted tissues. The exposure of pancreatic beta-  
53 cells to high glucose levels induces the c-Jun N-terminal kinase (JNK)- and extracellular signal-  
54 regulated kinase (ERK)1/2-mediated inhibitory serine phosphorylation of insulin receptor substrate-  
55 1 (IRS-1), resulting in inhibition of insulin signalling and consequent impairment of insulin’s ability  
56 to regulate its own biosynthesis (5). Similarly, chronic exposure of beta-cells to elevated free fatty  
57 acid levels, particularly long-chain saturated fatty acids (SFAs), can induce the JNK-mediated  
58 phosphorylation of IRS-1/2, inhibiting insulin-induced insulin gene transcription (6).

59 The p66<sup>Shc</sup> protein is a redox enzyme, capable of sensing and generating reactive oxygen species  
60 (ROS), that also plays a role in metabolic dysfunction. In previous work (7), we described for the  
61 first time the pro-survival apoptotic role of p66<sup>Shc</sup> in ~~pancreatic beta-~~INS-1E cells, which is  
62 dependent upon its Ser<sup>36</sup> phosphorylation. Moreover, p66<sup>Shc</sup> expression levels were found to be  
63 increased in ~~pancreatic beta-~~INS-1E cells exposed to SFAs, in pancreatic islets isolated from mice  
64 fed a high-fat diet, and in human pancreatic islets isolated from overweight/obese subjects (7).  
65 Previously, p66<sup>Shc</sup> has been implicated in the development of obesity-induced insulin resistance

66 through the S6K/IRS-1/protein kinase B (AKT) pathway in endothelial cells and adipose tissue (8,  
67 9).

68 To date, the effects of SFAs and excess body fat on insulin action and signalling in pancreatic beta-  
69 cells have not been explored, and the role of p66<sup>Shc</sup> in this process has not been investigated. In this  
70 study, we provide evidence that p66<sup>Shc</sup> is crucially involved in lipotoxicity-induced beta-cell insulin  
71 resistance.

## 72 **Research Design and Methods**

### 73 **Pancreatic islet isolation and culture**

74 Human islets were isolated from pancreata obtained from multi-organ donors at the Islet Cell  
75 Laboratory of the University of Pisa, Italy, or from pancreatic fragments obtained from patients  
76 undergoing pancreatectomy to treat tumors in the ampulla of Vater at the Division of General  
77 Surgery of the University Hospital Polyclinic of Bari, Italy. Human pancreatic tissues were  
78 processed with the approval of the local Ethics Committee after informed consent was obtained.  
79 Anonymised patient information is provided in the **Human Islets Checklist**. Twenty male, 4–8-  
80 week-old, C57BL/6 mice were purchased from Charles River Laboratories (Calco, Lecco, Italy).  
81 Animal experiments were conducted after obtaining approval from the Ethics Committee of the  
82 Genetic Research Centre “Gaetano Salvatore”, Biogem, Ariano Irpino, Italy, in accordance with the  
83 Guide for the Care and Use of Laboratory Animals, Eighth Edition (2011) and the regulations  
84 established in Italy and the EU for animal experiments. Mouse and human pancreatic islets were  
85 isolated and cultured as ~~previously~~ described [in 7,10, 11, and Supplemental Methods](#).

### 86 ~~Pancreatic beta-cell~~ **[INS-E cells](#) culture**

87 Rat insulin-secreting INS-1E cells (passage 15–30) were a kind gift from C. B. Wollheim,  
88 University of Geneva, Switzerland. INS-1E cells were cultured as ~~previously~~ described [in](#)  
89 **[Supplemental Methods \(7\)](#)**.

### 90 **Pancreatic islets and ~~beta-cell~~ [INS-1E cells](#) treatment**

91 Human (preparation 13-17 in **Human Islets Checklist**) and mouse islets and INS-1E cells were  
92 treated with 0.5 mmol/l palmitate [or oleate](#) solution (Sigma-Aldrich Inc., St Louis, MO, USA),  
93 ~~prepared as previously reported (7)~~, or with the same volume of a 10% w/v fatty acid [FA](#)-free BSA  
94 solution, as a control. After 24 h, islet and cells were stimulated with 10–100 nmol/l human  
95 recombinant insulin (Roche Diagnostics, Mannheim, Germany) for different times. To achieve



96 *p66<sup>Shc</sup>* knockdown, INS-1E cells were transfected with 100 nmol/l *p66<sup>Shc</sup>*-targeted short interfering  
97 RNA (siRNA, Qiagen, Hilden, Germany) using Lipofectamine<sup>®</sup> RNAiMAX Transfection Reagent  
98 and Opti-MEM<sup>®</sup> medium (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 48 h (7).  
99 The overexpression of *p66<sup>Shc</sup>* was achieved by transducing INS-1E cells with recombinant  
100 adenoviruses carrying a construct encoding the *p66<sup>Shc</sup>* protein (7). ~~In addition~~ Where indicated, INS-  
101 1E cells were transfected with an adenoviral *p66<sup>Shc</sup>* construct harboring a Ser<sup>36</sup> to Ala<sup>36</sup> mutation.  
102 An empty adenovirus vector was used as control (mock). See Supplemental Methods for further  
103 details.

#### 104 **Measurement of insulin content and secreted C-peptide levels**

105 After a wash with PBS 1X, murine and human pancreatic islets and INS-1E cells were incubated in  
106 Krebs-Ringer bicarbonate HEPES buffer (KRBH, 0.1% w/v BSA, 3 mmol/l glucose, 114 mmol/l  
107 NaCl, 4.4 mmol/l KCl, 1 mmol/l MgSO<sub>4</sub>, 29.5 mmol/l NaHCO<sub>3</sub>, 1.28 mmol/l CaCl<sub>2</sub>, and 10 mmol/l  
108 HEPES, all from Sigma-Aldrich, pH 7.4.) (12) for 100 min, followed by stimulation with insulin  
109 (10–100 mol/l) diluted in fresh KRBH solution for 40 min. To evaluate insulin content, cells were  
110 washed twice with PBS 1X, then mechanically lysed in a non-denaturing lysis buffer (13). The level  
111 of C-peptide released in the medium, as a measure of endogenously produced insulin, and insulin  
112 contents were measured using enzyme-linked immunosorbent assays (ELISAs) specific for mouse  
113 (Shibayagi Co., Ltd, Ishihara, Japan), humans, and rats (Merckodia AB, Sylveniusgatan, Uppsala,  
114 Sweden).

#### 115 **Immunoblotting**

116 Cells lysates were obtained and analysed by immunoblotting assays, ~~as previously described~~ (13  
117 and Supplemental Methods) (7). A list of the antibodies used is shown in Supplemental Table 1.

**118 Quantitative Real-Time PCR**

119 RNA isolation, cDNA synthesis and quantitative Real-Time PCR were conducted as previously  
120 reported [in 13 and Supplemental Methods \(7, 13\)](#). Primers sequences are available in  
121 [Supplemental Table 2](#).

**122 Statistical analysis**

123 Data are presented as the mean  $\pm$  SEM. Statistical analysis was performed using the two-tailed  
124 unpaired Student's t-test. Significance was set at  $p < 0.05$ .

**125 Data and Resource Availability**

126 ~~The datasets generated and/or analyzed during the current study are available from the~~  
127 ~~corresponding author upon reasonable request. No applicable resources were generated or analyzed~~  
128 ~~during the current study.~~

129 **Results**130 **Insulin increases its own content and C-peptide release in INS-1E cells and human and**  
131 **murine islets**

132 Insulin stimulation increased insulin content and C-peptide secreted levels in INS-1E cells (Fig. 1A  
133 and B), and human (Fig. 1C and D) and murine (Fig. 1E and F) pancreatic islets. This was  
134 associated with increased gene expression levels of *Insulin 1*, but not *Insulin 2*, in INS-1E cells  
135 (Supplemental Fig. 1).

136 **~~Palmitate impairs insulin augmentation of insulin content and stimulation of C-peptide~~**  
137 **~~secretion in pancreatic beta-cells and islets~~ effects in INS-1E cells and human and murine**  
138 **islets**

139 ~~Insulin stimulation resulted in increased insulin content and C-peptide secretion in INS-1E cells~~  
140 ~~(Fig. 1A and B), and in human (Fig. 1I and J) and murine (Supplemental Figure 1A and B)~~  
141 ~~pancreatic islets. In contrast, Prior exposure of cells and islets to the SFA palmitate impaired the~~  
142 ~~stimulatory effects of insulin on its own cellular content (Fig. 1A, C, E) ~~I~~, and Supplemental~~  
143 ~~Figure 1A) and C-peptide secretion (Fig. 1B, D, F) ~~J~~, and Supplemental Figure 1B). This~~  
144 ~~occurred also in INS-E cells exposed to the unsaturated FA oleate (Supplemental Fig. 2A and B).~~

145 **Palmitate-induced impairment of insulin effects in INS-1E cells is mediated by p66<sup>Shc</sup>**

146 The *p66<sup>Shc</sup>* silencing approach resulted in a 40%–50% reduction of p66<sup>Shc</sup> protein levels in all  
147 experimental conditions compared with control cells treated with vehicle only (Fig. ~~1C~~2A). Under  
148 these conditions, the palmitate-induced impairment of the insulin stimulatory effect on its own  
149 cellular content was largely prevented (Fig. ~~1D~~2B). In addition, *p66<sup>Shc</sup>* knockdown resulted in  
150 increased insulin-induced C-peptide secretion both in the absence and presence of palmitate,  
151 compared with control cells (Fig. ~~1E~~2C).

152 Conversely, p66<sup>Shc</sup> overexpression in INS-1E cells resulted in a marked increase of p66<sup>Shc</sup> protein  
153 levels (**Fig. 1F2D**), and in the inhibition of the ability of insulin to augment insulin content (**Fig.**  
154 **1G2E**) and secreted C-peptide levels (**Fig. 1H2F**) in cells not exposed to palmitate; in the presence  
155 of palmitate, these effects of insulin were impaired further (**Fig. 1G** and **1H2E and F**).

156 Following overexpression of the p66<sup>Shc</sup>Ala<sup>36</sup> mutant protein (**Fig. 1F2D**), which is unable to  
157 undergo phosphorylation at the key Ser<sup>36</sup> site (7), the palmitate-induced impairment of the effects of  
158 insulin to enhance its own content (**Fig. 1G2E**) and C-peptide secretion (**Fig. 1H2F**), respectively,  
159 were no longer observed.

#### 160 **Islets from overweight and obese subjects show elevated p66<sup>Shc</sup> mRNA expression and** 161 **reduced response to insulin**

162 In islets (preparation 1-12 in **Human Islets Checklist**) obtained from overweight/obese subjects,  
163 p66<sup>Shc</sup> mRNA levels were higher than in islets from lean subjects (**Fig. 1K2G**). In addition, the  
164 effects of insulin to increase insulin content (**Fig. 1L2H**) and secreted C-peptide (**Fig. 1M2I**) were  
165 blunted.

#### 166 **Role of JNK, p53 protein, and oxidative stress in SFAs-induced insulin resistance in INS-1E** 167 **cells**

168 **The inhibition of JNK (Fig. 3A) or p53 protein activity (Fig. 3E) reduced palmitate-induced p66<sup>Shc</sup>**  
169 **phosphorylation at Ser<sup>36</sup> (Fig. 3B) and p66<sup>Shc</sup> protein expression (Fig. 3F), respectively, and**  
170 **prevented the palmitate effects on insulin-induced insulin content (Fig. 3C and G) and C-peptide**  
171 **secretion (Fig. 3D and H). Of note, the reduction in ROS levels impaired insulin effects both under**  
172 **basal and palmitate-stimulated conditions (Supplemental Fig. 3).**

#### 173 **p66<sup>Shc</sup> mediates the palmitate-induced alterations of insulin signalling via S6K/IRS-1/AKT in** 174 **pancreatic beta INS-1E cells**

175 Palmitate treatment increased S6K Thr<sup>389</sup> phosphorylation (**Fig. 2A4A**) and IRS-1 Ser<sup>307</sup>  
176 phosphorylation, resulting both in reduced IRS-1 protein levels (**Fig. 2B4B**) and impaired ability of  
177 insulin to stimulate AKT Ser<sup>473</sup> phosphorylation (**Fig. 2C4C**). However, in beta-cells with *p66<sup>Shc</sup>*  
178 knockdown, the effects of palmitate on phosphorylation of S6K (**Fig. 2A4A**) and IRS-1 (**Fig.**  
179 **2B4B**) proteins were abrogated, and this enhanced insulin-stimulated AKT Ser<sup>473</sup> phosphorylation  
180 (**Fig. 2C4C**). Conversely, *p66<sup>Shc</sup>* overexpression did not alter S6K Thr<sup>389</sup> phosphorylation (**Fig.**  
181 **2D4D**) and exacerbated the palmitate-mediated IRS-1 Ser<sup>307</sup> phosphorylation and reduction of IRS-  
182 1 protein levels (**Fig. 2E4E**), leading to complete abrogation of insulin-stimulated AKT Ser<sup>473</sup>  
183 phosphorylation (**Fig. 2F4F**). Of note, *p66<sup>Shc</sup>* overexpression increased IRS-1 Ser<sup>307</sup>  
184 phosphorylation, reduced IRS-1 protein levels (**Fig. 2E4E**), and impaired insulin-stimulated AKT  
185 Ser<sup>473</sup> phosphorylation (**Fig. 2F4F**), also in cells not exposed to palmitate.

## 186 Discussion

187 ~~In this study, we~~ We show that acute insulin stimulation increased the cellular insulin content and  
188 extent of secreted C-peptide release in multiple cellular models of pancreatic beta-cells, including  
189 ~~rat INS-1E cells and human and murine pancreatic islets. Notably, t~~ in rat INS-1E cells and human  
190 and murine pancreatic islets. This occurred independently of glucose interference, as all  
191 experiments were conducted under low-glucose conditions. Moreover, both exposure to palmitate *in*  
192 *vitro* and presence of excess of body fat *in vivo* resulted in the impaired ability of insulin to  
193 stimulate its biosynthesis and secretion in beta-cells and murine and human pancreatic islets *in vitro*  
194 and in human pancreatic islets *ex vivo*, respectively. These results mirror and corroborate the  
195 previous observation of blunted insulin-induced insulin gene transcription following exposure of  
196 mouse pancreatic islets to palmitate (6). Our results in INS-1E cells indicate that palmitate alters the  
197 insulin signalling pathway by activating the phosphorylation of Thr<sup>389</sup> in the S6K protein and the  
198 inhibitory phosphorylation of Ser<sup>307</sup> in IRS-1, which are known to result in reduced IRS-1 protein  
199 levels and impairment of insulin-induced AKT activation (9, 14). These results support the  
200 emerging concept that lipotoxicity is capable to induce an insulin-resistant state in pancreatic beta-  
201 cells, similarly to other classical insulin target tissues.

202 Importantly, we show for the first time that p66<sup>Shc</sup> plays a key role in this lipotoxicity-mediated  
203 insulin resistance in pancreatic beta-cells, since *p66<sup>Shc</sup>* knockdown or overexpression respectively  
204 prevented or worsened palmitate-induced insulin resistance by reducing or boosting palmitate-  
205 induced activation of the S6K/IRS-1/AKT pathway (Fig. 24G). These results resemble the effects  
206 of lipotoxicity in endothelial and adipose cells (8, 9). ~~Moreover, we observed that p66<sup>Shc</sup>~~  
207 ~~phosphorylation at Ser<sup>36</sup> is necessary to mediate the detrimental effects of palmitate, as divergent~~  
208 ~~effects were found following overexpression of the wild-type and mutant (Ala<sup>36</sup>) forms of p66<sup>Shc</sup>,~~  
209 ~~respectively. Phosphorylation at Ser<sup>36</sup> was previously demonstrated to be necessary for the harmful~~  
210 ~~effects of p66<sup>Shc</sup> under various stressful conditions, including lipotoxicity-induced beta-cell~~

211 ~~apoptosis (7).~~ Experiments with overexpression of the wild-type and mutant (Ala<sup>36</sup>) forms of p66<sup>Shc</sup>  
212 suggested that p66<sup>Shc</sup> phosphorylation at Ser<sup>36</sup> is required to mediate the effects of palmitate on  
213 insulin action. Furthermore, we found that palmitate-induced insulin-resistance in INS-1E cells is  
214 mediated by p53 and JNK proteins. These results resemble the mechanisms already demonstrated  
215 for palmitate-induced beta-cell apoptosis (7).

216 The *ex vivo* experiments performed in human pancreatic islets isolated from donors without  
217 diabetes support the involvement of p66<sup>Shc</sup> in lipotoxicity-induced beta-cell insulin resistance in  
218 human obesity, as both elevated p66<sup>Shc</sup> mRNA expression and impaired insulin effects were  
219 observed in islets obtained from overweight/obese subjects, in comparison with islets obtained from  
220 lean subjects. ~~SFAs are known to increase p66<sup>Shc</sup> levels and phosphorylation of Ser<sup>36</sup>, through the~~  
221 ~~activation of the tumor suppressor p53 protein and the kinase JNK, respectively (7), and this~~  
222 ~~represents a key event for SFAs-induced apoptosis in beta-cells (Fig. 2G).~~ Overall, this evidence  
223 identifies p66<sup>Shc</sup> can be identified as a crucial mediator of lipotoxicity-promoted beta-cell failure in  
224 the context of human obesity. Interestingly, elevated p66<sup>Shc</sup> levels have also been found in the liver  
225 of patients with alcoholic and non-alcoholic steatohepatitis (15, 16), in the heart in response to  
226 cardiac dysfunction (17) and hyperglycemia (18), and in endothelial cells exposed to dyslipidemia  
227 (19), hyperglycemia (20), and pro-inflammatory cytokines (21). Taken together, this evidence  
228 suggests that p66<sup>Shc</sup> is ubiquitously involved in multi-organ damage in response to metabolic  
229 stressors.

230 The physiological significance of the effects of autocrine basal insulin *in vivo* are still debated (2, 4,  
231 22-24), even though under low-glucose conditions insulin could regulate its own content and  
232 release, representing a physiological mechanism to replenish insulin stores and make beta-cells  
233 prone to rapidly satisfy insulin demand in response to secretagogues (3, 22, 25-27). Of note, insulin  
234 signaling in beta-cells can be triggered by insulin binding at high concentrations to insulin receptor  
235 (IR), insulin-like growth factor-1 receptor (IGF-1R), or IR/IGF-1R hybrids (28). It has been

236 suggested that insulin promotes its own biosynthesis and secretion mainly through IR/IRS-1  
237 signaling (29-31). On the other hand, the insulin binding to IGF-1R mainly leads to the activation of  
238 IRS-2, which could play a major role in the regulation of beta-cell mass (32-33).

239 In conclusion, ~~this study identified p66<sup>Shc</sup> as a mediator of~~ mediates lipotoxicity-induced beta-cell  
240 insulin resistance, an emerging mechanism for beta-cell failure in obesity and type 2 diabetes. Of  
241 interest, glucagon-like peptide 1 receptor agonists, which reportedly inhibit palmitate-induced  
242 apoptosis by preventing JNK phosphorylation (~~22~~34), also inhibited p66<sup>Shc</sup> phosphorylation on  
243 Ser<sup>36</sup> (7), and may thus use this mechanism as well to enhance insulin secretion. Since the loss of  
244 pancreatic beta-cell functional mass is a necessary and early event in type 2 diabetes (1), the  
245 combined effects of p66<sup>Shc</sup> on beta-cell secretory-function and survival suggest that this protein may  
246 represent a potential ~~promising pharmacological~~ target for the prevention or treatment type 2  
247 diabetes onset or progression.



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249 **Author Contributions**

250 All of authors contributed to conception and design, acquisition of data, analysis, and interpretation  
251 of data; they drafted the final article or revised it critically for important intellectual content and  
252 finally approved the version to be published.

253 Conceptualization, F.G. and A.N.; data curation, G.B., N.M., L.D., A.N., and F.G.; formal analysis  
254 and investigation G.B., N.M., L.D., A.B., [M.R.](#), R.D., V.A.G., C.C.; project administration, F.G. and  
255 A.N.; resources, I.P., P.M. and L.V.; supervision, L.L., F.G., and A.N.; visualization, A.C., S.P. and  
256 L.L.; writing—original draft, A.N.; writing—review and editing, F.G.

257 **Guarantor Statement**

258 F.G. had full access to all the data in the study and takes responsibility for the integrity of the data  
259 and the accuracy of the data analysis.

260 **Conflict of Interest Statement**

261 No potential conflicts of interest relevant to this article were reported.

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269 Data have been previously presented at scientific meetings (79<sup>th</sup> ADA Scientific Session, 2019; 40<sup>th</sup>  
270 National Meeting of the Italian Society of Endocrinology, 2019; 27<sup>th</sup> National Meeting of the Italian  
271 Society of Diabetology, 2018; 53<sup>th</sup> EASD Annual Meeting, 2017; International Symposium on Insulin  
272 Receptor and Insulin Action, 2017; 39<sup>th</sup> National Meeting of the Italian Society of Endocrinology,  
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275 The p66<sup>Shc</sup> protein mediates saturated fatty acid-induced insulin resistance in pancreatic beta cells. G.  
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280 P. Marchetti, L. Laviola, F. Giorgino. Diabetes 2019 Jun; 68(Supplement 1).

### 281 **Data and Resource Availability**

282 The datasets generated and/or analyzed during the current study are available from the corresponding  
283 author upon reasonable request. No applicable resources were generated or analyzed during the  
284 current study.

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391 **Tables**392 **Table 1.** Characteristics of antibodies used for immunoblotting.

Specificity	Antibody	Dilution used
Phospho-AKT (Ser473)	Cell Signaling Technology Inc., #9271S	1:1000
Total AKT	Cell Signaling Technology Inc., #4691S	1:1000
Beta-actin	Santa Cruz Biotechnology, sc-47778	1:1000
Phospho-IRS-1 (Ser307)	Cell Signaling Technology Inc., #2381S	1:500
Total IRS-1	Millipore, #06-248	1:500
Phospho-p70-S6K (Thr389)	Cell Signaling Technology Inc., #9205	1:1000
Total She	Merck Millipore, #06-203	1:1000

393

394 **Table 2.** Primers used for quantitative real-time PCR.

Gene	Species	Primer sequences
<i>RNAI8S5</i>	Homo sapiens	FOR: 5'-CGAACGTCTGCCCTATCAACTT-3' REV: 5'-ACCCGTGGTCACCATGGTA-3'
<i>p66<sup>She</sup></i>	Homo sapiens	FOR: 5'-CCCCAAGCCCAAGTACAA-3' REV: 5'-GACCCAGAAGCCCCTTCCT-3'

395

396 **Figure Legends**

397 **Figure 1.** Role of p66<sup>She</sup> protein in lipotoxicity-induced beta-cell insulin resistance. **A, B, I, J:** INS-1E cells (**A** and **B**) and human pancreatic islets (**I** and **J**) were cultured in the presence of 0.5  
398 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, followed by stimulation  
399 with 10 (**A, B**) or 100 (**I, J**) nmol/l insulin for 40 minutes. **A** and **I:** Insulin content was measured by  
400 enzyme-linked immunosorbent assay (ELISA), normalised to protein concentration, and expressed  
401 as a percentage of untreated control (n = 4 independent experiments); the fold-increase of insulin-  
402 stimulated insulin content over control (not treated with palmitate) is also shown. **B** and **J:** Secreted  
403 C-peptide levels were measured by ELISA, normalised against total protein concentration, and  
404 expressed as a percentage of the untreated control (n = 4 independent experiments); the fold-  
405 increase of insulin-stimulated secreted C-peptide levels over control (not treated with palmitate) is  
406 also shown. \*p < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate. **C–E:**  
407 INS-1E cells were transfected with p66<sup>She</sup> siRNA for 24 h, then cultured in the presence of 0.5  
408 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for an additional 24 h, and finally  
409 stimulated with 10 nmol/l insulin for 40 minutes. The transfection reagent only was used as control.  
410 **C:** Representative immunoblot of p66<sup>She</sup> and beta-actin protein levels with the densitometric  
411 analysis of the bands, expressed as relative optical density. Values were corrected using total beta-  
412 actin levels as a loading control and normalised against untreated control (n = 4 independent  
413 experiments). **D:** Insulin content was measured with a specific ELISA, normalised to protein  
414 concentration, and shown as fold-increase of insulin-stimulated insulin content over control (not  
415 treated with palmitate) (n = 5 independent experiments). **E:** Secreted C-peptide levels were  
416 measured by ELISA, normalised to total protein concentration, and expressed as a percentage of the  
417 untreated control (n = 4 independent experiments). \*p < 0.05 vs control without insulin; †p < 0.05  
418 vs control without palmitate; ‡p < 0.05 vs transfection reagent. **F–H:** INS-1E cells were infected  
419 with a recombinant adenoviral vector encoding p66<sup>She</sup> (Ad/p66<sup>She</sup>), a phosphorylation-defective  
420

421 p66<sup>She</sup> protein containing a Ser<sup>36</sup> to Ala mutation (Ad/Ala<sup>36</sup>), or with empty adenovirus (Ad/mock)  
 422 for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white  
 423 bars), as a control, for 24 h, and then stimulated with 10 nmol/l insulin for 40 minutes. **F:**  
 424 Representative immunoblot of p66<sup>She</sup> and beta-actin protein levels with the densitometric analysis  
 425 of bands, expressed as the relative optical density, corrected using total beta-actin levels as a  
 426 loading control, and normalised against control (n = 8 independent experiments). **G:** Insulin content  
 427 was measured by ELISA, normalised to total protein concentration, and shown as fold-increase over  
 428 control (not treated with palmitate) (n = 5 independent experiments). **H:** Secreted C-peptide levels  
 429 were measured by ELISA, normalised to total protein concentration, and expressed as a percentage  
 430 of the control (n = 8 independent experiments). \*p < 0.05 vs control without insulin; †p < 0.05 vs  
 431 control without palmitate; ‡p < 0.05 vs Ad/mock; §p < 0.05 vs Ad/p66<sup>She</sup>. **K:** p66<sup>She</sup> mRNA levels  
 432 in pancreatic islets from lean (BMI < 25 kg/m<sup>2</sup>, white bars) and overweight/obese (BMI ≥ 25 kg/m<sup>2</sup>,  
 433 grey bars) subjects. †p < 0.05 vs BMI < 25 kg/m<sup>2</sup> (n = 4 independent experiments). **L and M:**  
 434 Pancreatic islets were isolated from lean or overweight/obese subjects and then stimulated with 100  
 435 nmol/l insulin for 40 min. **L:** Insulin content was measured by ELISA, normalised to total protein  
 436 concentration, and expressed as a percentage of the untreated control (n = 4 independent  
 437 experiments). **M:** Secreted C-peptide levels were measured by ELISA, normalized to total protein  
 438 concentration, and expressed as a percentage of the untreated control (n = 4 independent  
 439 experiments). \*p < 0.05 vs control; †p < 0.05 vs BMI < 25. Data are expressed as the mean ± SEM.  
 440 Palm, palmitate.

441 **Figure 1.** Effects of insulin and palmitate on insulin content and C-peptide release in INS-1E cells  
 442 and human and murine islets. INS-1E cells (A, B), human islets (C, D) and murine islets (E, F)  
 443 were cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control,  
 444 for 24 h, then incubated in KRBH buffer for 100 min, and finally stimulated with 10 (A, B) or 100  
 445 (C-F) nmol/l insulin in fresh KRBH buffer for 40 minutes. A, C, E: Insulin content was measured



446 by enzyme-linked immunosorbent assay (ELISA), normalized to protein concentration, and  
447 expressed as a percentage of untreated control (at least n = 4 independent experiments); the fold-  
448 increase of insulin-stimulated insulin content over control (not treated with palmitate) is also shown.  
449 **B, D, F:** Secreted C-peptide levels were measured by ELISA, normalized against total protein  
450 concentration, and expressed as a percentage of the untreated control (at least n = 3 independent  
451 experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not  
452 treated with palmitate) is also shown. \*p < 0.05 vs control without insulin; †p < 0.05 vs control  
453 without palmitate. Data are expressed as the mean ± SEM. Palm, palmitate.

454 **Figure 2.** Role of p66<sup>Shc</sup> protein in lipotoxicity-induced beta-cell insulin resistance. **A– C:** INS-1E  
455 cells were transfected with p66<sup>Shc</sup> siRNA for 24 h, then cultured in the presence of 0.5 mmol/l  
456 palmitate (grey bars) or BSA (white bars), as a control, for an additional 24 h. Successively, cells  
457 were incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh  
458 KRBH buffer for 40 minutes. The transfection reagent only was used as control. **A:** Representative  
459 immunoblot of p66<sup>Shc</sup> and beta-actin protein levels with the densitometric analysis of the bands,  
460 expressed as relative optical density. Values were corrected using total beta-actin levels as a loading  
461 control and normalized against untreated control (n = 4 independent experiments). **B:** Insulin  
462 content was measured with a specific ELISA, normalized to protein concentration, and shown as  
463 fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) (n = 5  
464 independent experiments). **C:** Secreted C-peptide levels were measured by ELISA, normalized to  
465 total protein concentration, and expressed as a percentage of the untreated control (n = 4  
466 independent experiments). **D–F:** INS-1E cells were infected with a recombinant adenoviral vector  
467 encoding p66<sup>Shc</sup> (Ad/p66<sup>Shc</sup>), a phosphorylation-defective p66<sup>Shc</sup> protein containing a Ser<sup>36</sup> to Ala  
468 mutation (Ad/Ala<sup>36</sup>), or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the  
469 presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, then  
470 incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh

471 KRBH buffer for 40 minutes. **D:** Representative immunoblot of p66<sup>Shc</sup> and beta-actin protein levels  
472 with the densitometric analysis of bands, expressed as the relative optical density, corrected using  
473 total beta-actin levels as a loading control, and normalized against control (n = 8 independent  
474 experiments). **E:** Insulin content was measured by ELISA, normalized to total protein  
475 concentration, and shown as fold-increase over control (not treated with palmitate) (n = 5  
476 independent experiments). **F:** Secreted C-peptide levels were measured by ELISA, normalized to  
477 total protein concentration, and expressed as a percentage of the control (n = 8 independent  
478 experiments). **G:** p66<sup>Shc</sup> mRNA levels in pancreatic islets from lean (BMI < 25 kg/m<sup>2</sup>, white bars)  
479 and overweight/obese (BMI ≥ 25 kg/m<sup>2</sup>, grey bars) subjects (n = 4 independent experiments). **H** and  
480 **I:** Pancreatic islets were isolated from lean or overweight/obese subjects, then incubated in KRBH  
481 buffer for 100 min, and finally stimulated with 100 nmol/l insulin in fresh KRBH medium for 40  
482 min. **H:** Insulin content was measured by ELISA, normalized to total protein concentration, and  
483 expressed as a percentage of the untreated control (n = 4 independent experiments). **I:** Secreted C-  
484 peptide levels were measured by ELISA, normalized to total protein concentration, and expressed  
485 as a percentage of the untreated control (n = 4 independent experiments). \*p < 0.05 vs control  
486 without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05  
487 vs Ad/mock; || p < 0.05 vs Ad/p66<sup>Shc</sup>; ¶ p < 0.05 vs BMI < 25 kg/m<sup>2</sup>. Data are expressed as the  
488 mean ± SEM. Palm, palmitate.

489 **Figure 3.** Role of JNK kinase and p53 in palmitate effects on insulin-induced insulin content and C-  
490 peptide release in INS-1E cells. **A, B:** Cells were stimulated with 10 or 30 μmol/l JNK inhibitor  
491 (SP600125) or dimethyl sulfoxide (DMSO) as control for 2 h, then cultured in the presence of 0.5  
492 mmol/l palmitate (or BSA, as a control) for 24 h. **A:** Representative immunoblot of c-Jun (Ser<sup>63</sup>)  
493 phosphorylation and beta-actin protein levels, quantified by densitometric analysis of the bands,  
494 expressed as relative optical density. C-Jun (Ser<sup>63</sup>) phosphorylation values were corrected using  
495 total beta-actin levels as a loading control and normalized against untreated control (n = 3

496 independent experiments). **B**: Representative immunoblot of p66<sup>Shc</sup> (Ser<sup>36</sup>) phosphorylation and  
497 protein levels, quantified by densitometric analysis of the bands, expressed as relative optical  
498 density. p66<sup>Shc</sup> (Ser<sup>36</sup>) phosphorylation values were normalized against p66<sup>Shc</sup> protein, then against  
499 untreated control (n = 3 independent experiments). **C, D**: Cells were stimulated with 30  $\mu$ mol/l JNK  
500 inhibitor (SP600125) or dimethyl sulfoxide (DMSO) as control for 2 h, then cultured in the  
501 presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h prior to be incubated in KRBH  
502 buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40  
503 minutes. **E, F**: Cells were stimulated with 30  $\mu$ mol/l pifithrin-alpha (PFT- $\alpha$ ) or dimethyl sulfoxide  
504 (DMSO) as control for 1 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a  
505 control) for 24 h. **E**: Representative immunoblot of p53 (Lys<sup>382</sup>) acetylation and beta-actin protein  
506 levels, quantified by densitometric analysis of the bands, expressed as relative optical density. P53  
507 (Lys<sup>382</sup>) acetylation values were corrected using total beta-actin levels as a loading control and  
508 normalised against untreated control (n = 6 independent experiments). **F**: Representative  
509 immunoblot of p66<sup>Shc</sup> protein levels, quantified by densitometric analysis of the bands, and  
510 expressed as relative optical density. P66<sup>Shc</sup> values were normalized against beta-actin levels, then  
511 against untreated control (at least n = 5 independent experiments). **G, H**: Cells were stimulated with  
512 30  $\mu$ mol/l pifithrin-alpha (PFT- $\alpha$ ) or dimethyl sulfoxide (DMSO) as control for 1 h, then cultured in  
513 the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h prior to be incubated in KRBH  
514 buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40  
515 minutes. **C, G**: Insulin content was measured by ELISA, normalized to total protein concentration,  
516 and expressed as a percentage of the untreated control (at least n = 3 independent experiments). **D,**  
517 **H**: Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration,  
518 and expressed as a percentage of the untreated control (at least n = 3 independent experiments). \*p  
519 < 0.05 vs control without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs control  
520 without inhibitor. Data are expressed as the mean  $\pm$  SEM. Palm, palmitate; JNKi, JNK inhibitor  
521 (SP600125); PFT- $\alpha$ , pifithrin-alpha.

522 **Figure 24.** Molecular mechanisms by which p66<sup>Shc</sup> mediates palmitate-induced insulin resistance in  
523 beta-cells. **A–C:** INS-1E cells were transiently transfected with p66<sup>Shc</sup> siRNA for 24 h, then  
524 cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for an additional 24 h, and  
525 then stimulated with 100 nmol/l insulin for 10 min. The transfection reagent only was used as a  
526 control. S6K (Thr<sup>389</sup>) (**A**), IRS-1 (Ser<sup>307</sup>) (**B**), and AKT (Ser<sup>473</sup>) (**C**) phosphorylation was measured  
527 by immunoblotting and quantified by densitometric analysis of the related bands, normalized  
528 against beta-actin (**A**), total IRS-1 (**B**), and total AKT (**C**), respectively. The resulting relative  
529 optical density values are expressed as percentages of the untreated control. **D–F:** INS-1E cells  
530 were infected with recombinant adenoviral vector encoding p66<sup>Shc</sup> (Ad/p66<sup>Shc</sup>) or with empty  
531 adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (or  
532 BSA, as a control) for 24 h and stimulated with 100 nmol/l insulin for 10 minutes. S6K (Thr<sup>389</sup>) (**D**),  
533 IRS-1 (Ser<sup>307</sup>) (**E**) and AKT (Ser<sup>473</sup>) (**F**) phosphorylation was measured by immunoblotting and  
534 quantified by densitometric analysis of the related bands, normalized against beta-actin (**D**), total  
535 IRS-1 (**E**), and total AKT (**F**), respectively. The resulting relative optical density values are  
536 expressed as percentages of the untreated control. **A** and **C**: n = 5 independent experiments; **B** and  
537 **E**: n = 3 independent experiments; **D**: n = 13 independent experiments; **F**: n = 8 independent  
538 experiments. Data are expressed as the mean ± SEM. \*p < 0.05 vs control without insulin; †p <  
539 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05 vs Ad/mock. Palm,  
540 palmitate. **G:** Saturated fatty acids increase p66<sup>Shc</sup> levels and its phosphorylation in Ser<sup>36</sup>, via the  
541 p53 protein and JNK kinase, respectively, thus inducing apoptosis **and insulin resistance** in  
542 pancreatic beta-cells (7). In addition, p66<sup>Shc</sup> activation induces the phosphorylation of Thr<sup>389</sup> in the  
543 S6K protein and the inhibitory phosphorylation of Ser<sup>307</sup> in IRS-1, which in turn reduce IRS-1  
544 protein levels and impair insulin-induced AKT activation, thus impairing the ability of insulin to  
545 increase its own cellular content and C-peptide secretion in pancreatic beta-cells. AKT, protein  
546 kinase b; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; p53, tumor suppressor

- 547 protein 53; p66<sup>Shc</sup>, SHC (Src homology 2 domain-containing) transforming protein 1, 66 kDa
- 548 isoform; S6K, ribosomal protein S6 kinase.

**Title page****Full title:**

The p66<sup>Shc</sup> protein mediates insulin resistance and secretory dysfunction in pancreatic beta-cells  
under lipotoxic conditions

**Short running title:**

p66<sup>Shc</sup> in beta-cell insulin resistance

**Authors:**

Giuseppina Biondi<sup>1</sup>, Nicola Marrano<sup>1</sup>, Lucia Dipaola<sup>1</sup>, Anna Borrelli<sup>1</sup>, Martina Rella<sup>1</sup>, Rossella  
D'Oria<sup>1</sup>, Valentina A. Genchi<sup>1</sup>, Cristina Caccioppoli<sup>1</sup>, Immacolata Porreca<sup>2</sup>, Angelo Cignarelli<sup>1</sup>,  
Sebastio Perrini<sup>1</sup>, Piero Marchetti<sup>3</sup>, Leonardo Vincenti<sup>4</sup>, Luigi Laviola<sup>1</sup>, Francesco Giorgino<sup>1</sup>,  
Annalisa Natalicchio<sup>1</sup>

**Affiliation:**

<sup>1</sup>Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy.

<sup>2</sup>Genetic Research Centre "Gaetano Salvatore" (IRGS), Biogem, Ariano Irpino (AV), Italy.

<sup>3</sup>Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

<sup>4</sup> University Hospital Polyclinic, Division of General Surgery, Bari, Italy.

**Corresponding author:**

Francesco Giorgino, M.D., Ph.D.; Section of Internal Medicine, Endocrinology, Andrology and  
Metabolic Diseases; Department of Emergency and Organ Transplantation; University of Bari Aldo  
Moro; ORCID iD: 0000-0001-7372-2678; Piazza Giulio Cesare, 11, I-70124 Bari, Italy;  
Phone +39 080.5593522 080.5478689 080.5478152; E-mail: [francesco.giorgino@uniba.it](mailto:francesco.giorgino@uniba.it).

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**23 Abstract**

24 We evaluated the role of the p66<sup>Shc</sup> redox adaptor protein in pancreatic beta-cell insulin resistance  
25 that develops under lipotoxic conditions and with excess body fat. Prolonged exposure to palmitate  
26 in vitro or the presence of overweight/obesity augmented p66<sup>Shc</sup> expression levels and caused an  
27 impaired ability of exogenous insulin to increase cellular insulin content and secreted C-peptide  
28 levels in INS-1E cells and human and murine islets. In INS-1E cells, p66<sup>Shc</sup> knockdown resulted in  
29 enhanced insulin-induced augmentation of insulin content and C-peptide secretion and prevented  
30 the ability of palmitate to impair these effects of insulin. Conversely, p66<sup>Shc</sup> overexpression  
31 impaired insulin-induced augmentation of insulin content and C-peptide secretion both in the  
32 absence and presence of palmitate. Under lipotoxic condition, the effects of p66<sup>Shc</sup> are mediated by  
33 p53-induced increase in p66<sup>Shc</sup> protein levels and JNK-induced p66<sup>Shc</sup> phosphorylation at Ser<sup>36</sup> and  
34 appear to involve the phosphorylation of the ribosomal protein S6 kinase at Thr<sup>389</sup> and of insulin  
35 receptor substrate-1 at Ser<sup>307</sup>, resulting in the inhibition of insulin-stimulated protein kinase b  
36 phosphorylation at Ser<sup>473</sup>. Thus, the p66<sup>Shc</sup> protein mediates the impaired beta-cell function and  
37 insulin resistance induced by saturated fatty acids and excess body fat.

## 38 **Introduction**

39 The loss of pancreatic beta-cell functional mass is a necessary and early event during the  
40 development of type 2 diabetes, as well as a potential target for the treatment and potential cure of  
41 type 2 diabetes (1). Insulin-secreting beta-cells are targeted by insulin itself, which acts in an  
42 autocrine manner to promote beta-cell viability and function (reviewed in 2). Constitutively  
43 secreted insulin is necessary to maintain beta-cell glucose sensitivity (3), and alterations in insulin  
44 receptor and insulin receptor substrate-1 (IRS-1) result in secretory dysfunction and glucose  
45 intolerance (reviewed in 2). However, the physiological relevance of autocrine insulin activity  
46 remains somewhat controversial due to the different insulin doses and glucose levels used to assess  
47 beta-cell secretory function (2, 4).

48 Several metabolic ‘stressors’ can result in impaired insulin activity or ‘insulin resistance’ in  
49 pancreatic beta-cells, as shown in other insulin-targeted tissues. The exposure of pancreatic beta-  
50 cells to high glucose levels induces the c-Jun N-terminal kinase (JNK)- and extracellular signal-  
51 regulated kinase (ERK)1/2-mediated inhibitory serine phosphorylation of insulin receptor substrate-  
52 1 (IRS-1), resulting in inhibition of insulin signalling and consequent impairment of insulin’s ability  
53 to regulate its own biosynthesis (5). Similarly, chronic exposure of beta-cells to elevated free fatty  
54 acid levels, particularly long-chain saturated fatty acids (SFAs), can induce the JNK-mediated  
55 phosphorylation of IRS-1/2, inhibiting insulin-induced insulin gene transcription (6).

56 The p66<sup>Shc</sup> protein is a redox enzyme, capable of sensing and generating reactive oxygen species  
57 (ROS), that also plays a role in metabolic dysfunction. In previous work (7), we described for the  
58 first time the pro-apoptotic role of p66<sup>Shc</sup> in INS-1E cells, which is dependent upon its Ser<sup>36</sup>  
59 phosphorylation. Moreover, p66<sup>Shc</sup> expression levels were found to be increased in INS-1E cells  
60 exposed to SFAs, in pancreatic islets isolated from mice fed a high-fat diet, and in human pancreatic  
61 islets isolated from overweight/obese subjects (7). Previously, p66<sup>Shc</sup> has been implicated in the



62 development of obesity-induced insulin resistance through the S6K/IRS-1/protein kinase B (AKT)  
63 pathway in endothelial cells and adipose tissue (8, 9).

64 To date, the effects of SFAs and excess body fat on insulin action and signalling in pancreatic beta-  
65 cells have not been explored, and the role of p66<sup>Shc</sup> in this process has not been investigated. In this  
66 study, we provide evidence that p66<sup>Shc</sup> is crucially involved in lipotoxicity-induced beta-cell insulin  
67 resistance.

## 68 **Research Design and Methods**

### 69 **Pancreatic islet isolation and culture**

70 Human islets were isolated from pancreata obtained from multi-organ donors at the Islet Cell  
71 Laboratory of the University of Pisa, Italy, or from pancreatic fragments obtained from patients  
72 undergoing pancreatectomy to treat tumors in the ampulla of Vater at the Division of General  
73 Surgery of the University Hospital Polyclinic of Bari, Italy. Human pancreatic tissues were  
74 processed with the approval of the local Ethics Committee after informed consent was obtained.  
75 Anonymised patient information is provided in the **Human Islets Checklist**. Twenty male, 4–8-  
76 week-old, C57BL/6 mice were purchased from Charles River Laboratories (Calco, Lecco, Italy).  
77 Animal experiments were conducted after obtaining approval from the Ethics Committee of the  
78 Genetic Research Centre “Gaetano Salvatore”, Biogem, Ariano Irpino, Italy, in accordance with the  
79 Guide for the Care and Use of Laboratory Animals, Eighth Edition (2011) and the regulations  
80 established in Italy and the EU for animal experiments. Mouse and human pancreatic islets were  
81 isolated and cultured as described in **10**, **11**, and **Supplemental Methods**.

### 82 **INS-E cells culture**

83 Rat insulin-secreting INS-1E cells (passage 15–30) were a kind gift from C. B. Wollheim,  
84 University of Geneva, Switzerland. INS-1E cells were cultured as described in **Supplemental**  
85 **Methods**.

### 86 **Pancreatic islets and INS-1E cells treatment**

87 Human (preparation 13-17 in **Human Islets Checklist**) and mouse islets and INS-1E cells were  
88 treated with 0.5 mmol/l palmitate or oleate solution (Sigma-Aldrich Inc., St Louis, MO, USA), or  
89 with the same volume of a 10% w/v FA-free BSA solution, as a control. After 24 h, islet and cells  
90 were stimulated with 10–100 nmol/l human recombinant insulin (Roche Diagnostics, Mannheim,  
91 Germany) for different times. To achieve *p66<sup>Shc</sup>* knockdown, INS-1E cells were transfected with

92 100 nmol/l *p66<sup>Shc</sup>*-targeted short interfering RNA (siRNA, Qiagen, Hilden, Germany) using  
93 Lipofectamine<sup>®</sup> RNAiMAX Reagent and Opti-MEM<sup>®</sup> medium (ThermoFisher Scientific, Waltham,  
94 Massachusetts, USA) for 48 h (7). The overexpression of *p66<sup>Shc</sup>* was achieved by transducing INS-  
95 1E cells with recombinant adenoviruses carrying a construct encoding the *p66<sup>Shc</sup>* protein (7). Where  
96 indicated, INS-1E cells were transfected with an adenoviral *p66<sup>Shc</sup>* construct harboring a Ser<sup>36</sup> to  
97 Ala<sup>36</sup> mutation. An empty adenovirus vector was used as control (mock). See **Supplemental**  
98 **Methods** for further details.

### 99 **Measurement of insulin content and secreted C-peptide levels**

100 After a wash with PBS 1X, murine and human pancreatic islets and INS-1E cells were incubated in  
101 Krebs-Ringer bicarbonate HEPES buffer (KRBH, 0.1% w/v BSA, 3 mmol/l glucose, 114 mmol/l  
102 NaCl, 4.4 mmol/l KCl, 1 mmol/l MgSO<sub>4</sub>, 29.5 mmol/l NaHCO<sub>3</sub>, 1.28 mmol/l CaCl<sub>2</sub>, and 10 mmol/l  
103 HEPES, all from Sigma-Aldrich, pH 7.4.) (12) for 100 min, followed by stimulation with insulin  
104 (10–100 mol/l) diluted in fresh KRBH solution for 40 min. To evaluate insulin content, cells were  
105 washed twice with PBS 1X, then mechanically lysed in a non-denaturing lysis buffer (13). The level  
106 of C-peptide released in the medium, as a measure of endogenously produced insulin, and insulin  
107 contents were measured using enzyme-linked immunosorbent assays (ELISAs) specific for mouse  
108 (Shibayagi Co., Ltd, Ishihara, Japan), humans, and rats (Merckodia AB, Sylveniusgatan, Uppsala,  
109 Sweden).

### 110 **Immunoblotting**

111 Cells lysates were obtained and analysed by immunoblotting assays (13 and **Supplemental**  
112 **Methods**). A list of the antibodies used is shown in **Supplemental Table 1**.

### 113 **Quantitative Real-Time PCR**

114 RNA isolation, cDNA synthesis and quantitative Real-Time PCR were conducted as reported in 13  
115 and **Supplemental Methods**. Primers sequences are available in **Supplemental Table 2**.

116 **Statistical analysis**

117 Data are presented as the mean  $\pm$  SEM. Statistical analysis was performed using the two-tailed  
118 unpaired Student's t-test. Significance was set at  $p < 0.05$ .

## 119 **Results**

### 120 **Insulin increases its own content and C-peptide release in INS-1E cells and human and** 121 **murine islets**

122 Insulin stimulation increased insulin content and C-peptide secreted levels in INS-1E cells (**Fig. 1A**  
123 and **B**), and human (**Fig. 1C and D**) and murine (**Fig. 1E and F**) pancreatic islets. This was  
124 associated with increased gene expression levels of *Insulin 1*, but not *Insulin 2*, in INS-1E cells  
125 (**Supplemental Fig. 1**).

### 126 **Palmitate impairs insulin effects in INS-1E cells and human and murine islets**

127 Prior exposure of cells and islets to the SFA palmitate impaired the stimulatory effects of insulin on  
128 its own cellular content (**Fig. 1A, C, E**) and C-peptide secretion (**Fig. 1B, D, F**). This occurred also  
129 in INS-E cells exposed to the unsaturated FA oleate (**Supplemental Fig. 2A and B**).

### 130 **Palmitate-induced impairment of insulin effects in INS-1E cells is mediated by p66<sup>Shc</sup>**

131 The *p66<sup>Shc</sup>* silencing approach resulted in a 40%–50% reduction of p66<sup>Shc</sup> protein levels in all  
132 experimental conditions compared with control cells treated with vehicle only (**Fig. 2A**). Under  
133 these conditions, the palmitate-induced impairment of the insulin stimulatory effect on its own  
134 cellular content was largely prevented (**Fig. 2B**). In addition, *p66<sup>Shc</sup>* knockdown resulted in  
135 increased insulin-induced C-peptide secretion both in the absence and presence of palmitate,  
136 compared with control cells (**Fig. 2C**).

137 Conversely, p66<sup>Shc</sup> overexpression in INS-1E cells resulted in a marked increase of p66<sup>Shc</sup> protein  
138 levels (**Fig. 2D**), and in the inhibition of the ability of insulin to augment insulin content (**Fig. 2E**)  
139 and secreted C-peptide levels (**Fig. 2F**) in cells not exposed to palmitate; in the presence of  
140 palmitate, these effects of insulin were impaired further (**Fig. 2E and F**).

141 Following overexpression of the p66<sup>Shc</sup>Ala<sup>36</sup> mutant protein (**Fig. 2D**), which is unable to undergo  
142 phosphorylation at the key Ser<sup>36</sup> site (**7**), the palmitate-induced impairment of the effects of insulin  
143 to enhance its own content (**Fig. 2E**) and C-peptide secretion (**Fig. 2F**), respectively, were no longer  
144 observed.

#### 145 **Islets from overweight and obese subjects show elevated p66<sup>Shc</sup> mRNA expression and** 146 **reduced response to insulin**

147 In islets (preparation 1-12 in **Human Islets Checklist**) obtained from overweight/obese subjects,  
148 p66<sup>Shc</sup> mRNA levels were higher than in islets from lean subjects (**Fig. 2G**). In addition, the effects  
149 of insulin to increase insulin content (**Fig. 2H**) and secreted C-peptide (**Fig. 2I**) were blunted.

#### 150 **Role of JNK, p53 protein, and oxidative stress in SFAs-induced insulin resistance in INS-1E** 151 **cells**

152 The inhibition of JNK (**Fig. 3A**) or p53 protein activity (**Fig. 3E**) reduced palmitate-induced p66<sup>Shc</sup>  
153 phosphorylation at Ser<sup>36</sup> (**Fig. 3B**) and p66<sup>Shc</sup> protein expression (**Fig. 3F**), respectively, and  
154 prevented the palmitate effects on insulin-induced insulin content (**Fig. 3C and G**) and C-peptide  
155 secretion (**Fig. 3D and H**). Of note, the reduction in ROS levels impaired insulin effects both under  
156 basal and palmitate-stimulated conditions (**Supplemental Fig. 3**).

#### 157 **p66<sup>Shc</sup> mediates the palmitate-induced alterations of insulin signalling via S6K/IRS-1/AKT in** 158 **INS-1E cells**

159 Palmitate treatment increased S6K Thr<sup>389</sup> phosphorylation (**Fig. 4A**) and IRS-1 Ser<sup>307</sup>  
160 phosphorylation, resulting both in reduced IRS-1 protein levels (**Fig. 4B**) and impaired ability of  
161 insulin to stimulate AKT Ser<sup>473</sup> phosphorylation (**Fig. 4C**). However, in beta-cells with p66<sup>Shc</sup>  
162 knockdown, the effects of palmitate on phosphorylation of S6K (**Fig. 4A**) and IRS-1 (**Fig. 4B**)  
163 proteins were abrogated, and this enhanced insulin-stimulated AKT Ser<sup>473</sup> phosphorylation (**Fig.**  
164 **4C**). Conversely, p66<sup>Shc</sup> overexpression did not alter S6K Thr<sup>389</sup> phosphorylation (**Fig. 4D**) and

165 exacerbated the palmitate-mediated IRS-1 Ser<sup>307</sup> phosphorylation and reduction of IRS-1 protein  
166 levels (**Fig. 4E**), leading to complete abrogation of insulin-stimulated AKT Ser<sup>473</sup> phosphorylation  
167 (**Fig. 4F**). Of note, p66<sup>Shc</sup> overexpression increased IRS-1 Ser<sup>307</sup> phosphorylation, reduced IRS-1  
168 protein levels (**Fig. 4E**), and impaired insulin-stimulated AKT Ser<sup>473</sup> phosphorylation (**Fig. 4F**),  
169 also in cells not exposed to palmitate.

## 170 Discussion

171 We show that acute insulin stimulation increased the cellular insulin content and C-peptide release  
172 in rat INS-1E cells and human and murine pancreatic islets. This occurred independently of glucose  
173 interference, as all experiments were conducted under low-glucose conditions. Moreover, both  
174 exposure to palmitate *in vitro* and presence of excess of body fat *in vivo* resulted in the impaired  
175 ability of insulin to stimulate its biosynthesis and secretion in beta-cells and murine and human  
176 pancreatic islets *in vitro* and in human pancreatic islets *ex vivo*, respectively. These results mirror  
177 and corroborate the previous observation of blunted insulin-induced insulin gene transcription  
178 following exposure of mouse pancreatic islets to palmitate (6). Our results in INS-1E cells indicate  
179 that palmitate alters the insulin signalling pathway by activating the phosphorylation of Thr<sup>389</sup> in the  
180 S6K protein and the inhibitory phosphorylation of Ser<sup>307</sup> in IRS-1, which are known to result in  
181 reduced IRS-1 protein levels and impairment of insulin-induced AKT activation (9, 14). These  
182 results support the emerging concept that lipotoxicity is capable to induce an insulin-resistant state  
183 in pancreatic beta-cells, similarly to other classical insulin target tissues.

184 Importantly, we show for the first time that p66<sup>Shc</sup> plays a key role in this lipotoxicity-mediated  
185 insulin resistance in pancreatic beta-cells, since *p66<sup>Shc</sup>* knockdown or overexpression respectively  
186 prevented or worsened palmitate-induced insulin resistance by reducing or boosting palmitate-  
187 induced activation of the S6K/IRS-1/AKT pathway (Fig. 4G). These results resemble the effects of  
188 lipotoxicity in endothelial and adipose cells (8, 9). Experiments with overexpression of the wild-  
189 type and mutant (Ala<sup>36</sup>) forms of p66<sup>Shc</sup> suggested that p66<sup>Shc</sup> phosphorylation at Ser<sup>36</sup> is required to  
190 mediate the effects of palmitate on insulin action. Furthermore, we found that palmitate-induced  
191 insulin-resistance in INS-1E cells is mediated by p53 and JNK proteins. These results resemble the  
192 mechanisms already demonstrated for palmitate-induced beta-cell apoptosis (7).

193 The *ex vivo* experiments performed in human pancreatic islets isolated from donors without  
194 diabetes support the involvement of p66<sup>Shc</sup> in lipotoxicity-induced beta-cell insulin resistance in



195 human obesity, as both elevated  $p66^{Shc}$  mRNA expression and impaired insulin effects were  
196 observed in islets obtained from overweight/obese subjects, in comparison with islets obtained from  
197 lean subjects. Overall,  $p66^{Shc}$  can be identified as a crucial mediator of lipotoxicity-promoted beta-  
198 cell failure in the context of human obesity. Interestingly, elevated  $p66^{Shc}$  levels have also been  
199 found in the liver of patients with alcoholic and non-alcoholic steatohepatitis (**15, 16**), in the heart in  
200 response to cardiac dysfunction (**17**) and hyperglycemia (**18**), and in endothelial cells exposed to  
201 dyslipidemia (**19**), hyperglycemia (**20**), and pro-inflammatory cytokines (**21**). Taken together, this  
202 evidence suggests that  $p66^{Shc}$  is ubiquitously involved in multi-organ damage in response to  
203 metabolic stressors.

204 The physiological significance of the effects of autocrine basal insulin *in vivo* are still debated (**2, 4,**  
205 **22-24**), even though under low-glucose conditions insulin could regulate its own content and  
206 release, representing a physiological mechanism to replenish insulin stores and make beta-cells  
207 prone to rapidly satisfy insulin demand in response to secretagogues (**3, 22, 25-27**). Of note, insulin  
208 signaling in beta-cells can be triggered by insulin binding at high concentrations to insulin receptor  
209 (IR), insulin-like growth factor-1 receptor (IGF-1R), or IR/IGF-1R hybrids (**28**). It has been  
210 suggested that insulin promotes its own biosynthesis and secretion mainly through IR/IRS-1  
211 signaling (**29-31**). On the other hand, insulin binding to IGF-1R mainly leads to the activation of  
212 IRS-2, which could play a major role in the regulation of beta-cell mass (**32-33**).

213 In conclusion,  $p66^{Shc}$  mediates lipotoxicity-induced beta-cell insulin resistance, an emerging  
214 mechanism for beta-cell failure in obesity and type 2 diabetes. Of interest, glucagon-like peptide 1  
215 receptor agonists, which reportedly inhibit palmitate-induced apoptosis by preventing JNK  
216 phosphorylation (**34**), also inhibited  $p66^{Shc}$  phosphorylation on Ser<sup>36</sup> (**7**), and may thus use this  
217 mechanism as well to enhance insulin secretion. Since the loss of pancreatic beta-cell functional  
218 mass is a necessary and early event in type 2 diabetes (**1**), the combined effects of  $p66^{Shc}$  on beta-

219 cell secretory-function and survival suggest that this protein may represent a potential target for the  
220 prevention or treatment type 2 diabetes onset or progression.

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223 All of authors contributed to conception and design, acquisition of data, analysis, and interpretation  
224 of data; they drafted the final article or revised it critically for important intellectual content and  
225 finally approved the version to be published.

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229 L.L.; writing—original draft, A.N.; writing—review and editing, F.G.

**230 Guarantor Statement**

231 F.G. had full access to all the data in the study and takes responsibility for the integrity of the data  
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**233 Conflict of Interest Statement**

234 No potential conflicts of interest relevant to this article were reported.

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#### 254 **Data and Resource Availability**

255 The datasets generated and/or analyzed during the current study are available from the corresponding  
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364 **Figure Legends**

365 **Figure 1.** Effects of insulin and palmitate on insulin content and C-peptide release in INS-1E cells  
366 and human and murine islets. INS-1E cells (**A, B**), human islets (**C, D**) and murine islets (**E, F**)  
367 were cultured in the presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control,  
368 for 24 h, then incubated in KRBH buffer for 100 min, and finally stimulated with 10 (**A, B**) or 100  
369 (**C-F**) nmol/l insulin in fresh KRBH buffer for 40 minutes. **A, C, E:** Insulin content was measured  
370 by enzyme-linked immunosorbent assay (ELISA), normalized to protein concentration, and  
371 expressed as a percentage of untreated control (at least  $n = 4$  independent experiments); the fold-  
372 increase of insulin-stimulated insulin content over control (not treated with palmitate) is also shown.  
373 **B, D, F:** Secreted C-peptide levels were measured by ELISA, normalized against total protein  
374 concentration, and expressed as a percentage of the untreated control (at least  $n = 3$  independent  
375 experiments); the fold-increase of insulin-stimulated secreted C-peptide levels over control (not  
376 treated with palmitate) is also shown. \* $p < 0.05$  vs control without insulin; † $p < 0.05$  vs control  
377 without palmitate. Data are expressed as the mean  $\pm$  SEM. Palm, palmitate.

378 **Figure 2.** Role of p66<sup>Shc</sup> protein in lipotoxicity-induced beta-cell insulin resistance. **A– C:** INS-1E  
379 cells were transfected with *p66<sup>Shc</sup>* siRNA for 24 h, then cultured in the presence of 0.5 mmol/l  
380 palmitate (grey bars) or BSA (white bars), as a control, for an additional 24 h. Successively, cells  
381 were incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh  
382 KRBH buffer for 40 minutes. The transfection reagent only was used as control. **A:** Representative  
383 immunoblot of p66<sup>Shc</sup> and beta-actin protein levels with the densitometric analysis of the bands,  
384 expressed as relative optical density. Values were corrected using total beta-actin levels as a loading  
385 control and normalized against untreated control ( $n = 4$  independent experiments). **B:** Insulin  
386 content was measured with a specific ELISA, normalized to protein concentration, and shown as  
387 fold-increase of insulin-stimulated insulin content over control (not treated with palmitate) ( $n = 5$   
388 independent experiments). **C:** Secreted C-peptide levels were measured by ELISA, normalized to



389 total protein concentration, and expressed as a percentage of the untreated control (n = 4  
390 independent experiments). **D–F:** INS-1E cells were infected with a recombinant adenoviral vector  
391 encoding p66<sup>Shc</sup> (Ad/p66<sup>Shc</sup>), a phosphorylation-defective p66<sup>Shc</sup> protein containing a Ser<sup>36</sup> to Ala  
392 mutation (Ad/Ala<sup>36</sup>), or with empty adenovirus (Ad/mock) for 24 h. Cells were then cultured in the  
393 presence of 0.5 mmol/l palmitate (grey bars) or BSA (white bars), as a control, for 24 h, then  
394 incubated in KRBH buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh  
395 KRBH buffer for 40 minutes. **D:** Representative immunoblot of p66<sup>Shc</sup> and beta-actin protein levels  
396 with the densitometric analysis of bands, expressed as the relative optical density, corrected using  
397 total beta-actin levels as a loading control, and normalized against control (n = 8 independent  
398 experiments). **E:** Insulin content was measured by ELISA, normalized to total protein  
399 concentration, and shown as fold-increase over control (not treated with palmitate) (n = 5  
400 independent experiments). **F:** Secreted C-peptide levels were measured by ELISA, normalized to  
401 total protein concentration, and expressed as a percentage of the control (n = 8 independent  
402 experiments). **G:** p66<sup>Shc</sup> mRNA levels in pancreatic islets from lean (BMI < 25 kg/m<sup>2</sup>, white bars)  
403 and overweight/obese (BMI ≥ 25 kg/m<sup>2</sup>, grey bars) subjects (n = 4 independent experiments). **H** and  
404 **I:** Pancreatic islets were isolated from lean or overweight/obese subjects, then incubated in KRBH  
405 buffer for 100 min, and finally stimulated with 100 nmol/l insulin in fresh KRBH medium for 40  
406 min. **H:** Insulin content was measured by ELISA, normalized to total protein concentration, and  
407 expressed as a percentage of the untreated control (n = 4 independent experiments). **I:** Secreted C-  
408 peptide levels were measured by ELISA, normalized to total protein concentration, and expressed  
409 as a percentage of the untreated control (n = 4 independent experiments). \*p < 0.05 vs control  
410 without insulin; †p < 0.05 vs control without palmitate; ‡p < 0.05 vs transfection reagent; §p < 0.05  
411 vs Ad/mock; || p < 0.05 vs Ad/p66<sup>Shc</sup>; ¶ p < 0.05 vs BMI < 25 kg/m<sup>2</sup>. Data are expressed as the  
412 mean ± SEM. Palm, palmitate.

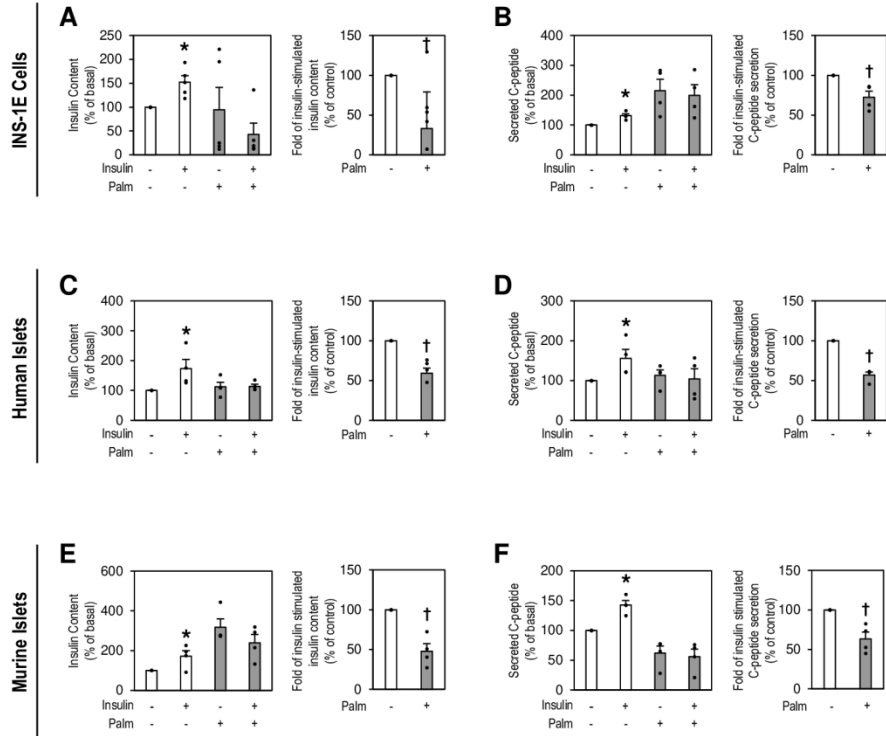
413 **Figure 3.** Role of JNK kinase and p53 in palmitate effects on insulin-induced insulin content and C-  
414 peptide release in INS-1E cells. **A, B:** Cells were stimulated with 10 or 30  $\mu\text{mol/l}$  JNK inhibitor  
415 (SP600125) or dimethyl sulfoxide (DMSO) as control for 2 h, then cultured in the presence of 0.5  
416 mmol/l palmitate (or BSA, as a control) for 24 h. **A:** Representative immunoblot of c-Jun (Ser<sup>63</sup>)  
417 phosphorylation and beta-actin protein levels, quantified by densitometric analysis of the bands,  
418 expressed as relative optical density. C-Jun (Ser<sup>63</sup>) phosphorylation values were corrected using  
419 total beta-actin levels as a loading control and normalized against untreated control (n = 3  
420 independent experiments). **B:** Representative immunoblot of p66<sup>Shc</sup> (Ser<sup>36</sup>) phosphorylation and  
421 protein levels, quantified by densitometric analysis of the bands, expressed as relative optical  
422 density. p66<sup>Shc</sup> (Ser<sup>36</sup>) phosphorylation values were normalized against p66<sup>Shc</sup> protein, then against  
423 untreated control (n = 3 independent experiments). **C, D:** Cells were stimulated with 30  $\mu\text{mol/l}$  JNK  
424 inhibitor (SP600125) or dimethyl sulfoxide (DMSO) as control for 2 h, then cultured in the  
425 presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h prior to be incubated in KRBH  
426 buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40  
427 minutes. **E, F:** Cells were stimulated with 30  $\mu\text{mol/l}$  pifithrin-alpha (PFT- $\alpha$ ) or dimethyl sulfoxide  
428 (DMSO) as control for 1 h, then cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a  
429 control) for 24 h. **E:** Representative immunoblot of p53 (Lys<sup>382</sup>) acetylation and beta-actin protein  
430 levels, quantified by densitometric analysis of the bands, expressed as relative optical density. P53  
431 (Lys<sup>382</sup>) acetylation values were corrected using total beta-actin levels as a loading control and  
432 normalised against untreated control (n = 6 independent experiments). **F:** Representative  
433 immunoblot of p66<sup>Shc</sup> protein levels, quantified by densitometric analysis of the bands, and  
434 expressed as relative optical density. P66<sup>Shc</sup> values were normalized against beta-actin levels, then  
435 against untreated control (at least n = 5 independent experiments). **G, H:** Cells were stimulated with  
436 30  $\mu\text{mol/l}$  pifithrin-alpha (PFT- $\alpha$ ) or dimethyl sulfoxide (DMSO) as control for 1 h, then cultured in  
437 the presence of 0.5 mmol/l palmitate (or BSA, as a control) for 24 h prior to be incubated in KRBH  
438 buffer for 100 min, and finally stimulated with 10 nmol/l insulin in fresh KRBH buffer for 40

439 minutes. **C, G:** Insulin content was measured by ELISA, normalized to total protein concentration,  
440 and expressed as a percentage of the untreated control (at least  $n = 3$  independent experiments). **D,**  
441 **H:** Secreted C-peptide levels were measured by ELISA, normalized to total protein concentration,  
442 and expressed as a percentage of the untreated control (at least  $n = 3$  independent experiments). \* $p$   
443  $< 0.05$  vs control without insulin; † $p < 0.05$  vs control without palmitate; ‡ $p < 0.05$  vs control  
444 without inhibitor. Data are expressed as the mean  $\pm$  SEM. Palm, palmitate; JNKi, JNK inhibitor  
445 (SP600125); PFT- $\alpha$ , pifithrin-alpha.

446 **Figure 4.** Molecular mechanisms by which  $p66^{Shc}$  mediates palmitate-induced insulin resistance in  
447 beta-cells. **A–C:** INS-1E cells were transiently transfected with  $p66^{Shc}$  siRNA for 24 h, then  
448 cultured in the presence of 0.5 mmol/l palmitate (or BSA, as a control) for an additional 24 h, and  
449 then stimulated with 100 nmol/l insulin for 10 min. The transfection reagent only was used as a  
450 control. S6K (Thr<sup>389</sup>) (**A**), IRS-1 (Ser<sup>307</sup>) (**B**), and AKT (Ser<sup>473</sup>) (**C**) phosphorylation was measured  
451 by immunoblotting and quantified by densitometric analysis of the related bands, normalized  
452 against beta-actin (**A**), total IRS-1 (**B**), and total AKT (**C**), respectively. The resulting relative  
453 optical density values are expressed as percentages of the untreated control. **D–F:** INS-1E cells  
454 were infected with recombinant adenoviral vector encoding  $p66^{Shc}$  (Ad/ $p66^{Shc}$ ) or with empty  
455 adenovirus (Ad/mock) for 24 h. Cells were then cultured in the presence of 0.5 mmol/l palmitate (or  
456 BSA, as a control) for 24 h and stimulated with 100 nmol/l insulin for 10 minutes. S6K (Thr<sup>389</sup>) (**D**),  
457 IRS-1 (Ser<sup>307</sup>) (**E**) and AKT (Ser<sup>473</sup>) (**F**) phosphorylation was measured by immunoblotting and  
458 quantified by densitometric analysis of the related bands, normalized against beta-actin (**D**), total  
459 IRS-1 (**E**), and total AKT (**F**), respectively. The resulting relative optical density values are  
460 expressed as percentages of the untreated control. **A** and **C:**  $n = 5$  independent experiments; **B** and  
461 **E:**  $n = 3$  independent experiments; **D:**  $n = 13$  independent experiments; **F:**  $n = 8$  independent  
462 experiments. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$  vs control without insulin; † $p <$   
463  $0.05$  vs control without palmitate; ‡ $p < 0.05$  vs transfection reagent; § $p < 0.05$  vs Ad/mock. Palm,

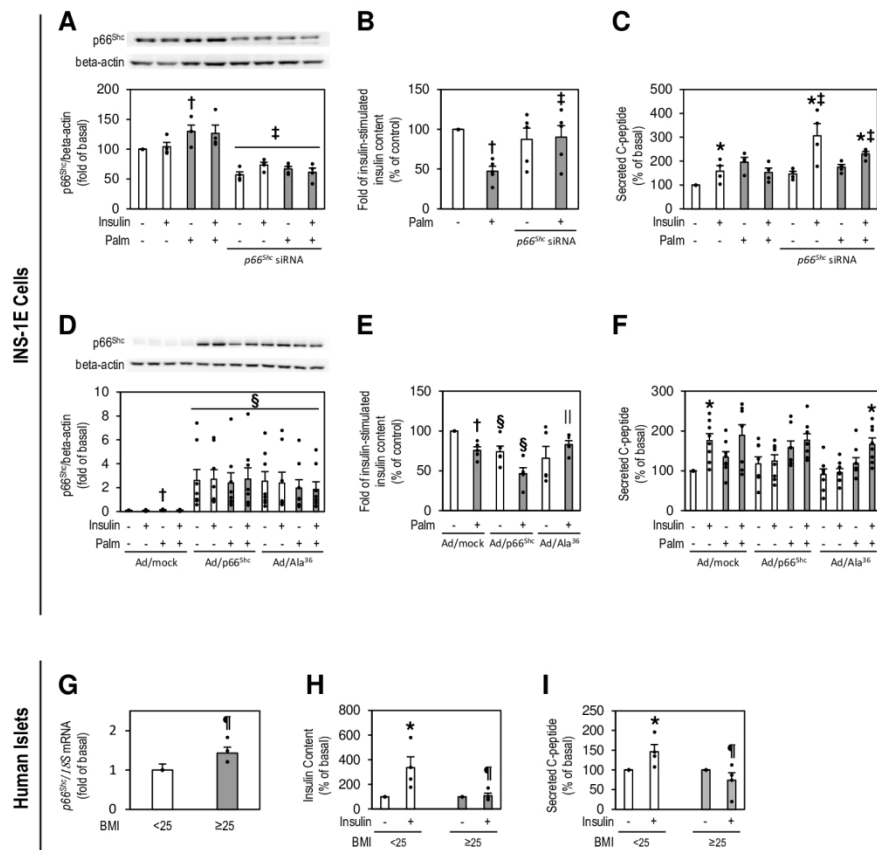
464 palmitate. **G:** Saturated fatty acids increase p66<sup>Shc</sup> levels and its phosphorylation in Ser<sup>36</sup>, via the  
465 p53 protein and JNK kinase, respectively, thus inducing apoptosis and insulin resistance in  
466 pancreatic beta-cells (7). In addition, p66<sup>Shc</sup> activation induces the phosphorylation of Thr<sup>389</sup> in the  
467 S6K protein and the inhibitory phosphorylation of Ser<sup>307</sup> in IRS-1, which in turn reduce IRS-1  
468 protein levels and impair insulin-induced AKT activation, thus impairing the ability of insulin to  
469 increase its own cellular content and C-peptide secretion in pancreatic beta-cells. AKT, protein  
470 kinase b; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; p53, tumor suppressor  
471 protein 53; p66<sup>Shc</sup>, SHC (Src homology 2 domain-containing) transforming protein 1, 66 kDa  
472 isoform; S6K, ribosomal protein S6 kinase.

Figure 1



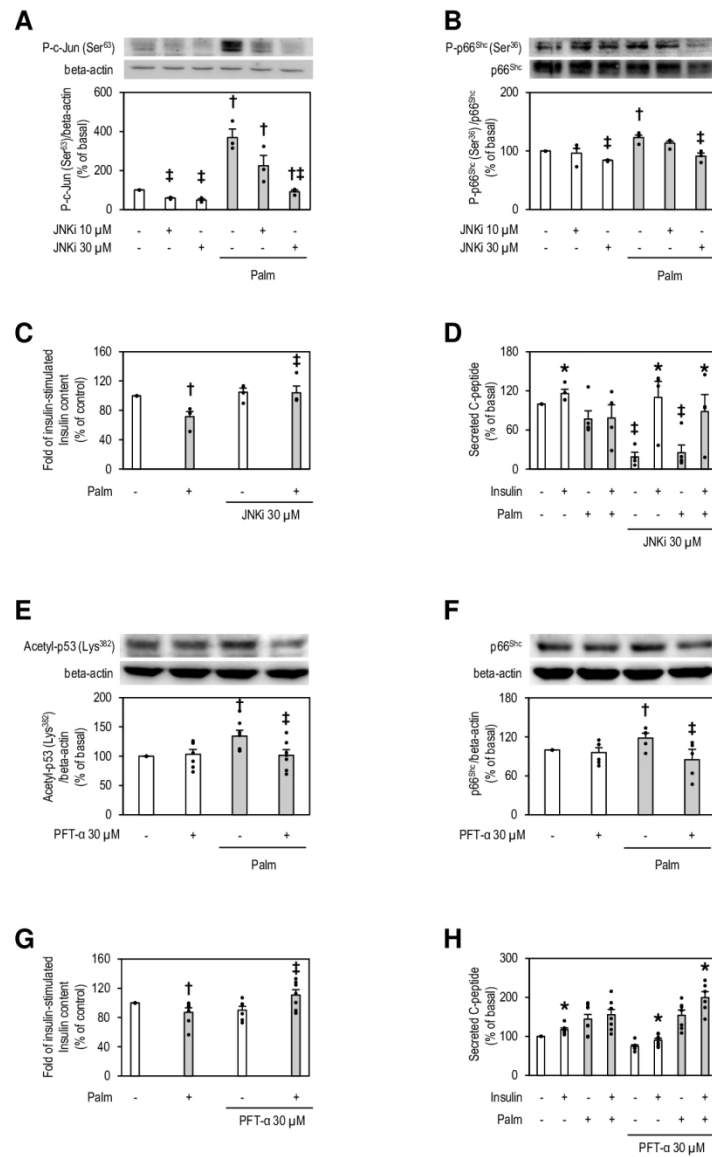
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Figure 2



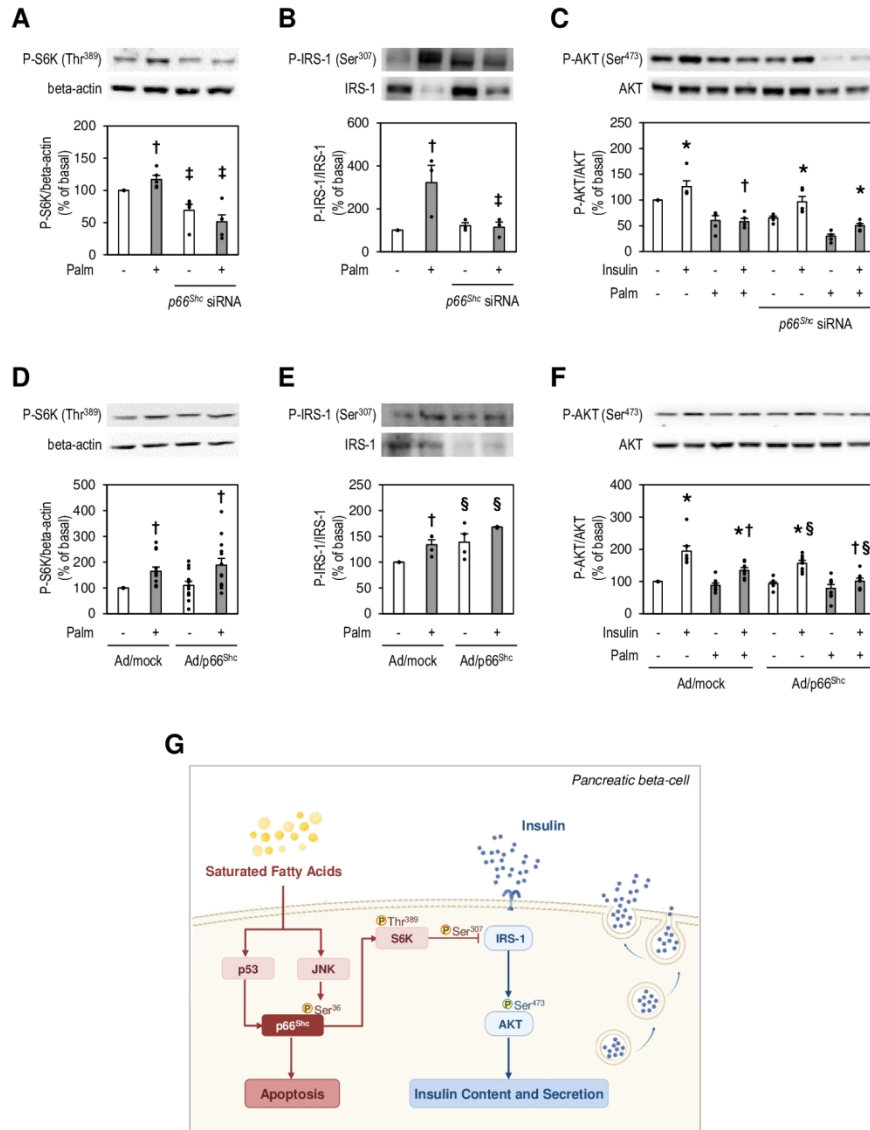
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Figure 3



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Figure 4



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## 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% CO<sub>2</sub>  
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<sup>Shc</sup> 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<sup>Shc</sup> 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<sup>Shc</sup> 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% CO<sub>2</sub> 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<sup>Shc</sup> construct harboring a  
36 Ser<sup>36</sup> to Ala<sup>36</sup> mutation. An empty adenovirus vector was used as control (mock). Viral titers used

37 were:  $5.2 \times 10^7$  PFU/ml for Ad/mock,  $2.5 \times 10^5$  PFU/ml for Ad/p66<sup>Shc</sup>, and  $1.2 \times 10^5$  PFU/ml for  
38 Ad/p66<sup>Shc</sup> (Ala<sup>36</sup>).

39 Where indicated, INS-1E cells were treated with 30  $\mu$ mol/l SP600125 for 2 h or 30  $\mu$ mol/l 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  $\mu$ 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  $2^{-\Delta\Delta Ct}$   
68 method. p66<sup>Shc</sup> 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  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub>  
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  $\mu$ 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).

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

<b>Specificity</b>	<b>Antibody</b>	<b>Dilution used</b>
Phospho-AKT (Ser <sup>473</sup> )	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 <sup>307</sup> )	Cell Signaling Technology Inc., #2381	1:500
Total IRS-1	Millipore, #06-248	1:500
Phospho-p70 S6K (Thr <sup>389</sup> )	Cell Signaling Technology Inc., #9205	1:1000
Total Shc	Merck Millipore, #06-203	1:1000
Phospho-p66 <sup>Shc</sup> (Ser <sup>36</sup> )	Invitrogen, #44828M	1:500
Phospho-c-Jun (Ser <sup>63</sup> ) II	Cell Signaling Technology Inc., #9261	1:1000
Acetyl p53 (Lys <sup>382</sup> )	Cell Signaling Technology Inc., #2525	1:500

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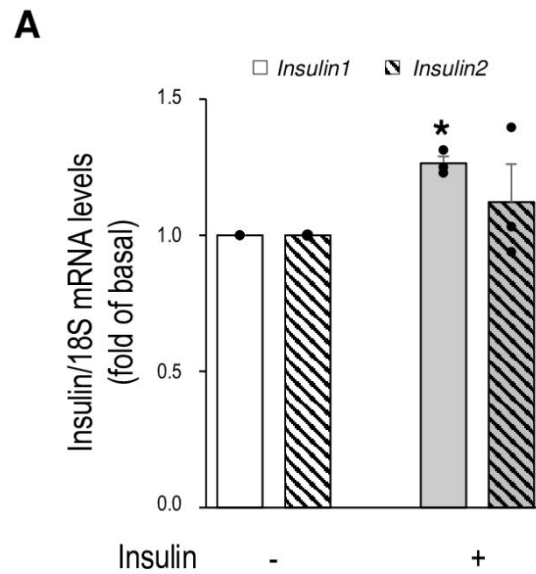
81 **Supplemental Table 2.** Primers used for quantitative real-time PCR analysis.

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

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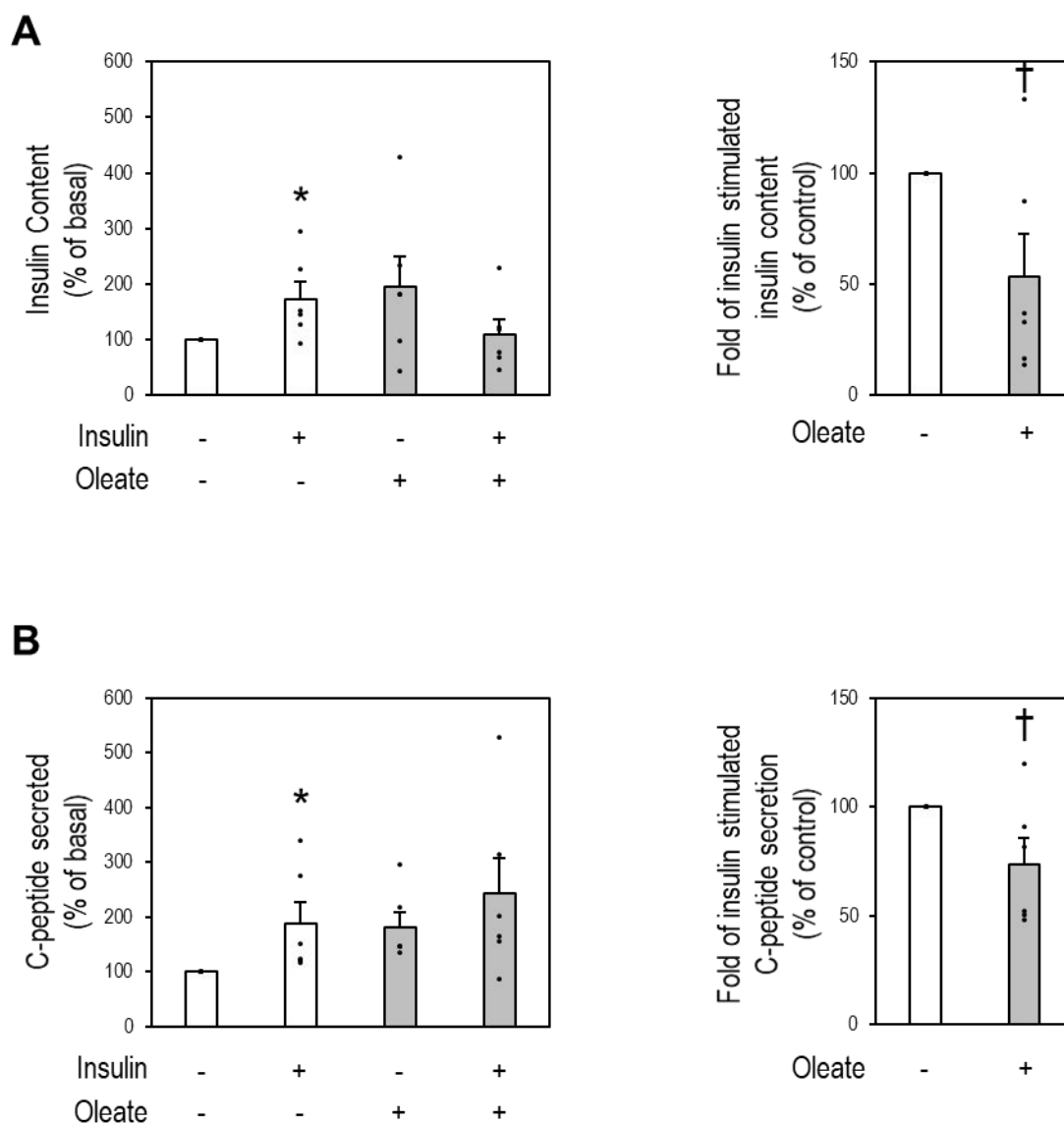
83 **Supplemental Figures**

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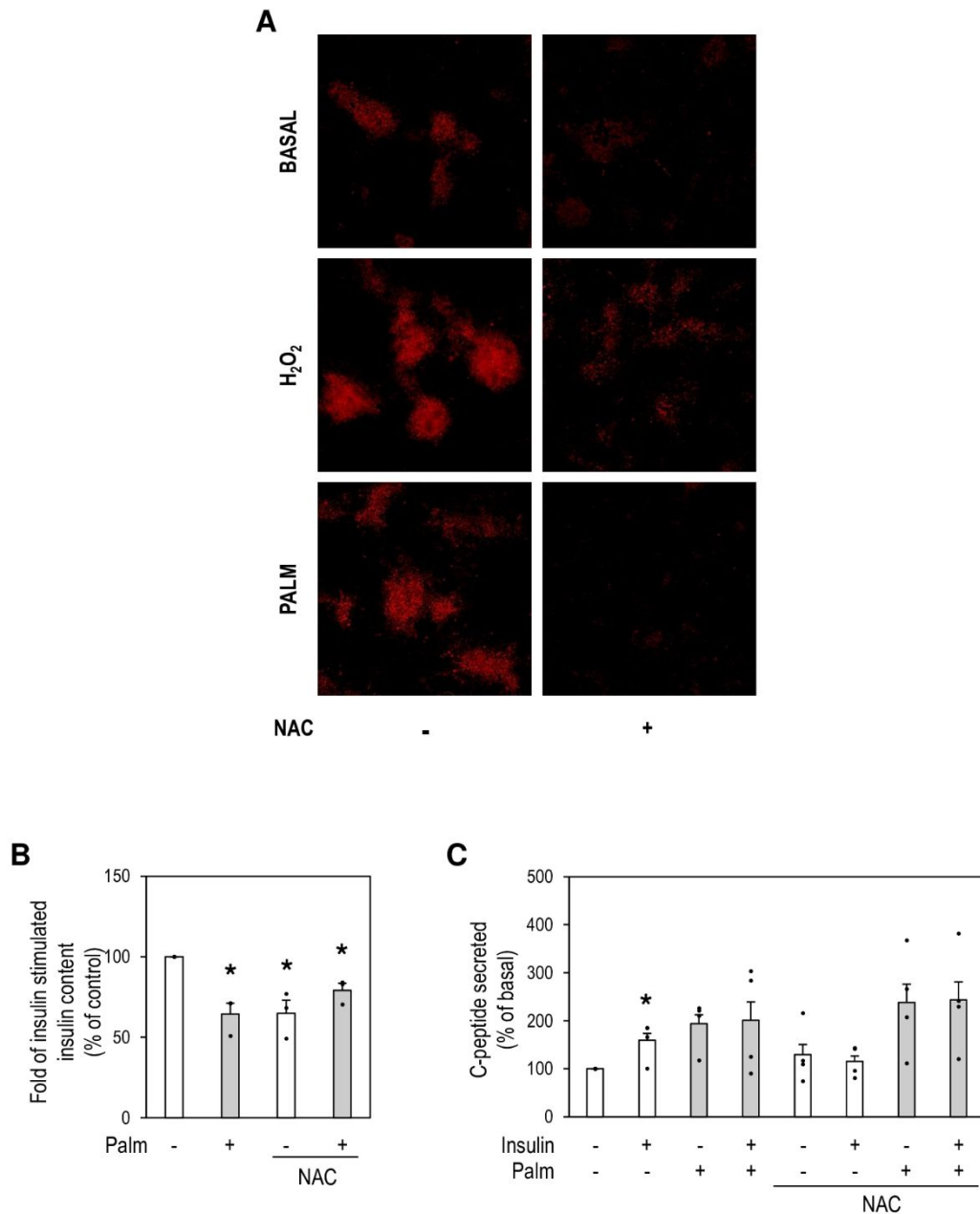
86 **Supplemental Figure 1.** Insulin increases *Insulin 1* gene expression levels in INS-1E cells. **A:** Cells  
87 were incubated in KRBH buffer for 100 min, then stimulated with 10 nM insulin in fresh KRBH  
88 buffer for 40 min. *Insulin 1* and *Insulin 2* gene expression was evaluated by quantitative 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 H<sub>2</sub>O<sub>2</sub> 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; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.



## Checklist for Reporting Human Islet Preparations Used in Research

Adapted from Hart NJ, Powers AC (2018) Progress, challenges, and suggestions for using human islets to understand islet biology and human diabetes. *Diabetologia* <https://doi.org/10.1007/s00125-018-4772-2>.

<b>Manuscript DOI:</b> <a href="https://doi.org/10.2337/db21-1066">https://doi.org/10.2337/db21-1066</a>	
<b>Title:</b> The p66 <sup>Shc</sup> protein mediates insulin resistance in pancreatic beta-cells under lipotoxic conditions	
<b>Author list:</b> Giuseppina Biondi, Nicola Marrano, Lucia Dipaola, Anna Borrelli, <b>Martina Rella</b> , Rossella D'Oria, Valentina Annamaria Genchi, Cristina Caccioppoli, Immacolata Porreca, Angelo Cignarelli, Sebastio Perrini, Piero Marchetti, Leonardo Vincenti, Luigi Laviola, Francesco Giorgino, Annalisa Natalicchio	
<b>Corresponding author:</b> Francesco Giorgino	<b>Email address:</b> francesco.giorgino@uniba.it

Islet preparation	1	2	3	4	5	6	7	8
<b>MANDATORY INFORMATION</b>								
Unique identifier	F65ND19.53	M70ND20.20	F55ND25.26	M71ND22.46	F67ND21.48	F61ND20.00	M77ND31.25	M78ND26.99
Donor age (years)	65	70	55	71	67	61	77	78
Donor sex (M/F)	F	M	F	M	F	F	M	M
Donor BMI (kg/m <sup>2</sup> )	19.53	20.20	25.26	22.46	21.48	20.00	31.25	26.99

Fasting glycemia (mg/dl)	96	81	95	88	96	90	91	84
Origin/source of islets <sup>b</sup>	Bari	Bari	Bari	Bari	Bari	Bari	Bari	Bari
Islet isolation centre	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari
Donor history of diabetes? Yes/No	No	No	No	No	No	No	No	No
<b>If Yes, complete the next two lines if this information is available</b>								
Diabetes duration (years)								
Glucose-lowering therapy at time of death <sup>c</sup>								

<b>RECOMMENDED INFORMATION</b>
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Donor cause of death	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Warm ischaemia time (h)								
Cold ischaemia time (h)	<1	<1	<1	<1	<1	<1	<1	<1

Estimated purity (%)	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Estimated viability (%)								
Total culture time (h) <sup>d</sup>	24	24	24	24	24	24	24	24
Glucose-stimulated insulin secretion or other functional measurement <sup>e</sup>	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Handpicked to purity? Yes/No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Additional notes								

Islet preparation	9	10	11	12	13	14	15	16
<b>MANDATORY INFORMATION</b>								
Unique identifier	F71ND30.47	M57ND25.10	F63ND28.28	UPI_T2DSyst_304	UPI_T2DSyst_266	F66ND19.38	UPI_T2DSyst_235	M64ND21.80
Donor age (years)	71	57	63	80	58	66	52	64

Donor sex (M/F)	F	M	F	F	F	F	F	M
Donor BMI (kg/m <sup>2</sup> )	30.47	25.10	28.28	24.80	25.80	19.38	25.40	21.80
Fasting glycemia (mg/dl)	126	116	86	122	104	82	85	162
Origin/source of islets <sup>b</sup>	Bari	Bari	Bari	Pisa	Pisa	Bari	Pisa	Bari
Islet isolation centre	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Department of Emergency and Organ Transplantation- University of Bari	Islet Laboratory – University of Pisa	Islet Laboratory – University of Pisa	Department of Emergency and Organ Transplantation- University of Bari	Islet Laboratory – University of Pisa	Department of Emergency and Organ Transplantation- University of Bari
Donor history of diabetes? Yes/No	No	No	No	No	No	No	No	No
<b>If Yes, complete the next two lines if this information is available</b>								
Diabetes duration (years)								
Glucose-lowering therapy at time of death <sup>c</sup>								

### RECOMMENDED INFORMATION

Donor cause of death	N/A	N/A	N/A	Cerebral hemorrhage	Post-anoxic encephalopathy	N/A	Ischaemic stroke	N/A
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Warm ischaemia time (h)								
Cold ischaemia time (h)	<1	<1	<1	36	7	<1	15	<1
Estimated purity (%)	N/D	N/D	N/D	90	60	N/D	60	N/D
Estimated viability (%)								
Total culture time (h) <sup>d</sup>	24	24	24	72	72	24	72	24
Glucose-stimulated insulin secretion or other functional measurement <sup>e</sup>	N/D	N/D	N/D	Yes, evaluated by batch incubation	Yes, evaluated by batch incubation	N/D	Yes, evaluated by batch incubation	N/D
Handpicked to purity? Yes/No	Yes	Yes	Yes	No	No	Yes	No	Yes
Additional notes								

<b>Islet preparation</b>	<b>17</b>							
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**MANDATORY INFORMATION**

Unique identifier	M68ND24.71							
Donor age (years)	68							
Donor sex (M/F)	M							
Donor BMI (kg/m <sup>2</sup> )	24.71							
Fasting glycemia (mg/dl)	81							
Origin/source of islets <sup>b</sup>	Bari							
Islet isolation centre	Department of Emergency and Organ Transplantation- University of Bari							
Donor history of diabetes? Yes/No	No							
<b>If Yes, complete the next two lines if this information is available</b>								
Diabetes duration (years)								
Glucose-lowering therapy at time of death <sup>c</sup>								

RECOMMENDED INFORMATION								
Donor cause of death	N/A							
Warm ischaemia time (h)								
Cold ischaemia time (h)	<1							
Estimated purity (%)	N/D							
Estimated viability (%)								
Total culture time (h) <sup>d</sup>	24							
Glucose-stimulated insulin secretion or other functional measurement <sup>e</sup>	N/D							
Handpicked to purity? Yes/No	Yes							
Additional notes								