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Salt-inducible kinases are required for glucose uptake and insulin signaling in human adipocytes

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3 **Salt-inducible kinases are required for glucose uptake and insulin signaling in human**
4 **adipocytes**
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10 Johanna Säll^{1,a}, Maria Lindahl¹, Andreas M. Fritzen², Claes Fryklund¹, Franziska Kopietz¹,
11 Emma Nyberg^{1,b}, Anna Warvsten¹, Björn Morén^{1,c}, Marc Foretz³, Bente Kiens², Karin G.
12 Stenkula¹, Olga Göransson^{1*}
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18

19 ¹ Department of Experimental Medical Science, Lund University, SWEDEN.

20
21 ² The August Krogh Section for Molecular Physiology, Department of Nutrition, Exercise
22 and Sports, Faculty of Science, University of Copenhagen, DENMARK.
23

24
25 ³ Institut Cochin, INSERM, CNRS, Department of Endocrinology, Metabolism and Diabetes,
26 Université Paris Cité, FRANCE.
27
28

29
30 *Corresponding author
31
32
33

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54

55 ^a Present address: Lund University, Department of Clinical Sciences (Malmö), Diabetes and Epigenetics, CRC
56 91-12, Jan Waldenströms gata 35, 21428 MALMÖ, SWEDEN

57 ^b Present address: Lund University, Department of Experimental Science, Experimental Dementia Research
58 Unit, BMC B11, Klinikgatan 26, 22242 LUND, SWEDEN

59 ^c Present address: Umeå University, Department of Integrative Medical Biology, Biologihuset, hus H, Johan
60 Bures väg 12, 90187 UMEÅ, SWEDEN

1
2
3 **Contact info corresponding author:** Olga Göransson, Lund University, Protein
4
5 Phosphorylation Research Group, BMC C11, Klinikgatan 28, 22242 LUND, SWEDEN,
6
7 Telephone: +46(0)46-2229552, E-mail: olga.goransson@med.lu.se
8
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Study importance

What is already known about this subject?

- The AMPK-related kinase salt-inducible kinase 2 (SIK2) is abundantly and preferentially expressed in adipose tissue
- SIK2 is downregulated in adipose tissue from individuals with obesity or insulin resistance, but the effect of rodent obesity on SIK2 expression is not clear
- SIK isoforms regulate glucose uptake in rodent and cultured human adipocytes but the underlying mechanism and the role in primary mature human adipocytes is not known

What are the new findings in your manuscript?

- SIK activity is required for normal glucose uptake in primary mature human and mouse adipocytes, and the underlying mechanism involves direct effects on the insulin signaling pathway
- SIK activity is required for membrane translocation of the PKB/Akt PH domain
- SIK2 is downregulated in adipose tissue from *ob/ob* mice and SIK2 is required for intact glucose uptake in mouse adipocytes

How might your results change the direction of research or the focus of clinical practice?

We have shown that SIK isoforms can regulate glucose uptake in human cells through mechanisms other than changes in gene expression. Future studies need to pinpoint the direct SIK2 target responsible for alterations in the insulin signaling pathway. All in all, our results support the possibility that downregulation of SIK2 plays a role in the development of obesity-induced insulin resistance, and that upregulation of SIK2 levels in adipocytes offers potential as a therapeutic measure.

Abstract

Objective

Salt-inducible kinase 2 (SIK2) is abundantly expressed in adipocytes and downregulated in adipose tissue from individuals with obesity or insulin resistance. The main aims of this work were to investigate the involvement of SIKs in the regulation of glucose uptake in primary mature human adipocytes, and to identify mechanisms underlying this regulation.

Methods

Primary mature adipocytes were isolated from human, rat, or mouse adipose tissue and treated with pan-SIK inhibitors. We also used adipocytes isolated from wild type, *ob/ob* and SIK2 knockout mice. Glucose uptake was examined by glucose tracer assay. The insulin signaling pathway was monitored by western blotting, co-immunoprecipitation and total internal reflection fluorescence (TIRF) microscopy.

Results

We demonstrate that SIK2 is downregulated in obese *ob/ob* mice, and SIK activity is required for intact glucose uptake in primary human and mouse adipocytes. The underlying mechanism involves direct effects on the insulin signaling pathway, likely at the level of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) generation or breakdown. Moreover, lack of SIK2 alone was sufficient to attenuate glucose uptake in mouse adipocytes.

Conclusions

SIK2 is required for insulin action in human adipocytes, and the mechanisms include direct effects on the insulin signaling pathway.

Introduction

Salt-inducible kinase (SIK) 1, SIK2 and SIK3 are related to AMP-activated protein kinase (AMPK) by sequence similarities in their kinase domains (1), and have been implicated in the regulation of metabolic processes in various tissues (2). SIK2 is abundantly and preferentially expressed in adipose tissue, whereas SIK1 and SIK3 isoforms have a lower expression level, compared to SIK2, in adipocytes (3, 4, 5, 6). We have demonstrated that SIK2 expression and kinase activity are markedly reduced in adipose tissue and adipocytes of individuals with obesity (5). Moreover, *SIK2* expression in adipose tissue displays a strong inverse correlation to insulin resistance, independently of BMI and age (5). We and others have shown that SIK2 activity is required for glucose uptake in primary rat adipocytes, and in mouse and human adipocytes differentiated *in vitro* (5, 7, 8). However, it is not known to what extent SIK2 is of importance for glucose uptake in primary mature human adipocytes. Findings of lower expression of glucose transporter 4 (GLUT4) in adipose tissue from SIK2 KO mice (7, 8) imply that an SIK2-mediated regulation of glucose uptake could be via transcriptional regulation of GLUT4. Such a mechanism might be mediated through inhibition of cAMP-responsive element-binding protein (CREB)-regulated transcription coactivators (CRTC) and/or histone deacetylase 4 (HDAC4), which are substrates of SIK2 (7, 8, 9) and reported to suppress GLUT4 expression in mouse adipocytes (7, 8, 10, 11). However, in cultured human adipocytes we found that glucose uptake was clearly attenuated after SIK inhibition or siRNA silencing, while GLUT1 or GLUT4 protein levels were not (5). Moreover, we discovered that one hour of SIK inhibition in these cells was sufficient to reduce basal and insulin-stimulated glucose uptake (5), which further suggests that SIK isoforms regulate glucose uptake in human cells through more acute and direct mechanisms other than changes in gene expression downstream of HDAC4 and CRTC.

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3 Previous reports have described contrasting roles for SIK2 in the regulation of insulin
4 signaling (3, 5, 8, 12, 13, 14, 15). A few studies reported that SIK2 inhibits insulin signaling
5 by interacting with IRS1 and/or phosphorylating it at Ser789 (3, 12), or by promoting
6 dephosphorylation and inactivation of protein kinase B (PKB/Akt) (15). On the other hand,
7 several other studies, including our own in *in vitro* differentiated human adipocytes,
8 demonstrated that SIK2 is required for the activating phosphorylation of PKB/Akt (5, 8, 13,
9 14), potentially by phosphorylating the regulatory p85 subunit of phosphoinositide 3-kinase
10 (PI3K) (13) or by affecting expression levels of PKB/Akt phosphatases (15).
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22 With this as a background, the aims of the current study were to investigate if SIK isoforms
23 regulate glucose uptake in an acute manner also in primary mature human adipocytes, and to
24 examine the individual role of SIK2 using SIK2 knockout (KO) mice. Moreover, we aimed to
25 explore the mechanisms underlying the effect of SIKs on glucose uptake, including effects on
26 the insulin signaling pathway. Together, this will ultimately give a better understanding of
27 whether downregulation of SIK2 could play a causal role in obesity-induced insulin
28 resistance in human adipose tissue.
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Methods

Chemicals and reagents, Definition of common buffers, Western blotting, and Antibodies

Sections describing these methods can be found in the online Supporting Information.

Ethics statement

All subjects were given written and oral information about the study before providing their written informed consent. Human studies were approved by the Regional Ethical Review Boards at Lund University (approval number 2017/920). Animal experiments were approved by the Regional Ethical Committee on Animal Experiments in Malmö/Lund (approval number 5.8.18-18569/2018) and the Danish Animal Experimental Inspectorate (2010/63/EU) and complied with the *European Convention for the Protection of Vertebrate Animals used for Experiments and other Scientific Purposes*.

Collection of adipose tissue and isolation of primary adipocytes

Abdominal subcutaneous adipose tissue was collected from women who underwent reconstructive breast surgery at Scania University Hospital (a total of $n=28$ individuals, body mass index (BMI)= 25.3 ± 3.1 kg/m² [mean \pm SD], age= 55.6 ± 8.6 years [mean \pm SD]). After excision, the adipose tissue was placed in PBS at room temperature and immediately transported to the laboratory for isolation of adipocytes. Epididymal adipose tissue was excised from 6-week-old male Sprague-Dawley rats (Charles River, Sulzfeld, Germany), from 9-week-old male C57Bl6/J mice (Taconic Biosciences, Ejby, Denmark), or from SIK2 KO and corresponding WT mice (12-24 weeks of age, see below). Adipose tissue was minced and digested with collagenase (1 mg/ml) in a shaking incubator at 37°C. Digests were filtered and washed in KRH buffer with BSA, to isolate primary mature adipocytes.

Electroporation of adipocytes

For the introduction of cDNA encoding GFP-PKB-PH or GFP-p85 α (WT or Ser154Ala), human, mouse or rat adipocytes were electroporated, as described before (16). Briefly, 100 μ l cells were mixed with 3.2 μ g of cDNA in a Bio-Rad GenePulser 0.4 cm cuvette, and electroporated using the following conditions; 400 V, 12 ms, one pulse (mouse and human adipocytes), 200 V, 12 ms, three pulses (rat adipocytes), in a total volume of 400 μ l DMEM containing 100 U penicillin/0.1 mg streptomycin per mL (P/S, for human cells), or 0.1 mg gentamicin per mL (for mouse and rat cells), and 200 nM PIA. Thereafter, cells were washed and resuspended in DMEM containing P/S (human) or gentamicin (mouse and rat), 200 nM PIA and 3.5% (wt./vol.) BSA, and incubated \approx 20 hours at 37°C, 5% CO₂, to allow for expression of the transgenes. The next day, cells were stimulated and lysed as described below.

Culture, stimulation and lysis of adipocytes

For stimulation and generation of lysates, isolated primary adipocytes were either incubated overnight as described above (human) or stimulated directly, without overnight recovery (rat and mouse). Cells were washed and resuspended in KRH with BSA, and treated with HG-9-91-01 or YKL-05-099 followed by insulin, at 37°C in a shaking (80 rpm) water bath. After stimulation, cells were washed in KRH (without BSA), and lysed in lysis buffer. Lysates were centrifuged at 13 000 g for 15 min (4°C) and protein concentration in the supernatant was determined by the Bradford assay.

TIRF microscopy of GFP-PKB-PH and immunolabelled pPKB Ser473

Human and mouse adipocytes expressing GFP-PKB-PH were equilibrated for 1 hour in KRBH buffer and treated with insulin and HG-9-91-01 as indicated in the figure legend. After treatment, adipocytes were fixed using 4% paraformaldehyde (PFA), and a subset of

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3 cells were additionally labelled with pPKB/Akt-Ser473 antibody (CST RRID:AB_2315049,
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5 dilution 1:200) and with Alexa Fluor™ 568 secondary antibody (Thermo Fisher Scientific
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7 RRID:AB_10563566, dilution 1:300) in KRH with BSA, supplemented with 1% goat serum
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9 and 0.05% saponin. Imaging with TIRF microscopy was performed using the same settings
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11 for all samples. For TIRF imaging we used a commercial system based on a Nikon Ti-E
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13 eclipse microscope equipped with a 100× Apo TIRF DIC oil immersion objective NA of 1.49
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15 (Nikon Instruments Inc.), an iXon Ultra DU-897 EMCCD camera (Andor Technology Ltd.),
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17 and four main laser lines, 405 (Cube, Coherent Inc), 488 (Melles-Griot), 561 (Sapphire,
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19 Coherent Inc), and 640 (Cube, Coherent Inc) with corresponding filter sets. The GFP-PKB-
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21 PH was detected with the 488 nm laser line and pPKB/Akt Ser473 was detected with the 561
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23 nm laser line. ~30 images were acquired per condition and average greyscale values were
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25 measured for each cell using FIJI.
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31 *Glucose uptake assay*

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35 Isolated primary human adipocytes cultured overnight, or rat and mouse adipocytes isolated
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37 the same day, were washed 3 times in KRBH buffer with BSA. Cell suspensions were diluted
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39 to equal concentration and divided equally in vials. A volume of 400 µl 5% [vol./vol.] cell
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41 suspension per vial (except for **Figure S1a** where 2.5% [vol./vol.] cell suspension was used)
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43 was treated with HG-9-91-01 or YKL-05-099 for 1 hour at 37°C in a shaking (80 rpm) water
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45 bath before the addition of insulin, or 10 µM cytochalasin B. Adipocytes were stimulated
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47 with insulin for 30 min followed by addition of 100 µl KRBH buffer containing 0.25 µl
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49 (0.0625 µCi) ¹⁴C-glucose. The reactions were stopped after another 30 min by aliquoting 300
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51 µl of the total 500 µl adipocyte suspension to Beckman microtubes containing 75 µl
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53 dinonylphthalate. The adipocyte suspension was centrifuged at 6000 x g and frozen at -20°C
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55 before the adipocytes were collected and subjected to scintillation counting. The assay was
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3 performed in triplicates for each condition. The analysis of glucose uptake was the primary
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5 outcome measure to determine the sample size in the SIK2 KO mouse study (mice further
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7 described below). Sample size was decided based on previous experience from work with the
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9 SIK2 mouse model and measurements of glucose uptake in isolated adipocytes. The analysis
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11 was carried out on $n=10$ different cell suspensions (5 containing cells from males, 5
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13 containing cells from females) per genotype (WT and SIK2 KO). Each cell suspension was
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15 prepared from 1-3 mice (12-24 weeks of age), which were age- and sex-matched within each
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17 suspension and between genotypes. A total of 18 mice per genotype were analyzed. Values
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19 are presented as counts per minute (CPM) after subtraction of the counts for each respective
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21 Cytochalasin B-treated negative control sample. *A priori* exclusion criteria included the
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23 absence of insulin effect on the glucose uptake and/or if a CPM value deviated more than
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25 50% from the mean of the other two CPM values in the same triplicate. Data from one out of
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27 10 paired WT/SIK2 KO cell suspensions (female) was excluded due to the absence of insulin
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29 effect at 0.01 nM insulin in the WT cell suspension (**Figure 7i**). The experimental group
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31 allocation was not blinded.
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39 *Immunoprecipitation of PI3K GFP-p85 α and IRS1*

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42 For IP of IRS1, human adipocyte lysates containing 100-250 μ g total protein were incubated
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44 for 1.5 hour with 5 μ g of anti-IRS1 antibody (Cell Signaling Technology [CST]
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46 RRID:AB_2800253) conjugated with 5 μ l packed protein-G-sepharose (GE Healthcare,
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48 Uppsala Sweden). For IP of GFP-p85 α , rat adipocyte lysates containing 700-1500 μ g total
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50 protein were incubated for 1 hour with 7.5 μ l packed GFP-Trap (Chromotek, Munich,
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52 Germany). Subsequently, the immunoprecipitates were washed twice with lysis buffer
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54 supplemented with 0.5 M (GFP-p85 α) or 0.15 M (IRS1) NaCl and 1 mM DTT, and twice
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56 with 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA and 1 mM DTT.
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SIK2 knockout (KO) mice

12-24-week-old male whole-body SIK2 KO mice were used as previously described (7). Briefly, these mice were generated by crossing SIK2^{+/lox} mice with deleter EIIa-CRE transgenic mice to produce SIK2^{+/-} mice, which subsequently were crossed to produce WT and SIK2^{-/-} mice (7, 9, 17). Routine genotyping was carried out as described (7). Mice were bred (courtesy of Bente Kiens and Erik Richter, University of Copenhagen, Denmark) and sacrificed under a protocol approved by the Danish Animal Experimental Inspectorate and that complied with the European Convention for the Protection of Vertebrate Animals used for Experiments and other Scientific Purposes (2010/63/EU). Mice were housed in temperature-controlled (22±1°C) facilities, maintained on a 12:12 light:dark cycle and received standard chow (Altromin, cat. No. 1324; Brogaarden, Lyngø, Denmark) and water *ad libitum*.

Body composition

Mouse body composition was assessed by MRI-scan (EchoMRI-4 in 1TM for Live Animals, Echo Medical System LLC, Texas, USA) according to manufacturer's instructions.

Glucose tolerance test (GTT)

Mice were fasted for 5 hours (9:00am – 2:00pm) and injected intraperitoneally with 2 g/kg body weight D-glucose dissolved in saline. Injection volume for all GTTs was 10 µl per gram mouse body weight. Blood glucose concentrations in mixed tail blood were measured with a glucometer just before injection (0 min) and at 20, 40, 60, 90 and 120 minutes post-injection. On a separate day, during the same fasting conditions, mixed tail blood was collected into heparinized capillary tubes just before and at 15 minutes after glucose injection, and assayed with an insulin ELISA kit (Mouse Ultrasensitive Insulin ELISA, #80-INSMSU-E01, Alpco, Salem, USA).

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3 *Statistical analysis*
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6 All values are presented as means (\pm SD). Statistical tests were performed using GraphPad
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8 Prism 9 (La Jolla, CA, USA) as indicated in figure legends.
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Results

SIK activity is required for glucose uptake in primary human adipocytes.

In our previous work, we demonstrated that inhibition of SIK isoforms using the highly selective pan-SIK inhibitor HG-9-91-01 overnight or for one hour in human adipocytes differentiated *in vitro*, resulted in attenuation of basal and insulin-stimulated glucose uptake (5). Here we investigated the effect of acute SIK inhibition (one hour) on glucose uptake in primary human adipocytes. In line with what we observed in cultured human adipocytes, one hour of pre-treatment with increasing doses of HG-9-91-01 had an overall inhibitory effect on glucose uptake as determined by a two-way ANOVA (**Figure 1a**, *** $p=0.0003$ for HG-9-91-01 dose). Treatment with 3 and 10 μM HG-9-91-01 inhibited glucose uptake both in the basal state (**Figure 1b**) and in the presence of a sub-maximal (0.1 nM) dose of insulin (**Figure 1c**). Maximally stimulated (10 nM insulin) glucose uptake was also inhibited by HG-9-91-01 (**Figure S1a-b**). To confirm the efficacy of the one-hour SIK inhibition, we monitored the phosphorylation of the known SIK substrate HDAC4 (**Figure 1d** and **Figure S1c**). As a complement to HG-9-91-01, we also used another pan-SIK inhibitor, YKL-05-099, which has higher IC₅₀ values for inhibition of SIK2 activity *in vitro* (40 nM for YKL-05-099 vs 3.5 nM for HG-9-91-01) (18). Compared to HG-9-91-01, YKL-05-099 resulted in an almost two times lower efficiency in reducing the phosphorylation of HDAC4 in rat adipocytes (**Figure S1d-e**). Similar to HG-9-91-01, YKL-05-099 had an overall inhibitory effect on glucose uptake (**Figure 1e**, *** $p=0.0003$ for YKL-05-099 dose). Treatment with 30 μM YKL-05-099 inhibited both basal (**Figure 1f**) and sub-maximally (0.1 nM insulin) stimulated glucose uptake (**Figure 1g**) in primary human adipocytes.

SIK activity is required for phosphorylation of the Rab-GTPase activating protein AS160 and PKB/Akt in primary human adipocytes.

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3 Phosphorylation of the Rab-GTPase activating protein AS160 by PKB/Akt at Thr642 is
4 important for the control of GLUT4 translocation and its stimulation by insulin (19). As shown
5 in **Figure 2a-b**, the insulin-induced (0.01 nM) phosphorylation of AS160 at Thr642 was
6 slightly attenuated by one hour of HG-9-91-01 pre-treatment in human adipocytes, potentially
7 contributing to the effect of SIK inhibition on insulin-induced glucose uptake. In search for
8 mechanisms underlying the reduced phosphorylation of AS160 we studied the activating
9 phosphorylation sites of its upstream kinase PKB/Akt in the same experimental setup. Indeed,
10 the insulin-induced phosphorylation of PKB/Akt at Thr308 (**Figure 2c-d**) and Ser473 (**Figure**
11 **2e-f**) was markedly reduced by SIK inhibition.

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25 *SIK inhibition does not affect IRS1 phosphorylation or its interaction with PI3K p85.*

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28 To elucidate upstream mechanism(s) whereby SIKs regulate PKB/Akt signaling in adipocytes,
29 we monitored the effect of SIK inhibition on several phosphorylation sites in IRS1, shown to
30 either inhibit or activate IRS1 function (20). We detected a minor decrease in the insulin-
31 induced (0.01 nM) phosphorylation of IRS1 at the activating site Tyr612 (Tyr608 in mouse/rat)
32 in human adipocytes (**Figure 3a-b**). However, inhibition of SIKs did not induce any changes
33 in the phosphorylation of IRS1 at the activating site Ser307 (Ser302 in mouse/rat, **Figure 3c**),
34 nor at the inhibitory sites Ser1101 (Ser1100 in mouse/rat, **Figure 3d**) or Ser789 (Ser794 in
35 human, **Figure 3e**) – the latter proposed to be phosphorylated by SIK2 (3, 12). In all, we did
36 not find robust evidence that the upstream mechanism whereby SIK2 regulates PKB/Akt
37 signaling is through the control of IRS1 phosphorylation.

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40 An important outcome of insulin-induced phosphorylation of IRS1 is its binding to the
41 regulatory p85 α subunit of PI3K. As a complement to the IRS1 phosphorylation analysis, we
42 thus studied whether inhibition of SIKs affected IRS1-p85 α interaction in human adipocytes.
43 As shown in **Figure 4a** and **4b**, insulin induced the co-immunoprecipitation of p85 α and IRS1,
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3 as well as the phosphorylation of IRS1 on Tyr612, which is known to be important for p85 α
4 binding (21). However, inhibition of SIK isoforms did not affect the interaction of p85 α with
5 IRS1 (**Figure 4a**), nor the phosphorylation of IRS1 at Tyr612 (**Figure 4b**), although it
6 markedly blunted the phosphorylation of the known SIK substrate HDAC4 (**Figure 4c**) and
7 the phosphorylation of PKB/Akt at Ser473 (**Figure 4d**). Together, these data suggest that SIKs
8 likely affect the insulin signaling pathway downstream IRS1.
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18 *SIK inhibition does not affect PI3K p85 α phosphorylation but disrupts translocation of the*
19 *PKB/Akt PH domain to the plasma membrane.*
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24 SIK2 has been suggested to phosphorylate p85 α on Ser154, thereby promoting PI3K activity
25 in ovarian cancer cells (13). Available p85 α Ser154 phospho-specific antibodies do not detect
26 endogenous levels of phosphorylated p85 α . Thus, to study potential SIK2-mediated
27 phosphorylation of p85 α , we used rat adipocytes in which we expressed GFP-p85 α by means
28 of electroporation. As in human adipocytes, inhibition of SIK activity by one hour of HG-9-
29 91-01 pre-treatment resulted in a reduction in glucose uptake, and in the phosphorylation of
30 PKB/Akt at Ser473 in rat adipocytes, although to a smaller degree than in human cells (**Figure**
31 **S2a-f**). The level of phosphorylation of GFP-p85 α at Ser154 in HG-9-91-01-treated cells
32 varied in relation to the control but was on average not significantly changed (**Figure 5a**),
33 although the phosphorylation of HDAC4 at Ser246 (**Figure 5b**) and PKB/Akt at Ser473
34 (**Figure 5c**) was consistently reduced in the same experiments. The phospho-specificity of the
35 p85 α Ser154 antibody was demonstrated by the use of a Ser154Ala mutant form of p85 α , for
36 which the phospho-signal was markedly lower than wild type (WT) p85 α (**Figure S2g**). A
37 correlation analysis revealed no correlation between the phosphorylation of GFP-p85 α and
38 HDAC4 (the latter used as a readout for the degree of SIK inhibition) (**Figure 5d**), whereas
39 there was a strong positive correlation between the phosphorylation of PKB/Akt and HDAC4
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3 (Figure 5e). Collectively, these results do not support that SIK activity is important for the
4 phosphorylation of p85 α at Ser154 – at least not in primary rat adipocytes.
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9 We next analyzed the requirement of SIK activity for the presence of a PKB/Akt PH domain
10 at the plasma membrane – as a proxy measurement of the level of phosphatidylinositol (3, 4,
11 5)-trisphosphate (PIP3), the product of PI3K. To do so, we electroporated primary human
12 adipocytes with a construct encoding a GFP-fused PH domain (from PKB/Akt), after which
13 the cells were treated with HG-9-91-01 and insulin, and analyzed by total internal reflection
14 fluorescence (TIRF) microscopy. As shown in Figure 6a (displaying mean quantifications
15 from three individual experiments/human donors), Figure 6b (displaying one cell per
16 condition from a representative experiment) and Figure 6c (displaying quantification of 30
17 individual cells per condition from a representative experiment), insulin induced an increase
18 of GFP-PKB-PH in the TIRF zone in control cells, whereas in cells treated with HG-9-91-01,
19 this insulin-induced translocation of GFP-PKB-PH to the plasma membrane was absent.
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34 Similar to what we had previously observed by western blotting, the level of insulin-induced
35 Ser473 phosphorylation of endogenous PKB/Akt, here measured by fluorescence
36 immunocytochemistry, was also blunted in HG-9-91-01-treated cells (Figure 6d-e). All in
37 all, these data suggest that the effect of SIK inhibition on insulin signaling takes place at the
38 level of PIP3 formation, breakdown, or localization.
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47 *SIK2 is downregulated in a genetic mouse model of obesity and required for normal glucose*
48 *uptake in murine adipocytes.*
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52 To further address the requirement of SIK activity for insulin signaling and glucose uptake, as
53 well as to investigate the specific contribution of SIK2, we used different mouse models. As a
54 starting point, we studied the expression of SIK2 in the *ob/ob* mouse model of obesity, as well
55 as the effect of HG-9-91-01 in primary mouse adipocytes from WT mice. In line with what we
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3 have previously shown for human obesity (5), but in contrast to what was reported for adipose
4 tissue from *db/db* mice (3), SIK2 protein levels were markedly downregulated in adipocytes
5 isolated from *ob/ob* mice, compared to control (*ob/-*) mice (**Figure 7a**). The effect of one hour
6 HG-9-91-01 pre-treatment of primary WT mouse adipocytes was reminiscent of that in human
7 adipocytes, with an inhibition of glucose uptake (**Figure 7b**) both in the basal state (**Figure 7c**)
8 and in the presence of a sub-maximal (0.1 nM) dose of insulin (**Figure 7d**), and an overall
9 decrease in the phosphorylation of PKB/Akt at Thr308 (**Figure 7e**, ** $p=0.0077$ for HG-9-91-
10 01 dose) and Ser473 (**Figure 7f**, ** $p=0.0012$ for HG-9-91-01 dose) as determined by a two-
11 way ANOVA. The phosphorylation of HDAC4 was used to demonstrate the efficiency of SIK
12 inhibition (**Figure 7g**). Moreover, pre-treatment with HG-9-91-01 reversed the insulin-induced
13 (0.1 nM) increase in GFP-PKB-PH present at the plasma membrane (**Figure 7h**).

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30 A previous study reported that glucose uptake and PKB/Akt phosphorylation were reduced in
31 adipocytes, which were differentiated *in vitro* from the stromal vascular fraction of global SIK2
32 KO mice (8). Effects on primary mature adipocytes from these mice were, however, not
33 investigated. To this end, we analyzed glucose uptake in primary adipocytes isolated from an
34 independent line of SIK2-deficient mice (7, 9). In these cells, loss of SIK2 was associated with
35 a trend towards lower glucose uptake (**Figure 7i**, $p=0.0831$ for SIK2 KO vs WT). However,
36 when analyzing younger (**Figure 7j**, <20 weeks old) and older (**Figure 7k**, >20 weeks old)
37 mice separately it was clear that SIK2-deficiency resulted in reduced basal and insulin-
38 stimulated uptake of glucose in the older mice (**Figure 7k**). The phosphorylation of PKB/Akt
39 at Thr308 was modestly reduced (18% lower compared to WT) in the presence of a sub-
40 maximal insulin concentration (0.1 nM) (**Figure S3a** and **3c**), while the phosphorylation at
41 Ser473 was reduced in three mice and increased in two mice – with no statistical difference in
42 the mean phosphorylation (30% lower compared to WT) (**Figure S3b** and **3c**). In line with the
43 previously described SIK2 KO mice (8), there were no differences in total body weight (**Figure**

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3 **S3d**), or in lean and fat mass of the mice (**Figure S3e-f**). Furthermore, mice lacking SIK2
4 displayed normal glucose tolerance, and insulin levels before and 15 min after glucose
5 administration (**Figure S3g-h**).
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10 **Discussion**

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14 In the present study, we demonstrate for the first time that SIK activity is required for glucose
15 uptake in primary mature human adipocytes and that the mechanism most likely involves
16 positive effects on the insulin signaling pathway at the level of PIP3 generation, breakdown,
17 or localization. An important novelty of our work is that pharmacological inhibition of SIKs
18 for as short as one hour is sufficient to markedly reduce glucose uptake and attenuate the
19 membrane translocation and phosphorylation of PKB/Akt in primary human adipocytes.
20 Similar results were observed in primary mouse adipocytes. This indicates that SIK isoforms
21 can regulate insulin signaling in an acute and direct manner that likely does not involve changes
22 in gene expression. Another important result of our study is that we did not find evidence
23 supporting a role for SIKs in the regulation of IRS1 Ser789 (Ser794 in human) or PI3K p85
24 Ser154 phosphorylation (using phospho-specific antibodies) in mature adipocytes – which was
25 suggested based on work *in vitro* and in cell lines (3, 12, 13).
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43 The main rationale for studying the role of SIK2 in adipocytes is our previous finding that
44 adipocyte SIK2 is downregulated in human obesity and insulin resistance (5), and that this
45 might play a causal role in the development of insulin resistance. In contrast to our results from
46 humans, early reports described that SIK2 was *up*-regulated in white adipose tissue from the
47 obese and diabetic *db/db* mouse model, suggesting distinct differences in the regulation of
48 SIK2 expression in human vs rodent obesity (3). However, our novel data showing a marked
49 reduction of SIK2 levels in adipocytes from *ob/ob* mice, as well as a recent paper showing
50 reduced SIK2 expression in white adipose tissue from high fat diet-fed mice (22), indicate that
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3 SIK2 is indeed *down*-regulated also in rodent obesity and that there are technical reasons to the
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5 previously contrasting data. Based on pan-SIK inhibition in primary adipocytes from humans,
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7 mice and rats, our study also supports that SIK isoforms are functionally similar in human and
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9 rodent adipocytes – at least when it comes to effects on insulin signaling and glucose uptake.
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13 Given the lack of isoform-specific SIK inhibitors and that gene silencing is extremely
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15 challenging in primary mature adipocytes, our main tool for studying the role of SIK2 in
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17 these cells was the highly selective pan-SIK inhibitor HG-9-91-01. The dose-dependency for
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19 the effects of HG-9-91-01 on basal and insulin-stimulated glucose uptake were similar to the
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21 ones on SIK activity – measured as the phosphorylation of SIK substrate HDAC4. The effect
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23 of pan-SIK inhibition on glucose uptake was replicated using the alternative inhibitor YKL-
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25 05-099 – albeit with YKL-05-099 having a lower efficiency – further strengthening the
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27 importance of SIKs in promoting glucose uptake in adipocytes. As a further way to
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29 investigate the requirement of SIK isoforms for glucose uptake and to address the individual
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31 role of SIK2, we also used adipocytes isolated from global SIK2 KO mice. Our metabolic
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33 phenotyping of these mice indicated that loss of SIK2 did not impact whole-body glucose
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35 homeostasis (measured by GTT) at 12-16 weeks of age. This is in contrast to the previously
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37 described SIK2 KO mouse line, which displayed mild glucose and insulin intolerance on
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39 chow diet at 8 weeks of age (8). However, while SIK isoforms show significant redundancy
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41 in other tissues, for example in the liver (9), it was interesting to note that SIK2 deficiency
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43 alone was sufficient to reduce phosphorylation of SIK substrates (7) and glucose uptake in
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45 primary mouse adipocytes (present study), both in the basal and insulin-stimulated state.
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48 Ultimately, it is not surprising that a lower uptake of glucose in adipocytes did not translate
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50 into alterations in whole-body glucose tolerance since adipose tissue is not a major site for
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52 the uptake of glucose during an intraperitoneal GTT. Moreover, given that the glucose uptake
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3 phenotype was particularly clear in 24-week old mice, it would be interesting to also measure
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5 glucose tolerance at this age.
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8 All in all, we believe that the results from our current study together with our previous findings
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10 using pan-SIK inhibition and siRNA silencing in cultured human adipocytes (5), as well as the
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12 results from the Koo group in an independent line of SIK2 KO mice (8), strongly support that
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14 SIK isoforms are required for normal uptake of glucose into both human and mouse adipocytes.
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18 A possible mechanism for the reduction of glucose uptake in the SIK2 KO mouse model is the
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20 downregulation of GLUT4 mRNA and protein in SIK2 KO adipocytes, which we and others
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22 have reported previously (7, 8). This is because, in contrast to what was observed after acute
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24 pan-SIK inhibition, we only found a modest reduction in PKB/Akt signaling, at the lowest
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26 concentration of insulin, in SIK2 KO adipocytes. Interestingly, we have not observed any
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28 changes in the expression of GLUT4 after modulation of SIK isoforms in human adipocytes
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30 (5). Indeed, few genes have been shown to be regulated downstream of SIK(2) in human cells,
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32 although the transcriptional co-regulators CRTC3 and class II HDACs are clearly substrates of
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34 SIKs in these cells (5, 7).
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40 An important focus of our current study was to pin-point mechanisms whereby short term (one
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42 hour) inhibition of SIK isoforms affects glucose uptake and insulin signaling. Based on
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44 previous reports, suggesting that SIK2 can both interact with and phosphorylate IRS1 (at
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46 Ser794 in human, Ser789 in mouse/rat), leading to *impaired* insulin signaling (3, 12), we first
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48 focused our investigation on IRS1. We found a small reduction of the phosphorylation of IRS1
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50 at Tyr612 (Tyr608 in mouse/rat), which is needed for full activation of IRS1 (23) when
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52 inhibiting SIKs. However, none of the other regulatory sites that we analyzed (using phospho-
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54 specific antibodies) were consistently affected, including the suggested SIK2 site Ser794
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56 (Ser789 in mouse/rat) (3). A challenge when studying IRS phosphorylation is that multiple
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3 phosphorylation sites exist, both activating and inhibitory (20), and the functional outcome of
4 the different phosphorylations can be difficult to predict. Moreover, the technical quality is
5 dependent on the availability of good phospho-specific antibodies. Therefore, as a way to
6 assess the overall effect of SIK inhibition on IRS1, we analyzed the interaction of IRS1 with
7 the regulatory PI3K p85 α subunit, using co-immunoprecipitation, but again found no effect of
8 SIK inhibition that could explain the reduced PKB/Akt phosphorylation or glucose uptake.
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11 Although the interaction of p85 α with IRS is thought to be the main way in which the catalytic
12 p110 subunit of PI3K is activated, PI3K p85 α has also been suggested to be regulated by
13 phosphorylation of Ser154 – a site shown to be phosphorylated by SIK2 *in vitro* resulting in
14 PI3K activation (13). Studying this site was challenging as the phospho-specific antibody we
15 had access to only detected phosphorylation of exogenously expressed p85 α , and there was
16 large variation in the effect that SIK inhibition had on the phosphorylation level. However, we
17 did not find any link between the magnitude of Ser154 phosphorylation and that of SIK activity
18 (measured as pHDAC4) or PKB/Akt phosphorylation, arguing against Ser154 being a substrate
19 for SIK2 in adipocytes. However, we do not exclude the possibility that SIK2 directly or
20 indirectly regulates PI3K activity by other means, such as for example through phosphorylation
21 of other sites. The potential effect of SIK2 on IRS1 or PI3K phosphorylation in adipocytes
22 could be addressed in future studies, for example using *in vitro* phosphorylation and
23 phosphopeptide mapping. A critical outcome of the PI3K reaction is the translocation of
24 PKB/Akt to the plasma membrane in insulin-stimulated adipocytes (24), due to the generation
25 of PIP3. A striking result of our study was the requirement of SIK activity for the translocation
26 of the PKB/Akt PH domain to the plasma membrane. While the approach we used is powerful
27 in that it provides a relatively “pure” system for monitoring PIP3 levels and PH domain
28 translocation, in future studies it would also be interesting to confirm this effect using
29 alternative approaches and on endogenous PKB/Akt. Moreover, the underlying mechanisms,
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3 including the direct SIK target, remain to be identified. Future studies could include unbiased
4 phospho-proteomic analysis of adipocytes after SIK inhibition, or could focus on assessing
5 PI3K and PTEN activity. Identifying the contribution of different SIK isoforms to the effect of
6 pan-SIK inhibitors in human adipocytes is also important.
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12 **Conclusions**

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16 In summary, we conclude that SIK activity is required for normal glucose uptake in primary
17 human adipocytes. The mechanism is likely via direct effects on the insulin signaling
18 pathway at the level of PIP3 generation or breakdown. This conclusion is based on the
19 observation that one hour of SIK inhibition was sufficient to reduce the insulin-induced
20 translocation of a PKB/Akt PH domain to the plasma membrane as well as the
21 phosphorylation of PKB/Akt and AS160, while the interaction of IRS1 with PI3K p85 was
22 preserved. In mice, we observed that the specific loss of SIK2 resulted in reduced glucose
23 uptake, pointing to an important role for this isoform. All in all, our results support the
24 possibility that downregulation of SIK2 plays a role in the development of obesity-induced
25 insulin resistance.
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49 p85 α expression, respectively. Ahmed Ashour Ahmed is acknowledged for kindly sharing anti-
50 p-p85 α Ser154 antibody.
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Figure legends

Figure 1. Acute (one hour) inhibition of SIKs attenuates basal- and insulin-induced glucose uptake in primary human adipocytes.

(a-g) Primary human adipocytes were pre-treated with increasing doses of HG-9-91-01 **(a-d)** or YKL-05-099 **(e-g)** for 1 hour, and then stimulated with 0.1 nM insulin for 30 min. **(a-c, e-g)** Basal and insulin-stimulated uptake of ^{14}C -glucose **(a-c; n=5** individuals, BMI=25.7±4.5 kg/m², age=55.8±10.3 years, **e-g; n=5** individuals (for insulin-stimulated samples $n=4$ individuals), BMI=23.8±1.4 kg/m², age=50.8±2.9 years). **(a, e)** displays basal and insulin-stimulated glucose uptake (CPM values), and the induction of glucose uptake in response to insulin in the control cells was 2.0- **(a)** and 1.7-fold **(e)**, respectively. **(b-c, f-g)** display the uptake expressed as % of the control cells, basal and insulin-stimulated samples in separate panels. **(d)** Basal phosphorylation of HDAC4 at Ser246 ($n=3$ individuals, BMI=23.1±1.9 kg/m², age=58.7±10.0 years). Values for BMI and age presented as mean±SD. Statistical significance was determined by repeated measures two-way **(a, e)** or one-way **(b-d, f-g)** ANOVA. Differences between individual groups were determined with Holm-Šidák's multiple comparisons post test **(b-d, f-g)**. In order to assess the effect of YKL-05-099 alone, the reference group treated with 10 μM HG-9-91-01 was excluded from statistical analysis **(e)**.

Figure 2. Acute (one hour) inhibition of SIKs by HG-9-91-01 attenuates PKB/Akt signaling in adipocytes.

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3 **(a-f)** Primary human adipocytes were pre-treated for 1 hour with 10 μ M HG-9-91-01 , followed
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5 by stimulation with increasing doses of insulin for 10 min. Phosphorylation of AS160 at
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7 Thr642 **(a-b)**, $n=5$ individuals (for samples stimulated with 0.01 nM insulin $n=4$ individuals),
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9 BMI= 27.3 ± 2.3 kg/m², age= 57.6 ± 12.5 years), PKB/Akt at Thr308 **(c-d)** or Ser473 **(e-f)**, $n=6$
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11 individuals (for samples stimulated with 0.01 nM insulin $n=5$ individuals), BMI= 27.6 ± 2.2
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13 kg/m², age= 57.8 ± 11.2 years) determined by western blotting, and normalized to the total
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15 protein levels of each respective protein. **(a, c, e)** display the specific phosphorylation
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17 expressed as % of the control cells stimulated with 0.1 nM insulin. **(b, d, f)** display the specific
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19 phosphorylation in the HG-9-91-01-treated samples relative to the control cells (dotted line) at
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21 each concentration of insulin. Values for BMI and age presented as mean \pm SD. Statistical
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23 significance was determined by repeated measures two-way ANOVA **(a, c, e)** or one sample t-
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25 test **(b, d, f)**. In order to increase clarity, only the groups treated with 0.1 and 1 nM insulin were
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27 visualized in **(d)** and **(f)** as the PKB/Akt phosphorylations in the basal and 0.01 nM insulin
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29 were virtually zero.
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40 ***Figure 3. SIK inhibition does not affect the phosphorylation of IRS1 at Tyr612, Ser307,***
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42 ***Ser1101 or Ser789 (Ser794 in human).***
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46 **(a-d)** Primary human or rat **(e)** adipocytes were pre-treated for 1 hour with 10 μ M HG-9-91-
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48 01, followed by stimulation with increasing doses of insulin for 10 min. Phosphorylation of
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50 IRS1 at Tyr612 **(a-b)**, $n=6$ individuals (for basal samples $n=5$ and samples stimulated with 0.01
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52 nM insulin $n=4$ individuals), BMI= 27.6 ± 2.2 kg/m², age= 57.8 ± 11.2 years), Ser307 **(c)**, $n=5$
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54 individuals (for samples stimulated with 0.01 nM insulin $n=4$ individuals), BMI= 26.9 ± 1.6
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56 kg/m², age= 57.0 ± 12.3 years), Ser1101 **(d)**, $n=4$ individuals (for basal and samples stimulated
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3 with 0.01 nM insulin $n=3$ individuals), BMI= 27.1 ± 1.9 kg/m², age= 57.5 ± 14.2 years) and
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5 Ser789 (**e**, $n=5$ experiments) determined by western blotting, and normalized to loading control
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7 (**a, c-d**) or the total protein levels of IRS1 (**e**). (**a, c-e**) display the specific phosphorylation
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9 expressed as % of the control cells stimulated with 0.1 nM insulin. (**b**) displays the specific
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11 phosphorylation in the HG-9-91-01-treated samples relative to the control cells (dotted line) at
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13 each concentration of insulin. Values for BMI and age presented as mean \pm SD. Statistical
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15 significance was determined by two-way ANOVA (**a, c-e**) or one sample t-test (**b**).
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24 ***Figure 4. Preserved interaction between IRS1 and p85 after SIK inhibition.***

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27 Primary human adipocytes were pre-treated for 1 hour with 10 μ M HG-9-91-01 followed by
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29 stimulation with 0.1 or 1 nM insulin for 10 min. The interaction between IRS1 and p85 α was
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31 analyzed by co-immunoprecipitation and western blotting (**a**, $n=5-6$ individuals,
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33 BMI= 24.8 ± 3.0 kg/m², age= 52.8 ± 11.7 years). The effects of insulin and HG-9-91-01 on the
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35 phosphorylation of IRS1 at Tyr612 (**b**, $n=5$ individuals, BMI= 24.9 ± 3.3 kg/m², age= 56.0 ± 9.8
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37 years), HDAC4 at Ser246 (**c**, $n=6$ individuals, BMI= 24.8 ± 3.0 kg/m², age= 52.8 ± 11.7 years) and
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39 PKB/Akt at Ser473 (**d**, $n=6-7$ individuals, BMI= 25.0 ± 3.0 kg/m², age= 54.3 ± 9.6 years) was also
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41 monitored. Values for BMI and age presented as mean \pm SD. Statistical significance was
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43 determined by two-way ANOVA with Holm-Šidák's multiple comparisons post test (**a, b, d**)
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45 or one sample t-test (**c**).
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55 ***Figure 5. SIK inhibition does not affect the phosphorylation of PI3K p85 α at Ser154.***

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3 Primary rat adipocytes expressing GFP-p85 α were treated for 1 hour with 10 μ M HG-9-91-01.
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5 GFP-p85 α IPs, or whole-cell lysates were then analyzed by western blotting for the
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7 phosphorylation of p85 α on Ser154 (**a**, n=11 independent experiments), HDAC4 at Ser246 (**b**,
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9 n=11 independent experiments) and PKB/Akt at Ser473 (**c**, n=11 independent experiments).
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11 Signal intensities for the phosphorylation were normalized to the total protein levels of each
12
13 respective protein and expressed as % of the control cells. The association between SIK
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15 inhibition (measured as HDAC4 phosphorylation) and the phosphorylation of p85 α at Ser154
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17 (**d**) or PKB/Akt at Ser473 (**e**) was also monitored. Statistical significance was determined by
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19 one sample t-test (**a-c**) and Pearson correlation (**d-e**).
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29 ***Figure 6. Acute (one hour) inhibition of SIKs by HG-9-91-01 attenuates membrane***
30 ***translocation of PKB/Akt.***
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34 Primary human adipocytes expressing a GFP-fused PH domain from Akt/PKB (GFP-PKB-PH
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36 were pre-treated for 1 hour with 10 μ M HG-9-91-01 followed by stimulation with 1 nM insulin
37
38 for 10 min. Cells were fixed and the presence of GFP-PKB-PH at the membrane was analyzed
39
40 by TIRF microscopy (**a-c**). (**a**) displays the mean quantification of 30 cells per condition from
41
42 three individuals (BMI=23.3 \pm 2.4 kg/m² [mean \pm SD], age=58.7 \pm 3.8 years [mean \pm SD]). In
43
44 parallel, the phosphorylation of PKB/Akt at Ser473 was analyzed by immunofluorescence (**d-**
45
46 **e**, only insulin-stimulated samples shown in (**e**)). (**b-e**) display data from one representative
47
48 experiment. Statistical significance was determined by two-tailed paired (**a**) or unpaired (**c, e**)
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50 Student's *t*-test. Scale bar corresponds to 20 μ m.
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60 ***Figure 7. SIK2 and the regulation of insulin action in mouse adipocytes.***

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3 **(a)** The protein levels of SIK2 were analyzed by western blotting in white adipocyte lysates
4 from *ob/-* (n=6) or *ob/ob* (n=7) mice. SIK2 protein levels were normalized to HSP90. **(b-g)**
5
6 Primary mouse adipocytes (WT) were pre-treated for 1 hour with increasing doses of HG-9-
7 91-01 and then analyzed for basal and insulin-induced (15 min, 0.1 nM) uptake of ¹⁴C-glucose
8
9 **(b-d)**, phosphorylation of PKB/Akt at Thr308 **(e)** and Ser473, and **(f)** phosphorylation of
10 HDAC4 at Ser246 **(g)** (**b-g**, n=3 independent experiments). **(b)** displays basal and insulin-
11 stimulated glucose uptake (CPM values), and the induction of glucose uptake in response to
12 insulin in the control cells was 3.1-fold. **(c-d)** display the uptake expressed as % of the control
13 cells, basal and insulin-stimulated samples in separate panels. Phosphorylations were
14 normalized to the total protein levels of each respective protein. **(e-f)** display the specific
15 phosphorylation expressed as % of the control cells stimulated with 0.1 nM insulin. **(g)** display
16 the specific phosphorylation expressed as % of the control cells. **(h)** Primary mouse adipocytes
17 (expressing GFP-PKB-PH) were pre-treated for 1 hour with increasing doses of HG-9-91-01,
18 followed by 0.1 nM insulin for 10 min. The presence of GFP-PKB-PH at the membrane was
19 analyzed by TIRF microscopy (n=2 independent experiments, each in which 30 cells per
20 condition were quantified. One representative experiment is shown). **(i-k)** Basal and insulin-
21 stimulated uptake of ¹⁴C-glucose in primary adipocytes isolated from WT or SIK2 KO mice
22 (n=9 different cell suspensions/genotype (5 containing cells from males, 4 containing cells
23 from females), each prepared from 1-3 mice (in total 18 mice per genotype), which were age-
24 and gender-matched within each suspension and between genotypes). **(i)** displays basal and
25 insulin-stimulated glucose uptake (CPM values), and the induction of glucose uptake in
26 response to the different doses of insulin in WT cells was 1.4- and 2.4-fold. **(j, k)** display
27 glucose uptake in SIK2 KO cells relative to WT cells (dotted line) at each concentration of
28 insulin separated by age (mice <20 weeks in **(j)**; mice >20 weeks in **(k)**). Statistical significance
29 was determined by two-tailed unpaired Student's *t*-test **(a)**, repeated measures two-way **(b, e-**
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3 **f, i** or one-way **(c-d, g)** ANOVA, or ordinary two-way **(h)** ANOVA, or one sample *t*-test **(k)**.
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5 Differences between individual groups were determined with Holm-Šidák's multiple
6 comparisons post test **(c-d, g-h)**.
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Figure 1

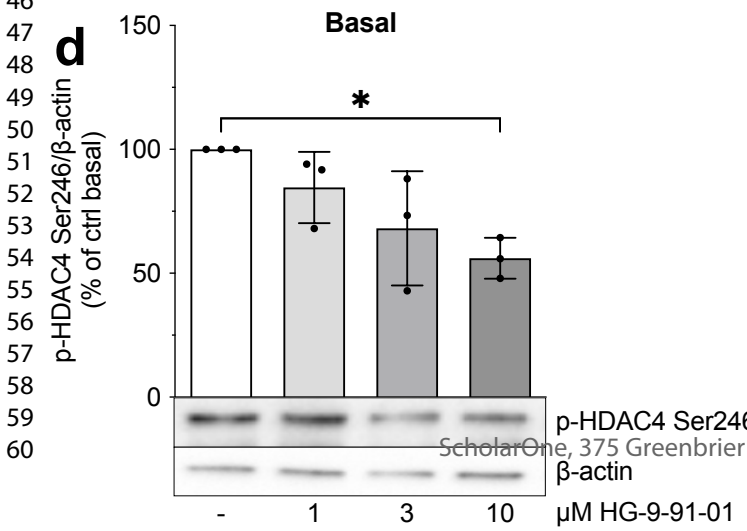
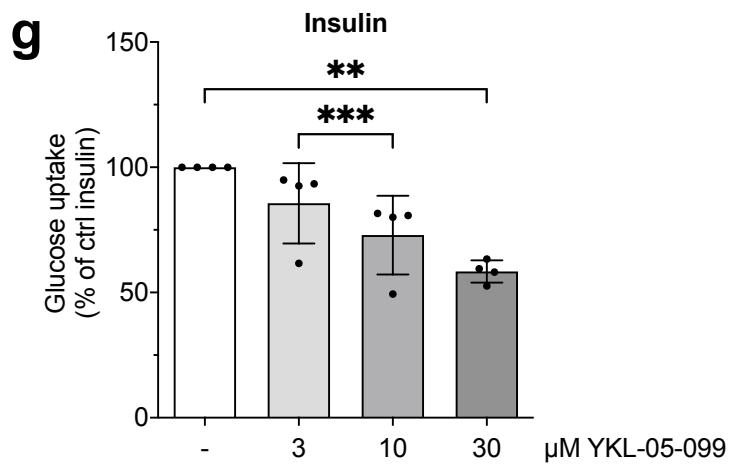
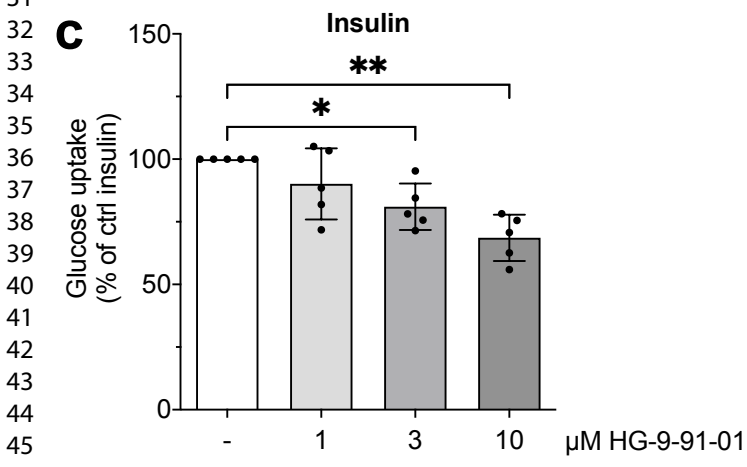
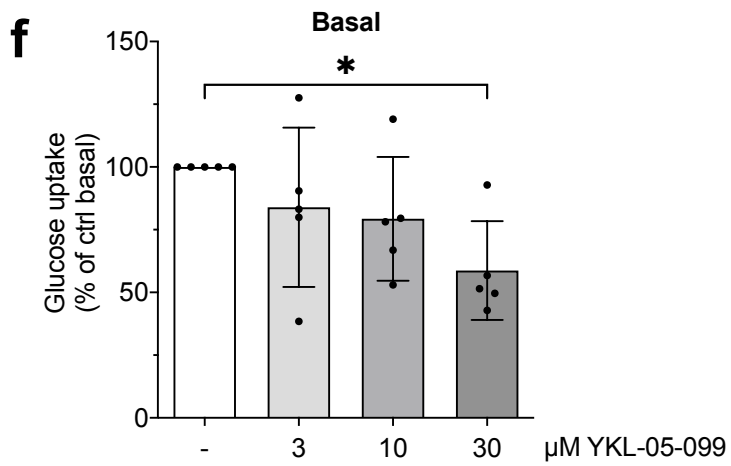
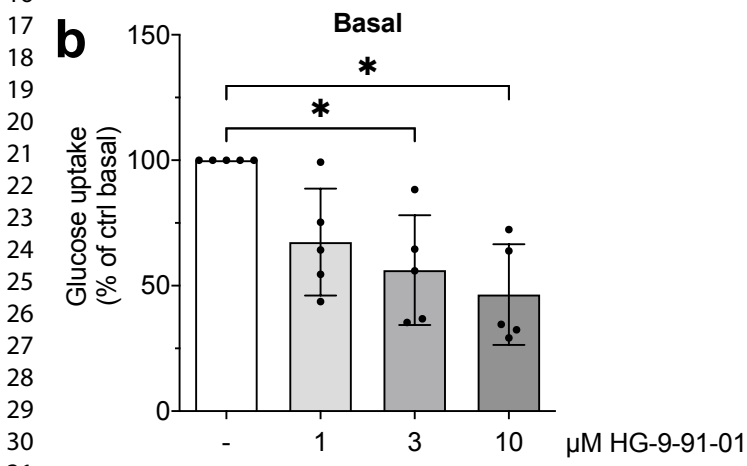
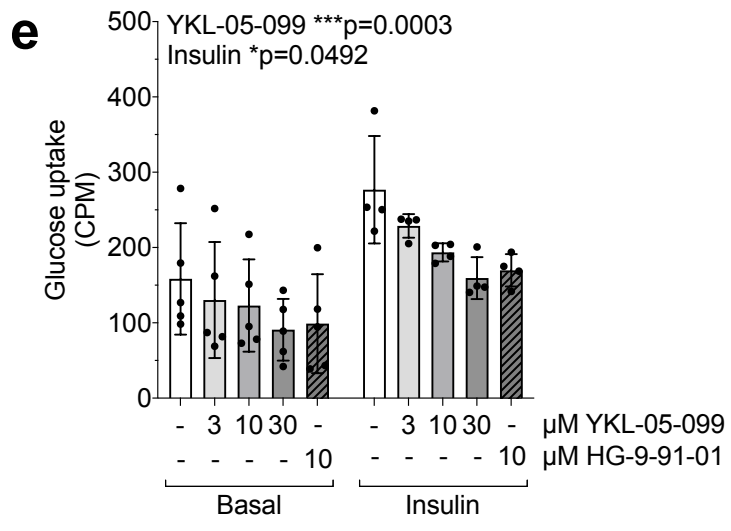
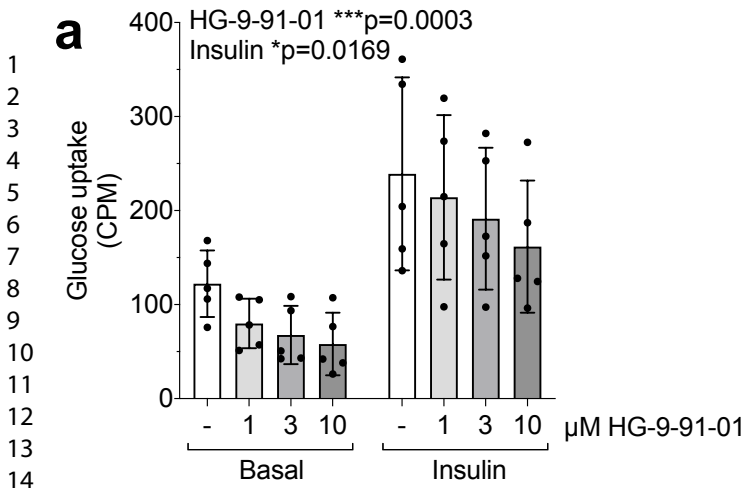
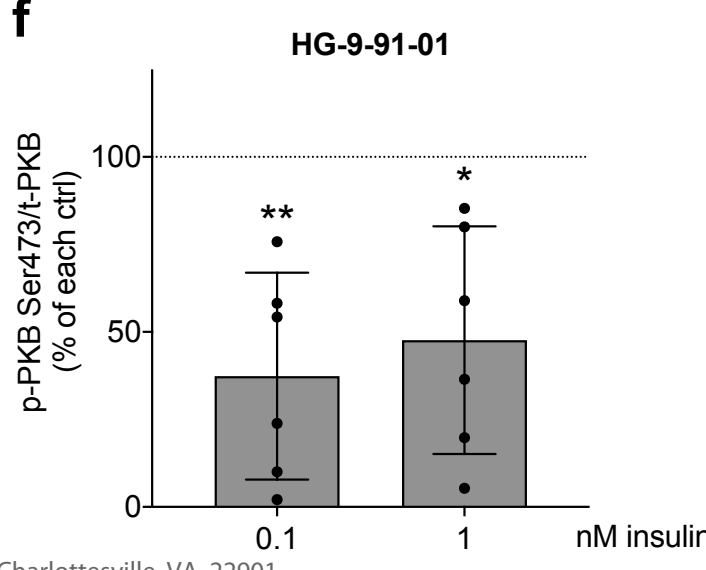
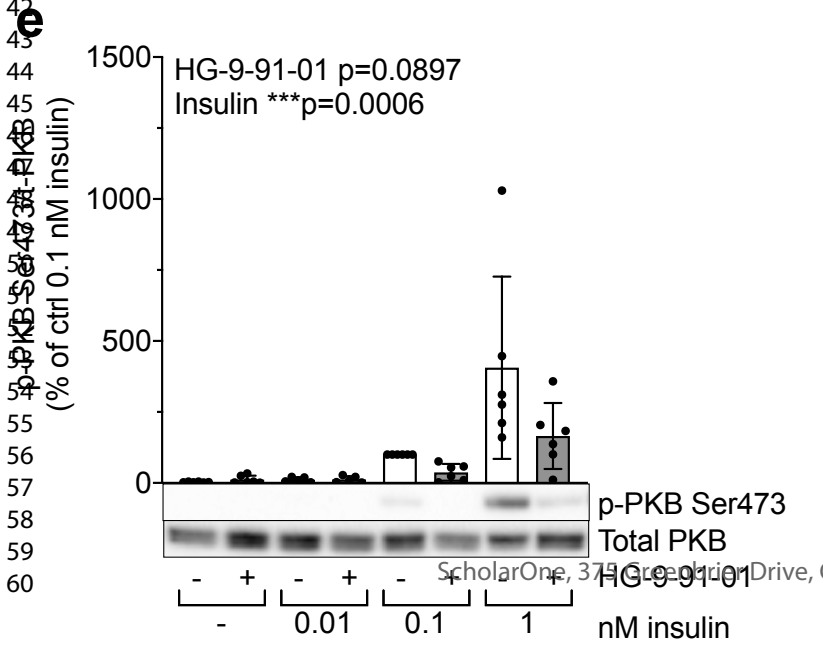
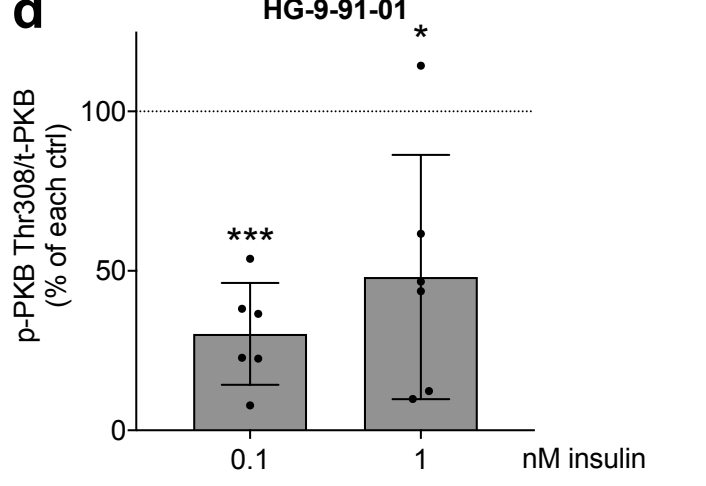
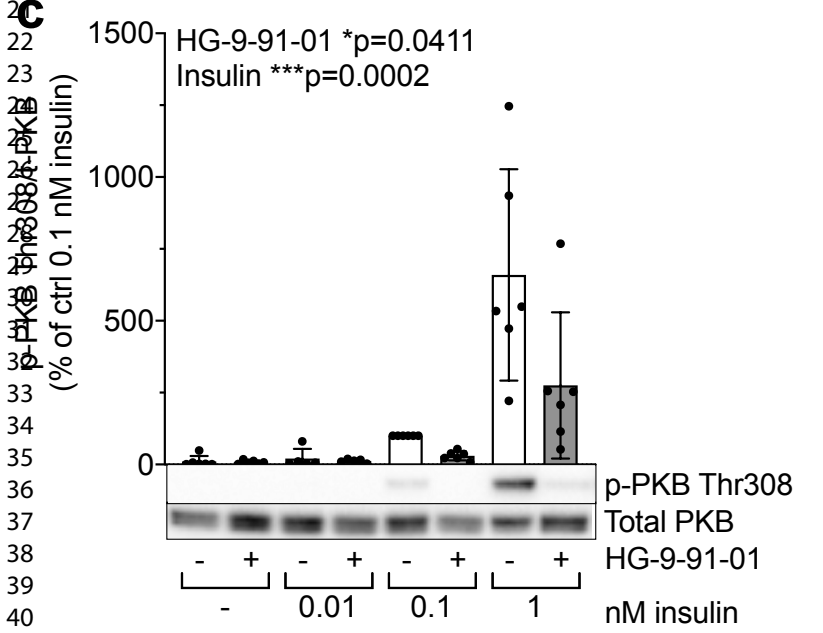
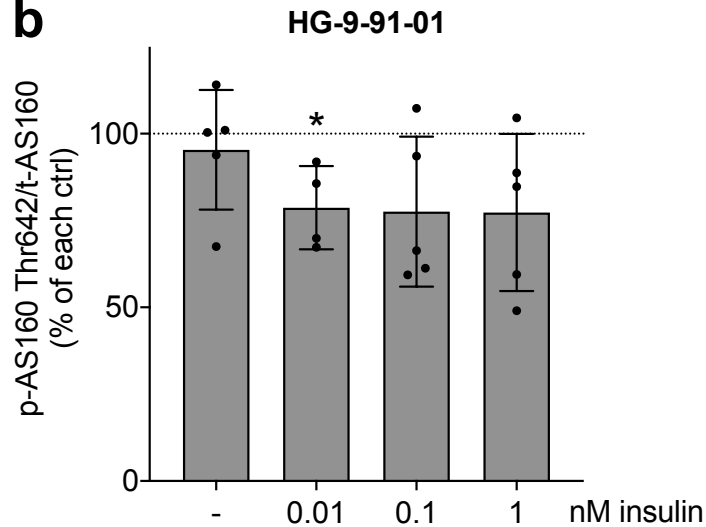
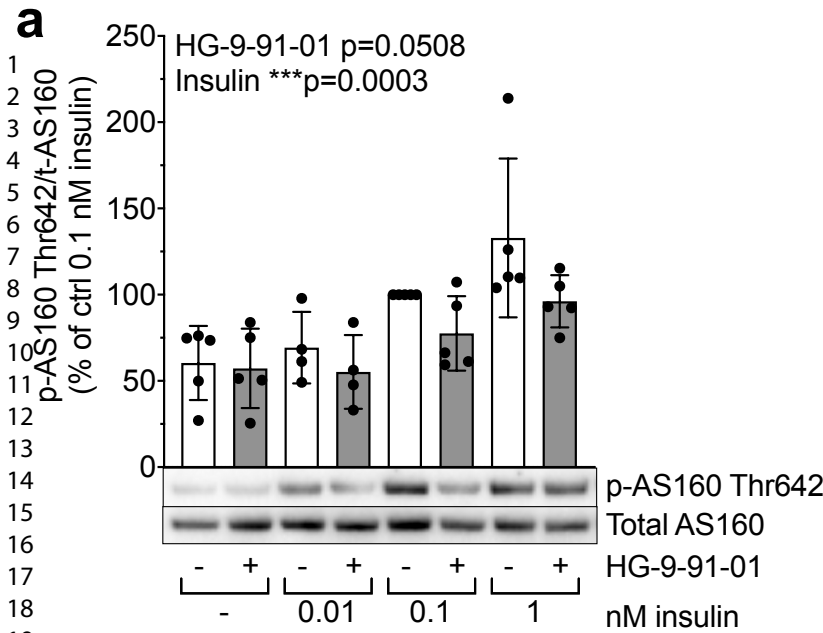
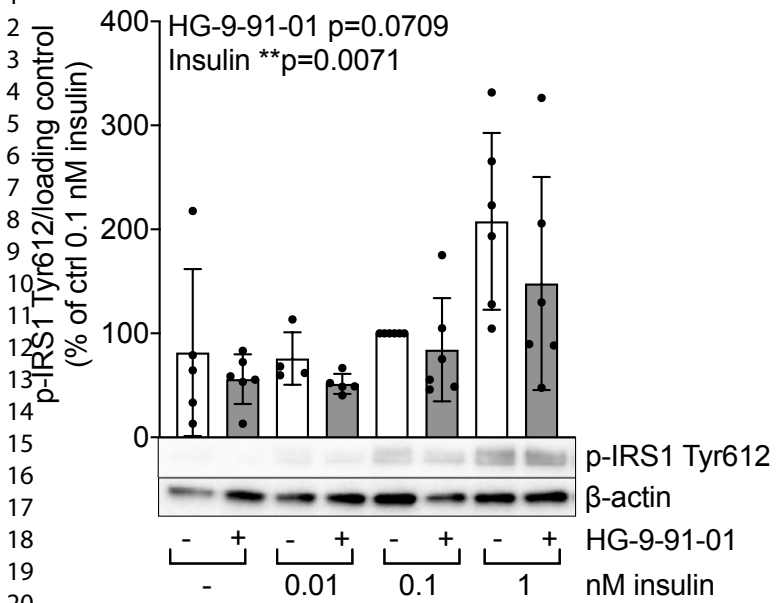


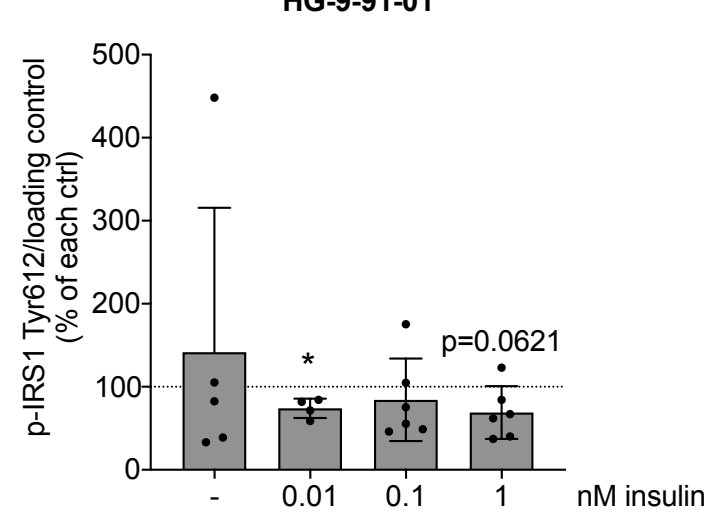
Figure 2



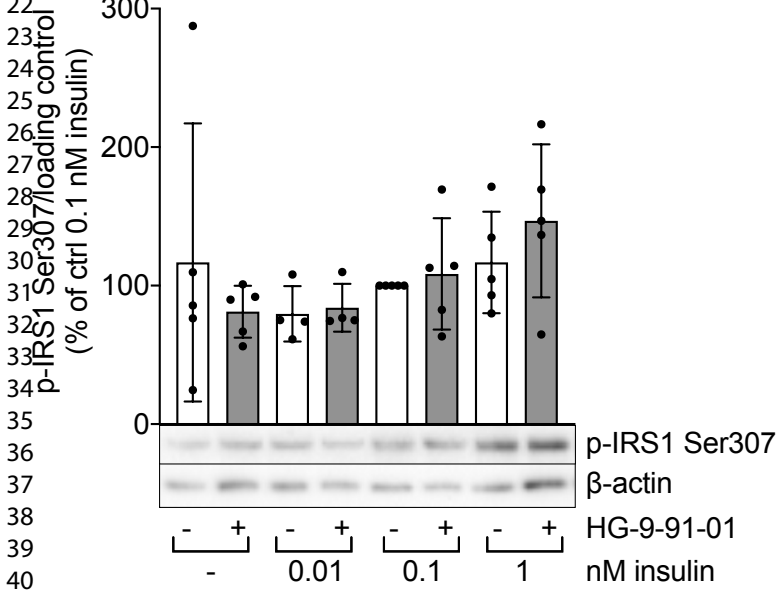
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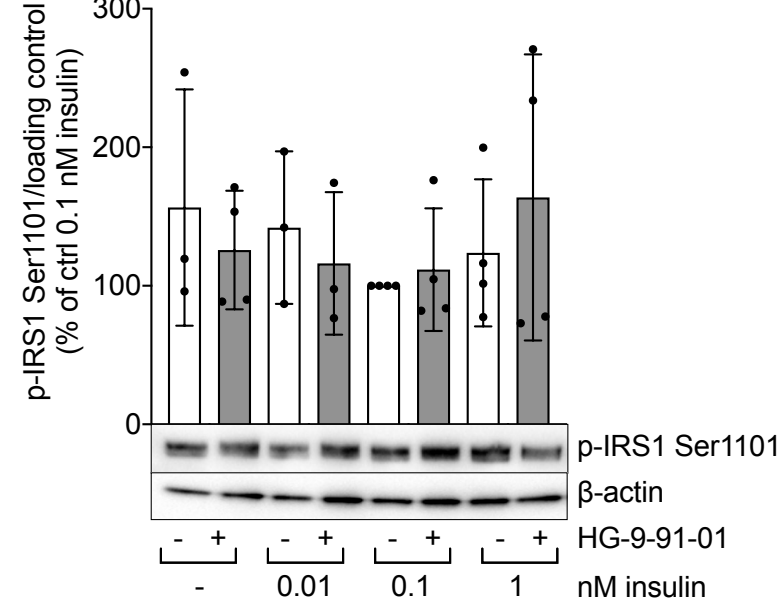
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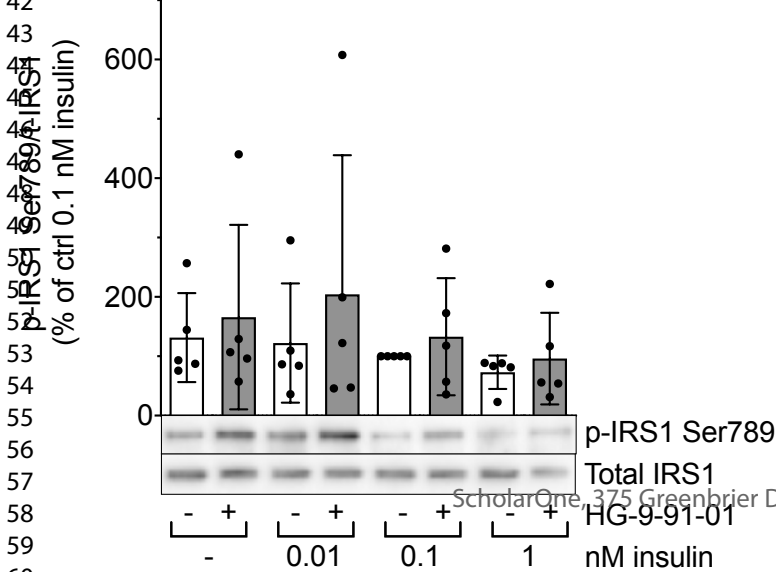
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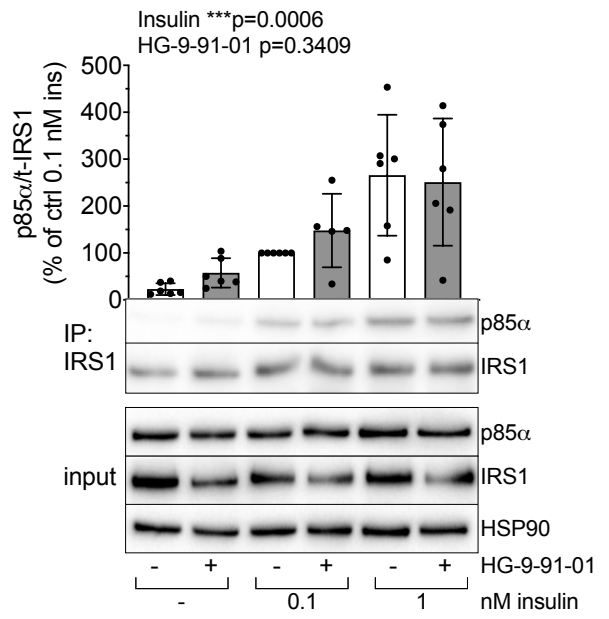


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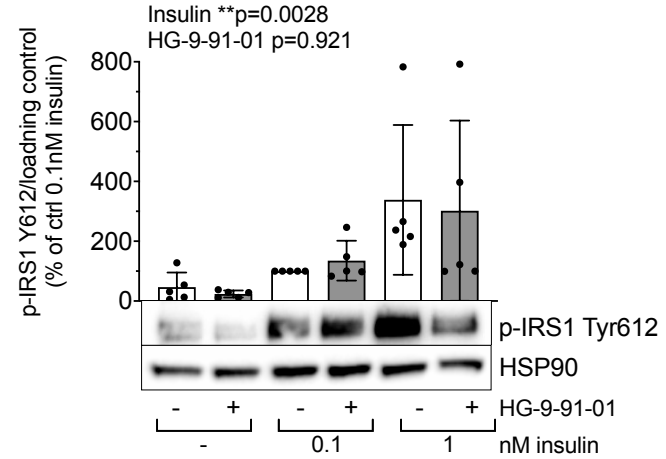


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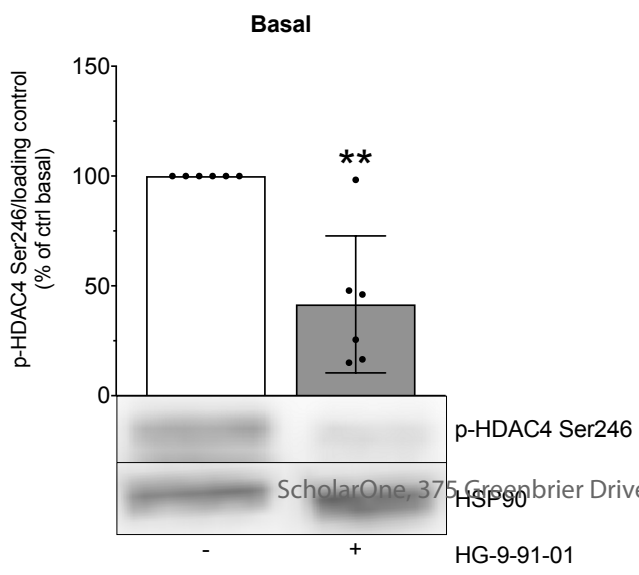
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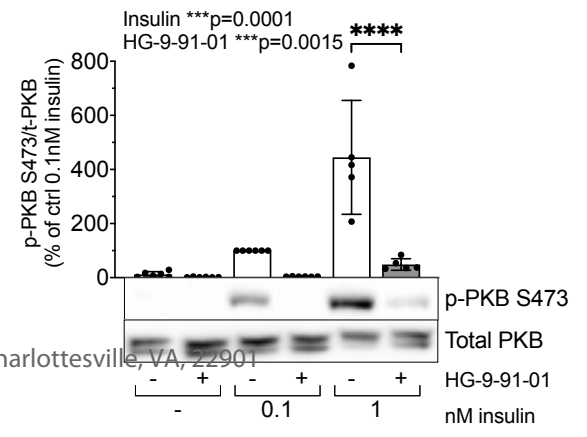
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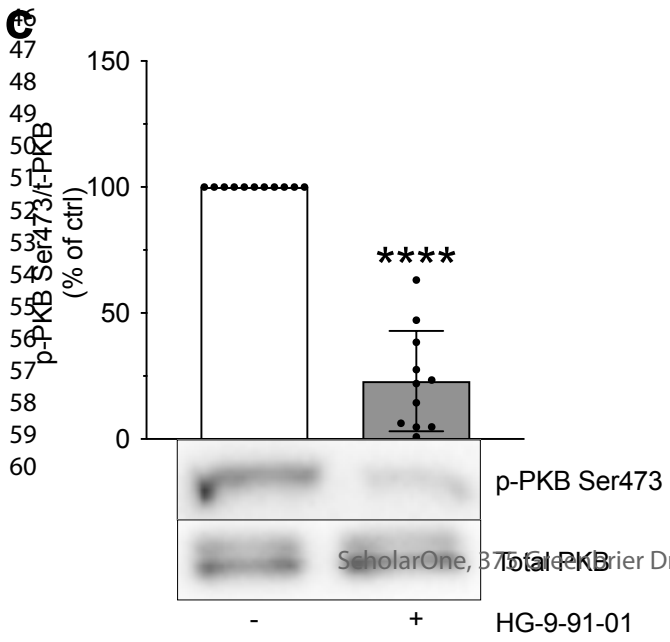
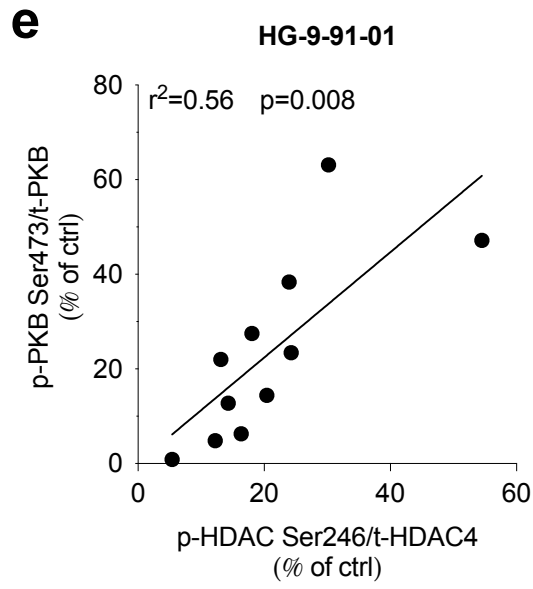
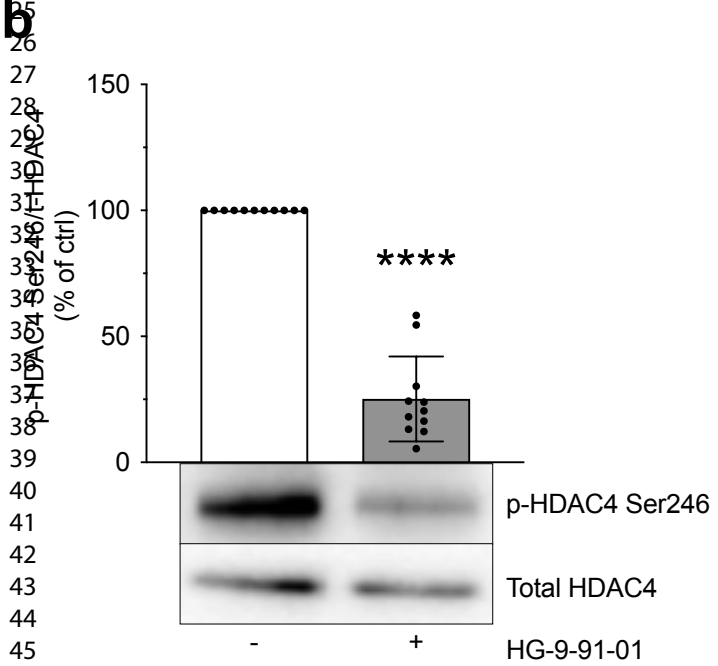
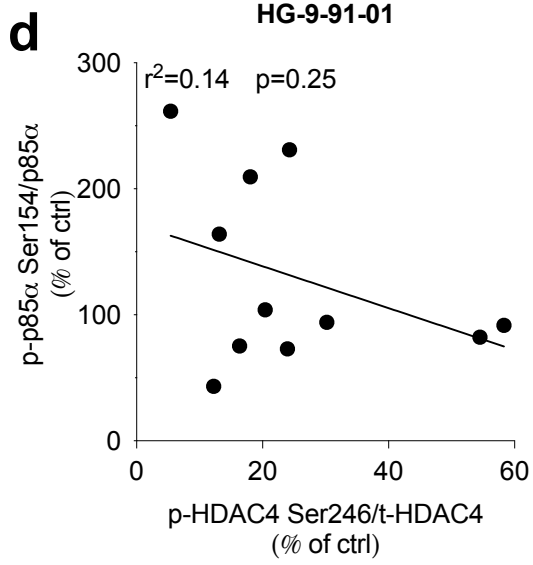
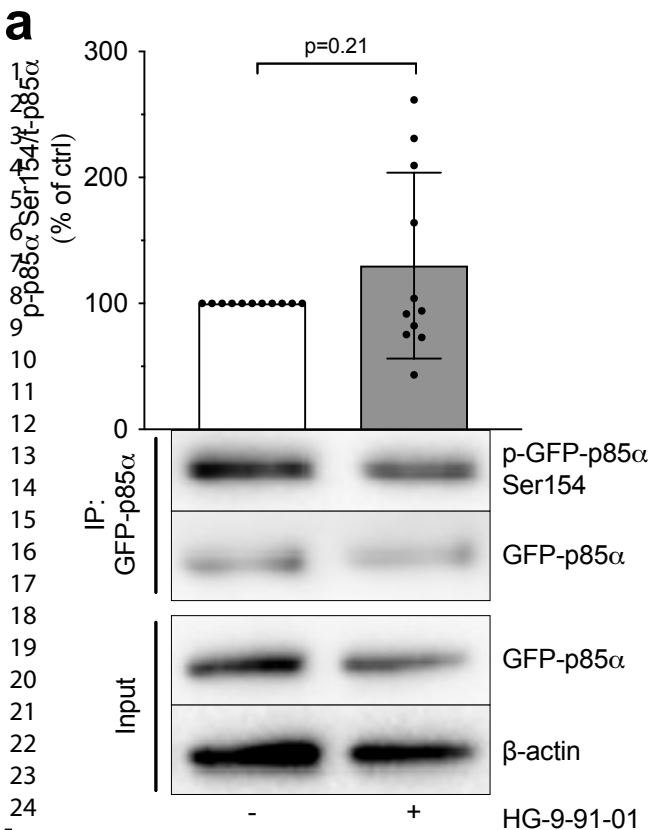


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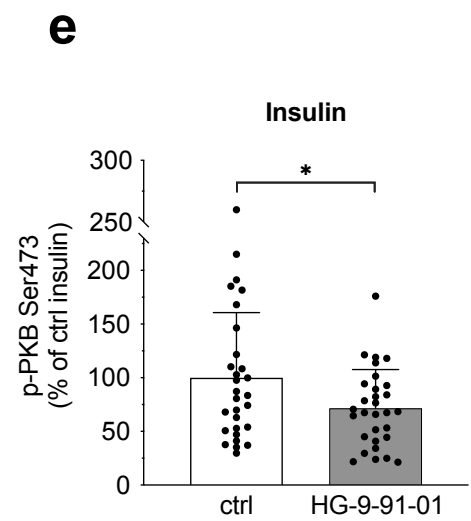
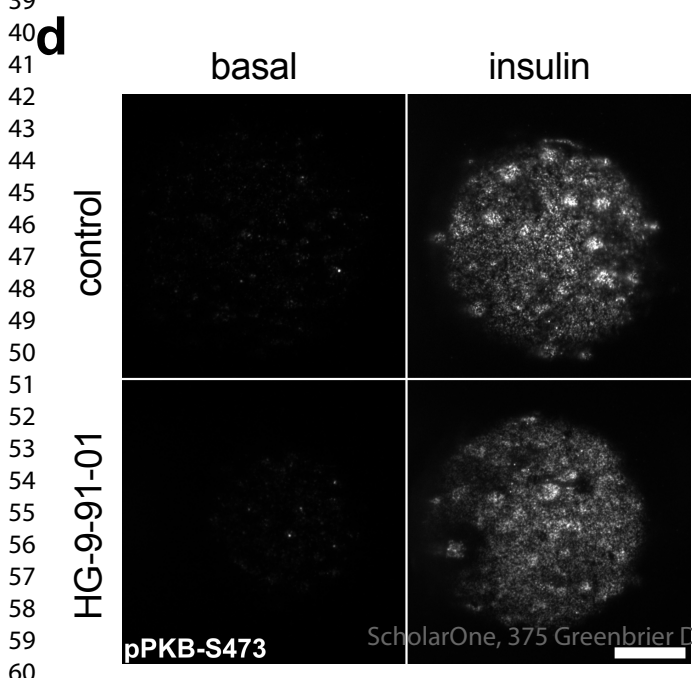
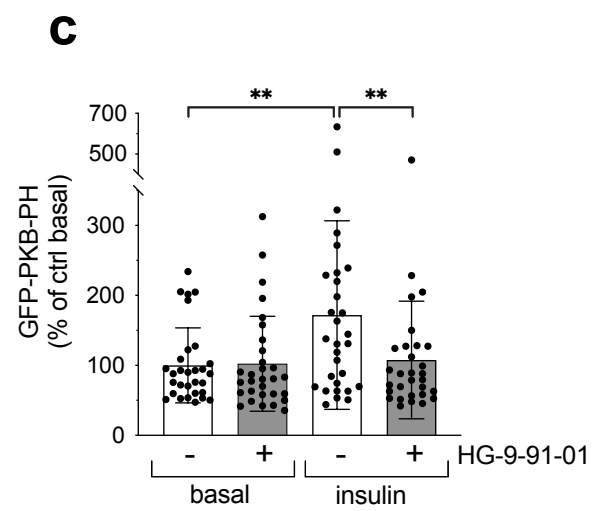
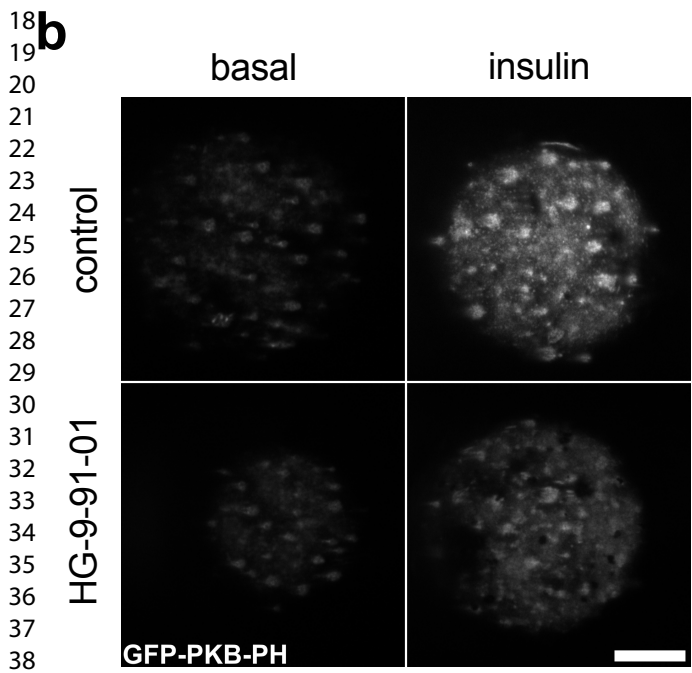
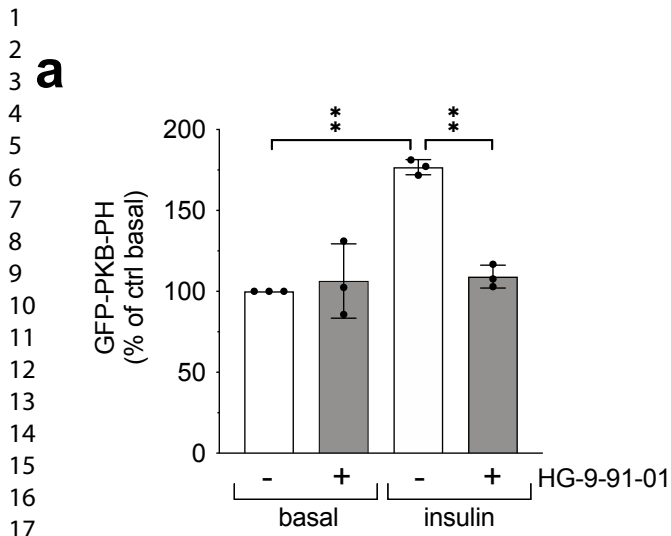
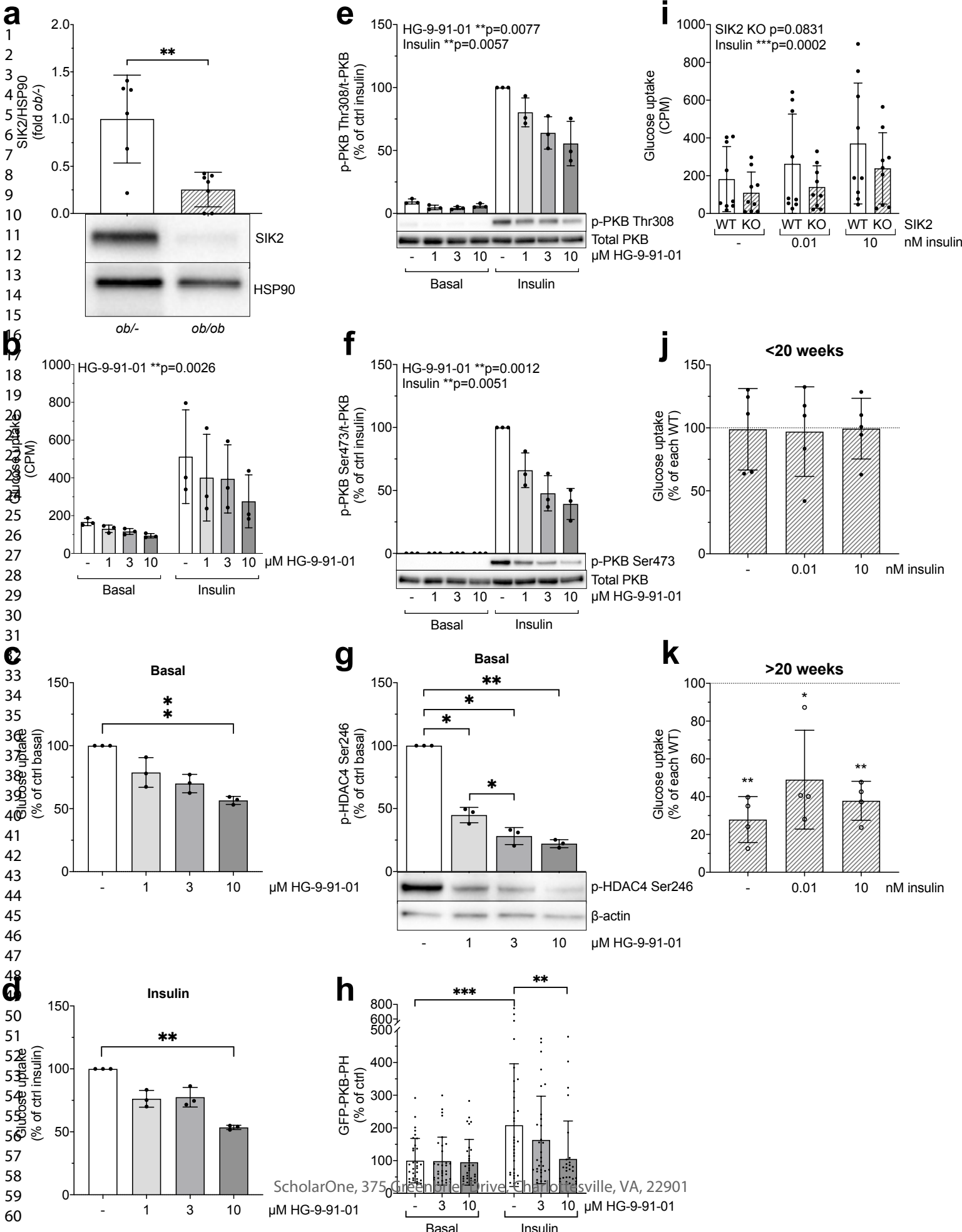


Figure 7



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3 **SUPPORTING INFORMATION to**
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7 **Salt-inducible kinases are required for glucose uptake and insulin signaling in human**
8 **adipocytes**
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12 Johanna Säll¹, Maria Lindahl¹, Andreas M. Fritzen², Claes Fryklund¹, Franziska Kopietz¹,

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14 Emma Nyberg¹, Anna Warvsten¹, Björn Morén¹, Marc Foretz³, Bente Kiens², Karin G.

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17 Stenkula¹, Olga Göransson^{1*}
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21
22 ¹Department of Experimental Medical Science, Lund University, SWEDEN.

23
24 ²The August Krogh Section for Molecular Physiology, Department of Nutrition, Exercise
25 and Sports, Faculty of Science, University of Copenhagen, DENMARK.

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27
28 ³Institut Cochin, INSERM, CNRS, Department of Endocrinology, Metabolism and Diabetes,
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30 Université Paris Cité, FRANCE.

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33 *Corresponding author
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Supplemental methods

Chemicals and reagents

The following reagents were used: complete protease inhibitor cocktail (Roche, Mannheim, Germany), ¹⁴C-glucose (Perkin Elmer NEC042x250UC), HG-9-91-01 (either kindly provided by Kristopher Clark, MRC Protein Phosphorylation Unit, University of Dundee (1), or purchased from MedChemExpress, Monmouth Junction, NJ, USA), YKL-05-099 (MedChemExpress), insulin (NovoRapid® (insulin aspart), Novo Nordisk, Bagsværd, Denmark), bovine serum albumin (BSA), cytochalasin B, Dulbecco's Modified Eagle's Medium (DMEM), gentamicin, PBS, phenylisopropyl adenosine (PIA) (all Sigma-Aldrich, St. Louis, MO, USA). All other standard chemicals were from Sigma-Aldrich. cDNA construct for the expression of GFP-PKB-PH (2) was kindly provided by Anders Tengholm (Uppsala University, Sweden). cDNA construct for GFP-p85 α (wild type [WT] and Ser154Ala mutant (3)) was kindly provided by Arne Ittner (Flinders University, AUS).

Definition of common buffers

To generate protein extracts for western blotting and immunoprecipitation (IP) experiments, cells were lysed in 50 mM TRIS-HCl (pH 7.5), 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM dithiothreitol, 1 % (wt./vol.) NP-40 and complete protease inhibitor cocktail (one tablet/50 ml) (lysis buffer).

Isolation and stimulation of primary adipocytes for generation of protein lysates was carried out in Krebs-Ringer medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄) containing 25 mM HEPES (pH 7.4), 200 nM adenosine, 2 mM glucose and 1% (wt./vol.; rat and human cells) or 3% (wt./vol.; mouse) BSA (KRH buffer).

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3 Glucose uptake assays were carried out in 30 mM HEPES (pH 7.4), 120 mM NaCl, 4 mM
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5 KH₂OPO₄, 1 mM MgSO₄, 0.75 mM CaCl₂, 10 mM NaHCO₃, 200 nM adenosine, and 1%
6
7 (wt./vol.; rat and human cells) or 3% (wt./vol.; mouse) BSA (KRBH buffer).
8
9

10 11 *Western blotting*

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14 Cell lysates (5-20 µg protein) or immunoprecipitates were analyzed by SDS-PAGE and
15
16 western blotting as described previously (4). Detection of primary antibodies was performed
17
18 using horseradish peroxidase (HRP)-conjugated secondary antibodies, and SuperSignal® West
19
20 Pico or Femto Chemiluminescent Substrates (Thermo Fisher Scientific, Waltham, MA, USA).
21
22 Luminescence signals were visualized in a ChemiDoc XRS+ (Bio-Rad, Hercules, C, USA) and
23
24 quantified by densitometry using the software Image Lab™ 5.1 (Bio-Rad). Signals were
25
26 normalized to the total levels of each respective protein, or to the loading controls β-actin or
27
28 HSP90.
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32 33 *Antibodies*

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37 The following primary antibodies were used for western blotting: mouse anti-β-actin (Sigma-
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39 Aldrich RRID:AB_476744, dilution 1:5000), rabbit anti-AS160 (Millipore RRID:AB_492639,
40
41 Billerica, MA, USA, dilution 1:1000), rabbit anti-p-AS160 Thr642 (Thermo Fisher Scientific
42
43 RRID:AB_2533564, dilution 1:1000), rabbit anti-p-HDAC4/5/7 Ser246/Ser259/Ser155 (CST
44
45 RRID:AB_2118723, dilution 1:1000), rabbit anti-IRS1 (CST RRID:AB_330333, dilution
46
47 1:1000), rabbit anti-p-IRS1 Ser307 (mouse/rat Ser302) (CST RRID:AB_330360, dilution
48
49 1:1000), rabbit anti-p-IRS1 Ser794 (mouse/rat Ser789) (CST RRID:AB_330353, dilution
50
51 1:1000), rabbit anti-p-IRS1 Ser1101 (mouse/rat Ser1100) (CST RRID:AB_330363, dilution
52
53 1:1000), rabbit anti-p-IRS1 Tyr612 (mouse/rat Tyr608) (Thermo Fisher Scientific
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55 RRID:AB_2533768, dilution 1:1000), rabbit anti-p85α (Abcam RRID:AB_2891324, dilution
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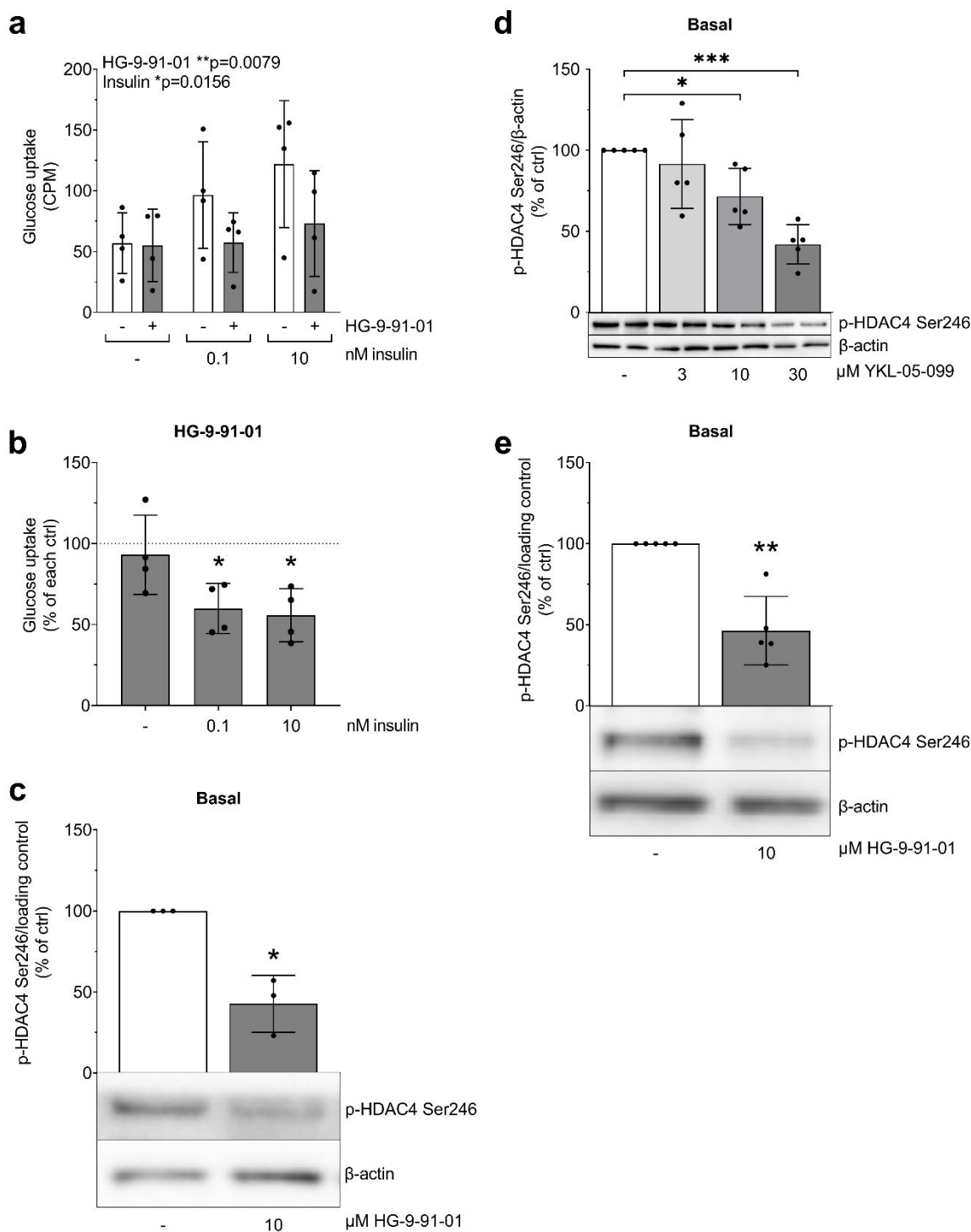
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3 1:1000), rabbit anti-PKB/Akt (pan) (CST RRID:AB_329827, dilution 1:1000), rabbit anti-p-
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5 PKB/Akt Thr308 (CST RRID:AB_329828, dilution 1:1000) and Ser473 (Thermo Fisher
6
7 Scientific RRID:AB_2533699, dilution 1:5000). The SIK2 antibody was raised in rabbit and
8
9 affinity-purified by Innovagen (Lund, Sweden, RRID:AB_2923162, dilution 0.5 µg/ml) (5).
10
11 The anti-p-p85α Ser154 antibody was kindly provided by Prof. Ahmed Ashour Ahmed
12
13 (University of Oxford, UK) (6). The following HRP-conjugated secondary antibodies were
14
15 used: anti-mouse (GE Healthcare RRID:AB_772210, dilution 1:2000) and anti-rabbit (Thermo
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17 Fisher Scientific RRID:AB_2534770, dilution 1:2500).
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Supplemental figures

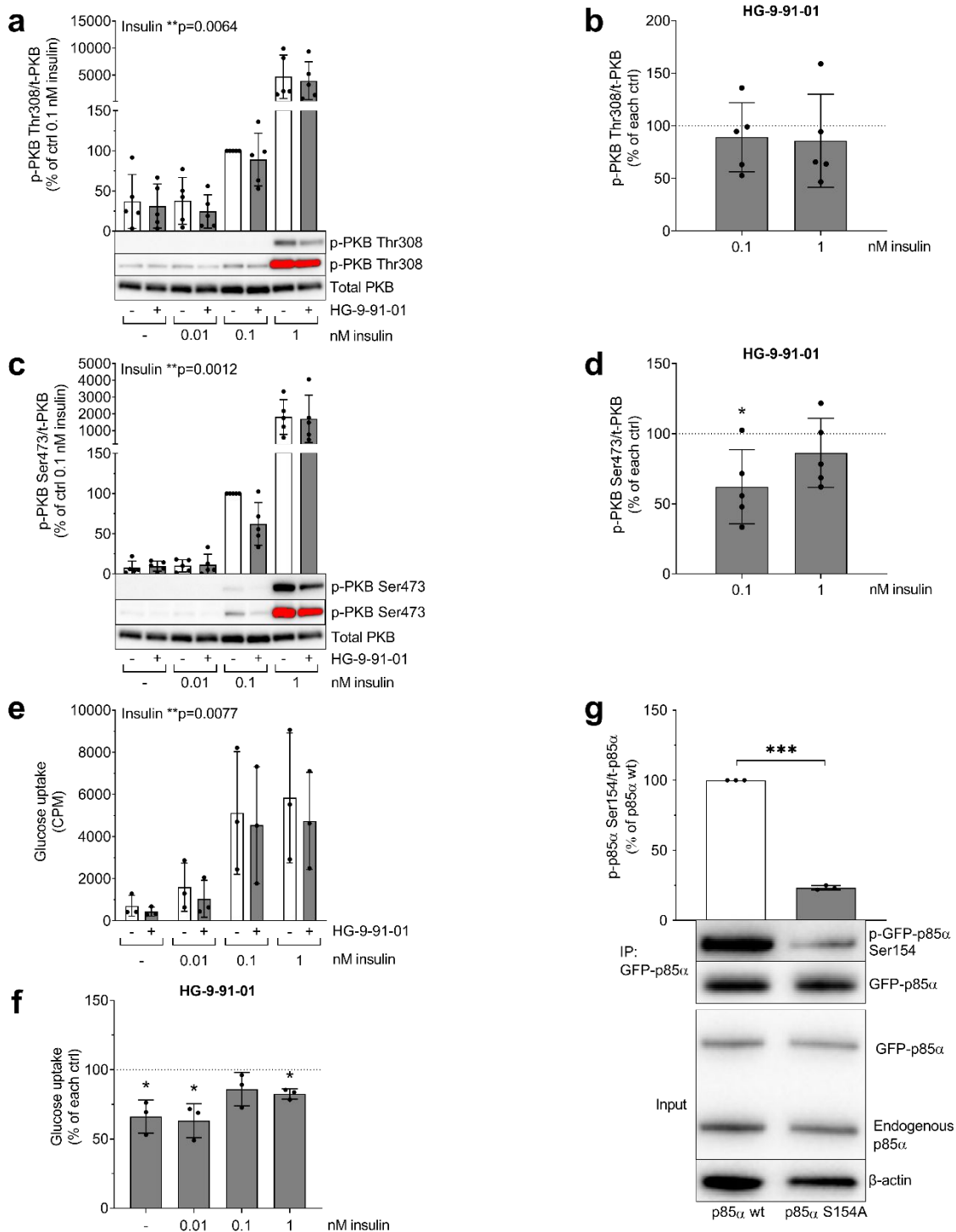
Figure S1



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3 **Figure S1. Effects of HG-9-91-01 and YKL-05-099 on insulin-induced glucose uptake and**
4 **the phosphorylation of HDAC4.**
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9 Primary human **(a-c)** or rat **(d-e)** adipocytes were pre-treated with 10 μ M HG-9-91-01 or
10 increasing doses YKL-05-099 for 1 hour. **(a-b)** Basal and insulin-stimulated uptake of 14 C-
11 glucose in adipocytes from $n=4$ individuals (BMI= 27.3 ± 1.7 kg/m 2 , age= 60.3 ± 5.1 years). **(a)**
12 displays basal and insulin-stimulated glucose uptake (CPM values), and the induction of
13 glucose uptake in response to the different doses of insulin in the control cells was 1.9- and
14 2.2-fold, respectively. **(b)** displays the glucose uptake relative to the control cells (dotted line)
15 at each concentration of insulin. **(c-e)** Basal phosphorylation of HDAC4 at Ser246 in primary
16 human **(c, n=3** individuals, BMI= 26.9 ± 2.3 kg/m 2 , age= 54.0 ± 15.1 years) or rat **(d-e, n=5**
17 independent experiments) adipocytes. Phosphorylation was normalized to loading control and
18 expressed as % of control cells. Values for BMI and age presented as mean \pm SD. Statistical
19 significance was determined by repeated measures two-way **(a)** or one-way **(b)** ANOVA, or
20 one sample t-test **(b, c, e)**. Differences between individual groups were determined with Holm-
21 Šidák's multiple comparisons post test **(d)**.
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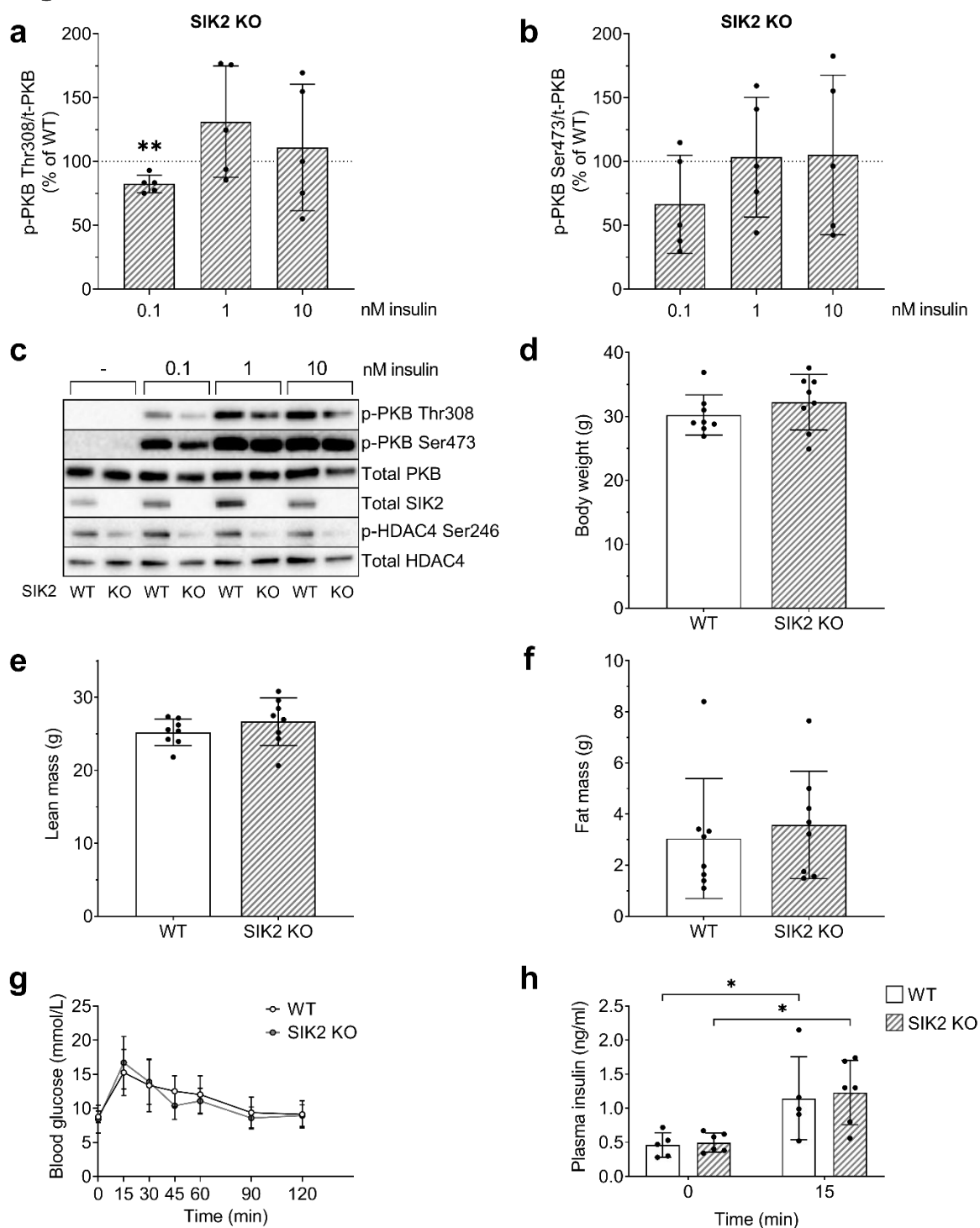
Figure S2



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3 **Figure S2. Acute (one hour) inhibition of SIKs by HG-9-91-01 mildly attenuates insulin-**
4 **induced glucose uptake and phosphorylation of PKB/Akt in primary rat adipocytes.**
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9 **(a-f)** Primary rat adipocytes were pre-treated for 1 hour with 10 μ M HG-9-91-01, followed by
10 stimulation with increasing doses of insulin for 10 min. Phosphorylation of PKB/Akt at Thr308
11 **(a-b)** or Ser473 **(c-d)** analyzed by western blotting ($n=5$ independent experiments).
12 Phosphorylations were normalized to the total protein levels of PKB/Akt. **(b, d)** display the
13 specific phosphorylation in the HG-9-91-01-treated cells relative to the control cells (dotted
14 line) at each concentration of insulin. In order to increase clarity, only the groups treated with
15 0.1 and 1 nM insulin were visualized in **(b)** and **(d)** as the PKB/Akt phosphorylations in the
16 basal and 0.01 nM insulin were virtually zero. **(e-f)** Basal and insulin-stimulated uptake of 14 C-
17 glucose ($n=3$ independent experiments). **(e)** displays basal and insulin-stimulated glucose
18 uptake (CPM values), and the induction in response to the different doses of insulin in the
19 control cells was 2.4-, 8.1- and 9.4-fold, respectively. **(f)** displays glucose uptake in the HG-
20 91-01-treated cells relative to the control cells (dotted line) at each concentration of insulin. **(g)**
21 Primary rat adipocytes were electroporated with cDNA encoding WT or S154A GFP-p85 α ,
22 and the expression and phosphorylation of p85 α was studied in GFP-IPs and in lysates by
23 western blotting ($n=3$ independent experiments). Statistical significance was determined by
24 repeated measures two-way ANOVA **(a, c, e)**, or one sample t-test **(b, d, f-g)**.
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Figure S3



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3 **Figure S3. Modest reduction of pPKB/Akt but no differences in body weight or glucose**
4 **tolerance between WT and SIK2 KO mice.**
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9 Phosphorylation of PKB/Akt at Thr308 **(a)** and Ser473 **(b)** in primary mouse adipocytes
10 isolated from male WT or SIK2 KO mice ($n=5$ different cell suspensions/genotype, each
11 prepared from 2-3 male mice, which were age-matched within each suspension and between
12 genotypes). Phosphorylations were normalized to the total protein levels of PKB/Akt and
13 expressed as % of WT cells. **(a-b)** display the specific phosphorylation in the SIK2 KO cells
14 relative to the WT cells (dotted line) at each concentration of insulin. **(c)** Representative
15 western blots from a matched WT and SIK2 KO mouse pair. Body weight **(d)**, lean mass **(e)**
16 and fat mass **(f)** measured by MR-scanning in 12–16-week-old male WT or SIK2 KO mice
17 ($n=8$ mice/genotype). **(g-h)** Blood glucose levels following injections of glucose (2 g/kg body
18 weight) dissolved in saline during a glucose tolerance test (GTT) **(g)** and plasma insulin levels
19 before and 15 min post-injection of glucose **(h)** in 12-16 week old male WT or SIK2 KO mice
20 ($n=6$ mice/genotype). Statistical significance was determined by one sample t-test **(a, b)** or
21 two-way ANOVA with Holm-Šidák's multiple comparisons post test **(h)**.
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