## ONLINE APPENDIX - SUPPLEMENTAL INFORMATION

# G PROTEIN-COUPLED RECEPTOR KINASE 2 (GRK2) PLAYS A RELEVANT ROLE IN INSULIN RESISTANCE AND OBESITY

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## SUPPLEMENTAL RESEARCH DESIGN AND METHODS

### Materials

LipofectAMINE™, culture media and sera were from Invitrogen (Paisley, UK). Autoradiographic films and 2-deoxy-D[1-$^3$H]-glucose (11.0 Ci/mmol) were from GE Healthcare (Rainham, UK). Insulin and anti-®-actin antibody were from Sigma-Aldrich (St. Louis, MO). Antibodies against total and phosphorylated AKT (Ser473) and ERK1/2(Thr202/Tyr204) were from Cell Signaling (Beverly, MA. IRS1 and PIRS1( Tyr612/608) antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Antibody against GRK2 (sc-562) was from Santa Cruz (Palo Alto, CA). TNF⟨ was from PeproTech, Inc. (Rocky Hill, NY). The plasmids pcDNA3-hygro, pcDNA3- GRK2-neo and the adenoviral ad-shGRK2-RNAi construct were previously used in our laboratory (1). All other reagents used were of the purest grade available.

### Magnetic Nuclear Resonance Imaging (MNRI)

Wt and GRK$^{+/-}$ 9 month-old male mice were anaesthetized with ECG and respiration continuously monitored and fat was measured by MNRI. The coil was positioned over epididymal fat in the abdomen. MNRI measurements were performed using a Bruker BIOSPEC 47/40 spectrometer (Bruker GmBH, Ettlingen, Germany) operating at 4.7 Tesla (200MHz) superconducting magnet (Oxford Instruments Ltd., Oxford, UK) and high-performance actively shielded gradients with a maximum gradient strength of 50 mT/m. Data were collected as 256×128 matrices using the standard Bruker RARE_MOD (Fast spin-echo) sequence, which yields $T_2$-weighted images.

### Animal Protocols

Experiments were performed on Wt and hemizygous-GRK2 (GRK2$^{+/-}$) mice maintained on the hybrid 129/SvJ C57BL/6 background. The animals were bred and housed on a 12-hour light/dark cycle with free access to food and water. At 4 weeks of age, mice were continued on a standard chow diet (2018S Harlan-Teklad, containing 18% protein and 5% fat with an energy density of 3.4 Kcal/g) or fed a HFD (93975 Harlan-Teklad, containing 21.2% protein and 54.8% fat and an

energy density of 4.8 Kcal/g) and were monitored for 12 weeks. Body weight and food intake were measured daily and weekly, respectively. All animal experimentation described in this study was conducted in accordance with accepted standards of human animal care and approved by the University Committee.

A group of Wt and GRK2+/- 12-14 week-old male mice were treated for 48h with TNF⟨ (ip. 0.1 ⌠g/g body weight) or vehicle (100 ⌠l PBS-0.1% BSA), as we previously described (2). At the end of treatments, GTTs and ITTs were determined. Animals were treated or not for 15 min with insulin (1 IU/Kg body weight) and tissue samples (epididymal, retroperitoneal and inguinal adipose tissue; skeletal muscle and liver) were removed. Tissue samples were immediately weighted and frozen in liquid nitrogen and stored at -80°C (2).

## Adipocyte Size Determination

Adipose tissues collected from Wt or GRK2+/- 9-month-old mice, and mice fed a chow or a HF diet were prepared for morphological analysis. Samples were fixed in 4% paraformaldehyde for 24h, cut into small pieces and embedded in paraffin for histological analysis. The samples were cut by microtome (5 μm thick), mounted on Dpolylisinated glass slides and stained with haematoxylin and eosin for the evaluation of adipocyte size. For each experimental group, five sections per animal were stained.

Digital images of adipose tissue sections were captured using a light microscope (Olympus, Germany) at 10X magnification. For each group, three fields from each of five different haematoxylin-eosin stained sections per animal were analyzed. Individual adipocyte areas within each field were determined using image analysis software (ImageJ). Relative adipocyte size was calculated in arbitrary fields, by quantitation of twenty cells in at least three different randomly chosen fields per mouse.

## Subjects

According to institutional guidelines, subjects were aware of the research nature of the study and agreed to participate. The study was carried out in accordance with the Helsinki Declaration, and the Ethical Committee of the University Clinic of Navarra approved the protocol. The studied population was selected from unrelated individuals consecutively referred to University Clinic of Navarra for routine medical work-up after a 12-hour overnight fast, and consisted of 25 Metabolic Syndrome (MetS) patients and 10 age-matched healthy control individuals. The diagnosis of MetS was established when 3 or more of the AHA/NHLBI criteria for defining MetS (3) were present. Central obesity (waist circumference ε102 cm in men and ε88 cm in women); hypertriglyceridemia (triglycerides ε1.7 mM or use of treatment to reduce triglycerides); low HDL cholesterol (HDL cholesterol <1.03 mM in men and <1.3 mM in women or use of treatment to increase HDL cholesterol); high blood pressure (systolic blood pressure (SBP) ε130 mm Hg, diastolic blood pressure (DBP) ε85 mm Hg or use of antihypertensive medication); high fasting glucose (glucose ε5.55 mM or use of treatment to reduce glucose). In all subjects, absence of history of coronary disease, stroke, or peripheral arterial disease was recorded; additional exclusion criteria were diabetes (those with glucose levels >125 mg/dL o under anti-diabetic treatment), the presence of severely impaired renal function, arthritis, connective tissue diseases, alcohol abuse, or use of non-steroidal, anti-inflammatory drugs in the 2 weeks before entering the study. Clinical screenings were based on medical history, physical examination and routine analytical tests. The homeostasis model assessment (HOMA) index (fasting glucose [mM]×fasting insulin [μU/mL]/22.5) was used as an index of insulin sensitivity.

## Data analysis

Results are means ± S.E. from 4 to 10 independent experiments. Statistical significance was tested with the unpaired Student's t test or with the one-way ANOVA followed by the protected least-significant different test. Differences were considered statistically significant when P values <0.05. Differences in the demographic and clinical characteristics and in the GRK2 expression between control individuals and metabolic syndrome patients were assessed by Student's t test or Mann-Whitney´s U tests.

Statistical significance was established as $P<0.05$.

## Supplemental Reference List

1. Penela,P, Ribas,C, Aymerich,I, Eijkelkamp,N, Barreiro,O, Heijnen,CJ, Kavelaars,A, Sanchez-Madrid,F, Mayor,F, Jr.: G protein-coupled receptor kinase 2 positively regulates epithelial cell migration. *EMBO J* 27:1206-1218, 2008

2. Nieto-Vazquez,I, Fernandez-Veledo,S, de Alvaro,C, Rondinone,CM, Valverde,AM, Lorenzo,M: Protein-Tyrosine Phosphatase 1B-Deficient Myocytes Show Increased Insulin Sensitivity and Protection Against Tumor Necrosis Factor-{alpha}-Induced Insulin Resistance. *Diabetes* 56:404-413, 2007

3. Grundy,SM, Cleeman,JI, Daniels,SR, Donato,KA, Eckel,RH, Franklin,BA, Gordon,DJ, Krauss,RM, Savage,PJ, Smith,SC, Jr., Spertus,JA, Costa,F: Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 112:2735-2752, 2005

Supplementary Table 1. Quantitative analysis of GRK2 levels and phosphorylated *vs* total AKT protein expression in eWAT, liver and muscle from Wt and GRK2+/- 3 and 9-month old male mice, fed on a HFD for 12 weeks or adult male mice treated with TNFα as in Fig. 4 are shown. Results were expressed as percentage of stimulation over 3-month old Wt mice. Differences between Wt *vs* GRK2+/- mice are expressed by *, differences between 3- month-old mice *vs* 9-month-old mice, fed on a HFD or treated with TNFα are expressed by §, and differences between control *vs* insulin are expressed by ‡. *, §, ‡ p<0.05

| | | 3 months | | | | 9 months | | | | HFD | | | | TNFα | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Wt | | GRK2+/- | | Wt | | GRK2+/- | | Wt | | GRK2+/- | | Wt | | GRK2+/- | |
| | | Crtl | Ins | Crtl | Ins | Crtl | Ins | Crtl | Ins | Crtl | Ins | Crtl | Ins | TNFα | TNFα+ins | TNFα | TNFα+ins |
| **GRK2** | Adipose | 100 ± 6.2 | | 44 ± 6.9* | | 133 ± 9.1§ | | 70 ± 5.9*§ | | 184 ± 10§ | | 56 ± 6.1* | | 182 ± 25§ | | 74 ± 12*§ | |
| | Liver | 100 ± 3.7 | - | 60 ± 7.4* | - | 94 ± 3.6 | - | 53 ± 3.7* | - | 126 ± 9§ | - | 67 ± 3.0* | - | 93 ± 12 | - | 72 ± 11* | - |
| | Muscle | 100 ± 1.0 | | 68 ± 2.5* | | 168 ± 17.2§ | | 71 ± 2.2* | | 163 ± 15§ | | 67 ± 2.2* | | 142 ± 21§ | | 66 ± 2* | |
| **P-AKT** | Adipose | 100 ± 1 | 385 ± 82‡ | 63 ± 10 | 397 ± 78‡ | 204 ± 41 | 180 ± 31 | 65 ± 11 | 537 ± 120‡ | 150 ± 32 | 250 ± 48‡ | 103 ± 9 | 410 ± 21‡ | 117 ± 10 | 99 ± 9 | 90 ± 11 | 372 ± 27‡ |
| | Liver | 100 ± 1 | 392 ± 63‡ | 76 ± 7 | 384 ± 50‡ | 105 ± 8 | 118 ± 9 | 69 ± 5 | 373 ± 28‡ | 94 ± 6 | 101 ± 3 | 82 ± 7 | 232 ± 17‡ | 94 ± 6 | 104 ± 7 | 78 ± 17 | 340 ± 23‡ |
| | Muscle | 100 ± 2 | 340 ± 36‡ | 79 ± 8 | 265 ± 30‡ | 110 ± 7 | 124 ± 8 | 80 ± 3 | 263 ± 18‡ | 152 ± 13 | 140 ± 6 | 125 ± 11 | 390 ± 41‡ | 167 ± 87 | 224 ± 49 | 117 ± 20 | 390 ± 41‡ |

*** Wt *vs* GRK2+/-; *P<0.05***

**§ 3 months *vs* 9 months, HFD, TNFα; *P<0.05***

**‡ Ctrl *vs* Ins; *P<0.05***

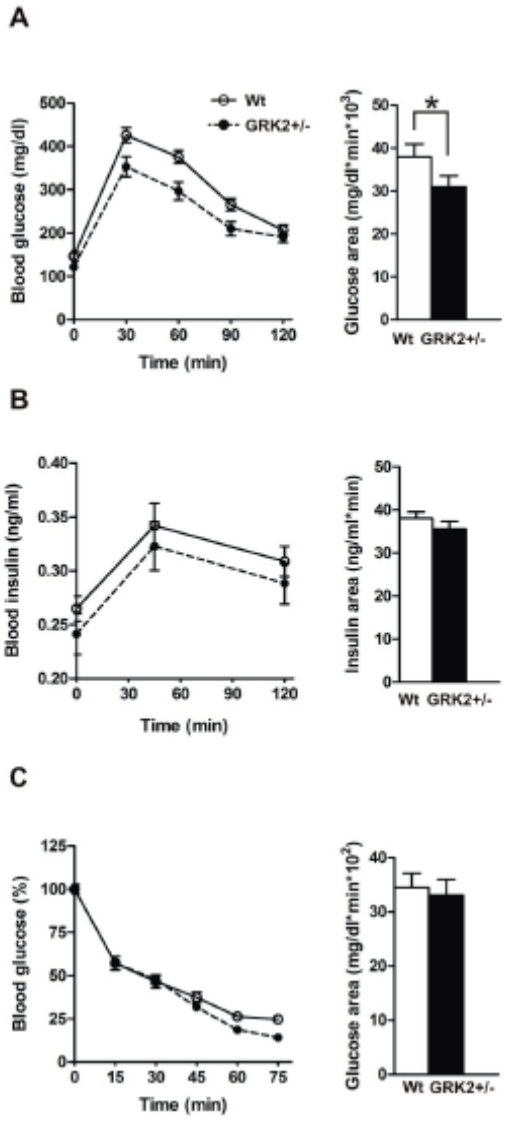
**Supplemental Table 2.** Demographical and clinical characteristics of the subjects included in the study.

| Parameter | Insulin-sensitive Individuals | Insulin-resistant patients |
|---|---|---|
| **BMI. kg/m$^2$** | 22.5±0.7 | 31.9±0.8* |
| **Age, yr** | 50±2 | 51±1 |
| **HOMA** | 0.99±0.10 | 4.81±0.57* |
| **Waist circumference, cm** | 81±4 | 107±3* |
| **Glucose, mg/dL** | 83.2±2.0 | 107.1±2.4* |
| **SBP, mmHg** | 105±4 | 133±3* |
| **DBP, mmHg** | 69±3 | 84±2* |
| **Plasma insulin, pmol/L** | 30.0±2.3 | 116.3±13.3* |
| **HDL-cholesterol, mg/dL** | 70±3 | 46±2* |
| **LDL-cholesterol, mg/dL** | 117±17 | 140±8 |
| **Total cholesterol, mg/dL** | 194±16 | 217±9 |
| **Triglycerides, mg/dL** | 67±10 | 150±10* |
| **cIMT, mm** | 0.45±0.08 | 0.71±0.04^ |
| **Sex, m/f** | 8/2 | 21/4 |
| **Smoking, %** | 20 | 24 |
| **Medication** | | |
| **Antihypertensives (%)** | --- | 36 |
| **Statins (%)** | --- | 16 |

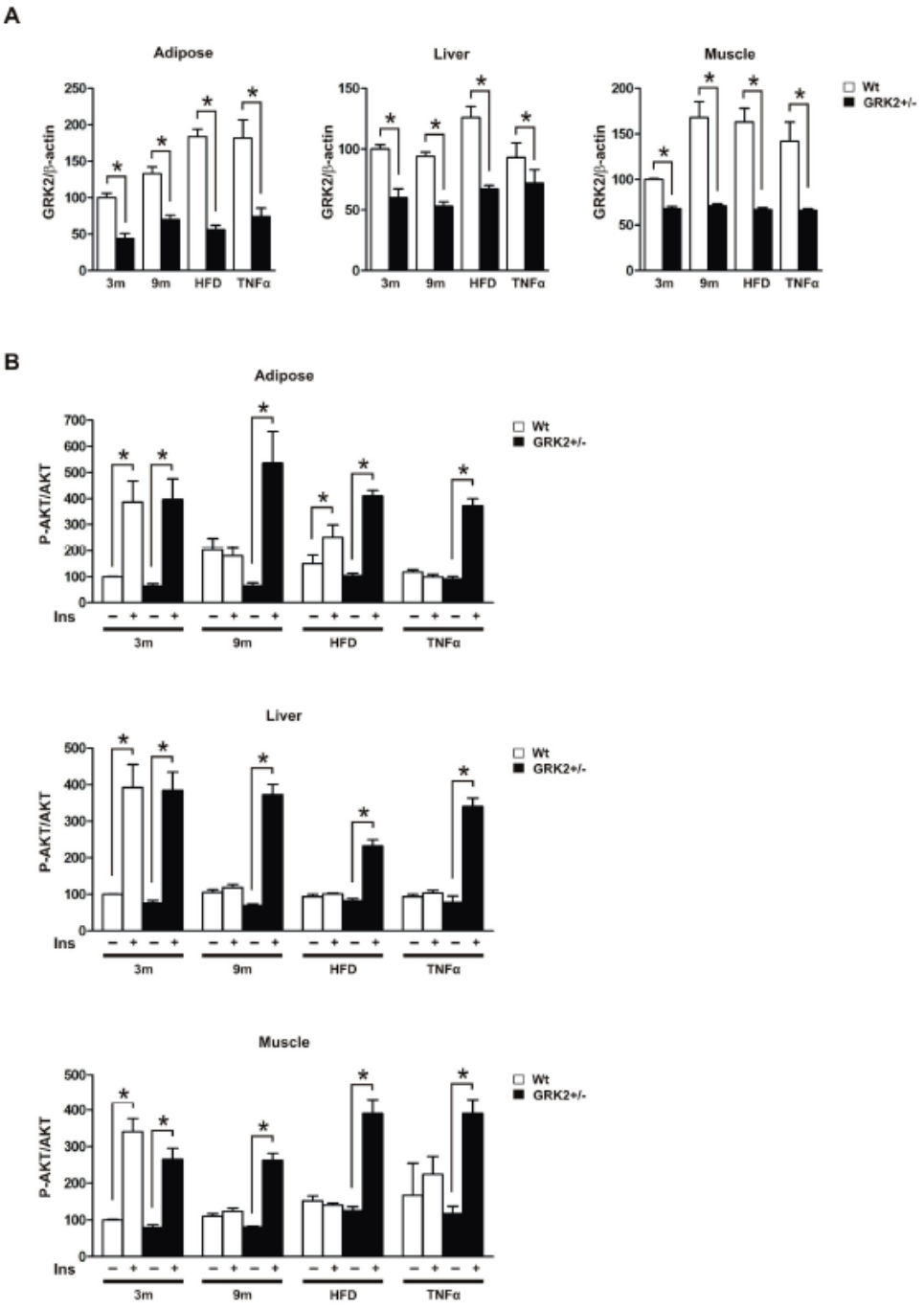
*** Insulin resistant vs insulin-sensitive; p< 0.001**

**^ Insulin resistant vs insulin sensitive; p=0.015**

**Supplemental Figure S1.** **(A)** GTTs were performed on Wt (white circles, solid lines) and GRK2+/- (black circles, dotted lines) 5-month-old male mice as in Fig. 4A. **(B)** Circulating insulin levels during the GTTs. **(C)** ITTs were performed on Wt (white circles, solid lines) and GRK2+/- (black circles, dotted lines) 5-month-old male mice as in Fig. 4C. Results are means ± SEM of 8 animals for each group. *, $p < 0.01$.

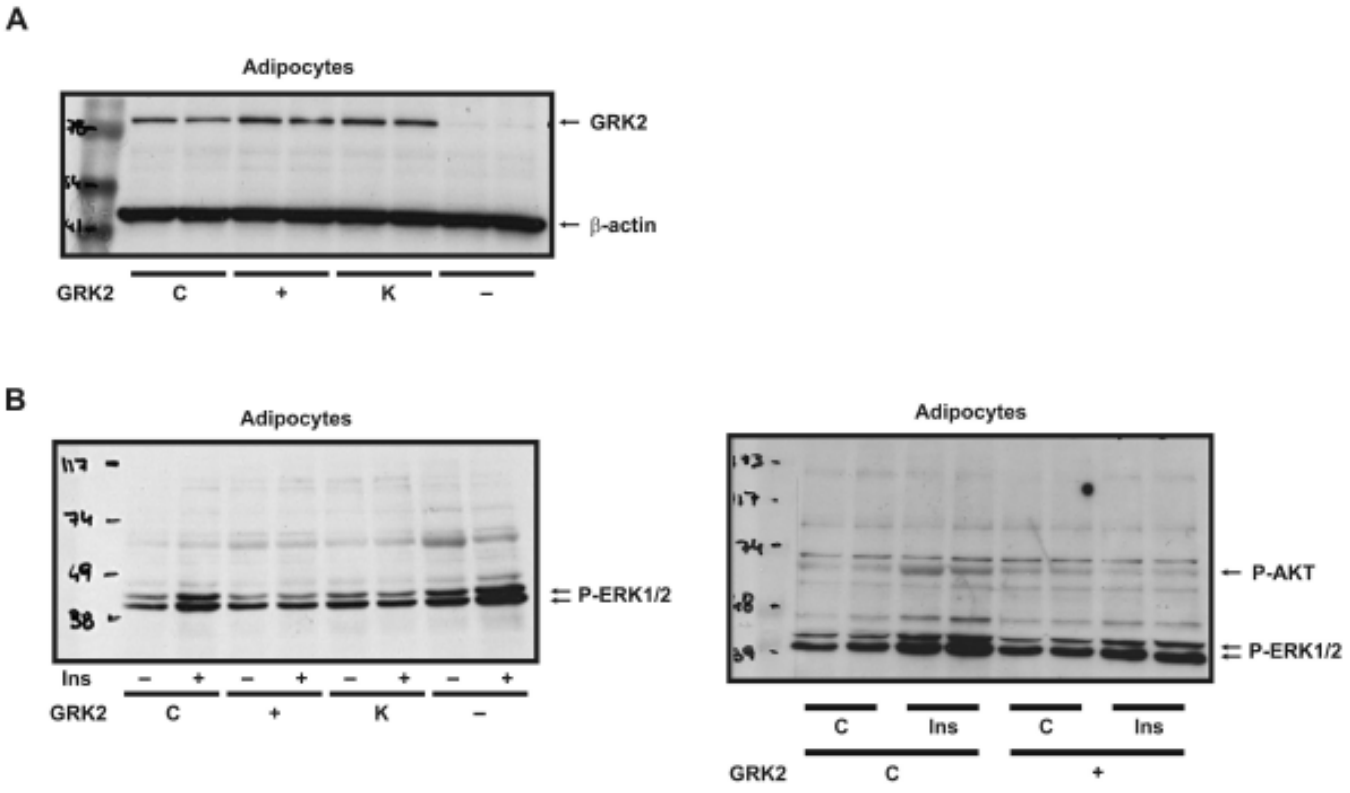


**Supplemental Figure S2.** **(A)** Densitometric analysis of GRK2 expression normalized with ®-actin levels in eWAT, liver and muscle from Wt (white bar) and GRK2+/- (black bar) 3 and 9-month old male mice, fed on a high-fat diet (HFD) for 12 weeks or adult male mice treated with TNFα as in Fig. 4 are shown. **(B)** Densitometric analysis of phosphorylated *vs* total AKT protein expression in presence or absence of insulin in eWAT, liver and muscle from Wt (white bar) and GRK2+/- (black bar) 3 and 9-month old male mice, fed on a HFD for 12 weeks or adult male mice treated with TNFα as in Fig. 4 are shown. Data were normalized with the indicated controls, and results obtained in non-stimulated 3-month old Wt mice taken as 100%. * $p < 0.05$.



**Supplemental Figures S3 and S4.** Full-length blots are shown corresponding to experiments performed for figures 1 and 4 of the manuscript including markers (Pro Sieve Color Protein Markers, Cat No.50550 from Lonza, www.lonza.com) and controls. Blots shown in **S3(A)** correspond to experiments performed for Figure 1A; **S3(B)** for Figure 1C; **S4**(A, B and C) for Figure 4D.

## Supplementary Figure S3



# Supplementary Figure S4