

Supplemental Information

Double-stranded RNA-dependent Protein Kinase Links Pathogen

Sensing with Stress and Metabolic Homeostasis

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Supplementary Figures

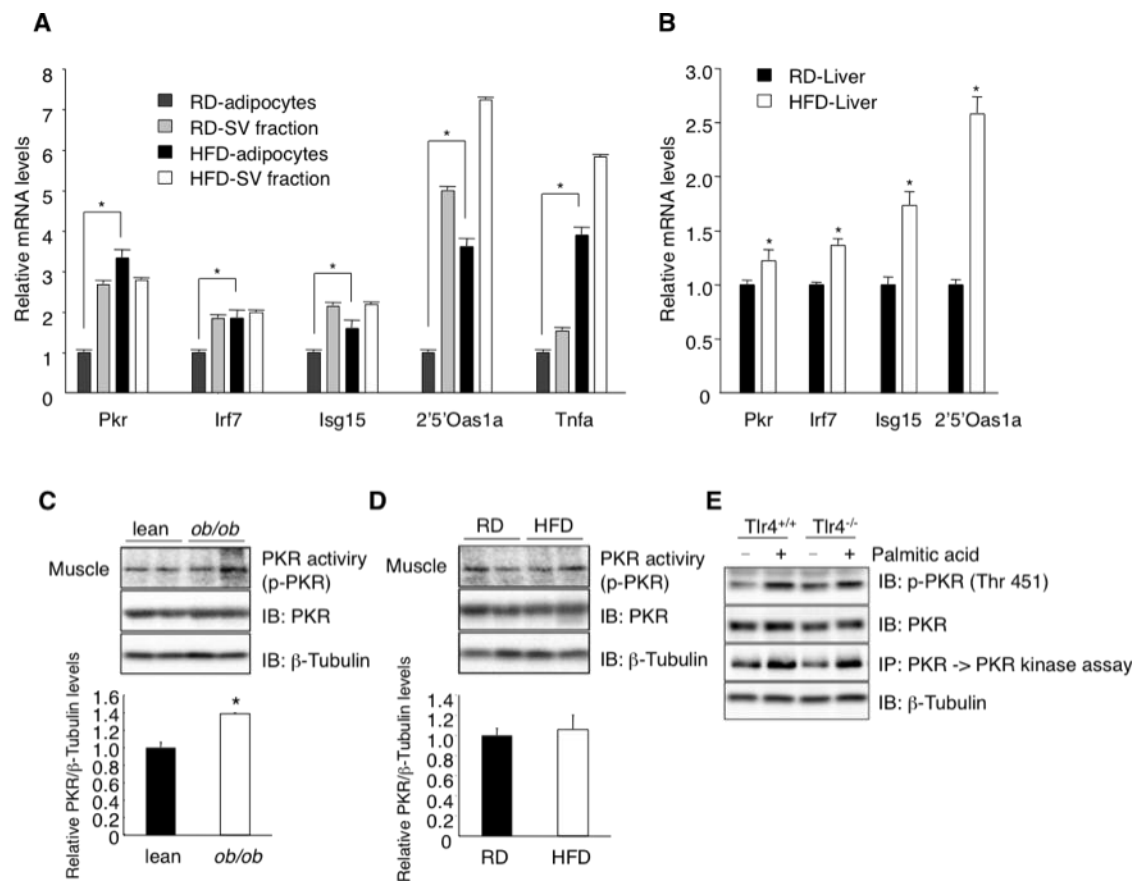


Figure S1, related to Figure 1

(A and B) Expression of PKR and other interferon α/β target genes in adipocytes, stromal vascular (SV) fraction (A) and liver (B) of WT mice fed high fat diet for 8 weeks.

Adipocytes and SV fraction were isolated from white adipose tissues. Data are shown as the mean $\pm$ SEM. * $P < 0.05$.

(C and D) Regulation of PKR activity and expression in skeletal muscle. A genetic (*ob/ob*) (C) and dietary (D) mouse models of obesity were used to examine PKR activity by a kinase assay using immunopurified PKR and ATP [γ - ^{32}P] in skeletal muscle with age- and sex- matched lean controls. β -tubulin protein is shown as controls. The graphs show the quantification of the data. * $P < 0.05$.

(E) Palmitic acid-induced PKR activation in Toll like receptor 4 (TLR4)-deficient MEFs. Primary *Tlr4*^{+/+} and *Tlr4*^{-/-} MEFs were treated with 0.5 mM palmitic acid for 90 minutes. Phosphorylation level of PKR was examined with anti-phospho-PKR (Thr451) antibody. PKR activity was assessed by autophosphorylation level of PKR using ATP [γ - ^{32}P].

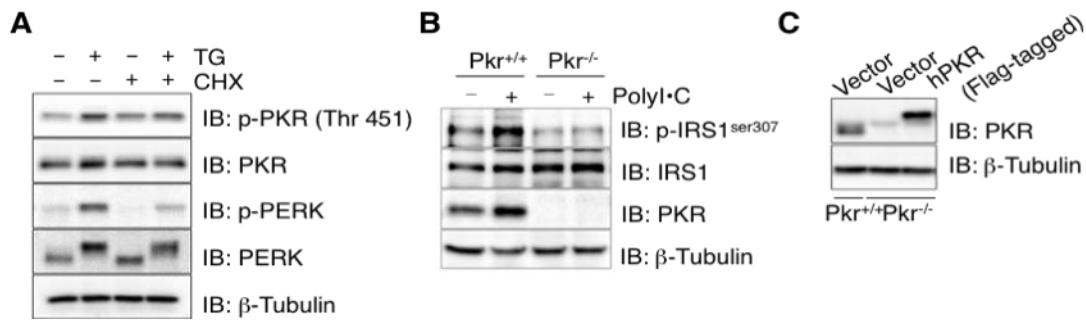


Figure S2, related to Figure 2.

(A) Thapsigargin-induced PKR expression and activation in the presence of cycloheximide (CHX). Wild type MEFs were pretreated with 20 μ g/ml CHX for 30 minutes before addition of 100 nM thapsigargin for 1 hour. Cell lysates were analyzed by western blot analyses with antibodies as indicated. Phosphorylation level of PKR was examined with anti-phospho-PKR (Thr451) antibody.

(B) Polyinosinic-polycytidylic acid (polyI•C)-induced IRS-1 phosphorylation in PKR-dependent manner. Induction of IRS-1 phosphorylation after 100 μ g/ml polyinosinic-polycytidylic acid (polyI•C) treatment for 2 hours in *Pkr*^{+/+} and *Pkr*^{-/-} MEFs. Phosphorylation level of IRS-1 on serine 307 was examined with anti-phospho-IRS-1 antibody.

(C) Expression level of retrovirus-mediated Flag-tagged PKR in *Pkr*^{-/-} MEFs. Flag-tagged PKR was introduced by retrovirus-mediated gene transfer in *Pkr*^{-/-} MEFs. After puromycin selection, the cell lysates were analyzed by western blot analyses with antibodies as indicated.

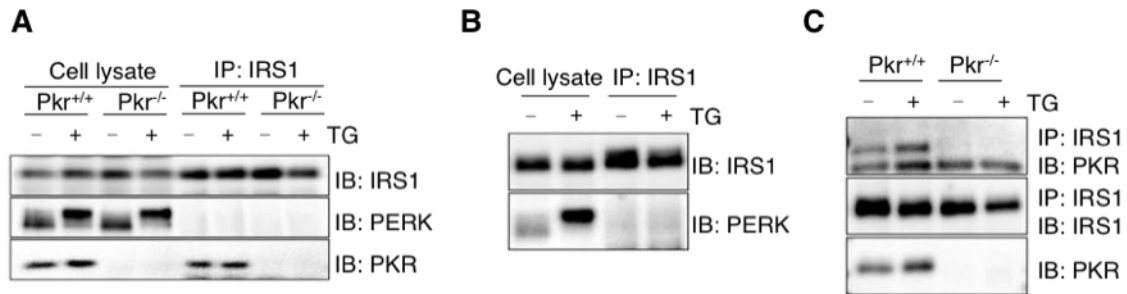


Figure S3, related to Figure 3.

Association of IRS-1 with PKR but not with PERK. (A) Cell lysates were prepared from 300 nM thapsigargin-treated or non-treated MEFs for 3 hours followed by immunoprecipitation with anti-IRS-1 antibody and western blot analyses with anti-PKR or anti-PERK antibodies. (B and C) Cell lysates were prepared from 100 nM thapsigargin-treated or non-treated MEFs for 1 hour followed by immunoprecipitation with anti-IRS-1 antibody and western blot analyses with anti-PKR or anti-PERK antibodies.

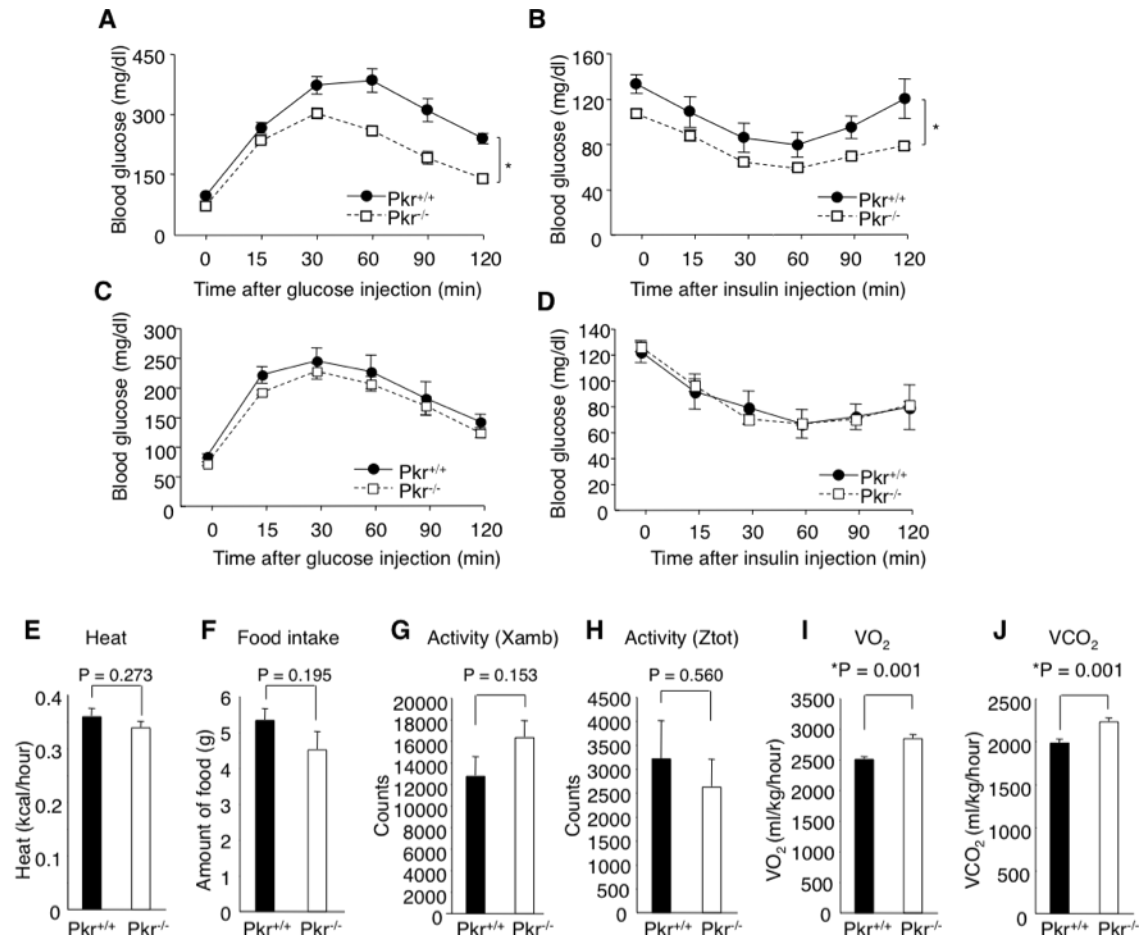


Figure S4, related to Figure 4.

(A-D) Glucose/Insulin tolerance tests in $Pkr^{+/+}$ and $Pkr^{-/-}$ mice on RD and HFD. Glucose tolerance tests were performed in $Pkr^{+/+}$ ($n = 5$) and $Pkr^{-/-}$ mice ($n = 6$) on HFD (A) or RD (C) for 14 weeks. Insulin tolerance tests were performed in $Pkr^{+/+}$ ($n = 5$) and $Pkr^{-/-}$ mice ($n = 6$) on HFD (B) or RD (D) for 16 weeks.

(E-J) Metabolic studies in $Pkr^{+/+}$ and $Pkr^{-/-}$ mice on HFD. Metabolic studies were performed in $Pkr^{+/+}$ ($n = 6$) and $Pkr^{-/-}$ ($n = 7$) mice on HFD for 10 weeks. (E) Heat production, (F) Daily food intake, (G and H) Physical activity determined by automatic sensors on x- (G) and z-axes (H), (I and J) Rates of oxygen consumption (I) and carbon dioxide production (J).

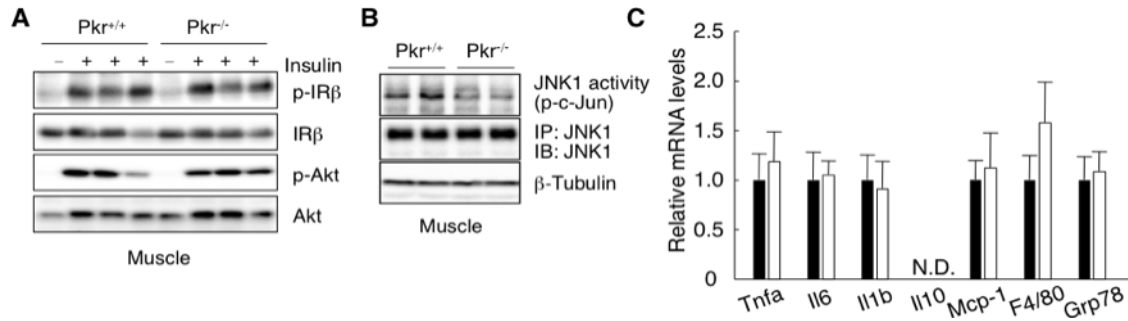


Figure S5, related to Figure 5.

(A and B) PKR expression and activation in skeletal muscle in obese mouse models. (A) Insulin-stimulated IRβ tyrosine 1162/1163 and Akt serine 473 phosphorylation in skeletal muscle of *Pkr*^{+/+} and *Pkr*^{-/-} mice on HFD. Tissue lysates were analyzed by western blot with antibodies as indicated. (B) JNK1 activity was detected by a kinase assay using immunopurified JNK1, ATP [^γ-³²P] and recombinant c-Jun protein as substrate. β-Tubulin is shown as a control.

(C) Gene expression in WAT including proinflammatory cytokine levels in *Pkr*^{+/+} and *Pkr*^{-/-} mice on RD. Data are shown as the mean ± SEM.

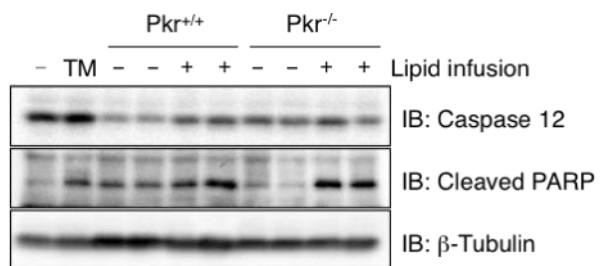


Figure S6, related to Figure 6.

Apoptotic gene expression after lipid-infusion in liver of *Pkr*^{+/+} and *Pkr*^{-/-} mice. Tissue lysates were analyzed by western blot analyses with antibodies as indicated. Tunicamycin (TM) treated liver sample was used as a control for induction of liver injury.

Table S1. Primers for real-time PCR

Gene	Species	Accession #	Forward		Reverse	
Tnfa	Mouse	NM_013693	5'-	CCC TCA CAC TCA GAT CAT CTT CT	-3' 5'-	GCT ACG ACG TGG GCT ACA G
Il6	Mouse	NM_031168	5'-	ACA ACC ACG GCC TTC CCT ACT T	-3' 5'-	CAC GAT TTC CCA GAG AAC ATG TG
Il1b	Mouse	NM_008361	5'-	GCA ACT GTT CCT GAA CTC AAC T	-3' 5'-	ATC TTT TGG GGT CCG TCA ACT
Il10	Mouse	NM_010548	5'-	GCT CTT ACT GAC TGG CAT	-3' 5'-	CGC AGC TCT AGG AGC ATG TG
Mcp-1	Mouse	NM_011333	5'-	CCA CTC ACC TGC TGC TAC TCA	-3' 5'-	TGG TGA TCC TCT TGT AGC TCT CC
F4/80	Mouse	NM_010130	5'-	CCC CAG TGT CCT TAC AGA GTG	-3' 5'-	GTG CCC AGA GTG GAT GTC T
Grp78	Mouse	NM_022310	5'-	TCA TCG GAC GCA CTT GGAA	-3' 5'-	CAA CCA CCT TGA ATG GCA AGA
PKR	Mouse	NM_011163	5'-	AAA ACA AGG TGG ATT GTC ACA CG	-3' 5'-	GTT GGG CTC ACA CTG TTC ATA AT
IRF7	Mouse	NM_016850	5'-	CTG GAG CCA TGG GTA TGC A	-3' 5'-	AAG CAC AAG CCG AGA CTG CT
ISG15	Mouse	NM_015783	5'-	TGG GAC CTA AAG GTG AAG ATG CTG	-3' 5'-	TCA GGC GCA AAT GCT TGA TCA C
2'SOAS1A	Mouse	NM_145211	5'-	GAG GTT CAG CAT GAG AGA CGT T	-3' 5'-	ACA CAG TTG GTA CCA GTG CTT G
36B4	Mouse	NM_007475	5'-	CAC TGG TCT AGG ACC CGA GAA	-3' 5'-	AGG GGG AGA TGT TCA GCA TGT
GAPDH	Mouse	NM_008084	5'-	ATC ACC ATC TTC CAG GAG CG	-3' 5'-	CCA TGC CAT CAC TGC CAC CC

Supplemental Experimental Procedures

Materials

Anti-IRS-1 and anti-phospho-IRS-1 (Ser307) were from Upstate Biotechnology (Lake Placid, NY). Antibody against PKR, JNK1, Akt, phospho-Akt, insulin receptor β subunit, β -tubulin, Fatty acid synthase (FAS), and Actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-eIF2 α (Ser52) antibody was purchased from Invitrogen (Carlsbad, CA). Anti-phospho-insulin receptor (Tyr1162/1163) was purchased from Calbiochem (Gibbstown, NJ). Anti-phospho-JNK (Thr183/Tyr185) antibody was purchased from Cell Signaling Technology (Danvers, MA). Recombinant IRS-1, agarose-conjugated PKR, and anti-phospho PKR antibody were purchased from Millipore (Billerica, MA). Active PKR recombinant protein was purchased from SignalChem (Richmond, BC, Canada).

Determination of metabolic parameters in Hyperinsulinemic-euglycemic clamp study

For the determination of plasma ^3H -glucose and 2- ^{14}C -DG concentrations, plasma was deproteinized with ZnSO_4 and Ba(OH)_2 , dried to remove $^3\text{H}_2\text{O}$, resuspended in water and counted in scintillation fluid for detection of ^3H and ^{14}C . The plasma concentration of $^3\text{H}_2\text{O}$ was determined by the difference between ^3H counts with and without drying. Tissue 2- ^{14}C -DG-6-phosphate content was determined in homogenized samples that were subjected to an ion-exchange column to separate 2- ^{14}C -DG-6-phosphate from 2- ^{14}C -DG. Rates of basal hepatic glucose production and insulin-stimulated whole-body glucose

uptake were determined as the ratio of the ^3H -glucose infusion rate to the specific activity of plasma glucose at the end of the basal period and during the final 30 min of clamps, respectively. Hepatic glucose production during the hyperinsulinemic-euglycemic clamps was determined by subtracting the glucose infusion rate from the whole-body glucose uptake. Glucose uptake in individual tissues was calculated from the plasma 2- ^{14}C -DG profile, which was fitted with an exponential curve, and tissue 2- ^{14}C -DG-6-phosphate content.

PKR pull down assay

An agarose-conjugated PKR (Millipore) was mixed with recombinant full-length IRS-1 protein (Millipore) in interaction buffer [5 mM Tris-HCl (pH 7.4), 25 mM KCl, 1 mM MgCl_2 , 0.25% Triton X] and then agitated at 4°C. After the agitation, the agarose-conjugated PKR was pelleted by centrifugation and washed with the interaction buffer followed by SDS-PAGE. One part of supernatant was kept to detect unbound IRS-1.

PKR kinase assay with recombinant protein

For kinase assay with recombinant protein, full-length IRS-1 (Millipore) was dephosphorylated by λ protein phosphatase (New England BioLabs) and used as a substrate. The kinase assay was performed with an active PKR (SignalChem) and the dephosphorylated IRS-1 in kinase buffer [5 mM Tris-HCl (pH 7.4), 25 mM KCl, 1 mM MgCl_2 , 0.25% Triton X, 10 mM ATP, 10 μCi ^{32}P - γATP] followed by SDS-PAGE.

JNK kinase assay

For JNK kinase assay, tissue lysates containing 500 µg of protein were mixed with JNK1 antibody (Santa Cruz) and protein G sepharose beads. The mixture was agitated at 4°C, pelleted by centrifugation and washed with the lysis buffer followed by additional washes with JNK kinase buffer [25 mM Hepes (pH 7.4), 20 mM MgCl₂, 20 mM β-glycerophosphate, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na₃VO₄, 1 mM PMSF] for equilibration. The beads were incubated in kinase buffer containing 10 µCi ³²P-γATP and c-Jun protein at 30°C for 20 min followed by SDS-PAGE.

3.0 ■

2.5

2.0

1.5

1.0

0.5

0

Figure S2, related to Figure 2

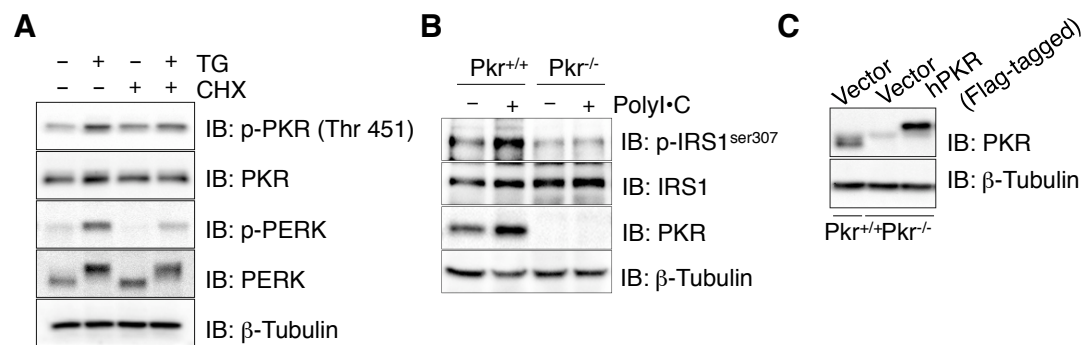


Figure S3, related to Figure 3

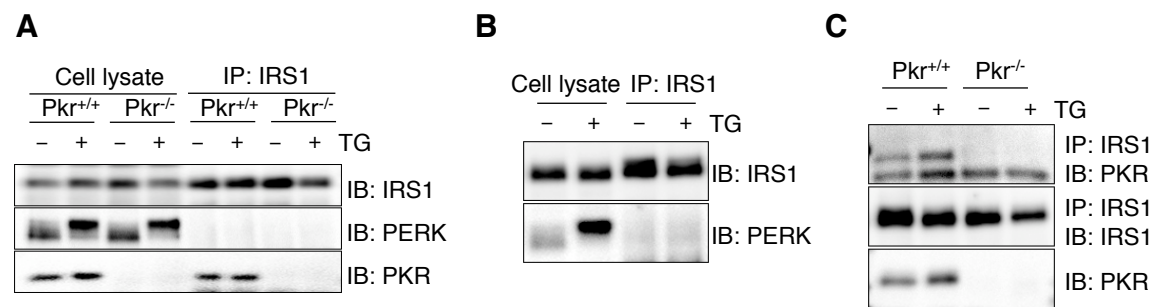


Figure S4, related to Figure 4

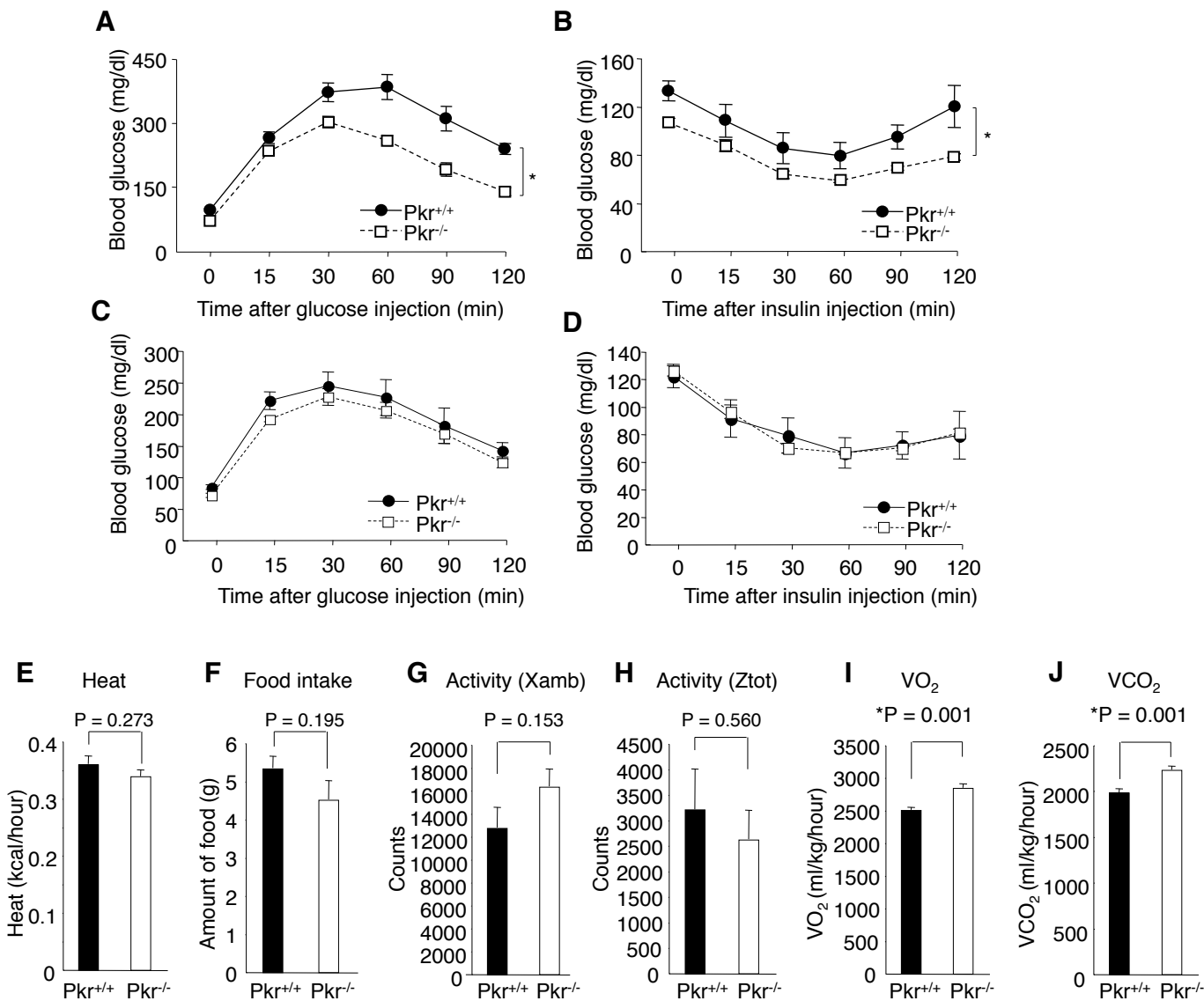


Figure S5, related to Figure 5

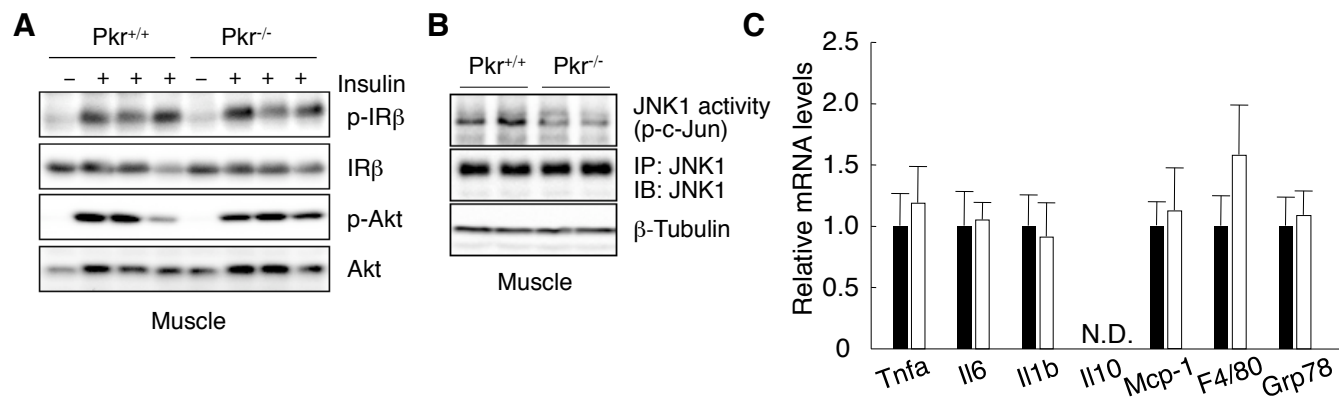


Figure S6, related to Figure 6

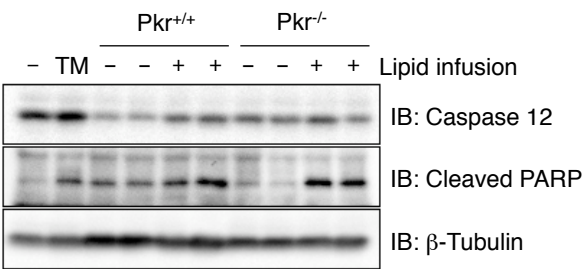


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GAPDH	Mouse	NM_008084	5'- ATC ACC ATC TTC CAG GAG CG	-3' 5'- CCA TGC CAT CAC TGC CAC CC