Figure S1. The effect of APPL1 expression on insulin sensitivity (Related to Figure 1).

(A) Identification of APPL1 knockout mice by Southern blot analysis. The genomic DNA was digested with SacI and hybridized with specific β-geo DNA probe that detected a 12.5 kb fragment from knockout (KO) mouse, but not the sample from wild type (WT) mouse.

(B) Identification of APPL1 knockout mice by PCR genotyping. Tail DNA from mouse was performed by PCR using specific primers for β-geo and GAPDH, respectively. +/+ (wild type), +/- (heterozygous), -/- (homozygous).

(C) Identification of APPL1 knockout mice by Western blot analysis. APPL1 proteins from liver, fat, and skeletal muscle tissue homogenates of wild type (WT) or knockout (KO) mice were detected by Western blot using specific APPL1 antibody.

(D) Oxygen consumption of WT and APPL1 KO mice, n=10/group.

(E) Weight of organs from 32 weeks of age male wild type, heterozygous and homozygous APPL1 KO mice. Data represent the percentage tissue weight of body weight under normal chow. n=12 (WT); 10 (Het); 13 (Homo).

(F) Respiratory quotient of WT and APPL1 KO mice, n=10/group.

(G) Activity of wild type (WT) and APPL1 knockout (KO) mice during light and dark cycle, 10 mice/group.

(H) Core body temperature of wild type (WT) and APPL1 knockout (KO) mice. n=12 (WT) and n=10 (KO).

(I) The expression levels of UCP-1 or UCP-2 in brown adipose tissue from wild type (WT) and APPL1 knockout (KO) mice.

(J) Metabolic profiles of APPL1 KO mice. Metabolic parameters of wild-type and APPL1-KO mice under fed-condition. Serum from 32 week–old of wild type or APPL1 KO male mice was analyzed with indicated assays, n=5-10/ group.
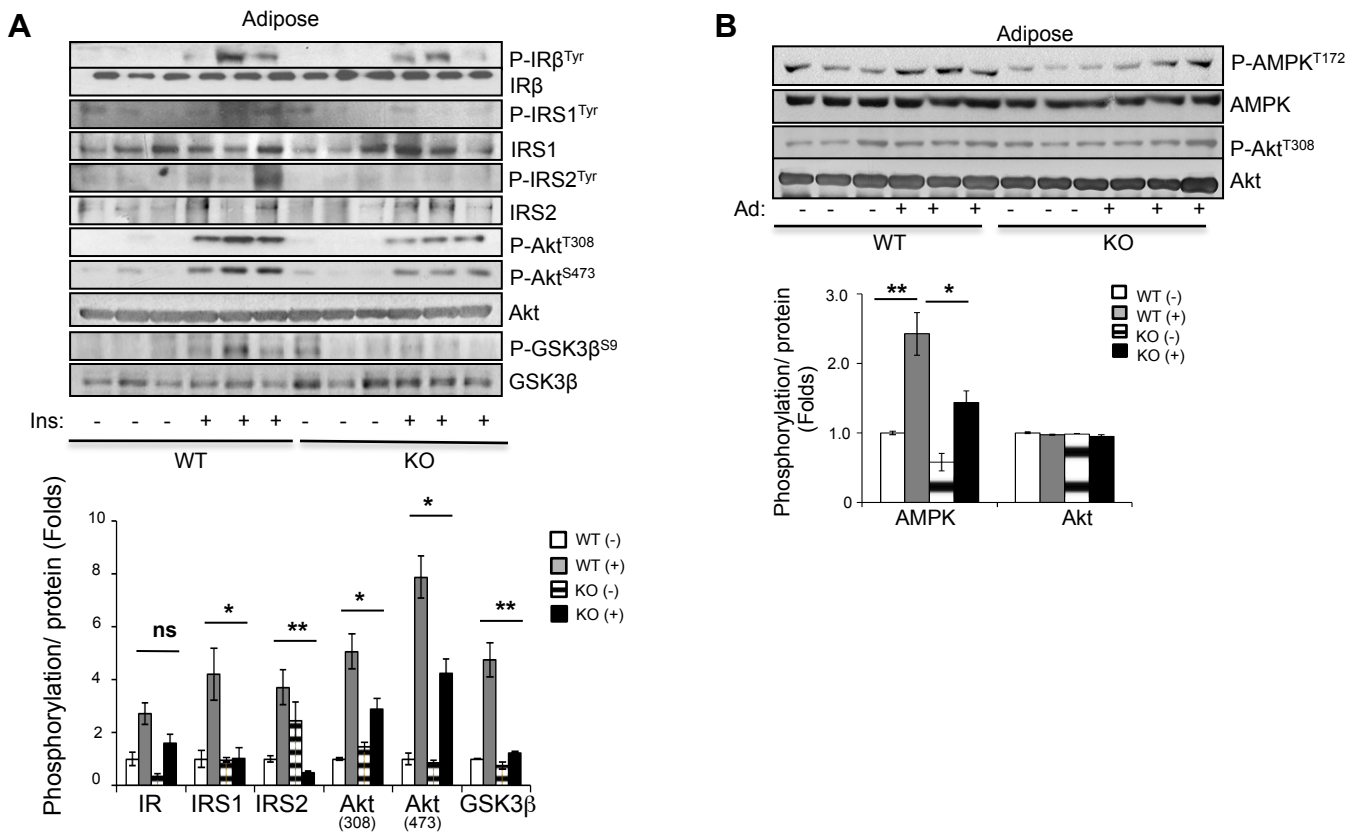
Figure S1. Ryu J., et al.

A

Adipose

P-IRβ^Tyr
IRβ
P-IRS1^Tyr
IRS1
P-IRS2^Tyr
IRS2
P-Akt^T308
P-Akt^S473
Akt
P-GSK3β^S9
GSK3β

Ins: -   -   -   +   +   +   -   -   -   +   +   +

WT   KO

B

Adipose

P-AMPK^T172
AMPK
P-Akt^T308
Akt

Ad: -   -   -   +   +   +   -   -   -   +   +   +

WT   KO

Figure S2. The *in vivo* role of APPL1 in regulating insulin and adiponectin signaling (Related to Figure 2).

(A) The effect of APPL1 expression on insulin signaling in peripheral insulin responsive tissues. Fasted wild type (WT) and APPL1 knockout (KO) mice were injected (i.p.) with insulin (0.5 U/kg of body weight) for 3 minutes for detecting the effect in adipose tissues. Bar graphs represent the ratios of insulin-stimulated phospho-IRβ, IRS1, IRS2, Akt and GSK3β over their total protein levels. Data are shown as mean ± s.e.m. *, $p < 0.05$ and ** and $P < 0.01$. n=4 for each group.

(B) The effect of APPL1 expression on adiponectin signaling in peripheral insulin responsive tissues. Overnight fasted wild type (WT) and APPL1 knockout (KO) mice were re-fed for 4 hrs and then injected (i.p.) with adiponectin (Ad) (5 μg/ g of body weight) for 30 minutes for detecting the effect in adipose tissues (B). Phosphorylation of AMPK and Akt and the protein expression were detected with specific antibodies as indicated. Bar graphs represent the ratios of phosphorylated AMPK and Akt over their total protein levels. Data are shown as mean ± s.e.m. *, $p < 0.05$ and **, $P < 0.01$ *vs.* WT with adiponectin stimulation. n=6 for each group.
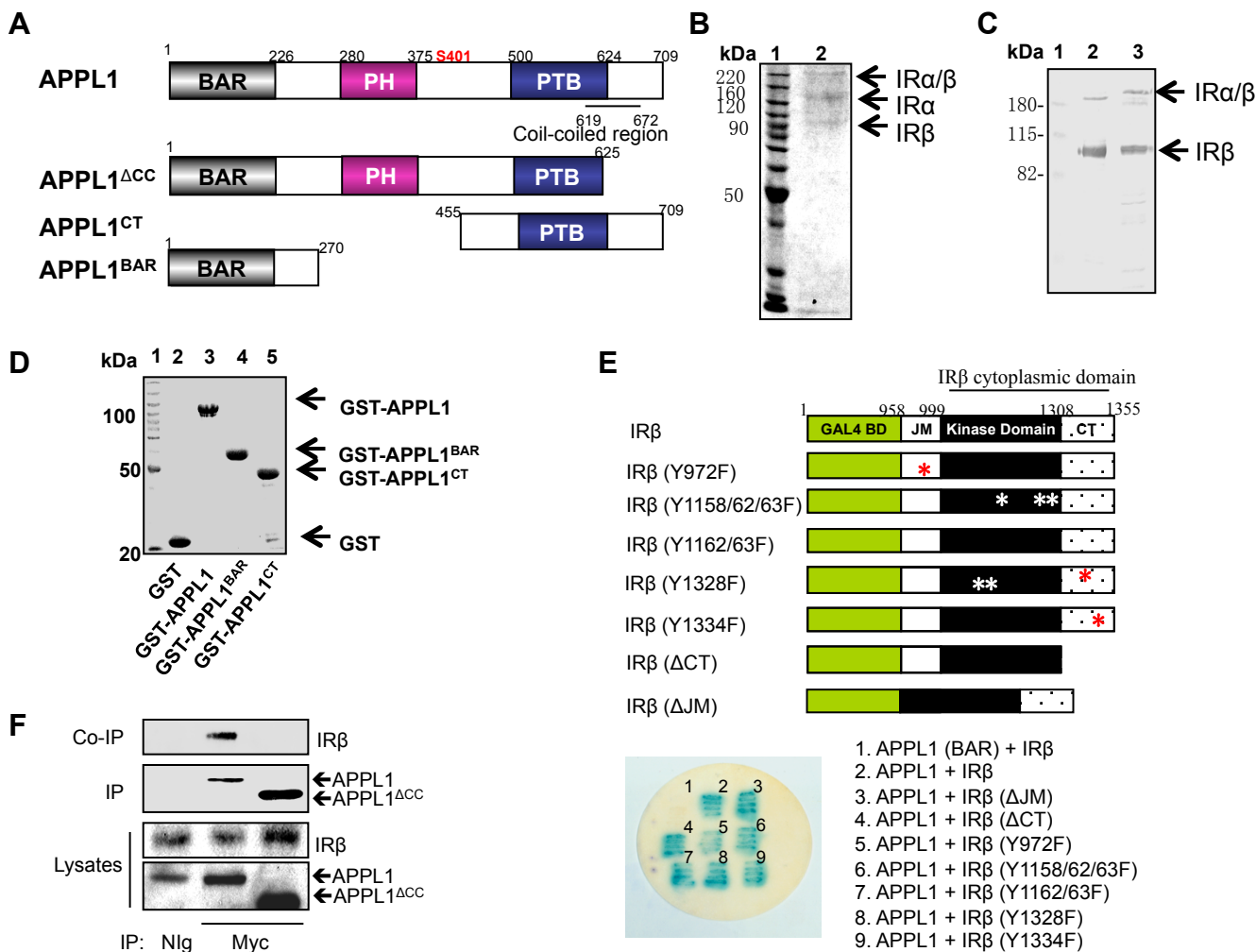
Figure S3. Interaction of APPL1 with insulin receptor (Related to Figure 3).

(A) Graphic presentation of the domain structures of human APPL1. Full-length APPL1: amino acid 1~709; APPL1$^{\Delta CC}$: amino 1~625; APPL1^{CT}: amino acid 455~709 and APPL1^{BAR}: amino acid 1~270. BAR, Bin-amphiphysin-Rvs domain; PH, pleckstrin homology domain; PTB domain, phospho-tyrosine binding domain; CT, carboxyl-terminus; CC, coil-coiled region.

(B) Coomassie blue staining of purified IR. To purify IRβ, lysates of CHO/IR cells were incubated with wheat germ agglutinin-agarose overnight at 4 ℃ with gentle rotation. The beads were packed into a small column and the bound IR was eluted with 0.3 M N-acetylglucosamine buffer (50 mM Tris-HCl, pH 7.4, 0.3 M N-acetyglucosamine, 0.1% Triton X-100, and 0.1 mM PMSF) after extensive washing the column with an ice-cold buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, and 1% Triton X-100). The eluted IR (Lane 2) was checked on SDS-PAGE followed with Coomassie blue staining. Three major bands were detected which correspond to the α subunit (IRα, M_r=130 kDa), β subunit (IRβ, M_r=90 kDa), and an un-processed precursor of the IR α and β subunits (IRα/β, M_r=220 kDa).

(C) Purified IRβ (lane 2) and CHO/IR cell lysates (lane 3, as a positive control) were detected by Western blot with an antibody to IRβ to confirm that the 90 kDa band is the β subunit of IR and the 220 kDa band is the IR precursor.

(D) Purity of GST-APPL1 fusion proteins. GST (lane 2), GST-APPL1 fusion protein (lane 3), GST-APPL1^{BAR} fusion protein (amino acids 5 to 270) (lane 4) and GST-APPL1^{CT} fusion protein (amino acid 455-693) (lane 5), were affinity purified with glutathione agarose and the purity of these fusion proteins were checked on SDS-PAGE and stained with Coomassie blue.

(E) Interaction of APPL1 with the IRβ in the Yeast Two-Hybrid System. Yeast two-hybrid plasmids pGBT9 containing deletions in either the juxtamembrane domain (JM) or the carboxyl-terminal (CT) of the receptors as well as mutants on essential tyrosine residues (showed as ∗) were co-transfected with pGADT7/APPL1 (full length or BAR domain only). Yeast cells were grown on selective media without tryptophan and leucine. Interactions were detected by lift colony assay.

(F) Coiled-coil (CC) region of APPL1 is essential for the interaction between APPL1 and IRβ. C2C12 myocytes transfected with or without Myc-tagged APPL1 or APPL1$^{\Delta CC}$ truncation mutant. Overexpressed APPL1 or APPL1$^{\Delta CC}$ truncation was immunoprecipitated by anti-Myc antibody. The co-immunoprecipitated endogenous IRβ was detected with specific antibodies as indicated.
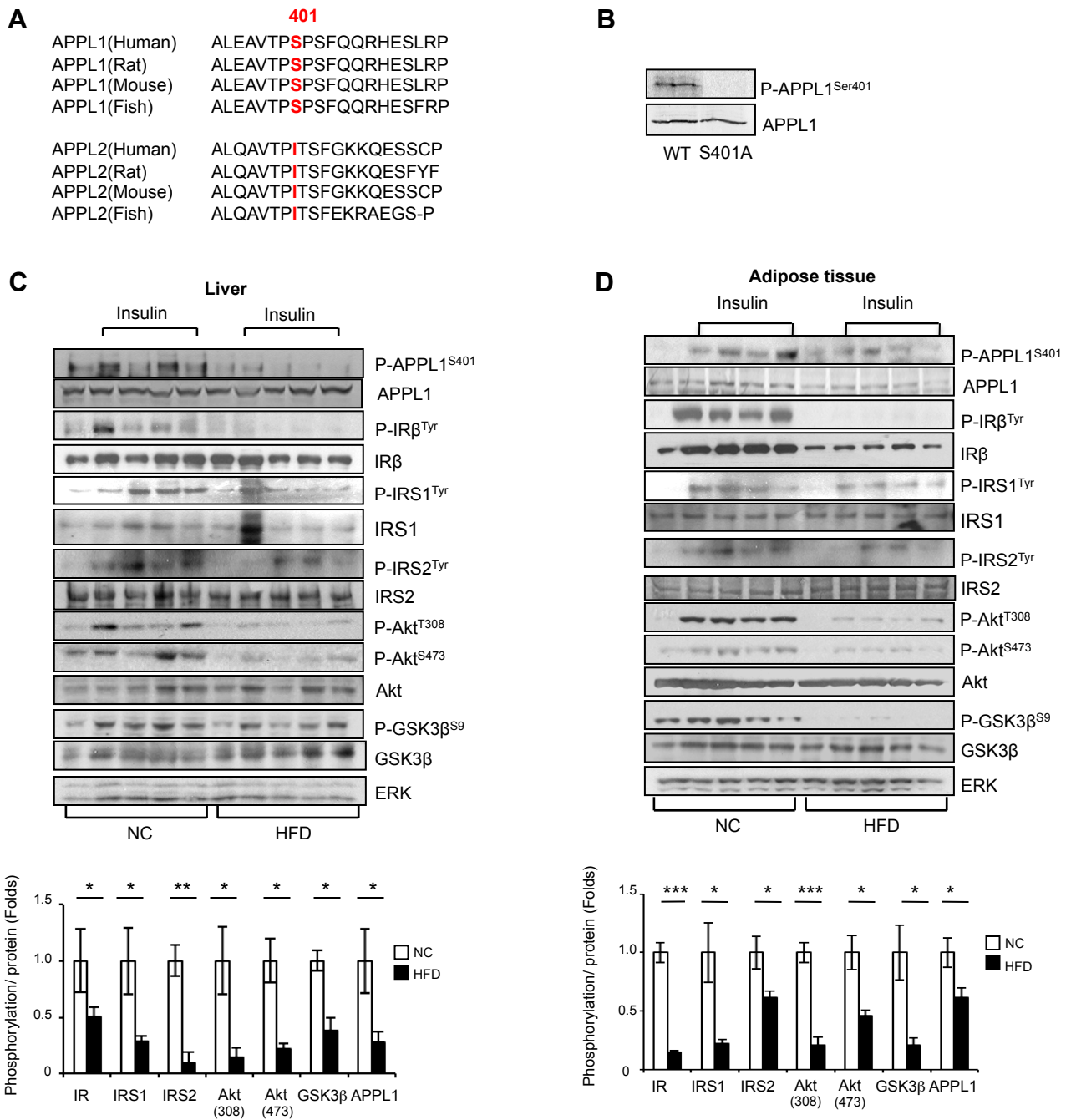
Figure S3. Ryu J., et al.

A

401

APPL1(Human)	ALEAVTP**S**PSFQQRHESLRP
APPL1(Rat)	ALEAVTP**S**PSFQQRHESLRP
APPL1(Mouse)	ALEAVTP**S**PSFQQRHESLRP
APPL1(Fish)	ALEAVTP**S**PSFQQRHESFRP

APPL2(Human)	ALQAVTP**I**TSFGKKQESSCP
APPL2(Rat)	ALQAVTP**I**TSFGKKQESFYF
APPL2(Mouse)	ALQAVTP**I**TSFGKKQESSCP
APPL2(Fish)	ALQAVTP**I**TSFEKRAEGS-P

B

P-APPL1^{Ser401}
APPL1
WT S401A

C Liver

Insulin   Insulin

P-APPL1^{S401}
APPL1
P-IRβ^{Tyr}
IRβ
P-IRS1^{Tyr}
IRS1
P-IRS2^{Tyr}
IRS2
P-Akt^{T308}
P-Akt^{S473}
Akt
P-GSK3β^{S9}
GSK3β
ERK

NC   HFD

D Adipose tissue

Insulin   Insulin

P-APPL1^{S401}
APPL1
P-IRβ^{Tyr}
IRβ
P-IRS1^{Tyr}
IRS1
P-IRS2^{Tyr}
IRS2
P-Akt^{T308}
P-Akt^{S473}
Akt
P-GSK3β^{S9}
GSK3β
ERK

NC   HFD

Bar graph C: Phosphorylation/ protein (Folds), * * ** * * * *, categories: IR, IRS1, IRS2, Akt(308), Akt(473), GSK3β, APPL1. NC (white), HFD (black).

Bar graph D: Phosphorylation/ protein (Folds), *** * * *** * * *, categories: IR, IRS1, IRS2, Akt(308), Akt(473), GSK3β, APPL1. NC (white), HFD (black).

Figure S4. Detection of APPL1 phosphorylation at Ser^{401} (Related to Figure 6).

(A) Alignment of APPL1 and APPL2 amino acid sequence through different species.

(B) Specificity of phospho-Ser^{401} antibody. The phosphospecific antibody against APPL1 Ser^{401} was generated by injecting rabbits with phosphorylated 13-mer peptides (Ac-C-Ahx-AVTP-[pS]-PSFQQR-amide) that are chemically synthesized. Wild type or S401A mutant of APPL1 were overexpressed in C2C12 myocytes. Overexpressed APPL1 protein was immunoprecipitated with Myc antibody and phosphorylation on Ser^{401} was detected by Western blot using the antibody against phosphor-Ser^{401} of APPL1.

(C) And (D) Normal chow (NC) and high fat diet (HFD)-fed male C57BL/6 mice were fasted overnight and treated with or without insulin (0.5 U/kg of body weight, 3 min). The liver (C) and adipose (D) tissues were homogenized and phosphorylation of APPL1 at Ser^{401} and APPL1 protein were detected by Western blot analysis. Tubulin was used as a loading control. Tyrosine phosphorylation of IRβ, IRS1, and IRS2 were detected after immunoprecipitation with indicated antibodies, respectively. Phosphorylation of Akt, GSK3β, and the protein expression were detected with specific antibodies as indicated. Bar graphs represent the ratios of insulin-stimulated phospho-APPL1, IRβ, IRS1, IRS2, AKT and GSK3β over their total protein levels. Data are shown as mean ± s.e.m. *, $p < 0.05$ and **, $P < 0.01$ and ***, $p < 0.001$. n=4 for each group.

Figure S4. Ryu J., et al.

A

P-APPL1^{S401}
APPL1
P-AMPK^{T172}
AMPK

Ad: 0 1 5 10 15 20 (min.)

B

1 2 3 4 5 6

P-APPL1^{S401}
APPL1
P-Akt^{T308}
Akt

Gö6983	-	-	+	-	-	-
LiCl	-	-	-	+	-	-
PD98059	-	-	-	-	+	-
LY-294002	-	-	-	-	-	+

Insulin

C

1 2 3 4 5 6

P-APPL1^{S401}
APPL1
P-AMPK^{T172}
AMPK

Gö6983	-	-	+	-	-	-
LiCl	-	-	-	+	-	-
PD98059	-	-	-	-	+	-
LY-294002	-	-	-	-	-	+

Insulin

D

1 2 3 4 5 6

P-APPL1^{S401}
APPL1
Tubulin

PMA 0 1 3 5 10 30 (min.)

E

1 2 3 4 5 6

P-APPL1^{S401}
APPL1
P-AMPK^{T172}
AMPK
P-Akt^{T308}
Akt

Insulin	-	+	-	-	+	-
Adiponectin	-	-	+	-	-	+
Gö6976		-			+	

F

Co-IP: APPL1, IRβ
IP: IRS1
Lysates: IRS1, IRβ, APPL1, P-AMPK^{T172}, AMPK

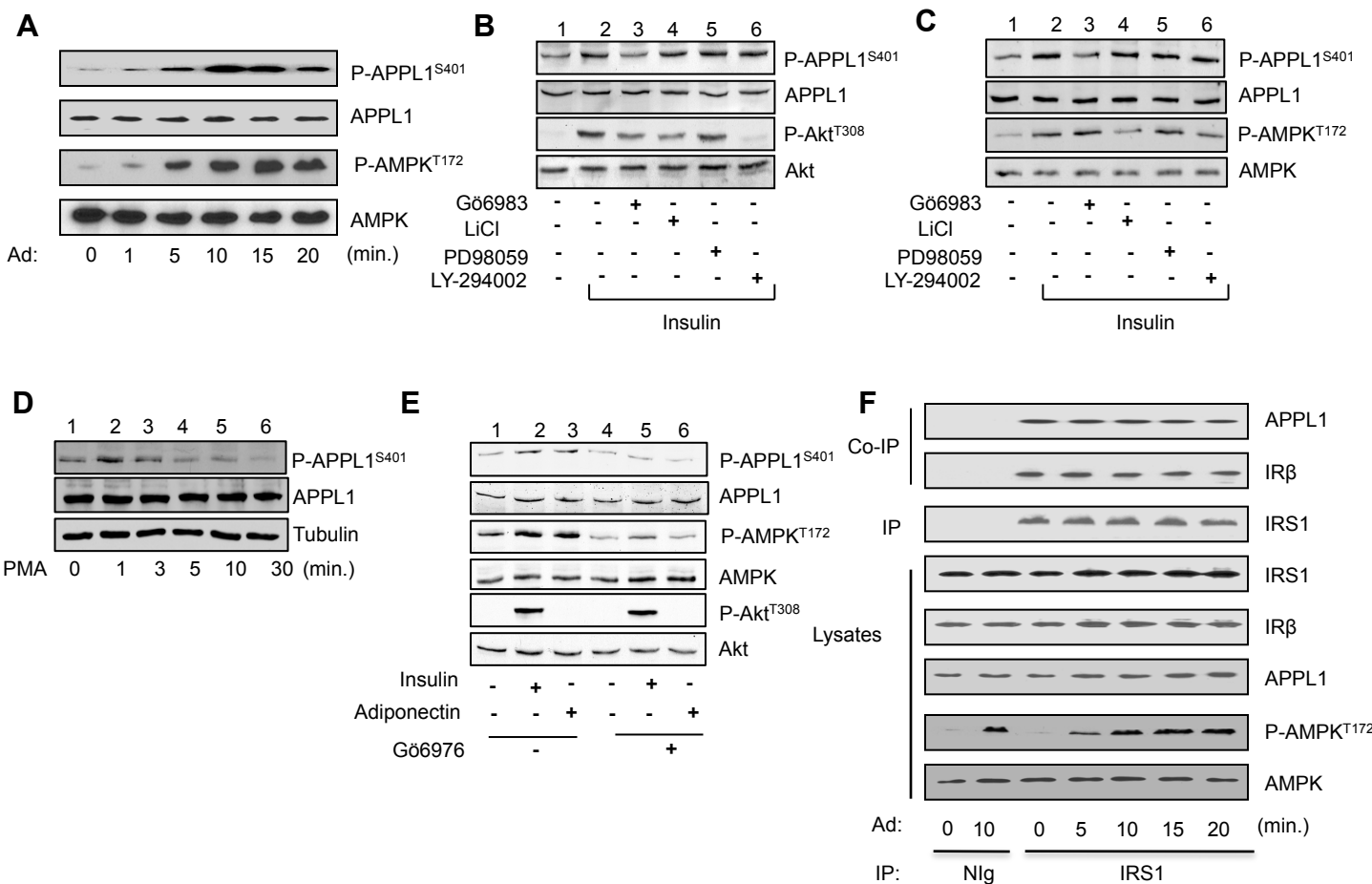Ad: 0 10 | 0 5 10 15 20 (min.)

IP: NIg | IRS1

Figure S5. . The effect of adiponectin on APPL1 phosphorylation and the interaction between APPL1 with IRS1 (Related to Figure 7).

(A) C2C12 myotubes were serum-starved overnight and treated with adiponectin (1 μg/ml) for indicated times. The cells were lysated and phosphorylation of APPL1 at Ser[401] and AMPK at Thr[172] were detected by Western blot analysis with specific antibodies as indicated.

(B) Inhibition of insulin-stimulated APPL1 phosphorylation with kinase inhibitors. C2C12 myotubes were serum-starved and pre-treated with different kinase inhibitors as indicated (2 μM of Gö6983 for PKC isoforms; 5 mM of LiCl for GSK3β; 50 μM of PD98056 for MEK; 25 μM of LY-294002 for PI 3-kinase) for one hour and treated with insulin (10 nM, 3 min). The cells were lysed and phosphorylation of APPL1 (Ser[401]), Akt (Thr[308]) and their protein levels were detected by Western blot analysis.

(C) Inhibition of adiponectin-stimulated APPL1 phosphorylation with kinase inhibitors. C2C12 myotubes were serum-starved and pre-treated with different kinase inhibitors as indicated (2 μM of Gö6983 for PKC isoforms; 5 mM of LiCl for GSK3β; 50 μM of PD98056for MEK; 25 μM of LY-294002 for PI 3-kinase) for one hour and treated with adiponectin (1 μg/ml, 10 min). The cells were lysed and phosphorylation of APPL1 (Ser[401]), AMPK (Thr[172]) and their protein levels were detected by Western blot analysis.

(D) PMA stimulates APPL1 phosphorylation. C2C12 myotubes were serum-starved and stimulated with PMA (1μM), for different time as indicated. The phosphorylation of APPL1-Ser[401] and APPL1 protein levels were detected by Western blot analysis.

(E) Gö6976 inhibits insulin and adiponectin stimulated APPL1 phosphorylation. C2C12 myotubes were serum-starved and pre-treated with Gö6976 as indicated for 1 hour and treated with insulin (10 nM, 3 min) or adiponectin (1 μg/ml, 10 min). The cells were lysed and phosphorylation of APPL1 (Ser[401]), Akt (Thr[308]), AMPK (Thr[172]) and their protein levels were detected by Western blot analysis.

(F) Adiponectin has no effect on the interaction of IRS1 with APPL1 and IRβ. Serum starved C2C12 myotubes were treated with or without 1 μg/ml adiponectin for indicated times. Endogenous IRS1 was immunoprecipitated and co-immunoprecipitated endogenous IRβ and APPL1 were detected by Western blot analysis with the specific antibodies as indicated.

Figure S5. Ryu J., et al.