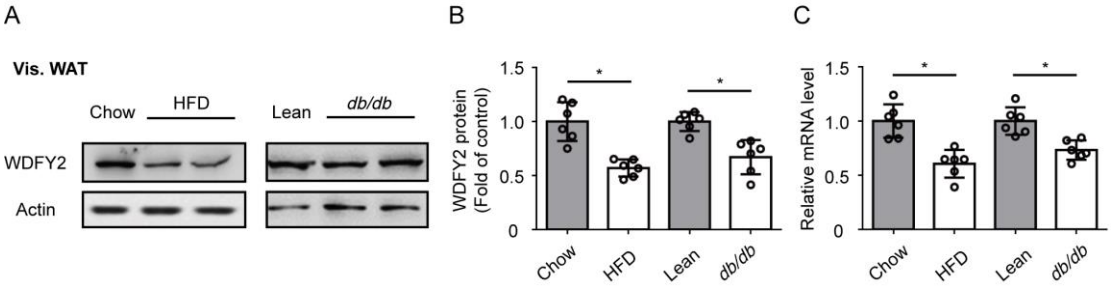
Supplementary Figures

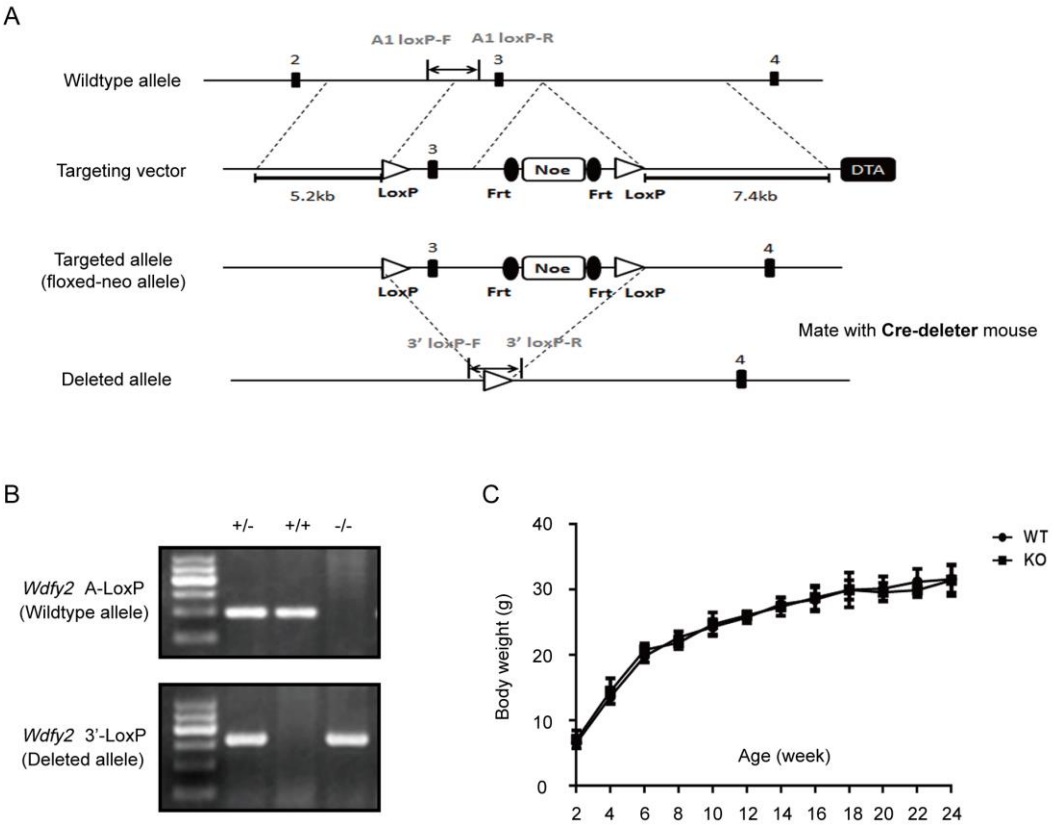
Supplementary Fig. 1



Supplementary Figure 1. WDFY2 expression is decreased in the adipose tissues of metabolic disorder mice (related to Fig. 1.).

Western blots (*A*) and the associated quantified WDFY2 expression levels (*B*) in epididymal adipose tissue isolated from mouse models of insulin resistance and metabolic disorders. Comparisons are made to the respective age-matched and sex-matched controls (n = 6 for all of the groups). (*C*) Real-time PCR analysis of *Wdfy*2 mRNA levels in mouse models of metabolic disorders. The data are normalized to β-actin (n = 6 for all of the groups). The data represent the means ± SD. *p* values were calculated by Student's t test (*$p < 0.05$ versus the respective control).

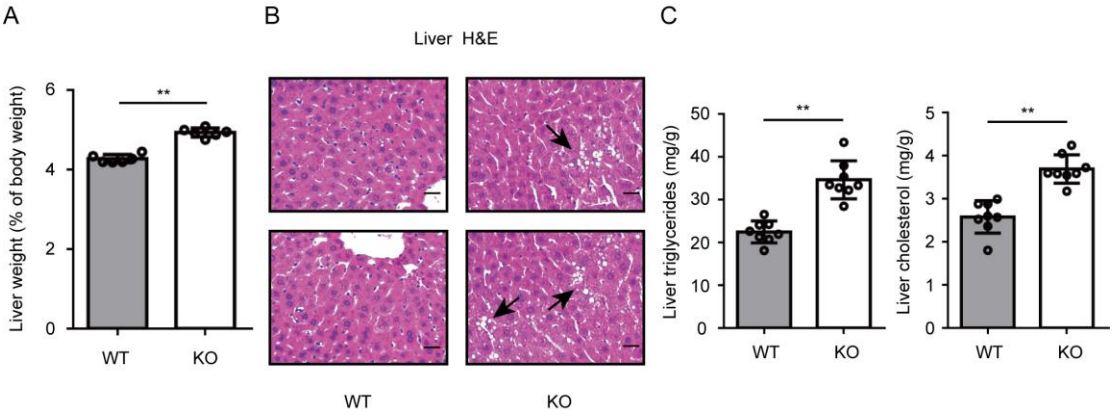
Supplementary Fig. 2



Supplementary Figure 2. Generation of systemic *Wdfy2* deficient mouse model (related to Fig. 2.).

A: Schematic representation of the *Wdfy2* gene. Exon 3 is flanked by loxP sites (triangle) to allow for removal by Cre recombinase. Exon 3 deletion leads to disrupted *Wdfy2* gene transcription and expression. *B*: Identification of wild-type (WT) (+/+), heterozygous (+/-) and *Wdfy2* knockout (KO) (-/-) mice by PCR-based genotyping from tail DNA. *C*: Body weight changes in WT and *Wdfy2* KO mice were monitored over 24 weeks under normal chow conditions (n = 10 mice for each genotype). The data represent the means ± SD.

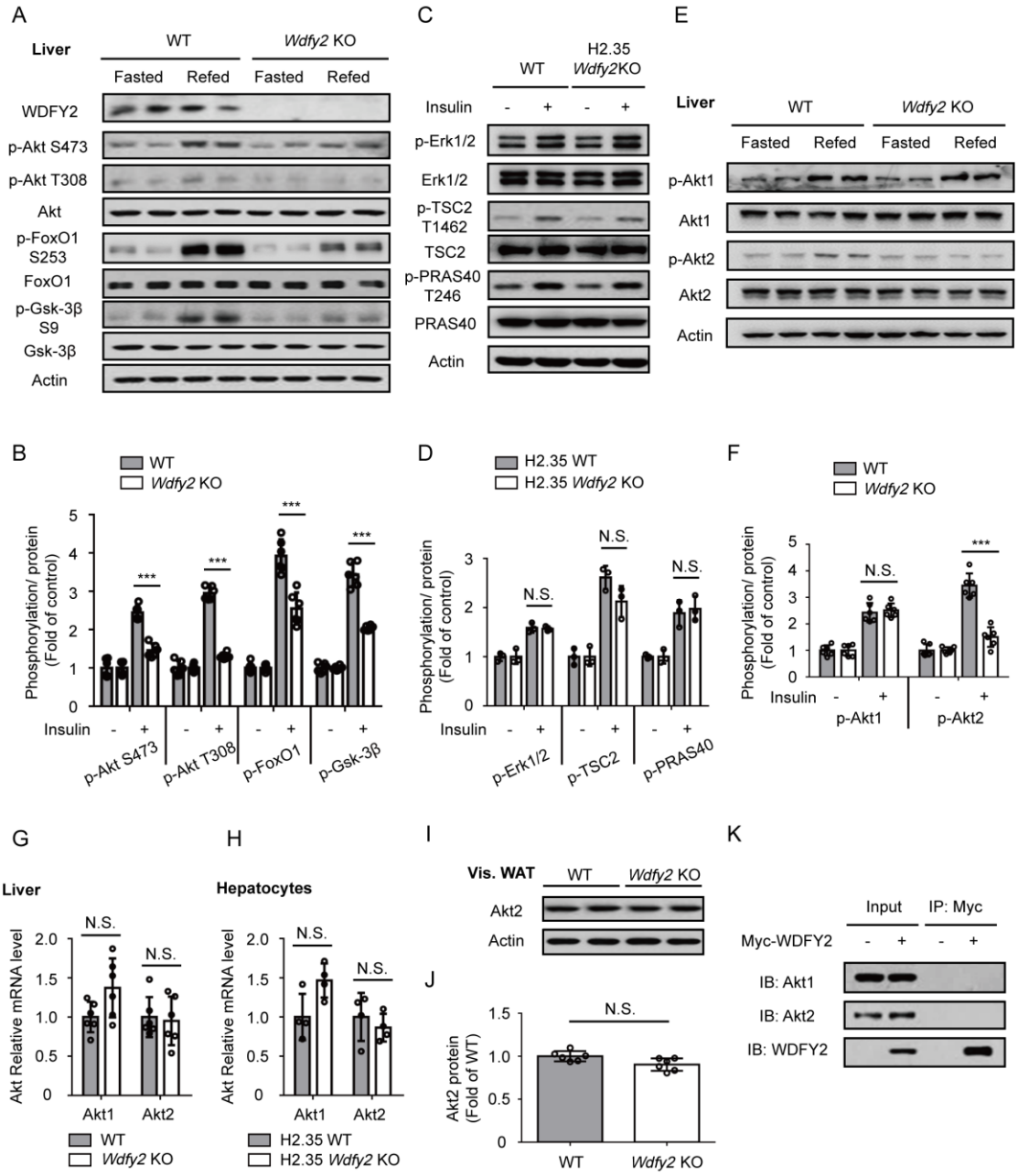
Supplementary Fig. 3



Supplementary Figure 3. Loss of *Wdfy2* leads to increased lipid deposition in the liver of old-aged mice. (related to Fig. 3.).

A: Liver weights of 8-month-old male wild-type (WT) and *Wdfy2* knockout (KO) mice fed a normal chow diet. The data represent the tissue weight as a percentage of the total body weight (n = 6 mice for each genotype). *B*: Representative histological liver sections stained with Hematoxylin and Eosin, from 8-month-old WT and *Wdfy2* KO mice. Scale bar, 20 μm (n = 6 mice for each genotype). *C*: Liver triglyceride or cholesterol contents in 8-month-old WT and *Wdfy2* KO mice (n = 8 mice for each genotype). The data represent the means ± SD. *p* values were calculated by Student's t test (**$p < 0.01$ versus the respective control).

Supplementary Fig. 4

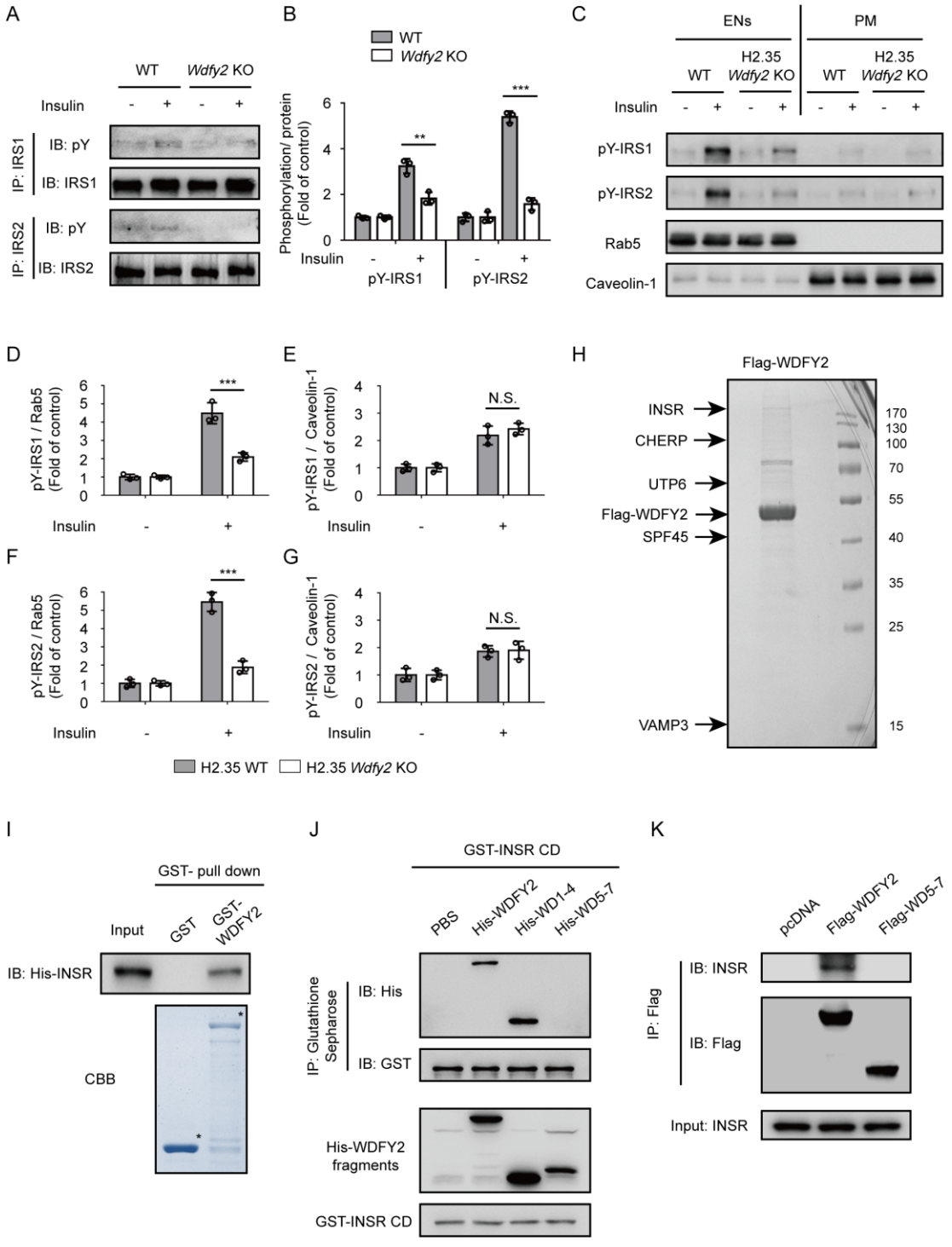


Supplementary Figure 4. WDFY2 has no effect on Akt expression (related to Fig. 4.).

A and *B*: Wild-type (WT) and *Wdfy2* knockout (KO) 3-month-old mice were fasted overnight or fasted and then allowed to refeed with normal chow for 4 h. Representative western blots (*A*) and the associated quantifications (*B*) show

phosphorylated Akt, FoxO1 and GSK-3β levels in liver lysates (n = 6 mice in each group). *C* and *D*: WT and *Wdfy2* KO H2.35 hepatocytes were serum-starved overnight and treated with 10 nM insulin for 15 min. Representative western blots (*C*) and the associated quantifications (*D*) show phosphorylated Erk1/2, TSC2 and PRAS40 levels in hepatocyte lysates (n = 3). *E* and *F*: Wild-type (WT) and *Wdfy2* knockout (KO) 3-month-old mice were fasted overnight or fasted and then allowed to refeed with normal chow for 4 h. Representative western blots (*E*) and quantification (*F*) show phosphorylated Akt1 and Akt2 levels in liver lysates (n = 6 mice in each group). *G*: Real-time PCR analysis of *Akt1 and Akt2* mRNA levels in WT and *Wdfy2* KO liver (n = 6 mice in each group). *H:* Real-time PCR analysis of *Akt1 and Akt2* mRNA levels in WT and *Wdfy2* KO liver and hepatocytes (n = 4).Western blots (*I*) and quantified WDFY2 expression levels (*J*) in Vis. WAT isolated from WT and *Wdfy2* KO H2.35 mice (n= 6 for each group). *K*: H2.35 hepatocytes were transiently transfected with Myc-WDFY2 or empty vectors. The interaction between Myc-WDFY2 and endogenous Akt1 or Akt2 was analyzed by IP with a Myc-tag antibody and IB with Akt1, Akt2 and Myc antibodies. The data represent the means ± SD. *p* values were calculated by two-way ANOVA analysis with Tukey's *post hoc* test (*A*-*F*) and Student's t test (*G*-*J*) (***$p < 0.001$ versus the respective control, N.S., not significant).

Supplementary Fig. 5



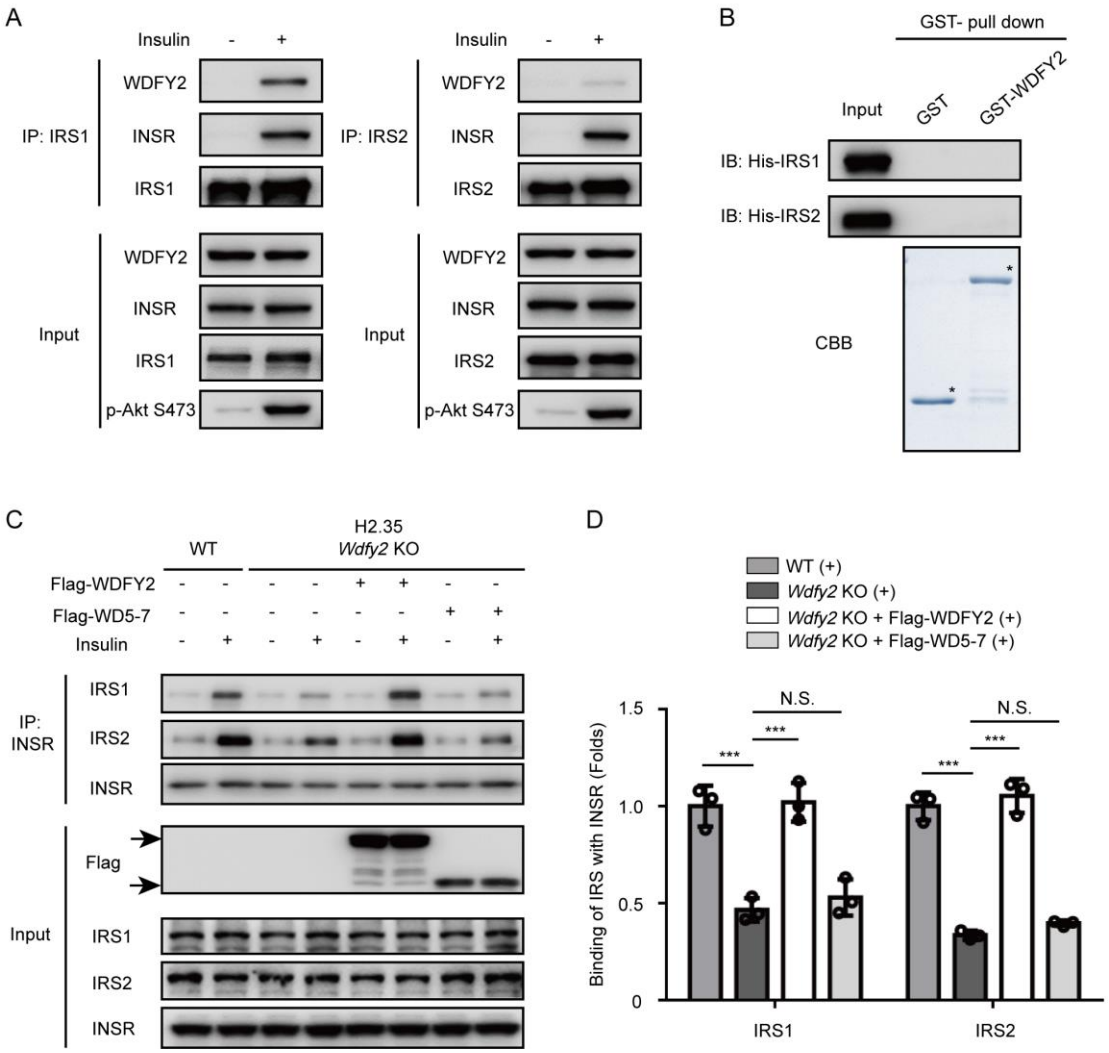
Supplementary Figure 5. INSR is identified as a novel WDFY2 binding partner (related to Fig. 5.).

A and *B*: Wild-type (WT) and *Wdfy2* knockout (KO) 3-month-old mice were fasted

overnight and injected intraperitoneally with either insulin (0.75 U/kg of body weight) or saline for 5 min. Representative western blots (*A*) and the associated quantifications (*B*) show insulin-induced tyrosine phosphorylation of INSR, IRS1 and IRS2 in liver lysates (n = 3). *C*-*G*: Analysis of total and phosphorylated IRS1 and IRS2 levels in the endosomes (ENs) and plasma membrane (PM) from WT and *Wdfy2* KO H2.35 hepatocyte lysates. Representative western blots (*C*) and the associated quantifications (*D*-*G*) show phosphorylated IRS1 and IRS2 levels in the ENs and PM fraction (n = 3). *H*: Cellular extracts from HepG2 cells expressing Flag-WDFY2 were immunopurified on anti-Flag affinity columns and eluted with a Flag peptide. The eluates were resolved by SDS-PAGE and then stained with Coomassie brilliant blue (CBB). The protein bands were retrieved and analyzed by mass spectrometry. *I*: Full-length GST-WDFY2 and His-INSR proteins were purified from *E coli*; then, the same molar quantities of the proteins were mixed and incubated overnight. All samples were resolved by SDS-PAGE followed by CBB staining or immunoblotting (IB) with the indicated antibodies (*, specific protein bands). *J*: GST-INSR CD (cytoplasmic domain) His-WDFY2, His-WD1-4 and WD5-7 proteins were purified from *E coli*. The same molar quantities of GST-INSR CD and His-tag proteins were mixed and incubated overnight. All samples were resolved by SDS-PAGE followed by IB with the indicated antibodies. *K*: H2.35 hepatocytes were transduced with an empty vector, Flag-WDFY2 or Flag-WD5-7 by retrovirus system for 48 h. The interactions between WDFY2 or WD5-7 and endogenous INSR were analyzed by immunoprecipitation (IP) with a Flag-tag antibody and IB with INSR and Flag-tag

antibodies. The data represent the means ± SD. *p* values were calculated by two-way ANOVA analysis with Tukey's *post hoc* test (**$p < 0.01$, ***$p < 0.001$ versus the respective control, N.S., not significant).

Supplementary Fig. 6

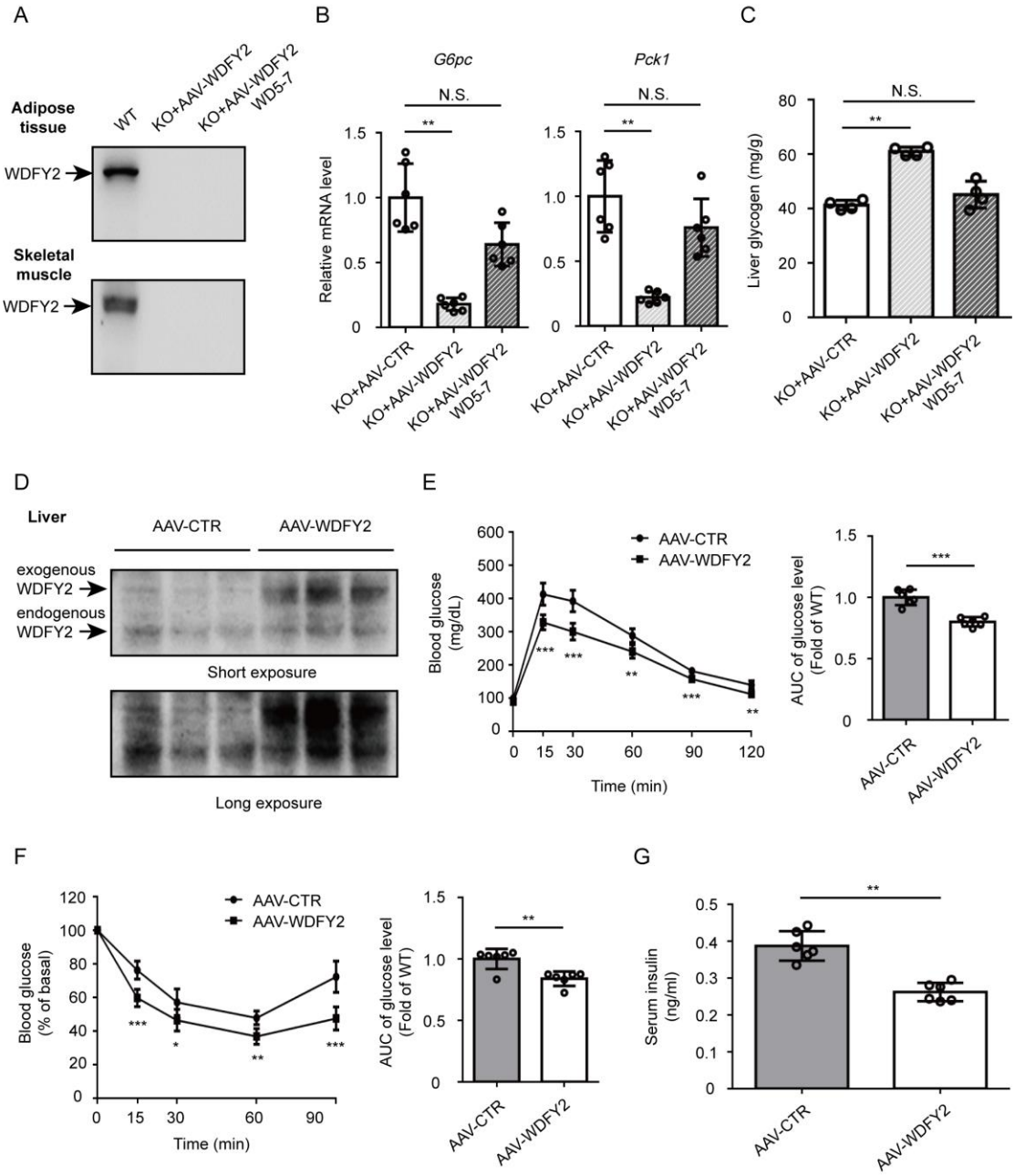


Supplementary Figure 6. WDFY2 has no direct interaction with IRS1/2 (related to Fig. 6.).

A: Wild-type (WT) and *Wdfy2* knockout (KO) H2.35 hepatocytes were stimulated with 10 mM insulin for 15 min or unstimulated before lysis. The stimulation-dependent interactions between WDFY2 or INSR and IRS1/2 were analyzed by IP with the indicated antibodies. Input data are shown as the expression controls, and p-Akt (Ser473) demonstrates insulin stimulation of the cells. *B*: Full-length GST-WDFY2 and His-IRS1 or His-IRS2 proteins were purified from *E.*

coli. The same molar quantities of the proteins were mixed and incubated overnight. All samples were resolved by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining or immunoblotting (IB) with the indicated antibodies (*, specific protein bands). *C* and *D*: *Wdfy2* KO H2.35 hepatocytes were transduced with an empty vector, Flag-WDFY2 or Flag-WD5-7 by retrovirus system for 48 h. WT and *Wdfy2* KO H2.35 hepatocytes were stimulated with 10 mM insulin for 5 min or unstimulated before lysis. Representative western blots (*C*) and the associated quantifications (*D*) show the interaction between INSR and IRS1 or IRS2 in hepatocyte lysates (n = 3). The data represent the means ± SD. *p* values were calculated by one-way ANOVA with Tukey's *post hoc* test (***$p < 0.001$ versus respective control, N.S., not significant).

Supplementary Fig. 7



Supplementary Figure 7. Hepatic WDFY2 over-expression ameliorates glucose utilization in both insulin-resistant and healthy animals (related to Fig. 7.).

A: Immunoblotting (IB) to detect the protein levels in the adipose and skeletal muscle tissues of *Wdfy2* KO mice at day 21 after infection with an adeno-associated virus encoding WDFY2 or a WD5-7 mutant. Protein from wild-type (WT) mice was used

as a positive control. *B*: Real-time PCR analysis of *G6pc* and *Pck1* mRNA levels in *Wdfy2* KO, *Wdfy2* KO with WDFY2 overexpressed and *Wdfy2* KO with WD5-7 overexpressed liver extracts in mice that were fasted overnight and then allowed to refeed for 4 h. The data are normalized to β-actin (n = 6 for each group). *C*: Hepatic glycogen content, determined by hydrolysis in livers obtained from *Wdfy2* KO, *Wdfy2* KO overexpressed and *Wdfy2* KO with WD5-7 overexpressed in mice that were fasted overnight and then allowed to refeed for 4 h (n = 4 for each group). *D*: 2-month-old wild-type (WT) mice were infected with an adeno-associated virus encoding WDFY2. Immunoblotting (IB) to detect protein levels of endogenous and exogenous WDFY2 in the liver tissue of mice at day 21 after infection. *E* and *F*: Glucose tolerance (*E*) and insulin tolerance (*F*) tests were performed in 3-month-old WT and WT with WDFY2 overexpressed mice at the indicated time points (left). The area under the curve (AUC) for the glucose tolerance tests (right) is shown (n = 6 mice for each group). *G*: Serum insulin levels in 3-month-old WT and WT with WDFY2 overexpressed mice under fasting conditions (n = 6 mice for each group). The data represent the means ± SD. *p* values were calculated by one-way ANOVA with Tukey's *post hoc* test (*B* and *C*) and Student's t test (*E*-*G*) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the respective control, N.S., not significant).

Supplementary Tables

Supplementary Table S1. Antibodies used for this study.

Antibody	Company	Cat. No.
WDFY2	Thermo Fisher	PA5-67604
MYC	MBL	M047-3
GFP	MBL	M048-3
HIS	MBL	PM032
FLAG	Sigma-Aldrich	F3165
GST	APPLYGEN	C1303
β-Actin	Santa Cruz	sc-47778
Rab5	Abcam	ab218624
Caveolin-1	Abcam	Ab2910
phospho-Erk1/2 (Thr202/Tyr204)	Cell Signaling	4370
phospho-Akt (Ser473)	Cell Signaling	4060
phospho-Akt (Thr308)	Cell Signaling	4056
phospho-Akt1 (Ser473)	Cell Signaling	9018
phospho-Akt2 (Ser474)	Cell Signaling	8599
phospho-FoxO1 (Ser256)	Cell Signaling	9461
phospho-Gsk-3β (Ser9)	Cell Signaling	9323
phospho-TSC2 (Thr1462)	Cell Signaling	3617
phospho-PRAS40 (Thr246)	Cell Signaling	2997
Erk1/2	Cell Signaling	4695
Akt	Cell Signaling	9272
Akt1	Cell Signaling	2938
Akt2	Cell Signaling	3063
FoxO1	Cell Signaling	2880
Gsk-3β	Cell Signaling	9315
PRAS40	Cell Signaling	2691
INSRβ	Cell Signaling	3020
IRS1	Cell Signaling	3407
IRS2	Cell Signaling	3089
phospho-Tyr-100	Cell Signaling	9411
TSC2	Proteintech	20004-1-AP

Supplementary Table S2. Primers used for genotyping.

The wild-type allele was detected using the following primers:

A1-Loxp-F	5’-CTCTCTGGTAGCTCAGTTGTCC-3’
A2-Loxp-R	5’-GCTTTGTGCTGTTACAGCTTTGA-3’

The deleted allele was detected using the following primers:

3'-Loxp-F	5’-CTCTCTGGTAGCTCAGTTGTCC-3’
3'-Loxp-R	5’-CCCTCAAGACCTACAATCAATGGATCTAAC-3’

Supplementary Table S3. Primers used for real-time PCR.

Transcript	Primers
Wdfy2	forward: 5’-CTTCAGAGGGCACACAGGA-3’ reverse: 5’-CGGAACTGCCTGAGAATAGC-3’
G6pc	forward: 5’-TCTGTCCCGGATCTACCTTG-3’ reverse: 5’-GAAAGTTTCAGCCACAGCAA-3’
Pck1	forward: 5’-CTGCATAACGGTCTGGACTTC-3’ reverse: 5’-CAGCAACTGCCCGTACTCC-3’
Actb	forward: 5’-CTAAGGCCAACCGTGAAAAG-3’ reverse: 5’-ACCAGAGGCATACAGGGACA-3’