Science Advances
AAAS

Supplementary Materials for

TSC22D4 interacts with Akt1 to regulate glucose metabolism

Sevgican Demir *et al.*

Corresponding author: Bilgen Ekim Üstünel, bilgen.ekimuestuenel@med.uni-heidelberg.de

Sci. Adv. **8**, eabo5555 (2022)
DOI: 10.1126/sciadv.abo5555

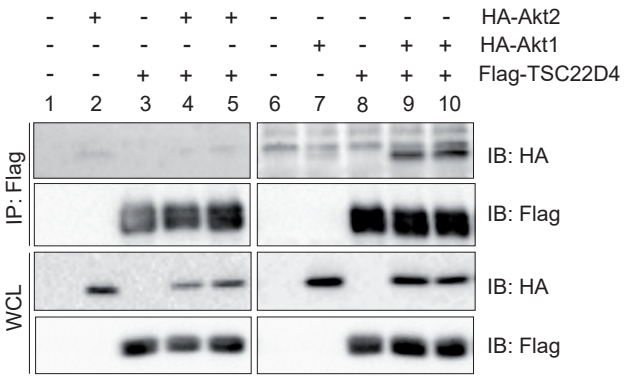
This PDF file includes:

Figs. S1 to S7

Supplementary Figure 1

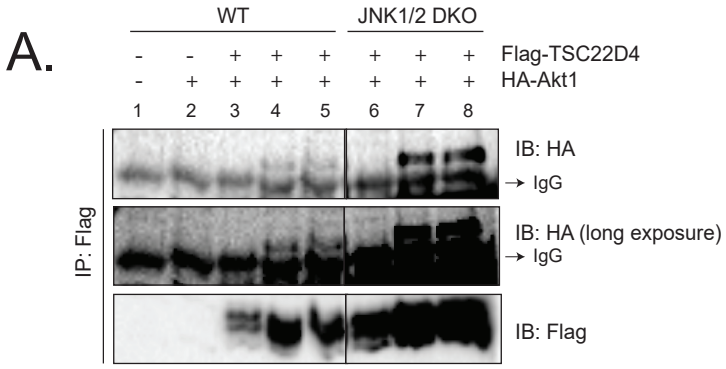
A.

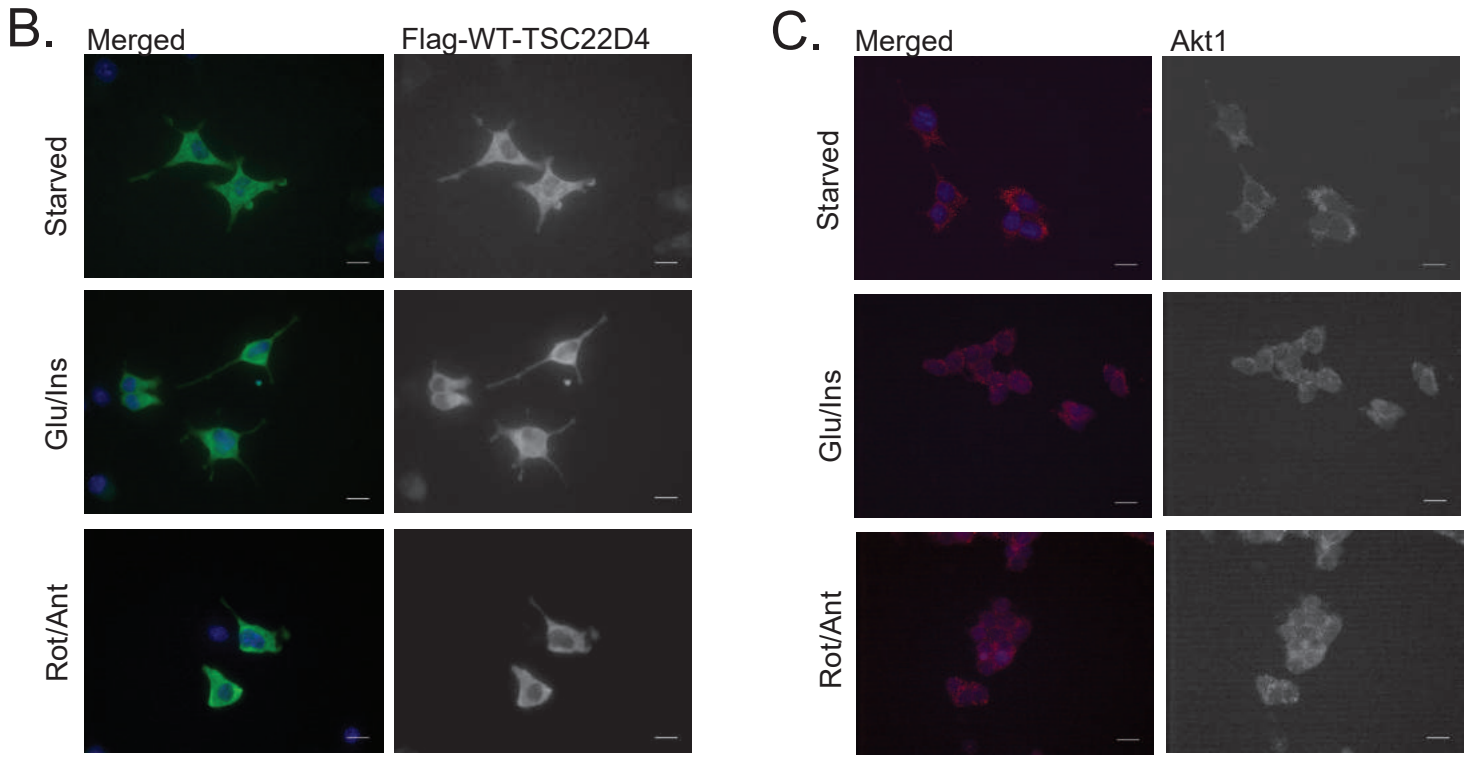
B.



Supplementary Figure 1. Evoutionarily conserved TSC22D4 protein interacts more specifically with Akt1. **A.** CLUSTAL O multiple sequence alignment analysis of TSC22D4. Highlighted in blue: Highly evolutionarily conserved regions on TSC22D4 primary amino acid sequence. Highlighted in pink: Leucine Zipper Motif. **B.** Hepa 1-6 cells were transiently transfected with vector control or co-transfected with Flag-TSC22D4 (2,5 µg) and HA-Akt1 or HA-Akt2 (2,5 µg) plasmids. 30 h posttransfection cells were serum and glucose starved overnight. After cell lysis the next day, Flag-TSC22D4 was immunopecipitated (IP) with anti-Flag affinity gel and the IPs and whole cell lysates (WCL) were immunblotted (IB) with indicated antibodies.

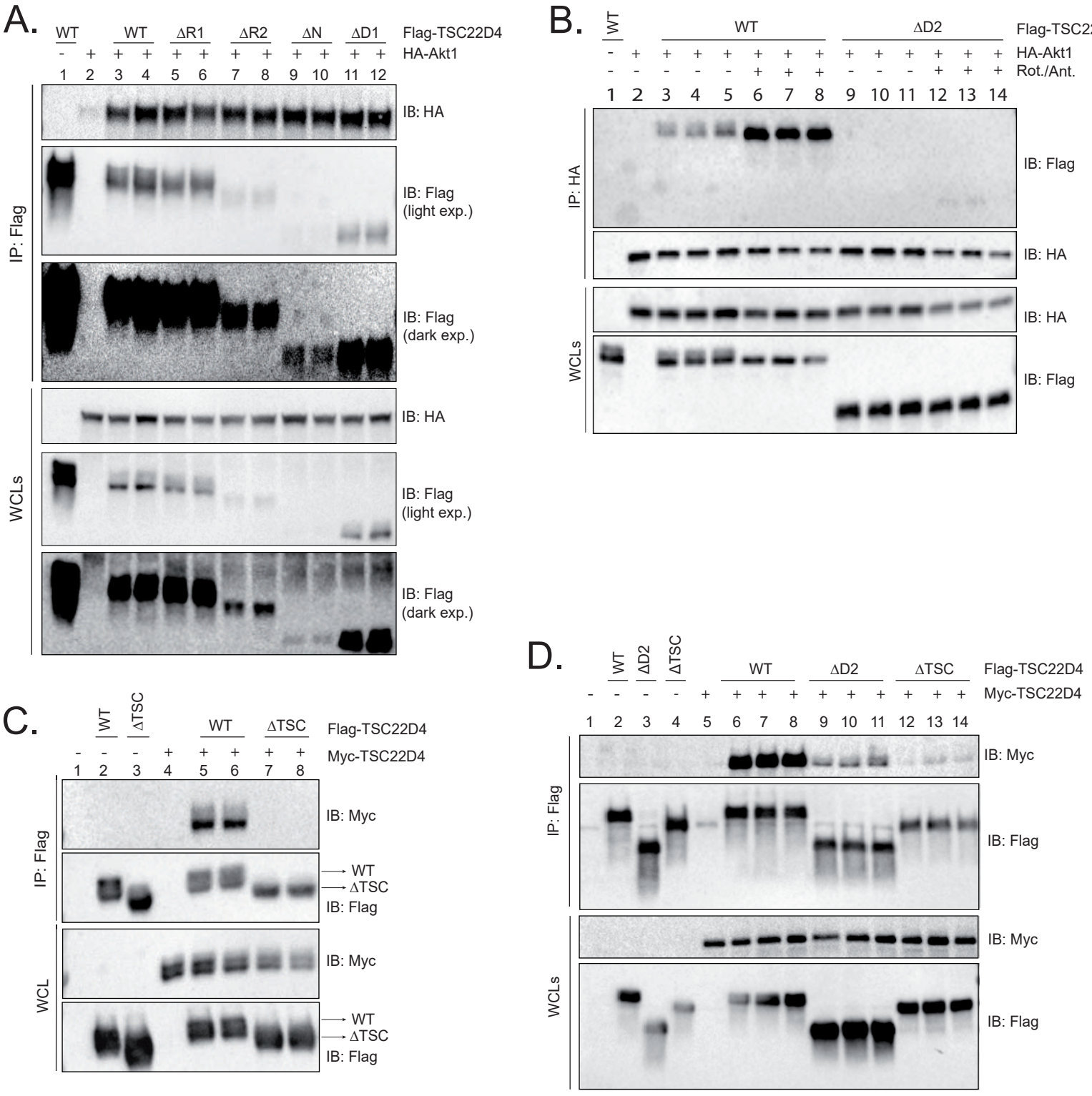
Supplementary Figure 2





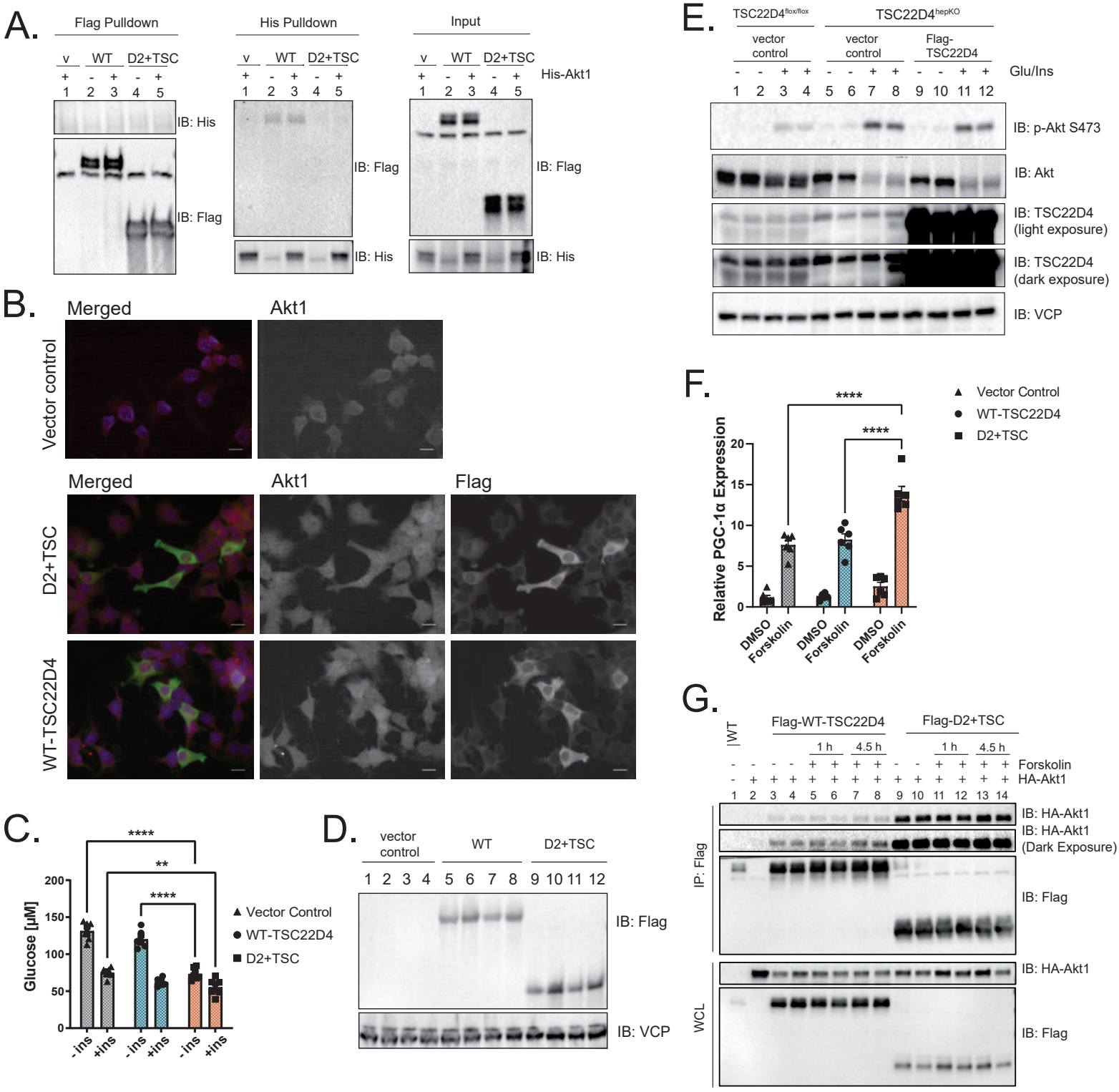
Supplementary Figure 2. TSC22D4-Akt1 interaction and TSC22D4, Akt1 subcellular localization remain unchanged in the absence of JNK1/2 and in response to metabolic and stress signals, respectively. A. Wild type (WT) or JNK1/2 double knockout (DKO) mouse embryonic fibroblasts (MEFs) were transiently co-transfected with Flag-TSC22D4-WT (2,5 µg) and HA-Akt1 (2,5 µg). Flag-TSC22D4 was immunoprecipitated (IP) with anti-Flag affinity gel and the IPs and WCL were immunblotted (IB) with indicated antibodies. **B and C.** Flag-TSC22D4 transfected **(B)** or not transfected Hepa 1-6 **(C)** cells were grown on chamber slides and starved overnight and treated either with glucose [20 mM]/insulin [100 nM] or rotenone [1 µM]/antimycin [1 µM] for 1 hour the following day. Next cells were fixed, permeabilized, blocked (5% horse serum in PBS) and stained with anti-Flag **(B)** or Akt1 **(C)** antibody. Alexa Fluor 488 (anti-Flag) or Alexa Fluor 568 (anti-Akt1) were used as secondary antibodies. Mounting medium with DAPI was used to detect the nucleus (blue). Zeiss fluorescence microscope and ZEN software was used to capture the images (40x, scale bar: 20 µM). Fiji, Image J was used for analyzing the images.

Supplementary Figure 3



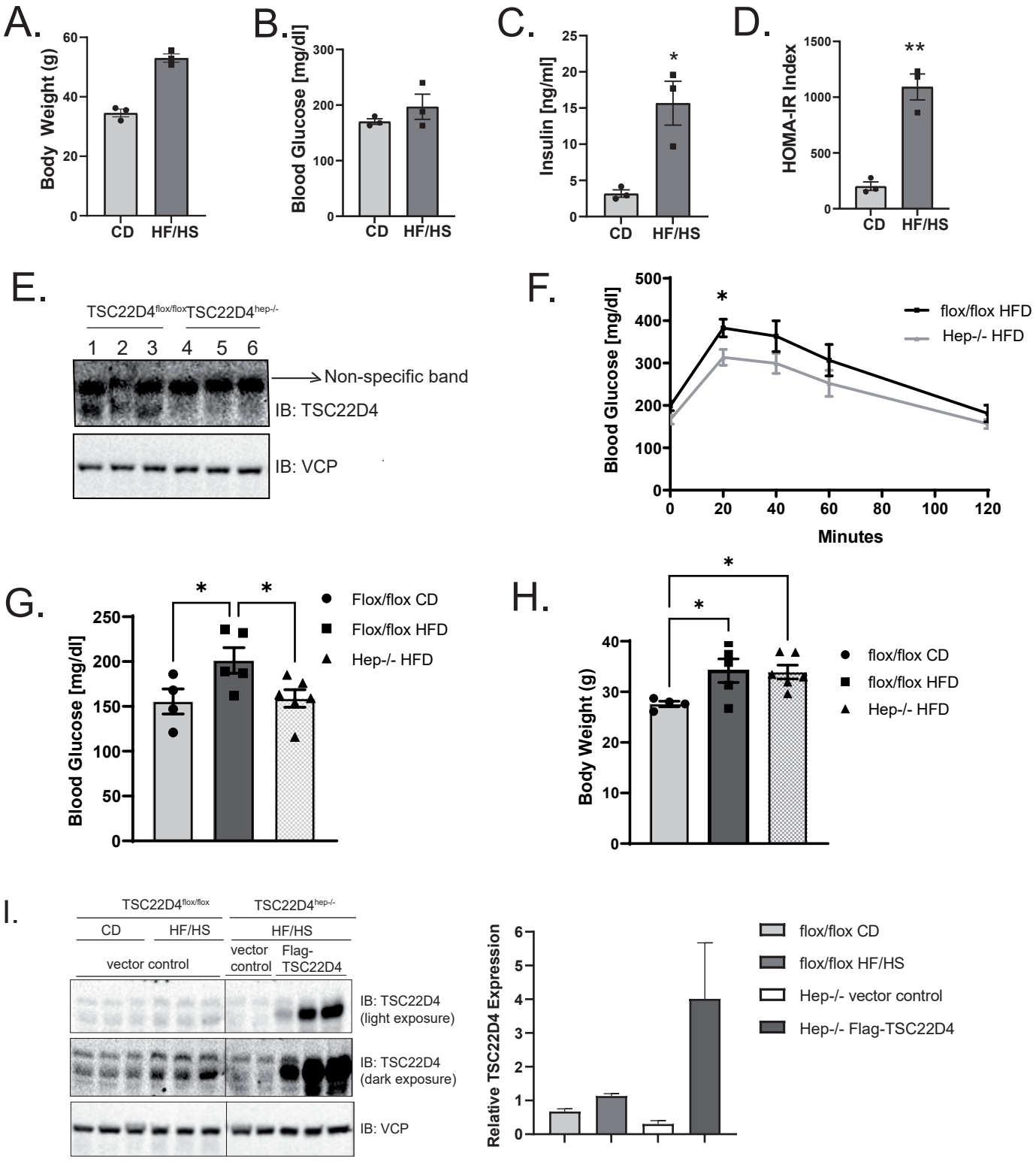
Supplementary Figure 3. Mapping the Akt1 interaction domains on TSC22D4. A. Hepa1-6 cells were transiently co-transfected with Flag-TSC22D4-WT (2,5 µg), -ΔR1 (2,5 µg), -ΔR2 (2,5 µg), -ΔN (2,5 µg) or -ΔD1 (2,5 µg) deletion mutants and HA-Akt1(2,5 µg). Flag-TSC22D4 was immunopecipitated (IP) with anti-Flag affinity gel and the IPs and WCLs were immunblotted (IB) with indicated antibodies. **B.** Hepa1-6 cells were transiently co-transfected with Flag-TSC22D4-WT (2,5 µg) or -ΔD2 (2,5 µg) and HA-Akt1 (2,5 µg). 30 h post transfection, cells were serum and glucose starved overnight and the next day, they were treated with DMSO vehichle control or with Rotenone/Antimycin [1 µM] for 1 h prior to cell lysis. IPs were performed with anti-HA antibody. IPs and WCLs were immunblotted (IB) with indicated antibodies. **C.** Hepa1-6 cells were transiently co-transfected with Flag-TSC22D4-WT (2,5 µg) or -ΔTSC (2,5 µg) and Myc-TSC22D4-WT (2,5 µg). Flag-TSC22D4 alleles were immunoprecipitated with anti-Flag affinity gel (IP) and WCLs were immunoblotted (IB) with indicated antibodies. **D.** Hepa1-6 cells were transiently co-transfected with Flag-TSC22D4-WT (2,5 µg), -ΔD2 (2,5 µg) or -ΔTSC (2,5 µg) and Myc-TSC22D4-WT (2,5µg). Flag IPs and WCLs were immunoblotted (IB) with indicated antibodies.

Supplementary Figure 4



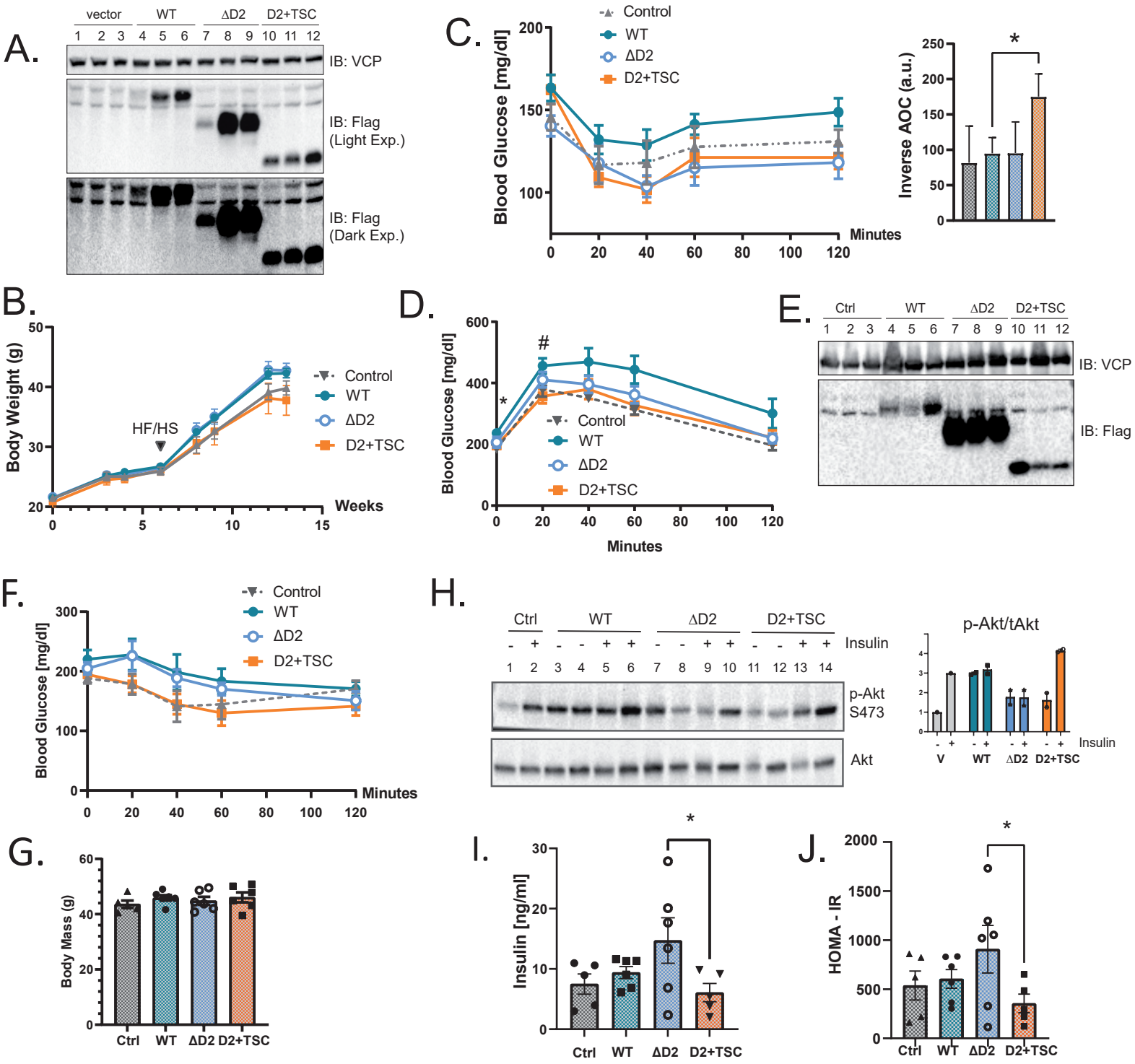
Supplementary Figure 4. Investigating the function of TSC22D4 D2+TSC mutant in primary hepatocytes.
A. In vitro binding assay: Lysates of cells that were transiently transfected with vector control, Flag-TSC22D4-WT or the Flag-D2+TSC alleles were incubated with or without recombinant His-Akt1 followed by a pull down reaction with anti-His or anti-Flag antibodies. The pulldown reactions and the input were immunblotted (IB) with indicated antibodies. **B.** Subcellular localization of Akt1 in Hepa 1-6 cells that are transiently transfected with vector control, Flag-WT-TSC22D4 or Flag-D2+TSC. **C.** Glucose secretion assay with TSC22D4 KO primary hepatocytes transduced with adenoviruses containing vector control, Flag-WT-TSC22D4 and Flag-D2+TSC alleles. **D.** Western blot analysis of Flag and VCP expression in primary hepatocytes transduced with vector control, Flag-WT-TSC22D4 and Flag- D2+TSC alleles for gluconeogenesis assay presented in Fig. 4C. **E.** Western blot analysis of flox/flox control wild type vs TSC22D4 KO primary hepatocytes transduced with indicated adenoviruses (AV). Cells were starved overnight before glucose [20 mM] and insulin [100 nM] stimulations the next day. **F.** WT or AV transduced TSC22D4 KO primary hepatocytes were starved overnight followed by DMSO or Forskolin [100 μM] treatment in the presence or absence of Akti 1/2 [5 μM] for 4.5 h. Cells were collected in Trizol for RNA isolation and gene expression was measured by qRT-PCR. **G.** Hepa1-6 cells were co-transfected with Flag-WT-TSC22D4 or Flag-D2+TSC together with HA-Akt1. The cells were starved overnight followed by a DMSO control or Forskolin [100 μM] treatment for 1 h or 4.5 h. IPs and whole cell lysates (WCLs) were immunoblotted with indicated antibodies.**Statistical Analysis (C, E-G):** Two-way ANOVA with Tukey's Multiple Comparisons Test. */#: p<0.05, **/## p<0.01, ***/### p<0.001, ****/####: p<0.0001.

Supplementary Figure 5



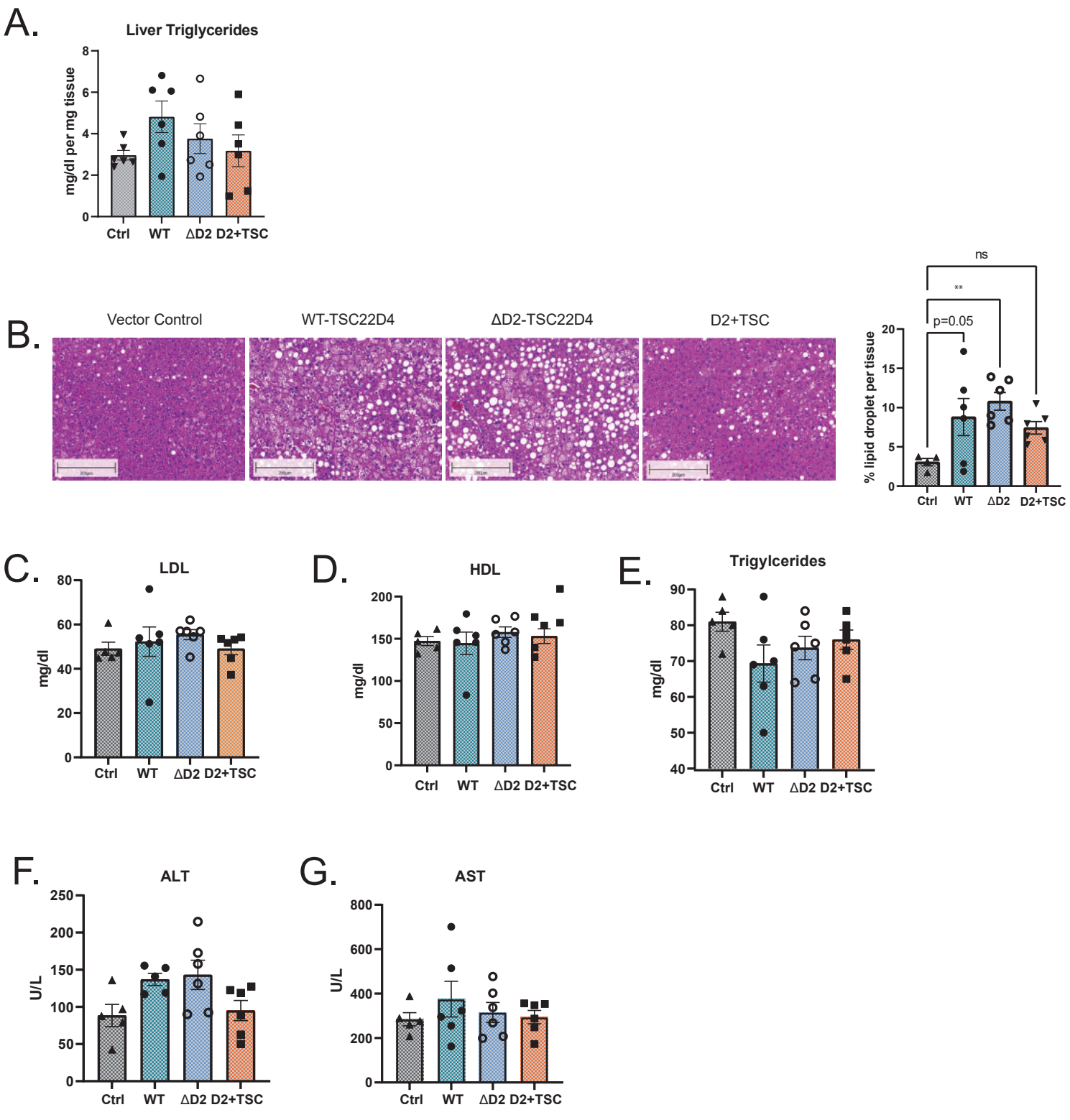
Supplementary Figure 5. **Hepatic loss of TSC22D4 improves glucose metabolism. A.** Body weight of mice fed with control diet (CD) or HF/HS diet for 26-27 weeks **B.** Blood glucose levels of mice as in A after a brief starvation for 3 h. **C.** Serum insulin levels of same mice as in A. **D.** HOMA-IR index of same mice as in A. **E.** Western blot analysis of the mouse liver lysates from TSC22D4$^{flox/flox}$ control mice or TSC22D4$^{hep-/-}$ mice immunoblotted with indicated antibodies. **F.** Intraperitoneal glucose tolerance test (i.p. GTT) performed with mice as in A subjected to high fat diet (HFD) for 6 weeks. Mice were fasted for 6 h of followed by glucose solution injection at a dose of 1g/kg mouse weight. n=5-6 per group. Statistical analysis: 2-way ANOVA with repeated measures and Fisher's LSD test. **G.** Fasting blood glucose levels of TSC22D4$^{flox/flox}$ and TSC22D4$^{hep-/-}$ mice on 9 weeks of control diet (CD) or HFD challenge . Statistical analysis One-way ANOVA and Fisher's LSD test. **H.** Body weight of mice as in C. Statistical analysis One-way ANOVA followed by Dunnet's multiple comparisons test. *: $p<0.05$ **I.** Western blot analysis of liver lysates from TSC22D4 flox/flox control mice on control vs. High Fat/High Sucrose (HF/HS) diet and injected with indicated adeno associated viruses (AAV). *Right*: TSC22D4 signal intensity (light exposure) was quantified and normalized to that of VCP.

Supplementary Figure 6



Supplementary Figure 6. AAV-mediated introduction of Flag-TSC22D4 alleles to TSC22D4$^{hep-/-}$ mice. A. Body weights of mice that were injected with adeno asscoiated viruses (AAVs) containing vector control, WT-TSC22D4, ΔD2-TSC22D4 or D2+TSC alleles and kept on chow diet for 6 weeks followed by a high fat/high sucrose (HF/HS) diet for 7 weeks (n=6). **B.** Liver lysates of mice in A were prepared at the end of the experiment and immunoblotted (IB) with indicated antibodies. **C.** ITT performed with mice as in A after 4 weeks of AAV administration. Mice were starved for 6 h in the morning follwed by an i.p. insulin solution injection [0.5 U/kg]. Right: Inverse Area of the Curve (AOC) was calculated by substracting basal blood glucose levels for each mice (n=6). **D.** GTT performed with mice as in A after 7 weeks of HF/HS diet. Glucose solution was i.p. injected [1g/kg] after mice were starved for 6 h in the morning.*: WT-TSC22D4 vs. vector control, #: WT-TSC22D4 vs. D2+TSC. **E.** Representative Western Blots for Flag-TSC22D4 expression from the liver lysates of mice challenged with HF/HS diet for 12 weeks (2nd cohort). **F.** ITT was performed in AAV injected mice after 12 weeks of HF/HS diet challenge (n=5-6). Mice were starved for 6 h in the morning and i.p. injected with insulin solution [1.3 U/kg]. **G** Body weights of mice injected with indicated AAVs and challenged with HF/HS diet for 12 weeks (n=5-6). **H.** Representative immunoblots of liver lysates of mice as in E, which were injected with control saline or insulin solution [1.3 U/kg] 10 minutes after a 6 h starvation. *Right:* Quantification of the p-Akt/Akt signal ratio. **I.** Serum insulin levels of mice as in I (n=5-6). **J.** HOMA-IR index of mice as in E (n=5-6). *Statistical Analysis: C. Kruskal Wallis Test followed by Dunnett's multiple comparison test D. Two-way ANOVA with repeated measures followed by Dunnett's multiple comparisons test. I, J: One-way ANOVA with Sidak's multiple comparisons test, */#: p<0.05, **/## p<0.001, **** p<0.0001*

Supplementary Figure 7



Supplementary Figure 7. Investigating the role of TSC22D4-Akt interaction on liver lipid metabolism. A. Liver lipids of mice injected with vector control, WT-, ΔD2-TSC22D4 or D2+TSC alleles were extracted and triglycerides were measured. **B.** H&E staining of liver sections of of AAV expressing TSC22D4$^{hep-/-}$ mice. Right: Quantification of lipid droplets. Statistical Analysis: One-way ANOVA followed by Dunnet's Multiple Comparisons Test **: p<0.01. **C, D, E, F, G.** Serum was collected from the AAV expressing TSC22D4$^{hep-/-}$ mice during the sacrifice and serum LDL, HDL, Triglyceride, ALT and AST levels were determined by serum analyzer