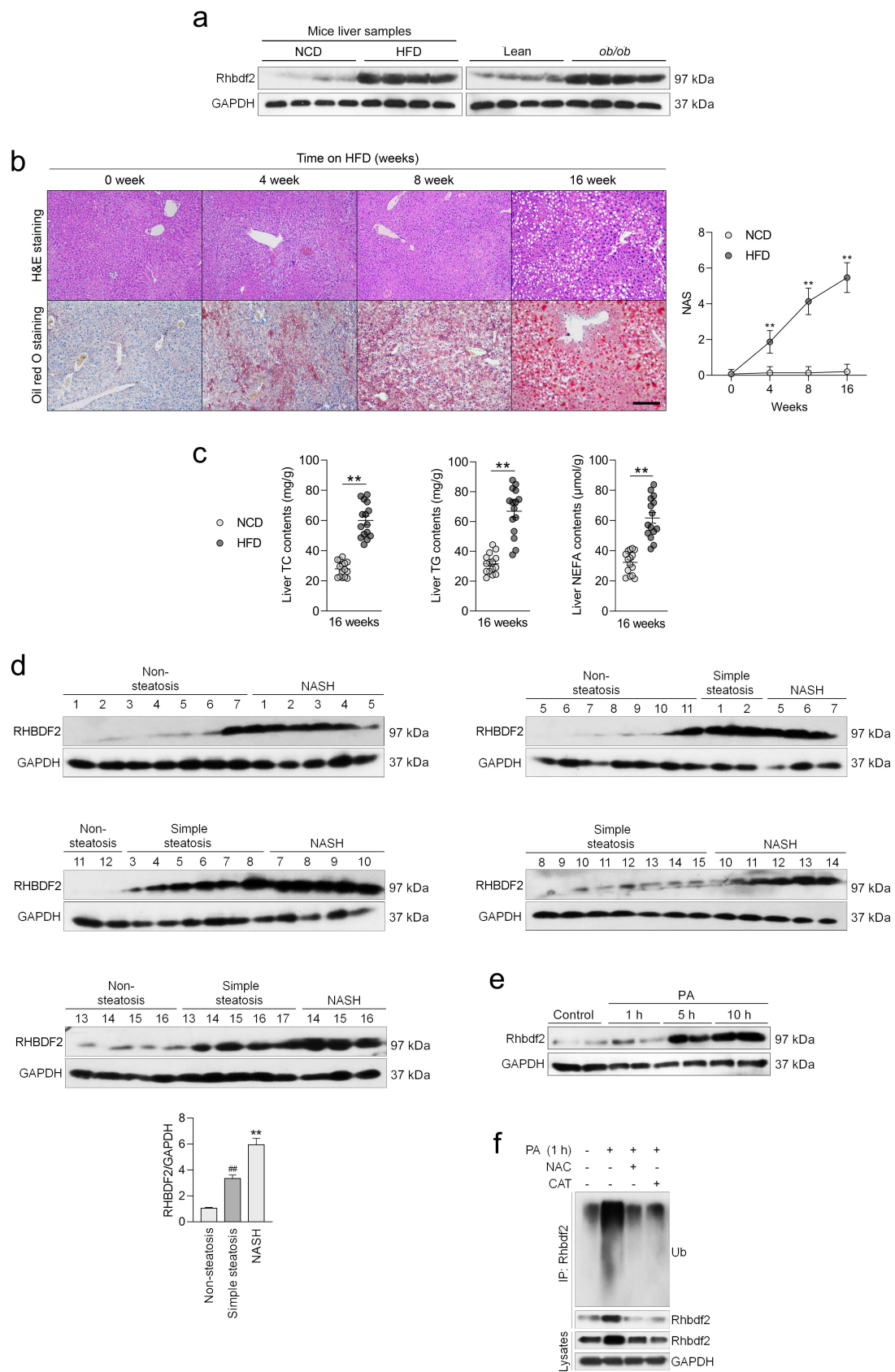
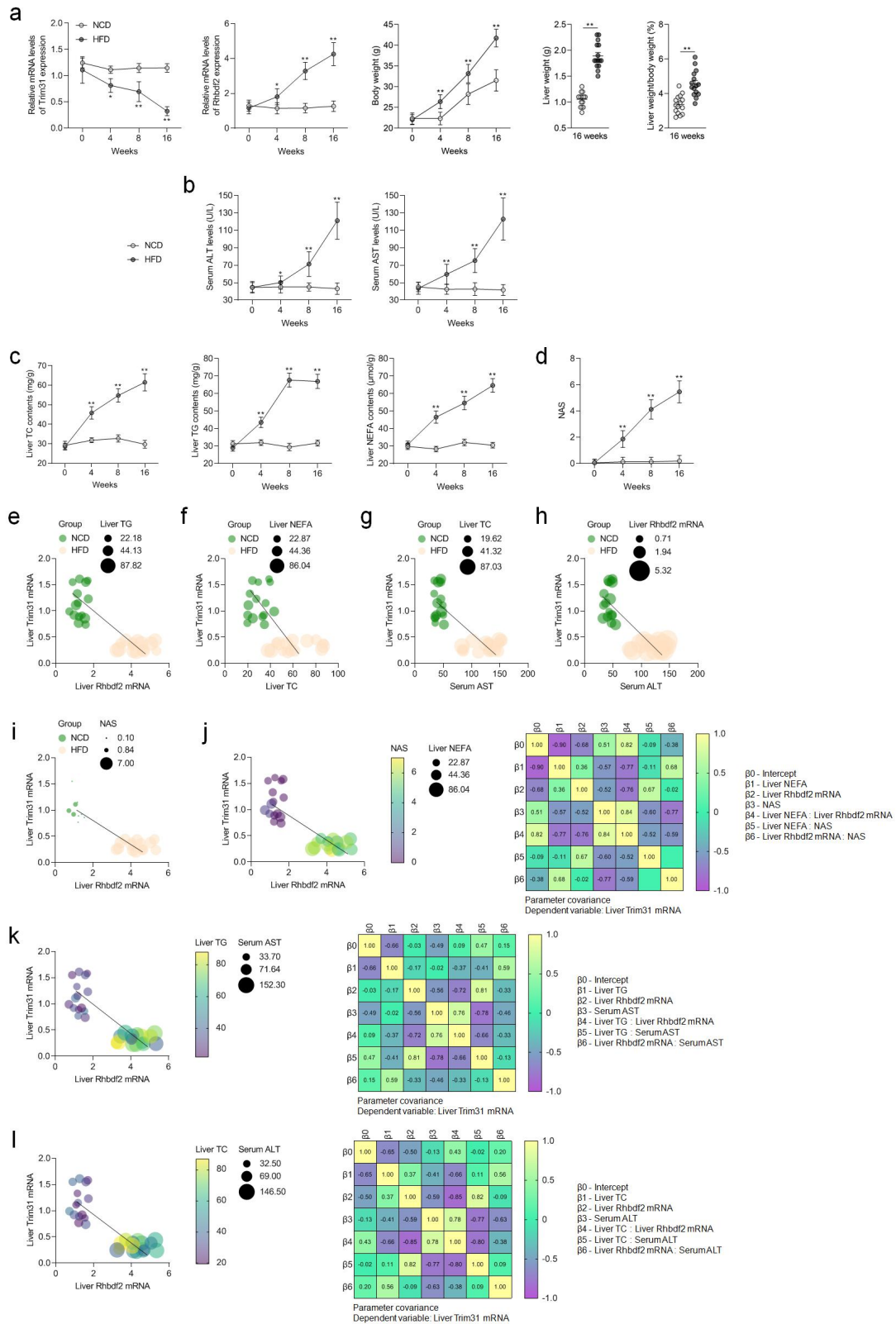


**The E3 ubiquitin-protein ligase Trim31 alleviates non-alcoholic fatty
liver disease by targeting Rhbdf2 in mouse hepatocytes**

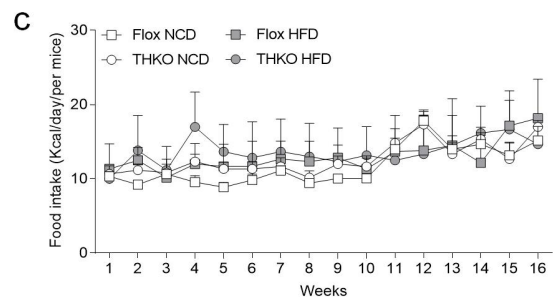
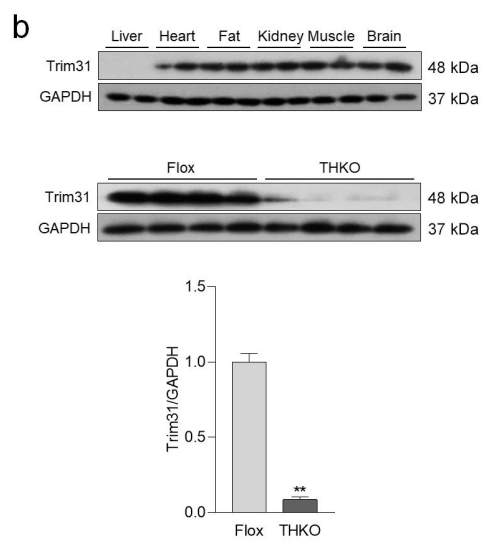
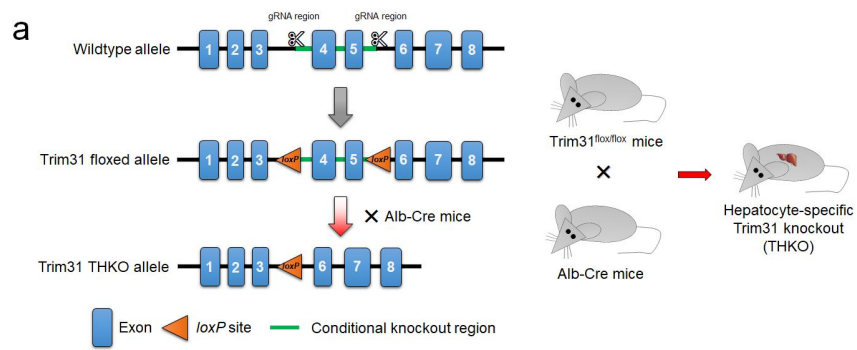
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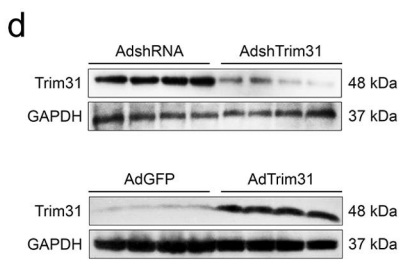
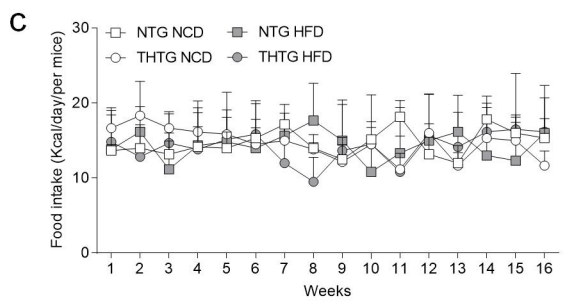
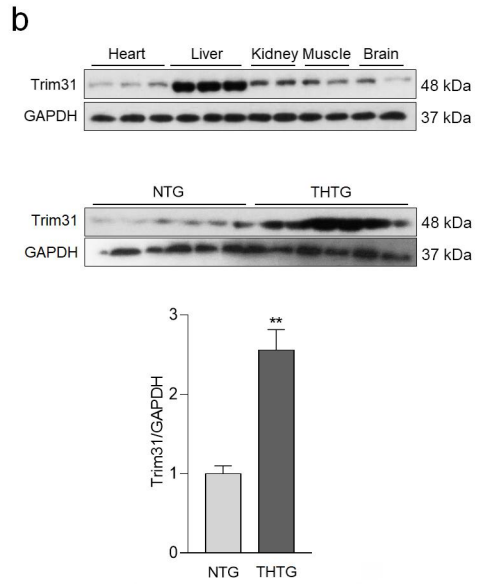
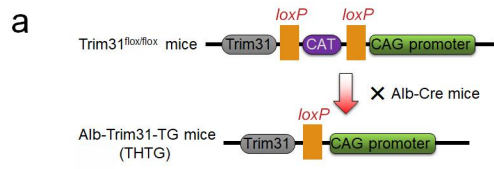
Supplementary Figure 1. Rhbdf2 is ubiquitinated, and Rhbdf2 protein expression is elevated in fatty liver. (a) Representative immunoblotting bands of Rhbdf2 expression in the liver samples isolated from C57BL/6N mice that were treated with a NCD or HFD for uninterrupted 16 weeks, or from *ob/ob* or lean mice ($n=4$ per experiment). (b) Representative images of H&E staining, Oil red O staining and NAS score evaluation for histological changes in the liver samples from HFD-fed mice (Magnification, 100 $\times$; $n=5$ images per group for each staining; $n=15$ for NAS score analysis) ($**P < 0.01$ vs. NCD groups). (c) Records for the liver lipid contents including TG, TC and NEFA after 16 weeks-HFD fed mice ($n=15$ per experiment) ($**P < 0.01$ vs. NCD groups). (d) Representative immunoblotting bands and relative expression levels of RHBDF2 in the liver samples of donors with non-steatosis ($n=16$ samples), simple steatosis ($n=17$ samples) or NASH ($n=16$ samples) phenotype ($**P < 0.01$ vs. simple steatosis groups, $^{##}P < 0.01$ vs. non-steatosis). (e) Representative immunoblotting bands of Rhbdf2 expression in cultured primary hepatocytes that were incubated with 400 μ M palmitate (PA). The BSA was treated as controls ($n=4$ per experiment). (f) Representative immunoblotting results for ubiquitinated Rhbdf2 in cultured primary hepatocytes. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis (b, c) and One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test (d).



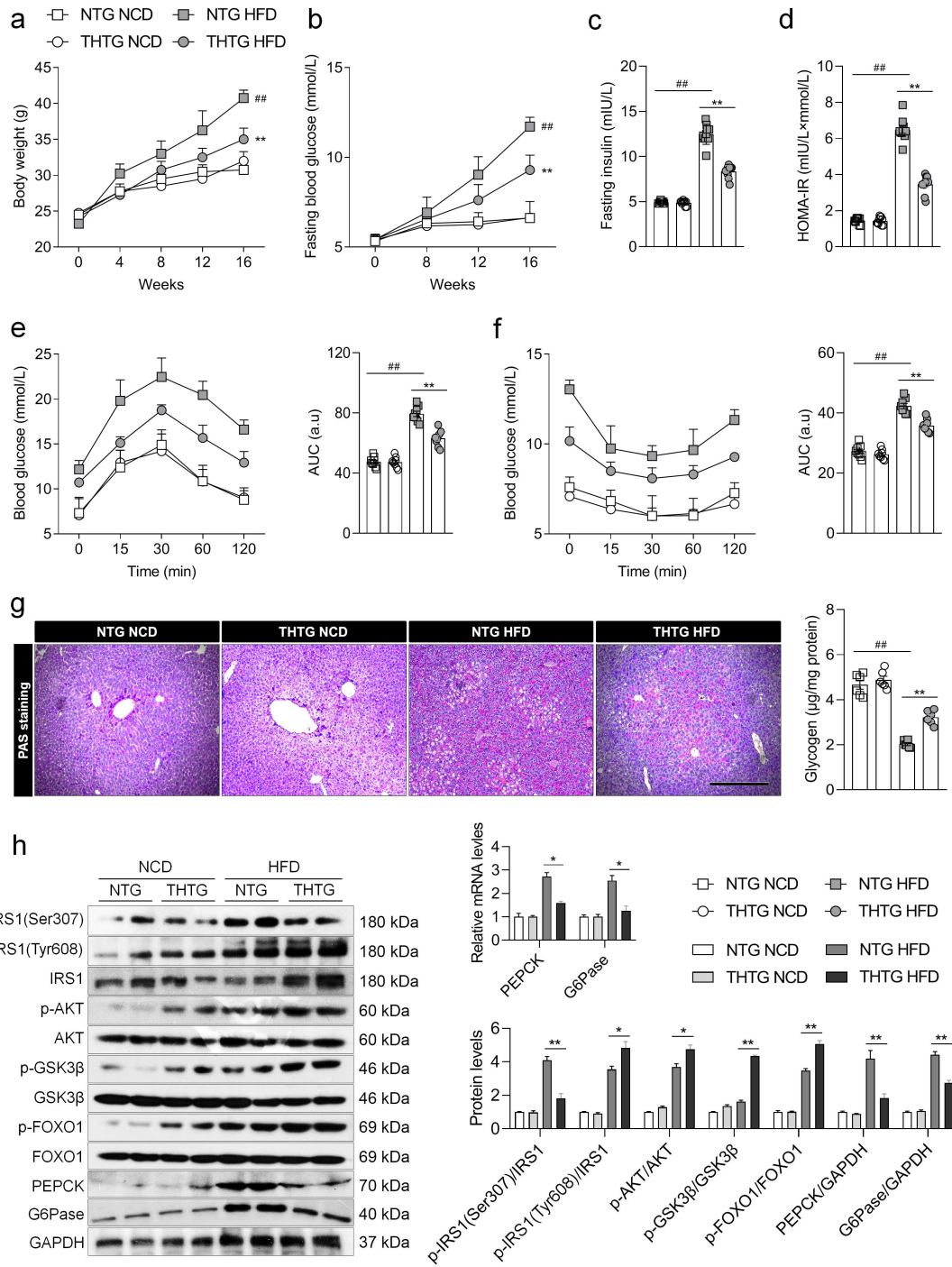
Supplementary Figure 2. Correlation analysis of Trim31 expression levels-related parameters in HFD-fed mouse model. (a-d) Analysis results of relative Trim31 and Rhbdf2 mRNA expression levels, body weight, liver weight and liver weight/body weight ratio (a), serum ALT and AST contents (b), liver TG, TC and NEFA levels (c), and NAS score analysis in the liver samples isolated from C57BL/6N mice that were treated with a HFD over time ($n=15$ mice for each time point) ($*P < 0.05$ and $**P < 0.01$ vs. NCD groups). (e-i) Pearson correlation analyses indicating the correlations between mice liver Trim31 mRNA levels and Rhbdf2 mRNA levels (e), liver Trim31 mRNA levels and liver TC contents (f), liver Trim31 mRNA levels and serum AST levels (g), liver Trim31 mRNA levels and serum ALT levels (h), and liver Trim31 mRNA levels and NAS score (i), mRNA expression is normalized to GAPDH levels. $P < 0.001$ for all of these correlations ($n=15$ per indicator for each group; $n=90$ in total). (j-l) Multiple linear regression showing the correlations between alteration of mice liver samples Trim31 and Rhbdf2 mRNA levels and parameter indexes associating with liver NEFA/NAS score (j), liver TG/serum AST (k), and liver TC/serum ALT. Relative liver Trim31 mRNA expression is treated as dependent variable. $P < 0.001$ for all of these correlations ($n=15$ per indicator for each group; $n=120$ in total). Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis.



Supplementary Figure 3. Liver-specific Trim31 deletion is unable to significantly alter energy consumption during HFD administration. (a) Schematic diagram showing the protocol for the generation of the hepatocyte-specific Trim31 deletion (THKO) mice. (b) Representative western blotting for the Trim31 expression in different organs (upper), and expression in the liver samples from Trim31-Flox and hepatocyte-specific Trim31 deletion (THKO) mice (lower) ($n=4$ per experiment) ($**P < 0.01$ vs. Flox groups). (c) Records for the food intake (Kcal/day/per mice) of indicated mice in a 16-weeks HFD treatment (no significant difference). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis.

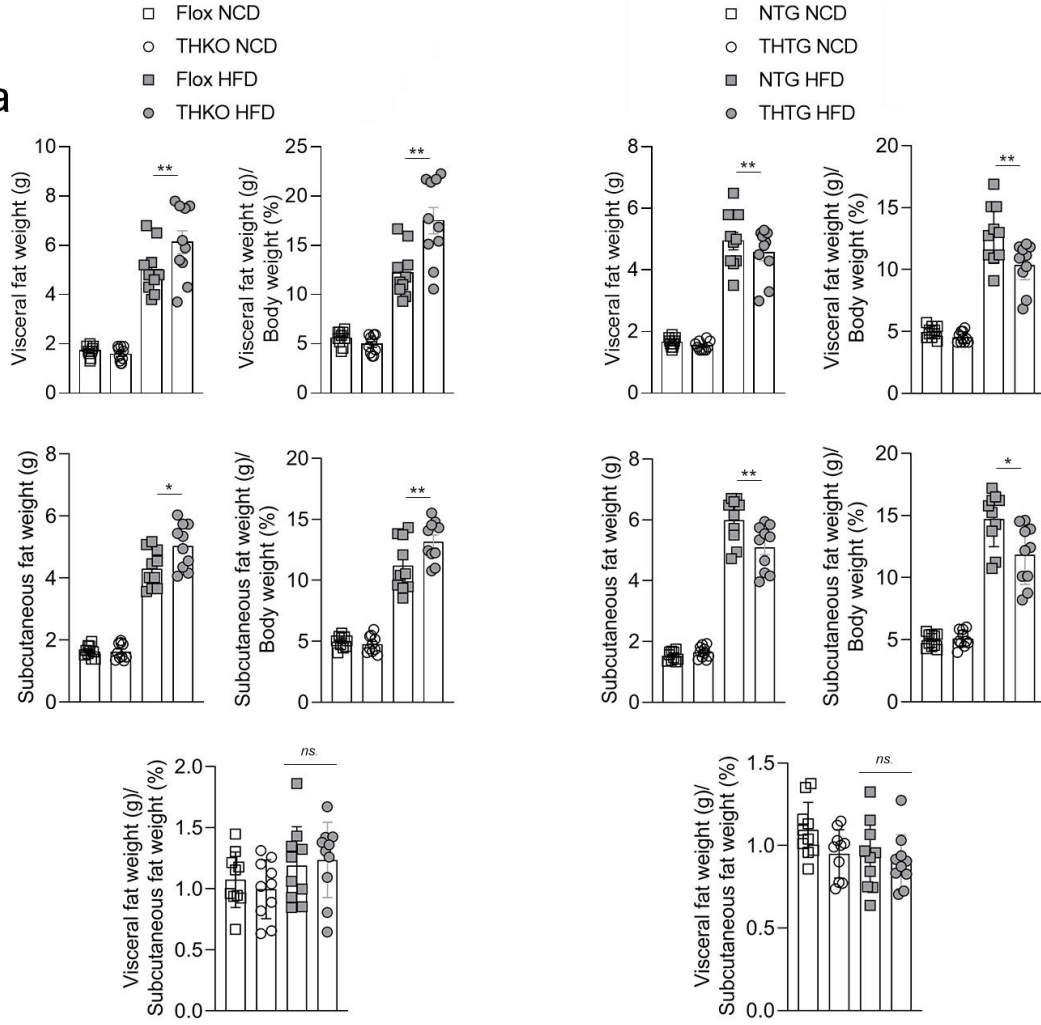


Supplementary Figure 4. Liver-specific Trim31 overexpression cannot change energy consumption during HFD treatment. (a) Schematic diagram showing the protocol for the generation of the hepatocyte-specific Trim31 transgenic (THTG) mice. (b) Representative western blotting for the Trim31 expression in different organs (upper) and in the liver samples from non-transgenic Trim31 (NTG) and THTG mice (lower) ($n=6$ per experiment) ($**P < 0.01$ vs. NTG groups). (c) Records for the food intake (Kcal/day/per mice) of indicated mice in a 16-weeks HFD treatment (no significant difference). (d) Representative western blotting for the Trim31 expression efficiency in AdshTrim31 or AdTrim31-transfected primary hepatocytes ($n=4$ per experiment). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis.

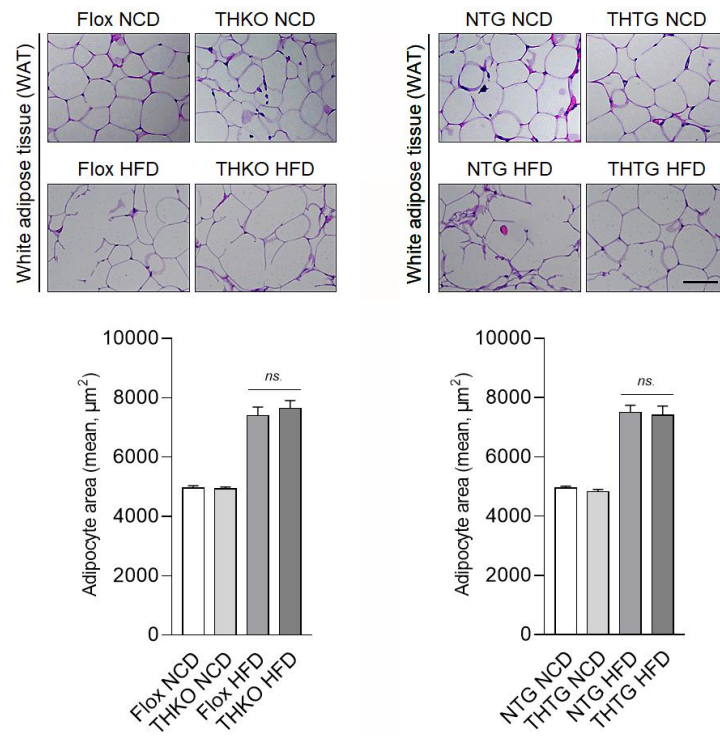


Supplementary Figure 5. Hepatocyte-specific Trim31 overexpression ameliorates HFD-triggered pathological phenotypes of insulin resistance. (a, b) Body weight records (a) and fasting blood glucose levels (b) of non-transgenic Trim31 (NTG) and hepatocyte-specific Trim31 transgenic (THTG) mice during 16 weeks of NCD or HFD administration ($n=15$ mice) ($**P < 0.01$ vs. NTG HFD groups, $^{##}P < 0.01$ vs. NTG NCD groups). (c, d) Fasting insulin levels (c) and corresponding HOMA-IR index (d) of NTG and THTG mice at the last week of NCD or HFD feeding ($n=10$ mice) ($**P < 0.01$ vs. NTG HFD groups, $^{##}P < 0.01$ vs. NTG NCD groups). (e, f) Records for the glucose tolerance test (GTT) (e) and insulin tolerance test (ITT) (f) in the NTG and THTG mice at the last week of NCD or HFD ingestion; a.u, arbitrary unit ($n=10$ mice) ($**P < 0.01$ vs. NTG HFD groups, $^{##}P < 0.01$ vs. NTG NCD groups). (g) Representative images of Periodic Acid-Schiff stain (PAS)-stained pathological section for glycogen storage changes in the liver samples from the NTG and THTG mice (Magnification, 100 $\times$; $n=6$ images per group for each staining) ($**P < 0.01$ vs. NTG HFD groups, $^{##}P < 0.01$ vs. NTG NCD groups). (h) Representative immunoblotting bands for expression alterations of total amounts and phosphorylated forms of critical indicators involving in insulin signaling, including IRS1, AKT, GSK3 β , FOXO1, PEPCK and G6Pase, in the liver of NTG and THTG mice after HFD feeding for 16 weeks ($n=4$ per experiment) ($*P < 0.05$ and $**P < 0.01$ vs. NTG HFD groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by two-way analysis of variance with general linear model procedures using a univariate approach.

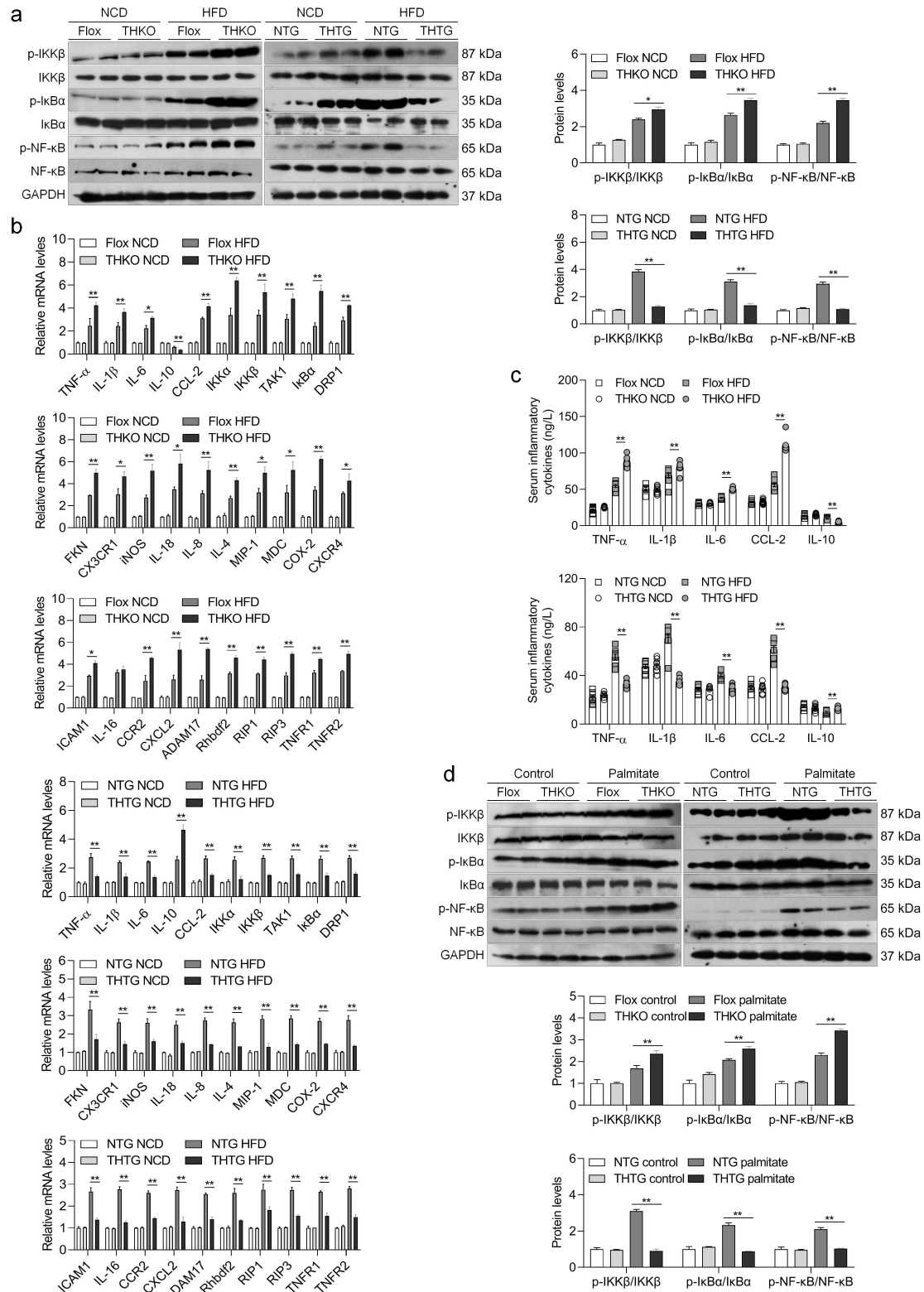
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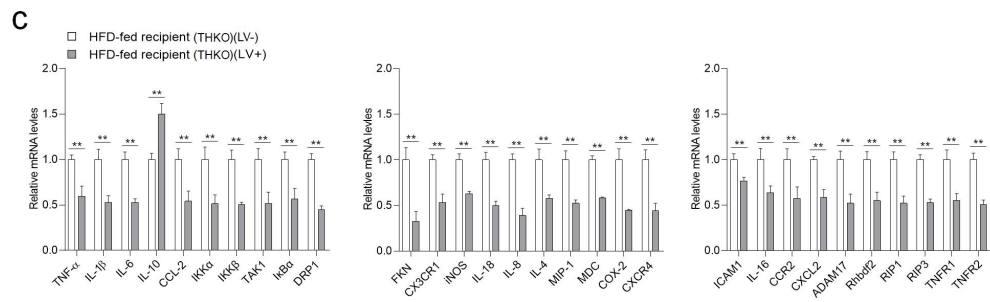
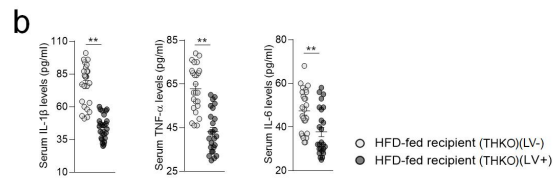
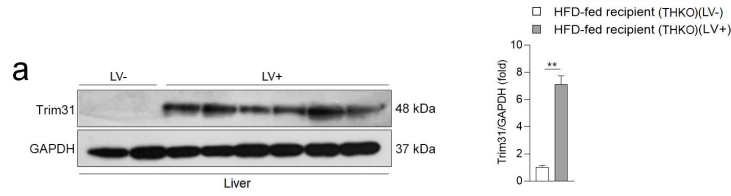
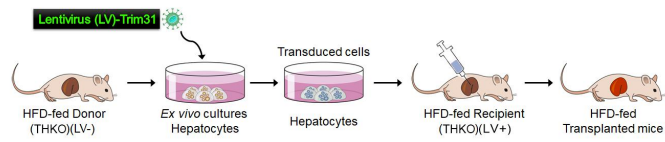
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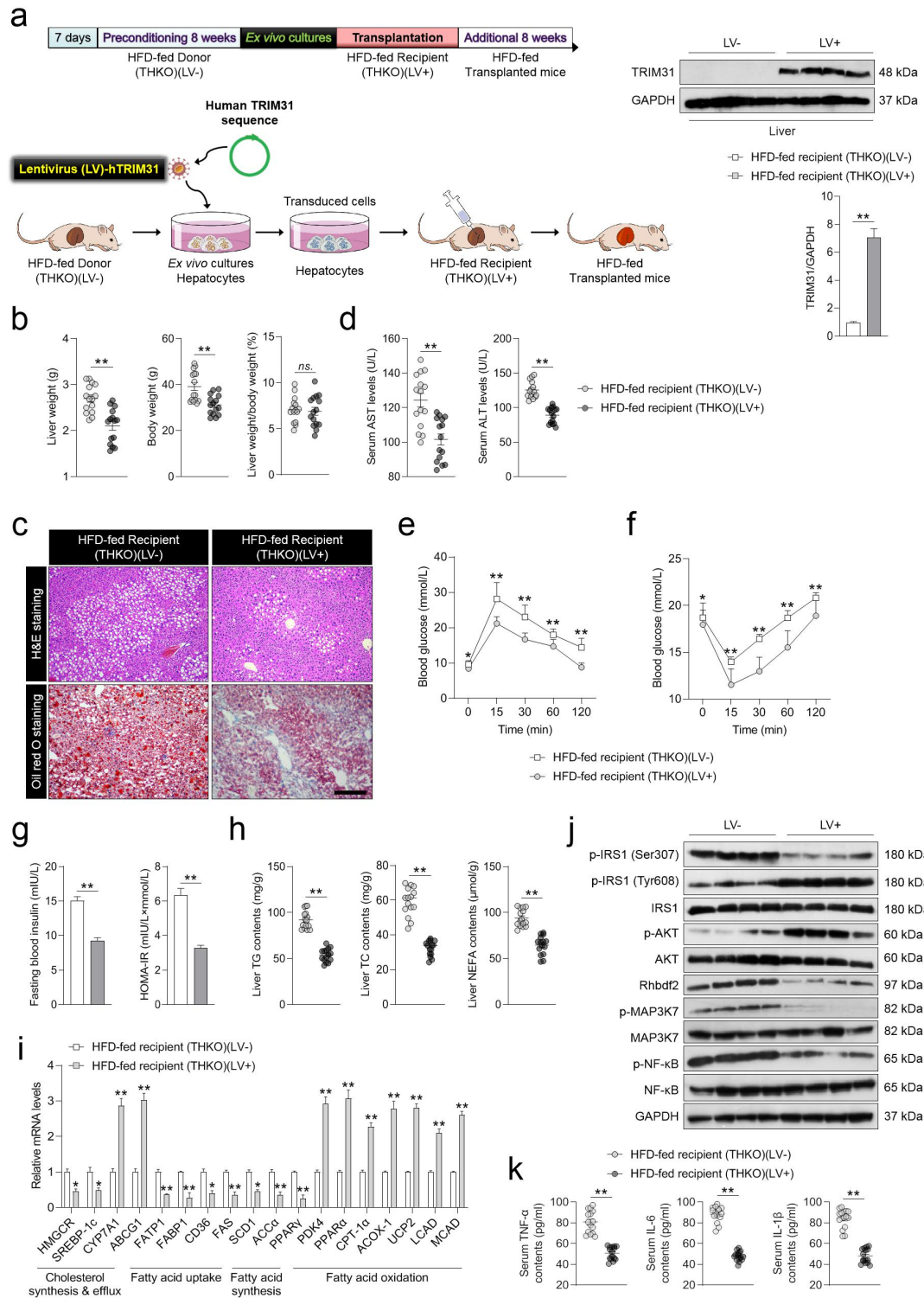
Supplementary Figure 6. Trim31 deficiency promotes weight gain of adipose tissue to facilitate HFD-triggered mice obesity. (a) Visceral fat weights, visceral fat weight/body weight ratio, subcutaneous fat weights, subcutaneous fat/body weight ratio and visceral fat weight/subcutaneous fat weight ratio in the hepatocyte-specific Trim31 deletion (THKO) mice, hepatocyte-specific Trim31 transgenic (THTG) mice, and their corresponding controls (Flox- and NTG-mice) after a 16-weeks of HFD or NCD treatment ($n=10$ mice per group) (* $P < 0.05$ and ** $P < 0.01$ vs. NTG HFD groups or Flox HFD groups, *ns.*, no significant difference). (b) The representative H&E staining pictures and the analyzed adipocytes area (μm^2) on the white adipose tissue (WAT) sections of mice in the indicated groups (Magnification, 200 $\times$; $n=10$ images per group for each staining) (*ns.*, no significant difference). Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by one-way ANOVA followed by Dunnett's multiple comparisons test.



Supplementary Figure 7. Functional loss of hepatic Trim31 aggravates HFD-induced liver inflammation. (a) Representative immunoblotting bands for expression alterations of total amounts or phosphorylated forms of critical indicators associating with inflammation signaling including IKK β , p-IKK β , I κ B α , p-I κ B α , NF- κ B and p-NF- κ B in the liver samples from THKO-, THTG-, NTG-, Flox mice after 16-weeks HFD or NCD feeding ($n=4$ per experiment). (b) qPCR analysis of the relative mRNA expression of genes associated with inflammation signaling in the liver samples from THKO-, THTG-, NTG-, Flox mice after 16-weeks HFD or NCD feeding ($n=10$ liver samples per group) (* $P < 0.05$ and ** $P < 0.01$ vs. Flox HFD groups or NTG HFD groups). (c) Representative pro-inflammatory cytokines and chemokines levels including TNF- α , IL-1 β , IL-6, CCL-2, and IL-10 in serum from THKO-, THTG-, NTG-, Flox mice after 16-weeks HFD or NCD feeding ($n=10$ mice per group) (* $P < 0.05$ and ** $P < 0.01$ vs. Flox HFD groups or NTG HFD groups). (d) Representative immunoblotting bands for expression changes of total amounts or phosphorylated forms of critical indicators associating with inflammation signaling including IKK β , p-IKK β , I κ B α , p-I κ B α , NF- κ B and p-NF- κ B in primary hepatocytes isolated from the Flox-, THKO- NTG- and THTG-mice that were incubated with 400 μ M palmitate (PA) for 10 hours ($n=4$ per experiment) (** $P < 0.01$ vs. Flox palmitate groups or NTG palmitate groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by one-way ANOVA followed by Dunnett's multiple comparisons test.



Supplementary Figure 8. Allogeneic hepatocyte transplantation using lentivirus-mediated Trim31 expression mitigates HFD-triggered liver inflammation. Preconditioned liver-specific Trim31 deletion (THKO) mice with an 8-weeks HFD treatment as donor were transduced (THKO)(LV-). The hepatocytes isolated from (THKO)(LV-) group mice were transduced with lentivirus-loaded full-length Trim31 sequence. The corresponding blank vector were transduced as controls. Then, the additional HFD-fed THKO mice as recipient were injected with transduced hepatocytes via portal vein. The HFD-fed transplanted (THKO)(LV+) mice were harvested for the further experimental detection. **(a)** Increased Trim31 expression in liver tissue after transplantation were detected by western blotting ($n=6$ per experiment) ($**P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(b)** Representative pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 in serum from HFD-fed transplanted (THKO)(LV+) and HFD-fed transplanted (THKO)(LV-) mice after 16-weeks HFD feeding ($n=25$ mice per group) ($**P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(c)** qPCR analysis of the relative mRNA expression of genes associated with inflammation signaling in the liver samples from HFD-fed transplanted (THKO)(LV+) and HFD-fed transplanted (THKO)(LV-) mice after 16-weeks HFD feeding ($n=10$ liver samples per group) ($**P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis.



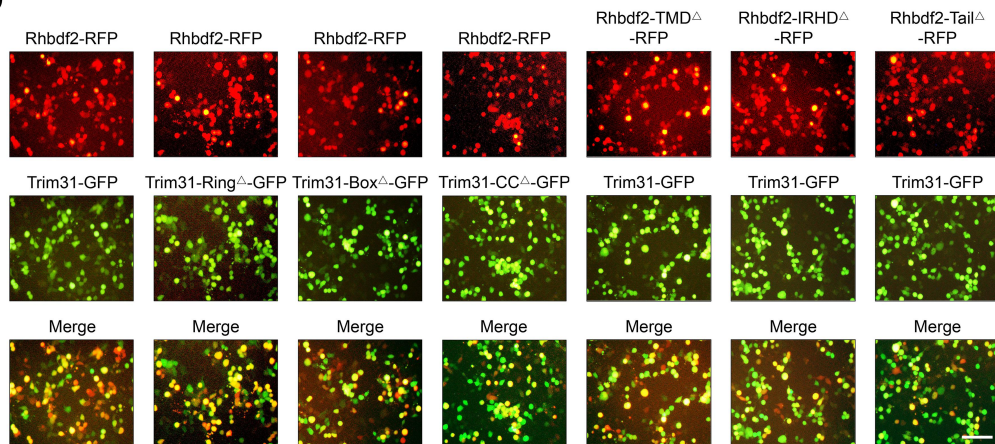
Supplementary Figure 9. Allogeneic hepatocyte transplantation using lentivirus-mediated human TRIM31 alleviates HFD-triggered hepatic steatosis, insulin resistance and inflammation. Preconditioned liver-specific Trim31 deletion (THKO) mice with an 8-weeks HFD treatment as donor were transduced (THKO)(LV-). The hepatocytes isolated from (THKO)(LV-) group mice were transduced with lentivirus-loaded human full-length TRIM31 sequence. The corresponding blank vector were transduced as controls. Then, the additional HFD-fed THKO mice as recipient were injected with transduced hepatocytes via portal vein. The HFD-fed transplanted (THKO)(LV+) mice were harvested for the further experimental detection. **(a)** The strategy diagram for *ex vivo* gene therapy used in the current study (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(b)** Records for the liver weight, body weight and the ratio of liver weight/body weight (%) of the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice at the last week of HFD treatment ($n=15$ mice per group) (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups, *ns.*, no significant difference). **(c)** Representative pictures for Oil red O staining and H&E staining-indicated liver histopathologic changes of the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after *ex vivo* experiment (Magnification, 100 $\times$; $n=10$ images per group for each staining). **(d)** Liver function markers ALT and AST levels were detected in transduced recipient mice ($n=15$ mice per group) (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(e-g)** Records for the glucose tolerance test (GTT) (e) and insulin tolerance test (ITT) (f), and the corresponding fasting insulin levels and HOMA-IR index (g) in the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after transplantation ($n=15$ mice per group) (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(h)** Liver lipid contents including TG, TC and NEFA of the transduced mice ($n=15$ mice per group) (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(i)** qPCR analysis of the relative mRNA expression of genes associated with fatty acid uptake, synthesis, and β -oxidation in the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after transplantation ($n=10$ liver samples per group) (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). **(j)** Representative immunoblotting bands for expression alterations of total amounts and phosphorylated forms of critical indicators involving in insulin signaling, including IRS1, p-IRS1(Ser307), p-IRS1(Tyr608), AKT, and p-AKT, and Rhbdf2-MAP3K7 axis and p-NF- κ B pathway in the liver of HFD-fed recipient (THKO)(LV-) and

(THKO)(LV+) mice. **(k)** Pro-inflammatory cytokines levels (TNF- α , IL-6 and IL-1 β) in serum in the liver of HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice (** $P < 0.01$ vs. HFD-fed recipient (THKO)(LV-) groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis.

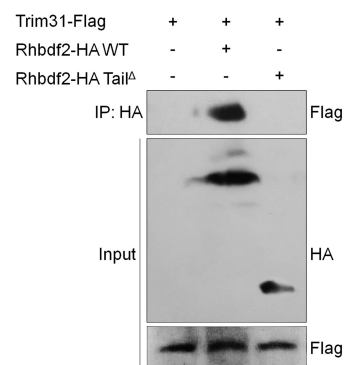
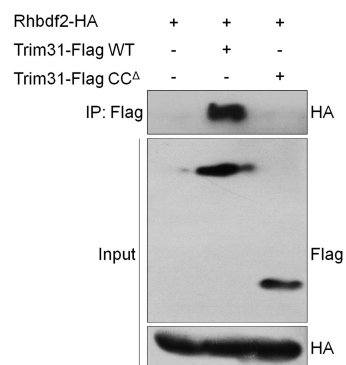
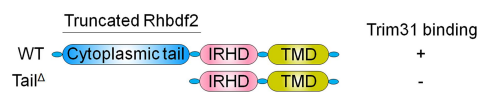
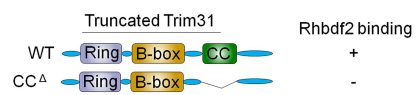
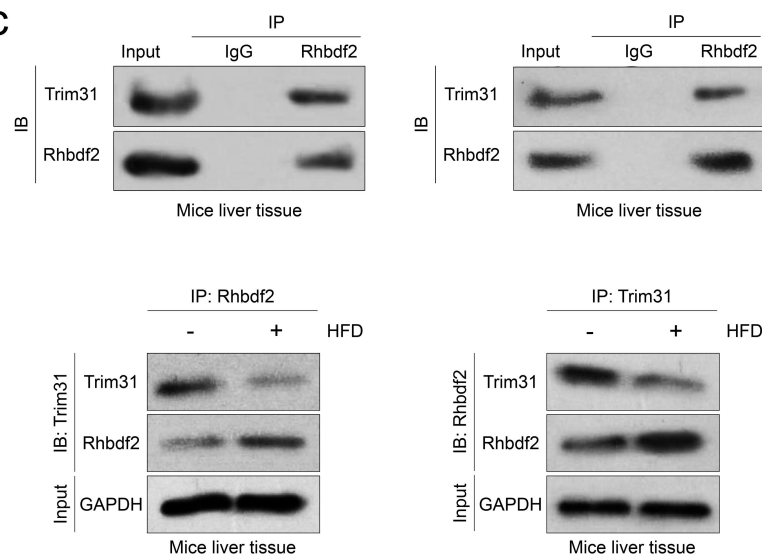
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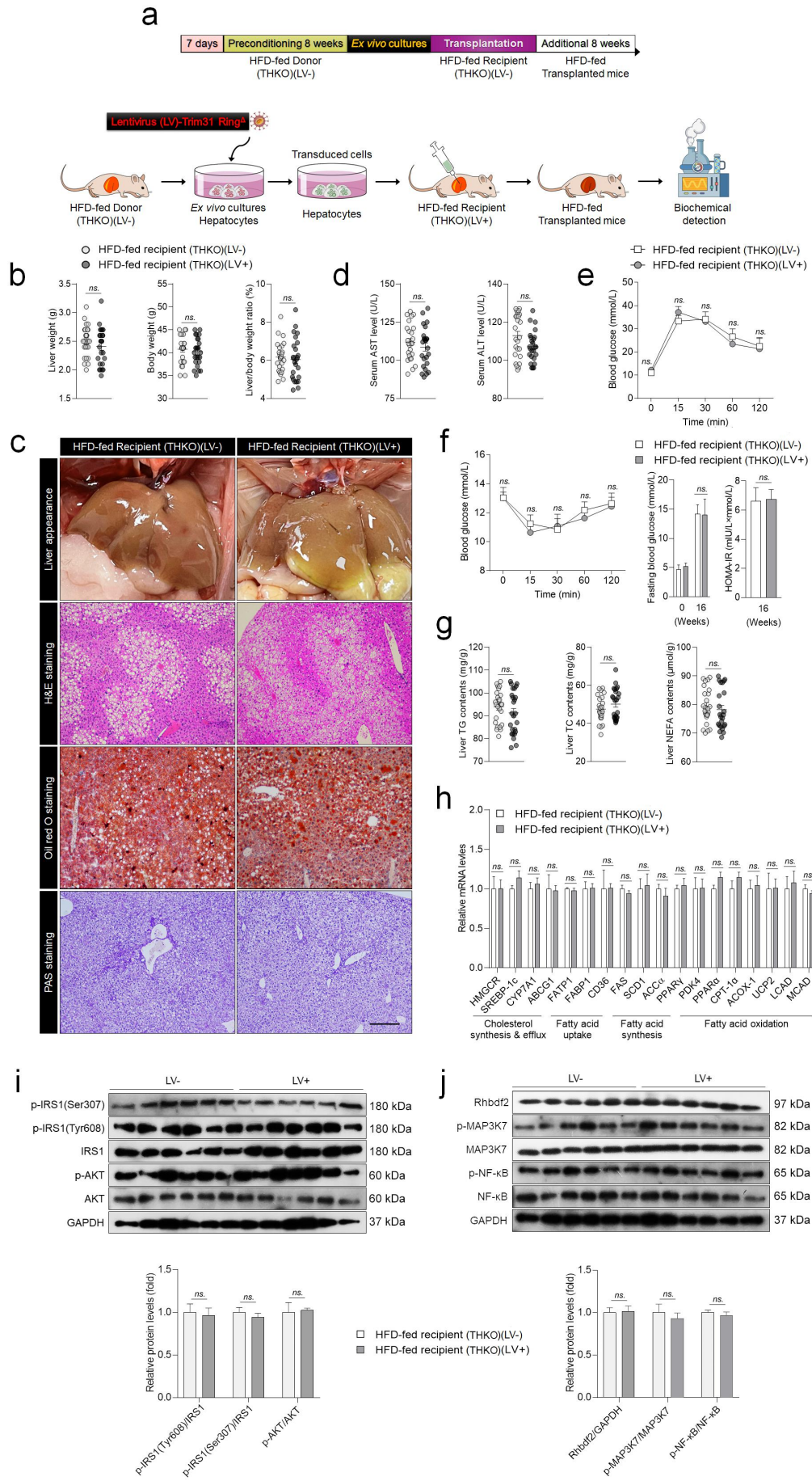
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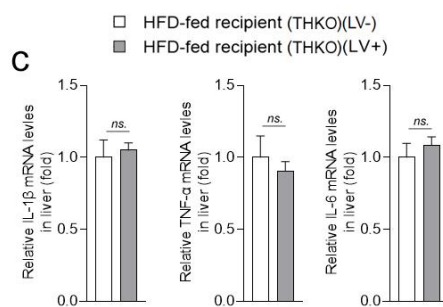
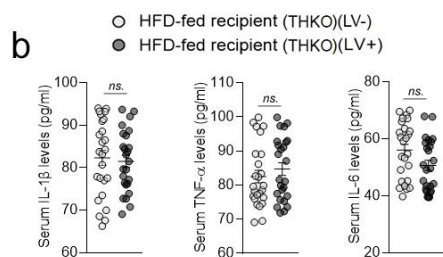
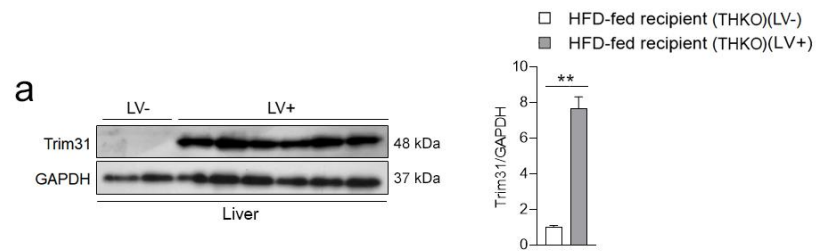
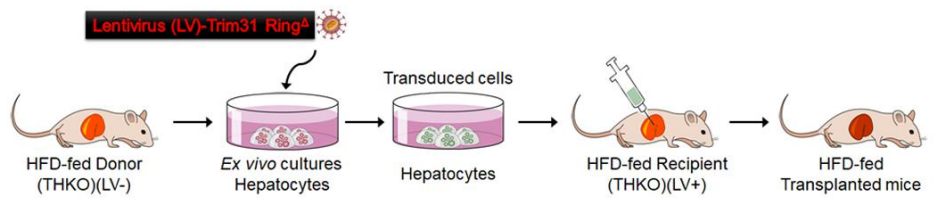


Supplementary Figure 10. The transfection efficiency of adenovirus-induced hepatocytes and protein interaction between mice Rhbdf2 and Trim31. (a) The primary hepatocytes isolated from the THKO or THTG mice were transfected with AdRhbdf2 or AdshRhbdf2. The upper western blotting bands shows the expression efficiency of Rhbdf2. The lower western blotting bands shows the inhibitory efficiency of Rhbdf2 ($n=4$ per experiment). (b) The L02 cells co-transfected with expression vectors for different sizes of the truncated target proteins (Magnification, 200 $\times$; $n=5$ images per group for each staining). (c) The binding domains of mice Rhbdf2 and Trim31 were detected using full-length and truncated Rhbdf2 or Trim31 expression vectors based on IP assays. Anti-Flag or anti-HA antibodies were used to confirm the binding sites of Rhbdf2 and Trim31, respectively.



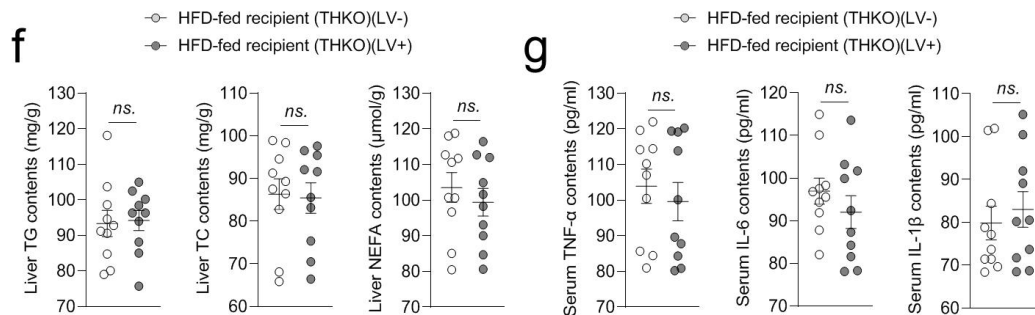
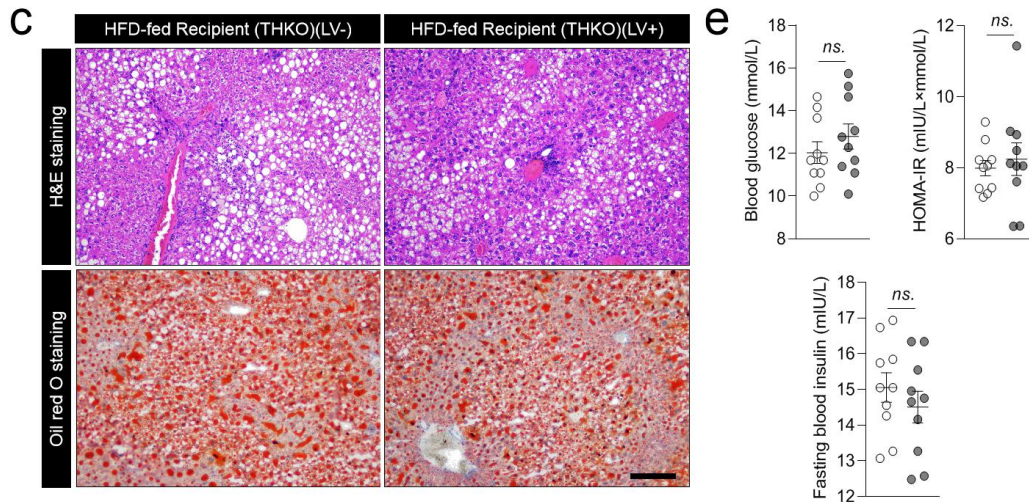
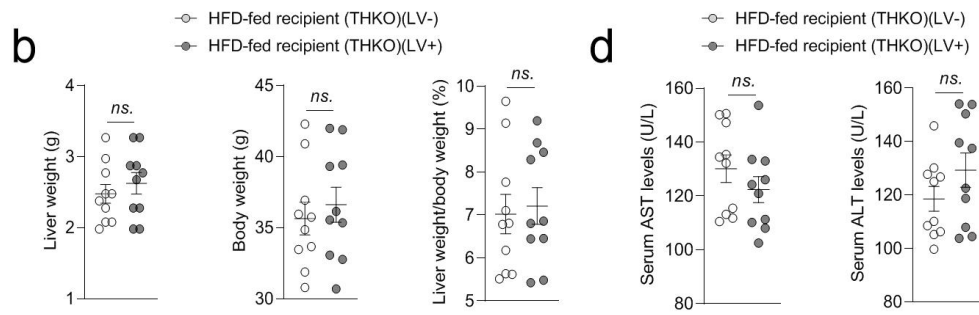
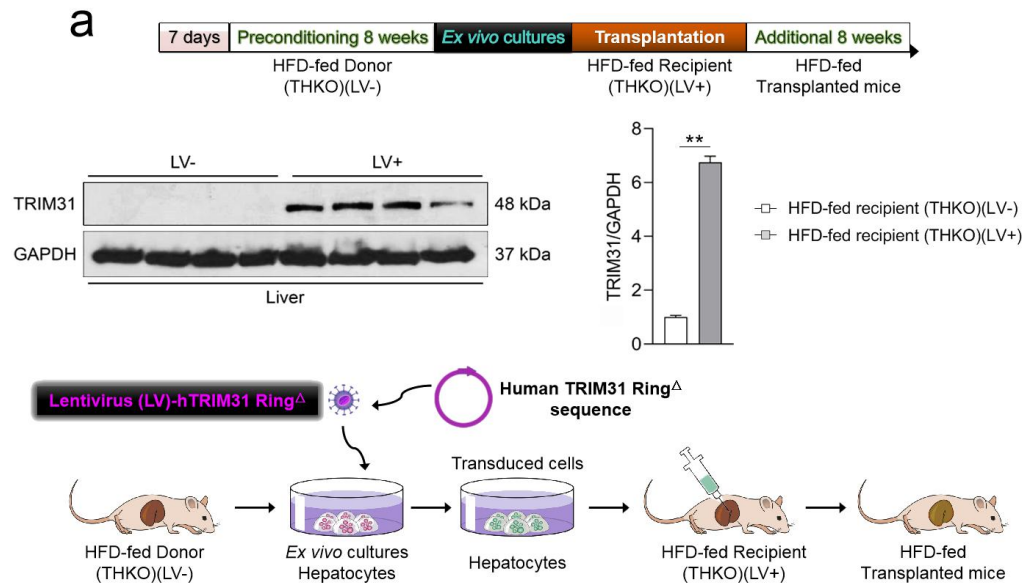
Supplementary Figure 11. Allogeneic hepatocyte transplantation using lentivirus-mediated RING domain deletion of Trim31 did not alleviate HFD-triggered impaired insulin signaling and hepatic steatosis. Preconditioned liver-specific Trim31 deletion (THKO) mice with an 8-weeks HFD treatment as donor were transduced (THKO)(LV-). The hepatocytes isolated from (THKO)(LV-) group mice were transduced with lentivirus-loaded Trim31 with RING domain mutant sequence (Trim31 RING^Δ). The corresponding blank vector were transduced as controls. Then, the additional HFD-fed THKO mice as recipient were injected with transduced hepatocytes via portal vein. The HFD-fed transplanted (THKO)(LV+) mice were harvested for the further experimental detection. **(a)** The strategy diagram for *ex vivo* gene therapy with LV- Trim31 RING^Δ used in the current study. **(b)** Records for the liver weight, body weight and the ratio of liver weight/body weight (%) of the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice at the last week of HFD treatment (*n*=25 mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(c)** Representative pictures for liver appearance and Oil red O staining, H&E staining, and PAS staining-indicated liver histopathologic changes of the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after *ex vivo* experiment (Magnification, 100×; *n*=10 images per group for each staining). **(d)** Liver function markers ALT and AST levels were detected in transduced recipient mice (*n*=25 mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(e, f)** Records for the glucose tolerance test (GTT) **(E)** and insulin tolerance test (ITT) **(F)**, and the corresponding fasting insulin levels and HOMA-IR index in the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after transplantation (*n*=25 mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(g)** Liver lipid contents including TG, TC and NEFA of the transduced mice (*n*=25 mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(h)** qPCR analysis of the relative mRNA expression of genes associated with fatty acid uptake, synthesis, and β -oxidation in the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after transplantation (*n*=10 liver samples per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(i, j)** Representative immunoblotting bands for expression alterations of total amounts and phosphorylated forms of critical indicators involving in insulin signaling **(i)**, including IRS1,

p-IRS1(Ser307), p-IRS1(Tyr608), AKT, and p-AKT, and Rhbdf2-MAP3K7 axis and p-NF- κ B pathway (**j**) in the liver of HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice ($n=4$ per experiment) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed *t*-test analysis.

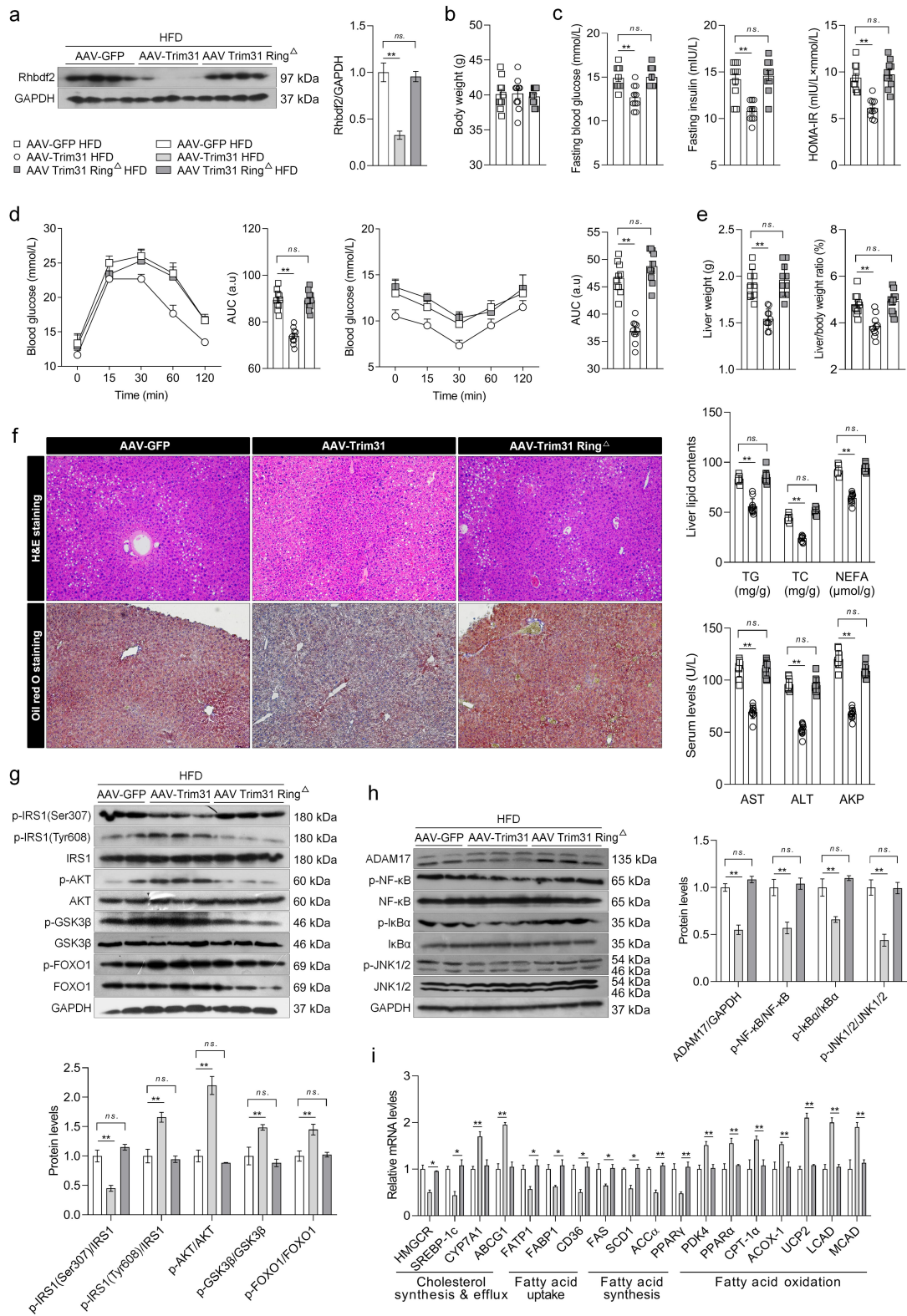


Supplementary Figure 12. Allogeneic hepatocyte transplantation using lentivirus-mediated RING domain deletion of Trim31 fails to mitigate HFD-triggered liver inflammation. (a)

Increased Trim31 expression in liver tissue after transplantation were detected by western blotting ($n=6$ per experiment) ($**P < 0.01$ vs. HFD-fed transplanted (THKO)(LV-) groups). **(b)** Representative pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 in serum from HFD-fed transplanted (THKO)(LV+) and HFD-fed transplanted (THKO)(LV-) mice after 16-weeks HFD feeding ($n=25$ mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(c)** qPCR analysis of the relative mRNA expression of genes associated with inflammation signaling including TNF- α , IL-1 β and IL-6 in the liver samples from HFD-fed transplanted (THKO)(LV+) and HFD-fed transplanted (THKO)(LV-) mice after 16-weeks HFD feeding ($n=10$ liver samples per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed *t*-test analysis.

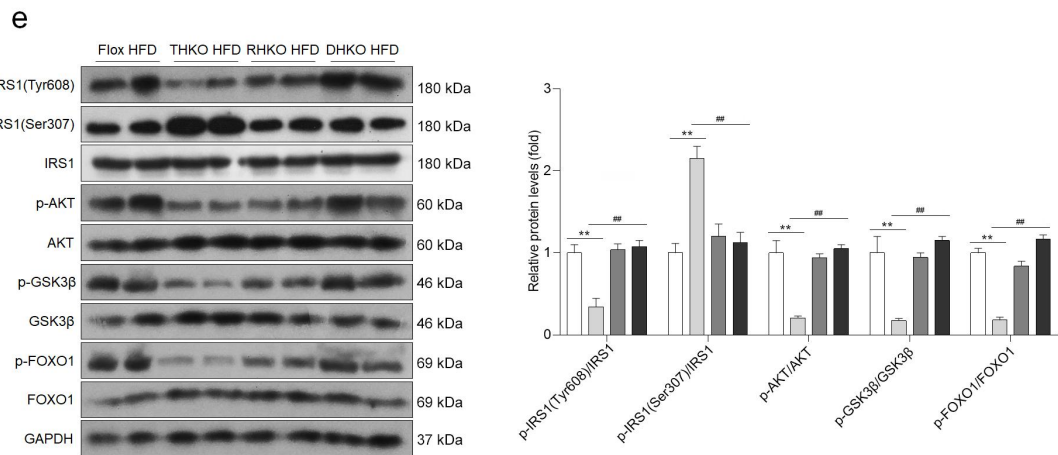
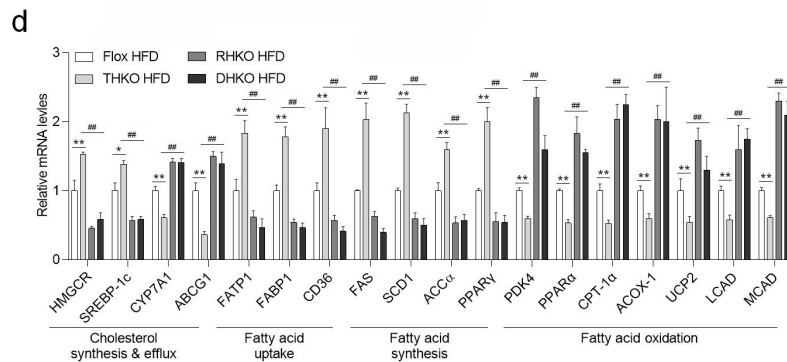
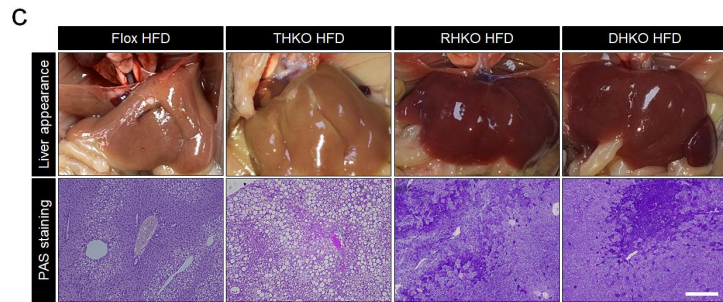
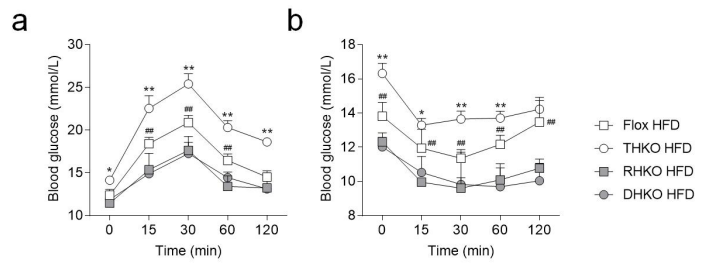


Supplementary Figure 13. Lentivirus-mediated human TRIM31 (RING Δ) did not alleviate HFD-triggered impaired insulin signaling and hepatic steatosis. Preconditioned liver-specific Trim31 deletion (THKO) mice with an 8-weeks HFD treatment as donor were transduced (THKO)(LV-). The hepatocytes isolated from (THKO)(LV-) group mice were transduced with lentivirus-loaded human TRIM31 with RING domain mutant sequence (TRIM31 RING Δ). Then, the additional HFD-fed THKO mice as recipient were injected with transduced hepatocytes via portal vein. The HFD-fed transplanted (THKO)(LV+) mice were harvested for the further experimental detection. **(a)** The strategy diagram for *ex vivo* gene therapy with LV-TRIM31 RING Δ used in the current study and representative western blotting bands for human TRIM31 expression in liver samples ($n=6$ per experiment) (** $P < 0.01$ vs. HFD-fed transplanted (THKO)(LV-) groups). **(b)** Records for the liver weight, body weight and the ratio of liver weight/body weight (%) of the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice at the last week of HFD treatment ($n=10$ mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(c)** Representative pictures for H&E staining and Oil red O staining-indicated liver histopathologic changes of the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after *ex vivo* experiment (Magnification, 100 $\times$; $n=10$ images per group for each staining). **(d)** Liver function markers ALT and AST levels were detected in transduced recipient mice ($n=10$ mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(e)** Records for the blood glucose, HOMA-IR index and fasting blood insulin levels in the HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice after transplantation ($n=10$ mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(f)** Liver lipid contents including TG, TC and NEFA of the transduced mice ($n=10$ mice per group) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). **(g)** The pro-inflammatory cytokines levels (TNF- α , IL-6 and IL-1 β) in serum (k) in the liver of HFD-fed recipient (THKO)(LV-) and (THKO)(LV+) mice ($n=10$ per experiment) (*ns.*, no significant differences compared with the HFD-fed recipient (THKO)(LV-) groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed *t*-test analysis.

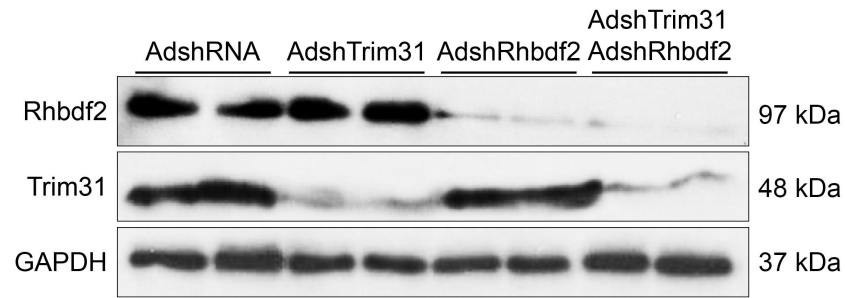


Supplementary Figure 14. RING-finger domain mutant of Trim31 fails to mediate Trim31-regulated hepatic steatosis, insulin resistance, and inflammation. To specifically over-expressed Trim31 in hepatocytes *in vivo* experiments, the adeno-associated virus serotype 8 (AAV8) encoding full-length Trim31 sequences (AAV-Trim31) and AAV8 encoding mutant Trim31 with RING domain deletion (AAV-Trim31 RING^Δ) delivery system were established according to a standard molecular procedure. The 6-weeks HFD-fed preconditioned WT mice were injected with AAV-Trim31 or AAV-Trim31 RING^Δ by tail vein with 100 μl of virus containing 2×10¹¹ vg of vectors and then fed with HFD for additional 10 weeks. The empty vector was injected into mice as the corresponding control. (a) Representative immunoblotting bands for Rbdf2 expression alterations after AAV-Trim31 or AAV-Trim31 RING^Δ injection at the last week of HFD-fed WT mice (*n*=4 per experiment) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences). (b, c) Records for the body weights (b), fasting blood glucose, fasting insulin levels and HOMA-IR index (c) of the AAV-injected WT mice at the last week of HFD treatment (*n*=10 mice per group) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences). (d) Records for the glucose tolerance test (GTT) and insulin tolerance test (ITT) in the HFD-fed indicated mice after AAV injection (*n*=10 mice per group) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences). (e) Records for the liver weight, the ratio of liver weight/body weight (%), hepatic lipid contents (TG, TC and NEFA) and liver function indicators including AST, ALT and AKP levels of the HFD-fed indicated mice after AAV injection (*n*=10 mice per group) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences). (f) Representative images for H&E staining, Oil red O staining-indicated liver histopathologic changes, lipid contents and liver function-related indexes of the HFD-fed mice after AAV injection (Magnification, 100×; *n*=10 images per group for each staining) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences). (g) Representative immunoblotting bands for expression alterations of total amounts and phosphorylated forms of critical indicators involving in insulin signaling, including IRS1, AKT, GSK3β and FOXO1, in the liver of mice with AAV-Trim31 or AAV-Trim31 RING^Δ injection after HFD feeding for 16 weeks (*n*=4 per experiment) (***P* < 0.01 vs. AAV-GFP HFD groups, *ns.*, no significant differences). (h) Representative immunoblotting bands for expression alterations of

total amounts and phosphorylated forms of critical indicators associating with inflammation signaling, including ADAM17, I κ B α , p-I κ B α , JNK1/2, p-JNK1/2, NF- κ B and p-NF- κ B, in the liver of mice with AAV-Trim31 or AAV-Trim31 RING^Δ injection after HFD feeding for 16 weeks ($n=4$ per experiment) ($**P < 0.01$ vs. AAV-GFP HFD groups, *ns.*, no significant differences). (i) qPCR analysis of the relative mRNA expression of genes associated with fatty acid uptake, synthesis, and β -oxidation in the HFD-fed indicated mice after AAV injection ($n=10$ liver samples per group) ($*P < 0.05$ and $**P < 0.01$ vs. AAV-GFP HFD groups, *ns.*, no significant differences). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

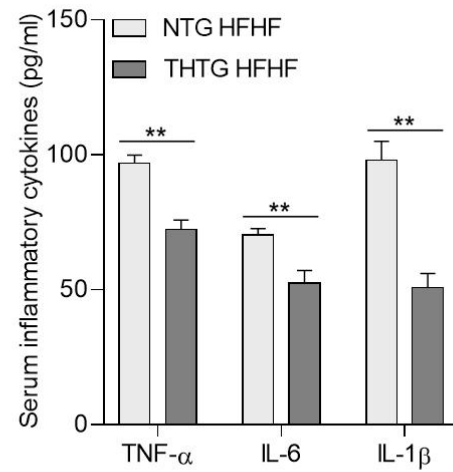
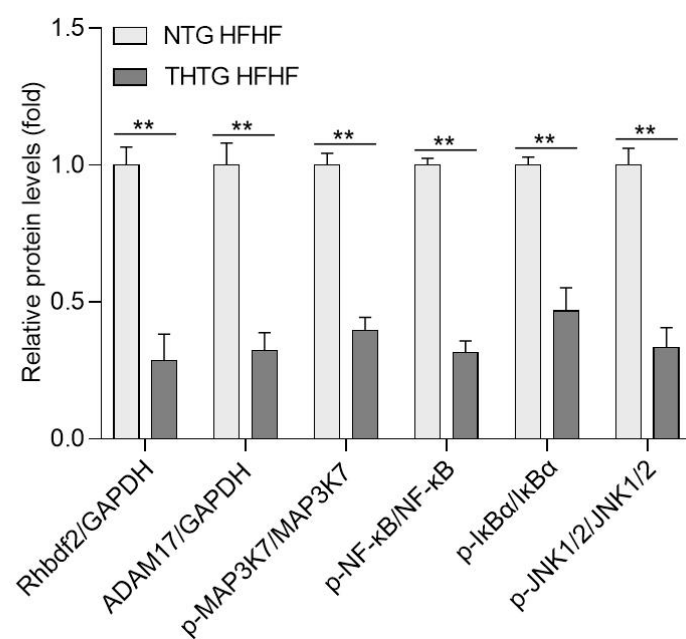
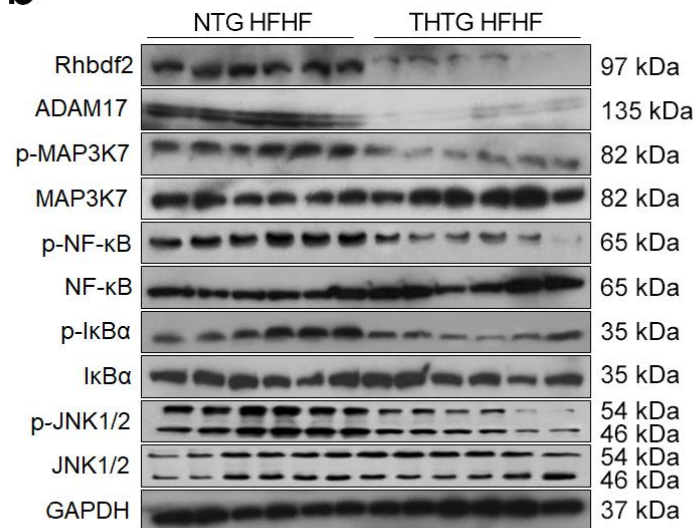


Supplementary Figure 15. Hepatocyte-specific Trim31 and Rhbdf2 double deletion mitigates HFD-induced insulin resistance. (a, b) Records for the glucose tolerance test (GTT) (a) and insulin tolerance test (ITT) (b) in the 16-weeks HFD-fed mice ($n=10$ mice per group) ($*P < 0.05$ and $**P < 0.01$ vs. Flox HFD groups, $^{##}P < 0.01$ vs. THKO HFD groups). (c) Representative images for liver appearance and PAS staining-indicated liver histopathologic changes of the HFD-fed mice (Magnification, 100 $\times$; $n=10$ images per group for each staining). (d) qPCR analysis of the relative mRNA expression of genes associated with fatty acid uptake, synthesis, and β -oxidation in the HFD-fed indicated mice after HFD treatment ($n=10$ liver samples per group) ($*P < 0.05$ and $**P < 0.01$ vs. Flox HFD groups, $^{##}P < 0.01$ vs. THKO HFD groups). (e) Representative immunoblotting bands for expression alterations of total amounts and phosphorylated forms of critical indicators involving in insulin signaling, including IRS1, AKT, GSK3 β and FOXO1, in the liver of mice after HFD feeding for 16 weeks ($n=4$ per experiment) ($**P < 0.01$ vs. Flox HFD groups, $^{##}P < 0.01$ vs. THKO HFD groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by two-way analysis of variance with general linear model procedures using a univariate approach.

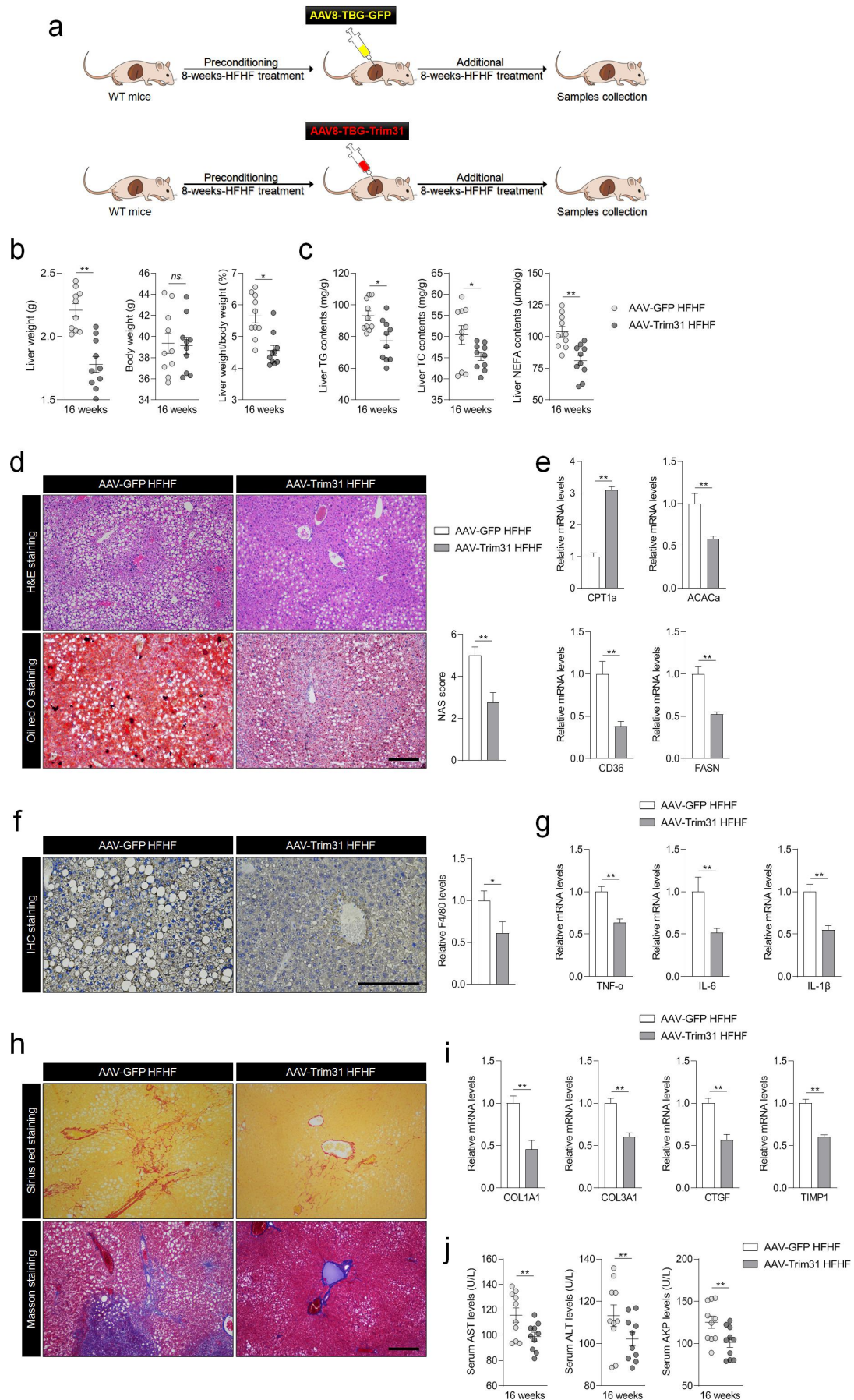


Supplementary Figure 16. The transfection efficiency of adenovirus-induced hepatocytes.

Representative western blotting bands of the primary hepatocytes that were transfected with AdshRhbd2 or AdshTrim31 or co-treated with AdshRhbd2/AdshTrim31 ($n=4$ per experiment).

a**b**

Supplementary Figure 17. Trim31 overexpression suppresses Rhbdf2/MAP3K7 axis-associated liver inflammation in HFHF-induced NASH model. (a) Representative pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 in serum from HFHF-fed NTG- and THTG mice ($n= 15$ mice per group) (** $P < 0.01$ vs. NTG HFHF groups). (b) Representative immunoblotting bands for expression alterations of total amounts and phosphorylated forms of critical indicators involving in inflammation signaling, including Rhbdf2-MAP3K7 and its downstream events cascades, in the liver of mice after HFHF feeding for 16 weeks ($n=6$ per experiment) (** $P < 0.01$ vs. NTG HFHF groups). The GAPDH was used as loading control. Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed t -test analysis.



Supplementary Figure 18. AAV8-mediated Trim31 gene therapy protects against HFHF-induced nonalcoholic steatohepatitis (NASH) *in vivo*. (a) An experiment design of AAV8-mediated Trim31 gene therapy in HFHF-induced NASH mouse model. (b) Records for liver weight, body weight and ratio of liver weight/body weight in AAV-GFP and AAV-Trim3 groups after a 16-weeks HFHF administration ($n=10$ mice per group) ($*P < 0.05$ and $**P < 0.01$ vs. AAV-GFP HFHF groups, *ns.*, no significant difference). (c) Liver lipid contents including TG, TC and NEFA of the indicated mice after a 16-weeks HFHF ingestion ($n=10$ mice per group) ($*P < 0.05$ and $**P < 0.01$ vs. AAV-GFP HFHF groups). (d) Representative pictures for H&E staining and Oil red O staining-indicated liver histopathologic changes of a 16-weeks HFHF-fed mice (Magnification, 100 $\times$; $n=5$ images per group for each staining) ($**P < 0.01$ vs. AAV-GFP HFHF groups). (e) qPCR analysis of the relative mRNA expression of genes in the livers associated with fatty acid metabolism after a 16-weeks HFHF administration ($n=10$ liver samples per group) ($**P < 0.01$ vs. AAV-GFP HFHF groups). (f) Immunohistochemistry staining of F4/80 analysis showing the histopathologic changes of the liver samples in indicated mice (Magnification, 200 $\times$; $n=5$ images per group for each staining) ($*P < 0.05$ vs. AAV-GFP HFHF groups). (g) qPCR analysis of the relative mRNA expression of genes in the livers associated with inflammation after a 16-weeks HFHF administration ($n=10$ liver samples per group) ($**P < 0.01$ vs. AAV-GFP HFHF groups). (h) Representative pictures for Sirius red staining and Masson staining showing the collagen deposition levels of the liver samples in indicated mice (Magnification, 100 $\times$; $n=5$ images per group for each staining). (i, j) qPCR analysis of the relative mRNA expression of genes in the livers associated with collagen synthesis after a 16-weeks HFHF administration (i) ($n=10$ liver samples per group); and hepatic function indicator AST, ALT and AKP levels in the 16-weeks HFHF-fed AAV-GFP or AAV-Trim3 injected mice (j) ($n=10$ mice per group) ($**P < 0.01$ vs. AAV-GFP HFHF groups). Data are expressed as mean $\pm$ SEM. The relevant experiments presented in this part were performed independently at least three times. Significance determined by Student's 2-tailed *t*-test analysis.

Supplementary Table 1. The clinical information of non-steatosis, simple steatosis and NASH patients.

Items	Sex	Age (Years)	BMI (Kg/m ²)	AST (U/L)	ALT (U/L)	TG (mmol/L)	TC (mmol/L)	Fasting blood glucose (mmol/L)	Serum TNF- α (ng/ml)	Serum IL-6 (pg/ml)	NAS
Non-steatosis	Male/Female (11/5)	35.1 (21-47)	19.6 (17.9-21.6)	24.8 (15.6-30.7)	21.9 (10.7-27.5)	1.7 (1.47-1.6)	3.8 (3.73-4.78)	5.0 (4.1-6.1)	9.9 (8.62-11.37)	33.5 (24.51-52.16)	0
Simple steatosis	Male/Female (10/7)	35.7 (32-51)	25.2 (24.6-26.1)	54.2 (41.5-62.5)	45.5 (30.7-57.7)	2.1 (1.78-2.5)	4.2 (3.33-5.10)	5.7 (4.8-7.1)	13.4 (8.54-18.70)	52.9 (35.28-74.54)	1.7
NASH	Male/Female (8/8)	35.9 (22-55)	30.1 (24.4-37.4)	51.9 (36.6-64.4)	42.1 (31.8-57.2)	2.3 (1.67-3.54)	4.4 (3.12-5.96)	6.2 (5.0-7.9)	21.2 (13.09-28.29)	55.9 (36.63-82.28)	4.3

Data were expressed as mean for all items. The values in bracket indicate the minimum and maximum value in corresponding item.

Supplementary Table 2. The primer sequences for the targeted genes used in this study.

Primers	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Rhbf2	CACAGCCCAGTGGTTTGGGGTCA	GAGGGCGGCGGCTGCCTGAAAGCT
Trim31	CCAGAGTCAAACCGTGAGCG	GGCAACTTGGAGCCCGAA
TNFR1	TACATTGCAGCCTCTGCCTC	AGAGCTTGGACTTCCACCGT
TNFR2	ACATCAGACGTGGTGGTGCAA	CCAAGTGAAGAGCCAAGTC
TNF- α	ACCTGGCCTCTCTACCTTGT	CCCGTAGGGCGATTACAGTC
IL-1 β	AGCAAAGTGATAGGCCGAC	GCGTCGCAATTAGTGTTGTG
IL-6	GGGCAACAAGATTCCGATATA	AGCCACTACATGGAATCTAAT
MCP-1	CTGGTCCGAGTGAGACAAAG	AGATCAGGCTCTGATGGAGAA
iNOS	CCAACCGGAGAAGGGGACGAAC	GGAGGGTGGTGGCGCTGGAC
CXCR1	GCTGCCCACTGGAGATTATTC	TATGCCTGGCGGAAGATAGC
CCL1	GGCTGAACAAAGGTAGAGAAAGC	TGGAGGACTGAGGGAAACTG
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAATTCTC
FKN	ACCTATGGCCCTGACATCATCAC	CTTGCCAGCCCTCAGAATCAC
MDC	TCTGATGCAGGTCCCTATGGT	TTATGGAGTAGCTTCTTCAC
MIP-1	CCCGACTGCCTGCTGCTTCTCC	CAAAGGCTGCTGGTCTCAAA
COX-2	CCACCTCTGCGATGCTCTTC	ACATTCCCCACGGTTTTGAC
CX3CR1	CCGGTCTCATTTCAGGCTTA	TGGGAATTGAACTTGGGACCTC
IL-18	CAAGGCTGGTCCATGCTCC	TGCTATCACTTCCTTCTGTTC
CXCR4	GAAGCTCTTGGTGAAAAGG	GAGTCGATGCTGATCCCAAT
CCR2	CCTTGGAATGAGTAACTGTGTGA	ACAAAGGCATAAATGACAGGATTAATG
CXCL2	CGGTCAAAAAGTTTGCCTTG	TCCAGGTCAGTTAGCCTTGC
ICAM1	TAAGAGGACTCGGTGGATGG	ATGAGGCTCGATTGTTTCAGC
Col4	TGTCATGGTGTGAAGGGACA	TCTCCAGCATCACCTTTTG
SREBP-1c	AGGGGTAGGGCCAACGGCCT	CAAGCTGCCTGGGAGCTGGTA
HMGCR	TGTCCTTGATGGCAGCCTTG	CGCGCTTCAGTTCAGTGTCAG
ABCG1	GATTGGCTTCAGGATGTCCATGTTGGAA	GTATTTTTCGAAGGCTACCAGTTACATTTGACAA
UCP2	GTTCTCTGTCTCGTCTTGC	GGCCTTGAAACCAACCA
CYP7A1	CTGTGTTCACTTTCTGAAGCCATG	CCCAGGCATTGCTCTTTGAT
FATP1	GATGTGCTCTACGACTGTCTG	CAGCCCATAGATGAGACACTG
FABP1	GCAGAGCCAGGAGAACTTTG	GGGTCCATAGGTGATGGTGAG
CD36	AGCAACTGGTGGATGGTTTC	TCAAGGGAGAGCACTGGTTT
CPT-1 α	TCTTGACAGTCGACTCACCTT	TCCACAGGACACATAGTCAGG
PPAR α	CAAGGCCTCAGGGTACCACT	TTGCAGCTCCGATCACACTT
PPAR- γ	CAAGACTACCCTTTACTGAA	CTACTTTGATCGCACTTTGGT
ACC α	GGCCAGTGCTATGCTGAGAT	AGGGTCAAGTGCTGCTCCA
FAS	CTGCGGAACTTCAGGAAATG	GGTTCGGAATGCTATCCAGG
SCD1	CTTCTTGCGATACACTCTGG	TGAATGTTCTTGTCGTAGGG
PKD4	TGTGATGTGGTAGCAGTAGTC	GTGGTGAAGGTGTGAAGGA

ACOX-1	GAATTTGGCATCGCAGACCC	GATCTCCAGATTCCAGGCCG
LCAD	AAGGATTTATTAAGGGCAAGAAGC	GGAAGCGGAGGCGGAGTC
MCAD	GAGCCTGGGAACCTCGGCTTGA	GCCAAGGCCACCGCAACTTT
GAPDH	GGTGAAGGTCGGTGTGAACG	CCCGTAGGGCGATTACAGTC

Uncropped western blotting bands used in this study

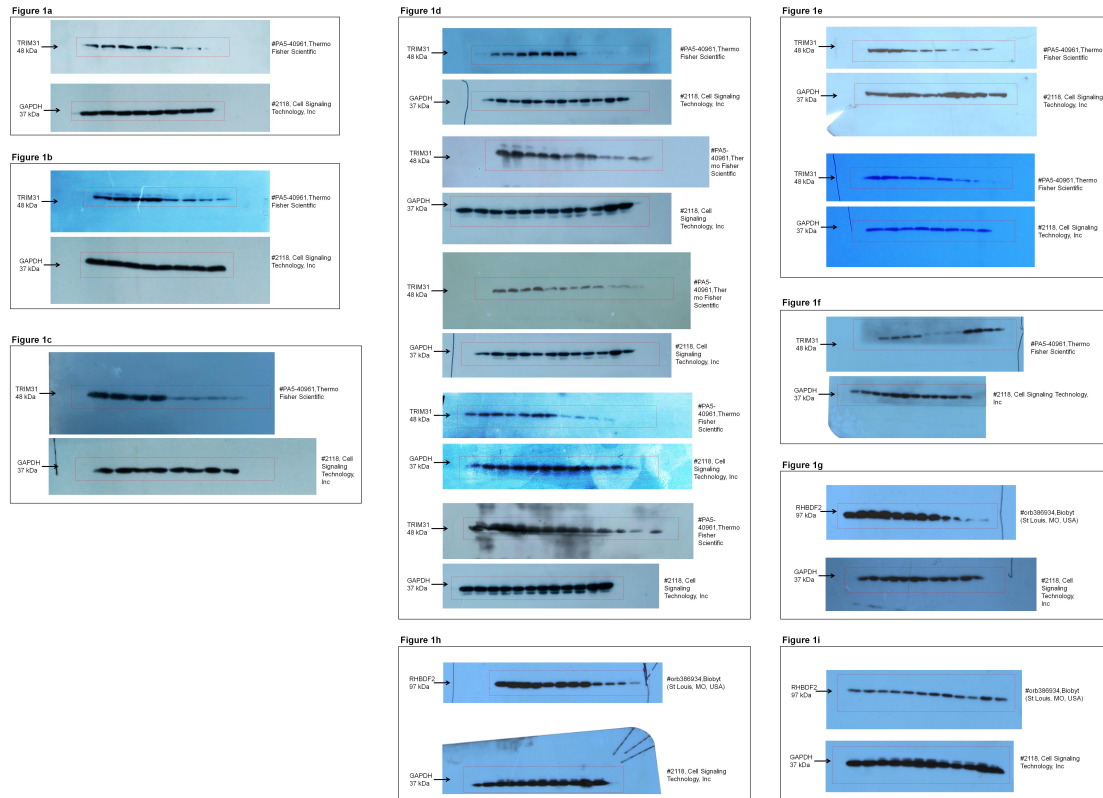


Figure 2h

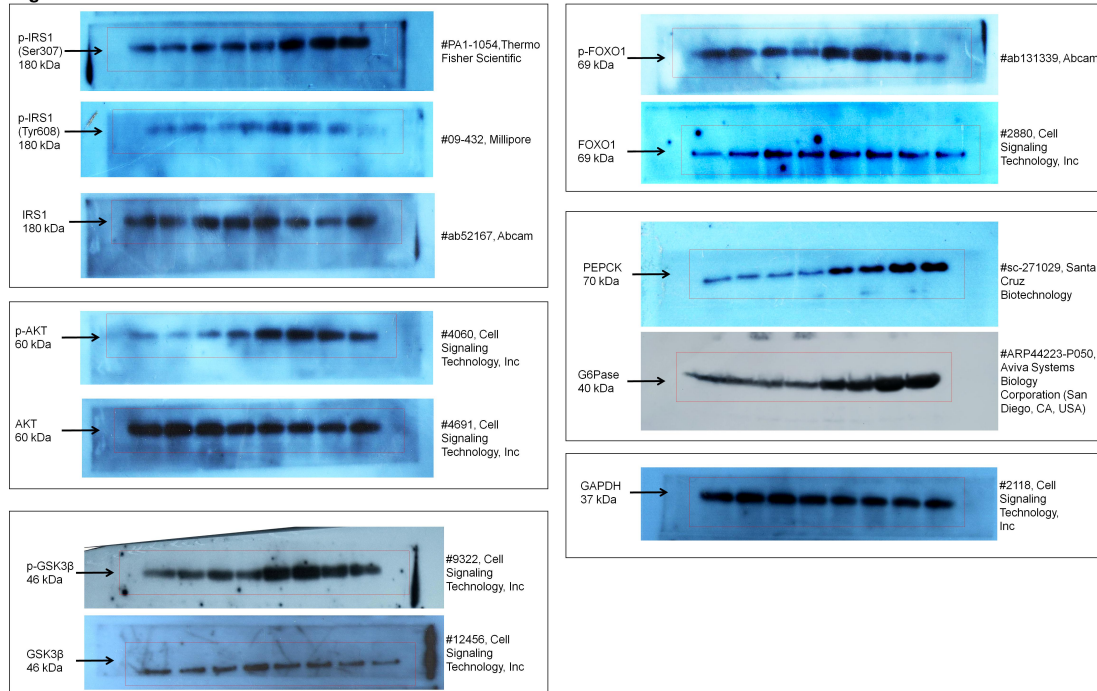


Figure 4i

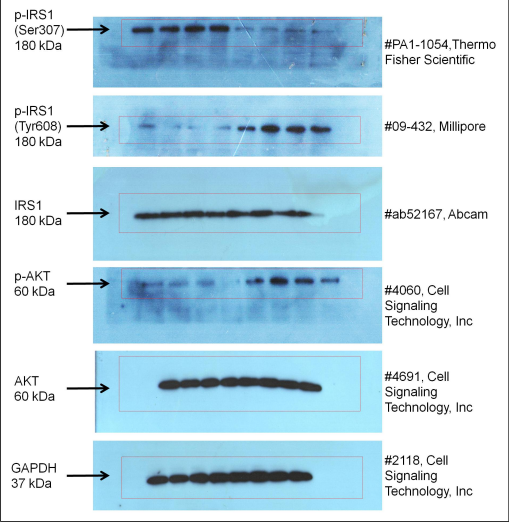


Figure 4j

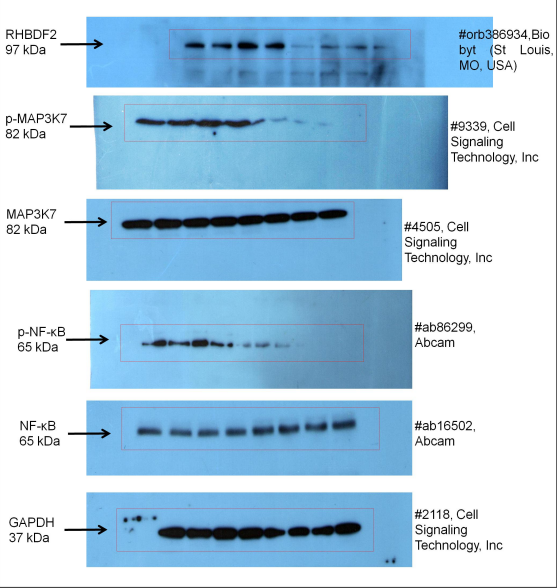


Figure 5b

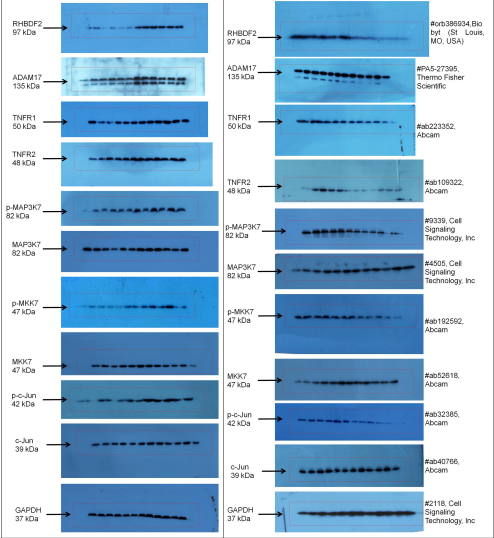


Figure 5c

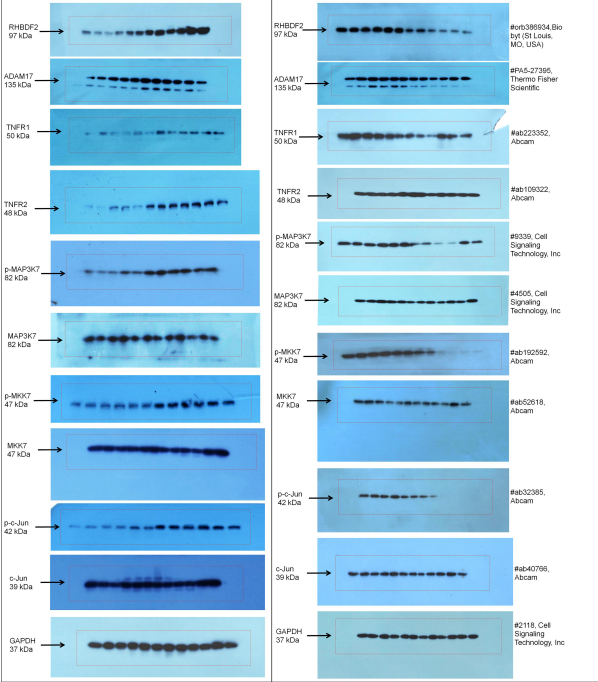


Figure 5d

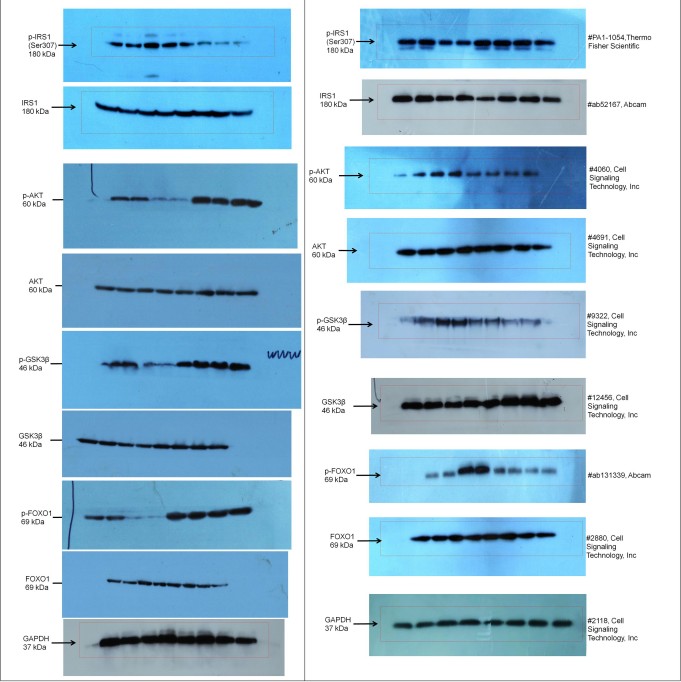


Figure 6

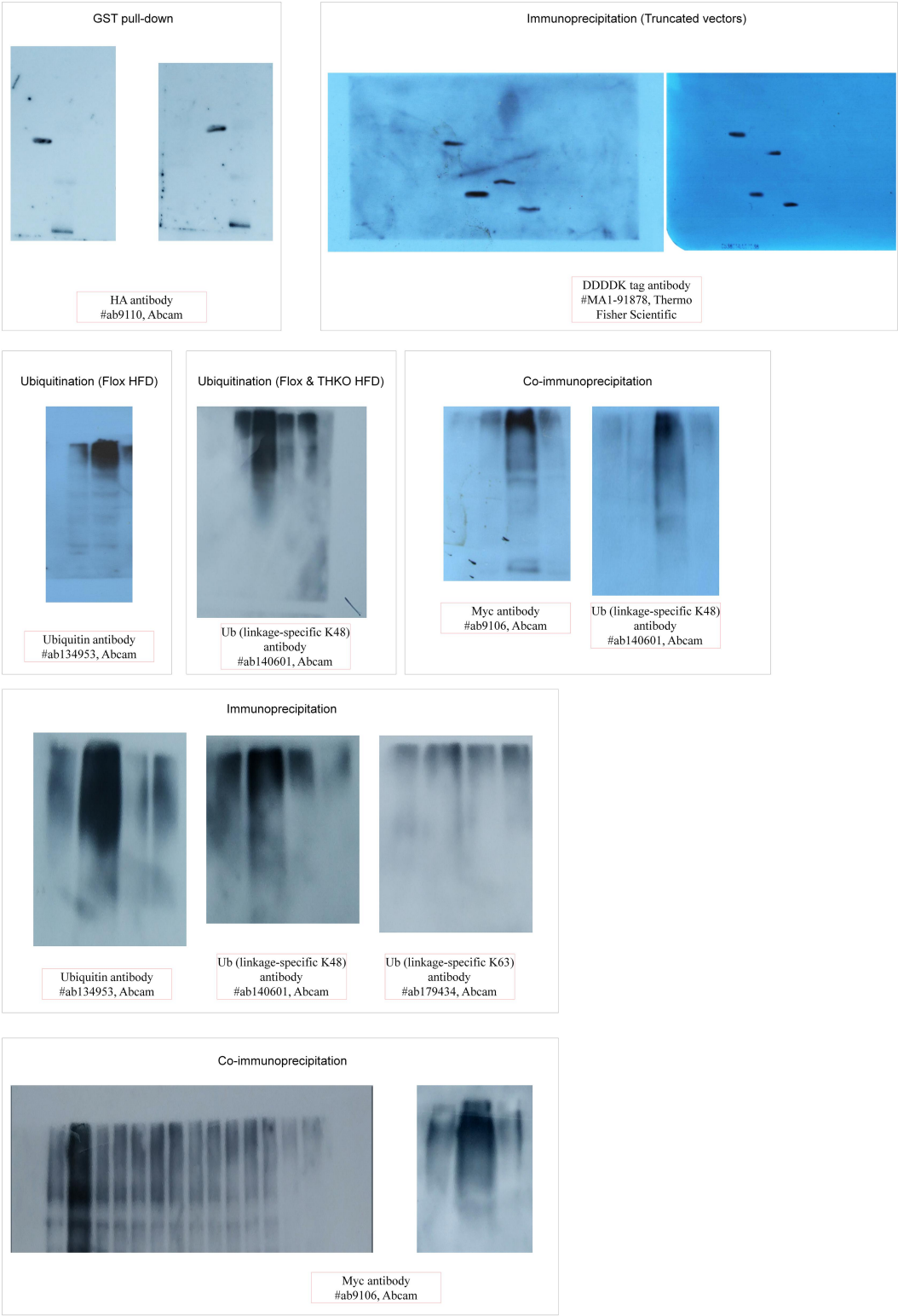
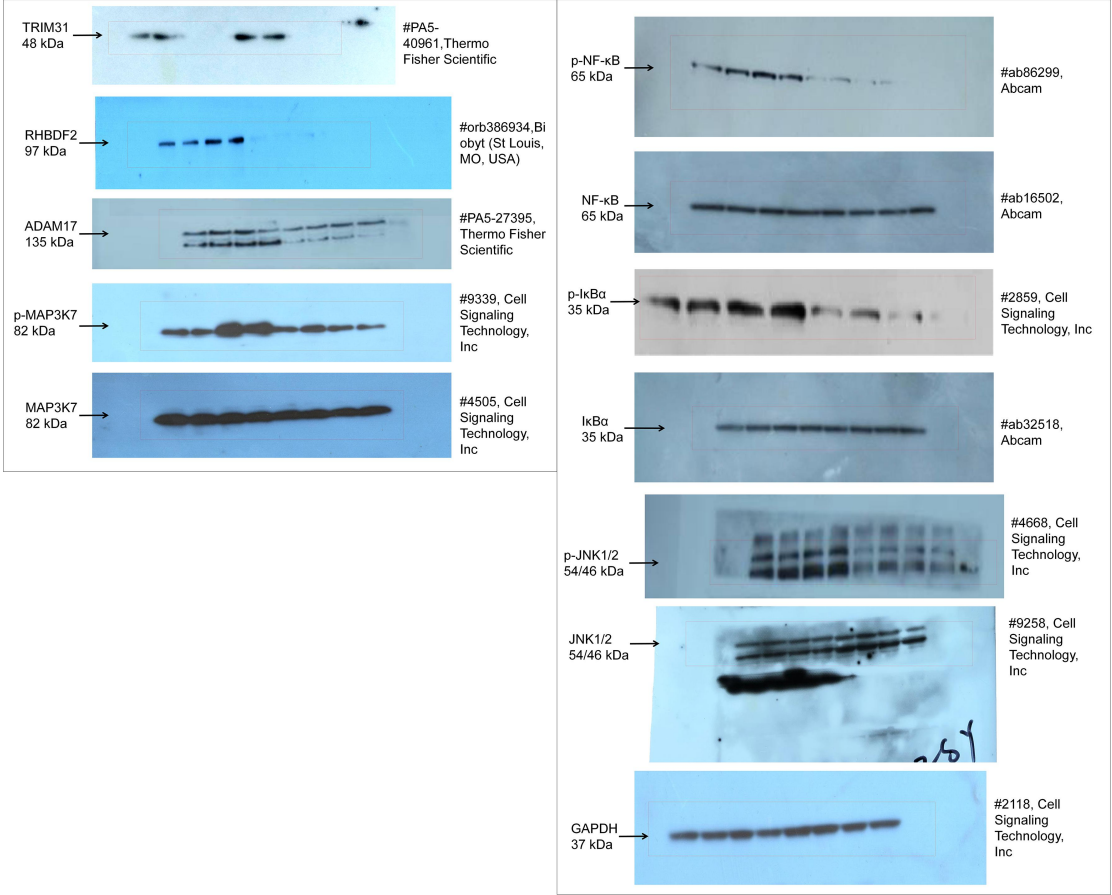
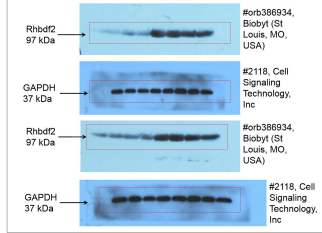


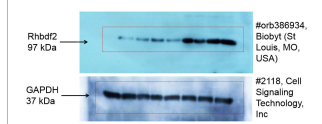
Figure 7a



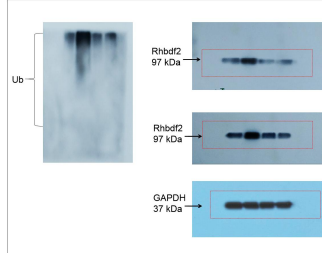
Supplementary Figure 1a



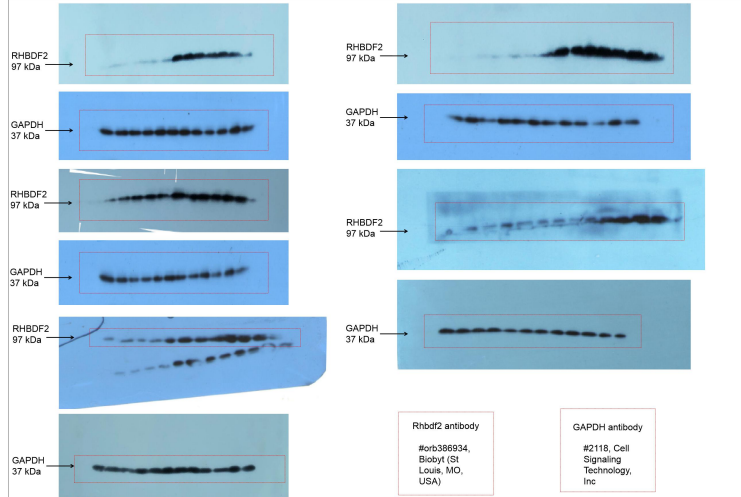
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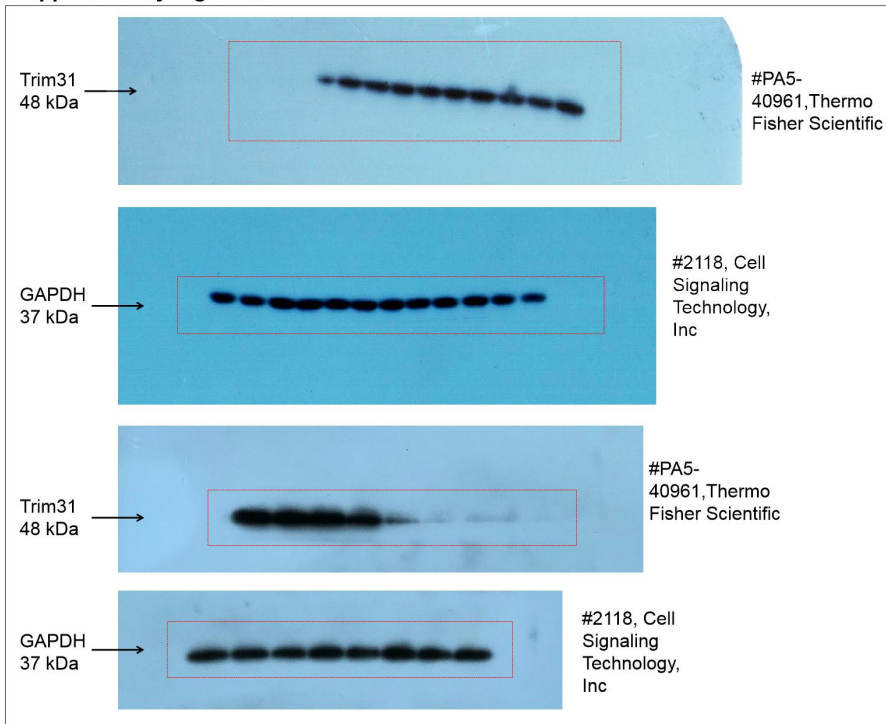
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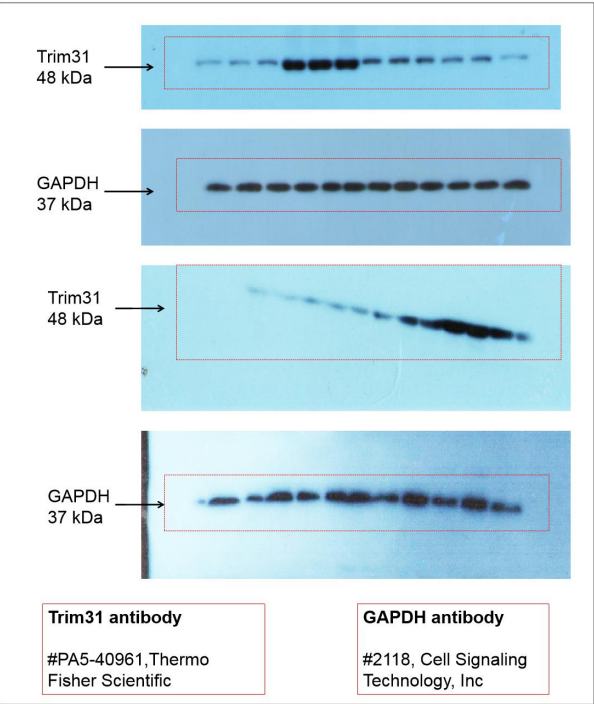
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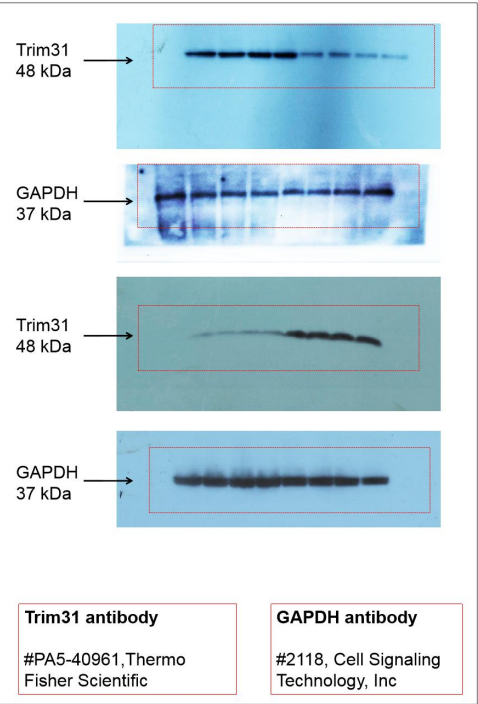
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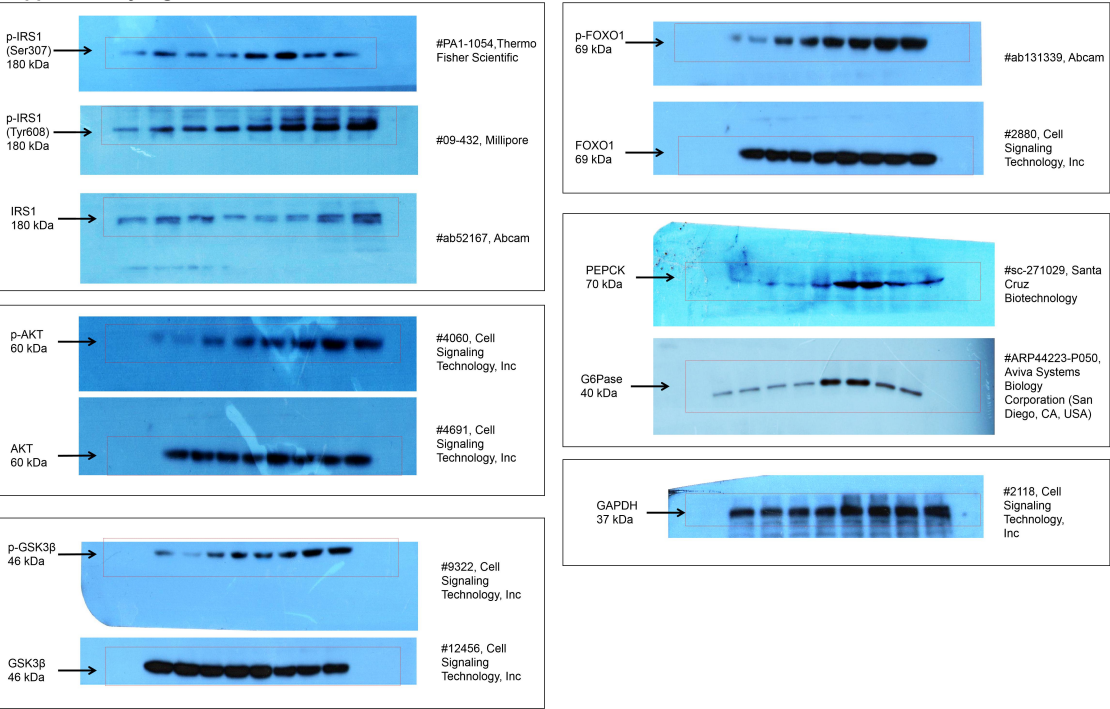
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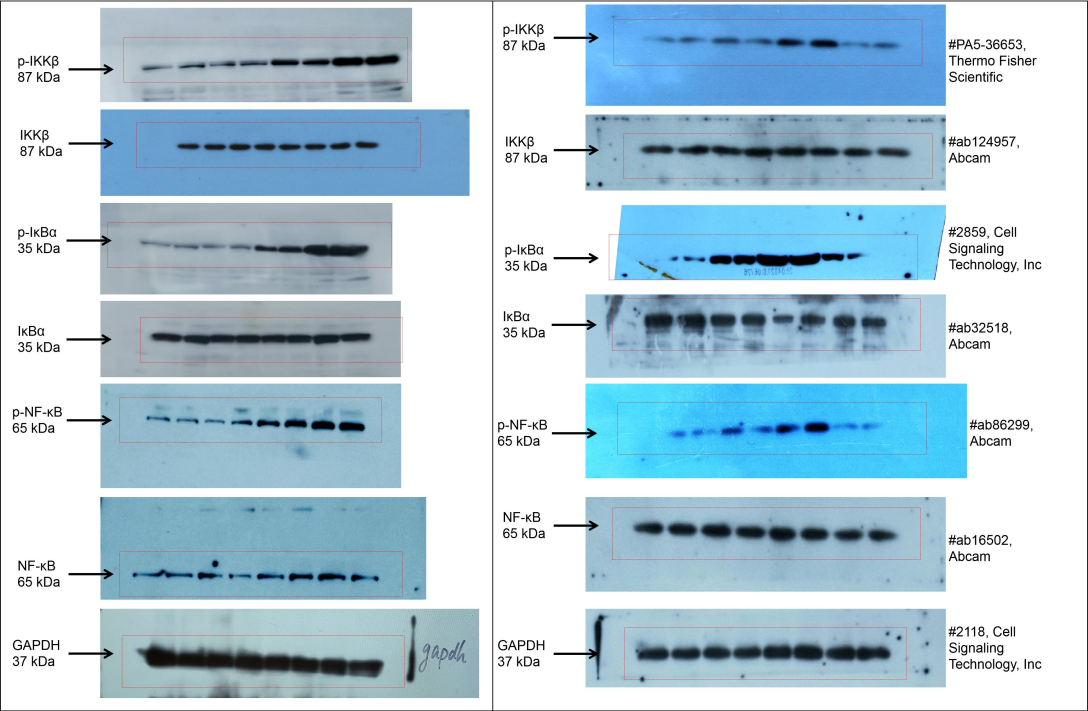
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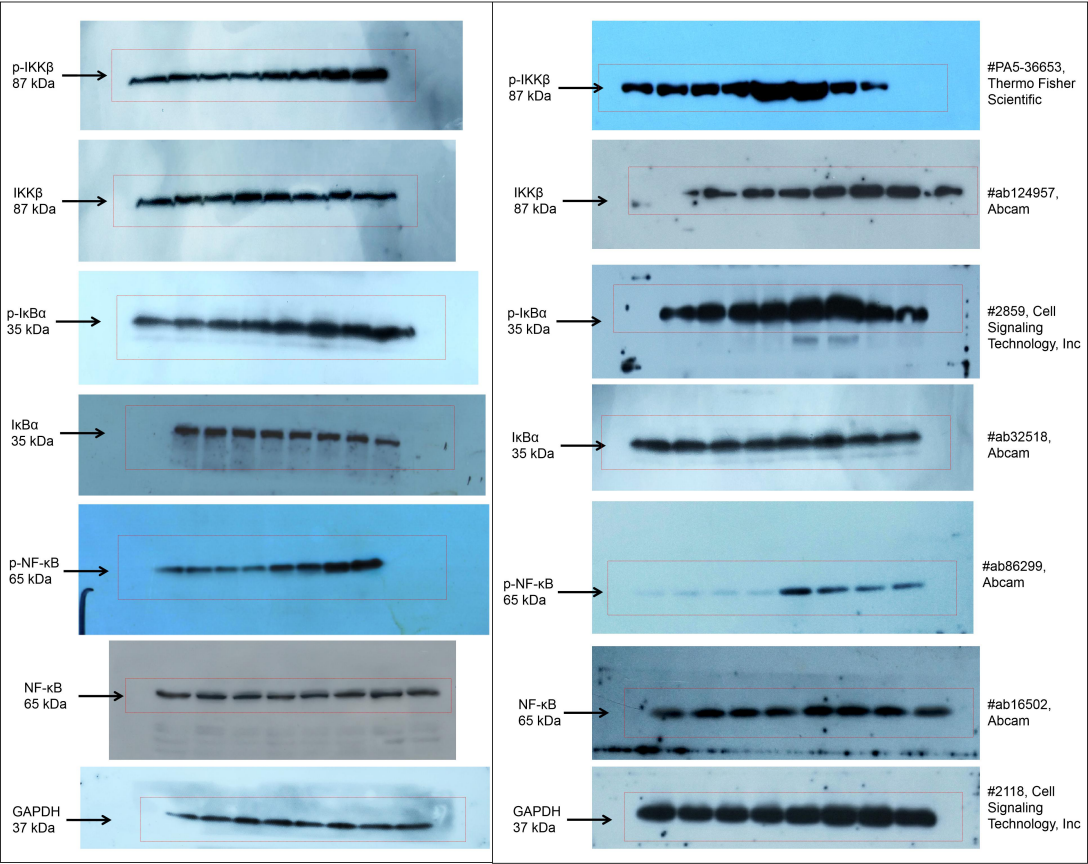
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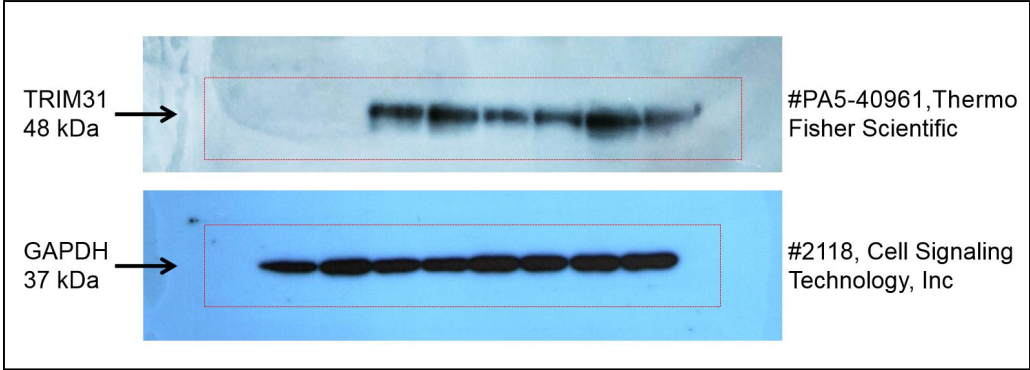
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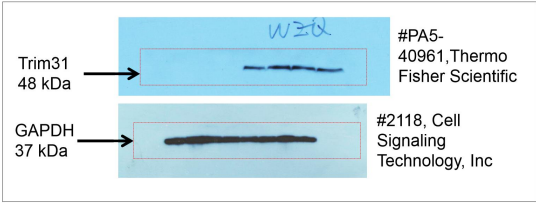
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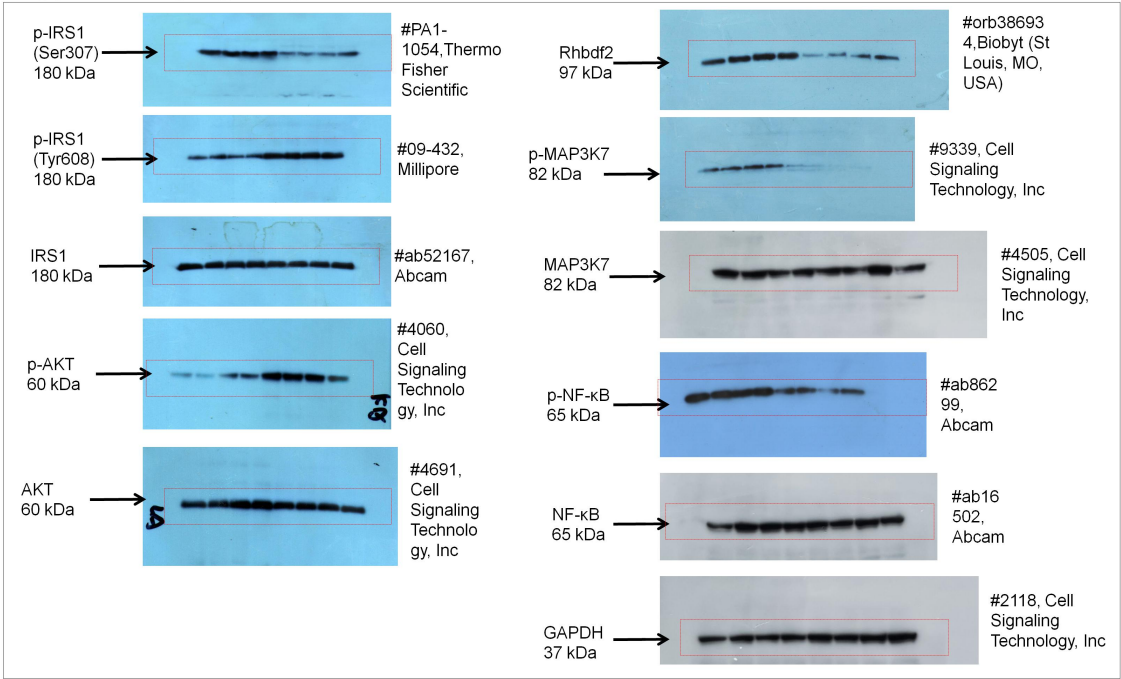
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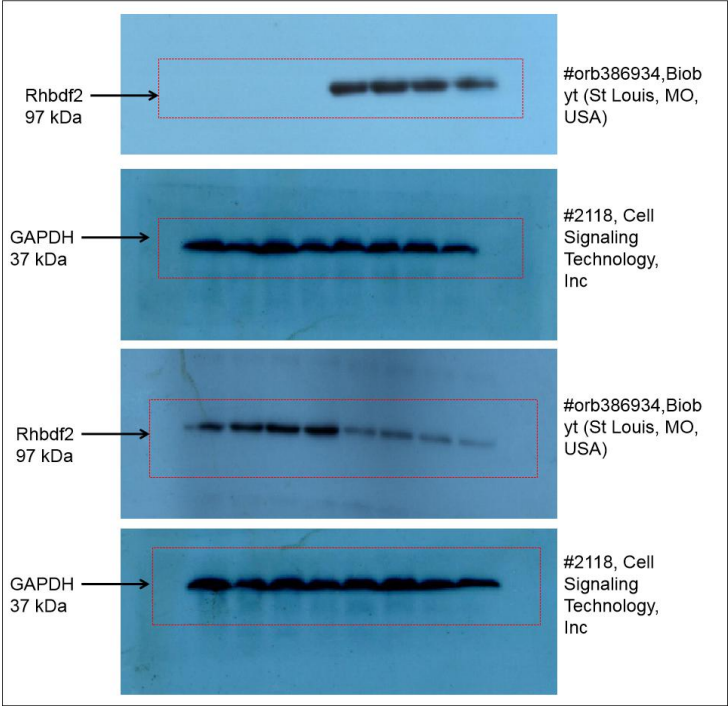
Supplementary Figure 9a



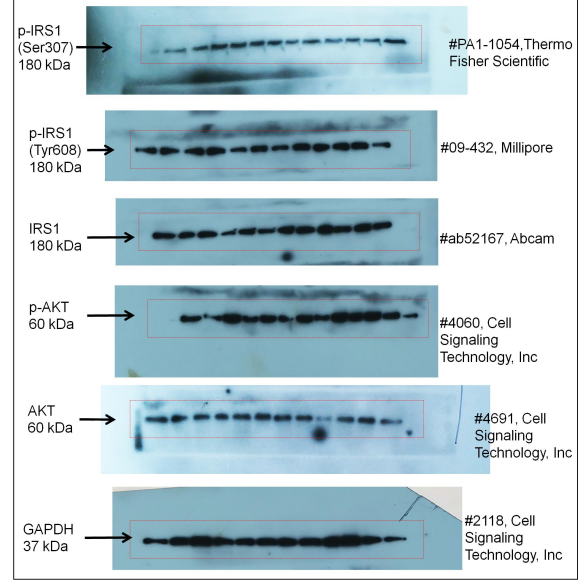
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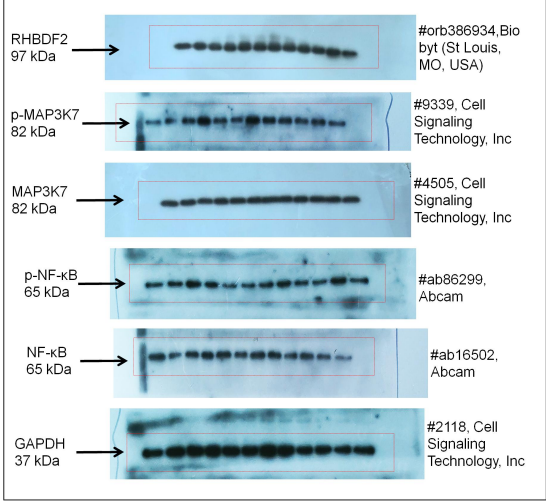
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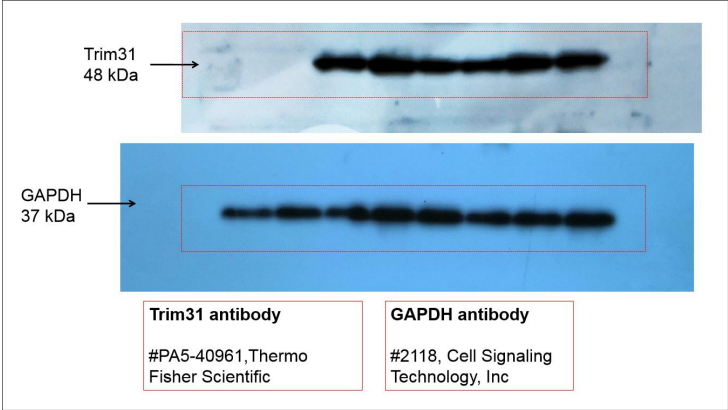
Supplementary Figure 11i



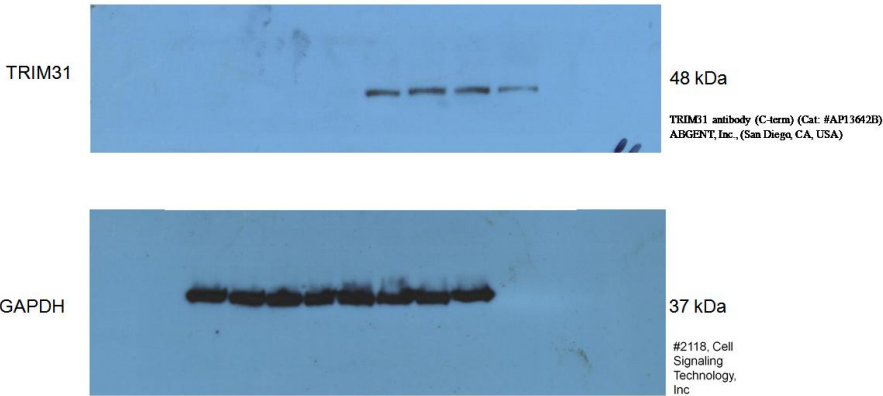
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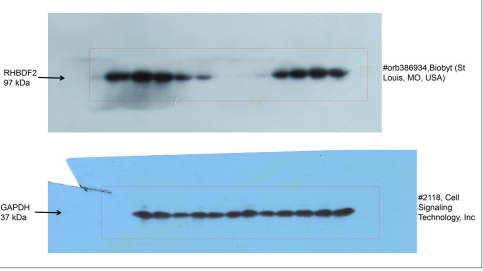
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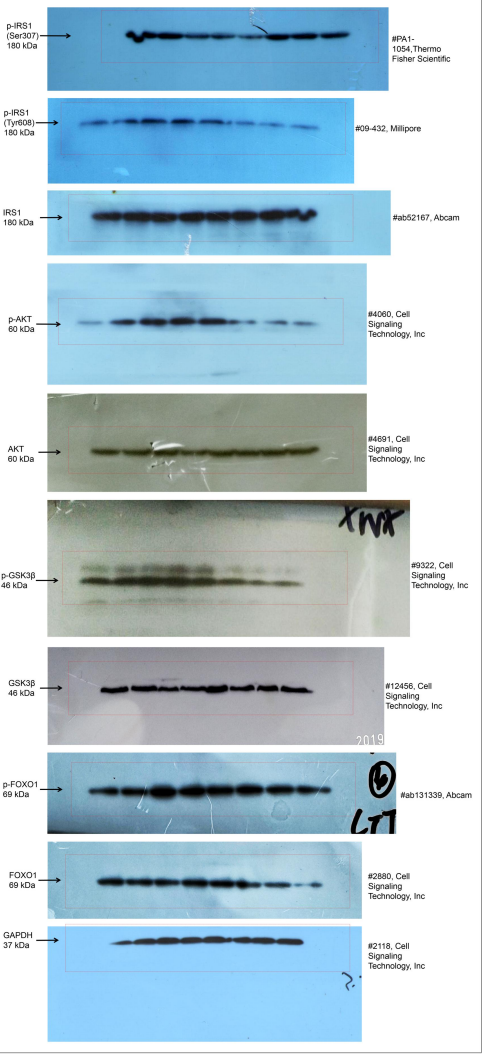
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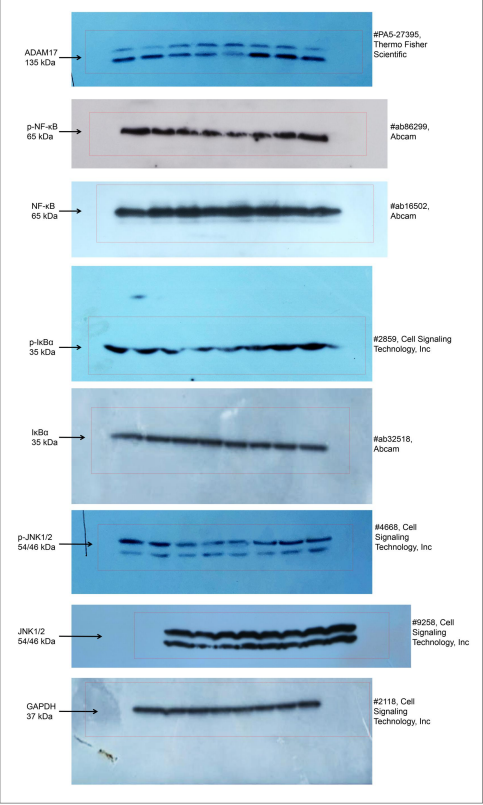
Supplementary Figure 14a



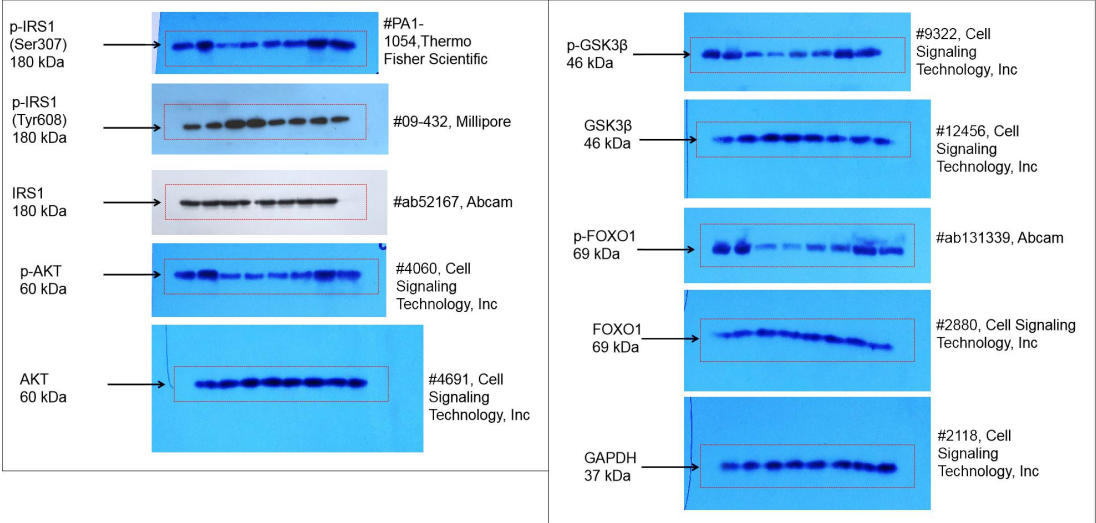
Supplementary Figure 14g



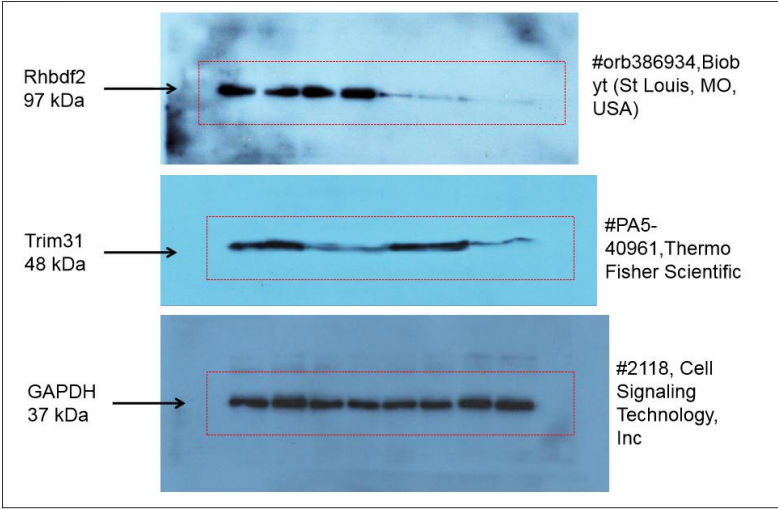
Supplementary Figure 14h



Supplementary Figure 15e



Supplementary Figure 16



Supplementary Figure 17b

