

E3 ligase FBXO2-mediated protein stability of insulin receptor regulates adipogenesis and metabolic health in obesity

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Abstract

Adipogenesis, a crucial physiological process, serves to safely sequester lipids, thereby preventing lipotoxicity in peripheral organs and preserving metabolic health during obesity. While insulin signaling plays a pivotal role in adipogenesis, regulating factors especially the braking mechanism governing this process warrants further investigation. Our study identified proteasome-dependent degradation of the insulin receptor (IR) during the early stages of adipogenesis as a critical event for the mitotic clonal expansion phase of the adipocyte differentiation program. A series of studies confirmed that the ubiquitinated modification of IR is regulated by E3 ligase FBXO2 and this is based on its phosphorylation. We further elucidated that the FBD domain of FBXO2 is indispensable for its function in catalyzing p-IR ubiquitination. Gain or loss of function of Fbxo2 inhibited or promoted adipocyte progenitors proliferation and adipogenesis both *in vitro* and *In vivo*, which regulated adipose hyperplasia and plasticity of adipose tissue. Moreover, FBXO2 played an important role in regulating metabolic health of mice when subjected to caloric excess. Collectively, our findings unveil FBXO2 as a negative regulator of adipogenesis by impairing insulin signaling pathway.

Introduction

Obesity is characterized by the expansion of white adipose tissue (WAT), which primarily occurs in one of two ways: adipocyte size increase (hypertrophy) or adipocyte number rise (hyperplasia) (1–3). Hypertrophy involves the enlargement of existing mature adipocytes, accompanied by lipid accumulation, necrosis, inflammatory cell infiltration, and tissue fibrosis, leading to adipose tissue dysfunction and complications such as insulin resistance. Conversely, hyperplasia refers to the formation of new adipocytes through precursor differentiation during adipogenesis. Shifting adipose tissue expansion from hypertrophy to hyperplasia could potentially prevent pathological remodeling and dysfunction of adipose tissue in response to excess caloric intake. Adipogenesis plays a crucial role in maintaining the normal physiological function of adipose tissue and overall metabolic health (4–6). Therefore, research aimed at identifying new targets that promote adipogenesis holds significant potential for clinical interventions aimed at addressing obesity-related complications.

Numerous signaling hormones and ligands, including insulin, glucocorticoids, BMP, WNT, and hedgehog signaling, modulate the process of adipogenesis (1, 7). Insulin, in particular, is a crucial component of *in vitro* adipogenic differentiation medium (8, 9). Upon binding to its receptor, insulin initiates a signaling cascade involving IR, IRS1/2, PI3K, AKT1 or AKT2, mTOR, and the FOXO family. Activation of insulin signaling leads to the transcription of peroxisome proliferator-activated receptor- γ (PPAR γ) and/or CCAAT/enhancer-binding protein- α (C/EBP α), facilitating preadipocyte differentiation (7, 10). Given the physiological role of insulin in glucose metabolism, an interesting implication is that insulin serves as a permissive signal to adipogenesis under the condition of overnutrition (11, 12, 13). It is of great significance to explore new approaches that regulate adipogenesis induced by insulin signaling. It has been reported that post-translational modifications of IR and IRS play crucial roles in regulating insulin signal transduction, particularly through ubiquitination and degradation (14). For instance, the E3 ligase

CHIP could catalyze IR ubiquitinated modification while this effect is weakened during the process of aging and results in inhibition of longevity genes (15). Inhibition of CULLIN significantly prolongs the half-life of IRS protein and enhances the activity of the insulin signaling pathway, resulting in lowered blood glucose levels (16). Therefore, the stability of IR and/or IRS is closely linked to the activity of insulin signaling, making them pivotal targets for insulin signaling regulation.

E3 ligases play a pivotal role as they confer substrate specificity in the ubiquitination pathway (17–19). They are involved in various cellular processes, including protein degradation, cell cycle, DNA repair, and signal transduction (20–22). It was reported that E3 ligase APPBP2 degrades PRDM16 by ubiquitination and inhibits the regeneration of beige fat (23). The deubiquitinating enzyme OTUD3 prevents obesity and diabetes by stabilizing PPAR δ (24). Additionally, CUL4B in adipocytes negatively regulates PPAR γ -mediated adipose tissue expansion and insulin sensitivity (25). Therefore, exploring the roles of E3 ubiquitin ligases in regulating adipogenesis holds significant promise for improving metabolic health.

This study aimed to identify novel factors associated with insulin signaling that modulate adipogenesis. We discovered that ubiquitination-dependent degradation of the insulin receptor occurs during the early stage of adipogenic differentiation. Through a series of ubiquitination experiments, we confirmed that this process is regulated by the E3 ligase FBXO2. Gene ectopic studies conducted both in vitro and in vivo validated FBXO2 as an inhibitor of adipogenesis. FBXO2 emerged as a negative regulator of metabolically beneficial WAT plasticity by ubiquitinating the insulin receptor and reducing insulin signaling activity. Our findings suggest FBXO2 as a potential therapeutic target for inhibiting metabolic complications such as insulin resistance and fatty liver.

Research Design and Methods

Animal Models

In this study, *Fbxo2*-Flox mice, *Fabp4*-Cre mice, and *Adipoq*-Cre mice were purchased from Gempharmatech Co., Ltd (Nanjing China). *Pdgfra*-Cre mice were kindly provided by Dr. Tongjin Zhao in Chinese Academy of Sciences Shanghai Institute of Materia Medica (58). *Fbxo2*^{flox/flox} mice were bred to *Pdgfra*-Cre, *Fabp4*-Cre and *Adipoq*-Cre mice to generate *Fbxo2*^{Pdgfra-/-} mice, *Fbxo2*^{Fabp4-/-} mice and *Fbxo2*^{Adipoq-/-} mice, respectively. All mice used in this study were on a C57BL/6J genetic background. Male mice at 6–8 weeks of age were used for experiments. Mice were housed in a temperature (22 $\pm$ 2°C) and humidity (40–60%) controlled facility with regular 12:12 light/dark cycle and fed a rodent chow with free access to water. All animal experiments were performed in the animal facility of Capital Medical University (Beijing, China) and approved by the Animal Use and Care Committee of Capital Medical University. *Fbxo2*^{Pdgfra-/-} mice, *Fbxo2*^{Fabp4-/-} mice and *Fbxo2*^{Adipoq-/-} mice developed as normally as wildtype control mice and did not suffer from any spontaneous abnormalities when fed NCD. For HFD feeding experiments, 6–8 weeks old male mice were fed with HFD (60 kcal%, Research Diets, D12492) diets for up to 12 weeks. Mouse genotyping was performed using genomic DNA isolated from mouse tails.

Cell Culture and Adipocyte Differentiation

HEK 293T cells, 3T3-L1 cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in the presence of 5% CO₂ and 95% air. For differentiation, the day when cells reached confluence was designated as day - 2. For 3T3-L1 cells, two days after confluence (day 0), they were induced to differentiate with DMEM containing 10% FBS and 1% penicillin/streptomycin, 5 µg/mL insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, and 1 mmol/L dexamethasone for 2 days (day 2); and maintained in the culture medium supplemented with 5 µg/mL insulin until the end (the medium was changed every 2 days).

Primary SVF Isolation and Differentiation

SVF were isolated from the epididymal and subdermal fat pads of male mice, respectively (denoted as SVF eWAT and SVF sWAT, respectively). The epididymal or subdermal fat pads were washed with Krebs-Ringer solution and minced in Krebs-Ringer solution containing 0.8 mg/mL collagenase type I and 1% free fatty acid-free bovine serum albumin. The fat pads were then digested on a horizontal shaking at 37°C for 50 min. The digestive mixture was filtered through 80 steel mesh and then 40 mesh cell strainers. After centrifugation at 1000 × g for 10 min, cell pellets were collected, suspended, and counted. The isolated cells were maintained in DMEM/F12 medium containing 10% FBS at 37°C under an atmosphere of 5% CO₂ and 95% air. For adipogenic differentiation, cells were incubated with differentiation medium I (DMI, DMEM/F12 supplemented with 5 µg/mL insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 mmol/L dexamethasone, 3 mmol/L indomethacin, 1% penicillin/streptomycin and 10% FBS) for 2 days and differentiation medium II (DMII, DMEM/F12 supplemented with 5 µg/mL insulin, 1% penicillin/streptomycin and 10% FBS) for another 2 days. After the induction of adipogenic differentiation, cells were cultured in maintaining media with 5 µg/mL insulin for another 4 days. The day of adding DMI was defined as the first day (day 0). In addition, cells were pretreated with MG132 at 20 µM for 2 h.

Administration of adeno-associated viral (AAV) vectors

AAV-FBXO2 was used to overexpress FBXO2 in WAT, while AAV-GFP was used as the control. Inguinal WAT (iWAT) pads on both sides of mice were injected with the AAV. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). For AAV delivery in iWAT, longitudinal incisions were made on the skin around the inguinal areas, followed by exposition of the fat pad using tweezers. AAV was injected into each fat pad in multiple spots (8–10 spots per fat pad). The total volume was 50 µl, and the total virus titer was 1×10^{11} viral genomes (vg) for each pad. For AAV delivery in eWAT, laparotomy was performed to expose the eWAT, and the AAV was injected as described above. Then, the abdomen was rinsed with sterile saline solution and closed with a two-layer suture.

Plasmid Construction and Cell Transfection

For stable RNA interference, shCON and shFBXO2 were purchased from Genechem Co., Ltd (Shanghai, China). Plasmid encoding IR-Flag, FBXO2-Myc, FBXO2-Flag, Del1 (1-124), Del2 (1-95), Del3 (1-54), FBD (55-95), IR (Y1179A), IR (Y1180A), and IR (Y1179, 1180A) were purchased from Sangon Co., Ltd (Shanghai, China). Plasmid encoding HA-UB were kindly provided by Dr. Ping Xie at Capital Medical University. siRNA for mouse FBXO2 and negative control siRNA (siNC) were purchased from Sangon Co., Ltd (Shanghai, China). For cell transfection: the transient expression of genes was achieved using lipofectamine with plus reagents according to the instructions from the manufacturer. Transient siRNA transfection into SVF was performed *in vitro* using Lip2000 according to the manufacturer's instructions. 48 hours after transfection, SVF were used for experiments.

LC-MS Analyses

HEK 293T cells were transfected with NC, IR-Flag/or FBXO2-Myc. Before harvest, cells were treated with 20 mM MG132 for 6 hr. Cell pellets were lysed in NP-40 lysis buffer (50 mM HEPES pH8.0, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, and 0.1% Tween 20 with protease and phosphatase inhibitors). After centrifugation, the supernatant was applied for immunoprecipitation with anti-FLAG® M2 affinity gel (B23102, Bimake) overnight. The following day, beads were washed and pulled-down proteins were eluted out using Flag peptides (F3290, Sigma-Aldrich). Eluted proteins were separated by SDS PAGE gel and visualized with Pierce™ Silver Stain for Mass Spectrometry (24612, Thermo Fisher Scientific Inc). Excised bands were destained and sent out for LC-MS/MS in Capital Medical University. The data were analysed and proteins with at least two unique peptides were retained for further analysis.

Cell Viability Assay

Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. Briefly, cells were plated at a density of 104 cells/well in 96-well plates. Cell viability was analyzed by using the CCK-8 kit according to the manufacturer's protocol.

Flow Cytometry Analysis

Adherent cells were trypsin-digested into single-cell suspensions, terminated with complete medium, and centrifuged at 1000 rpm for 5 min. Pellets were resuspended in pre-cooled 1×PBS for counting, then re-centrifuged. Cells were fixed in 0.5 mL pre-cooled 70% ethanol at 4°C overnight, centrifuged, and washed with PBS to remove fixative. PI staining solution (per manufacturer's instructions) was added (0.5 mL) for 37°C dark incubation (30 min), then stored at 4°C protected from light. Diluted, filtered samples were analyzed via calibrated BD flow cytometer (10,000 cells/sample), detecting PI fluorescence (617 nm excitation, FL3 channel). FlowJo software quantified G1/G0, S, G2/M phases; n = 3, statistically analyzed.

Real-Time RT-PCR

Total RNAs were isolated from tissues or cells using TRIzol reagent (Takara Bio, China) according to the manufacturer's instructions and converted into cDNA using a cDNA synthesis kit (Vazyme, China). RNA concentration was about 1500–3000 ng/μl determined with NanoDrop2000. Real-time PCR analysis was performed using SYBR Green Master Mix (Vazyme, China) in CFX96 Real-time System, C1000 Thermal Cycler (Bio Rad). The primer sets we used were as Table S3.

Western Blot and Immunoprecipitation

Total protein samples were isolated by treating tissue or cell samples with RIPA lysis buffer (65 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail tablets (P1265-2, Applygen, Beijing, China) and phosphatase inhibitor tablets (P1260-5, Applygen, Beijing, China)). The BCA Protein Assay Kit (23225, Thermo Fisher Scientific) was used to measure protein concentrations. Proteins were separated using 10% SDS-PAGE gels and then transferred to PVDF membranes (IPVH00010, Millipore). After the membranes were blocked in 5% skim milk, they were incubated overnight at 4°C with primary antibodies and then for 1 h at room temperature with the corresponding secondary antibodies. The primary antibodies we used were as Table S4. After 3 washes in TBST, the immune complexes were detected using the ECL detection reagents (SQ201, Epizyme, Shanghai, China). Protein expression levels were quantified using Image Lab software and normalized to the levels of GAPDH, which was used as a loading control. For Immunoprecipitation was performed using Pierce™ IP kit (26146, Thermo) following manufacture's guidelines.

Ubiquitination Assay

HEK 293T cells were co-transfected with IR-Flag, HA-ubiquitin, and/or FBXO2-Myc. Before harvest, cells were treated with 20 mM MG132 for 6 hr. Cells were lysed in IP lysis buffer. Then, subsequent experiments were performed using the Pierce™ IP Kit (26146, Thermo) following the manufacturer's guidelines. Samples were boiled in SDS loading buffer and subjected to western blot using anti-HA antibody.

Histology and Immunohistochemistry

When animals were sacrificed, WAT and liver tissues were dissected and immediately fixed in 4% paraformaldehyde. Tissues were then routinely processed for paraffin embedding, and 5 mm sections were cut and mounted on glass slides. For hematoxylin-eosin, Masson trichrome, IHC, and EDU staining, the liver, sWAT, and eWAT were fixed overnight in 4% formalin, embedded in paraffin, and sectioned into sections 5 mm in thickness. The sections were stained using the corresponding kits (Masson trichrome staining kits, G1340, Solebao, Beijing, China; EDU staining kit, C0071S/C0078S, Beyotime, Shanghai, China) according to the manufacturers' instructions. Immunohistochemistry were performed on paraffin embedded WAT and liver sections. Antigen retrieval was performed in citrate buffer using a pressure cooker. Immunohistochemistry (IHC) analysis was performed using IHC detection kit (PV-9001, ZSGB-

Bio, China) following manufacture's guidelines. The sections were visualized with DAB (ZLI-9018; ZSGB-Bio, China) and counterstained with hematoxylin.

Oil Red O Staining

Differentiated SVF and 3T3-L1 cells were fixed with 4% formaldehyde in PBS for 30 min and then washed 3 times in PBS for 10 min. For Oil-Red-O staining, the cells were stained with 0.3% Oil Red O for 30 min in the dark at room temperature. Then washed 3 times in PBS for 10 min. Oil-Red-O stained cells were directly imaged using an inverted microscope or lipid content was quantified after Oil-Red-O extraction with isopropanol by determination of light absorbance at 490 nm.

Glucose and Insulin Tolerance test

To determine glucose tolerance, 16-h-fasted mice were intraperitoneally administered with glucose (1.5 g/kg of body weight). The blood glucose levels were measured from the tail blood before and 15, 30, 60, and 120 min after glucose and insulin injections. To determine insulin sensitivity, 6-h-fasted mice were intraperitoneally administered with insulin (0.75 U/kg of body weight). The blood glucose levels were measured from the tail blood before and 15, 30, and 60 min after glucose and insulin injections. Blood glucose from tail-vein blood was quantified by a Sinocare glucometer at designated time after administration.

Biochemical Analysis

Serum TG (A110-1), TC (A111-1), FFA (A042-2-1), ALT (C009-3-1) and AST (C010-3-1) levels were measured with biochemical assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Elisa Assay

Serum insulin (CEA448Mu), leptin (SEA084Mu) and adiponectin (SEA605Mu) levels were measured with ELISA kits (Cloud-Clone, Wuhan, China).

Metabolic Assessment

We used β 3-adrenergic receptor agonist to activate brown fat. Mice were injected with CL316,243 (C5976; Sigma) at 1 mg/kg when needed. The core body temperature was monitored using a rectal probe (Omron, Dalian, China). Body temperature of mice was measured after CL316,243 injection. Body weight of mice was monitored weekly by a digital precision scale (accuracy 0.1 g). For energy metabolism analysis of mice normal chow diet (NCD) feeding or CL316,243 injection, a total of 96 h of monitoring was conducted, and the data of the latter 48 h were taken for analysis. Energy expenditure measurements were made using a Comprehensive Lab Animal Monitoring System (Columbus Instruments). Mice were acclimated to the Comprehensive Lab Animal Monitoring System (CLAMS) for at least 24 h prior to acquisition of data.

Statistical Analysis

The statistical significance of differences between groups was examined using unpaired two-tailed Student's *t* test or two-way ANOVA. All data are expressed as the mean $\pm$ SEM values and were analyzed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, California, USA). Differences with $p < 0.05$ were defined as significant. If not otherwise mentioned, each experiment was repeated independently with similar results at least three times.

Results

Insulin receptor protein levels decline during adipogenic differentiation via FBXO2 mediated proteasomal degradation

To explore the role of insulin receptor (IR) ubiquitination in adipogenesis, we examined IR expression at both transcriptional and translational levels during adipogenic differentiation of stromal vascular fraction (SVF) and 3T3-L1 cells. A significant decrease in IR protein levels was observed at day 2 of differentiation, while mRNA levels remained stable (Fig. 1A, B and fig. S1A, B). This reduction in IR protein was evident as early as 12 hours post-differentiation induction, with no change in mRNA levels (Fig. 1C and fig. S1C, D). Given that ubiquitination is a key post-translational modification for protein degradation, we investigated whether IR reduction was linked to proteasome-dependent degradation using the proteasome inhibitor MG132. MG132 treatment significantly increased IR protein levels during early adipogenic differentiation (Fig. 1D and fig. S1E).

Mass spectrometric analysis identified IR-interacting proteins, revealing that IR co-purified with FBXO2, Cullin1, and SKP1, components of the SCF^{FBXO2} complex (Fig. 1E and fig. S2A, S2B). Detailed data of the mass spectrometry analysis are provided in Supplementary Material Table S1. These interactions were confirmed in 3T3-L1 cells and subcutaneous white adipose tissue (sWAT) (Fig. 1F, G). Further mass spectrometry analysis identified FBXO2-interacting proteins (Fig. 1H and fig. S2C). Detailed data of the mass spectrometry analysis are provided in Supplementary Material Table S2. Overexpression of FBXO2 reduced IR protein levels and downstream insulin signaling molecules (IRS and AKT) without affecting *Insr* mRNA levels (Fig. 1I and fig. S2D, S2E). Conversely, FBXO2 knockdown increased IR and downstream molecule levels (fig. S2F, S2G). The reduction in IR protein caused by FBXO2 was rescued by MG132 treatment (Fig. 1J). These findings suggest that IR protein levels decrease during early adipogenic differentiation, likely regulated by FBXO2-mediated proteasomal degradation.

FBXO2 catalyzes ubiquitination and degradation of phosphorylated IR

We next tested whether IR is a direct substrate of FBXO2. *In vivo* ubiquitination assays confirmed that FBXO2 modifies IR ubiquitination (Fig. 2A). Notably, phosphorylated IR (p-IR) was more significantly altered than total IR (Fig. 1I, J). These results indicate that phosphorylated modification might be

essential for FBXO2-mediated IR ubiquitination. To test this, we generated IR mutants at tyrosine sites Y1179 and Y1180. Ubiquitination assays showed that IR ubiquitination was unaffected by single mutations at Y1179 or Y1180 but was nearly abolished with double mutations (Fig. 2B). Thus, FBXO2-mediated IR ubiquitination is on base of its phosphorylation.

FBXO2 contains four domains: PEST, F-box (FBD), linker, and sugar-binding (SBD). To elucidate the role of these domains, we constructed FBXO2 fragments (fig. S2H). Only fragments containing the FBD reduced p-IR/IR levels (Fig. 2C, D), highlighting its essential role for FBXO2. Ubiquitination assays confirmed that the FBD is crucial for IR multi-ubiquitination (Fig. 2E). These results demonstrate that phosphorylation is essential for IR ubiquitination and degradation, and the FBD is indispensable for FBXO2 function.

FBXO2 inhibits proliferation and adipogenic differentiation of preadipocytes by attenuating insulin signaling pathway *in vitro*

Given that FBXO2 weakens insulin signaling by degrading p-IR, we hypothesized that FBXO2 regulates adipogenic differentiation. *Fbxo2* mRNA levels increased during early adipogenic differentiation and peaked after 12 hours induction (Fig. 3A and fig. S3A, B). FBXO2 protein levels also increased during early differentiation (Fig. 3B, C and fig. S3C, D). The early stage of adipocyte differentiation is for preadipocytes mitotic clonal proliferation (30, 31). We observed that knockdown of FBXO2 decreased the G1 + S phase and increased the G2 + M phase preadipocytes (Fig. 3D-F), promoted cell viability by CCK8 (Fig. 3G), and enhanced cell proliferation by EDU staining (Fig. 3H, I). Meanwhile, knockdown of FBXO2 upregulated proliferation genes (*Ki67*, *Ly6a*, *Pdgfra*, *Pdgfrβ*), without affecting apoptosis genes (*Bax*, *Casp2*, *Puma*) (Fig. 3J, K). Conversely, FBXO2 overexpression reduced proliferation and downregulated proliferation genes, while apoptosis genes remained unchanged (Fig. 3L-O). FBXO2 knockdown also inhibited SVF proliferation (fig. S3E-I), underscoring its role in preadipocyte proliferation.

During adipogenic differentiation, FBXO2 knockdown enhanced early factor induction (*C/ebpβ*, *Klf5*, *Krox20*) and adipogenic gene expression (*C/ebpa*, *Adiponectin*, *Pparγ*) (Fig. 4A, B). Oil Red O staining showed increased lipid accumulation with FBXO2 knockdown (Fig. 4C, D). Conversely, FBXO2 overexpression inhibited adipogenic gene expression and lipid accumulation (Fig. 4E-H). FBXO2 knockdown increased p-IR/IR and p-AKT/AKT levels (Fig. 4I-K), while overexpression inhibited insulin signaling (Fig. 4L-N). FBD overexpression also inhibited adipogenic gene expression (Fig. 4O, P). Additionally, FBXO2 knockdown promoted adipogenic gene induction and lipid accumulation in sWAT but not eWAT (fig. S4A-E). Collectively, FBXO2 suppresses adipogenic differentiation by inhibiting insulin signaling *in vitro*.

FBXO2 deficiency in adipocyte progenitors enhances adipogenesis in sWAT by augmenting insulin signaling

To investigate the contribution of FBXO2 in adipogenesis *in vivo*, mice carrying the floxed-Fbxo2 allele containing two loxP sites were crossed with adipocyte progenitor-specific targeted Pdgfra-Cre to generate *Fbxo2*^{Pdgfra-/-} mice. Mice were respectively fed with a normal chow diet (NCD) or high-fat diet (HFD) for 12 weeks, it was shown that body weight and tissue weight ratio to the body including eWAT, BAT, and liver of *Fbxo2*^{Pdgfra-/-} mice remained comparable to those of control. While subcutaneous fat mass of *Fbxo2*^{Pdgfra-/-} mice exhibited a higher ratio in both NCD and HFD group (Fig. 5A, B). Histological analysis revealed a higher number of adipocytes with smaller size in sWAT from both NCD and HFD-fed *Fbxo2*^{Pdgfra-/-} mice compared to control, indicating the pro-adipogenic effect of Fbxo2 deficiency (Fig. 5C-E). These phenotypic traits were accompanied by upregulated mRNA levels of adipocyte progenitor markers and adipogenesis genes in sWAT of *Fbxo2*^{Pdgfra-/-} mice fed with NCD or HFD, while the apoptotic genes expression was comparable between two groups (Fig. 5F and fig. S5A, B). EdU tracing experiments revealed higher vitality of adipogenic progenitors in sWAT from *Fbxo2*^{Pdgfra-/-} mice compared to control mice under both NCD and HFD feeding (Fig. 5G-J). SVF isolated from sWAT of *Fbxo2*^{Pdgfra-/-} mice showed a greater number of lipid droplets accumulation by Oil Red O staining (Fig. 5K, L). Meanwhile, protein levels of p-IR/IR and p-AKT/AKT were increased in sWAT or primary SVF of *Fbxo2*^{Pdgfra-/-} mice compared to control (Fig. 5M and fig. S5C, D). In eWAT, the adipocyte size and number, mRNA levels of adipogenic factors, number of EdU positive progenitors and proliferation genes expression were not altered in *Fbxo2*^{Pdgfra-/-} mice compared with control under either NCD or HFD feeding (fig. S7A-G).

In another mouse model, we generated *Fbxo2*^{Fabp4-/-} mice by crossing Fbxo2-Flox with Fabp4-Cre mice. There was also no discernible difference between control and *Fbxo2*^{Fabp4-/-} mice in body or tissue weight when subjected to NCD or HFD feeding, except for that the mass-to-body-weight ratio of sWAT was mildly elevated in *Fbxo2*^{Fabp4-/-} mice (Fig. S6A, B). Notably, *Fbxo2*^{Fabp4-/-} mice exhibited an increased number of PDGFR α or EDU-positive adipocytes with smaller size in sWAT compared to control (Fig. S6C-J). Lipid accumulation was increased in SVF of *Fbxo2*^{Fabp4-/-} mice (Fig S6K, L). The expression of adipogenic genes in sWAT of *Fbxo2*^{Fabp4-/-} mice was higher than that of control (Fig. S6M and fig. S5E, F). FBXO2 deficiency led to higher protein levels of p-IR/IR and p-AKT/AKT, indicating an enhanced insulin signaling pathway in sWAT of *Fbxo2*^{Fabp4-/-} mice (Fig. 6N and fig. S5G, H). Additionally, adipogenesis in eWAT of *Fbxo2*^{Fabp4-/-} mice was similar with control (fig. S7H-N). All together, these results suggest that FBXO2 deficiency in preadipocytes promotes adipogenesis by enhancing insulin signaling pathway.

To further validate that the biological function of FBXO2 predominantly on regulating adipogenesis rather than mature adipocytes, we generated *Fbxo2*^{Adipoq-/-} mice by using Adipoq-Cre mice. A series of studies showed that there was no discernible difference on adipogenesis between *Fbxo2*^{Adipoq-/-} mice and control group (fig. S8A-P and fig. S7O-P).

FBXO2 deficiency improves metabolic health in obese mice

We next investigated whether the effects of FBXO2 in regulating adipogenesis have an impact on whole-body metabolism. It was shown that FBXO2 deficiency improved glucose tolerance and insulin sensitivity in *Fbxo2*^{Pdgfra^{-/-}} mice (Fig. 6A, B). Serum levels of triglyceride (TG), total cholesterol (TC), free fatty acid (FFA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and insulin were lower, while adiponectin was higher in *Fbxo2*^{Pdgfra^{-/-}} mice (Fig. 6C, D and fig. S9A-F). Next, we assessed fibrosis and inflammation in adipose and liver tissue by Masson staining and immunohistochemical assays. Notably, eWAT and sWAT of *Fbxo2*^{Pdgfra^{-/-}} HFD mice exhibited alleviative fibrosis and inflammation compared to control (Fig. 6E and fig. S9G). Additionally, the expression of genes associated with fibrosis (*Acta2*, *Col1a1*, *Tgfβ1*) and inflammation (*Il6*, *Il1β*, *Tnfa*) was markedly lower in eWAT and sWAT of *Fbxo2*^{Pdgfra^{-/-}} mice than control with an HFD feeding (Fig. 6F, G and fig. S9H, I). Histological examination showed a pronounced alleviative lipid infiltration, fibrosis, and inflammation in the liver of *Fbxo2*^{Pdgfra^{-/-}} HFD mice than control (Fig. 6H). The expression of genes associated with fibrosis, inflammation, mitochondrial, hypoxia, and macrophages marker was also markedly lower in the liver of *Fbxo2*^{Pdgfra^{-/-}} HFD mice than control mice (Fig. 6I, J and fig. S9J). In the other mouse model, FBXO2 deficiency also improved whole-body metabolism in *Fbxo2*^{Fabp4^{-/-}} mice, including glucose homeostasis and serum biochemical parameters (Fig. 6K-N and fig. S9K-P). *Fbxo2*^{Fabp4^{-/-}} mice also exhibited an improved metabolic state in adipose tissue and liver (Fig. 6O-T and fig. S9Q-T). In analysis of *Fbxo2*^{Adipoq^{-/-}} mice, it was shown that there was no obviously change in their whole body metabolism (fig. S10A-K). Taken together, these data suggest that FBXO2 deficiency in preadipocytes significantly contributes to the amelioration of systemic metabolic health.

Given the pivotal roles of brown and beige fat biogenesis in regulating metabolic health (34–38). We conducted a comprehensive assessment of various metabolic parameters in both *Fbxo2*^{Pdgfra^{-/-}} and control mice under basal condition or CL316,243 stimulation. Our analysis revealed no significant differences in thermogenesis, energy expenditure and whole body metabolism between control and *Fbxo2*^{Pdgfra^{-/-}} mice (fig. S11A-T).

FBXO2 overexpression inhibits adipogenesis and exacerbates metabolic syndrome

To further study the role of FBXO2 overexpression in regulating adipogenesis and metabolic health. We next conducted gene overexpression with Adeno-Associated Virus (AAV)-mediated *Fbxo2* gene transfer into adipose tissue of mice. The FBXO2-overexpression and control mice were subjected to a short or long term HFD feeding for 4 or 16 weeks. There was no discernible difference in body weight and fat mass between the two groups fed with short or long term HFD (fig. S12A-D). Histological analysis revealed that adipocytes in sWAT from AAV-FBXO2 mice with larger size compared to control with both

short and long term HFD feeding (Fig. 7A, B and fig. S12E). Furthermore, FBXO2 overexpression inhibited the mRNA levels of adipocyte progenitor markers and the adipogenic genes in sWAT of both short and long term HFD feeding mice, while the apoptotic genes expression was comparable between the two groups (Fig. 7C and fig. S12F). We also observed that fibrosis and inflammation were obviously aggravated in sWAT of AAV-FBXO2 mice with Masson, F4/80 and α -SMA staining (Fig. 7A). This was further validated by higher expression of inflammatory and fibrotic genes (Fig. 7D, E). FBXO2-overexpression mice showed impaired glucose tolerance and insulin sensitivity compared with control by glucose tolerance test (GTT) and insulin tolerance test (ITT) in both short and long term HFD feeding mice (Fig. 7F, G and fig. S12G, H). Serum levels of TG, TC, FFA, and leptin were significantly higher in HFD-fed AAV-FBXO2 mice than that of control, while adiponectin level was lower in AAV-FBXO2 mice (Fig. 7H-L). Further, we analyzed the biological function of eWAT and liver caused by FBXO2 overexpression. Histological analysis showed that eWAT of AAV-FBXO2 mice with larger adipocyte size, aggravated fibrotic and inflammatory state with HE, Masson, F4/80 and α -SMA staining (Fig. 8A). Higher expression of inflammatory and fibrotic genes was found in eWAT of FBXO2 overexpression mice (Fig. 8B, C). In the liver, FBXO2 overexpression obviously aggravated fat infiltration, fibrosis and inflammation by analysis of HE, Masson, F4/80 and α -SMA staining (Fig. 8D). Serum levels of ALT and AST were increased which indicated impaired liver function in FBXO2 overexpressed mice (Fig. 8E, F). Also, the expression of inflammatory and fibrotic genes was higher in the liver of FBXO2 overexpressed mice than control (Fig. 8G, H). Taken together, these data suggest that FBXO2 overexpression significantly inhibited adipogenesis and aggravated metabolic unhealthy of HFD induced obese mice.

Discussion

The emergence of hyperplastic adipocytes in de novo differentiation is often accompanied by the attenuation of fibrosis and inflammation within white adipose tissue during obesity (39, 40). There is a growing body of evidence suggesting that promoting adipogenesis contributes to the preservation of metabolic health and enhances glucose metabolism (36). Consequently, researchers are actively seeking novel regulators of adipogenesis to address obesity-related metabolic disorders. Notably, insulin serves as a central regulator of adipogenesis, with the insulin receptor (IR) playing a pivotal role in the insulin signaling pathway (26, 27). Post-translational modification of IR, particularly ubiquitination has been implicated in regulating insulin signal transduction. However, the precise relationship between IR post-translational modification and adipogenesis remains elusive. In our study, we demonstrate that FBXO2 serves as a potent repressor of adipogenesis both *in vitro* and *in vivo*, by catalyzing the ubiquitinated modification and subsequent degradation of the phosphorylated IR. FBXO2 exerts this inhibitory effect by suppressing the proliferation of adipocyte progenitors, leading to aberrant adipocyte expansion and dysfunction. Consequently, FBXO2 promotes the onset of insulin resistance, fatty liver, and other obesity-related complications (Fig. 8I). Our findings offer a novel theoretical foundation for further understanding the mechanism of adipogenesis.

It is reported that FBXO2 plays a multifaceted role in various biological processes by targeting diverse substrates, including those involved in tumor progression, non-alcoholic fatty liver disease, and viral and

bacterial infections (41–44). In this study, we firstly propose the role of FBXO2 in regulating adipose plasticity which is a braking mechanism of insulin signaling pathway in adipogenesis. Given that the substrate of FBXO2 is always a phosphorylated protein, we sought to elucidate whether FBXO2 specifically targets the phosphorylated form of IR. As expected, our investigation revealed that FBXO2 could no longer ubiquitinate IR if the phospho-sites (Y1179 and Y1180) are mutant. This indicates that FBXO2 effectively and forcefully involves in regulating insulin signaling by directly targeting p-IR ubiquitination and degradation. In other studies, it has been documented that the SBD domain is essential for FBXO2 to recognize certain glycoprotein substrates (43). Hence, we infer that FBXO2 catalyzes the ubiquitination of different substrate proteins via either the FBD or SBD domain in various physiological and pathological contexts. In this study, we elucidate the indispensable role of the F-box domain (FBD) for FBXO2 in catalyzing p-IR ubiquitination. In future, screening for small molecule compounds that target the FBD domain or p-IR ubiquitination could offer novel therapeutic strategies for promoting adipogenesis and ameliorating metabolic health in obesity.

Obesity often precipitates a decline in the plasticity of adipose tissue and may lead to fibrosis, inflammation, progenitor cell senescence, and catecholamine resistance (45, 46). The mechanisms governing adipose tissue expansion, including the proliferation of fat cells or the enlargement of existing fat cells, as well as its distribution (subcutaneous or visceral), profoundly influence metabolic health (47, 48). Notably, the proliferative activity of adipocyte progenitors serves as a key determinant of adipose tissue plasticity, orchestrating critical adaptive processes such as adipose tissue expansion, browning, and the maintenance of the fat cell population (39, 49). In mice, *Fbxo2* deficiency led to an increased number of newly generated fat cells in sWAT while its overexpression obviously inhibited adipocytes generation. In our study, we find that FBXO2 mainly regulated plasticity of subcutaneous fat but not in visceral fat. This may be attributed to regional difference of adipogenic potential between sWAT and eWAT (50). Recent studies have shed further light on the benefits of adipogenesis in maintaining metabolic health, particularly in obese individuals. Our findings indicate that FBXO2 deficiency in adipose progenitors give mice a metabolically healthy phenotype. Our work underscoring the role of FBXO2 as an inhibitor of adipogenesis and its potential use for regulating adipose plasticity and metabolic health.

Together, our study identifies FBXO2 as an inhibitor of adipogenesis. FBXO2 targets insulin receptor for ubiquitination based on its phosphorylation to brake insulin signaling in adipogenesis both *in vitro* and *in vivo*. Gene deletion of *Fbxo2* in mice promoted adipogenesis and improved remodeling of adipose tissue, thus ameliorating the overall metabolic health under the condition of excess nutrition. While *Fbxo2* overexpression showed an opposite function on regulating adipogenesis and metabolic health. Our findings suggest that targeting insulin receptor ubiquitination catalyzed by FBXO2 may represent a novel therapeutic approach for improving metabolic health in obesity.

Declarations

Competing interests

The authors declare no conflicts of interest.

Ethical approval

All animal experiments were approved by the Medical Ethics Committee of Capital Medical University.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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Author contributions

Y.G. and D.F. conceived the project and designed research. Y.X. mainly performed the experiments. J.L., Z.Z., and H.W. provided help in histological staining and genotyping. M.W., X.W., R.W., Q.W., X.J., L.W., H.S., X.L., M.Z., T.L., and C.Y. helped in mice tissue processing and other assistance. Y.X., D.F., and Y.G. analyzed the data and wrote the original draft.

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Figures

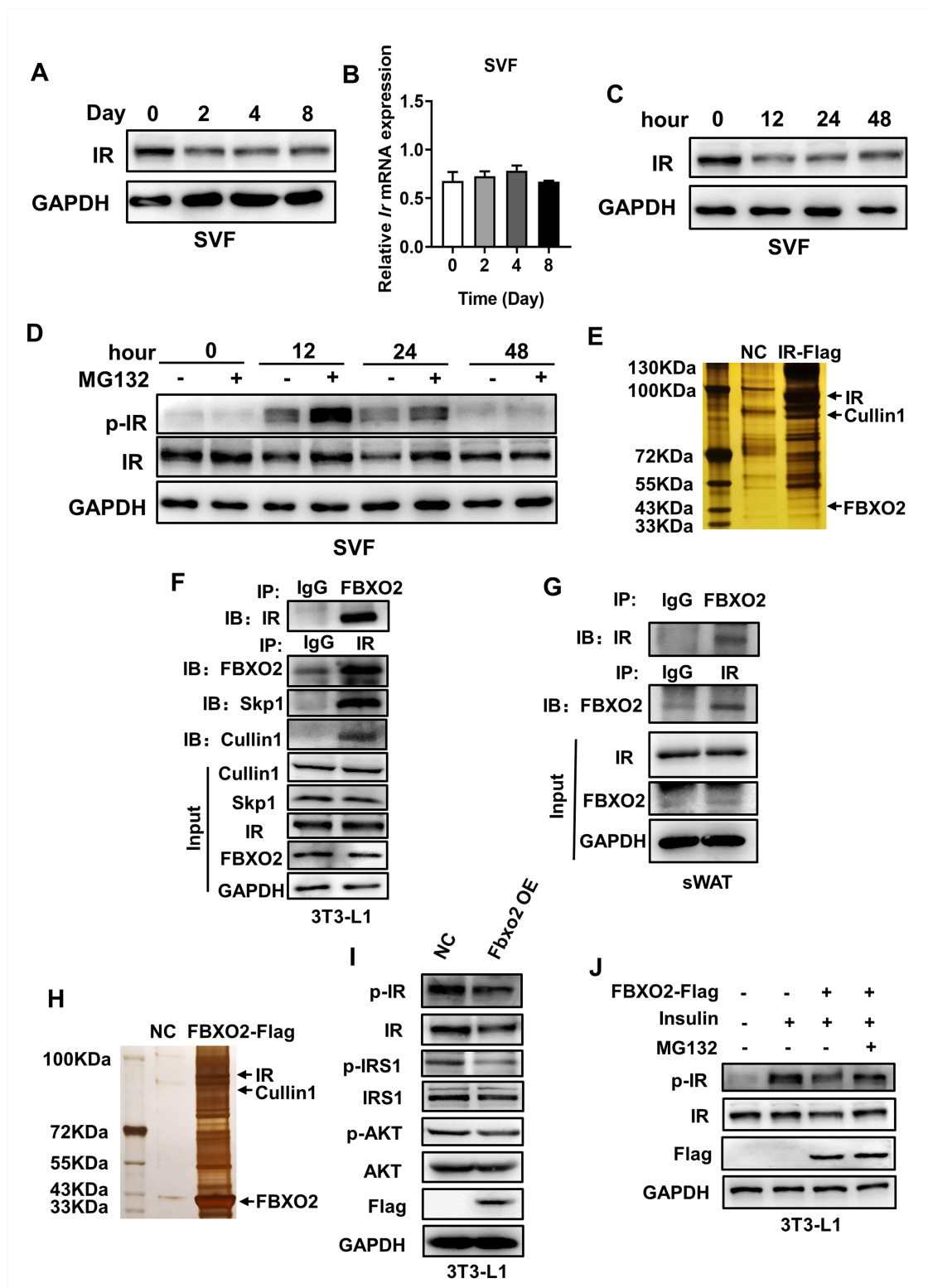


Figure 1

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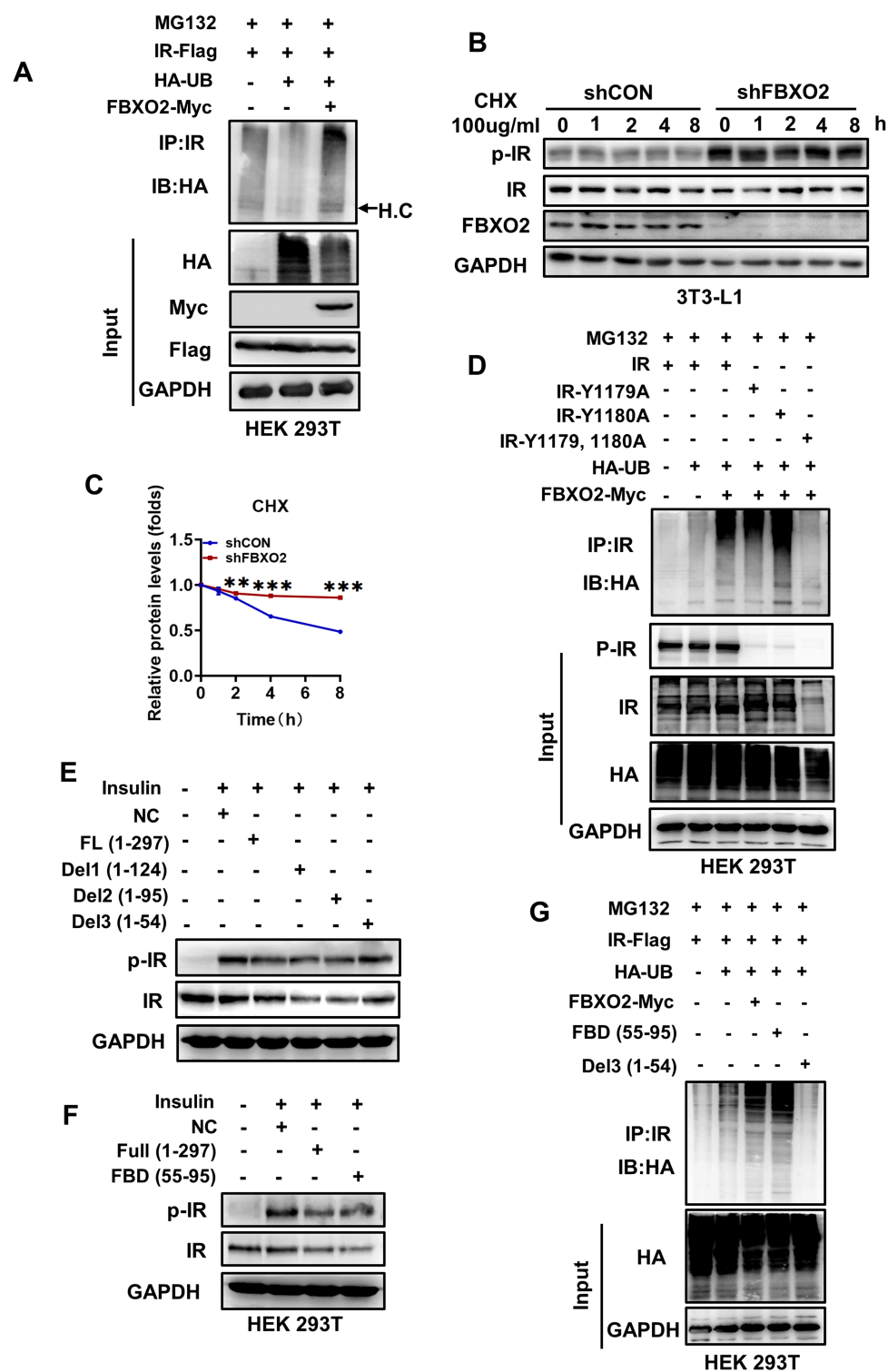


Figure 2

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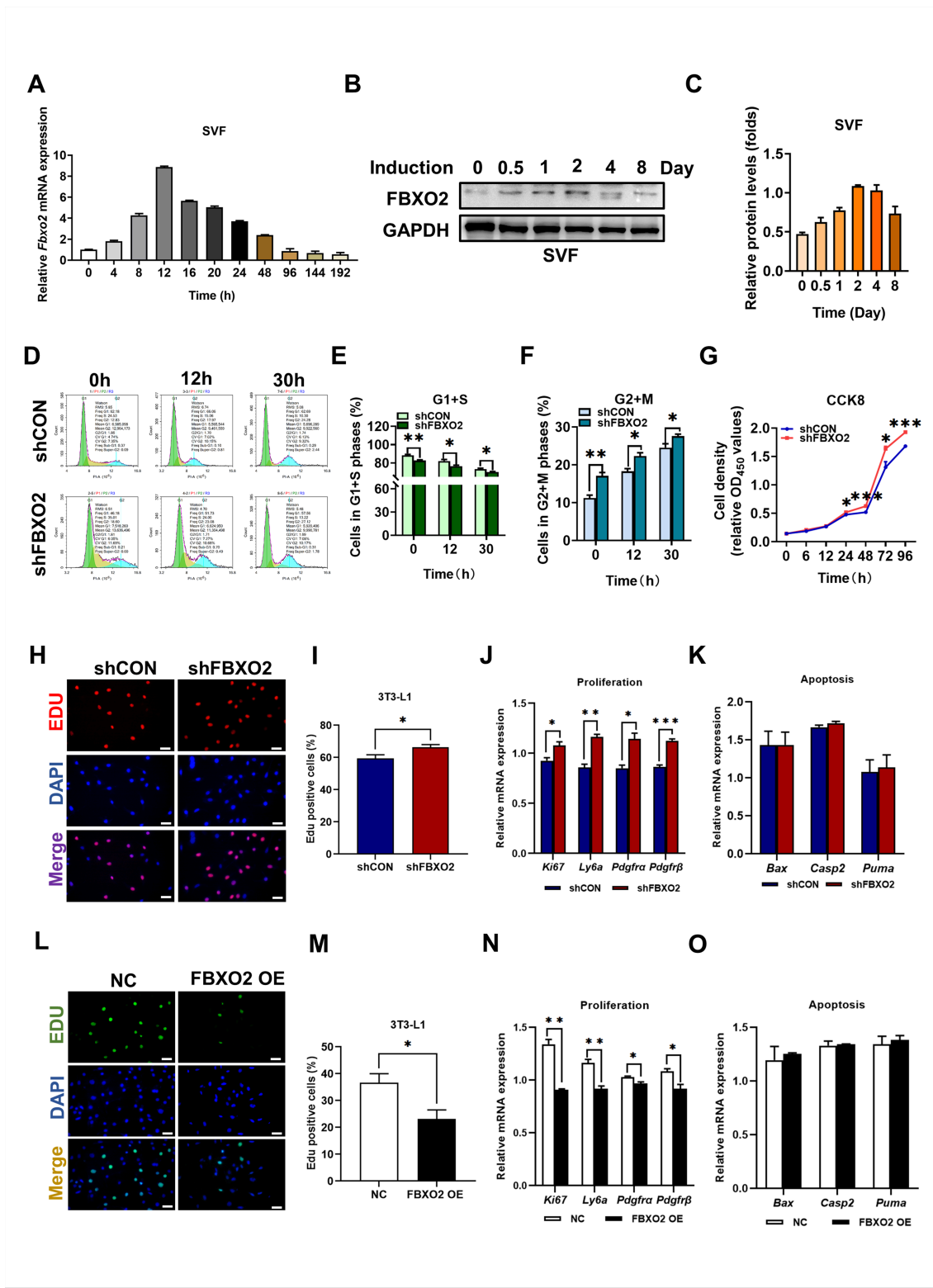


Figure 3

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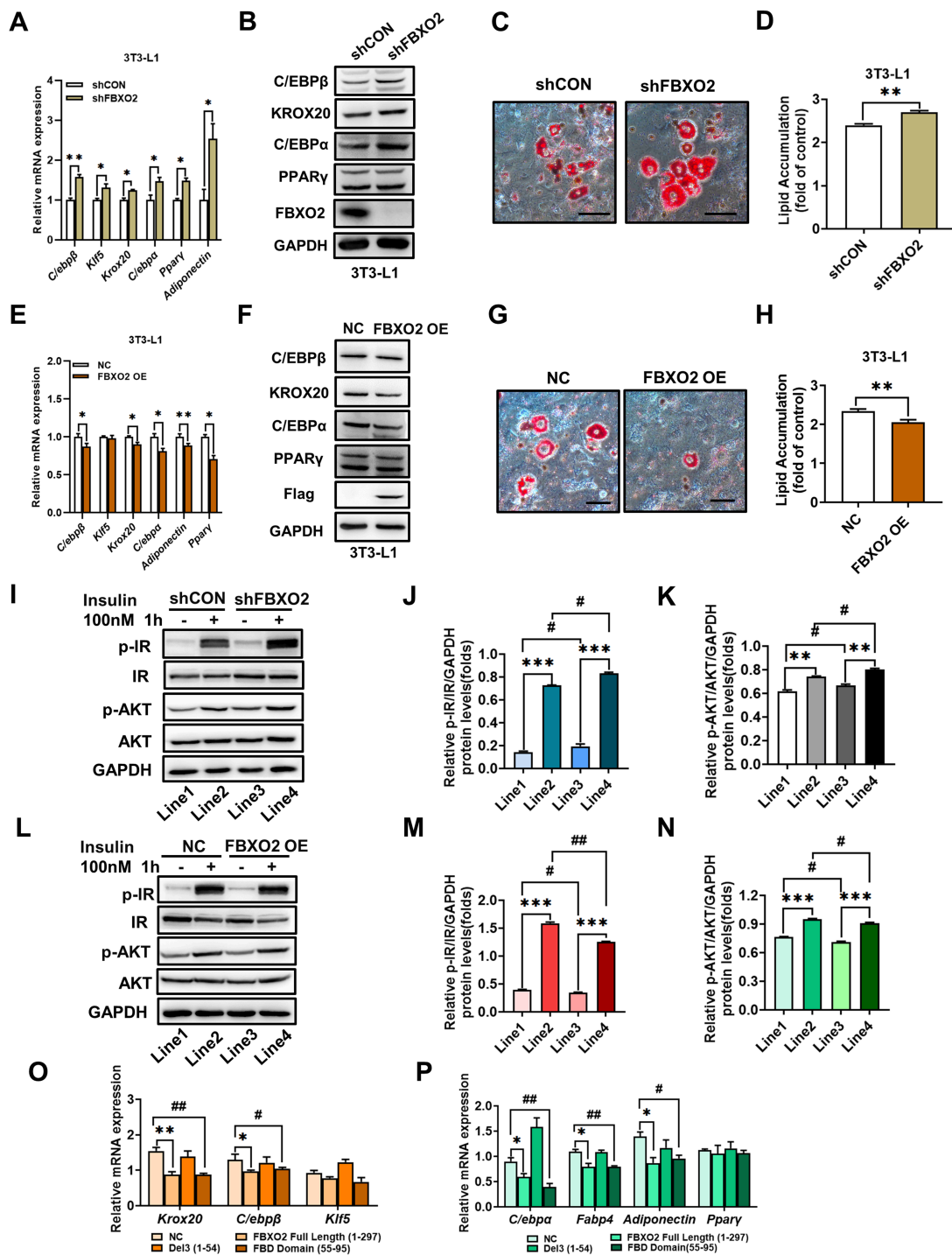


Figure 4

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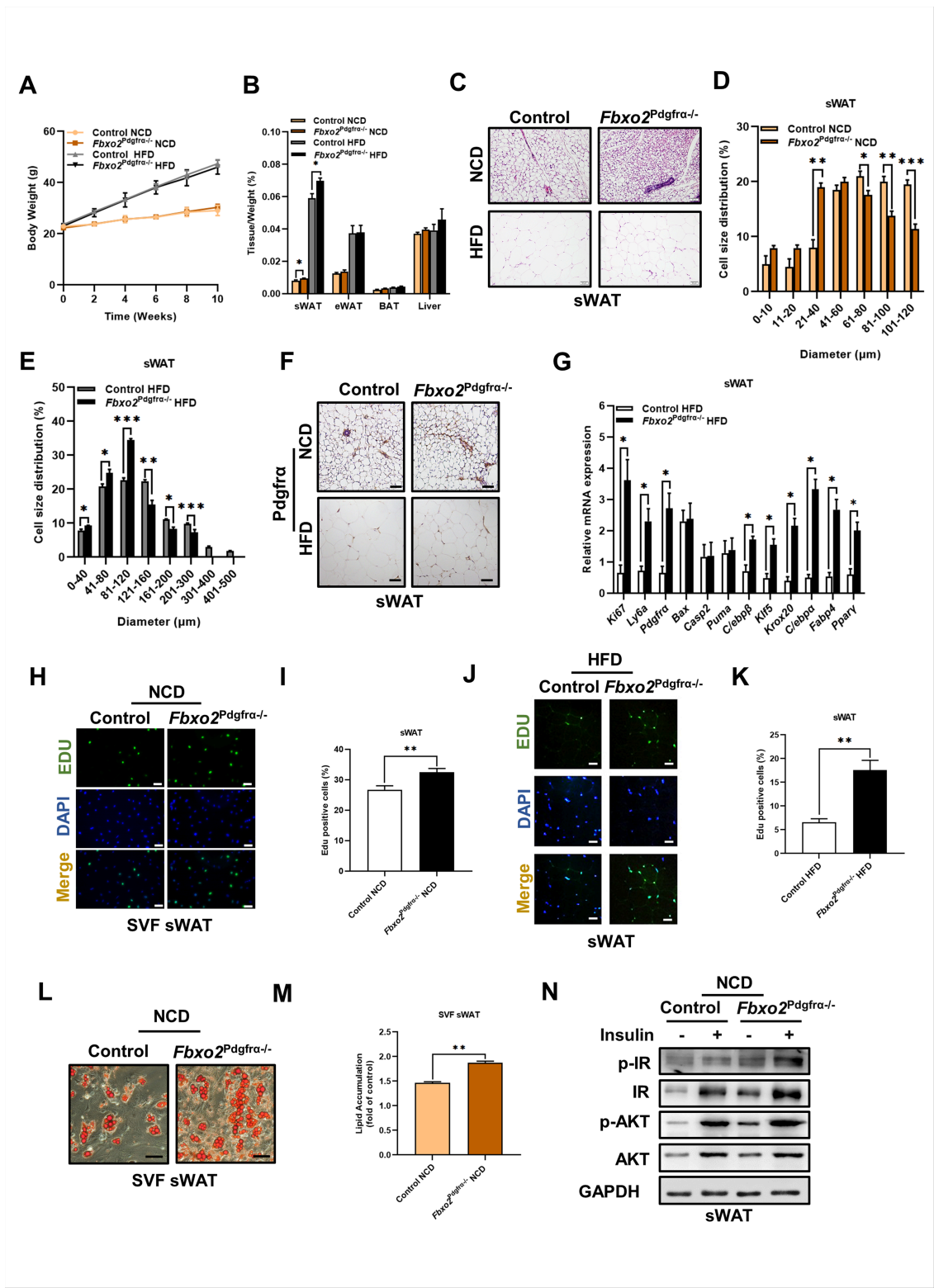


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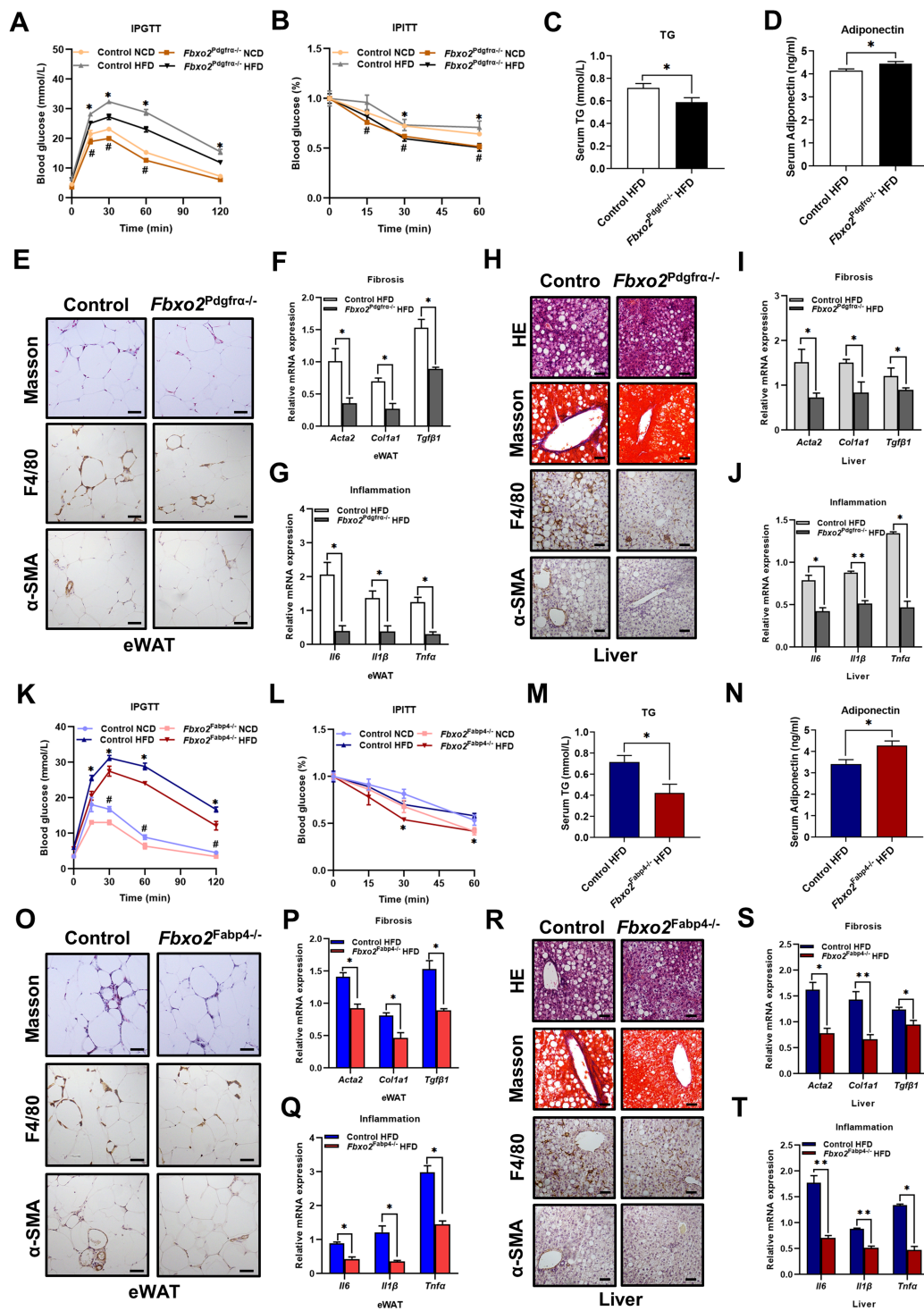


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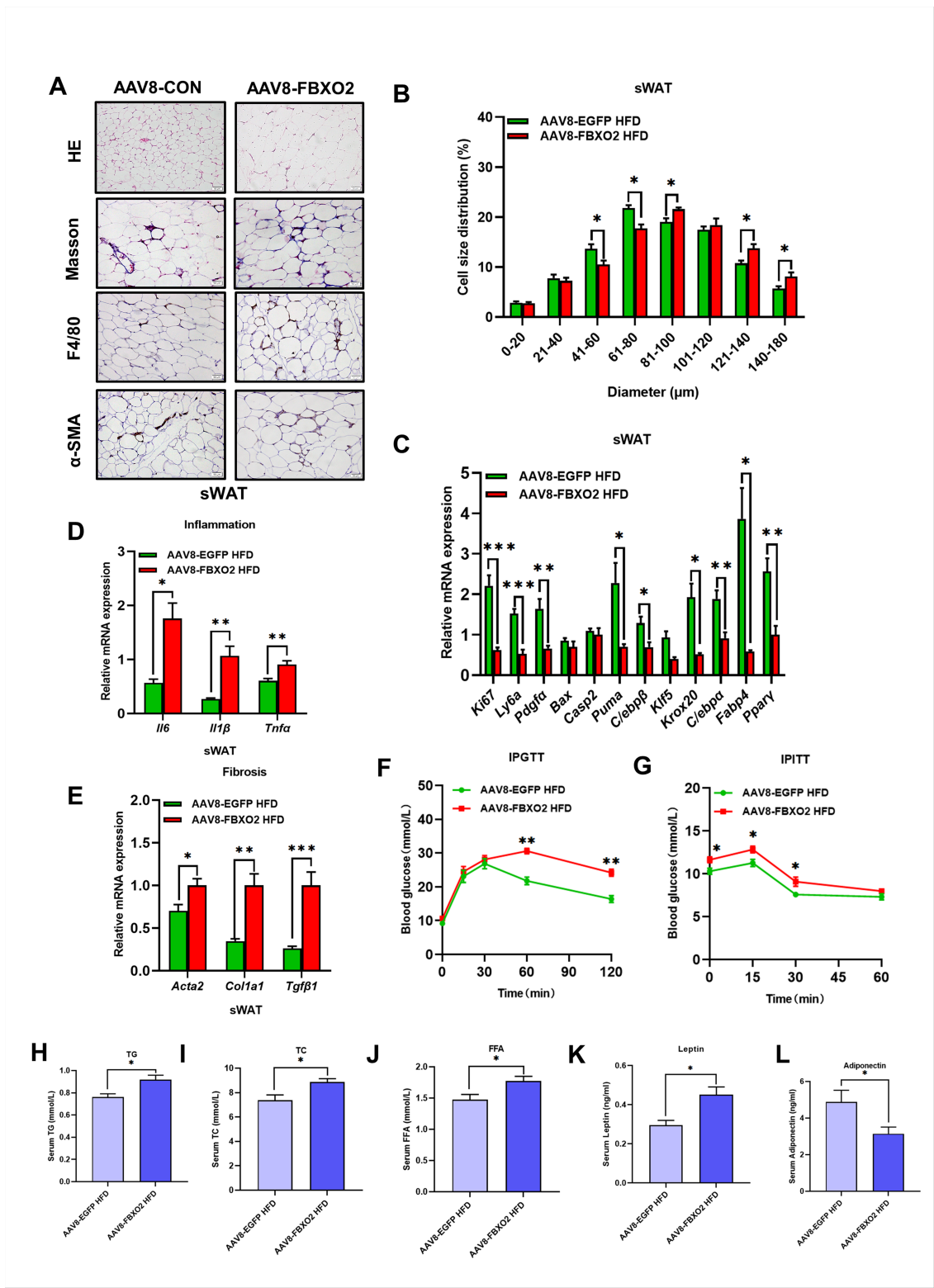


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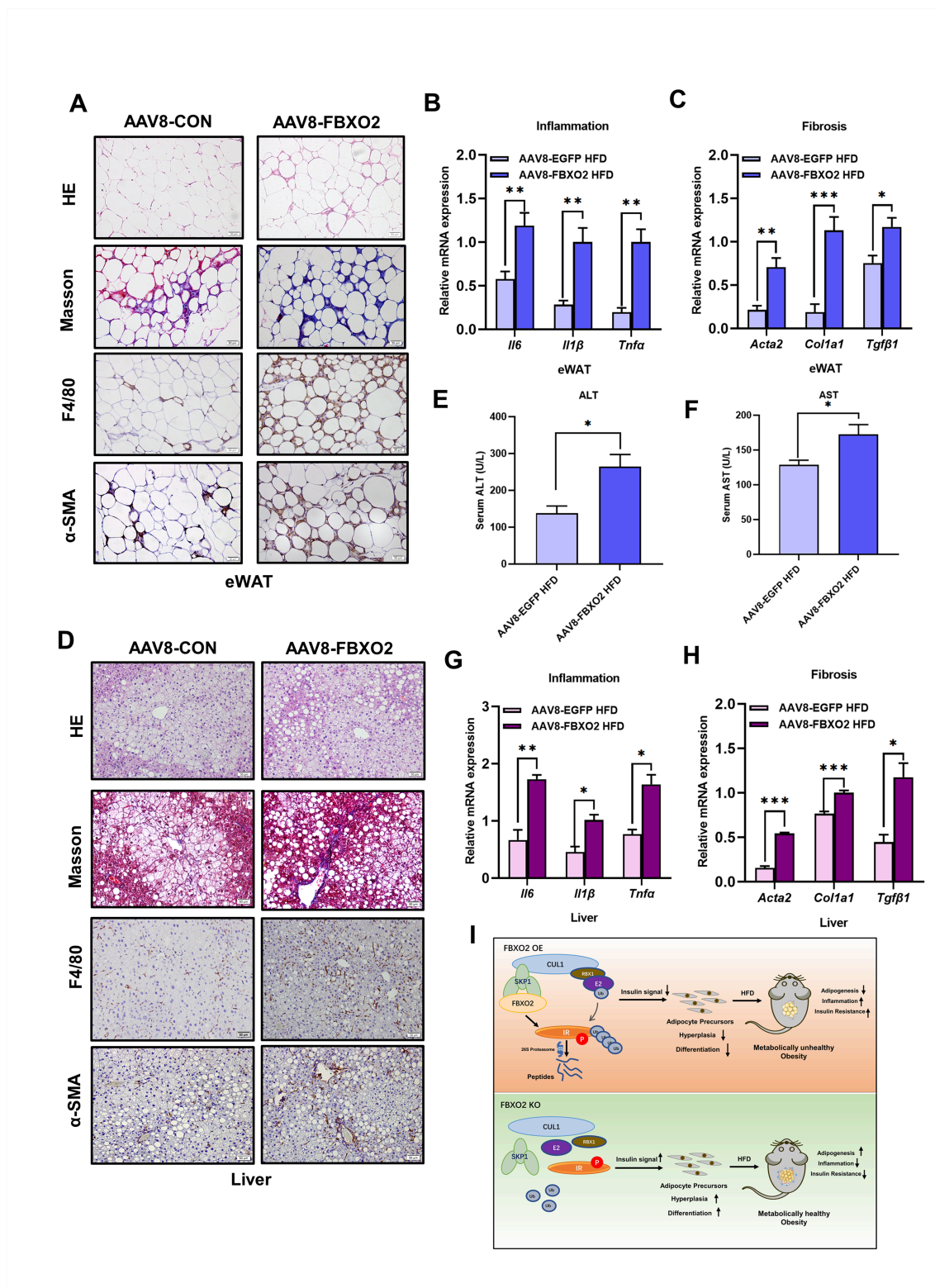


Figure 8

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