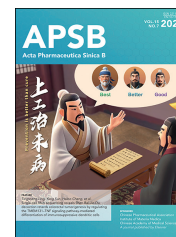




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ORIGINAL ARTICLE

RXR α modulates hepatic stellate cell activation and liver fibrosis by targeting CaMKK β –AMPK α axis



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KEY WORDS

K-80003;
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Abstract Hepatic stellate cells (HSCs) are the primary fibrogenic cells in the liver, and their activation plays a crucial role in the development and progression of hepatic fibrosis. Here, we report that retinoid X receptor-alpha (RXR α), a unique member of the nuclear receptor superfamily, is a key modulator of HSC activation and liver fibrosis. RXR α exerts its effects by modulating calcium/calmodulin-dependent protein kinase kinase β (CaMKK β)-mediated activation of AMP-activated protein kinase-alpha (AMPK α). In addition, we demonstrate that K-80003, which binds RXR α by a unique mechanism, effectively suppresses HSC activation, proliferation, and migration, thereby inhibiting liver fibrosis in the CCl₄ and

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Phase separation;
Mitophagy

amylin liver NASH (AMLN) diet animal models. The effect is mediated by AMPK α activation, promoting mitophagy in HSCs. Mechanistically, K-80003 activates AMPK α by inducing RXR α to form condensates with CaMKK β and AMPK α via a two-phase process. The formation of RXR α condensates is driven by its N-terminal intrinsic disorder region and requires phosphorylation by CaMKK β . Our results reveal a crucial role of RXR α in liver fibrosis regulation through modulating mitochondrial activities in HSCs. Furthermore, they suggest that K-80003 and related RXR α modulators hold promise as therapeutic agents for fibrosis-related diseases.

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1. Introduction

Nonalcoholic steatohepatitis (NASH), also known as metabolic dysfunction-associated steatohepatitis (MASH), is an advanced form of nonalcoholic fatty liver disease (NAFLD). It is characterized by liver steatosis, inflammation, and fibrosis, which can progress to cirrhosis, liver failure, and hepatocellular carcinoma^{1,2}. Members of the nuclear receptor family hold significant potential for treating metabolic diseases³. Among them, retinoid X receptor- α (RXR α), liver X receptor (LXR), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs), and thyroid hormone receptor (T₃R) are highly expressed in the liver and play crucial roles in regulating bile acid, lipid, and glucose homeostasis⁴. Several modulators targeting these nutrient-sensing nuclear receptors, including obeticholic acid (OCA; an FXR α agonist), pioglitazone (a PPAR γ agonist), T₃R β agonist (resmetirom), elafibranor (GFT505; a dual PPAR δ /PPAR α agonist), and lanifibranor (a pan-PPAR agonist), have been extensively evaluated in clinical trials for treating MASH. Resmetirom has recently become the first approved drug for the treatment of adults with MASH^{1,5-9}. However, improving the therapeutic index of these compounds remains a challenge. There is a need for nuclear receptor modulators with enhanced anti-fibrotic efficacy and fewer side effects to optimize NASH treatment outcomes.

Liver fibrosis, the primary determinant of mortality in MASH¹⁰, is reversible, making it a key target for therapeutic intervention. There is significant interest in developing new strategies to reverse liver fibrosis and/or prevent its progression to cirrhosis^{2,10}. The key fibrogenic effector cell type in the liver is the activated hepatic stellate cells (HSCs)^{2,10}. The activation of hepatic stellate cells (HSCs) plays a central role in fibrosis, as these cells transdifferentiate into myofibroblasts that produce extracellular matrix (ECM) proteins, driving the fibrogenic response to liver injury^{2,10}. A hallmark of HSC activation is the depletion of lipid droplets, which store 50%–80% of the body's vitamin A¹¹⁻¹³. Upon activation, vitamin A is released and subsequently converted into retinoic acids (RAs), which regulate HSC activation and fibrosis^{12,14,15}. RA and its natural and synthetic analogs (retinoids) are crucial in various biological processes, including cell growth, differentiation, apoptosis, lipid metabolism, and calcium signaling¹⁶⁻¹⁹. Their effects are primarily mediated by 2 classes of nuclear receptors, RA receptors (RARs) and RXRs, both belong to the nuclear receptor superfamily^{3,18,20-22}. Among the three RXR subtypes, RXR α is highly expressed in HSCs^{15,22,23}, yet its levels decline during liver fibrosis²⁴. Modulating RXR α activities with its ligands has been shown to inhibit

HSC activation and liver fibrosis^{23,25-27}. However, the precise role of RXR α in HSC activation remains obscure, warranting further investigation.

Members of the nuclear receptor superfamily contain a C-terminal ligand-binding domain (LBD), responsible for ligand-binding and receptor dimerization, and a DNA-binding domain (DBD), which enable specific DNA interaction²⁰. These receptors act as transcription factors, regulating target gene expression by binding to its cognate DNA response elements either as homodimers²⁸ or heterodimers^{29,30}. Additionally, nuclear receptors can act independently of their transcription regulation by interacting with various cytoplasmic signaling proteins³¹⁻³⁴. Beyond the LBD and DBD, nuclear receptors possess an intrinsically disordered region (IDR) at their N-terminal end. The IDR contributes to the complexity of nuclear receptor regulatory networks and cell- and tissue-specific signaling³⁵⁻³⁷. Proteins containing IDR often function as interaction hubs within signaling networks, playing central roles in pathway regulation and the assembly of molecular complexes implicated in the development and progression of cancer and diseases. As a result, they are increasingly recognized as potential drug targets and are under extensive investigation^{38,39}. Recent studies indicate that intrinsically disordered proteins participate in multivalent interactions, driving liquid–liquid phase separation (LLPS) and the formation of biomolecular condensates^{40,41}. We recently reported that the IDR of the orphan nuclear receptor Nur77 engages in multivalent interactions with p62, leading to condensate formation essential for mitophagy⁴². However, whether and how the IDR of RXR α contributes to LLPS remains unknown.

AMP-activated protein kinase- α (AMPK α) is a master regulator of metabolism that maintains cellular energy homeostasis⁴³⁻⁴⁷. It has emerged as an attractive therapeutic target for hepatic fibrosis. AMPK α activity is significantly lower in patients with advanced fibrosis or cirrhosis compared to healthy individuals⁴⁸. Both pharmacological and genetic activation of AMPK α in the liver have been shown to alleviate liver injury and attenuate hepatic fibrosis in various MASH models⁴⁶. In human HSCs, AMPK α activation by adiponectin or 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) suppresses platelet-derived growth factor (PDGF)-induced HSC activation, whereas AMPK α silencing enhances PDGF-induced HSC proliferation, migration, and activation⁴⁹. In the mouse carbon tetrachloride (CCl₄) model, treatment with the AMPK α agonist AICAR reduces HSC proliferation mitigates hepatic fibrosis, and improves liver function⁴⁹. Given its role in fibrosis suppression, understanding the mechanisms by which AMPK α is activated to inhibit HSC activation has become an area of active research.

We report here that K-80003, a small molecule that binds RXR α through a unique mechanism^{31–34}, effectively inhibited HSC activation *in vitro* and improved liver fibrosis *in vivo* by activating AMPK α . Mechanistically, K-80003 promoted the interaction of RXR α with calcium/calmodulin-dependent protein kinase kinase β (CaMKK β), an upstream kinase of AMPK α , in a RXR α phosphorylation-dependent manner. Furthermore, K-80003 binding facilitated the formation of IDR-dependent condensates of phosphorylated RXR α with CaMKK β and AMPK α through a two-phase LLPS process, which in turn resulted in AMPK α activation and subsequently mitochondrial autophagy (mitophagy) in HSCs. Our results reveal an RXR α –CaMKK β –AMPK α signaling axis that regulates HSC activation and hepatic fibrosis through phase separation and identifies K-80003 as a promising anti-fibrosis agent by targeting this pathway.

2. Materials and methods

2.1. Cell culture

Cell lines were passaged for fewer than 4 months after resuscitation in culture for this study. Human primary hepatic stellate cells (hHSC, iXCells Biotechnologies, San Diego, CA, USA, Cat. 10HU-210, Lot. 300074) used in this study were passaged less than twice. Mouse primary hepatic stellate cells (mHSC) were isolated from 16-week-old C57BL/6 mice. CFSC rat HSCs, LX-2 human HSCs, and primary HSCs were cultured in DMEM (Gibco, Carlsbad, CA, USA, Cat. C11965500BT) supplemented with 10% FBS (Sigma, St Louis, MO, USA, Cat. F0193) in a humidified atmosphere containing 5% CO₂ at 37 °C. Cell transfections were carried out by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA, Cat. 11668019) according to the manufacturer's instructions.

2.2. Plasmids

Myc-RXR α , Myc-RXR α -NESm, Myc-RXR α -S49A, Myc-RXR α -T53A, Myc-RXR α -S78A, Myc-RXR α -T82A, Myc-RXR α -S49/T53A, Myc-RXR α -S78/T82A, Myc-RXR α -1-235, Myc-RXR α -1-235-S78/T82A, Myc-RXR α -1-235-S78/T82D, Myc-RXR α -LBD, Flag-RXR α -LBD, Flag-CaMKK β , Flag-AMPK α , Myc-AMPK α , pET-28a-mEGFP-RXR α , pET-28a-mEGFP-RXR α -IDR, pET-28a-mEGFP-RXR α -LBD, pET-28a-mCherry-RXR α , pET-28a-mCherry-RXR α -IDR, pET-28a-mCherry-RXR α -DBD, pET-28a-mCherry-RXR α -LBD, pET-28a-mCherry-CaMKK β , pET-28a-6His-AMPK α , pmCherry-RXR α , pmCherry-RXR α - Δ IDR, pmCherry-RXR α -DBD, and pmCherry-RXR α -LBD were constructed with standard methods.

2.3. Compounds

All-*trans*-retinoic acid (ATRA) (Sigma, Cat. R2625); 9-*cis*-RA (Sigma, Cat. R4643); CD3254 (Tocris, Bristol, UK, Cat. 3302); malotilate (Target Mol, Shanghai, China, Cat. T6576); ocaliva (MedChemExpress, Monmouth Junction, NJ, USA, Cat. HY-135400); actinomycin D (Target Mol, Cat. T3356); leptomycin B (Beyotime Biotechnology, Haimen, China, Cat. S1726); STO-609 (MedChemExpress, Cat. HY-19805), metformin (MedChemExpress, Cat. HY-B0627), AICAR (MedChemExpress, Cat. HY-13417), cyclosporin A (MedChemExpress, Cat. HY-B0579). K-80003 was synthesized as described³¹.

2.4. Western blot (WB)

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample loading buffer, resolved by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. The membranes were blocked in 5% milk in Tris-buffered saline and Tween 20 [TBST: 10 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20] for 1 h at room temperature. After washing 3 times with TBST, the membranes were incubated with appropriate primary antibodies (Supporting Information Table S1) in TBST with 0.5% BSA for 4 °C overnight and then washed 3 times with TBST, probed with horseradish peroxidase-linked anti-immunoglobulin (1:10,000 dilution) for 1 h at room temperature. After 3 washes with TBST, immunoreactive products were visualized using enhanced chemiluminescence reagents. The recombinant proteins involved in the experiments included TGF- β (Novoprotein, Suzhou, China, Cat. CA59), PDGF-BB (Novoprotein, Cat. C199), and Calmodulin/CALM1 (Novoprotein, Cat. C911).

2.5. Isolation of mouse HSCs

Mouse primary HSCs were freshly isolated from the livers of mice (12-week-old) using pronase/collagenase methods⁵⁰. After anesthetizing C57BL/6 mice with isoflurane, their limbs were fixed, and the liver and gastrointestinal organs were exposed. A surgical needle and suture were inserted beside the inferior vena cava under the liver, and the suture was pulled behind the blood vessel. A catheter was inserted under the suture into the inferior vena cava. The needle was removed from the catheter, and the catheter was sutured and hand-tightened. The pump was turned on and connected to the catheter, followed by pinning the filter and tubing tightly. The liver was allowed to inflate for several seconds, and then the portal vein was cut to release pressure, ensuring that it was not overinflated to prevent liver rupture. The diaphragm was cut, and the upper part of the inferior vena cava under the heart was clamped. Perfusion was done for 4 min, followed by 5 min of pronase E digestion, and then 5 min of collagenase D (Sigma, Cat. V900893-1 g) digestion. The mouse was raised, and the fascia was cut to drop the liver into a culture dish, ensuring minimal liquid entry. The liver was kept moist in digestion solution (about 25 mL without DNase I). After preparing all livers, membranes were gently torn to release dissociated cells. The tissue was transferred to a 50 mL tube, with an additional digestion solution of 49 mL, and 1 mL DNase I (Roche, Basel, Switzerland, Cat. 10104159001), gently pipetting 5–10 times for further dissociation. Incubate at 37 °C for 10–15 min with gentle stirring. Filter the digested liver through a cell filter into a new 50 mL tube, centrifuge at 935 $\times g$ for 8 min, discard the supernatant, and resuspend the pellet, centrifuging again at 935 $\times g$ for 8 min. Resuspend the pellet in Nycodenz (Serumwerk Bernburg AG, Bernburg, Germany, Cat. 18003) solution and mix. Centrifuge at 2000 $\times g$ for 20 min without a brake. Remove the top fat layer and collect the upper white cell layer containing HSCs into a new 50 mL tube. Centrifuge at 800 $\times g$ for 7 min, collect the pellet, resuspend in 10% FBS DMEM medium, count cells, and plate for culture.

2.6. Animal studies

Protocols for animal studies were approved by the Animal Care and Use Committee of Xiamen University (approval No.

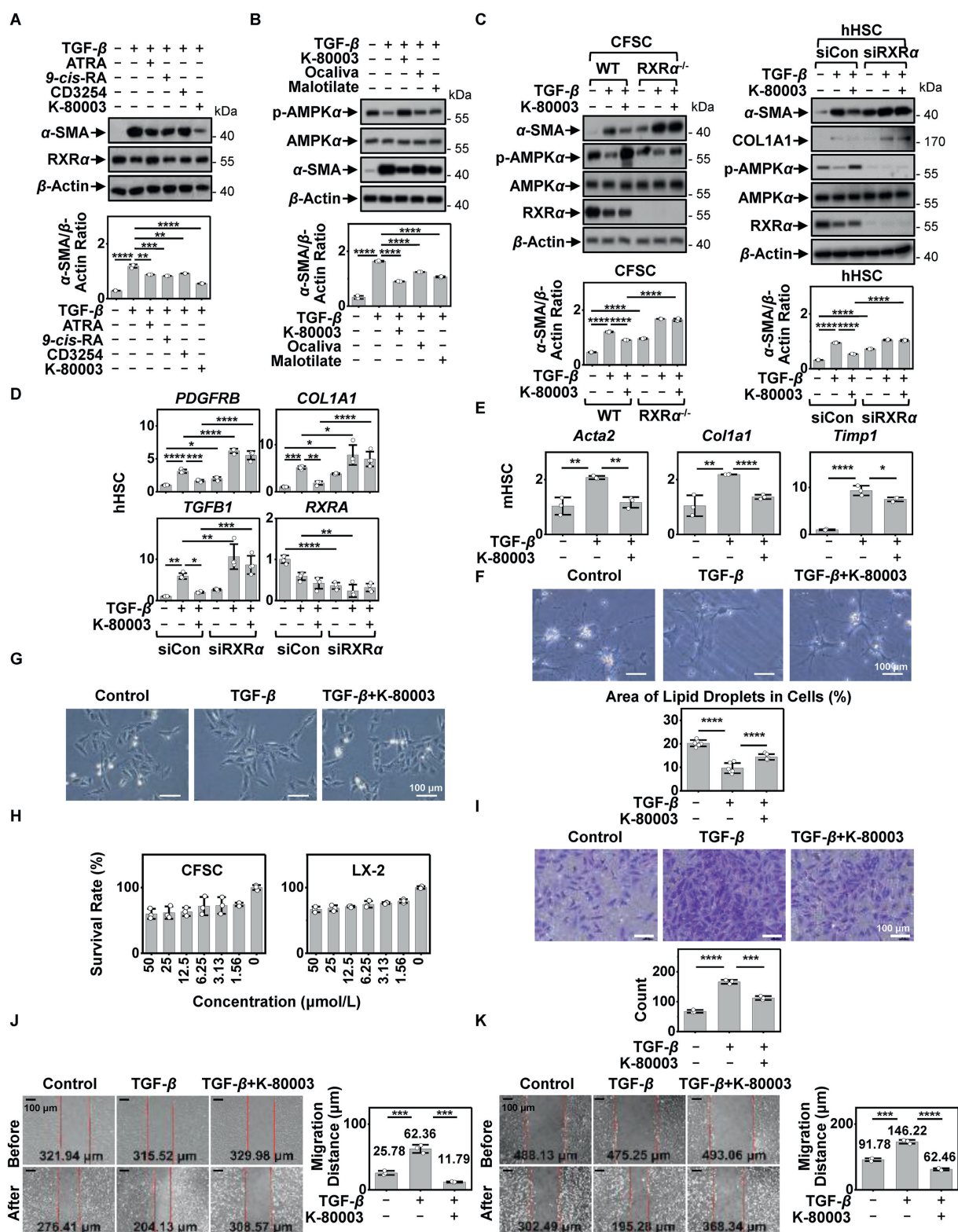


Figure 1 K-80003 inhibits HSC activation. (A) Effect of natural and synthetic retinoids. Serum-starved CFSCs treated with 100 nmol/L of ATRA, 9-*cis*-RA, or CD3254, of 5 μ mol/L K-80003 in the presence or absence of TGF- β (10 ng/mL) for 24 h were analyzed by Western blot. $n = 3$. (B) Effects of 5 μ mol/L of K-80003, malotilate, and ocaliva on inhibiting α -SMA expression in CFSCs were analyzed by Western blot. $n = 3$. (C) Role of RXR α . CFSCs depleted with RXR α (RXR $\alpha^{-/-}$) or hHSCs transfected with RXR α siRNA were treated with K-80003 and/or TGF- β for 24 h and analyzed by Western blot. $n = 3$. (D) hHSCs transfected with RXR α siRNA were treated with K-80003 and/or TGF- β for 24 h and analyzed by qRT-PCR, $n = 4$. (E, F) Effect of K-80003 in mouse primary HSCs (mHSCs). Serum-starved mHSCs treated with K-80003 and/or TGF- β for 24 h were analyzed by qRT-PCR (E) or microscopy for lipid droplet loss (F). $n = 3$ for (E) and $n = 6$ for (F). (G) Effect on HSC

XMULAC20230178), and all mice were handled in accordance with the “Guide for the Care and Use of Laboratory Animals” and the “Principles for the Utilization and Care of Vertebrate Animals”. All of the mice were maintained in the animal room with 12-h light/12-h dark cycles at the laboratory animal center at Xiamen University.

CaMKK2^{−/−} mice were obtained from The Jackson Laboratory, provided by Dr. Chen-Song Zhang.

For the CCl₄-induced liver fibrosis model, eight-week-old male C57BL/6 mice were treated with 0.5 mL/kg CCl₄ dissolved in corn oil (25% v/v, intraperitoneally) once per 3 days for 6 weeks. Mice were then treated with K-80003 (60 mg/kg, K-80003 with 5% Tween-80 dissolved in normal saline, gavage) for the last 2 weeks.

For AMLN diet-induced insulin-resistant obese NAFLD/NASH model, six-week-old male C57BL/6 mice were fed AMLN diet (40% fat kcal, 22% fructose and 2% cholesterol) for 29 weeks, and then mice were treated with K-80003 (50 mg/kg, K-80003 with 5% Tween-80 dissolved in normal saline, gavage) or OCA (25 mg/kg, OCA with 5% Tween-80 dissolved in normal saline, gavage) for 4 weeks.

Mice blood samples were bled from the eye and liver tissues were also collected for histological, serological, and gene expression analysis.

2.7. Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). The data between groups were performed with the OriginPro, and analyzed by *t*-test or one-way ANOVA. *P*-values less than 0.05 were considered statistically significant.

Additional materials and methods details are given in the Supporting Information.

3. Results

3.1. RXR α ligand K-80003 inhibits HSC activation in vitro

To study the role of RXR α in HSC activation, we evaluated the effect of several natural and synthetic RXR α ligands on TGF- β -induced α -smooth muscle actin (α -SMA) expression, a fibrosis marker, in rat CFSC stellate cells (Fig. 1A, Supporting Information Fig. S1A). TGF- β induction of α -SMA expression was inhibited by all-*trans*-retinoic acid (ATRA) that binds and activates RARs and can also activate RXRs as a result of its conversion to 9-*cis*-RA, a ligand of RXR α ⁵¹. Similarly, both 9-*cis*-RA and RXR-selective ligand CD3254⁵² inhibited TGF- β -induced α -SMA expression. These results demonstrated that RXR α modulation could be a promising approach for inhibiting TGF- β activation of HSCs. Notably, K-80003, a unique RXR α modulator derived from the non-steroidal anti-inflammatory drug sulindac^{31–34}, exhibited a strong inhibitory effect on TGF- β -induced α -SMA expression in CFSC cells (Fig. 1A, Fig. S1A). The inhibitory

effect of K-80003 was more pronounced than that of OCA, a ligand for FXR with promising anti-MASH activity in clinical studies⁵³, and comparable to malotilate, a 5-lipoxygenase inhibitor with anti-fibrotic activity⁵⁴ (Fig. 1B, Fig. S1B). Furthermore, K-80003 dose-dependently suppressed TGF- β -induced expression of not only α -SMA but also collagen α 1 (COL1A1), another HSC activation marker (Fig. S1C). These results identified K-80003 as a potent inhibitor of TGF- β signaling in HSCs, underscoring its potential as an anti-fibrotic agent.

To determine whether the inhibitory effect of K-80003 on HSC activation is RXR α dependent, we employed a CRISPR-Cas9 approach to knock out RXR α in CFSC cells⁵⁵. The resulting RXR α ^{−/−} CFSC cells, which lack RXR α expression, were evaluated for α -SMA expression in the absence or presence of TGF- β and/or K-80003. Notably, both basal and TGF- β -induced α -SMA expression were significantly elevated in RXR α ^{−/−} CFSC cells compared to their parental counterparts (Fig. 1C, Fig. S1D), indicating a crucial role for RXR α in modulating HSC activation. When the effect of K-80003 was examined, its ability to inhibit TGF- β induced α -SMA expression was markedly attenuated in RXR α ^{−/−} CFSC cells. Interestingly, K-80003-induced suppression of α -SMA expression coincided with AMPK α activation, as evidenced by the increased level of phosphorylated AMPK α (p-AMPK α) (Fig. 1B and C). Furthermore, this AMPK α activation was also RXR α dependent, suggesting that AMPK α plays a role in mediating the anti-fibrotic activity of K-80003. The effect of K-80003 was also evaluated in human primary hepatic stellate cells (hHSCs) (Fig. 1C). Consistent with the results in CFSC cells, K-80003 inhibited TGF- β -induced α -SMA expression in hHSCs while simultaneously activating AMPK α . To confirm the role of RXR α , RXR α was knocked down in hHSCs by transfecting RXR α siRNA. Similar to RXR α knockout, RXR α knockdown attenuated the ability of K-80003 to suppress TGF- β -induced expression of α -SMA and COL1A1 and to activate AMPK α (Fig. 1C). Thus, both the inhibition of TGF- β signaling and the activation of AMPK α by K-80003 are RXR α dependent, revealing the importance of RXR α in mediating the anti-fibrotic effects of K-80003.

To further determine the anti-fibrogenic effect of K-80003, we examined its impact on the expression of pro-fibrotic genes in HSCs following TGF- β stimulation. K-80003 significantly inhibited TGF- β -induced mRNA expression of *COL1A1*, *TGFBI*, and *PDGFRB* in hHSCs (Fig. 1D) and *Acta2*, *Col1a1* and *Timp1* in mouse primary HSCs (mHSCs) (Fig. 1E). K-80003 also antagonized the pro-fibrotic effects of TGF- β in both CFSCs and hHSCs (Fig. S1E and S1F). A hallmark of HSC activation is the depletion of lipid droplets^{11–13}. Treatment of primary HSCs with TGF- β led to a significant loss of lipid droplets, an effect largely prevented by co-treatment with K-80003 (Fig. 1F). Furthermore, K-80003 reversed TGF- β -induced morphological change in CFSC cells, restoring their long fusiform shape to a more quiescent phenotype (Fig. 1G). Since HSC activation promotes cell proliferation and migration⁵⁶, we also investigated the effect of K-80003 on these processes and found that K-80003 dose-

morphology. Morphological changes of CFSCs treated with K-80003 or TGF- β were revealed by microscopy. (H) Inhibitory effect of K-80003 on HSC proliferation. The proliferation of CFSCs and LX-2 cells cultured in the presence of the indicated concentrations of K-80003 was examined by MTT assays, *n* = 3. (I) Inhibitory effect of K-80003 on HSC migration. Migration of serum-starved CFSC cells in the presence or absence of K-80003 and TGF- β were examined by Transwell migration assay, *n* = 3. (J, K) The effect of K-80003 on the migration of serum-starved CFSC (J) and LX-2 (K) cells in the presence or absence of TGF- β was examined by scratch assay. The average migration distance was counted by ImageJ, *n* = 3. Data are presented as mean $\pm$ SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. (*t*-test for A, B, D, I, J, and K; one-way ANOVA for C, E, and F).

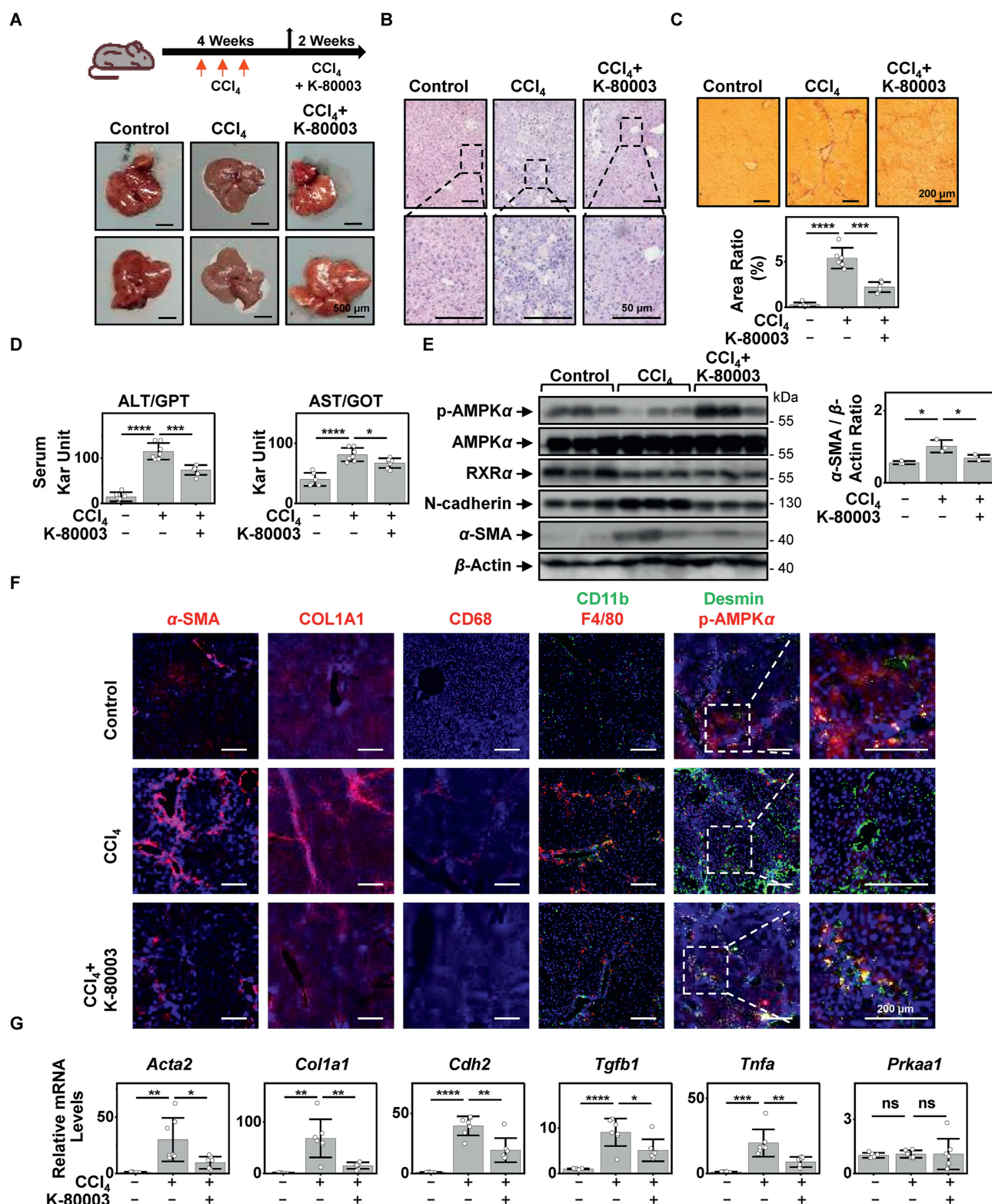


Figure 2 K-80003 ameliorates CCl₄-induced liver fibrosis in animals. (A) Evaluation of K-80003 in the CCl₄ mouse model. C57BL/6 mice were injected intraperitoneally with CCl₄ every three days for 6 weeks, and gavaged daily with K-80003 (60 mg/kg) for the last 2 weeks. Liver tissue morphology is shown. (B) Anti-inflammatory effect of K-80003. H&E staining was examined by microscopy at 400 × magnification. (C) Anti-fibrogenic effect of K-80003. Sirius red staining was examined by microscopy using ImageJ. (D) K-80003 improves liver function. Levels of ALT/GPT and AST/GOT in serum (D). (E–G) Effect of K-80003 on gene expression. The effect of K-80003 on gene expression in animals was determined by Western blot (E), immunofluorescence staining (F), and qRT-PCR (G). For (D–G), *n* represents the number of mice in each group, *n* = 6 for Control group; *n* = 7 for CCl₄ group and CCl₄+K-80003 group. Data are presented as mean ± SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, no significance between indicated groups. (*t*-test).

dependently inhibited the proliferation of CFSCs and human LX-2 HSCs (Fig. 1H). Transwell migration assay also revealed that K-80003 strongly suppressed TGF- β -induced CFSC migration (Fig. 1I), which was further confirmed by scratch assays demonstrating that K-80003 effectively inhibited TGF- β -induced migration of both CFSC cells (Fig. 1J) and LX-2 cells (Fig. 1K). Thus, K-80003 effectively inhibits HSC activation, proliferation, and migration.

3.2. Anti-fibrogenic effects of K-80003 in animals

To evaluate the anti-fibrogenic effects of K-80003 *in vivo*, we examined its impact on liver fibrosis using CCl₄-induced liver fibrosis mouse model⁵⁷. C57BL/6 mice were injected intraperitoneally with CCl₄ every three days for six weeks and gavaged daily with K-80003 (60 mg/kg) during the final two weeks. Morphological analysis revealed that the liver of mice lost luster and became rough after CCl₄ treatment, which was significantly improved after K-80003 treatment (Fig. 2A). Histological analysis showed that K-80003 reduced CCl₄-induced infiltration of inflammatory cells (Fig. 2B) and attenuated collagen deposition and fibrous morphology (Fig. 2C). K-80003 administration also decreased the levels of serum AST and ALT (Fig. 2D). Analysis of liver tissue samples demonstrated that K-80003 inhibited CCl₄-induced expression of pro-fibrotic genes, including α -SMA and N-cadherin, while simultaneously promoting AMPK α activation (Fig. 2E). Immunohistochemical staining further confirmed the inhibitory effect of K-80003 on the expression of pro-fibrotic genes and inflammatory markers as well as its activation of AMPK α (Fig. 2F). The anti-fibrogenic effect of K-80003 in the CCl₄ mouse model was verified by its downregulation of the mRNA expression of various pro-fibrotic genes (Fig. 2G). Similar results were obtained in a separate experiment where mice received **simultaneously** CCl₄ and K-80003 treatment for 4 weeks (Supporting Information Fig. S2). Thus, K-80003 effectively mitigates CCl₄-induced liver injury and liver fibrosis in animals.

We further evaluated the anti-fibrotic effects of K-80003 using an AMLN diet-induced insulin-resistant obese NAFLD/MASH model⁵⁸. Male C57BL/6 mice approximately 6 weeks of age were fed a high-*trans* fat, high-fructose, and high-cholesterol diet (AMLN) for 29 weeks, followed by daily treatment with K-80003 (50 mg/kg) for 4 weeks (Supporting Information Fig. S3A). For comparison, OCA (25 mg/kg) was used. AMLN-fed mice exhibited significant weight gain and liver enlargement, both of which were reduced by co-treatment with K-80003 and OCA (Fig. S3B). Like OCA, K-80003 effectively lowered the AMLN-induced elevations in serum AST and ALT levels (Fig. S3C). Histological analysis demonstrated that both K-80003 and OCA treatments significantly reduced hepatic lipid accumulation in AMLN-fed mice (Fig. S3D). K-80003 also effectively inhibited AMLN-induced hepatic inflammation, as evidenced by CD68 immunostaining, and reduced hepatic lipid accumulation, as shown by bodipy staining, with effects comparable to OCA (Fig. S3E). Evaluation of the anti-fibrogenic effect of K-80003 demonstrated that it strongly suppressed AMLN-induced α -SMA expression by immunostaining. Sirius red staining and collagen quantitation also revealed that K-80003 was more effective than OCA in reducing fibrosis in AMLN-fed mice (Fig. S3F). The anti-fibrosis effect of K-80003 was further illustrated by a marked reduction in α -SMA levels (Fig. S3G) and the **downregulation** of pro-fibrotic genes *Acta2* and *Colla1* (Fig. S3H). While treatment with K-80003 and OCA had no

significant effect on body weight in mice, K-80003 notably reduced the ratio of epididymal white adipose tissue (eWAT) weight to body weight (Fig. S3I and S3J). Additionally, K-80003 exhibited an inhibitory effect on serum TC and LDL-c levels (Fig. S3K and S3L). Thus, K-80003 is also effective in inhibiting fibrosis in the AMLN animal model.

3.3. K-80003 does not inhibit canonical TGF- β /Smad signaling pathway

To study the mechanism underlying the anti-fibrogenic effects of K-80003 in HSCs, we first examined its impact on the TGF- β /Smad signaling pathway, a key pathway for HSC activation⁵⁶. TGF- β signaling is initiated upon its binding to its receptors, followed by phosphorylation of Smad2 and Smad3 and their association with Smad4, which then translocate into the nucleus and activate fibrosis-related genes⁵⁹. However, K-80003 did not affect the intracellular localization (Supporting Information Fig. S4A) or phosphorylation of Smad2/3 (Fig. S4B). Additionally, K-80003 showed a similar inhibitory effect on TGF- β -induced α -SMA expression in both wild-type CFSC cells and Smad3^{-/-} CFSC cells (Fig. S4C). These results indicate that the anti-fibrotic effects of K-80003 are independent of the canonical TGF- β /Smad pathway, suggesting an alternative mechanism of action.

3.4. K-80003 modulates energy metabolism and mitochondrial function

To identify signaling pathways regulated by K-80003, we conducted RNA-Seq analysis using mHSCs treated with TGF- β in the presence or absence of K-80003 (Fig. 3A and B). KEGG pathway analysis (Fig. 3B, Fig. S4D) revealed that mitochondrial functions, including amino acid metabolism and biosynthesis of unsaturated fatty acids, were significantly suppressed by K-80003, whereas the glycolysis pathway remained largely unaffected. Consistently, K-80003 reduced intracellular ATP levels and increased the ADP/ATP ratio in an RXR α -dependent manner (Fig. 3C–E). Given the central role of mitochondrion in energy metabolism^{60–62}, we further assessed mitochondrial function using oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) assays, which measure mitochondrial respiration and glycolysis, respectively. TGF- β stimulation enhanced mitochondrial energy metabolism while having little effect on glycolysis in CFSC cells (Fig. 3F). However, K-80003 treatment strongly suppressed TGF- β -induced mitochondrial respiration without significantly affecting glycolysis (Fig. 3F). K-80003 also reduced mitochondrial membrane potential (MMP) (Fig. S4E) and increased mitochondrial reactive oxygen species (MitoROS) (Fig. S4F) in CFSC cells in an RXR α -dependent manner.

Recent studies reveal a critical role of mitophagy, the selective degradation of mitochondria by autophagy, in the control of both the quality and the number of mitochondria to meet cellular metabolic demands⁶³. Therefore, we studied whether K-80003 regulated mitophagy using the COX8–EGFP–mCherry reporter, which targets the inner mitochondrial membrane and serves as a widely used mitophagy marker⁶⁴. In this system, normal mitochondria appear yellow due to the presence of both green (EGFP) and red (mCherry) fluorescence. However, when mitochondria are engulfed by acidic lysosomes, the green fluorescence is quenched, leaving only red fluorescence as an indicator of mitophagy. While control or TGF- β -treated CFSC cells showed no mitophagy, as evidenced by the absence of red fluorescence, CFSC cells treated

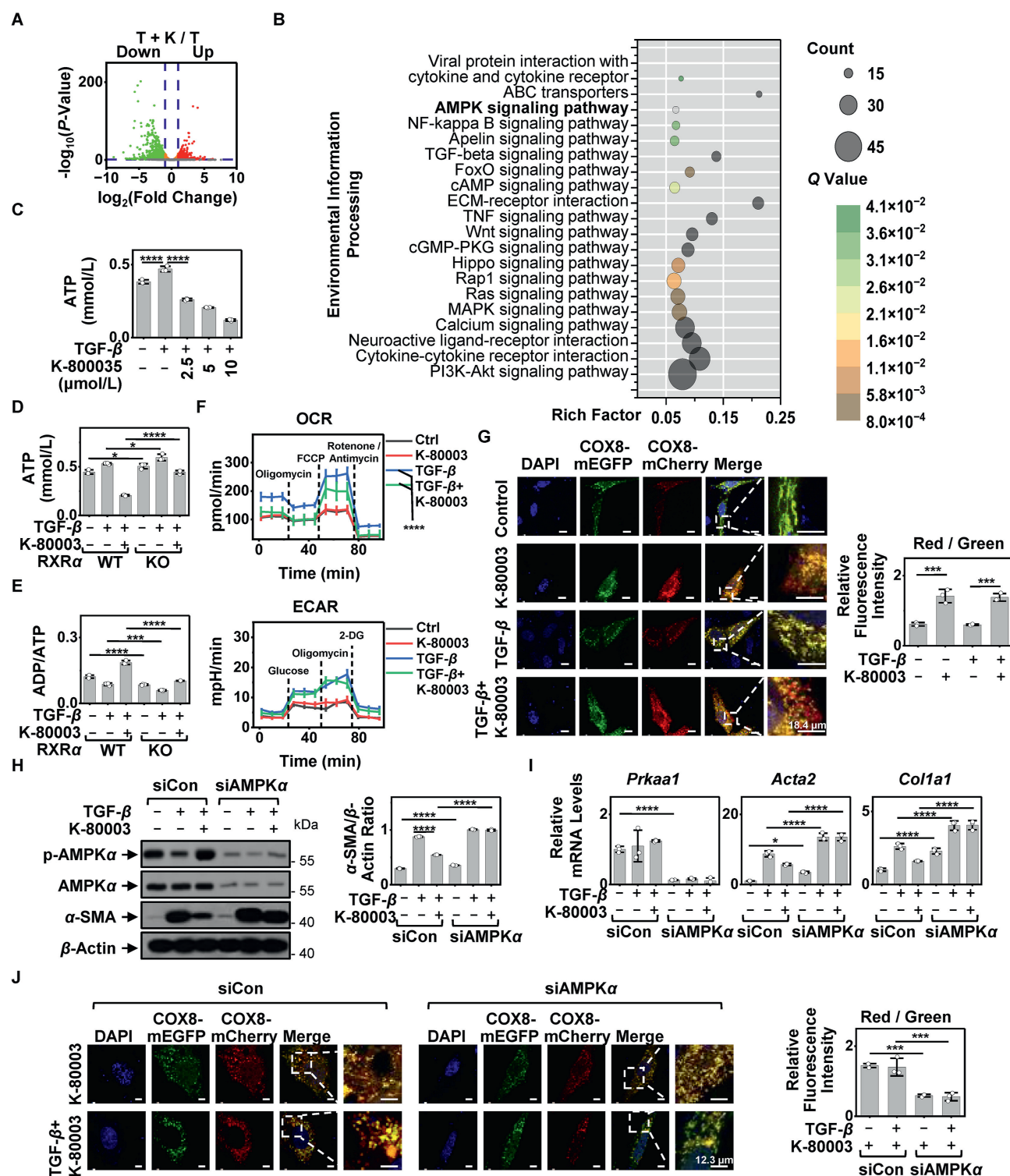


Figure 3 K-80003 regulates mitochondrial function and inhibits hepatic stellate cell activation by activating AMPK α . (A, B) RNA-Seq analysis. mHSC treated with K-80003 (5 $\mu\text{mol/L}$) and/or TGF- β (10 ng/mL) for 24 h were analyzed by RNA-Seq. Volcano plot (A) and KEGG analysis (B). TGF- β (T) + K-80003 (K)/TGF- β (T), filtered by fold change >2, $P < 0.05$. Data show that 1023 genes were up-regulated and 352 genes were down-regulated. The expression of genes involved in mitochondrial function (down panel). (C) K-80003 inhibits ATP production. CFSCs treated with the indicated concentration of K-80003 and TGF- β for 24 h were analyzed for the production of intracellular ATP. (D) CFSC (WT) and RXR $\alpha^{-/-}$ CFSC (KO) cells treated with K-80003 and TGF- β for 24 h were analyzed for the production of intracellular ATP. (E) ADP/ATP ratio was assessed in CFSC and RXR $\alpha^{-/-}$ CFSC cells treated with K-80003 and TGF- β for 24 h. (F) Effect of K-80003 on TGF- β -mediated mitochondrial respiration and glycolysis. Serum-starved CFSC cells were treated with K-80003 (5 $\mu\text{mol/L}$) in the presence or absence of TGF- β (10 ng/mL) for 24 h, and Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were analyzed using Seahorse.

with K-80003, either alone or in combination with TGF- β exhibited extensive red fluorescence, which was accompanied by a dramatic morphological change of mitochondria (Fig. 3G). In addition, K-80003 treatment led to a reduction in COXII/MTCO2, a mitochondrial protein, an effect that was mitigated by co-treatment of cyclosporin A, a known mitophagy inhibitor (Fig. S4I). Thus, K-80003 regulates mitochondrial function by inducing mitophagy in HSCs.

3.5. The anti-fibrogenic effect of K-80003 is mediated by its activation of AMPK α

K-80003 could activate AMPK α in both CFSC cells (Fig. 1) and animals (Fig. 2). This activation was also confirmed through KEGG pathway analysis of RNA-Seq data, showing a significant effect of K-80003 on the AMPK signaling pathway (Fig. 3B). To further investigate the role of AMPK α activation in mediating the anti-fibrogenic effect of K-80003, we conducted several experiments. Knocking down AMPK α expression using AMPK α siRNA in CFSC cells reduced the inhibitory effect of K-80003 on HSC activation. Specifically, AMPK α knockdown impaired the ability of K-80003 to suppress TGF- β -induced α -SMA protein expression (Fig. 3H) and to inhibit the mRNA expression of fibrosis-related genes *Acta2* and *Col1a1* (Fig. 3I). Moreover, K-80003-induced mitophagy was significantly diminished in CFSC cells transfected with AMPK α siRNA (Fig. 3J). Thus, AMPK α plays a crucial role in mediating both the anti-fibrotic and mitophagic effects of K-80003 in HSC cells. Furthermore, RXR α knockout in CSFC cells abrogated the effects of K-80003 on activating AMPK α and inhibiting TGF- β -induced α -SMA expression, demonstrating that RXR α is required for K-80003 activation of AMPK α . However, RXR α depletion did not affect AMPK α activation by malotilate, or by AMPK agonists AICAR and metformin (Fig. S4G). Additionally, PPAR γ knockdown had no impact on K-80003 activation of AMPK α (Fig. S4H). Thus, RXR α specifically mediates K-80003-induced AMPK α activation.

3.6. The effect of K-80003 is mediated by the extranuclear function of RXR α

Nuclear receptors are ligand-dependent transcriptional factors that regulate gene transcription by binding to target gene promoter^{3,18,20,21}. We investigated whether the transcription regulation of RXR α contributed to the anti-fibrotic effect of K-80003. Treatment of CFSC cells with the transcription inhibitor actinomycin D did not affect K-80003-induced activation of AMPK α (Fig. 4A). In contrast, actinomycin D completely abolished TGF- β -induced α -SMA expression (Fig. 4B). Thus, transcription regulation by K-80003 was not involved in its activation of AMPK α . This conclusion was further supported by data showing that K-80003 did not affect AMPK α protein expression despite inducing AMPK α phosphorylation (Fig. 1B and C). RXR α exerts its biological effects by acting both inside and outside the

nucleus^{18,21}. Notably, we observed that RXR α was diffusely distributed in both the nucleus and cytoplasm when CFSC cells were treated with K-80003 regardless of TGF- β presence (Fig. 4C). This observation prompted us to study whether the activation of AMPK α by K-80003 was mediated through RXR α 's cytoplasmic action. Indeed, treatment of cells with the nuclear export inhibitor leptomycin B not only prevented the cytoplasmic localization of RXR α (Fig. 4D) but also blocked K-80003-induced activation of AMPK α and inhibition of α -SMA expression (Fig. 4E). To further confirm the role of cytoplasmic RXR α in AMPK α activation, we mutated the nuclear export sequence (NES)⁶⁵ (Fig. 4F). The resulting mutant, RXR α -NESm, failed to translocate to the cytoplasm in response to K-80003 treatment, whereas wild-type RXR α remained diffusely distributed in both the nucleus and cytoplasm (Fig. 4G). The RXR α -NESm mutant also failed to restore the inhibitory effect of K-80003 on intracellular ATP levels (Fig. 4H). When RXR α -NESm was transfected into RXR α -depleted CFSC cells, it could not support K-80003-induced activation of AMPK α and inhibition of TGF- β -induced α -SMA expression. In contrast, transfection with wild-type RXR α successfully rescued these effects (Fig. 4I). To study whether the cytoplasmic localization of RXR α was required for K-80003-induced mitophagy, we conducted the COX8–EGFP–mCherry reporter assay in CFSC and RXR α ^{−/−} CFSC cells. The results showed K-80003-induced mitophagy in the absence or presence of TGF- β was lost in RXR α ^{−/−} CFSC cells. However, mitophagy was restored upon transfection with wild-type RXR α but not RXR α -NESm (Fig. 4J). Together, these results reveal a transcription-independent mechanism by which RXR α mediates AMPK α activation, α -SMA inhibition, and mitophagy induction by K-80003 in HSCs.

3.7. A unique interaction between RXR α and CaMKK β and its modulation by K-80003

AMPK α can be activated by LKB1, CaMKK β , and TAK1. However, the activation of AMPK α and the inhibition of TGF- β -induced α -SMA expression by K-80003 were unlikely to be mediated through LKB1 or TAK1, as K-80003 had no apparent effect on their expression or activation (Supporting Information Fig. S5A and S5B). We therefore focused on the role of CaMKK β in K-80003-induced AMPK α activation. Surprisingly, co-transfection of Flag-tagged CaMKK β (Flag-CaMKK β) and Myc-tagged RXR α (Myc-RXR α) into CFSC cells resulted in a significant shift of the Myc-RXR α band on SDS/PAGE (Fig. 5A), suggesting that Myc-RXR α might be modified by transfected Flag-CaMKK β . Similarly, endogenous RXR α protein in the liver exhibited a shift when mice were administered with CCl₄, which correlated with inhibition of AMPK α activation (Fig. 5B). These data suggested a crucial role of RXR α modification in regulating AMPK α activation during liver fibrosis.

Given that RXR α was modified upon CaMKK β co-transfection, we studied their interaction. Co-immunoprecipitation (CoIP) assay

(G) K-80003 induces mitophagy in CFSCs. CFSCs transfected with the COX8-mEGFP-mCherry plasmid were treated with K-80003 and/or TGF- β for 24 h, and examined by confocal microscopy. The ratio of red fluorescence to green fluorescence was analyzed by ImageJ. (H, I) AMPK α activation is required for K-80003 inhibition of α -SMA expression. CFSCs or CSFCs transfected with AMPK α siRNA were treated with K-80003 and/or TGF- β for 24 h, and examined by Western blot (H) or qRT-PCR (I). (J) AMPK α activation is required for K-80003-induced mitophagy. CFSCs transfected with control (siCon) or AMPK α siRNA were transfected with the COX8-mEGFP-mCherry plasmid, treated with K-80003 and/or TGF- β for 24 h, and examined by confocal microscopy. The ratio of red to green fluorescence was analyzed by ImageJ. Data are presented as mean $\pm$ SD; * P < 0.05, *** P < 0.001, **** P < 0.0001. (one-way ANOVA).

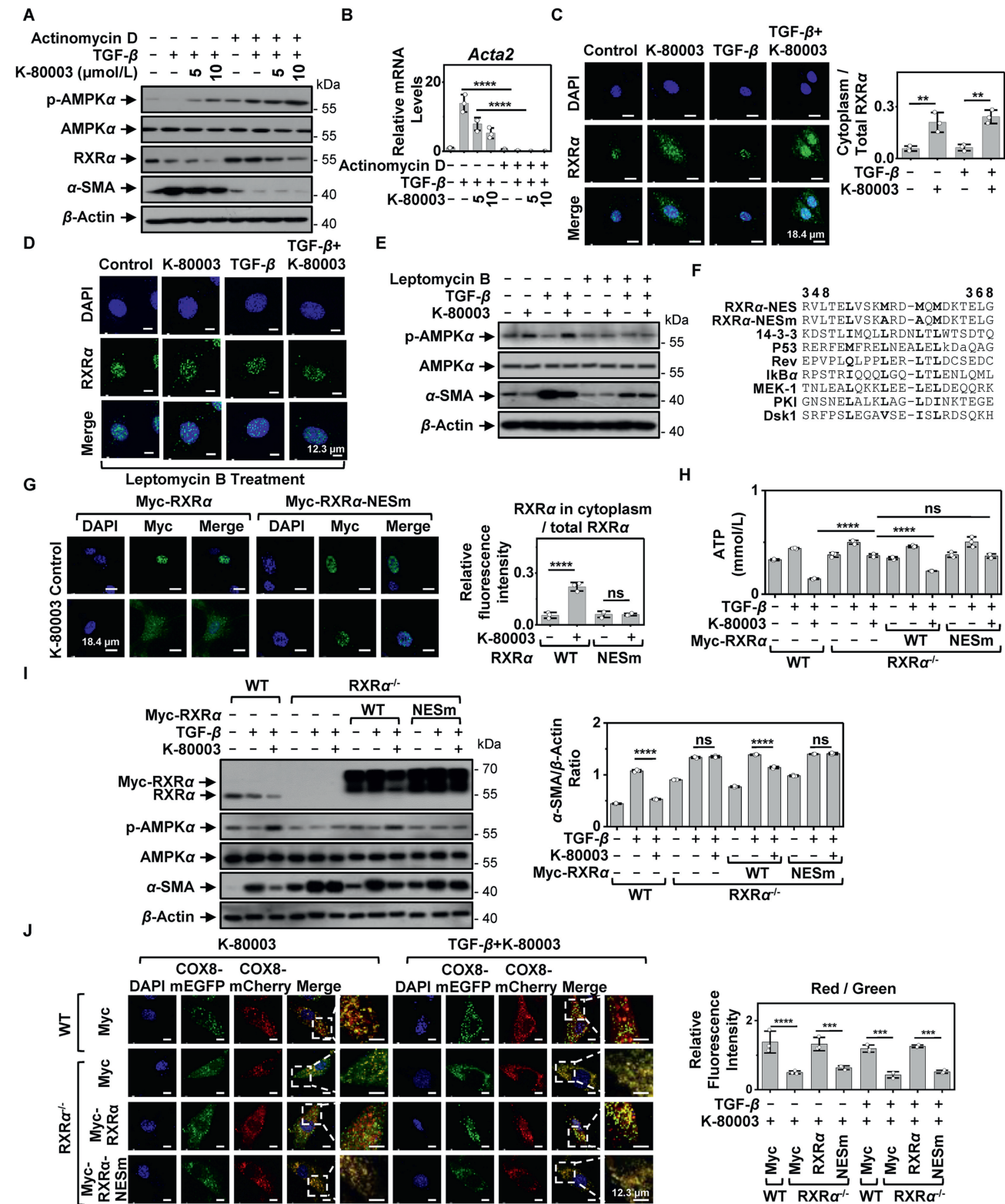


Figure 4 K-80003 inhibits HSC activation by regulating the extranuclear functions of RXR α . (A, B) Effect of actinomycin D. CFSCs treated with actinomycin D (2 μ mol/L) together with the indicated concentrations of K-80003 and/or TGF- β (10 ng/mL) for 24 h were analyzed by Western blot (A) or qRT-PCR (B). (C) Induction of RXR α cytoplasmic localization by K-80003. Serum-starved CFSCs treated with K-80003 (5 μ mol/L) and/or TGF- β for 24 h were examined by confocal microscopy. Relative fluorescence intensity was analyzed by ImageJ. The ratio of cytoplasmic RXR α to total RXR α was shown. (D, E) Effect of Leptomycin B. CFSCs treated with Leptomycin B (100 ng/mL) together with K-80003 and/or TGF- β for 24 h were analyzed by immunofluorescence assay (D) or Western blot (E). (F) Schematic representation of the NES of RXR α and its comparison with NESs of known genes. The boldface letters indicate conserved amino acid residues. (G–I) Role of RXR α nuclear

showed that the upshifted, but not the regular, Myc-RXR α strongly interacted with Flag-CaMKK β , and this interaction was slightly enhanced by K-80003 but not by TGF- β (Fig. 5A). This suggested a unique interaction between modified RXR α (m-RXR α) and CaMKK β . We also studied the interaction between endogenous RXR α and CaMKK β in cells treated with K-80003 and/or TGF- β for 24 h. Using anti-RXR α antibody for immunoprecipitation, we found that CaMKK β was pulled down only when the upshifted RXR α band was pulled down. In contrast immunoprecipitation of the regular RXR α band in the absence of K-80003 failed to pull down CaMKK β (Fig. 5C), reinforcing the specificity of the interaction between m-RXR α and CaMKK β .

The modification of RXR α and its interaction with CaMKK β depended on CaMKK β activation, as treatment of CFSC cells with the CaMKK inhibitor STO-609 blocked K-80003-induced precipitation of m-RXR α and its co-immunoprecipitation of CaMKK β by anti-RXR α antibody (Fig. 5D). Interestingly, K-80003 did not affect CaMKK β activation of other downstream kinases, such as CaMKI and CaMKIV⁶⁶ (Fig. S5C), suggesting that the K-80003-induced interaction between m-RXR α and CaMKK β specifically contributes to CaMKK β -mediated AMPK α activation. Such an effect of K-80003 might play a role in CaMKK β -mediated calcium signaling, as it induced fluctuations in intracellular calcium levels (Fig. S5D).

To further investigate the interaction between CaMKK β and RXR α , we conducted an immunostaining experiment in cells transfected with Flag-CaMKK β and treated with K-80003 and/or TGF- β (Fig. 5E, Fig. S5E). In control cells, RXR α resided predominantly in the nucleus. However, upon K-80003 but not TGF- β treatment, RXR α was redistributed to the cytoplasm, forming punctate structures that co-localized with Flag-CaMKK β . Collectively, these results demonstrate that K-80003-induced RXR α modification depends on CaMKK β activation and that the RXR α modification confers it with the ability to interact with CaMKK β in the cytoplasm, highlighting a novel mechanism of AMPK α activation.

3.8. RXR α is modified by CaMKK β through phosphorylation

Our observation that K-80003-induced RXR α modification depended on CaMKK β activation prompted us to investigate whether RXR α was phosphorylated by CaMKK β . Thus, lysates prepared from cells transfected with Flag-CaMKK β and Myc-RXR α were incubated with alkaline phosphatase FastAP. The Flag-CaMKK β -induced upshift of the Myc-RXR α band, detected using either anti-Myc or anti-RXR α antibody, was completely abolished following FastAP treatment (Fig. 5F), demonstrating that RXR α undergoes phosphorylation upon CaMKK β co-transfection. Consistently, inhibiting CaMKK β activity by STO-609 prevented CaMKK β -induced phosphorylation of RXR α (p-RXR α) (Fig. 5G). The induction of p-RXR α by K-80003, TGF- β , or PDGF-BB in CFSC cells or mHSCs was also inhibited by STO-609, further revealing the role of CaMKK β in RXR α phosphorylation (Fig. 5H).

To identify CaMKK β phosphorylation sites in RXR α , we conducted mutagenesis on several putative phosphorylation sites within its N-terminal A/B domain (Fig. 5I), as phosphorylation in this region may cause a mobility shift of RXR α on SDS/PAGE⁵⁵. Mutation of Ser78 or Thr82 resulted in mutants (RXR α -S78A and RXR α -T82A, respectively) that showed reduced phosphorylation upon CaMKK β co-transfection. Moreover, simultaneous mutation of both sites (RXR α -S78/T82A) completely abolished CaMKK β -induced phosphorylation (Fig. 5J). In contrast, mutations at other amino acid residues had either no effect or slightly enhanced phosphorylation, indicating Ser78 and Thr82 are the primary phosphorylation sites targeted by CaMKK β . Further, CoIP assay revealed that mutation of either Ser78 (RXR α -S78A) or Thr82 (RXR α -T82A) impaired the interaction between RXR α and CaMKK β , while the simultaneous mutation of both sites (RXR α -S78/T82A) completely abolished the interaction (Fig. S5F). Additionally, *in vitro* kinase assays confirmed that the RXR α -S78/T82A mutant was not phosphorylated by CaMKK β (Fig. 5K). Together, these data demonstrate that phosphorylation of RXR α at Ser78 and Thr82 is required for its interaction with CaMKK β , elucidating the mechanism by which CaMKK β regulates RXR α function.

3.9. Interaction between RXR α with CaMKK β promotes AMPK α activation by K-80003

To determine the role of RXR α interaction with CaMKK β in K-80003-induced AMPK α activation, we first examined the effect of CaMKK β knockdown. Transfection of CFSC cells with CaMKK β siRNA, which inhibited CaMKK β expression, significantly attenuated the ability of K-80003 to activate AMPK α and inhibit TGF- β -induced α -SMA expression (Fig. 6A). Similarly, pharmacological inhibition of CaMKK β activation using STO-609 (Fig. 6B) or the calcium chelator EGTA AM (Fig. 6C) also impaired K-80003-induced AMPK α activation. To further study the role of AMPK α activation in K-80003-induced mitophagy, we used the COX8-EGFP-mCherry reporter to assess mitophagy in response to STO-609 treatment. We found that STO-609 effectively blocked K-80003-induced mitophagy both in the presence or absence of TGF- β (Fig. 6D). Furthermore, mutation of Ser78 and Thr82, either individually or simultaneously, impaired the ability of RXR α to rescue the effects of K-80003 on activating AMPK α , inhibiting TGF- β -induced α -SMA expression (Fig. 6E), reducing intracellular ATP level (Fig. 6F), and promoting mitophagy (Fig. 6G) in RXR α -depleted cells. Thus, the phosphorylation-dependent interaction between RXR α and CaMKK β plays a critical role in K-80003-induced activation of AMPK α , induction of mitophagy, and inhibition of HSC activation.

Next, we studied the mechanism by which RXR α interaction with CaMKK β activates AMPK α . Since CaMKK β is known to activate AMPK α through direct interaction^{44,66}, we hypothesized that RXR α interaction with CaMKK β might facilitate its association with AMPK α , thereby enhancing AMPK α activation. To test

export. RXR α ^{-/-} CFSCs transfected with RXR α or RXR α -NESm were treated with K-80003 and/or TGF- β for 24 h, and analyzed by immunofluorescence assay (G), the production of intracellular ATP (H) or Western blot (I). For (G), *n* represents the number of fields of view, and for (H, I), *n* represents the number of independent experiments. (J) Cytoplasmic localization of RXR α is required for K-80003-induced mitophagy. RXR α ^{-/-} CFSCs were transfected with the COX8-GFP-mCherry plasmid together with RXR α or RXR α -NESm, treated with K-80003 and/or TGF- β for 24 h, and analyzed by confocal microscopy. Data are presented as mean $\pm$ SD; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, no significance between indicated groups. (one-way ANOVA).

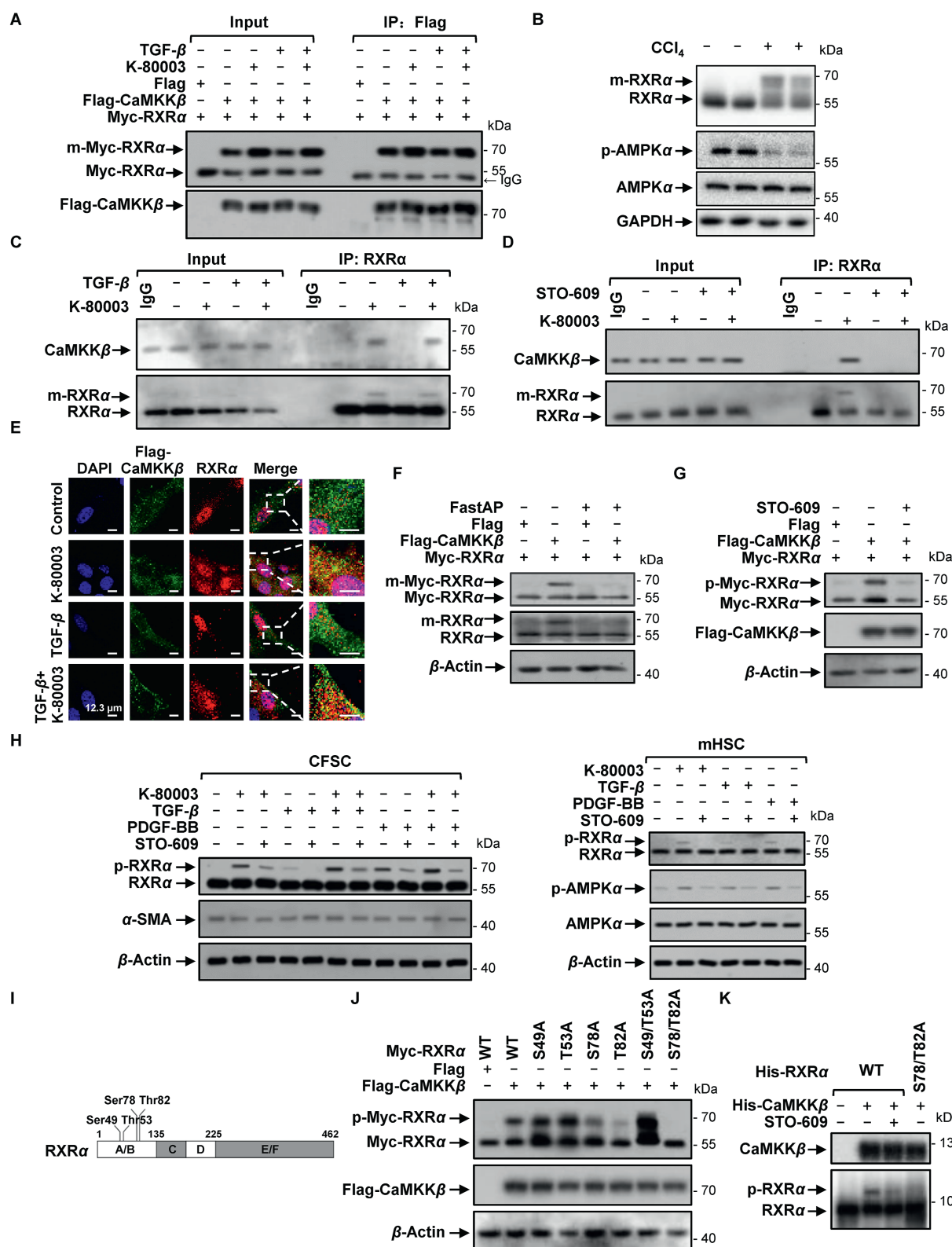


Figure 5 Characterization of RXR α interaction with CaMKK β . (A) Modified RXR α (m-RXR α) interacts with CaMKK β . CFSCs were transfected with Myc-RXR α and Flag-CaMKK β , treated with TGF- β (10 ng/mL) and/or K-80003 (5 μ mol/L) for 24 h, and analyzed by CoIP using anti-Flag antibody. Co-transfection of Flag-CaMKK β resulted in upshift of Myc-RXR α (m-Myc-RXR α), which was co-immunoprecipitated together with Flag-CaMKK β . (B) RXR α is modified by CCl₄ in the liver. Lysates of the liver from mice administered with 0.5 mL/kg CCl₄ intraperitoneally for 30 min were analyzed by Western blot. (C) Endogenous CaMKK β was co-immunoprecipitated only when m-RXR α was immunoprecipitated in the presence of K-80003. CFSCs treated with TGF- β and/or K-80003 for 24 h were analyzed by CoIP using anti-RXR α antibody. (D) Effect of STO-609. CFSCs treated with K-80003 and/or STO-609 (5 μ mol/L) for 24 h were analyzed by CoIP using anti-RXR α antibody.

the hypothesis, we examined whether K-80003 could promote the interaction between CaMKK β and AMPK α . Thus, CFSC cells were co-transfected with Myc-AMPK α and Flag-CaMKK β , and their interaction was assessed in the presence or absence of K-80003 and TGF- β . CoIP assay revealed that K-80003, but not TGF- β , significantly enhanced the interaction between Myc-AMPK α and Flag-CaMKK β (Fig. 7A). The effect of K-80003 was likely mediated by p-RXR α , as transfection with wild-type RXR α , but not the RXR α -S78/T82A mutant, enhanced the ability of CaMKK β to interact with AMPK α (Fig. 7B). Interestingly, immunoprecipitation of Flag-CaMKK β with an anti-Flag antibody not only pulled down Myc-AMPK α but also the up-shifted Myc-RXR α , suggesting the formation of a p-RXR α /CaMKK β /AMPK α complex. This hypothesis was further supported by CoIP data showing that immunoprecipitation of Myc-RXR α using an anti-Myc antibody resulted in the co-immunoprecipitation of both Flag-CaMKK β and Flag-AMPK α (Fig. 7C). Notably, Myc-RXR α did not interact with Flag-AMPK α in the absence of Flag-CaMKK β (Fig. 7C and Supporting Information Fig. S6), indicating that CaMKK β interaction with p-RXR α is essential for the formation of p-RXR α /CaMKK β /AMPK α complex. Further analysis revealed that K-80003 not only promoted the interaction between Flag-CaMKK β and Myc-AMPK α but also enhanced the association between Flag-CaMKK β and p-Myc-RXR α (Fig. 7D). Thus, K-80003 enhances AMPK α activation by inducing the interaction between p-RXR α and CaMKK β , which in turn strengthens the interaction between CaMKK β and AMPK α , ultimately leading to increased AMPK α activation.

To study how p-RXR α enhances the interaction between CaMKK β and AMPK α , we examined whether the C-terminal LBD (RXR α -LBD) or the N-terminal region of RXR α (RXR α -1–235) (Fig. 7E) interacted with CaMKK β . Co-transfection experiments revealed that CaMKK β strongly phosphorylated RXR α -1–235 but not RXR α -LBD. However, RXR α -LBD, rather than phosphorylated or unphosphorylated RXR α -1–235, interacted with CaMKK β (Fig. 7F). This interaction was further confirmed by the ability of RXR α -LBD to promote CaMKK β interaction with AMPK α , whereas RXR α -1–235 did not. Furthermore, transfection of RXR α -LBD, but not RXR α -1–235, into RXR α ^{−/−} CFSC cells promoted AMPK α activation and enhanced the inhibitory effect of K-80003 on α -SMA activation (Fig. 7G). These findings suggest that the C-terminal LBD of RXR α plays a key role in mediating CaMKK β -dependent AMPK α activation. To explore the mechanism by which CaMKK β -induced phosphorylation of RXR α promotes its interaction with CaMKK β , we hypothesized that phosphorylation disrupts an intramolecular interaction within RXR α ^{31,55}, which may normally conceal the CaMKK β -interaction site in RXR α -LBD. To test this, we examined the effect of RXR α

phosphorylation on the intramolecular interaction between RXR α -LBD and RXR α -1–235 (Fig. 7H). Consistent with our previous results^{31,55}, CoIP analysis revealed that Flag-RXR α -LBD efficiently pulled down the unmodified Myc-RXR α -1–235, demonstrating an intramolecular interaction between the N-terminal and C-terminal regions of RXR α . However, phosphorylated Myc-RXR α -1–235 failed to interact with Flag-RXR α -LBD, suggesting that phosphorylation disrupts this intramolecular interaction. Further analysis revealed that Flag-RXR α -LBD interacted with Myc-RXR α -1–235/S78/T82A mutant but not with the phosphomimetic mutant Myc-RXR α -1–235/S78/T82D (Fig. 7H), reinforcing the idea that phosphorylation of RXR α at its N-terminal region promotes its interaction with CaMKK β by disrupting the intramolecular N–C interaction within RXR α , thereby exposing the CaMKK β -binding site in RXR α -LBD (Fig. 7I).

3.10. RXR α phase separation provides hubs for AMPK α activation by recruiting CaMKK β

A major discovery in the past decade is the recognition that proteins, nucleic acids, or their mixture can demix into 2 phases through liquid–liquid phase separation (LLPS)⁶⁷. The condensed phase can give rise to membraneless organelles that facilitate various biological functions⁶⁸. For proteins, intrinsically disordered regions (IDRs) and low-affinity multivalent interactions can drive phase separation, providing a driving force for generating various biomolecular condensates⁶⁹. To study whether the N-terminal A/B domain, which is largely unstructured with a high disordered tendency (Fig. 8A), mediated RXR α interaction with CaMKK β , we examined whether RXR α could form condensates *in vitro* and *in vivo*. For *in vitro* analysis, we expressed and purified mEGFP-tagged RXR α (mEGFP-RXR α) and mCherry-tagged RXR α (mCherry-RXR α). Notably, mEGFP-RXR α readily formed condensates when NaCl concentration was reduced from 300 mmol/L to 50 mmol/L (Fig. 8B). At 50 mmol/L NaCl concentration, mCherry-RXR α and mEGFP-RXR α formed co-condensate (Fig. 8C). Additionally, mEGFP-RXR α condensates could fuse to form larger condensates, revealing condensate coalescence (Fig. 8D). Examination of the spatiotemporal dynamics of RXR α condensates by fluorescence recovery after photobleaching (FRAP) (Fig. 8E) revealed their liquid-like property. When added to droplet formation buffer containing a crowding agent (10% PEG-8000) to simulate the densely crowded environment of the nucleus, mEGFP-RXR α but not mEGFP formed micro-sized condensates in solution (Supporting Information Fig. S7A). Similar condensate formation was observed in the presence of other crowding agents such as ficoll-400, dextran-70, or highly concentrated lysozyme (1 mmol/L) (Fig. S7B), suggesting that the formation of RXR α condensates was a general

antibody. (E) K-80003 induces co-localization of RXR α with transfected Flag-CaMKK β in the cytoplasm. CFSCs transfected with Flag-CaMKK β were treated with TGF- β and/or K-80003 for 24 h, and analyzed by confocal microscopy. (F) Phosphorylation is responsible for CaMKK β modification of RXR α . Lysates prepared from CFSCs transfected with Myc-RXR α and Flag-CaMKK β were treated with or without FastAP and analyzed by Western blot. (G) STO-609 inhibits CaMKK β phosphorylation of RXR α . CFSCs transfected with Myc-RXR α and Flag-CaMKK β , treated with or without STO-609 were analyzed by Western blot. (H) STO-609 inhibits p-RXR α expression. Serum-starved CFSCs and mHSCs treated with K-80003, TGF- β , PDGF-BB (1 ng/mL) and STO-609 for 2 h were analyzed by Western blot. (I) Schematic representation of putative CaMKK β phosphorylation sites on RXR α . (J) Ser78 and Thr82 in RXR α are responsible for CaMKK β phosphorylation of RXR α . CFSCs transfected with Flag-CaMKK β and Myc-RXR α or mutant were analyzed by Western blot. (K) His-GFP-RXR α or His-GFP-RXR α -S78/T82A was incubated with His-mCherry-CaMKK β and STO-609 was analyzed by Western blot.

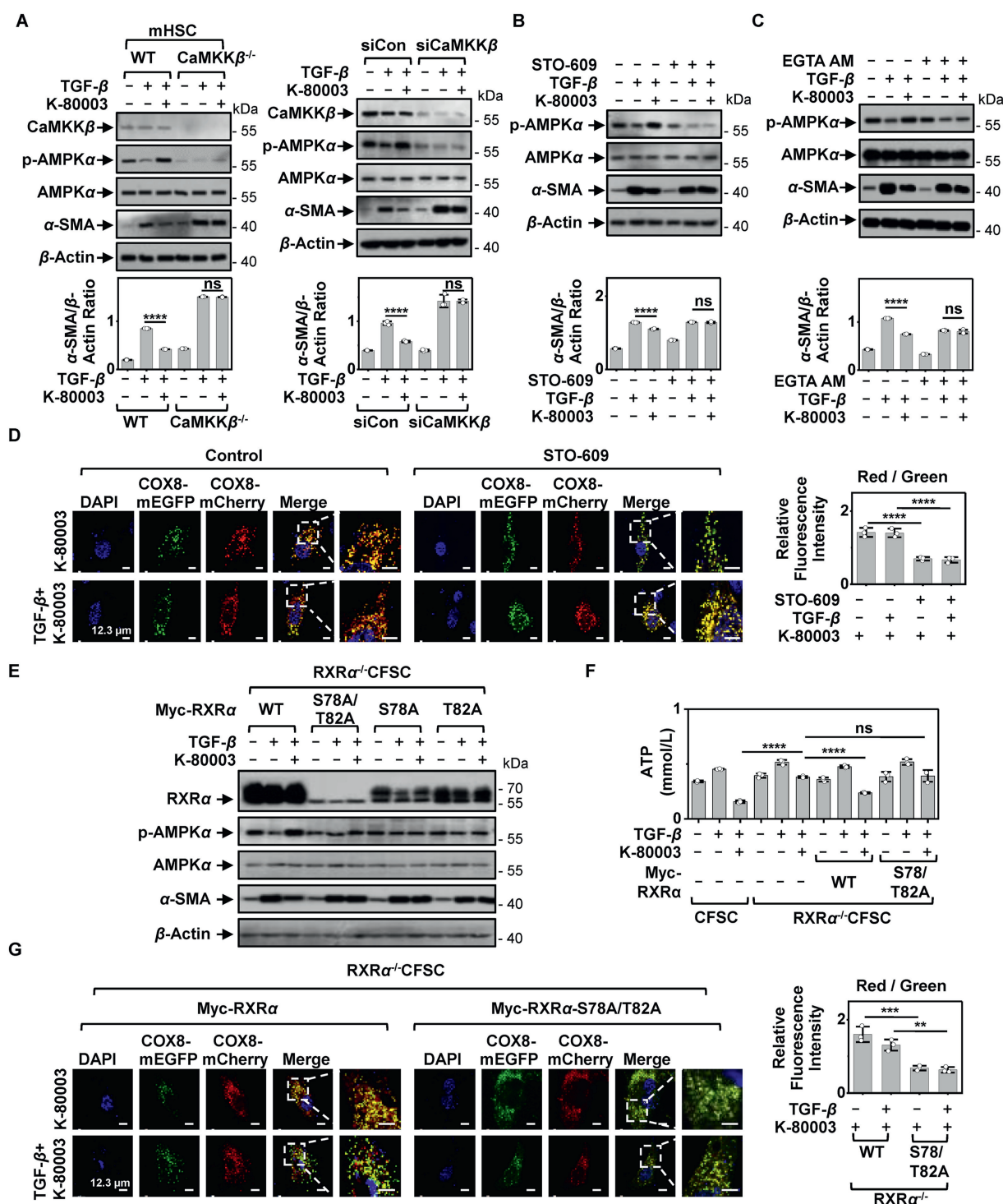


Figure 6 RXR α interaction with CaMKK β mediates K-80003 activation of AMPK α . (A) Role of CaMKK β . CFSCs transfected with control or CaMKK β siRNA were treated with K-80003 (5 μ M) in the presence or absence of TGF- β (10 ng/mL) for 24 h and analyzed by Western blot. (B) STO-609 inhibits K-80003 activity. CFSCs treated with K-80003, TGF- β (10 ng/mL), and/or STO-609 (5 μ M) for 24 h were analyzed by Western blot. (C) EGTA inhibits K-80003 activity. CFSCs treated with K-80003, TGF- β (10 ng/mL), and EGTA AM (5 μ M) for 24 h were analyzed by Western blot. (D) STO-609 inhibits K-80003 induction of mitophagy. CFSCs transfected with the COX8-GFP-mCherry were treated with K-80003, TGF- β , and STO-609 for 24 h and examined by confocal microscopy. Relative fluorescence intensity was analyzed by ImageJ. The ratio of red to green fluorescence is shown. (E, F) Mutating Ser78 and/or Thr82 impairs the effect of RXR α in mediating K-80003 activity. CFSCs or RXR $\alpha^{-/-}$ CFSCs transfected with Myc-RXR α or indicated Myc-RXR α mutants were treated with K-80003 and/or TGF- β for 24 h and analyzed by Western blot (E) or for the production of ATP (F). (G) Mutating Ser78 and/or Thr82 impairs the effect of RXR α in mediating K-80003 induction of mitophagy. RXR $\alpha^{-/-}$ CFSCs transfected with the COX8-GFP-mCherry plasmid together with RXR α or RXR α -S78/T82A were treated with K-80003 and/or TGF- β for 24 h and analyzed by confocal microscopy. Relative fluorescence intensity was analyzed by ImageJ.

result of macromolecular crowding rather than being an artifact of PEG. However, mEGFP-RXR α failed to form condensates in a 10% glycerol solution (Fig. S7B), indicating that molecular weight may affect droplet formation. Consistently, the formation of RXR α condensates was dependent on the molecular weight of PEG, with the highest efficiency observed at 8 kDa PEG (Fig. S7C). Similarly, the size distribution and turbidity of RXR α condensates increased in a protein and crowding agent concentration-dependent manner (Fig. S7D and S7E). Thus, these results indicate that RXR α forms a reversible and plasticity of phase-separated condensate.

When transfected into CFSC cells, mCherry-RXR α formed bright, liquid-like condensates within the nucleus (Fig. 8F). Similarly, endogenous RXR α in CFSC cells exhibited droplet-like spheres in the nucleus (Fig. 8G), indicating that these condensates were not an artifact of the mCherry tag. FRAP analysis demonstrated that mCherry-RXR α redistributed rapidly from the unbleached area to the bleached area in cells (Fig. 8H), revealing the dynamic nature of the condensates. These results demonstrated that RXR α forms liquid-like condensates in cells. To identify the domains of RXR α required for phase separation, we generated various RXR α mutants (Fig. 8I). Deletion of IDR (RXR α - Δ IDR) significantly impaired the ability of RXR α to form nuclear puncta, whereas neither the LBD nor the DBD alone was sufficient to form droplets in cells (Fig. 8J and K). We also purified mEGFP-RXR α -IDR and mEGFP-RXR α -LBD mutant proteins and tested their ability to undergo phase separation *in vitro*. Consistent with the results obtained from cells, mEGFP-RXR α -IDR readily formed condensates, whereas mEGFP or mEGFP-RXR α -LBD did not (Fig. 8L). Interestingly, mCherry-RXR α -LBD, but not mCherry-RXR α -DBD, was strongly incorporated into the condensates formed by mCherry-RXR α -IDR (Fig. 8M and N), suggesting that the LBD may interact with phase separated RXR α -IDR. Taken together, the N-terminal IDR is critical in driving RXR α phase separation.

We next determined whether RXR α phase separation enabled it to form condensates with CaMKK β , thereby serving as a reactive center for AMPK α activation. When purified RXR α and CaMKK β proteins were mixed *in vitro*, they co-assembled into phase separated condensates, where the core RXR α condensate was encapsulated by a spherical shell of CaMKK β . This two-phase LLPS, a condensed demixed phase, and a surrounding dilute aqueous phase, is a well-recognized mechanism for the formation of membraneless organelles to promote biological functions⁶⁹. K-80003 significantly promoted the enrichment of CaMKK β in the peripheral phase of these condensates (Fig. 8O and P). Similarly, when CaMKK β and AMPK α were mixed, K-80003 promoted the formation of AMPK α condensates that co-localized with CaMKK β (Fig. 8Q). These results suggested that RXR α phase separation may provide a localized environment for the activation of AMPK signaling pathway by CaMKK β . In the presence of K-80003, the phosphomimetic RXR α -S78/T82D mutant, but not the non-phosphorylatable RXR α -S78/T82A mutant, efficiently recruited a large amount of CaMKK β and AMPK α to its peripheral phase (Fig. 8R and S, Fig. S7F), demonstrating a pivotal regulatory role of RXR α

phosphorylation in the formation of CaMKK β condensates for AMPK α activation.

4. Discussion

Retinoid receptors are expressed in quiescent HSCs in both rodents and humans, with RXR α being highly abundant²². Previous studies have shown that RXR α and its ligands can suppress liver fibrosis by inhibiting HSC activation and proliferation²². However, the underlying molecular mechanisms remain unclear. Here, we provide *in vitro* and *in vivo* evidence demonstrating that RXR α is a key regulator of HSC activation by regulating AMPK α activation through its unique interaction with CaMKK β via phase separation. In investigating the therapeutic potential of RXR α , we identified K-80003, an RXR α modulator derived from the NSAID sulindac^{31,32,34}, as a potent inhibitor of HSC activation *in vitro* (Fig. 1) and liver fibrosis in both the CCl₄ and AMLN animal models (Fig. 2, Figs. S2 and S3). These results highlight RXR α as an attractive nuclear receptor target for developing new therapies against MASH and identified K-80003 as a promising lead compound for anti-fibrotic treatment.

Our results revealed a close correlation between the anti-fibrogenic effects of K-80003 and its activation of AMPK α in HSCs (Fig. 1). Knockdown of AMPK α in HSCs dramatically reduced the ability of K-80003 to antagonize TGF- β -induced expression of several fibrogenic genes (Fig. 3G and H), indicating that AMPK α activation is essential for the anti-fibrogenic effects of K-80003. This finding is consistent with recent studies showing a critical role of AMPK in regulating HSC activation *in vitro* and animals^{46,47,49}. The mechanism of AMPK α action in HSCs remains to be fully elucidated. AMPK α can antagonize TGF- β signaling by disrupting the interaction between Smad3 and its transcriptional coactivator p300⁷⁰. However, our results demonstrated that Smad3 knockout did not affect the ability of K-80003 to inhibit TGF- β -induced α -SMA expression (Fig. S4C), ruling out the involvement of AMPK α /Smad3 pathway. Recent studies have revealed a critical role of AMPK α in maintaining mitochondrial health and homeostasis during disease progression^{44,71}. Notably, AMPK activation has been linked to mitochondrial fragmentation in response to energy stress⁷². However, little is known about mitochondrial status in HSCs and its role in HSC activation and reversion. Our findings reveal that mitochondrial activity increases during TGF- β -induced HSC activation, as evidenced by elevated production of ATP (Fig. 3C–F). This is consistent with the high energy demand associated with HSC activation and is supported by previous reports showing that mitochondrial activity increases during HSC activation⁷³. Furthermore, mild mitochondrial uncouplers, which reduce ATP and ROS levels, have been shown to prevent HSC activation and proliferation⁷⁴.

Interestingly, we found that AMPK α activation by K-80003 is required for its induction of mitophagy, which coincides with its inhibition of HSC activation (Fig. 3I). In addition, inhibition of CaMKK β /AMPK α pathway by STO-609 suppressed mitophagy (Fig. 6D). Moreover, the RXR α -S75A/T82A mutant, which is incapable of mediating K-80003-induced AMPK α activation,

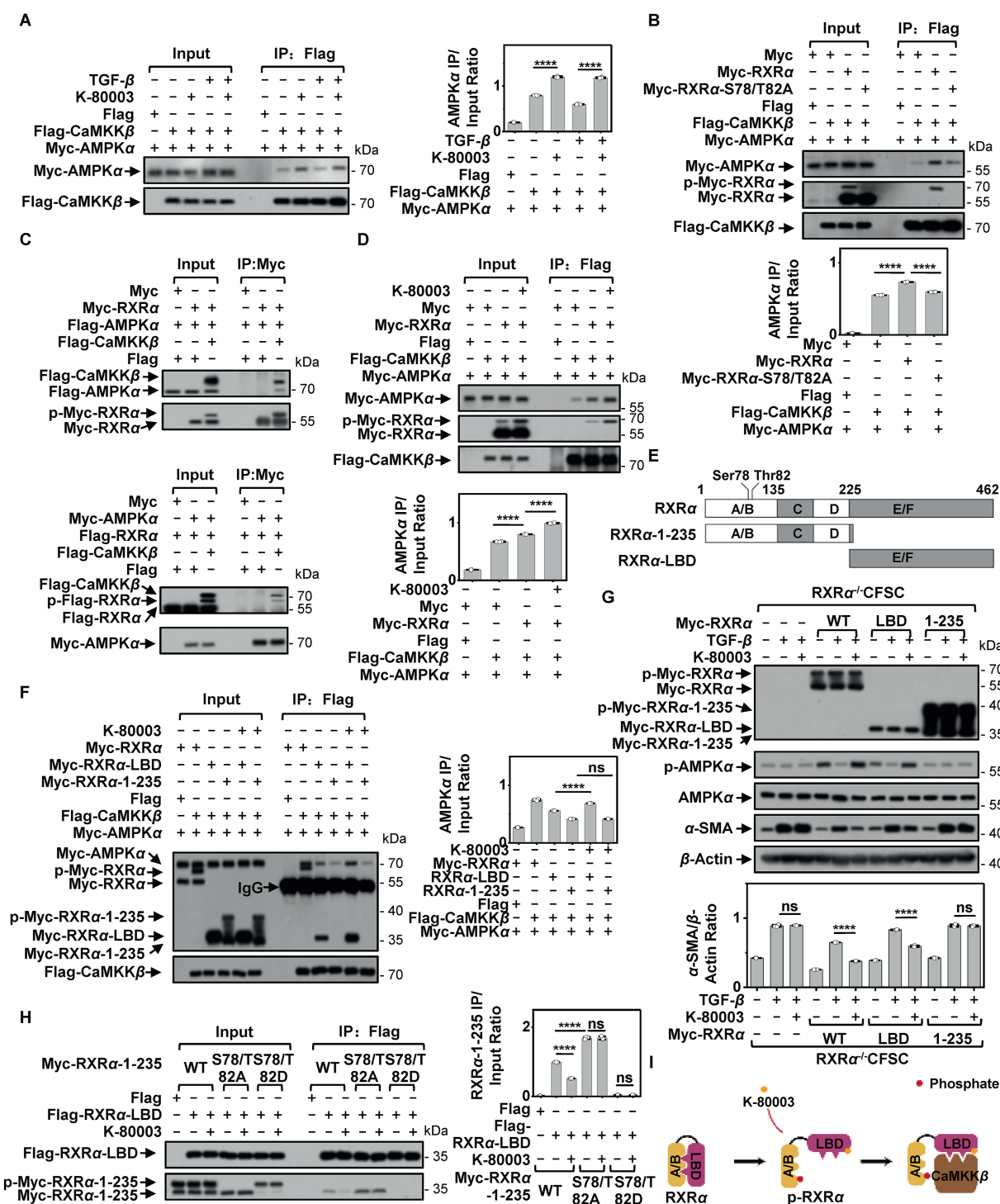


Figure 7 RXR α forms a complex with CaMKK β and AMPK α . (A) K-80003 enhances the interaction between CaMKK β and AMPK α . CFSCs transfected with Flag-CaMKK β and Myc-AMPK α were treated with TGF- β (10 ng/mL) and/or K-80003 (5 μ mol/L) and analyzed by CoIP using anti-Flag antibody. (B) RXR α but not RXR α -S78/T82A enhances CaMKK β interaction with AMPK α . CFSCs transfected with Flag-CaMKK β , Myc-AMPK α , and RXR α or RXR α -S78/T82A were analyzed by CoIP using anti-Flag antibody. (C) RXR α forms a complex with CaMKK β and AMPK α . CFSCs transfected with the indicated expression plasmids were analyzed by CoIP using anti-Myc antibody. (D) K-80003 promotes RXR α complex formation with CaMKK β and AMPK α . CFSCs transfected with the indicated plasmids were treated with or without K-80003 and analyzed by CoIP anti-Flag antibody. (E) Schematic representation of RXR α , RXR α -1-235 and RXR α -LBD. (F) LBD of RXR α is responsible for complex formation with CaMKK β and AMPK α . CFSCs transfected with the indicated plasmids were treated with or without K-80003 and analyzed by CoIP using anti-Flag antibody. (G) RXR α -LBD mediates the effects of K-80003. RXR α ^{-/-} CFSCs transfected with RXR α or mutants were treated with TGF- β and/or K-80003 and analyzed by Western blot. (H) Phosphorylation of Ser78 and Thr82 in RXR α impairs its intramolecular interaction. CFSCs transfected with the indicated plasmids were treated with or without K-80003 and analyzed by CoIP using anti-

failed to promote mitophagy in HSCs (Fig. 6G). Defective mitophagy has been linked to various MASH-related phenotypes⁷⁵. Melatonin has been shown to protect against liver fibrosis by upregulating mitophagy in rat⁷⁶. Although the role of mitophagy in eliminating dysfunctional mitochondria in hepatocytes is well recognized, its function in HSCs remains largely unknown. Mitophagy not only removes dysfunctional mitochondria but also regulates mitochondrial numbers to change cellular metabolic demands⁷⁷. A well-known example is the elimination of mitochondria *via* mitophagy during cellular differentiation of red blood cells^{78,79}. Mitophagy also serves to reduce mitochondrial mass in yeast cells when oxidative phosphorylation is no longer needed⁸⁰. We observed that RXR α -dependent inhibition of TGF- β -induced production of ATP and ROS was associated with AMPK α -dependent mitophagy and reduced mitochondrial mass (Fig. 3, Fig. S4). Thus, it is tempting to speculate that AMPK induction of mitophagy may contribute to the suppression of HSC activation by reducing mitochondrial number. It is noteworthy that a recent study reported that mitophagy in HSCs increases in parallel with apoptosis during the reversal of hepatic fibrosis⁸¹, suggesting an additional mechanism by which AMPK α inhibits HSC activation. Despite these insights, the precise mechanisms by which AMPK mediates the mitophagic effects of RXR α /K-80003 remain unknown. Given that both AMPK and RXR α can localize to mitochondria, further studies are needed to determine whether their mitochondrial localization plays a role in the induction of mitophagy.

Although energy stress-mediated and LKB1-mediated AMPK activation has been extensively characterized, the regulatory mechanisms governing calcium- and CaMKK2-mediated AMPK activation remain poorly understood. Several proteins, including IQGAP1⁸² and STIM2⁸³, have been shown to regulate calcium-mediated AMPK activation by modulating CaMKK β –AMPK interaction. Our study identifies RXR α as a novel modulator of this interaction in HSCs. We showed that K-80003 enhances AMPK activation by promoting RXR α interaction with CaMKK β , independent of the LKB1-mediated pathway (Fig. 5, Fig. S5). These results revealed a previously unrecognized mechanism of RXR α in promoting AMPK α activation. Traditionally, RXR α is considered a ligand-dependent transcription factor that modulates target gene transcription^{28,30}. However, our study revealed that RXR α activates AMPK α through direct interaction with CaMKK β in the cytoplasm, highlighting a novel non-transcriptional mechanism of RXR α action (Fig. 5). Notably, CaMKK β phosphorylated RXR α , and only the phosphorylated form of RXR α interacted with CaMKK β (Fig. 5), underscoring its role in an auto-regulatory mechanism that mediates CaMKK β activation of AMPK α . As RXR α is a critical mediator of vitamin A action, this mechanism may play a pivotal role in modulating HSC activation and maintaining liver homeostasis, particularly in environments rich in vitamin A and its derivatives.

The interaction between RXR α and CaMKK β is both unique and highly specific, as only phosphorylated RXR α interacts with CaMKK β (Fig. 5A). Interestingly, a similar modified RXR α was detected in the liver of mice treated with CCl₄, coinciding with reduced AMPK α activation (Fig. 5B). These observations suggest that RXR α phosphorylation may be a critical event in liver

fibrosis, positioning the modified RXR α as an attractive target for MASH therapeutics. The selective interaction of phosphorylated RXR α with CaMKK β indicates that this interaction is regulated by an intra-molecular interaction between the N- and the C-terminal segments of RXR α , which serves to mask the CaMKK β -interaction motif within the C-terminal LBD (Fig. 7H). CaMKK β -mediated phosphorylation at the N-terminal region of RXR α disrupts this N/C interaction, unveiling a regulatory paradigm for RXR α action. The N/C intramolecular interaction has been described for several nuclear receptors, including estrogen receptor⁸⁴, peroxisome proliferator-activated receptors⁸⁵, and androgen receptor⁸⁶. We previously reported that RXR α engages such N/C interaction that modulates its binding to the p85 α regulatory subunit of phosphoinositide 3-kinase (PI3K)³¹ and mitotic kinase polo-like kinase 1 (PLK1)⁵⁵. Thus, this mechanism plays an important role in the transcription-independent action of RXR α , orchestrating its crosstalk with other cellular signaling pathways and contributing to a complex RXR α -mediated signaling network.

Our study provides compelling evidence that RXR α phase separation is essential for CaMKK β -dependent activation of AMPK α through a two-phase condensation mechanism, in which RXR α forms condensates that are encapsulated by a spherical shell of CaMKK β . The assembly of this layered liquid, regulated by RXR α phosphorylation and K-80003, facilitates the recruitment and activation of AMPK α . These data suggest that layered condensates function as reaction hubs for AMPK α activation, providing new molecular insights into the regulation of AMPK α signaling. IDRs, especially those containing low-complexity domains, are known to drive the formation of intracellular membraneless organelles through LLPS for compartmentalized biochemical reactions^{41,67}. The N-terminal IDR of RXR α exhibits liquid-like properties, which are necessary not only for RXR α phase separation but also for the formation of RXR α /CaMKK β condensates and the subsequent recruitment of AMPK α . Similarly, we previously reported that the N-terminal IDR of the nuclear receptor Nur77 exhibits liquid-like properties, facilitating its condensation with p62 for mitophagy⁴². Furthermore, a recent study has shown that RXR β and RXR γ also undergo phase separation *via* their N-terminal IDR^{87,88}. The N-terminal IDRs of nuclear receptor family members play a crucial role in many nuclear receptor activities⁸⁹. Many nuclear receptor subtypes with distinct IDRs, arise through alternative splicing, translation initiation⁹⁰, and proteolytic cleavage³², contributing to the complexity of nuclear receptor regulatory networks and cell/tissue-specific signaling. Given that LLPS-mediated membraneless organelles have emerged as key platforms for diverse biological processes, IDR-mediated phase separation may represent a fundamental mechanism by which nuclear receptors regulate a wide range of cellular activities. Interestingly, the majority of phosphorylated residues in nuclear receptors are located within their N-terminal IDR regions⁹¹. Our findings reveal that the phosphorylation of Ser78 and Thr82 in RXR α IDR is critical for its phase separation with CaMKK β (Fig. 8R and S, Fig. S7F), revealing the pivotal role of phosphorylation in regulating nuclear receptor phase separation. This regulation likely occurs through the formation of charge blocks known to strongly induce LLPS in a spatiotemporal manner⁹².

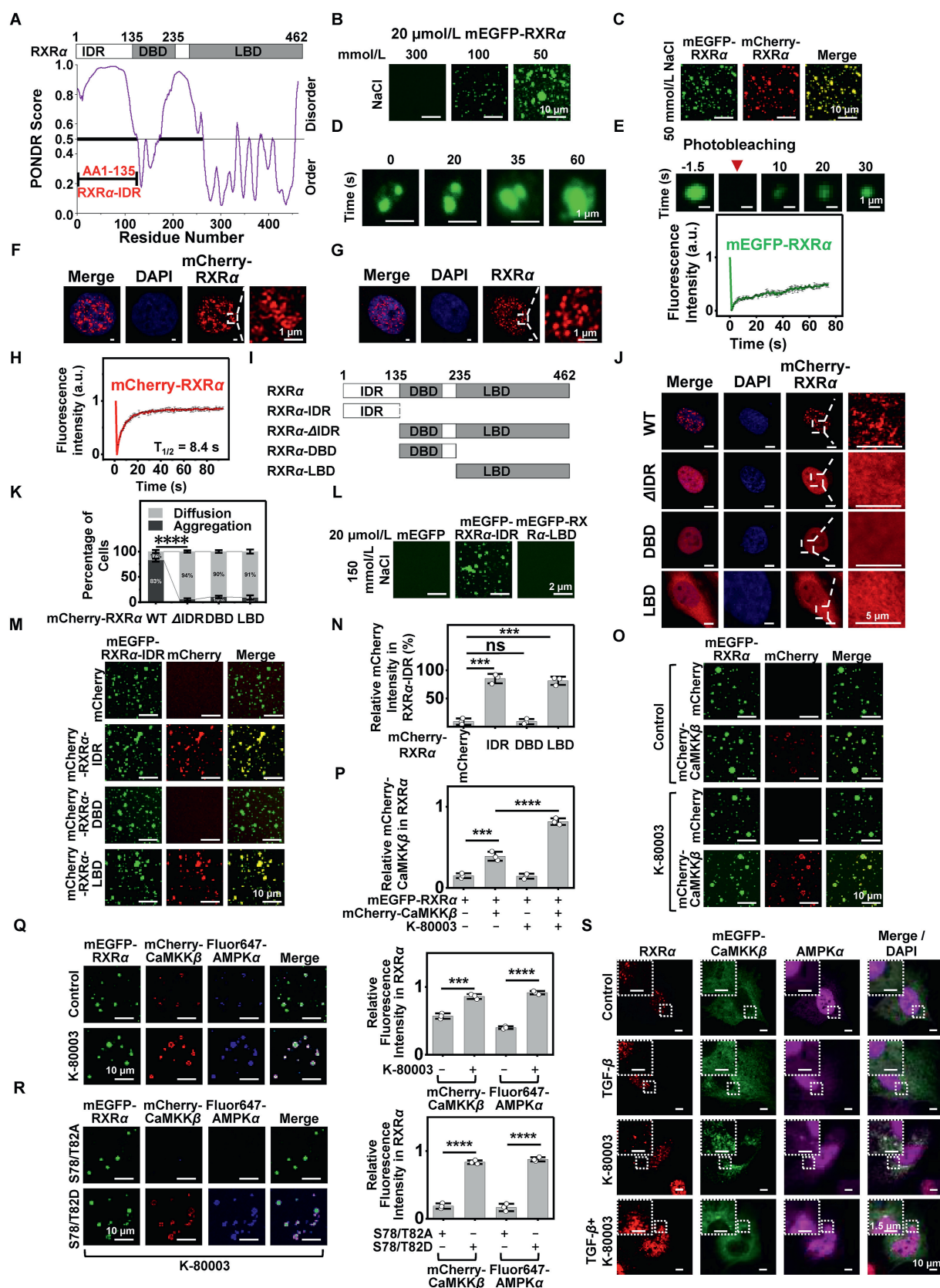


Figure 8 RXRα phase separation provides hubs for AMPKα activation by recruiting CaMKKβ. (A) Intrinsic disorder tendency of RXRα. IDR: intrinsically disordered region, DBD: DNA-binding domain, LBD: ligand-binding domain. (B) mEGFP-RXRα droplets fused into larger with salt concentration. (C) Representative images of mEGFP-RXRα were incubated with mCherry-RXRα phase separation. (D) mEGFP-RXRα droplets fused during the *in vitro* phase separation process. (E) Top: representative images of fluorescence recovery. Bottom: changes in fluorescence

5. Conclusions

Our study demonstrates that K-80003, a unique RXR α modulator, inhibits fibrosis by promoting AMPK α activation and subsequent mitophagy in hepatic stellate cells. We show that K-80003 enhances AMPK α activation by facilitating RXR α interaction with CaMKK β , a process driven by the phase separation of the N-terminal intrinsically disordered region of RXR α and regulated by phosphorylation. Our results highlight the critical role of RXR α in modulating liver fibrosis through mitochondrial regulation in hepatic stellate cells. Moreover, our study identifies K-80003 and related RXR α modulators as promising therapeutic agents for fibrosis-related diseases.

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Author contributions

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Xiaohui Chen: Data curation. Qi Zhao: Data curation. Ting Deng: Data curation. Lulu Chen: Data curation. Jingwei Su: Data curation. Luoyan Sheng: Data curation. Ying Su: Formal analysis. Ling-Juan Zhang: Funding acquisition, Formal analysis. Fu-Quan Jiang: Supervision, Resources, Funding acquisition, Conceptualization. Xiao-Kun Zhang: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Conflicts of interest

Xindao Zhang, Baohuan Lian, Zhibin Su, Yuqi Zhou, and Ying Su are employees and/or shareholders of NucMito Pharma. All other authors declare no competing interests.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.05.023>.

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intensity of mEGFP-RXR α droplets after photobleaching were plotted over time. Data are presented as mean values $\pm$ SD ($n = 3$ independent experiments). (F) Fixed imaging of mCherry-RXR α in CFSCs. (G) Endogenous RXR α puncta were detected using anti-RXR α antibody in CFSCs. (H) Quantification of fluorescence intensity recovery in the bleached region of mEGFP-RXR α droplets in CFSCs. Data are presented as mean values $\pm$ SD ($n = 3$ independent experiments). (I) Schematic illustration of RXR α and its mutants. (J) CFSCs were transfected with mCherry-RXR α (WT), mCherry-RXR α - Δ IDR (Δ IDR), mCherry-RXR α -DBD (DBD), and mCherry-RXR α -LBD (LBD), and analyzed by confocal microscopy. mCherry-RXR α phase separated into liquid-like droplets, whereas mCherry-RXR α - Δ IDR, mCherry-RXR α -DBD and mCherry-RXR α -LBD failed. (K) Quantification of RXR α mutant droplets formation for (J) in CFSCs. Data are presented as mean values $\pm$ SD ($n = 3$ independent experiments). (L) Phase-separation assay of RXR α mutants *in vitro*. mEGFP-RXR α -IDR fragment phase separated into liquid-like droplets, whereas mEGFP-RXR α -LBD failed. (M) Role of RXR α -IDR. Purified mEGFP-RXR α -IDR were incubated with mCherry-RXR α , mCherry-RXR α -LBD, mCherry-RXR α -IDR and mCherry as control. (N) Quantification of RXR α -IDR in modulating phase separation of RXR α -DBD and RXR α -LBD *in vitro*. Data are presented as mean values $\pm$ SD ($n = 3$ independent experiments). (O) Fluorescence microscopy images showing a shell-like formation of CaMKK β around RXR α droplets in the presence of 0.5 mmol/L EDTA, 10 μ mol/L calmodulin, 5 mmol/L MgCl₂ and 2.5 mmol/L ATP. (P) Quantification of K-80003 promotes CaMKK β recruitment by RXR α . (Q) K-80003 promotes AMPK α recruitment to the surface of RXR α droplets. (R) Effect of RXR α phosphorylation for recruiting CaMKK β /AMPK α . (S) K-80003 induces co-localization of RXR α , AMPK α with transfected mEGFP-CaMKK β in the cytoplasm. CFSC cells transfected with mEGFP-CaMKK β were treated with TGF- β (10 ng/mL) and/or K-80003 (5 μ mol/L) for 24 h and analyzed by confocal microscopy. Data are presented as mean $\pm$ SD ($n = 3$ independent experiments). Data are presented as mean $\pm$ SD; *** $P < 0.001$, **** $P < 0.0001$; ns, no significance between indicated groups. (one way ANOVA).

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