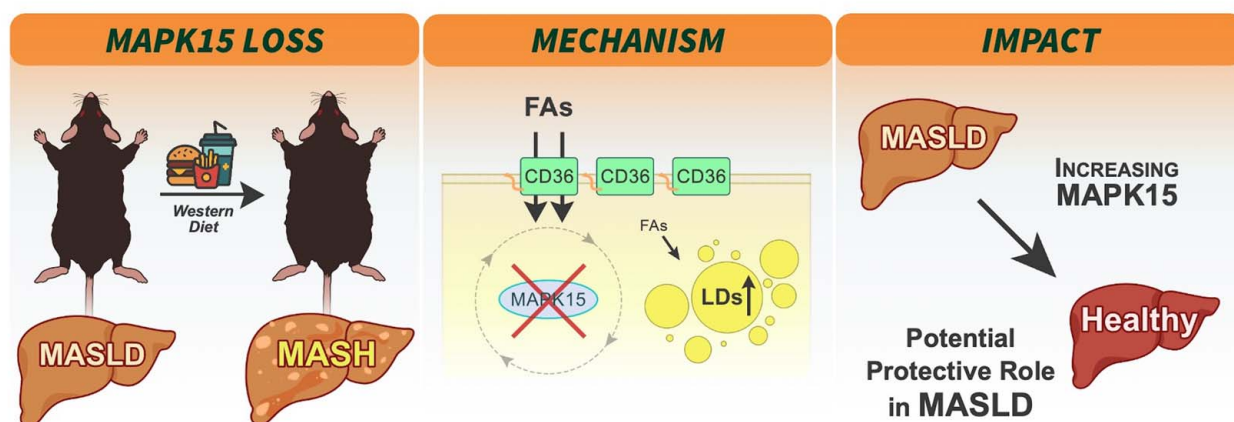


MAPK15 controls intracellular lipid uptake and protects mammalian liver from steatotic disease

VISUAL ABSTRACT

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ORIGINAL ARTICLE

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MAPK15 controls intracellular lipid uptake and protects mammalian liver from steatotic disease

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Abstract

Background: Accumulation of lipids in the liver characterizes metabolic dysfunction–associated steatotic liver disease (MASLD), the most prevalent chronic liver disease worldwide.

Methods: To explore the role of mitogen-activated protein kinase 15 (MAPK15) in mammalian lipid homeostasis, we created and characterized the first knockout mouse model for this gene. Hepatocellular in vitro models were also used to further investigate molecular mechanisms underlying MAPK15-dependent regulation of lipid metabolism in the liver.

Results: We observed that *Mapk15*^{-/-} mice exhibited liver steatosis in the context of a MASLD-like phenotype while hepatocellular in vitro models allowed to demonstrate that dysregulated accumulation of lipids was due to increased expression and membrane localization of the CD36 fatty acid translocase. Consistently, *Mapk15*^{-/-} mice exhibited elevated hepatic levels of CD36 and feeding them with a western-type diet significantly accelerated their progression to a steatohepatitis-like phenotype. Importantly,

Abbreviations: ACC1, acetyl-CoA carboxylase 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCS, body condition score; BW, body weight; CD36, cluster of differentiation 36; ChREBP, carbohydrate-responsive element-binding protein; DNL, de novo lipogenesis; ER, endoplasmic reticulum; FAO, fatty acid β -oxidation; FASN, fatty acid synthase; FAs, fatty acids; FER, food efficiency ratio; GSVA, Gene Set Variation Analysis; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HFD, high-fat diet; HOMA-IR, homeostatic model assessment for insulin resistance; HRI, hepatorenal index; IHC, immunohistochemistry; KO, knockout; LCFA, long-chain fatty acids; LDs, lipid droplets; LW, liver weight; MAFLD, metabolic dysfunction–associated fatty liver disease; MAPK15, mitogen-activated protein kinase 15; MASL, metabolic dysfunction–associated steatotic liver; MASLD, metabolic dysfunction–associated steatotic liver disease; MASH, metabolic dysfunction–associated steatohepatitis; MTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NRF2, NF-E2–related factor 2; OA, oleic acid; PA, palmitic acid; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SASP, senescence-associated secretory phenotype; SD, standard diet; SCD1, stearoyl-CoA desaturase-1; SRE1, sterol regulatory element-1; SREBP, sterol regulatory element-binding protein; SSO, sulfo-N-succinimidyl oleate; TAG, triacylglycerol; TG, triglyceride; T2D, type 2 diabetes mellitus; VLCFA, very-long-chain fatty acids; VLDL, very-low-density lipoproteins; WD, Western diet; WT, wild type.

Giovanni Inzalaco and Sara Gargiulo contributed equally to this article.

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transcriptomic analysis of human cohorts revealed increased liver expression of MAPK15 in MASLD patients, suggesting a compensatory role in disease progression. In this context, overexpression of this kinase efficiently opposed lipid accumulation in a MASLD hepatocellular model, opening to the possibility of counteracting hepatic steatosis in humans by pharmacologically or genetically activating this MAP kinase.

Conclusions: Presented data highlight a critical role for MAPK15 in liver physiopathology, by contributing to maintaining physiological intracellular levels of lipids in this tissue.

Keywords: CD36, knockout, MAP kinases, steatohepatitis, western diet

HIGHLIGHTS

- *Mapk15*^{-/-} mice develop a spontaneous MASLD-like phenotype.
- MAPK15 loss increases CD36 expression and membrane localization.
- Western diet fed *Mapk15*^{-/-} mice readily progress to liver inflammation and fibrosis.
- MAPK15 protects the liver from lipid accumulation.

INTRODUCTION

Lipid metabolism maintains a continuous state of dynamic equilibrium, regulated by dietary intake, de novo lipogenesis (DNL), mobilization from adipose tissue, and the consumption of lipids, to ultimately meet body energy demands.^[1–3] Disruption of the balance within these pathways, through increased fatty acids (FAs) uptake or enhanced DNL, are potential primary causes of excessive lipid storage, leading to significant deleterious effect and the development of various human diseases.^[4] Among them, metabolic dysfunction–associated steatotic liver disease (MASLD), formerly known as metabolic dysfunction–associated fatty liver disease (MAFLD) or non-alcoholic fatty liver disease (NAFLD), has become the most common liver disorder worldwide and represents a leading cause of cirrhosis and hepatocellular carcinoma (HCC).^[5] Indeed, MASLD occurrence is continuously rising, primarily due to poor dietary habits, with current estimation indicating a global prevalence exceeding 30%.^[5] Importantly, men exhibit a higher incidence and prevalence of MASLD compared with pre-menopausal women, but not in relation to post-menopausal women.^[6] This suggests that female sex hormones may play a protective role in the pathogenesis of the disease.^[6]

Primary criteria for defining MASLD include the presence of hepatic steatosis, characterized by the

accumulation of triglycerides (TGs) in hepatocytes, and the absence of secondary causes such as excessive alcohol consumption, use of steatogenic drugs, or inborn metabolic disorders.^[5] MASLD encompasses a spectrum of liver conditions, ranging from simple liver steatosis (metabolic dysfunction–associated steatotic liver, MASL) to more severe forms such as metabolic dysfunction–associated steatohepatitis (MASH), which can further progress to fibrosis and cirrhosis. This disease spectrum is often accompanied by an increasing rate of obesity, metabolic syndrome, and type 2 diabetes mellitus (T2D).^[5] Unfortunately, resmetirom, an orally active, liver-directed, thyroid hormone receptor agonist, is the only drug for the treatment of MASH that has obtained positive results in a phase III clinical trial. Therefore, most pharmacological therapies for MASLD patients are currently directed to the management of their cardiometabolic comorbidities (eg, T2D and obesity).^[5]

Mitogen-activated protein kinase 15 (MAPK15; ERK8; ERK7) is an atypical MAP kinase implicated in several cellular processes, such as cell proliferation,^[7–9] genomic integrity,^[9–11] secretion,^[12,13] autophagy and mitophagy,^[7,14,15] oxidative stress, aging, and cellular senescence.^[15,16] Interestingly, few studies have also suggested a role for *MAPK15* in human diseases related to lipid metabolism. Among them, a genome-wide association study identified a polymorphism in this gene as a possible risk allele for childhood obesity^[17] while, more recently, *MAPK15* has been found associated with obesity-related traits,^[18] consolidating evidence for its contribution to local fat distribution and obesity phenotypes. Ultimately, MAPK15 has been recently demonstrated to control nutrient homeostasis and lipid storage in *Drosophila* larvae^[19] while its expression is stimulated, in mouse livers, upon high-fat diet (HFD)-induced MASLD.^[20]

Here, we characterized the first *Mapk15* knockout (KO, *Mapk15*^{-/-}) mouse model and demonstrated a key role for this gene in controlling the accumulation of lipids

in mouse hepatocytes, determining liver steatosis associated with an overweight/obesity phenotype, insulin resistance, and dyslipidemia, ultimately supporting the existence of a MASLD-like phenotype. Importantly, we demonstrated that gene deletion in mice or downregulation in cultured cells led to increased hepatocyte expression and membrane localization of cluster of differentiation 36 (CD36, also known as SR-B2 or FAT), a transmembrane glycoprotein playing a key role in the cellular uptake of extracellular FAs. Ultimately, by feeding *Mapk15*^{-/-} mice with a lipid-rich and carbohydrate-rich western-style diet (WD), we demonstrated that the loss of this gene greatly accelerated the progression of liver steatosis toward more advanced pathological features of steatohepatitis, such as hepatocyte damage, liver inflammation, and fibrosis.

METHODS

Mouse models

Mapk15^{-/-} mice were obtained from Lexicon Pharmaceuticals. In this model, the full coding sequence of *Mapk15* has been substituted with a *LacZ/Neo* gene. The mutation has been generated in 129SvEvBrd strain-derived embryonic stem cells. Chimeric mice have been bred to C57BL/6J albino mice to generate F1 heterozygous animals on a 129SvEvBrd/C57BL/6J background. Heterozygous and homozygous mice are vital and show no signs of suffering. Mice used in this study are on a pure C57BL/6J background after crossing into it for at least 10 generations. Inbred C57BL/6J control *Mapk15* wild-type (WT, *Mapk15*^{+/+}) mice (JAX stock #000664) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) via Charles River (Calco, LC, Italy) at 7 weeks of age (see Supplemental Reagents and tools info Table S1.1, <http://links.lww.com/HC9/C239>). All mice were socially housed in groups of up to 4 mice per cage under standard conditions (12 h light cycle, ambient temperature of 20–23 °C), and free access to food and water. Mice in the WD group were switched from the standard diet [SD: 3% fat, 4RF21, Mucedola, Italy; 18.5% kcal from protein; 3% from fat; 53.5% from carbohydrates (3% sucrose); 3.150 kcal/g] to the lipid-rich diet [WD: 0.2% cholesterol and 21% butter, Western U8958 version 35, SAFE, France; 14.4% kcal from protein; 38.1% from fat; 47% from carbohydrates (33% sucrose); 4.2594 kcal/g] starting from 8 up to 24 weeks of age. The WD was stored at 4 °C and replaced once per week to avoid lipid peroxidation. All WD-fed mice appeared healthy and active throughout the diet intervention period, and no mouse had to be euthanized before 17 weeks of feeding. Mice in the control group were fed with SD from weaning until 24 weeks of age. Body weight (BW)

and food consumption were monitored twice per week, immediately after food replenishment. Food pellets were weighed twice per week, and the amount of food left in the cages was subtracted from the initially recorded amount. For each mouse, the average daily food weight intake per week was calculated. According to NIH-MMPCs guidelines, we calculated the BW change from the initial measurements to analyze the overall effect of diet on this parameter. Furthermore, the BCS was monitored at relevant time points (every 8 wk) over a 17-week period, as recommended. The food intake of mice was analyzed as the daily amount of food (g) consumed by a single mouse for each experimental week. Mice were sacrificed and analyzed at 24 weeks of age. Detailed methodologies for in vivo animal procedures have been previously described.^[21] Animal experiments were performed in accordance with Directive 2010/63/EU and D.L. 26/2014 and with NIH and ARRIVE guidelines for the use and care of live animals and approved by the Animal Welfare Board of Fondazione Toscana Life Sciences, Siena, Italy (protocol code: 9AECF.34) and by the Italian Ministry of Health (protocol code: 175/2021-PR). Supplementary methods, reagents and info tables can be found in the Supplementary Materials and Methods section, <http://links.lww.com/HC9/C239>.

RESULTS

Mapk15 gene deletion induces a MASLD-like phenotype

To characterize the specific phenotype of a newly generated *Mapk15* KO (*Mapk15*^{-/-}) mouse model in the C57BL/6J genetic background (Supplemental Figures S1A, B, <http://links.lww.com/HC9/C230>), we started our analysis by examining its baseline morphometric traits and blood chemistry profile. Upon feeding with a standard diet (SD), male *Mapk15*^{-/-} mice showed a significantly higher body weight (BW) when compared with age-matched and sex-matched wild type (*Mapk15*^{+/+}, WT) mice (Figure 1A). Indeed, male *Mapk15*^{-/-} mice showed an “overweight” phenotype, as demonstrated by visual examination (Figure 1B), paralleled by a significantly higher body condition score (BCS) when 24 weeks old (Supplemental Figure S2, <http://links.lww.com/HC9/C230>), and by the evidence of more abundant visceral adipose deposits upon necroscopic analysis (Figure 1C). Twenty-four-week-old male *Mapk15*^{-/-} and *Mapk15*^{+/+} mice showed equivalent fasting serum levels of TGs and transaminases (alanine aminotransferase, ALT, and aspartate aminotransferase, AST), but significantly higher values of fasting serum cholesterol in *Mapk15*^{-/-} animals (Figure 1D). Male *Mapk15*^{-/-} mice also showed significantly higher fasting serum levels of glucose and

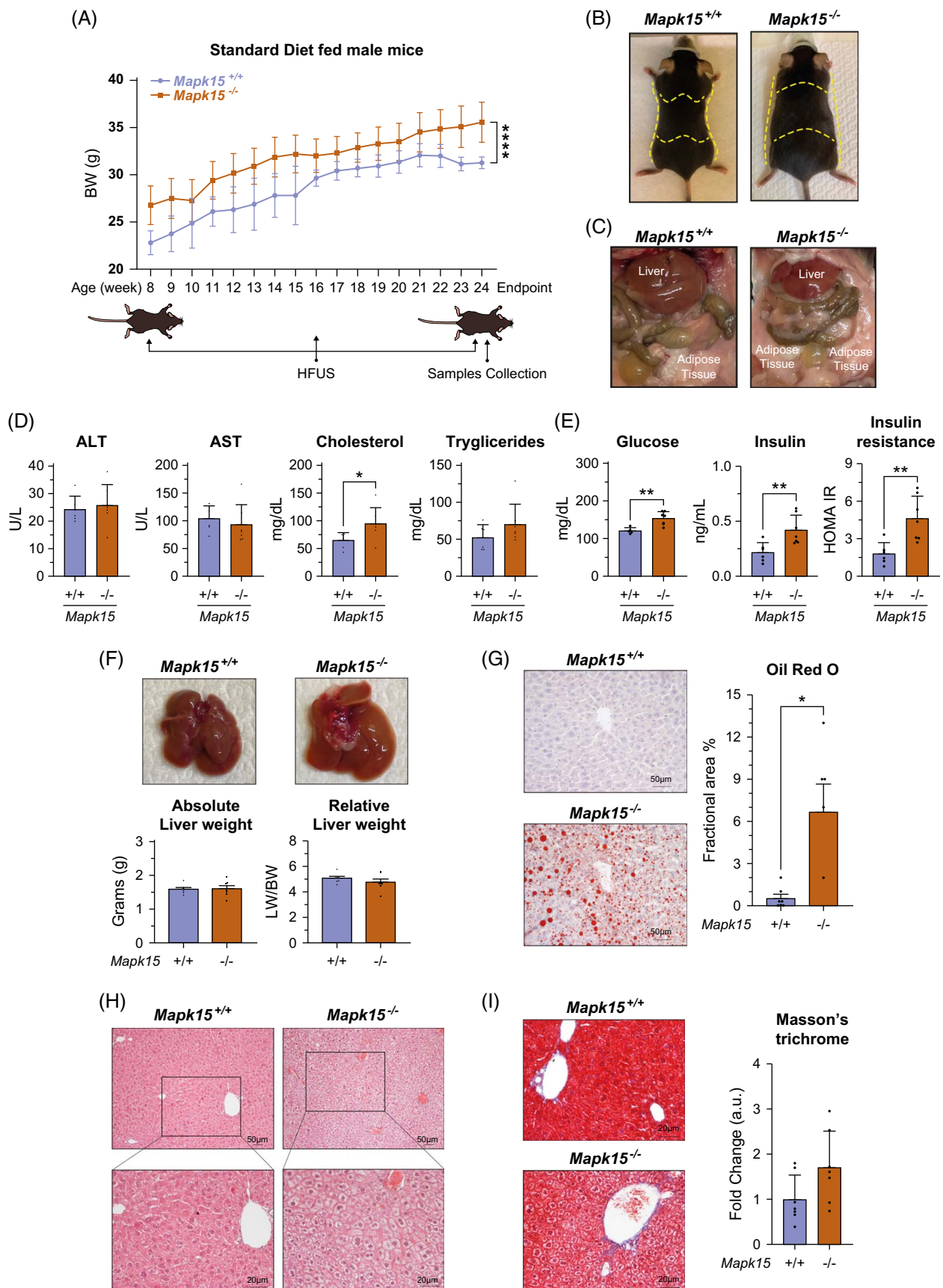


FIGURE 1 *Mapk15* KO mice show metabolic syndrome associated with hepatic steatosis. (A) *Mapk15*^{+/+} and *Mapk15*^{-/-} mice were fed with a standard diet (SD), and data were collected from 8 to 24 weeks of age. Mice were weighed every week, visceral ultrasound data were acquired at

8, 16, and 24 weeks of age, while blood samples were collected at the end of the study. Two-way mixed-effect ANOVA model (repeated measures, interaction age $\times$ genotype: **** $p \leq 0.0001$). (B) Representative images of the appearance of *Mapk15*^{+/+} and *Mapk15*^{-/-} mice at 24 weeks of age. (C) Necroscopic analysis of *Mapk15*^{+/+} and *Mapk15*^{-/-} SD-fed mice at 24 weeks of age. (D) Blood samples from 24-week-old *Mapk15*^{+/+} and *MAPK15*^{-/-} SD-fed mice were collected and analyzed for the indicated markers: ALT, AST, cholesterol, and TG. (E) Same as in (D), but analyzing glucose and insulin. Insulin resistance values (HOMA-IR) were also calculated. (F) Representative gross liver pictures and absolute (liver weight, LW) and relative (body weight/liver weight, BW/LW) liver weights. (G) Representative Oil Red O staining of histological samples from the livers of *Mapk15*^{+/+} and *Mapk15*^{-/-} male mice. The accompanying graph scores the fractional area of Oil Red O staining. (H) Representative haematoxylin and eosin staining of histological samples from *Mapk15*^{+/+} and *Mapk15*^{-/-} SD-fed male mice livers. (I) Representative Masson trichrome staining of histological sections from *Mapk15*^{+/+} and *Mapk15*^{-/-} SD-fed mice livers. The accompanying graph scores the blue collagen fibers as fold changes comparing WT (n = 7) and KO (n = 7) liver. One-way ANOVA—Mann–Whitney nonparametric test: * $p \leq 0.05$; ** $p \leq 0.01$. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; HOMA-IR, homeostatic model assessment for insulin resistance; KO, knockout; SD, standard diet; TG, triglyceride; WT, wild type.

insulin than *Mapk15*^{+/+} animals, demonstrating increased insulin resistance index, evaluated by the homeostatic model assessment for insulin resistance (HOMA-IR) method^[22] (Figure 1E). Overall, our data therefore supported the existence of important metabolic dysfunctions (“metabolic syndrome”)^[23] resulting from the deletion of the *Mapk15* gene.

Ultrasound analysis of SD-fed male *Mapk15*^{-/-} mice, compared with their *Mapk15*^{+/+} counterparts, also showed, at different time points, in vivo changes of hepatic features indicative of mild parenchymal steatotic alterations (Supplemental Figure S3A, <http://links.lww.com/HC9/C230>). Indeed, 24-week-old SD-fed *Mapk15*^{-/-} male mice showed a diffuse increase of parenchymal echogenicity and echotexture heterogeneity, and frequent (43%) incidence of isoechoic appearance of the caudate liver lobe compared with the right kidney cortex (Supplemental Figure S3A, <http://links.lww.com/HC9/C230>), although differences in parametric values such as hepatorenal index (HRI) and liver echogenicity did not reach statistical significance (Supplemental Figures S3B, C, <http://links.lww.com/HC9/C230>). Therefore, to definitely ascertain hepatic accumulation of lipids in *Mapk15*^{-/-} male mice, these were sacrificed when 24 weeks old and livers, which appeared paler although of similar weights when compared with *Mapk15*^{+/+} mice (Figure 1F), were subjected to histological analysis with a specific fat-soluble dye for intracellular lipid stain (ie, Oil Red O), ultimately revealing widespread hepatocellular lipid accumulation (Figure 1G). Therefore, our findings of overweight phenotype, insulin resistance, and dyslipidaemia in the presence of hepatic accumulation of lipids strongly suggested that deletion of the *Mapk15* gene promoted the onset of a MASLD-like phenotype in mice.^[5] Importantly, the histological evaluation of livers from male *Mapk15*^{-/-} mice did not reveal evident accumulation of inflammatory cells (Figure 1H) and/or liver fibrosis (Figure 1I), thus excluding a more advanced liver disease.^[24] To investigate the potential role of sex in the development of MASLD,^[6] we performed equivalent studies in female mice. Interestingly, while female *Mapk15*^{-/-} mice also showed an increased tendency to accumulate lipids in their livers, this phenotype was milder than in their male counterparts, as demonstrated by ex vivo non-significant differences in the histological appearance of livers between

Mapk15^{-/-} and *Mapk15*^{+/+} mice at Oil Red O (Supplemental Figure S4A, <http://links.lww.com/HC9/C230>) and hematoxylin and eosin (H&E) (Supplemental Figure S4B, <http://links.lww.com/HC9/C230>) staining. Therefore, our data confirm available literature showing that female mice have a reduced tendency to accumulate hepatic TGs and develop steatosis,^[6,25] recapitulating the observation of a higher prevalence of MASLD in men when compared with premenopausal women (but not to those in post-menopausal status).^[6]

MAPK15 depletion stimulates the accumulation of lipid droplets in hepatocellular in vitro models

We next decided to use hepatocellular in vitro models to further investigate molecular mechanisms underlying MAPK15-dependent regulation of lipid metabolism, with the aim of uncovering key pathways contributing to the phenotype in *Mapk15*^{-/-} mice. As lipid droplets (LDs) are the most important site for intracellular storage of lipids,^[26] we used the BODIPY 493/503 probe to evaluate the amount of these organelles in HepG2 cells transiently depleted for MAPK15 expression. Indeed, HepG2 cells transfected with siRNA against MAPK15 (siMAPK15) (Supplemental Figure S5, <http://links.lww.com/HC9/C230>) showed a significant increase in LD amounts when analyzed by confocal microscopy, compared with control siRNA-transfected cells (siSCR) (Figure 2A). Importantly, this effect was completely rescued by overexpressing a siRNA-resistant form of MAPK15 (MAPK15^{Res}), ultimately demonstrating the specificity of our experimental approach (Figure 2B). These results were also confirmed by flow cytometry analysis quantifying LDs' mean fluorescence intensity, using 2 different MAPK15-specific siRNAs (Supplemental Figure S6A, <http://links.lww.com/HC9/C230>) and by inhibiting its kinase activity by a specific MAPK15 pharmacological inhibitor, Ro-318220^[27] (Figure 2C). Importantly, to confirm that such mechanism was applicable also to more physiologically relevant cellular models, we used the hepatoma HepaRG cell line, an immortalized hepatic cell line that retains many characteristics of primary human

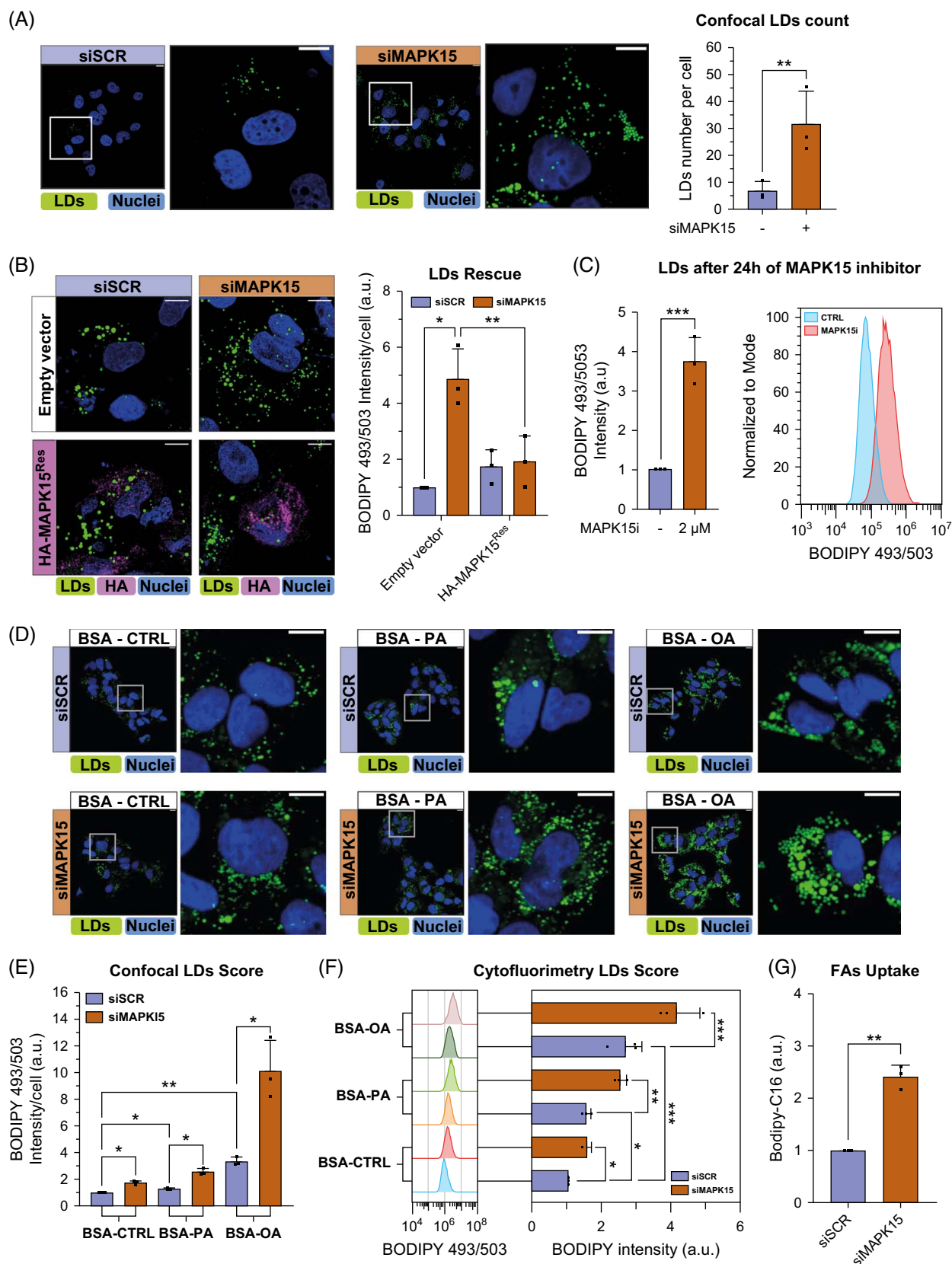


FIGURE 2 MAPK15 depletion increases LD content in hepatocellular model systems. (A) Representative confocal microscopy images showing lipid LDs content in MAPK15-interfered (72 h) HepG2 cells (siMAPK15), stained with BODIPY 493/503. The accompanied graph shows quantification of LDs $\pm$ SD. Ratio paired *t* test was used to assess differences between the siMAPK15 and siSCR conditions across 4 independent experiments (** $p \leq 0.01$). (B) Representative confocal microscopy images showing LDs content in siMAPK15 (72 h) with HA-

MAPK15^{Res} (a mutant of MAPK15 resistant to siMAPK15^[9]) expression rescued (48 h) by transfection in HepG2 cells. LDs were stained with BODIPY 493/503, HA-MAPK15^{Res} with Alexa Fluor 647, and DAPI for nuclei. Scale bar 10 μ m. The accompanying graph shows quantification of LDs per cell $\pm$ SD, using the quantification Module of the Velocity software. Ratio paired *t* test was used to assess differences between the conditions across 3 independent experiments (** $p \leq 0.01$). (C) LDs accumulation in HepG2 cells treated for 24 hours with 2 μ M of Ro-318220 (MAPK15 pharmacological inhibitor—MAPK15i) and stained with BODIPY 493/503, scored by flow cytometry analysis. Paired *t* test: *** $p \leq 0.001$. (D) Representative confocal microscopy images showing LDs after 24 hours of treatment with 200 μ M of FAs [BSA-palmitic acid (BSA-PA) and BSA-oleic acid (BSA-OA); BSA as control], in MAPK15-interfered (72 h) cells and relative controls. (E) Densitometric analysis of (D). BODIPY 493/503 fluorescence was shown as signal per cell $\pm$ SD. Two-way ANOVA with lipid treatment (BSA-CTRL, BSA-PA, BSA-OA) and MAPK15 downregulation (siSCR and siMAPK15) as factors (Treatment: $p = 0.0036$; Downregulation: $p = 0.0002$) followed by the Tukey post hoc test (* $p \leq 0.05$, ** $p \leq 0.01$). (F) Same as in (D) but using flow cytometry analysis to score LDs cellular content fluorescence after treatments with FAs. Two-way ANOVA with lipid treatment (BSA-CTRL, BSA-PA, BSA-OA) and MAPK15 downregulation (siSCR and siMAPK15) as factors. (Treatment: $p < 0.0001$; Downregulation: $p < 0.0001$) followed by the Tukey post hoc test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (G) Cytofluorometric quantification of exogenous FAs uptake using BODIPY-C16 (2 μ M). Scale bar 10 μ m. Paired *t* test: ** $p \leq 0.01$. Abbreviations: FAs, fatty acids; LDs, lipid droplets; SD, standard diet.

hepatocytes,^[28] and demonstrated that, when interfered for MAPK15 expression (Supplemental Figure S6B, <http://links.lww.com/HC9/C230>), they also increased LDs content, by both confocal microscopy (Supplemental Figure S6C, <http://links.lww.com/HC9/C230>) and cytofluorimetric (Supplemental Figure S6D, <http://links.lww.com/HC9/C230>) approaches. Similarly, we also validated the impact of MAPK15 on hepatocellular lipid accumulation by using primary hepatocytes isolated from *Mapk15*^{-/-} and *Mapk15*^{+/-} mice, demonstrating also in these cells that deletion of this gene determined increased LDs content, as ascertained by both confocal microscopy (Supplemental Figure S7A, <http://links.lww.com/HC9/C230>) and cytofluorimetric (Supplemental Figure S7B, <http://links.lww.com/HC9/C230>) approaches. Next, as MASLD induction can be modeled in HepG2 cells in vitro by short-term treatments with palmitic acid (PA) and/or oleic acid (OA),^[6] LDs content was also examined, in siMAPK15-treated cells, by confocal microscopy (Figures 2D, E) and flow cytometry analysis (Figure 2F) after 24 hours treatment with these FAs, demonstrating also in these conditions increased number and size of LDs. Importantly, this result was further confirmed in primary hepatocytes from *Mapk15*^{-/-} and *Mapk15*^{+/-} mice (Supplemental Figure S7C, <http://links.lww.com/HC9/C230>), overall suggesting that an increased uptake of exogenous FAs could be responsible for the enhanced accumulation of lipids inside hepatic cells. Indeed, we demonstrated an increased cellular uptake of exogenously added fluorescent palmitate (BODIPY-C16) in siMAPK15 HepG2 cells, when compared with their siSCR counterpart (Figure 2G).

Accumulation of LDs, in MAPK15-depleted cells, depends on the increased expression and membrane localization of the CD36 fatty acid translocase

Approximately 59% of hepatic fat originates from circulating free FAs, while de novo lipogenesis accounts for about 26%, and dietary sources for the remaining 15%.^[29] Several FAs transport proteins, particularly FATP1-6 (SLC27 protein family) and CD36, have been

implicated in mediating the transport of exogenous long and very-long-chain fatty acids (LCFA and VLCFA, respectively) through the cellular membrane^[3] (Figure 3A). Importantly, CD36 increases FAs uptake, and, in the liver, drives hepato-steatosis onset and contributes to its progression to MASH.^[30] Therefore, we hypothesized that the increase in LDs content observed in siMAPK15 HepG2 cells may be attributed to enhanced uptake of exogenous free FAs, potentially mediated by CD36. Indeed, upon downregulation of MAPK15, HepG2 cells showed increased expression of CD36 at both mRNA (Figure 3B) and protein (Figure 3C) levels.

Subcellular localization of CD36 at the plasma membrane contributes to determining its efficacy in mediating free FAs uptake, and CD36 palmitoylation mediated by ZDHHC5 is required for its plasma membrane localization.^[31] As we also observed increased ZDHHC5 protein levels in MAPK15 downregulated cells (Figure 3C), we next evaluated CD36 membrane localization in these experimental conditions. Indeed, increased CD36 membrane localization was demonstrated in HepG2 cells interfered with MAPK15 expression, when scored by both confocal microscopy (Figure 3D) and flow cytometry (Figure 3E) approaches. Next, to demonstrate a causal role for CD36 in the observed increase of LDs in MAPK15-interfered cells, we used a specific CD36 inhibitor, sulfo-N-succinimidyl oleate (SSO).^[32] Indeed, SSO significantly rescued palmitate uptake induced by MAPK15 downregulation in HepG2 cells (Figure 3F). CD36 mRNA and protein expression were also similarly increased by downregulating MAPK15 expression in HepaRG cells (Supplemental Figure S8A, <http://links.lww.com/HC9/C230>), and SSO prevented the increase of palmitate uptake induced by MAPK15 downregulation also in these cells (Supplemental Figure S8B, <http://links.lww.com/HC9/C230>). As an additional and independent confirmation of our pharmacological rescue approach, we also interfered with the expression of CD36 in HepG2 cells by specific siRNA (siCD36) to rescue the effect induced by MAPK15 downregulation

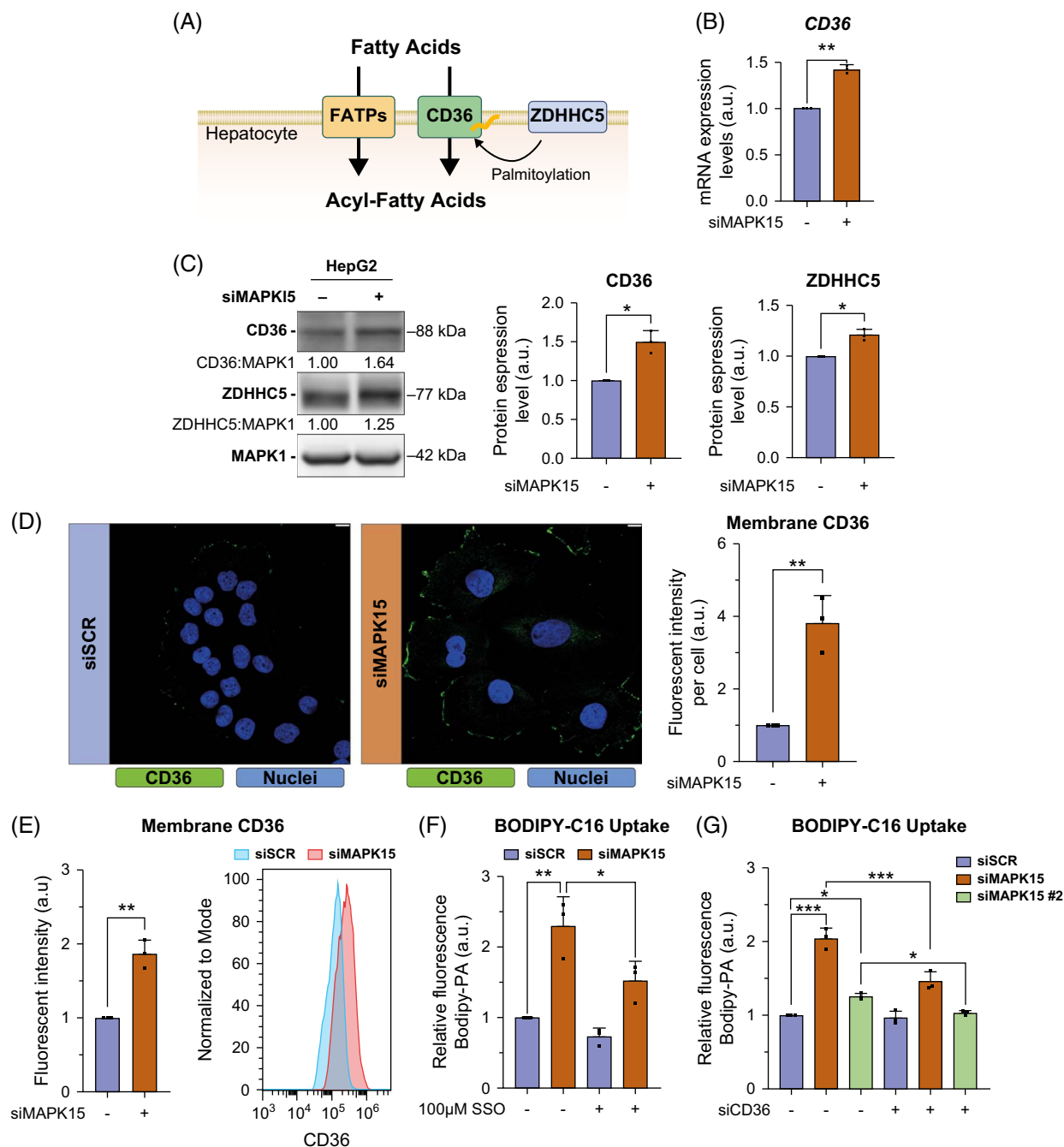


FIGURE 3 MAPK15 depletion increases CD36 expression and membrane localization. (A) A cartoon illustrating the transport of exogenous long-chain and very-long-chain fatty acids across the cellular membrane. (B) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to monitor mRNA expression of CD36 from HepG2 cells interfered with for MAPK15 expression (72 h). Paired t test: $**p \leq 0.01$. (C) Representative immunoblots of CD36 and ZDHHC5 protein expression from HepG2 cells interfered with MAPK15 expression (72 h). The accompanying graph shows densitometric analysis of average protein expression levels; paired t test ($*p \leq 0.05$). (D) Representative confocal microscopy images of membrane-localized CD36 from HepG2 cells interfered with MAPK15 expression (72 h). Intensitometric analysis of CD36 fluorescence signal per cell $\pm$ SD is shown in the accompanying histogram. Paired t test: $**p \leq 0.01$. (E) Cytofluorometric quantification of the membrane-localized CD36 protein, in HepG2 cells, interfered with MAPK15 expression (72 h). Paired t test: $**p \leq 0.01$. (F) Cytofluorometric quantification of BODIPY-C16 (2 μ M) in HepG2 cells interfered with MAPK15 expression (72 h) and was treated with 100 μ M SSO in the absence of FBS for 6 hours. Two-way ANOVA with SSO treatment and MAPK15 downregulation as factors (Treatment: $p = 0.0064$; Downregulation: $p < 0.0001$) followed by Tukey post hoc test ($*p \leq 0.05$, $**p \leq 0.01$). (G) Same as in (F), but treating MAPK15-interfered HepG2 cells with a specific siRNA against CD36. Two-way ANOVA with siCD36 treatment and MAPK15 downregulation as factors (Treatment: $p < 0.0001$; Downregulation: $p < 0.0001$) followed by Tukey post hoc test ($*p \leq 0.05$, $***p \leq 0.001$). Abbreviations: FBS, fetal bovine serum; SSO, sulfo-N-succinimidyl oleate.

(Supplemental Figure S9, <http://links.lww.com/HC9/C230>). Indeed, we observed a reduction in the cellular uptake of palmitate also in these experimental conditions (Figure 3G), overall indicating that the observed increase in the expression of CD36 is necessary for enhanced accumulation of exogenous FAs in hepatic cells. Accordingly, mouse livers from *Mapk15*^{-/-} mice also expressed increased amounts of *Cd36* mRNA as compared with livers from *Mapk15*^{+/+} mice, while expression of other genes involved in transmembrane fatty acid transport (ie, *Fatp1*, *Fatp2*, *Fatp5*, *Fatbp1*, and *Fatb4*) were not significantly affected by *Mapk15* gene deletion (Figure 4A). Ultimately, livers from *Mapk15*^{-/-} mice expressed higher protein levels of both CD36 and ZDHHC5 (Figure 4B), and immunohistochemistry (IHC) analysis showed increased CD36 localization at the plasma membrane (Figure 4C), overall supporting also an in vivo role for CD36 in the lipid metabolic rewiring governed by MAPK15 in the liver. Interestingly, we also observed increased CD36 expression in isolated primary *Mapk15*^{-/-} hepatocytes as compared to *Mapk15*^{+/+} hepatocytes both at mRNA (Figure 4D) and protein level (Figure 4E), supporting our results obtained in cultured, siMAPK15-downregulated, immortalized cells.

DNL does not contribute to the intracellular accumulation of lipids in MAPK15-depleted/deleted cells

DNL is largely controlled at the transcriptional levels by 2 master transcription factors, that is, sterol regulatory element-binding protein 1 (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP), which stringently regulate the expression of key rate-limiting lipogenic enzymes, for example, acetyl-CoA carboxylase 1 (ACC), fatty acid synthase (FASN), and stearoyl-CoA desaturase-1 (SCD1)^[2,33] (Figure 5A). Importantly, together with the uptake of exogenous FAs, DNL represents another key mechanism driving hepatic FAs accumulation. Indeed, hepatic steatosis typically occurs when the rate of one or both these processes exceed the capacity of the liver to oxidize FAs or secrete TGs via very-low-density lipoproteins (VLDL).^[33] Still, MAPK15-interfered HepG2 cells showed significantly reduced lipid biosynthesis when pulsed with radiolabelled ¹⁴C-glucose (Figure 5B), suggesting that DNL did not contribute to the observed intracellular accumulation of lipids when reducing MAPK15 expression. Accordingly, we also observed that downregulation of MAPK15 in HepG2 cells strongly reduced the expression of SREBP-1 (Figure 5C) and its specific transactivating function, measured through the transfection of a reporter gene under the control of the sterol regulatory element (SRE)^[34] (Figure 5D). Similarly, the expression of the ChREBP transcription factor,

which acts complementarily to SREBP-1 to induce lipogenesis,^[2] was significantly reduced in MAPK15-interfered HepG2 cells (Figure 5E). Consequently, mRNA (Figure 5F) and protein (Figure 5G) levels of SREBP-1 and ChREBP transcriptional targets were also reduced in MAPK15-interfered HepG2 cells. Interestingly, the expression of *Srebp-1* and *Chrebp* (Figure 5H), and of their target genes (Figure 5I) did not significantly change in the livers of *Mapk15*^{-/-} mice, as compared with those from *Mapk15*^{+/+} mice, overall demonstrating that DNL does not contribute to the lipid accumulation observed in MAPK15-depleted hepatic cells. Similarly, we did not notice differences in the protein expression of apoB-100 and microsomal triglyceride transfer protein (MTP), 2 key regulators of VLDL assembly and secretion,^[35] in livers of MAPK15^{+/+} and MAPK15^{-/-} mice (Supplemental Figure S10, <http://links.lww.com/HC9/C230>), suggesting that also these processes are not significantly affected in vivo, in our experimental conditions.

Western-type diet synergizes with loss of MAPK15 to induce steatohepatitis in the mouse

To investigate a role for MAPK15 in protecting mammalian liver from steatogenic insults, we next tested the effect of a western-style diet (WD) on the livers of *Mapk15*^{-/-} mice in accelerating progression toward the development of more severe pathological features of MASLD. Indeed, in humans, a diet high in unhealthy fats is considered an independent risk factor for the development and progression of MASLD^[24] and, correspondingly, HFD-fed mice have recently emerged as the most balanced models in terms of metabolic, histologic, and transcriptomic similarities to human disease.^[36] Therefore, starting from the 8th up to the 24th week of age (total 16 wk), male *Mapk15*^{+/+} and *Mapk15*^{-/-} mice were switched to a lipid-rich WD and analyzed for different markers pathognomonic of human MASLD. WD-fed *Mapk15*^{-/-} mice showed an average BW significantly higher than age-matched *Mapk15*^{-/-} mice (Figure 6A) and a clearly overweight/obese phenotype, as demonstrated by visual examination (Figure 6B) and by a significantly higher BCS (Supplemental Figure S11, <http://links.lww.com/HC9/C230>). Visceral adipose deposits in *Mapk15*^{-/-} mice were increased when compared with *Mapk15*^{+/+} mice (Figure 6C). Liver ultrasound analysis of male *Mapk15*^{-/-} mice showed a higher incidence of more severe steatosis features both in the early and late experimental phases (Supplemental Figure S12A, <http://links.lww.com/HC9/C230>), with significantly higher HRI (Supplemental Figure S12B, <http://links.lww.com/HC9/C230>) and liver echogenicity (Supplemental Figure S12C, <http://links.lww.com/HC9/C230>) at

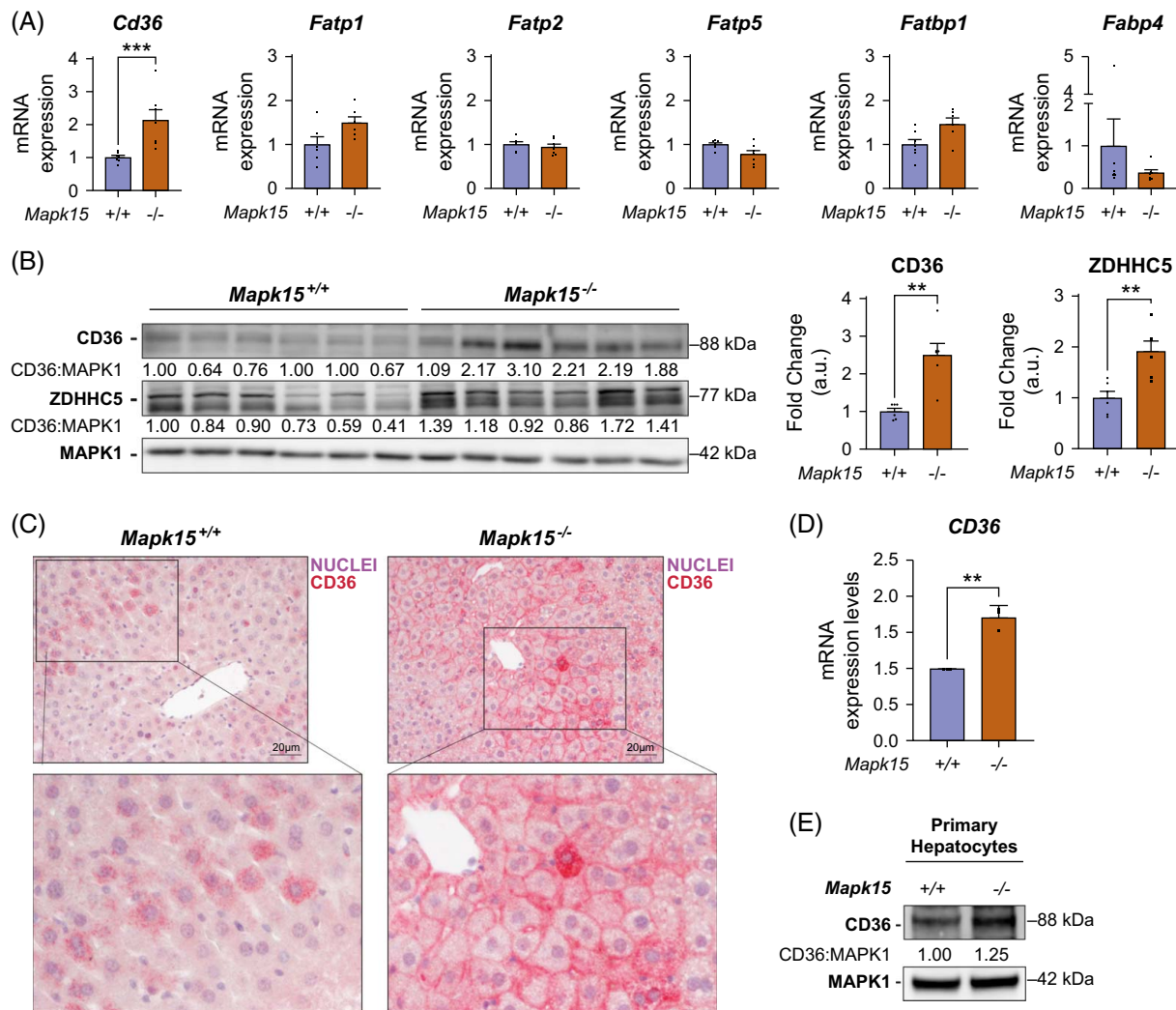


FIGURE 4 *Mapk15*^{-/-} mice show increased CD36 expression and membrane localization. (A) RT-qPCR was performed on livers from *Mapk15*^{+/+} and *Mapk15*^{-/-} mice to evaluate mRNA levels of indicated genes (*Cd36*, *Fatp1*, *Fatp2*, *Fatp5*, *Fatbp1*, and *Fabp4*). (B) Representative immunoblots of mouse liver protein extracts for indicated proteins (CD36, ZDHHC5, and MAPK1). Densitometric analysis of average protein expression levels in livers from *Mapk15*^{+/+} and *Mapk15*^{-/-} mice is shown in the accompanying graph. (C) IHC analysis of CD36 in liver sections of both genotypes. One-way ANOVA—Mann–Whitney nonparametric test: **p* ≤ 0.05, ***p* ≤ 0.01, and ****p* ≤ 0.001. (D) RT-qPCR to monitor mRNA expression of CD36 in isolated primary hepatocytes from *Mapk15*^{+/+} and *Mapk15*^{-/-} mice. Paired *t* test: ***p* ≤ 0.01. (E) Representative immunoblots of CD36 in isolated primary hepatocytes from *Mapk15*^{+/+} and *Mapk15*^{-/-} mice liver. Abbreviations: IHC, immunohistochemistry; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

the experimental endpoint, compared with *Mapk15*^{+/+} mice. These findings were next confirmed by necropsy at 24 weeks of age, showing in WD-fed *Mapk15*^{-/-} mice, a paler appearance of livers (Figure 6D), significantly higher absolute (LW) and relative (BW/LW) liver weights (Figure 6E), and much larger cytoplasmic lipid accumulation in their hepatocytes (Figure 6F). WD-fed *Mapk15*^{-/-} mice showed significantly higher baseline fasted serum values of cholesterol but a similar TG amount, and higher serum levels of ALT (Figure 6G), a specific biomarker of hepatic alterations such as steatosis, due to its greater concentration in the liver compared with other tissues.^[37] Ultimately, WD-fed *Mapk15*^{-/-} mice also showed significantly higher baseline fasted serum levels of glucose and insulin than *Mapk15*^{+/+} animals, with higher insulin resistance (HOMA-IR) (Figure 6H).

Importantly, WD-fed *Mapk15*^{-/-} mice still expressed significantly increased levels and plasma membrane localization of the CD36 protein compared with corresponding *Mapk15*^{+/+} animals, as demonstrated by western blot (Figure 6I) and IHC analysis (Figure 6L), respectively.

Based on the histological liver features in WD-fed *Mapk15*^{-/-} mice suggesting a progressive MAFLD-like phenotype, we next investigated the presence of specific markers of hepatic damage and inflammation, usually found in human MASH. Indeed, histological examination of their livers showed an increased number of lymphocytic infiltrates and frequent “ballooning” injury of the hepatocytes^[38] as compared with WD-fed *Mapk15*^{+/+} mice (Figure 7A). Interestingly, WD-fed *Mapk15*^{-/-} livers also exhibited increased local levels

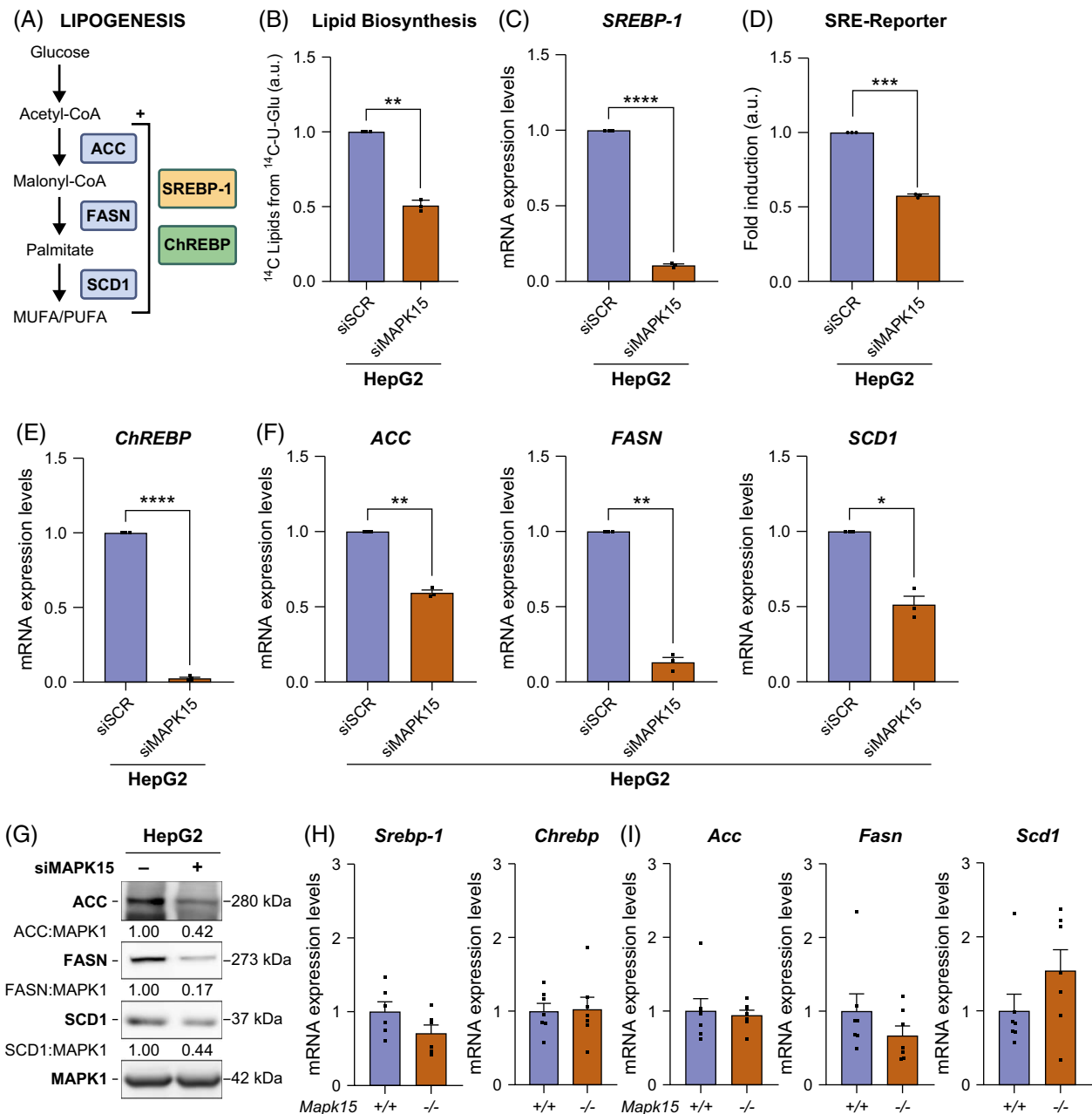


FIGURE 5 De novo lipogenesis does not contribute to LD accumulation in MAPK15-depleted cells. (A) A cartoon illustrating the main pathway for de novo lipogenesis. (B) Functional assay to evaluate lipid biosynthesis from ^{14}C -U-labeled glucose, in MAPK15-interfered (72 h) HepG2 cells. Lipids were extracted, and the radioactive signal was measured to track metabolite incorporation into lipids. Each value was normalized to cell counts. Paired t test: $**p \leq 0.01$. (C) RT-qPCR evaluation of *SREBP-1* mRNA level in MAPK15-interfered (72 h) HepG2 cells. Paired t test: $****p \leq 0.0001$. (D) Activation of a sterol regulatory element (SRE) luciferase reporter plasmid transfected in MAPK15-interfered (72 h) HepG2 cells. Paired t test: $***p \leq 0.001$. (E) Evaluation of ChREBP mRNA expression in MAPK15-interfered (72 h) HepG2 cells. Paired t test: $****p \leq 0.0001$. (F) RT-qPCR was performed to evaluate mRNA levels of *ACC*, *FASN*, and *SCD1* upon *MAPK15* RNA interference in HepG2 cells. Paired t test: $*p \leq 0.05$ and $**p \leq 0.01$. (G) Representative immunoblots of MAPK15-interfered (24 h) HepG2 cell extracts to evaluate the expression of *ACC*, *FASN*, and *SCD1* proteins. (H) RT-qPCR was performed on livers from *Mapk15*^{+/+} and *Mapk15*^{-/-} mice to evaluate mRNA levels of the *Srebp-1* and *Chrebp* genes. The graphs show the average expression of the different genes in the livers of *Mapk15*^{+/+} and *Mapk15*^{-/-} mice. (I) Same as in (H) but evaluating *Acc*, *Fasn*, and *Scd1* gene expression. Abbreviations: FASN, fatty acid synthase; LD, lipid droplet; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

of different proinflammatory cytokines when compared with both WD-fed *Mapk15*^{+/+} livers and also to livers from SD-fed *Mapk15*^{+/+} and *Mapk15*^{-/-} animals, namely TGF- β ,^[39] IL-1 α ,^[40] and MCP1,^[41] while TNF- α levels,

although increasing, did not reach statistical significance (Figure 7B). Importantly, *Mapk15*^{-/-} liver tissue trichrome stain showed evidence of periportal and perisinusoidal fibrosis compared with WD-fed

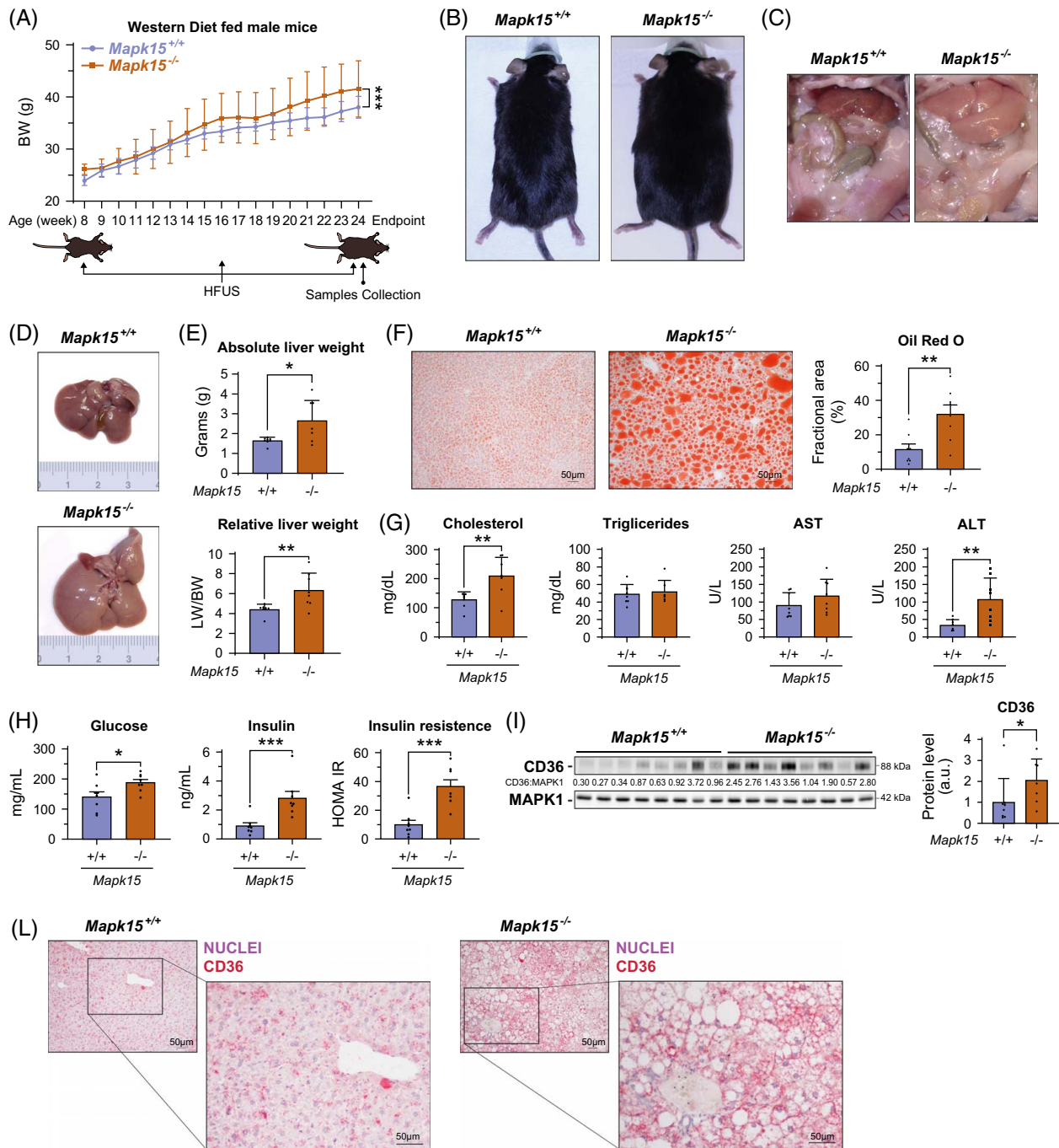


FIGURE 6 *Mapk15* gene deletion cooperates with a western-type diet to accelerate the progression of a MASLD-like phenotype. (A) From 8 to 24 weeks of age, *Mapk15*^{+/+} and *Mapk15*^{-/-} mice were fed with a western-type diet (WD). Mice were weighed every week, as reported in the graph, and visceral ultrasound data were acquired at 8, 16, and 24 weeks of age. Blood samples were collected at the endpoint (24 weeks). Two-way ANOVA mixed-effect model—repeated measures, interaction age × genotype: ****p* < 0.001. (B) Representative images of the appearance of *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. (C) Necropsy analysis of *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. (D) Representative gross liver pictures from *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. (E) Absolute (liver weight, LW) and relative (liver weight/body weight, LW/BW) liver weights of *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. (F) Representative Oil Red O staining of histological samples from livers of *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. The accompanying graph scores the fractional area of Oil Red O staining. (G) Blood samples from 24-week-old *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice were collected and analyzed for the indicated markers (cholesterol, triglycerides, AST, and ALT). (H) Same as (G) but for indicated markers (glucose and insulin). Insulin resistance values (HOMA-IR) were also calculated. (I) Immunoblot analysis of CD36 protein expression in livers from *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. Quantification of average expression in the different genotypes is also shown in the accompanying graph. (L) Representative IHC of CD36 in liver sections of both genotypes. One-way ANOVA—Mann–Whitney nonparametric test: **p* ≤ 0.05, ***p* ≤ 0.01, and ****p* ≤ 0.001. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; HOMA-IR, homeostatic model assessment for insulin resistance; MASLD, metabolic dysfunction–associated steatotic liver disease.

Mapk15^{+/+} mice (Figure 7C), overall demonstrating a faster progression of *Mapk15*^{-/-} mice to a MASH-like phenotype upon a typical steatogenic insult, that is, WD, ultimately suggesting a key protective role for this gene against MASLD pathogenetic stimuli.

MAPK15 gene expression is increased since the earliest MASLD stages in humans, and its overexpression reduces hepatocellular lipid accumulation

Based on our results in mice and human cell lines, we next sought to establish the potential clinical and

translational relevance of our data in humans. Therefore, we evaluated MAPK15 expression in MASLD using 2 publicly available patients' cohorts (Pantano and Govaere cohorts).^[42,43] Transcriptomic data and clinical covariates were collected for a total of 344 patients encompassing various stages of MASLD progression and stages of fibrosis, revealing that MAPK15 expression was significantly higher in livers' patients at all stages of the disease, compared with healthy controls (Figure 8A, Pantano cohort, two-way ANOVA $p=0.00024$; Figure 8B, Govaere cohort, two-way ANOVA $p=0.03504$). These differences, further examined in the Pantano cohort due to a comparable number of patients across groups, remained significant

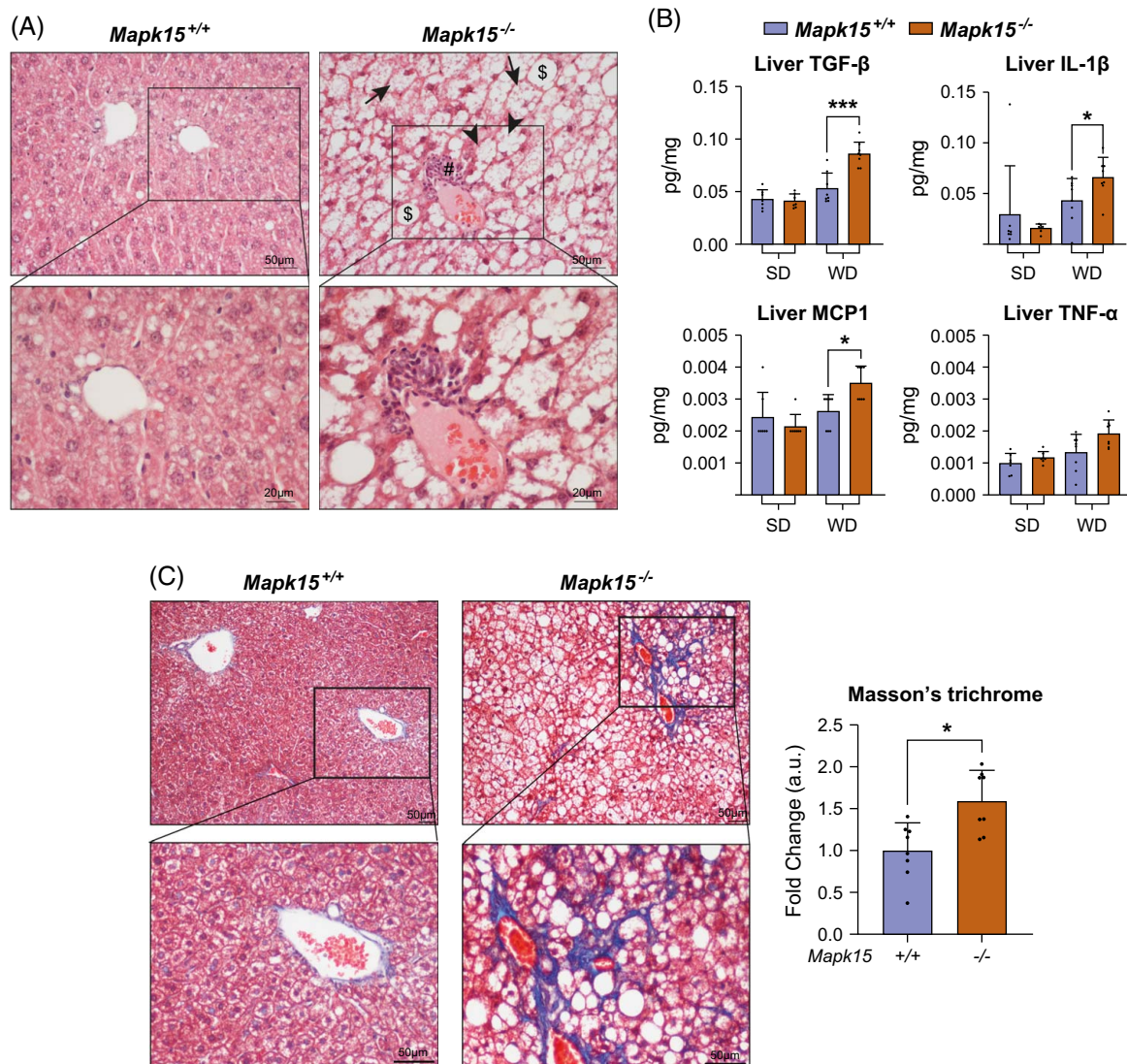


FIGURE 7 Loss of *Mapk15* in combination with WD leads to hepatic inflammatory damage. (A) Representative haematoxylin and eosin staining on histological samples from livers of *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. #: inflammatory infiltrates; arrows: ballooning hepatocytes; arrowheads: micro-vesicular steatosis; \$: macro-vesicular steatosis. (B) Local protein levels of indicated proinflammatory cytokines (TGF-β, IL-1β, MCP1, and TNF-α) from livers of *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. (C) Representative Masson trichrome staining of histological sections from *Mapk15*^{+/+} and *Mapk15*^{-/-} WD-fed mice. The accompanying graph scores the blue collagen fibers as fold changes comparing *Mapk15*^{+/+} (n=8) and *Mapk15*^{-/-} (n=8) liver. One-way ANOVA—Mann–Whitney nonparametric test: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. Abbreviations: SD, standard diet; WD, Western diet.

in pairwise comparisons between controls and each stage of fibrosis (Figure 8A). To extend our analysis and investigate the correlation of MAPK15 expression with transcriptional programs associated with disease progression, we next assembled a comprehensive gene set relevant to MASLD. This set was constructed by integrating genes described as significantly dysregulated in human and murine MASLD by Vacca et al.^[36] with additional key genes manually curated from the literature (the complete list of genes is provided in Supplemental Table S1, <http://links.lww.com/HC9/C230>) and validated its clinical relevance in our cohort using Gene Set Variation Analysis (GSVA).^[44] Indeed,

the resulting GSVA score effectively stratified patients according to both MASLD and Fibrosis stage, confirming the set's ability to capture disease-relevant biological signals (Supplemental Figure S13, <http://links.lww.com/HC9/C230>). Using this validated set, we then specifically investigated the correlational landscape surrounding MAPK15 by computing the Kendall tau correlation between MAPK15 and every other gene in the set for each cohort: the significance of these correlations, adjusted using the Benjamini–Hochberg method, is presented in volcano plots in Figure 8C and detailed in Supplemental Table S1, <http://links.lww.com/HC9/C230>.

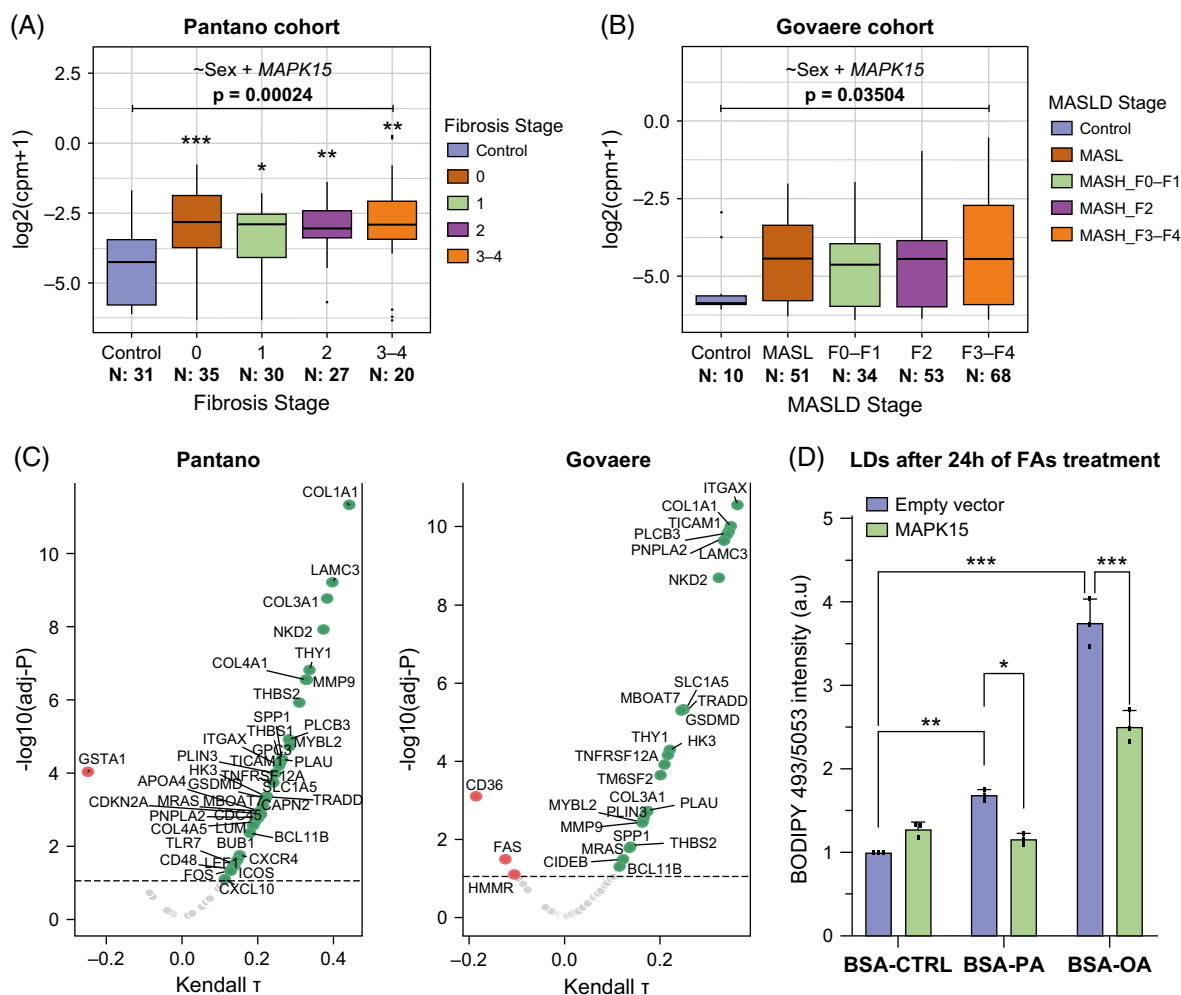


FIGURE 8 MAPK15 protects human hepatic cells from lipid accumulation and liver steatosis. (A) Comparison of MAPK15 expression levels in patients stratified by fibrosis stage, using classifications from Pantano et al.^[43] (ANOVA $p = 0.00024$). (B) Same as in (A) but using the classification of the MASLD stages scheme adopted in the work of Govaere et al.^[42] (ANOVA $p = 0.03504$). In each cohort, the ANOVA model compared the mean MAPK15 expression level between disease stages, adjusting for the effect of sex. The Tukey HSD pairwise comparisons between control and pathological groups reach significance only in the Pantano cohort ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$), while in the Govaere cohort, they did not reach statistical significance due to the small size of the control group, which emphasizes the measured variance. (C) Correlational landscape of MAPK15 expression within the steatosis gene set. Volcano plots show Kendall tau correlations between MAPK15 and all other genes in the set across patient cohorts, with significance adjusted using the Benjamini–Hochberg method. (D) Flow cytometry analysis to score LDs cellular fluorescence after 24 hours of treatment with 200 μ M of FAs (BSA-PA and BSA-OA; BSA as control), in MAPK15-overexpressing HepG2 cells and empty vector-transfected control cells. Data were analyzed using a two-way ANOVA with lipid treatment (BSA-CTRL, BSA-PA, BSA-OA) and MAPK15 overexpression (Empty, MAPK15) as factors. The Tukey post hoc test was used for multiple comparisons ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$). Abbreviations: FAs, fatty acids; LDs, lipid droplets; MASL, metabolic dysfunction–associated steatotic liver; MASLD, metabolic dysfunction–associated steatotic liver disease; OA, oleic acid; PA, palmitic acid.

As these data suggested that, similarly to mice, MAPK15 carries out protective functions against toxicity dependent on lipid accumulation in human livers, we next tested the possibility that its overexpression and, therefore, activation, may be able to prevent detrimental accumulation of LDs in our in vitro model of MASLD induction, based on fatty acid treatment of HepG2 cells.^[6] Indeed, we demonstrated that the hepatocellular accumulation of LDs induced by treatment with exogenous fatty acids (PA and OA) was prevented by MAPK15 overexpression (Figure 8D), ultimately suggesting potential for this kinase as a new actionable therapeutic target for the treatment of human steatosis and steatohepatitis, to limit toxicity due to excessive lipid accumulation in this organ, ultimately preventing or delaying liver fibrosis and irreversible liver cirrhosis.

DISCUSSION

MASLD is a pandemic metabolic disorder affecting nearly a fourth of the worldwide population, with very high human and socioeconomic costs.^[45] A better understanding of the pathogenesis of this disease is therefore extremely urgent to develop new therapies able to control its long-term deleterious effects. Indeed, while hepatic steatosis is often self-limited in the context of MASLD, under the pressure of environmental factors and/or genetic predisposition, up to 15% of affected individuals can progress and exhibit some degree of MASH, the inflammatory subtype of MASLD.^[29] Alarmingly, up to ~30% of MASH patients can, in turn, progress to cirrhosis, a serious premalignant condition often evolving in HCC or liver failure (up to 25% of cases), therefore requiring liver transplantation.^[29] Still, the heterogeneous, fluctuating, and slow natural history of the disease usually requires long-term studies to demonstrate the effects of an intervention on clinical outcomes, and such studies are currently limited. Therefore, preclinical mouse models for MASLD have become crucial tools to investigate mechanisms as well as novel treatment modalities during the progression from steatosis to MASH and subsequent HCC, in preparation for human clinical trials. Still, although there are numerous genetically-, toxins- and diet-induced models of MASH, not all of them faithfully phenocopy and mirror the human pathology very well, leading to huge efforts for defining their metabolic relevance to the human disease.^[36] Among such models, western-type diets, composed of saturated fats, carbohydrates, and 0.1%–2% cholesterol, akin to fast-food diets, are increasingly recognized as the best models for mimicking human MASH pathology and comorbidities.^[36] Still, while these diets can induce, in mice, obesity with dyslipidemia and elevated plasma liver enzymes and increased liver steatosis with extensive inflammatory infiltration and hepatocyte ballooning,^[46] they often require extended

durations to obtain significant fibrosis, especially at low cholesterol concentrations.^[36] Intriguingly, we have shown that, when associated with WD, deletion of the *Mapk15* gene strongly accelerated the insurgence of liver inflammation and fibrosis, closely mimicking the progression of the disease to MASH. This may enhance our ability to study the disease and test behavioral or pharmacological interventions aimed at slowing, halting, or even reversing the necro-inflammatory responses triggered by the lipotoxic insult caused by intracellular lipid accumulation.^[29] Furthermore, our mouse model also allows for direct testing of a role for MAPK15 in the progression of MASLD to HCC, the third leading cause of cancer-related deaths and the sixth most common cancer globally.^[47] This could prove to be particularly important because, besides lipotoxicity, HCC is associated with additional important etiologies traceable to other infective (HBV, HCV) or chemical (alcohol, drugs) causes of inflammatory hepatocyte stress, which could altogether take advantage of specific pharmacological approaches targeting MAPK15 for prevention or cure.

Fibrosis is the main histologic feature of MASH that predicts clinical outcomes of the disease.^[45] Therefore, accumulation of collagen fibers in WD-fed *Mapk15*^{−/−} mice strongly suggests an important role for this MAP kinase in protecting the liver from evolution into MASH and, possibly, liver cirrhosis. Since hepatocytes are a rich source of soluble signals driving stellate cell activation, the extremely high levels of accumulated lipids observed in *Mapk15*^{−/−} mice suggest that lipotoxicity deriving from this process could be a key factor driving cell injury and consequent release of paracrine signals (ROS, oxidized phospholipids, leptin, and different chemokines) with profibrotic activity.^[45] Still, current knowledge on MAPK15 cellular functions also points to different mechanisms working in conjunction with intracellular lipid accumulation to produce the profibrotic effects resulting from the deletion of this kinase. Among such mechanisms, we have in fact recently demonstrated a key role of MAPK15 in the control of intracellular oxidative stress by promoting mitochondrial fitness^[15] and by controlling the activity of the main antioxidant transcription factor, NF-E2-related factor 2 (NRF2).^[16] Consequently, its depletion may possibly further worsen oxidative stress in both hepatocytes and stellate cells, exacerbating fibrosis and potentially causing oxidative DNA damage. Moreover, MAPK15 depletion may also induce cellular senescence triggering a senescence-associated secretory phenotype (SASP),^[15] whose chemokine components often have profibrotic activities and may even alter the immune microenvironment ultimately supporting MASH development or even tumorigenesis.^[45,48] Ultimately, it was intriguing to observe more subtle differences in the tendency of female *Mapk15*^{−/−} mice to accumulate hepatic fat than their wild-type counterparts, although our results were perfectly in line with those obtained in

most laboratory mouse strains used to model human MASLD by high-fat diets.^[25] In turn, such results suggest that our *Mapk15* knockout model may represent a useful tool also to try to understand underlying hormonal causes for these differences between sexes,^[6] which we plan to tackle by next studying hepatic steatosis comparing pre- and post-menopausal and ovariectomized SD- and WD-fed female *Mapk15*^{-/-} mice.

Regarding the molecular mechanisms underlying MAPK15's ability to control lipid accumulation in hepatic cells, our combined genetic and pharmacological approaches indicate that increased expression and membrane localization of the CD36 fatty acid translocase, both in vitro and in vivo, are key events contributing to the accumulation of high amounts of exogenous lipids into hepatocellular LDs. Based on our current data, we next look forward to precisely deciphering MAPK15 effectors (eg, kinase substrates and/or direct protein interactors) able to control CD36 expression and membrane localization. Clarifying these molecular connections will be critical to exploit MAPK15-dependent pathways from the perspective of increasing our possibility to pharmacologically or genetically modulate MASLD progression in patients.

Overall, by taking advantage of the first *MAPK15* knockout mouse model, our current work describes a novel role for this gene in controlling lipid accumulation in mammalian cells, with important clinical and translational implications for human disease. Indeed, these mice readily manifest progressive MASLD, which closely mirrors the human disease, in terms of both histologic and biochemical characteristics, as well as sex-related differences in the severity of their manifestations. Importantly, we also identified a specific mechanism driving this phenotype, based on the CD36-dependent increase in intracellular lipid accumulation, disclosing a potentially novel and important role for a currently undervalued MAP kinase in protecting the liver from an "epidemic" disease for which very few pharmacological approaches are available. Accordingly, our analysis of *MAPK15* expression in livers from human cohorts showed an overall increase in the different stages of MASLD compared with the unaffected individuals, supporting a model in which liver cells induce the expression of this kinase to counteract the deleterious effects of lipotoxic stress due to different steatogenic stimuli. In conclusion, as preclinical studies have demonstrated that reverting hepatic steatosis is also able to resolve liver inflammation, liver fibrosis, and diabetes, our data provide a new actionable therapeutic target with potential for preventing or reverting MASH and, possibly, contribute to reducing its frequent debilitating and deadly consequences, such as cirrhosis and HCC.

DATA AVAILABILITY STATEMENT

The data associated with this paper and further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mario Chiariello (mario.chiariello@cnr.it).

AUTHOR CONTRIBUTIONS

Conceived and designed the project: Mario Chiariello. Supervised the project: Eugenio Bertelli, Romina D'Aurizio, Maria Grazia Andreassi, Giuseppina Basta, Amalia Gastaldelli, Andrea Morandi, Virginia Barone, and Mario Chiariello. Designed the animal experiments: Sara Gargiulo. Performed most of the experiments and analyzed data: Giovanni Inzalaco, Sara Gargiulo, Denise Bonente, Lisa Gherardini, Nicla Lorito, Serena Del Turco, and Danilo Tatoni. Helped in experiments and data analyses: Lorenzo Franci, Tiziana Tamborrino, and Federico Galvagni. Wrote the manuscript: Mario Chiariello. Edited the manuscript: Giovanni Inzalaco, Sara Gargiulo, Lisa Gherardini, Lorenzo Franci, Nicla Lorito, Romina D'Aurizio, Maria Grazia Andreassi, Federico Galvagni, Giuseppina Basta, Andrea Morandi, Virginia Barone, and Mario Chiariello. Discussed and interpreted the results and approved the manuscript: all authors.

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CONFLICTS OF INTEREST

Amalia Gastaldelli consults, advises, is on the speakers' bureau, and has received grants from Boehringer. She

consults, advises, and is on the speakers' bureau for Merck Sharp & Dohme. She advises and is on the speakers' bureau for Novo Nordisk. She consults and advises Regeneron. She consults and received grants from Eli Lilly. She is on the speakers' bureau and received grants from Madrigal. She received grants from Echosens.

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