Copper restoration by liver Ceruloplasmin ablation ameliorates NAFLD via SCO1-AMPK-LKB1 complex

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Article

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Abstract

Copper is an essential nutrient and a co-factor of numerous enzymes governing a wide range of intracellular processes. Copper deficiency has emerged to be associated with various lipid metabolism diseases, including non-alcoholic fatty liver disease (NAFLD). However, the molecular mechanisms of how copper regulates lipid metabolism and is sensed remain elusive. Here, we reveal that copper elevation caused by hepatic ceruloplasmin (CP) ablation enhances lipid catabolism by promoting the assembly of copper-load SCO1/AMPK complex. We report that overnutrition-mediated CP elevation results in hepatic copper loss, and that liver-specific CP ablation counteracts this reduction in copper levels and ameliorates NAFLD in mice. Mechanistically, SCO1 constitutively interacts with LKB1 even in the absence of copper, and copper-loaded SCO1 directly tethers LKB1 to AMPK, thereby activating AMPK and consequently promoting mitochondrial biogenesis and fatty acid oxidation in hepatocytes. Therefore, this study reveals an unexpected role for AMPK to sense copper alteration via SCO1 and uncovers a previously unidentified mechanism by which copper, as a signaling molecule, improves hepatic lipid catabolism, and indicates that targeting copper-AMPK signaling pathway ameliorates NAFLD development by modulating AMPK activity.

Introduction

Both macronutrients, including amino acids, glucose and lipids, and micronutrients, including trace metals, are all essential for human health¹. Copper is an essential dietary trace element that serves as a structural and enzymatic cofactor for proteins involved in critical cellular processes, including redox balance, mitochondrial function and iron metabolism^{2,3}. Although the mechanisms underlying glucose, amino acid, and lipid sensing by AMP-activated protein kinase (AMPK)⁴, mammalian target of rapamycin complex (mTOR)⁵, and CD36^{6,7}, respectively, are well documented, how copper levels are sensed in the cell and whether copper is involved in the metabolic regulation of macronutrients remains elusive.

Liver is the central organ for copper homeostasis, and hepatic copper levels need to be maintained within a narrow range^{8,9}. CP, a principal copper-binding protein secreted from the liver, plays a critical role in maintaining hepatic copper homeostasis by distributing copper from the liver to extrahepatic tissues¹⁰.

Circulating CP is elevated in patients with diabetes, obesity and NAFLD^{11–14}; in parallel, hepatic copper deficiency is strongly associated with metabolic diseases, including diabetes mellitus, metabolic syndrome and NAFLD^{15–17}. NAFLD is characterized by abnormal lipid metabolism, which results in lipid accumulation in hepatocytes, and can lead to non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma¹⁸. Despite that the worldwide prevalence of NAFLD is estimated at 25%¹⁸, no approved clinical therapies yet exist for NAFLD, underscoring the urgent need to identify novel pathogenic factors of NAFLD and therapeutic targets. These observations suggest the critical roles of copper and its transporter —CP in metabolic diseases. Despite these intriguing correlational findings in humans, little is known about how low hepatic copper causes metabolic derangements.

Here, we reveal that overnutrition-caused CP up-regulation leads to a reduction in hepatic copper content along with NAFLD; while restoration of hepatic copper levels by CP deletion, ameliorates HFD-induced NAFLD development. We identify that the SCO1-LKB1-AMPK complex senses the elevation of copper concentration, and hepatic copper is an intriguing modulator of lipid catabolism via AMPK activation through a novel signaling pathway containing copper binding protein, SCO1.

Results

Copper concentrations are lower and CP expression is higher in fatty liver and diabetes models than in controls

To investigate the roles of copper and CP in lipid metabolism, we then measured the expression of CP and copper content in human liver biopsies and mouse models of metabolic diseases. Notably, copper levels in human liver biopsies were significantly lower in NAFLD patients than healthy controls ($p < 0.05$) (Fig. 1a). The hepatic copper content in *ob/ob* and HFD-induced obese (DIO) mice was also lower than that in control mice (Fig. 1b, Extended Data Fig. 1a). These results suggest that a reduction in hepatic copper content might be associated with NAFLD in humans and mice.

Next, we analyzed the expressions of genes involved in copper metabolism and transportation in human liver biopsies, including *CP*, *CTR1* and *SOD1*, and found that the expression of *CP*, *SOD1*, *SOD2*, and *COX17* was higher in liver biopsies of NAFLD patients than controls (Fig. 1c). To verify which gene regulates hepatic copper abundance, we knocked down these four genes respectively in primary hepatocytes. Only knocking-down of *Cp* substantially increased the intracellular copper concentration (Fig. 1d), which is consistent with the essential role of CP in hepatic copper homeostasis². In addition, we found that expression levels of hepatic CP were significantly increased in all three NAFLD mouse models (Fig. 1e,f and Extended Data Fig. 1b-f).

In view of the close link between NAFLD and type 2 diabetes, CP expression was measured in diabetes models, including STZ-injected mice (Extended Data Fig. 1g-i) and mice with genetic inactivation of hepatic insulin receptor substrate *Irs1* and *Irs2* double knockout (LDKO) (Extended Data Fig. 1j-l). Similar to the obese NAFLD mouse models, CP was highly expressed in diabetic mice. These results suggest that excess hepatic CP might lead to reductions in hepatic copper level, and subsequently results in the development of NAFLD.

Acute CP-knockdown attenuates HFD-induced hepatic steatosis

To determine the function of CP on hepatic glucose and lipid metabolism and NAFLD development, we knocked down hepatic CP in DIO mice (Extended Data Fig. 2a). Relative to controls, knockdown of hepatic CP significantly reduced liver weight (Fig. 1g,h), ectopic lipid accumulation in the liver (Fig. 1i,j), and plasma free fatty acids (FFAs) (Extended Data Fig. 2b). Lipidomic analysis showed that the levels of

diacylglycerides (DAGs), FFAs and triglycerides (TGs) were substantially reduced in Ad-shCP-treated mouse livers (Fig. 1k). Moreover, glucose clearance and insulin sensitivity were substantially improved in CP knockdown mice (Extended Data Fig. 2c-f), but no differences in body weight or food intake were observed (Extended Data Fig. 2g,h).

Then, we used AAV (which is less immunogenic and widely used in clinical trials) to express Cre in mouse liver by transduction of CP^{flox/flox} mice with Cre^{AAV-TBG} viruses (CP^{flox/flox}-Cre^{AAV-TBG}). Consistent with Ad-shCP injected mice, CP^{flox/flox}-Cre^{AAV-TBG} mice displayed lower ectopic hepatic lipid accumulation (Extended Data Fig. 2i) and substantially less insulin resistance (Extended Data Fig. 2j). However, there were no differences in glucose tolerance between CP^{flox/flox}-Cre^{AAV-TBG} and CP^{flox/flox}-GFP^{AAV-TBG} mice (Extended Data Fig. 2k).

LKO mice are protected from HFD-induced hepatic steatosis

To confirm the results observed in mice with virus-mediated CP-knockdown, we generated liver-specific CP knockout (LKO) mice (Extended Data Fig. 2l). LKO mice exhibited less lipid accumulation in the liver, less liver weight and hepatic triglyceride (Fig. 1l-n). Glucose tolerance and insulin sensitivity were improved as well in LKO mice (Extended Data Fig. 2m,n). There were no significant differences in body weight and food intake between the two groups of mice (Extended Data Fig. 2o,p).

We also investigated the effect of CP knockout (CP-KO) on lipid accumulation in primary hepatocytes, and the result showed that CP-KO resulted in less palmitic acid (PA)-induced lipid droplet and TG accumulation in primary hepatocytes than controls (Fig. 1o-q).

Taken together, these data indicate that deletion of CP ameliorates HFD-induced hepatic steatosis, and improves insulin sensitivity and glucose homeostasis.

CP-knockdown increases mitochondrial biogenesis and FAO

Given that mitochondria play a key role in lipid metabolism, we examined whether CP ablation affected mitochondrial function. Electron microscopy showed that CP deletion in mouse liver ameliorated HFD-induced mitochondria swelling and deteriorations of mitochondrial morphology (Fig. 2a). In line with this, CP ablation substantially increased mitochondrial number (Fig. 2b) and the copy number of mitochondrial DNA in primary hepatocytes (Fig. 2c). Moreover, expressions of genes encoding mitochondrial respiratory chain complex was upregulated, including *Nd1*, *Nd2*, *Co1* and *Atp6* (Fig. 2d). Consistent with this, immunoblotting confirmed that CP deletion efficiently upregulated protein expression of the subunits of mitochondria respiratory chain complexes, such as ATP5A and UQCRC2 (Fig. 2e). These results suggest that CP deletion rescues mitochondrial morphology under HFD feeding and increases the number of healthy mitochondria.

Next, we explored the effects of CP ablation on mitochondrial function. Notably, CP-KO cells exhibited a significantly higher capacity for basal respiration, maximal respiration, spare respiration, and ATP production (Fig. 2f,g), indicating that CP-KO resulted in higher mitochondrial activity. In addition, mitochondrial FAO rate was also increased (Fig. 2h). Consistent with this, CP-KO hepatocytes showed significantly higher expressions of genes involved in mitochondrial FAO, including *Cpt1a*, *Cpt2* and *Hmgcs2* ($p < 0.01$) (Fig. 2i), but without significant changes in the levels of lipogenic genes, including *Srebp1c*, *Acc1*, *Fasn*, *Chrebpa*, *Chrebpβ*, and *Acly* (Extended Data Fig. 2q). Furthermore, we found that overexpressing CP in primary hepatocytes substantially suppressed the expressions of genes related to FAO (Fig. 2j,k). These results indicate that CP depletion substantially enhances mitochondrial FAO activity.

AMPK is required for amelioration of ectopic lipid accumulation caused by hepatic CP-KO

To elucidate the molecular mechanisms by which CP deletion reduces hepatic steatosis, we performed RNA-seq analysis of Ad-shCP or Ad-shCON treated mouse livers. Ingenuity Pathway Analysis showed that the AMPK pathway was one of the most enriched biological process terms (Fig. 3a). Incubating CP-KO primary hepatocytes with inhibitors against various pathways, reveals that only inhibitors of AMPK and SIRT1 (a direct downstream target of AMPK) blocked CP deletion-mediated upregulation of *Hmgcs2* and *Creb3l3* (Fig. 3b, Extended Data Fig. 3a,b). Moreover, AMPK was activated in CP-deleted hepatocytes (Fig. 3c) and LKO mice liver (Fig. 3d). In contrast, inhibition AMPK with Compound C (CC) impaired CP-deletion-induced up-regulation of mitochondrial respiration (Fig. 3e, Extended Data Fig. 3c). Consistent with this data, genetic ablation of AMPK attenuated CP-knockdown-mediated reduction of lipid accumulation in hepatocytes (Fig. 3f, Extended Data Fig. 3d,e), also suppressed CP-knockdown-caused increases in mitochondria mass (Fig. 3g) and the expressions of FAO genes (Fig. 3h) and other mitochondrial genes (Fig. 3i).

Collectively, these results indicate that AMPK is a critical downstream target of CP that regulates mitochondrial function and lipid metabolism in the liver.

CP deletion-induced upregulation of FAO is dependent upon the AMPK-PGC1α-PPARα axis

Previous reports show that AMPK increases mitochondrial FAO by enhancing transcriptional activity of PGC1α and PPARα¹⁹. Notably, we found that CP ablation decreased the abundance of acetylated PGC1α, an inactivated form of PGC1α (Fig. 3j); by contrast, overexpressing CP elevated acetylated PGC1α levels (Fig. 3k). Activated PGC1α promotes its interaction with PPARα, thereby increasing its co-activator ability²⁰. Here, we found that CP deletion efficiently enhanced the interaction between PGC1α and PPARα (Fig. 3l). By contrast, overexpression of CP substantially decreased the interaction of PGC1α and PPARα (Fig. 3m). We further examined PGC1α-PPARα-mediated transcription of FAO genes, and revealed that knocking down CP increased the activity of *PPARE-Luc*, *CPT1α-Luc* and *HMGCS2-Luc* reporters (Extended Data Fig. 4a), indicating that CP deletion enhances PPARα-transactivation. By contrast, overexpressing

CP suppressed the GW9578 (PPAR α agonist)-induced increase in promoter activities of these luciferase reporters (Extended Data Fig. 4b). In line with this, chromatin immunoprecipitation assays confirmed that CP deletion enhanced recruitment of PGC1 α (Fig. 3n) and PPAR α (Fig. 3o) to the promoters of PPAR α target genes; whereas knocking down PGC1 α (Extended Data Fig. 4c) or PPAR α (Extended Data Fig. 4d) completely blocked the elevated transcription of FAO genes caused by CP deletion. In line with this, inactivation of PPAR α via GW6471, an antagonist of PPAR α , abolished the effects of CP-knockdown on the reduction of lipid accumulation in hepatocytes (Extended Data Fig. 4e,f).

Restoration of hepatic copper content reduces excess lipid accumulation in liver through AMPK activation

Next, we explored how CP deletion activated AMPK. Unexpectedly, we didn't detect significant alteration in AMP/ATP or ADP/ATP in the livers of LKO mice (Extended Data Fig. 4g,h), suggesting that CP deletion activates AMPK via an AMP/ADP-independent mechanism. Notably, CP deletion efficiently prevents the decrease in hepatic copper levels caused by HFD feeding (Fig. 4a,b and Extended Data Fig. 5a). Interestingly, treating primary hepatocytes with a copper solution increased AMPK phosphorylation in a dose-dependent manner (Fig. 4c). Furthermore, depleting intracellular copper with copper cation-specific chelator, bathocuproinedisulfonic acid disodium salt (BCS), attenuated the effect of CP deletion on upregulation of *Hmgcs2* (Fig. 4d, Extended Data Fig. 5b) and *Cpt1a* (Fig. 4e, Extended Data Fig. 5b). By contrast, an iron cation-specific chelator, deferasirox (DFX), had little effect on *Hmgcs2* (Fig. 4d) or *Cpt1a* (Fig. 4e) mRNA levels. In addition, we performed similar experiments with additional copper chelator, tetrathiomolybdate (TM) and iron chelators, pyridoxal isonicotinoyl hydrazine (PIH) and deferoxamine (DFO) in primary hepatocytes, and obtained consistent conclusion that chelating copper, but not iron, blocked CP-deletion-upregulated FAO gene expressions (Fig. 4f, Extended Data Fig. 5c-e). Moreover, BCS and TM attenuated increases in phosphorylated AMPK by CP deletion in primary hepatocytes (Fig. 4g). Chelating intracellular copper with BCS also prevented CP ablation-induced reductions in lipid accumulation; whereas the iron chelator DFX failed to do so (Fig. 4h,i and Extended Data Fig. 5f,g).

The global CP KO (WKO) mouse is an animal model of aceruloplasminemia that exhibits excess iron accumulation in the liver²¹. We also observed an increased level of hepatic iron in the WKO mouse (Extended Data Fig. 5h). By contrast, Prussian blue staining of the LKO mouse liver did not reveal obvious iron accumulation (Extended Data Fig. 5h). Although hepatic iron levels of LKO mice (measured by ICP-MS) were modestly increased compared to controls (Extended Data Fig. 5i), they are comparable to hepatic iron levels in normal mice as previously reported²²⁻²⁴.

Taken together, these results indicate that the effect of CP deletion on the activation of AMPK and suppression of fatty liver is dependent on upregulation of hepatic copper content.

Copper upregulates hepatic FAO via SC01

Given that copper activates AMPK and that AMPK is not reported as a copper-binding protein²⁵, we hypothesized that a copper-binding protein mediates AMPK activation. To test this hypothesis, we used a

collection of siRNAs targeting key copper-binding proteins in primary hepatocytes, to identify candidate protein(s) mediating the effect of CP deletion on upregulation in FAO genes, including *Hmgcs2* and *Creb3l3* (Fig. 4j, Extended Data Fig. 6a-c).

We found that only knockdown of SCO1 consistently blunted the upregulation of FAO gene expression caused by CP deletion (Fig. 4j, Extended Data 6c). Moreover, we found that knocking down SCO1 entirely abrogated the effect of CP ablation on increasing phosphorylated AMPK levels in hepatocytes (Fig. 4k). Finally, SCO1 deficiency blocked CP knockout-dependent suppression of lipid accumulation in hepatocytes (Fig. 4l,m).

Taken together, these results suggest that SCO1 plays a critical role in copper-dependent AMPK activation and lipid catabolism.

SCO1 promotes AMPK activation by directly interacting with AMPK and LKB1

Next, we explored the mechanism by which SCO1 regulates AMPK activity. SCO1 was reported as a mitochondrial protein²⁶. However, we detected that some SCO1 signals were well overlapped with that of β -actin (Fig. 5a), suggesting that a number of SCO1 proteins localized in cytoplasm. Moreover, co-immunoprecipitated assay showed that exogenous SCO1 interacted with exogenous AMPK α 1 and endogenous LKB1 (Fig. 5b). Reverse co-immunoprecipitation further confirmed the interaction among AMPK, SCO1 and LKB1 (Fig. 5c). Besides, the endogenous interaction between SCO1 and AMPK was also detected in L02 cells (Fig. 5d). Furthermore, pull-down assays showed a direct interaction between SCO1 (Fig. 5e,f) and AMPK as well as between SCO1 and LKB1 (Fig. 5g,h). In addition, confocal images revealed the colocalization of AMPK and SCO1 in hepatocytes (Fig. 5i). Next, we performed similar co-immunoprecipitated assay in subcellular fractionation. The cytoplasm and mitochondria were respectively purified from primary hepatocytes (Extended Data Fig. 6d), and we only detected the interaction between SCO1 and AMPK in cytosol but not in the lysate of mitochondria (Fig. 5j,k). The co-immunoprecipitated assay in AMPK-DKO primary hepatocytes showed that SCO1 interacted with LKB1 even in the absence of AMPK, suggesting that SCO1 interacts with LKB1 independent of AMPK (Fig. 5l). Taken together, these results suggest that SCO1 acts as a scaffold protein to tether the activating kinase LKB1 to AMPK, resulting in AMPK phosphorylation. Next, we determined whether copper or CP regulates the assembly of the SCO1-LKB1-AMPK complex. A co-immunoprecipitation assay showed that overexpression of CP reduced the interaction between SCO1 and AMPK, but had little effect on SCO1-LKB1 interaction (Fig. 5m). Interestingly, we failed to detect CP protein in SCO1-LKB1-AMPK precipitation (Fig. 5m), which further confirmed that CP modulation of AMPK activity is not through its direct physical interaction with AMPK. Given that overexpression of CP might lead to a reduction in intracellular copper via copper excretion, we hypothesized that copper affects the assembly of the SCO1-LKB1-AMPK complex. To confirm this hypothesis, we depleted intracellular copper with BCS, and the co-immunoprecipitation result showed that BCS treatment significantly reduced the interaction between SCO1 and AMPK (Fig. 5n), indicating the important role of copper in the SCO1-AMPK interaction. In

another experiment, we constructed an SCO1 copper-binding deficient mutant (C152/156A) by substituting CyS152 and CyS156 with alanine, as reported^{27,28}. An obvious shift of the SCO1 (C152/156A) band (Fig. 5o, line 7), similar with BCS treatment (Fig. 5o, line 6), revealed that its copper-binding capacity was abolished. Notably, a co-immunoprecipitation assay showed that the C152/156A mutation in SCO1 impaired its interaction with AMPK, but had no effects on its interaction with LKB1 (Fig. 5p). Moreover, a pulldown assay demonstrated that the a truncated SCO1 (only containing SCO domain where the copper-binding motif resides, His-Truncated) displayed a strong interaction with AMPK, compared to full length SCO1 (Fig. 5q). We next performed a similar pull-down assay with copper-loaded His-Truncated (Cu-Truncated) or copper-deficient His-Truncated protein (apo-Truncated). An obvious up-shift of apo-Truncated band in non-reducing gel electrophoresis confirmed its copper-deficient status, and the pull-down result demonstrated that Cu-Truncated had a stronger binding affinity to AMPK than apo-Truncated (Fig. 5r).

Taken together, these results reveal that intracellular copper increase activates AMPK through promoting the assembly of the SCO1-LKB1-AMPK complex.

Adequate hepatic copper levels are necessary for CP deletion-induced amelioration of hepatic steatosis

Given that SCO1 deficiency blocked CP knockout-dependent suppression of lipid accumulation in hepatocytes (Fig. 4l,m), and depleting intracellular copper by BCS produced copper-deficient SCO1 (Fig. 5o, line 6), we thus deprived the copper of SCO1 by treating mice with BCS to explore its effect on lipid metabolism *in vivo* (Fig. 6a, Extended Data 7a). ICP-MS analyses showed that BCS treatment substantially blunted CP LKO-caused elevation of hepatic copper content (Fig. 6b). Consistently, BCS administration reduced AMPK phosphorylation levels upregulated by CP LKO (Fig. 6c, Extended Data Fig. 7b-d), presumably due to depletion copper from SCO1. In line with this, BCS treatment also abolished the effects of CP LKO on the amelioration of hepatic lipid accumulations (Fig. 6d-f). Moreover, BCS-treatment impaired the effects of CP-LKO on the upregulation of FAO (Fig. 6g) and other mitochondrial genes (Fig. 6h), as well as maintaining healthy mitochondria morphology in the liver (Fig. 6i). There were no significant differences in body weight and food intake between the two groups of mice (Extended Data Fig. 7e,f).

Taken together, these findings suggest that copper is required to increase AMPK activation and hepatic lipid catabolism in mouse liver with CP deletion.

Discussion

Copper is a unique transition metal and is required in a variety of essential biological processes. Conventionally, copper is regarded as a static cofactor within enzyme active sites; however, emerging evidence suggests non-canonical roles for copper. For example, copper serves as a modulator in autophagy signaling pathway^{29,30} and as an essential cofactor for the copper-dependent transcriptional

regulation³¹. The total copper content in the adult body is 50–120 mg, and maintaining the amount of copper in the liver and other tissues is key for the homeostasis of biological processes¹. Therefore, it is reasonable to postulate that at least one system exists to sense fluctuations in the amount of copper in the liver; however, this system and its downstream biological impact have not been defined.

Here, we demonstrate that AMPK is activated by copper in a dose-dependent manner and hepatic CP deletion restores hepatic copper levels and consequently activates AMPK via SCO1. Knockdown of SCO1 entirely blocks the activation of AMPK by CP deletion. Interestingly, we detected SCO1, but not CP, in AMPK-LKB1 complex, suggesting that CP regulates AMPK activation is directly mediated through SCO1. SCO1 is known as a copper chaperone for maturation of mitochondrial complex IV, the cytochrome c oxidase complex²⁶. However, knockdown of other copper-binding proteins that are essential to transferring copper to this complex has no effect on the AMPK activation by hepatic CP knockdown, demonstrating a unique effect of SCO1 on AMPK. Impairment of the copper-binding function of SCO1, either by mutation of the copper-binding motif of SCO1 or BCS-mediated intracellular copper depletion, blocks the interaction between SCO1 and AMPK, but has little effect on SCO1 binding to LKB1. Previous reports show that oxidation of SCO1 on cysteine residues induced by copper loading results in a disulfide band formation that changes the structure and conformation of SCO1³². Therefore, copper-mediated conformational changes in SCO1 likely regulate the interaction between AMPK and SCO1; whereas LKB1 might bind to a region of SCO1 that is distal from the copper-loading domain of SCO1. Thus, these findings show that the SCO1-LKB1-AMPK complex is a novel copper sensor.

In the canonical mechanism of AMPK activation, AMP binds three of the four cystathionine β -synthetase (CBS) sites of the γ subunit of AMPK trimeric complex and stimulates its kinase activity⁴. Recent studies revealed that AMPK also responds to glucose starvation via a non-canonical, AXIN-dependent mechanism, in which AXIN acts as an adaptor for LKB1 to interact with and phosphorylate AMPK^{33–35}. Here, we report that SCO1 is a novel “LKB1 adaptor”, which also promotes the interaction between LKB1 and AMPK, acts in response to alteration in hepatic copper levels.

The role of copper and copper-containing proteins in regulating lipid metabolism by activating AMPK is a novel finding. This mechanism may exist because adequate hepatic copper is indispensable in assembling functioning mitochondrial enzymes, such as COX, which support normal mitochondrial function. When the hepatic copper concentration is adequate, the SCO1-LKB1-AMPK complex may be activated, consequently promoting mitochondrial biogenesis and lipid catabolism. In parallel, copper also acts as a signaling modulator and triggers mitochondria biogenesis and lipid utilization via the SCO1-LKB1-AMPK complex. Moreover, our study reveals that 4 hours-fasting does not change the hepatic copper level, probably because the hepatic copper concentration is strictly controlled within a narrow range, although the precise mechanisms maintaining physiological hepatic copper content remain unclear. Thus, our results imply a role for low hepatic copper content led by high hepatic CP levels, to be concurrent with the high energy status in NAFLD, and that activation of AMPK by copper activates catabolic processes, including FAO and mitochondrial biogenesis and oxidative phosphorylation.

We found that a HFD substantially increases hepatic CP expression in mice, which exported copper out of liver and caused a reduction in hepatic copper content in these mice. Consistent with this, it is well documented that overnutrition results in low hepatic copper levels^{15,23,24,36,37}. The dysregulation of copper metabolism by overnutrition could lead to a downregulation of AMPK-mediated mitochondrial biogenesis and FAO. Therefore, our study supports a notion that overnutrition-caused hepatic copper deficiency mediates the development of NAFLD, which thereby adds a new layer to the “multi-hit” hypothesis of NAFLD development³⁸, such that disrupted hepatic copper homeostasis may present a previously unrecognized “hit” in the pathogenesis of NAFLD.

As a ferroxidase, intracellular iron export is impaired in mice and humans with aceruloplasminemia (loss of function of CP)²¹. Although CP is mainly produced by the liver, extrahepatic CP expression has been demonstrated in a wide arrange of tissues including spleen, lung, testis, and brain^{39,40}, as well as in many cell types, such as macrophages, glial cells, neurons and Sertoli cells^{41–43}. Consistent with this, we found that global CP knockout (WKO) leads to excess iron accumulation in the liver; whereas hepatocyte-specific CP deletion (LKO) only results in a moderate increase in hepatic iron content, which is within the normal physiological range^{22–24}. Taken together, our data suggest that LKO do not phenocopy mouse models of aceruloplasminemia and Wilson’s disease^{21,44}

In conclusion, the SCO1-LKB1-AMPK complex serves as a sensor for alterations in hepatic copper levels. Once copper binds to SCO1 and causes allosteric transformation, copper-SCO1-LKB1 anchors to AMPK and thus activates AMPK, stimulating mitochondrial biogenesis and lipid catabolism. Importantly, our findings reveal that restoration of hepatic copper content and targeting hepatic copper-binding proteins may present potentially novel therapeutic approaches for the treatment of NAFLD.

Limitations of study

We reveal that high fat feeding increases hepatic CP expression; our study however does not reveal the underlying mechanism. In addition, we report a novel function of AMPK in sensing hepatic copper content, however, whether AMPK regulates copper homeostasis deserves further investigation. Moreover, our results demonstrate that restoration of copper triggers assemble of SCO1-LKB1-AMPK complex that consequently results in an increase in mitochondrial biogenesis and FAO; however, the impact of SCO1-LKB1-AMPK complex on other AMPK regulated processes warrants future investigation.

Declarations

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AUTHOR CONTRIBUTIONS

J.L.L., J.M., L.P.X., Y.M.Y., S.M.X. and S.J.L. conceived and designed the study; L.P.X., Y.M.Y., S.M.X. and S.J.L. performed all the experiments with help from Y.P.W., Y.B.W., X.J.Z., S.Z.C., J.X.L., H.L.S., R.X.H., H.L.P., C.C.W., J.W., N.L. and W.P.J.; J.L.L provided funding; and J.L.L wrote the manuscript with assistance from J.M., L.P.X., Y.M.Y., S.J.L. and Morris F. White.

DECLARATION OF INTERESTS

The authors declare no conflict of interest associated with this study.

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Figures

Figure 1

CP-knockdown attenuates HFD-induced hepatic steatosis. (a) ICP-MS detected hepatic copper concentrations of individuals with NAFLD (n=27) and healthy controls (n=14). (b) Hepatic copper concentrations in DIO and control mice (n=6 in each group). (c) qPCR analysis of copper-binding proteins in livers of individuals with NAFLD (n=16) and healthy controls (n=14). (d) Copper concentrations in primary hepatocytes treated with siRNAs. (e-f) CP mRNA (e) and protein (f) levels in livers of DIO and

control mice. (g-k) DIO mice (fed a HFD for 13 weeks) were injected with Ad-shCP or Ad-shCON (n=10 in each group). (g) Representative images of liver morphology. (h) Liver weight (g). (i) Representative images of H&E-stained liver sections, (scale bar: 50 μ m). (j) Hepatic triglyceride concentrations. (k) Heat map of hepatic TAG, DAG and FFA content by LC-MS analysis; each column represents data from a single mouse and each row represents a lipid species. (l-n) Liver specific CP knockout mice (LKO) and control mice (CPf/f, CON) fed a HFD for 20 weeks. (l) Representative images of H&E-stained liver sections, (scale bar: 50 μ m). (m) Liver weight normalized to body weight. (n) Hepatic triglyceride concentrations. (o-q) Primary hepatocytes isolated from LKO and CON mice, were treated with PA. (o) Representative images of Oil Red O staining. (p) Lipid content. (q) TG concentrations. Data are presented as mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

Figure 2

CP-knockdown increases mitochondrial biogenesis and FAO. (a) Transmission electron microscopy of liver mitochondria in LKO and CON mice fed a HFD for 16 weeks, [scale bar: 1 μ m (line 1), 0.5 μ m (line 2), 0.2 μ m (line 3)]. (b-c, f-g) Primary hepatocytes from CON and LKO mice termed as CON and CP-KO. (d, h-k) Primary hepatocytes from C57BL/6J were treated with adenovirus as indicated. (b) Representative images of Mitotracker staining of CON and CP-KO primary hepatocytes, (scale bar: 10 μ m). (c) The ratio

of mitochondrial DNA amount to nuclear DNA total amount in primary hepatocytes. (d) mRNA expression of mitochondrial DNA in primary hepatocytes treated with Ad-shCON and Ad-shCP. (e) Immunoblot of subunit proteins of mitochondria respiratory chain complexes in CON and LKO mice liver. (f) Oxygen consumption rate (OCR) in CON and LKO primary hepatocytes. (g) Average OCR analyzed from (f). (h) Fatty acid oxidation rate of primary hepatocytes treated with Ad-shCON and Ad-shCP. (i) mRNA expression levels of FAO genes in Ad-shCON and Ad-shCP infected primary hepatocytes. (j-k) CP protein abundance (j) and mRNA levels of FAO genes (k) in primary hepatocytes treated with Ad-CP and Ad-GFP. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

Figure 3

CP regulates fatty acid oxidation genes via the AMPK-PGC1 α axis. (a) Ingenuity Pathway Analysis of RNA-seq data obtained from livers of Ad-shCP or Ad-shCON-injected DIO mice. (b) Hmgcs2 mRNA levels in Ad-shCP or Ad-shCON-infected and inhibitors-treated primary hepatocytes, JNK inhibitor (SP), PKA inhibitor (H89), SIRT1 inhibitor (Ex527) and AMPK inhibitor (Compound C, CC). (c) Immunoblots (protein indicated) in CP-KO primary hepatocytes. (d) Immunoblots (protein indicated) in livers of LKO mice. (e)

OCR of CON and CP-KO primary hepatocytes treated with CC. (f-l) Primary hepatocytes were isolated from AMPK α 1/ α 2 DKO (AMPK-DKO) or AMPK α 1 flox/flox and α 2 flox/flox (AMPKf/f) mice, followed by the infection with Ad-shCP or Ad-shCON. (f) Representative images of Oil Red O staining of primary hepatocytes, (scale bar: 50 μ m). (g) Representative images of Mitotracker staining of primary hepatocytes, (scale bar: 20 μ m). (h) mRNA levels of FAO genes in primary hepatocytes. (i) mRNA expression levels of mitochondrial DNA. (j-k) Acetylation of PGC1 α in L02 cells treated as indicated. (l-m) Immunoblotting for PGC1 α and Flag-tagged PPAR α proteins after immunoprecipitation of Flag-PPAR α from AML12 cells treated as indicated. (n-o) Chromatin immunoprecipitation (ChIP) assay-qPCR analysis of the promoter regions of FAO genes in with Ad-shCP-treated primary hepatocytes, with an anti-PGC1 α (n) or an anti-PPAR α (o) antibody. IgG, immunoglobulin. Data are presented as mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the control group.

Figure 4

CP regulates AMPK signaling by changing intracellular copper concentrations and the Cu-binding protein SCO1. (a) Hepatic copper concentrations of WT mice fed chow diet (NC) or a HFD and of the mice injected with indicated adenovirus. (b) Copper concentrations in livers of LKO and CON mice. (c) p-AMPKα and AMPKα in primary hepatocytes treated with CuSO₄ for 4 h. (D-E) mRNA levels of Hmgcs2 (d) and Cpt1α (e) in CP-knockdown (Ad-shCP) and control (Ad-shCON) primary hepatocytes treated with a

copper chelator (BCS) or an iron chelator (DFX). (f) mRNA levels of Hmgcs2 and Creb3l3 in CP-knockdown (Ad-shCP) and control (Ad-shCON) primary hepatocytes treated with a copper chelator (TM). (g) Protein levels of p-AMPK α and CP in BCS or TM-treated hepatocytes isolated from CON and LKO mice. (h-i) BCS-treated primary hepatocytes isolated from CON and LKO mice after PA treatment for 24 h. (h) Representative images of Oil Red O staining, (scale bar: 50 μ m). (i) Lipid contents. (j) mRNA levels of Hmgcs2 in Ad-shCP-infected primary hepatocytes after knockdown of copper-binding proteins with siRNA as indicated. (k) Protein expression of p-AMPK α , SCO1 and CP in LKO primary hepatocytes (CP-KO) treated with siRNA targeting SCO1 (siSCO1#1, siSCO1#2). (l-m) Primary hepatocytes isolated from CON and LKO mice treated with siSCO1 and PA or vehicle. (l) Representative images of Oil Red O staining, (scale bar: 50 μ m). (m) Lipid contents. Data are presented as mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001 compared with the control group.

Figure 5

SCO1 promotes AMPK activation by directly interacting with AMPK and LKB1. (a) Immunofluorescence of SCO1 and β -Actin. Cells were reacted with indicated antibodies. Nuclei were counterstained with DAPI. (b) Immunoblotting for LKB1, AMPK α and Flag-tagged SCO1 proteins after immunoprecipitation of SCO1 from HEK293T cells. Triangle indicates LKB1 (up), SCO1 (down). (c) Immunoblotting for SCO1, LKB1 and AMPK α proteins after immunoprecipitation of AMPK α 1 from HEK293T cells. (d) AMPK α and SCO1

protein levels after immunoprecipitation of SCO1 from L02 cells. (e-h) Direct interaction between SCO1 and AMPK complex. Pull-down assays were performed with Ni-NTA beads against His-SCO1 (e,g) or an anti-Flag antibody against Flag-tagged AMPK α (f,h), followed by immunoblotting with the antibodies as indicated. (i) Immunofluorescence of SCO1, AMPK. Cells were reacted with indicated antibodies. Nuclei were counterstained with DAPI. (j-k) Immunoblotting for endogenous SCO1 and AMPK α after immunoprecipitation of SCO1 in different subcellular fraction. (l) Protein levels of LKB1, AMPK α and SCO1 after immunoprecipitation of SCO1 from primary hepatocytes isolated from AMPK f/f and AMPK-DKO mice. (m) Immunoblotting for LKB1, AMPK α and Flag-tagged SCO1 proteins after immunoprecipitation of SCO1 from HEK293T cells overexpressing CP and Flag-tagged SCO1. (n) Immunoblotting for endogenous SCO1, AMPK α and LKB1 after immunoprecipitation of SCO1 from L02 cells treated with BCS for 24 h. (o) Immunoblotting in non-reducing SDS-PAGE for the protein levels of copper-loaded SCO1 (Cu-SCO1) and copper-deficient SCO1 (apo-SCO1) in HEK293T cells transfected with wild-type (WT) or mutant (C152/156A) SCO1, followed by treatment with CuSO₄ (50 mM) or BCS (200 mM) for 24 h. (p) Immunoblotting for AMPK α , LKB1 and Flag-tagged SCO1 proteins after immunoprecipitation of SCO1 from HEK293T cells transfected with indicated vectors; arrow indicates SCO1. (q) Direct interaction between AMPK α and full length SCO1 (His-SCO1) or truncated SCO1 (His-Truncated). Pull-down assays were performed with Ni-NTA beads against SCO1, followed by immunoblotting with the antibodies indicated. (r) Pull-down assays were performed between AMPK α with truncated SCO1 (His-Truncated) after depleting copper binding to His-truncated with EDTA, or loading copper to His-Truncated with CuSO₄. D-isoascorbic acid (DIA) were used to reduce Cu²⁺ to Cu¹⁺. Immunoblotting in non-reducing SDS-PAGE for the protein levels of copper-loaded His-Truncated (Cu-Truncated) and copper-deficient His-Truncated (apo-Truncated).

Figure 6

Copper chelation aggravates hepatic steatosis. (a) Workflow for administration of BCS in liver specific CP-knockout and control mice (n=10 in each group). (b) Copper concentrations in the livers of LKO and CON mice. (c) The quantified ratio of signals of p-AMPK α to total AMPK α in mouse livers using western blotting analysis in Figure S7B-S7D. (d-e) Representative images of H&E (d) and Oil Red O staining (e) of mouse liver sections, (H&E staining scale bar: 100 μ m; Oil Red O staining: 50 μ m). (f) Hepatic TG

concentrations. (g) mRNA levels of FAO genes in mice livers. (h) mRNA levels of mitochondrial DNA in mouse livers. (i) Transmission electron microscopy analysis of mitochondria in mice livers, [scale bar: 0.5 μm (line 1), 0.2 μm (line 2)]. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group. ns, not significant.

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