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## TAK1 regulates hepatic lipid homeostasis through SREBP

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### Abstract

Sterol regulatory element-binding proteins (SREBPs) are key transcription factors regulating cholesterol and fatty acid biosynthesis. SREBP activity is tightly regulated to maintain lipid homeostasis, and is modulated upon extracellular stimuli such as growth factors. While the homeostatic SREBP regulation is well studied, stimuli-dependent regulatory mechanisms are still elusive. Here we demonstrate that SREBPs are regulated by a previously uncharacterized mechanism through TGF- $\beta$  activated kinase 1 (TAK1), a signaling molecule of inflammation. We found that TAK1 binds to and inhibits mature forms of SREBPs. In an *in vivo* setting, hepatocyte-specific *Tak1* deletion upregulates liver lipid deposition and lipogenic enzymes in the mouse model. Furthermore, hepatic *Tak1* deficiency causes steatosis pathologies including elevated blood triglyceride and cholesterol levels, which are established risk factors for the development of hepatocellular carcinoma (HCC) and are indeed correlated with *Tak1*-deficiency-induced HCC development. Pharmacological inhibition of SREBPs alleviated the steatosis and reduced the expression level of the HCC marker gene in the *Tak1*-deficient liver. Thus, TAK1 regulation of SREBP critically contributes to the maintenance of liver homeostasis to prevent steatosis, which is a potentially important mechanism to prevent HCC development.

### Keywords

TAK1; SREBP; lipogenesis; liver; steatosis; hepatocellular carcinoma

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### AUTHOR CONTRIBUTIONS

SM, KS, EO, YI and JNT performed the experiments and analyzed the data. SM, KM and JNT designed the experiments and wrote the manuscript.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

## INTRODUCTION

Sterol regulatory element-binding proteins (SREBPs) are a basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family, which are the central regulators of lipogenesis by transcriptionally activating a number of genes involved in fatty acids, triglycerides and cholesterol synthesis.<sup>1</sup> Mammalian SREBP family has three isoforms, SREBP1a, SREBP1c and SREBP2.<sup>2</sup> SREBP1a and SREBP1c are splicing variants and are derived from *Srebf1* gene, and SREBP2 is encoded by *Srebf2*. SREBP1a and SREBP1c play predominant roles in fatty acid and triglyceride synthesis by upregulating a series of enzymes including acetyl CoA carboxylase, fatty acid synthase (FASN), and steadily CoA denatures (SCD). While SREBP1a and SREBP1c are functionally overlapped, SREBP1c is known to be the predominant form in the liver. SREBP2 is selectively involved in cholesterol synthesis and uptake by upregulating enzymes such as HMG CoA synthase, HMG CoA reductase and mevalonate kinase (MVK). SREBP2 can play compensatory roles to upregulate fatty acid and triglyceride synthesis when SREBP1 isoforms are genetically deleted.<sup>3</sup> Homeostatic SREBP activity is controlled by its unique sterol-dependent feedback loop.<sup>1</sup> Inactive precursor SREBPs are localized onto the endoplasmic reticulum (ER) membrane and interact with polytopic SREBP cleavage activated protein (SCAP) and insulin-induced gene protein (Insig).<sup>4</sup> When cholesterol levels drop, SREBPs are released from ER to the Golgi apparatus where SREBP undertakes protein processing to release the N-terminal bHLH-LZ domain. The mature bHLH-LZ SREBP enters the nucleus and binds as a dimer to SREBP responsive element (SRE) within promoters of its target genes. As SREBP2 activates cholesterol synthesis, cholesterol gradually builds up in the cell and activates negative feedback loop in which cells restore Insig-SCAP interaction to terminate the SREBP pathway.<sup>5</sup> Mature SREBPs are constantly degraded through a ubiquitin-proteasome system,<sup>6, 7</sup> which prevents sustained transcriptional activation of lipogenic genes and ensures a quick response to the negative feedback regulation.

In addition to the sterol-dependent feedback loop regulation, SREBP activity is modulated in response to extracellular stimuli. Growth factors enhance the activity of SREBP through the AKT-mTOR pathway, which provides membrane lipids as cell building materials to support cell growth.<sup>8-12</sup> Unfolded protein-induced stress conditions, ER stress, are also known to activate SREBP.<sup>13, 14</sup> in which upregulated lipogenesis supplies the ER membrane to alleviate ER stress conditions.<sup>15, 16</sup> Conversely, protein kinase AMPK, which is activated by nutrient stress (low cellular ATP), is reported to directly phosphorylate and inhibit SREBP, which reduces lipogenesis to restore the ATP level by limiting energy expenditure.<sup>17</sup> While the mechanism of sterol feedback loop regulation of SREBP is extensively studied, stimuli-induced regulatory mechanisms of SREBP are still elusive.

TAK1 is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family, and is activated by inflammatory cytokines such as IL-1, TNF $\alpha$  or Toll-like receptor ligands.<sup>18</sup> TAK1 signaling promotes inflammatory responses through its downstream targets including but not limited to transcription factors NF- $\kappa$ B and AP-1. Deletion of *Tak1* causes TNF $\alpha$ -induced cell death followed by tissue damage and animal mortality in the epithelial and endothelial-specific *Tak1*-deficient mouse models.<sup>19-21</sup> Hepatocyte-specific deletion of *Tak1* causes hepatocyte death resulting in liver injury,<sup>22-24</sup> which is also due to TNF $\alpha$ -

induced cell death.<sup>23, 25</sup> However, unlike epithelial- or endothelial-specific deletion of *Tak1*, hepatocyte-specific *Tak1* deletion does not cause acute animal mortality but induces hepatocellular carcinoma (HCC) around 2 months of age.<sup>22–24</sup> Several cancer-associated abnormalities have been implicated in *Tak1* deficiency-induced HCC, which include TGF- $\beta$ -induced fibrosis<sup>26</sup> and hyper-proliferation of hepatocytes.<sup>24</sup> One reasonable explanation of why *Tak1* deficiency induces HCC is compensatory hyper-proliferation of hepatocytes, which is initiated by TNF $\alpha$ -induced cell death. However, while ablation of TNF $\alpha$  signaling effectively rescues cell death,<sup>23, 25</sup> it does not abolish development of HCC as described in this report. Thus, TNF $\alpha$ -induced cell death is not solely the cause of HCC, and the mechanism by which *Tak1* deficiency induces HCC is still elusive.

HCC in the *Tak1*-deficient liver develops spontaneously and very quickly within several weeks, which is different from any abnormalities that are observed in mouse models harboring liver-specific deletion of NF- $\kappa$ B or AP-1 pathways.<sup>24, 27, 28</sup> For example, deletion of NF- $\kappa$ B slowly induces HCC at over 12 months of age.<sup>24, 29, 30</sup> Deletion of AP-1 activators, JNKs, in hepatocytes enhances chemical-induced HCC but no spontaneous HCC has been reported.<sup>27</sup> Thus, we postulate that TAK1 deficiency induces HCC predominantly not through impaired NF- $\kappa$ B or AP-1. Here we report an identification of SREBPs as new TAK1 targets. We show that *Tak1* deficiency caused hepatic steatosis through dysregulation of SREBP. Steatosis is recognized as a liver lesion, which can progress to or facilitate HCC. Our results demonstrate that TAK1 regulation of SREBP is critical in liver homeostasis by preventing hepatic steatosis, which could further associate with the prevention of HCC.

## RESULTS

### Tnfr1 deletion does not effectively block tumorigenesis in Tak1-deficient liver

Hepatocyte-specific *Tak1*-deficient mice have been generated and characterized in three independent studies,<sup>22–25</sup> which demonstrated that deletion of *Tak1* in the liver induces liver lesions characterized by hepatocyte death and early onset of HCC within 2 months of age. Ablation of TNF $\alpha$  signaling by deletion of *Tnfr1* has been demonstrated to abolish hepatocyte death in *Tak1*-deficient liver,<sup>23, 25</sup> indicating that TNF $\alpha$  is the cause of *Tak1*-deficient hepatocyte death. Cell death is known to induce compensatory proliferation, which is implicated in tumorigenesis.<sup>31</sup> Thus, we initially anticipated that TNF $\alpha$ -induced cell death is the cause of HCC. However, we unexpectedly observed liver tumors in mice having liver-specific deletion of *Tak1* in the *Tnfr1*<sup>-/-</sup> background (Tak1<sup>LKO</sup> Tnfr1<sup>-/-</sup>) at 5–8 months old (supplementary Fig. S1A). To validate this observation, we examined the levels of fetal liver gene expression, including a non-coding RNA, *H19*, alpha-fetoprotein (*Afp*) and *Rex3*, which are known to be upregulated at the early stage of HCC.<sup>32–34</sup> We previously reported that the Tak1<sup>LKO</sup> liver at 4–5 weeks of age does not have visually detectable tumors, but these HCC markers are highly increased.<sup>22</sup> In the Tak1<sup>LKO</sup> Tnfr1<sup>-/-</sup> livers, the levels of the HCC markers were still upregulated compared to the wild type littermate livers, although the levels were slightly or moderately lower compared to littermate mice having functional TNF signaling (Tak1<sup>LKO</sup> Tnfr1<sup>+/-</sup>) (Fig. 1A). At 4–5 months of age, the *H19*RNA level was highly varied but still upregulated in the Tak1<sup>LKO</sup> Tnfr1<sup>-/-</sup> liver compared to the control

littermate livers (supplementary Fig. S1B). Thus, TNF $\alpha$ -induced hepatocyte death is not solely the cause of tumorigenesis in the *Tak1*-deficient liver.

### **Tak1 deficiency causes liver steatosis through a previously unidentified mechanism**

*Tak1*-deficient liver also exhibited typical features of steatosis including lipid depositions (supplementary Fig. S2), which is consistent with a recent report by Seki's group.<sup>35</sup> The levels of both blood triglycerides and cholesterol were upregulated in *Tak1*<sup>LKO</sup> mice (Fig. 1B, 1C supplementary Fig. S3). Importantly, we found that deletion of *Tnfr1* did not reduce the levels of blood triglycerides or cholesterol (Fig. 1B and 1C). Given that *Tnfr1* deletion completely blocks the hepatocyte death in *Tak1*<sup>LKO</sup> mice,<sup>25</sup> the hepatic steatosis is independent on TNF-induced cell death in *Tak1*<sup>LKO</sup> liver. Many cellular processes not limited to excess lipogenesis or impaired breakdown of lipids have been implicated in hepatic steatosis.<sup>36, 37</sup> Accordingly, a diverse set of cell signaling molecules are known to be involved in the development of steatosis including well-known targets of TAK1, i.e. NF- $\kappa$ B and JNK. Liver-specific deletion of NF- $\kappa$ B or JNK causes hepatic steatosis.<sup>29, 38</sup> AMP-activated protein kinase (AMPK) is also a target of TAK1,<sup>39</sup> and was reported to inhibit lipogenesis.<sup>17</sup> An earlier study reports that deletion of *Tak1* reduces AMPK activation in cultured hepatocytes.<sup>35</sup> Thus, we next examined whether these targets of TAK1 were impaired in the *Tak1*-deficient liver *in vivo*. Unexpectedly, we found that activities of NF- $\kappa$ B and JNK were not pronouncedly altered by deletion of *Tak1* in the liver (supplementary Fig. S4). Furthermore, the level of AMPK activity was not reduced but rather increased in the *Tak1*-deficient liver (supplementary Fig. S4). Thus, these TAK1 targets are unlikely to be involved in the steatosis phenotype of the *Tak1*-deficient liver. These results lead us to explore new targets of TAK1.

### **TAK1 interacts with and phosphorylates SREBPs**

We previously performed a series of yeast-two hybrid screenings using TAK1 protein as a bait.<sup>40, 41</sup> Among the identified but not yet characterized binding partners of TAK1, we focused on SREBP2 as a potential target of TAK1, which could be involved in liver steatosis in the *Tak1*-deficient liver. The N-terminal region of mouse SREBP2 amino acid residues positioned between 114 and 395 was isolated as a binding region of TAK1, which is well conserved among SREBP1a, SREBP1c and SREBP2 (45% identical) (Fig. 2A). The TAK1 binding region of SREBP was mapped between the transactivation domain and the cleavage sites of SREBP, and included the entire region of bHLH-ZF domain, which covers most parts of the mature SREBPs (Fig. 2A). Thus, we examined whether mature forms of SREBP1a, SREBP1c and SREBP2 interact with TAK1 using the overexpression system in human embryonic kidney 293 cells. Exogenously expressed TAK1 was efficiently pulled down by immunoprecipitation against SREBPs (Fig. 2B, top panel), whereas endogenous TAK1 was moderately precipitated (2nd panel). Endogenous TAK1 may be pre-occupied with endogenous TAK1 binding partners including SREBPs, which might cause the less efficient co-precipitation of endogenous TAK1 with exogenously expressed SREBPs. Reciprocally, all matured forms of SREBP isoforms were pulled down with TAK1 (Fig. 2C), although SREBP2 co-precipitated TAK1 with a lesser efficiency compared to SREBP1a and SREBP1c. To examine the interaction between TAK1 and SREBPs under the physiological settings, we also conducted co-immunoprecipitation analysis using proteins from a human

HCC cell line, HepG2, and the mouse liver. Endogenous full-length SREBPs were co-precipitated with TAK1 (Fig. 2D and supplementary Fig. S5.). These results suggest that TAK1 interacts with SREBP in cells, and raise the possibility that TAK1 participates in modulation of SREBPs. Because TAK1 is a protein kinase, TAK1 potentially modulates SREBPs through phosphorylation. To test whether TAK1 phosphorylates SREBPs, we conducted an *in vitro* kinase assay. Activated TAK1 was prepared from HEK293 cells overexpressing the catalytic domain of TAK1 (TAK1<sup>C</sup>) together with TAK1-binding protein 1 (TAB1), which is an activating subunit of TAK1.<sup>42</sup> Bacterially purified recombinant GST-SREBP1a (mature form) was incubated with the TAK1-TAB1. SREBP1a was phosphorylated when incubated with activated TAK1, which was reduced in the presence of a selective inhibitor of TAK1, 5Z-7-Oxozeaenol (5Z) (Fig. 2E). Thus, TAK1 is a potential modulator of SREBPs through binding to and phosphorylating SREBPs.

### TAK1 inhibits SREBPs

We next examined whether TAK1 modulates activity of SREBPs. We co-expressed TAK1 or the kinase-negative form of TAK1(K63W) together with mature forms of SREBPs. We found that the protein levels of all SREBP isoforms were greatly increased by co-expression of catalytically active TAK1 (Fig. 3A), while kinase-dead TAK1, TAK1(K63W), did not alter the expression levels of SREBPs (Fig. 3B). The transfection efficiencies monitored by co-transfected a constitutively expressed reporter were comparable in all samples, indicating that increased amounts of SREBPs were not due to a higher transfection efficiency (Fig. 3A, bottom graph). An earlier study demonstrates that inhibition of the transcriptional activity of SREBP blocks proteasome-dependent degradation of SREBP,<sup>6</sup> which is one of the feedback regulations to maintain the homeostatic level of SREBP activity. Thus, catalytically active TAK1 may inhibit SREBP activity. Indeed, we found that transcriptional activity of SREBPs monitored by a sterol regulatory element (SRE)-driven reporter expression was greatly diminished (Fig. 3C). By contrast, expression of catalytically inactive TAK1 highly increased transcriptional activity of all SREBPs (Fig. 3D). TAK1(K63W) is known to act as a dominant negative inhibitor by preventing autophosphorylation of TAK1.<sup>42, 43</sup> Thus, endogenous TAK1 is likely to play an inhibitory role in SREBP activation. These results demonstrate that TAK1 binds to and inhibits mature SREBPs, and this regulation depends on its kinase activity. To obtain mechanistic insights into TAK1 regulation of SREBPs, we tested the possibility that TAK1 inhibits nuclear localization of mature SREBPs. HEK293 cells expressing the mature form of SREBP1a with and without TAK1 were fractionated into the nuclear and non-nuclear cytoplasmic fractions, and the localization of SREBP was analyzed by immunoblotting (supplementary Fig. S6A). We note here that, since TAK1 highly upregulates the amounts of SREBPs as shown in Fig. 3A, the vector amounts used for expression of SREBP with and without TAK1 co-expression were adjusted to achieve the similar expression levels of SREBP between samples. The mature form of SREBP1a was mostly localized to the nucleus, and overexpression of TAK1 did not alter the SREBP localization (supplementary Fig. S6A). We also examined the localization of SREBP2 in wild-type and Tak1<sup>LKO</sup> liver. SREBP2 was predominantly localized to the nucleus, and we found no observable alteration in the localization of SREBP2 by *Tak1* deficiency (supplementary Fig. S6B). These results indicate that TAK1 inhibits transcriptional activity of SREBPs not by blocking their nuclear localization.

### TAK1 is a regulator of SREBP

To investigate whether TAK1 modulates SREBP in the physiological settings, we examined whether downregulation of *Tak1* alters endogenous SREBP activity. We lowered the TAK1 protein level by utilizing three independent siRNAs targeted against *TAK1* mRNA, and transcriptional activity of endogenous SREBP was examined in 293 cells. The knockdown of TAK1 reduced the TAK1 protein ranging from moderately to effectively (Fig. 4A). Under the normal culture condition, SREBP activity was found to be low and was not detectably altered by knockdown of TAK1 (Fig. 4B). However, when endogenous SREBP was activated by depleting cellular cholesterol using methyl- $\beta$ -cyclodextrin, SREBP activity was augmented at higher levels in TAK1-knockdown cells compared to control cells (Fig. 4B). This suggests that endogenous TAK1 inhibits SREBP. To further examine whether TAK1 modulates SREBP in hepatocytes, the human HCC cell line, HepG2, was utilized. TAK1 was knocked down using an shRNA targeted against TAK1 (Fig. 4C, right panels), and transcriptional activity of exogenously expressed SREBPs was monitored by the reporter assay. Ablation of TAK1 was found to upregulate SREBP activity in HepG2 cells (Fig. 4C). These data demonstrate that endogenous TAK1 suppresses SREBP activity.

### TAK1 suppresses SREBP in the liver

Taking the fact that the liver is the central tissue of lipid synthesis, our results above prompted us to connect between TAK1 regulation of SREBP and *Tak1* deficiency-induced hepatic steatosis. We examined whether impaired TAK1-dependent SREBP suppression is associated with steatosis in the *Tak1*-deficient liver. We first examined endogenous liver SREBP activity by monitoring the specific binding ability to the sterol regulatory element consensus sequence (electron mobility shift assay). The SREBP DNA binding activity was increased in *Tak1*<sup>LKO</sup> liver compared to control littermate livers (Fig. 5A lanes 1–6). Consistent with the increased SREBP activity, SREBP target genes including *Fasn*, *Scd1*, *Mvk1* and glucose-6-phosphate dehydrogenase (*G6pd*) were upregulated in the *Tak1*-deficient liver (Fig. 5B). SREBP is known to be self-regulated, and SREBP gene (*Srebf*) expression showed a trend of increase in *Tak1*-deficient liver (Supplementary Fig. S7). To investigate whether increased SREBP activity is causally associated with steatosis in the *Tak1*-deficient liver, we treated *Tak1*<sup>LKO</sup> mice with an inhibitor of SREBP, fatostatin, which binds to SCAP and inhibits translocation of SREBP from the endoplasmic reticulum to the Golgi apparatus.<sup>44</sup> Treatment of fatostatin in *Tak1*<sup>LKO</sup> mice reduced the levels of blood triglyceride and cholesterol (Fig. 5C and 5D). These results suggest that TAK1 regulation of SREBP is critical to prevent hepatic steatosis. Finally, we ask whether the hepatic steatosis by activated SREBP is causally associated with HCC in *Tak1*<sup>LKO</sup> mice. We examined the level of HCC marker, H19, expression in the fatostatin treated mouse livers. We found that the reduced SREBP activity by fatostatin was correlated with the lower expression levels of the HCC marker (see Fig. 5A, bottom numbers, lanes 7 and 8), which suggest a potential causal association between activation of SREBP and HCC in *Tak1*<sup>LKO</sup> mice.

## DISCUSSION

SREBP is the master regulator of lipogenesis, and plays an indispensable role to maintain the proper levels of cellular lipids including triglycerides and sterols though the highly

sophisticated proteolytic processing mechanism.<sup>1</sup> SREBP is also modulated upon growth stimulation, in which growth factor-PI3K-mTOR pathway upregulates SREBP activity through promoting nuclear localization of SREBP.<sup>8–12</sup> Activated SREBP provides triglycerides and sterols to build new cell membranes. In the current study, we show that TAK1 inhibits mature SREBP, and impairment of this regulation induces hepatic steatosis. TAK1 is the inflammatory signaling intermediate, and is activated when cells are exposed to danger signals including inflammatory cytokines and microorganisms.<sup>18</sup> Inflammation and lipid homeostasis is closely associated.<sup>45</sup> Chronically inflamed conditions induce lipolysis in the adipose tissue and upregulate lipid biogenesis in the liver.<sup>46, 47</sup> We show here that ablation of TAK1 disrupts normal lipid homeostasis by increasing SREBP activity. The liver is at the front line to constantly deal with exogenous stressors such as moieties from microorganisms and chemicals, many of which are inflammatory stimuli and activators of TAK1. Thus, hepatic TAK1 is likely to be constantly activated by these stressors, which may be one of the critical mechanisms by which normal liver limits their lipogenesis.

In the current study, we identified SREBPs as novel binding partners of TAK1, and demonstrate that TAK1 inhibits SREBP activity. Although the detailed molecular mechanism is still elusive, our study shows several interesting aspects in TAK1 regulation of SREBP. Conventionally, maturation (processing) and subsequent nuclear localization of SREBP are the keys to SREBP activation.<sup>1</sup> However, we found that SREBP nuclear localization is not affected by either activation or ablation of TAK1 (supplementary Fig. S6). In contrast, we observed increased ability of SREBP to bind sterol-responsive element DNA sequence in the *Tak1*-deficient liver (Fig. 5A), suggesting that TAK1 modulates SREBP DNA binding. We also demonstrate that TAK1 can phosphorylate SREBP using an *in vitro* kinase assay. Furthermore, we found that endogenous TAK1 binds to full-length SREBP. Collectively, one possible scenario of TAK1 regulation of SREBP is that TAK1 binds to and phosphorylates ER- or Golgi-localized full-length SREBP in the cytoplasm, which does not prevent SREBP maturation or translocation into the nucleus, but the TAK1 modulated (phosphorylated) SREBPs possess diminished transcriptional activity due to their reduced ability of DNA binding. Alternatively or additionally, TAK1 modulation may block transcriptional activity of SREBP by modulating interaction between SREBPs and transcriptional co-factors such as CBP and p300.<sup>48, 49</sup> Further investigations to determine the mechanism for TAK1 regulation of SREBP will enhance our understanding in the molecular link between inflammation and lipogenesis.

Hepatic steatosis is a common liver disorder, which is induced by excess alcohol consumption, hepatitis viruses and metabolic syndromes. The mechanisms for development of hepatic steatosis are not yet entirely clear, but are clearly associated with elevated and chronic exposures to stressors. As protein kinase signaling pathways are generally regulated by negative feedback loops mediated by activation or induction of protein phosphatases,<sup>50</sup> TAK1 signaling is normally negatively regulated by sustained stimulation.<sup>51, 52</sup> Thus, chronic exposures to stressors may not chronically activate but rather impair TAK1 signaling. Given that ablation of TAK1 causes hepatic steatosis, one possible mechanism for hepatic steatosis is impaired TAK1 signaling under chronically stressed conditions, which warrants further investigations of potential causal relationships among chronic stress, TAK1 activity and hepatic steatosis.

Hepatic steatosis is recognized as a liver lesion, which could lead to cirrhosis, fibrosis and ultimately HCC. These pathogenic processes from steatosis to HCC normally take a considerable time in humans, and excess lipogenesis alone by overexpression of mature SREBP1c does not result in HCC in the mouse model.<sup>53–55</sup> Hyper-activation of PI3K-mTOR pathway by liver-specific deletion of PTEN effectively induces steatosis through activation of SREBP but develops HCC very slowly, which takes more than 9 months in the mouse model.<sup>56</sup> These suggest that not only activation of SREBP but also some additional aberrations are likely to be required for effective development of HCC. *Tak1*-deficient liver develops steatosis, fibrosis and HCC within 2 months,<sup>23, 24</sup> which is exceptionally effective; even more effective compared to the HCC models induced by transgenic expression of oncogenes such as K-Ras and  $\beta$ -catenin.<sup>57</sup> Thus, it is unlikely that steatosis alone is the cause of HCC in *Tak1*-deficient liver. Constantly occurring cell death induces inflammation, which is associated with genetic instability and quick development of HCC.<sup>58</sup> *Tak1* deficiency induces TNF $\alpha$ -dependent cell death in the liver.<sup>23, 25</sup> Combination of steatosis and chronic injury due to constantly occurring cell death may be the cause of HCC in *Tak1*-deficient liver. However, while ablation of TNF $\alpha$  signaling could rescue hepatocyte death in *Tak1*-deficient liver, it did not abolish HCC development (see Fig. 1A). Thus, TNF $\alpha$ -induced cell death facilitates but is not causally associated with HCC. It is noteworthy that a non-coding RNA H19 is highly expressed in *Tak1*-deficient liver even at 4–5 weeks of age when HCC is not yet clearly observed. Importantly, the level of H19 is only moderately reduced by deletion of *Tnfr1*, suggesting that H19 expression is not the result of cell death. H19 is considered as a marker of cell de-differentiation, which is known to be associated with tumor metastasis.<sup>32, 33, 59</sup> Thus, TAK1 may be involved in regulatory processes of hepatocyte differentiation. Nonetheless, TAK1-deficiency induced HCC is unique, which develops quickly but still shares key features of human HCC including steatosis and fibrosis, and warrants future studies.

## MATERIALS AND METHODS

### Mice and cell culture

*Tak1*-floxed (*Tak1*<sup>flox/flox</sup>) mouse was previously described.<sup>60</sup> *Alb.Cre* transgenic and *Tnfr1*<sup>-/-</sup> C57BL/6 mice were obtained from the Jackson Laboratories.<sup>61, 62</sup> All strains used were backcrossed at least 7 times to C57BL/6. Both male and female mice were used. Inhibition of SREBP was achieved by intraperitoneal (ip) injection of fatostatin (Millipore) (10 mg/kg mouse weight per day) for 30 consecutive days. All animal experiments were conducted with the approval of the North Carolina State University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering. Human embryonic kidney (HEK) 293 and HepG2 cells were cultured in DMEM with 10% bovine growth serum (Hyclone) and penicillin-streptomycin at 37°C in 5% CO<sub>2</sub>.

### Antibodies, reagents, plasmids and siRNAs

Anti-TAK1 antibody was described previously.<sup>63</sup> Anti-SREBP1 (2A4) (Santa Cruz Biotechnology), anti-SREBP2 (ab30682) (Abcam), anti-AMPK (Cell Signaling Technology), anti-phospho-AMPK (Thr172) (40H9) (Cell Signaling Technology), anti-JNK1/2 (sc571) (Santa Cruz Biotechnology), anti-phospho-JNK (Thr183/Tyr185) (Cell



Signaling Technology), anti-phospho-p38 (Thr180/Tyr182) (D3F4) (Cell Signaling Technology), anti-p38 (N-20) (Santa Cruz Biotechnology), anti-p65 NF- $\kappa$ B (C20) (Santa Cruz Biotechnology), anti-phospho-p65 (Ser276) (Cell Signaling Technology), anti- $\beta$ -actin (AC15) (Sigma), anti-lamin B (Ab-1) (Millipore), anti-lactate dehydrogenase (AB1222) (Millipore), anti-HA (HA.11) (Covance) and anti-FLAG (Sigma, M2) were used. The reagents used were methyl- $\beta$ -cyclodextrin (Sigma), lipopolysaccharide (LPS) (Sigma, from *Salmonella minnesota*) and Oil-red-O (Sigma). DsRed-tagged TAK1 expression vector and other TAK1 expression plasmids were described previously<sup>25</sup>. FLAG-tagged SREBP expression vectors were from Addgene.<sup>64</sup> To generate GST-tagged SREBP1a expression plasmid, FLAG-SREBP1a was subcloned into a pGEX vector (GE Healthcare). SRE-driven luciferase reporter (SRE-luciferase) and control EF1 $\alpha$  promoter-driven renilla luciferase (EF1 $\alpha$ -renilla luciferase) constructs were used for the transcription reporter assay.<sup>65, 66</sup> A retrovirus vector expressing shRNA targeted against *TAK1* (the same targeted sequence as TAK1 siRNA#1 described below) was described previously.<sup>67</sup> *TAK1* and non-targeting control siRNAs were obtained from Sigma (*TAK1* siRNA#1, 5'-GAGAUUCGACUACAAGGAGA-3'; *TAK1* siRNA#2, 5'-GGCAAAGCAACAGAGUGAAUCUGGA-3'; *TAK1* siRNA#3, 5'-GAGUGCUGACAUGUCUGAAAUAGAA-3' and Non-targeting siRNA, 5'-UUCUCCGAACGUGUCACGU-3').

### Immunoblotting

Whole cell extracts were prepared using an extraction buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM  $\beta$ -glycerophosphate, 1.5 mM MgCl<sub>2</sub>, 2 mM EGTA, 10mM NaF, 2 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 20  $\mu$ M aprotinin, and 0.5% Triton X-100. Cell extracts were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

### Immunoprecipitation

HEK293 cells were transfected with plasmids using the standard calcium phosphate method. Cell lysates were prepared in extraction buffer described above. DsRed-tagged TAK1, FLAG-tagged SREBP were immunoprecipitated with anti-TAK1 or anti-FLAG antibodies for overnight. The resulting immune complexes were washed with extraction buffer three times. Samples were resolved on SDS-PAGE and exposed to autoradiographic films.

### Subcellular fractionation

Nuclear Extract Kit (Active Motif) was used for cytoplasmic and nuclear fractionation. Briefly, cells were lysed in an ice-cold hypotonic buffer, and the lysates were centrifuged and separated into the supernatant (the cytoplasmic fraction including organelles) and the pellets. Nuclear proteins were extracted from the nuclei-containing pellet.

### In vitro kinase assay

HEK293 cells were transfected with expression vectors for HA-tagged catalytic domain of TAK1 (TAK1 C) together with TAB1 using the standard calcium phosphate method. Cell lysates were prepared in the extraction buffer described above. HA-TAK1 C-TAB1 complex was immunoprecipitated with anti-HA antibody for 2 h at 4°C. TAK1 was pre-activated by incubation in a kinase buffer (10 mM HEPES, pH 7.4, 1 mM DTT, and 5 mM MgCl<sub>2</sub>) with 1 mM ATP for 30 min at 30°C and washed once with the kinase buffer. The TAK1 C protein and bacterially purified GST-tagged mature form of SREBP1a were mixed and incubated in the kinase buffer supplemented with 200 μM ATP and 5 μCi γ-[<sup>32</sup>P]ATP for 30 min at 30°C. Samples were resolved on SDS-PAGE. Proteins were visualized by coomassie brilliant blue staining and the gel was exposed to autoradiographic films.

### Quantitative real time PCR Analysis

Total RNA was isolated from the liver using an RNeasy kit (Qiagen) and transcribed into cDNA using MultiScribe™ reverse transcriptase (Life Technologies). Expression levels of *Tak1*, *Srebp* and SREBP target genes were determined by quantitative real time PCR (qPCR) and normalized to the level of *Gapdh*. The following primers were used:

*Srebp1a*-forward, 5'-GGCCGAGATGTGCGAACT-3'; *Srebp1a*-reverse, 5'-TTGTTGATGAGCTGGAGCATGT-3';

*Srebp1c*-forward, 5'-GGAGCCATGGATTGCACATT-3'; *Srebp1c*-reverse, 5'-GGCCCGGAAGTCACTGT-3';

*Srebf2*-forward, 5'-GGATCCTCCCAAAGAAGGAG-3'; *Srebf2*-reverse, 5'-TTCCTCAGAACGCCAGACTT-3';

*Scd1*-forward, 5'-CTGACCTGAAAGCCGAGAAG-3'; *Scd1*-reverse, 5'-GCGTTGAGCACCAGAGTGTA-3';

*Fasn*-forward, 5'-AAGGCTGGGCTCTATGGATT-3'; *Fasn*-reverse, 5'-GGAGTGAGGCTGGGTTGATA-3';

*vk1*-forward, 5'-GGGACGATGTCTTCCTTGAA-3'; *Mvk1*-reverse, 5'-GAACTTGGTCAGCCTGCTTC-3';

*G6pd*-forward, 5'-CCTACCATCTGGTGGCTGTT-3'; *G6pd*-reverse, 5'-TGGCTTTAAAGAAGGGCTCA-3';

*Tak1* exon 2-forward 5'-AGGTTGTCCGGAAGAGGAGCT-3'; *Tak1* exon 2-reverse 5'-CTCCACAATGAAAGCCTTCC-3'; and

*Gapdh*-forward, 5'-GAAGGTCGCTGTGAACGGA-3'; *Gapdh*-reverse, 5'-GTTAGTGGGGTCTCGCTCCT-3'.

### Measurement of plasma Triglyceride and Cholesterol level

Peripheral blood was collected from the facial vein into 1.5 ml tube containing 1 μl of 0.5 M EDTA and centrifuged at 1000g for 10 min. Supernatant was kept as plasma. Plasma triglyceride and cholesterol amount was determined by triglyceride measurement kit (Wako)

and cholesterol measurement kit (Life Technologies), respectively. The assay was conducted according to the manufactured protocols.

### Oil-Red-O staining

Oil-Red-O staining was conducted on frozen section. Briefly, freshly cut frozen sections were dried using hair-dryer for 30 min. Sections were then fixed with 4% paraformaldehyde followed by wash with H<sub>2</sub>O. After the rinse with 60% isopropanol, sections were incubated in Oil-Red-O solution (5 mg/ml in 60% isopropanol) for 15 min. Samples were washed with 60% isopropanol and were counter stained with Hematoxylin.

### Electrophoresis mobility shift assay (EMSA)

Oligonucleotides for NF- $\kappa$ B (5'-AGTTGAGGGGACTTTCCCAGG-3') was purchased from Promega. Oligonucleotides for sterol regulatory element (SRE), wild type (5'-TTTGAAAATCACCCACTGCAAACCTCC-3') and mutant (5'-TTTGAAAGTCAAACCGTTGCAAACCTCC-3') sterol regulatory element (SRE) were synthesized.<sup>68, 69</sup> The binding reaction contained <sup>32</sup>P radiolabeled oligonucleotide probe, 10  $\mu$ g of cell extracts, 4% glycerol, 1mM MgCl<sub>2</sub>, 0.5mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 500 ng of poly (dI-dC) (GE Healthcare), and 10  $\mu$ g of bovine serum albumin to a final concentration of 15  $\mu$ l. The reaction mixture were incubated at 25°C for 30 min, separated by 5% (w/v) polyacrylamide gel, and visualized by autoradiography. The NF- $\kappa$ B band was confirmed by super-shift using anti-p65 NF- $\kappa$ B. Since antibodies against SREBPs are not suitable for super-shift of mouse SREBP, the SREBP band was confirmed by a competition with wild type and mutant SRE.

### Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed liver sections using anti-SREBP2 (Abcom). The bound antibodies were visualized with biotin-conjugated secondary antibody to rabbit IgG using the ABC/DAB reagents according to the manufacturer's protocol (Vector Laboratories). Sections incubated with non-immunized IgG are served as controls. Images were acquired on an upright microscope (BX41; Olympus). Scale bars, 20  $\mu$ m.

### Statistics

Results are shown as the mean  $\pm$  standard error or standard deviation as indicated. No statistical methods were used to predetermine sample size. Statistical significance was determined using a two-tailed unpaired Student's t test or one-way ANOVA (Tukey's post hoc test).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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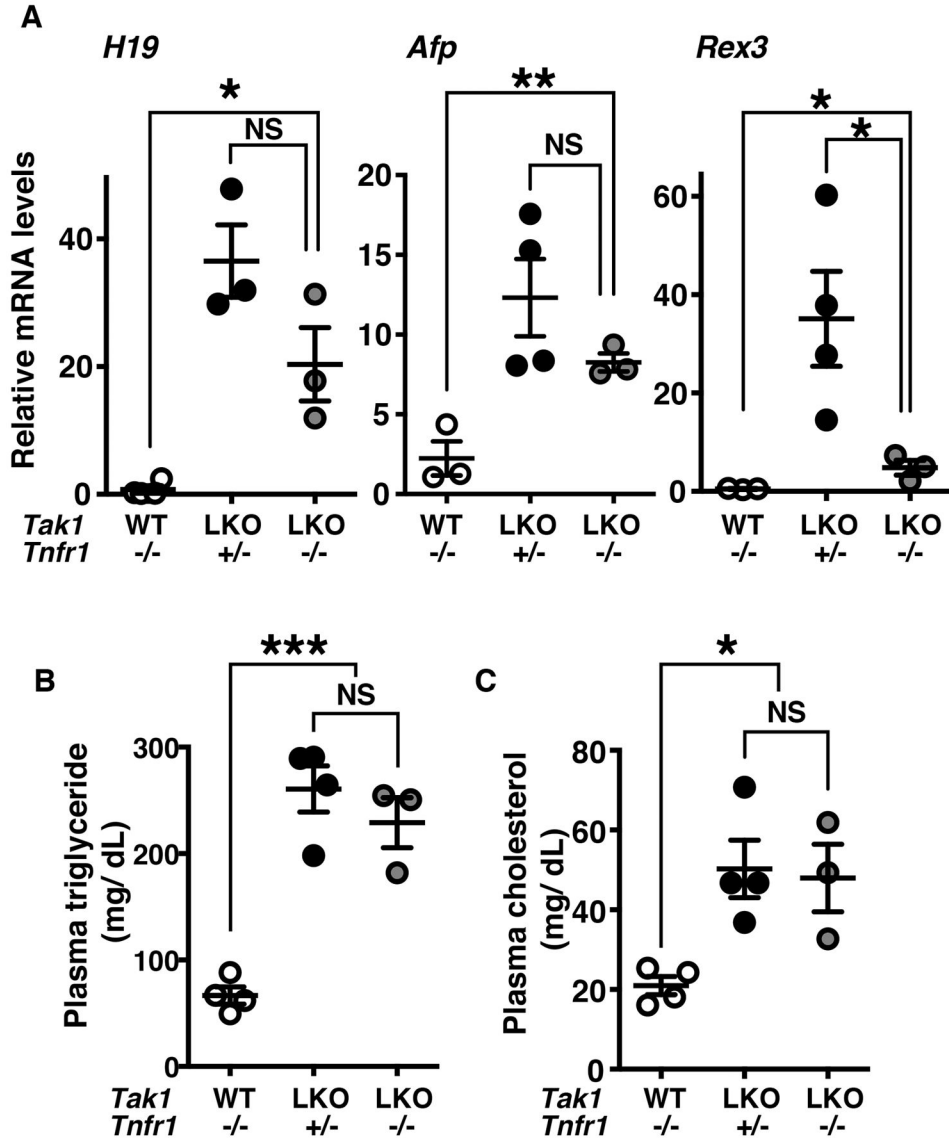
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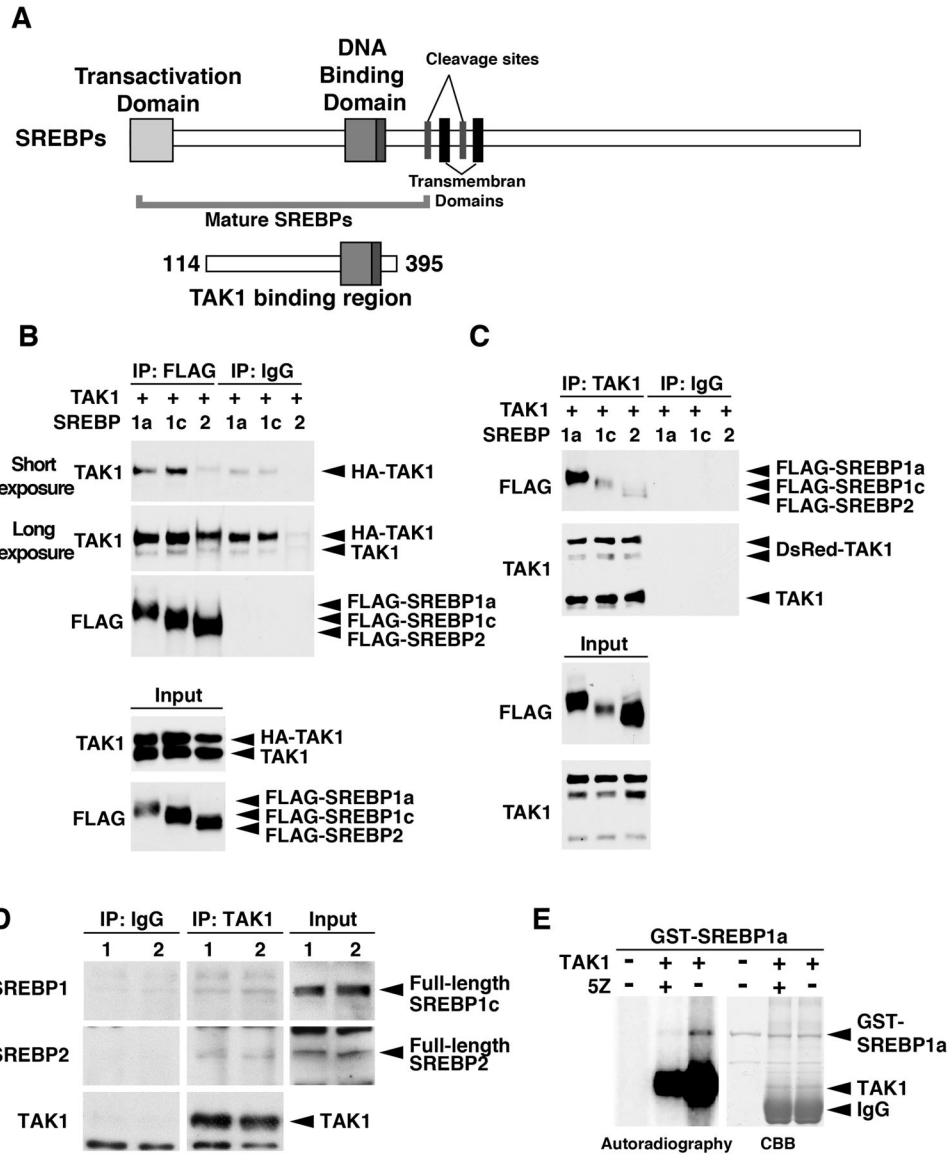
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**Figure 1. Deletion of *Tnfr1* does not prevent tumorigenesis or steatosis in the *Tak1*-deficient liver** (A) The liver was isolated from *Tak1*<sup>LKO</sup> (LKO) or control no-Cre (WT) littermate in the background of *Tnfr1*<sup>-/-</sup> or *Tnfr1*<sup>+/-</sup> mice at 6 weeks of age, and RNA was analyzed by the quantitative real time PCR. All data points and means  $\pm$  SEM of *H19*, *Afp* and *Rex3* mRNA levels relative to *Gapdh* are shown. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; NS, not significant (two-tailed unpaired Student's t test). (B, C) Blood was collected from the facial vein of the same mice as described (A), and the levels of plasma triglyceride and cholesterol were determined. All data points and means  $\pm$  SEM are shown. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ , NS, not significant (one-way ANOVA).





**Figure 2. TAK1 interacts with SREBP**

(A) Structure of SREBPs. The region of TAK1 binding domain isolated from the yeast two-hybrid screening is denoted by encompassing amino acid residues of SREBP2. (B, C) HEK293 cells were transfected with expression vectors for HA- (B) or DsRed- (C) tagged TAK1 and FLAG-tagged mature forms of SREBPs (N-terminal region), and proteins from cell lysates were immunoprecipitated by anti-FLAG (B) or anti-TAK1 (C) as well as non-immunized control IgG. Immunoprecipitates were analyzed by immunoblotting. The amounts of input proteins are also shown in bottom two panels. Shorter (B, top panel) and longer (B, second panel) exposures are shown to visualize less efficiently co-precipitated HA-TAK1 (3<sup>rd</sup> lane) and endogenous TAK1 (D) Cytoplasmic fractions including cytoplasmic organelle fractions from the mouse liver were immunoprecipitated by anti-TAK1 or control IgG. Immunoprecipitates were analyzed by immunoblotting. Each lane (lane 1 and 2) represents a sample from an individual mouse. (E) HEK293 cells were

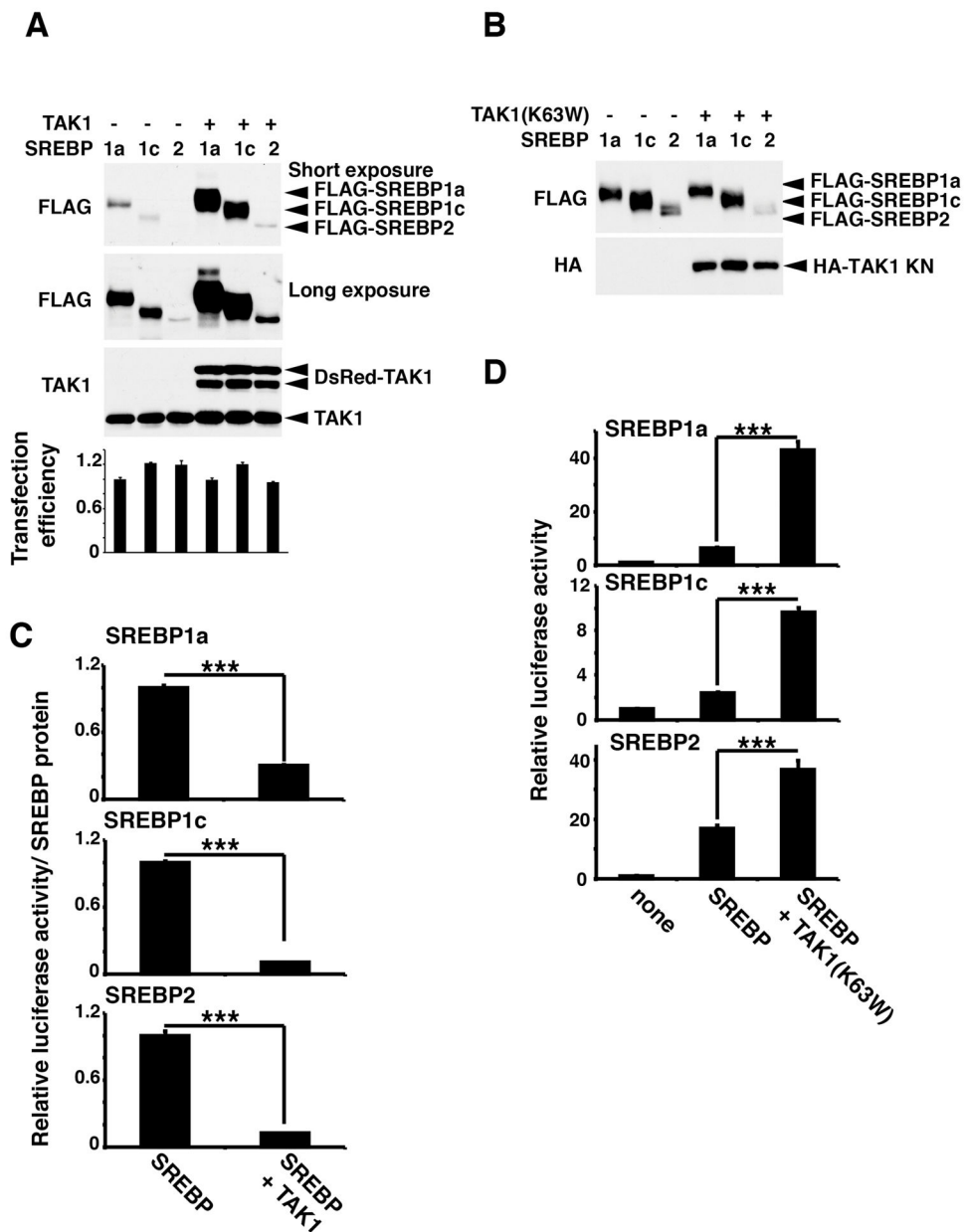
transfected with expression vectors for HA-tagged catalytic domain of TAK1 (TAK1 C) together with TAB1. HA-TAK1 C was purified from protein extracts by immunoprecipitation, and incubated with bacterially purified GST-tagged mature SREBP1a in the presence (+) or absence (-) of 5Z-7oxozeaenol (5Z) (500 nM). Phosphorylation of the proteins was detected by autoradiography. The protein amounts are shown by coomassie brilliant blue (CBB) staining.

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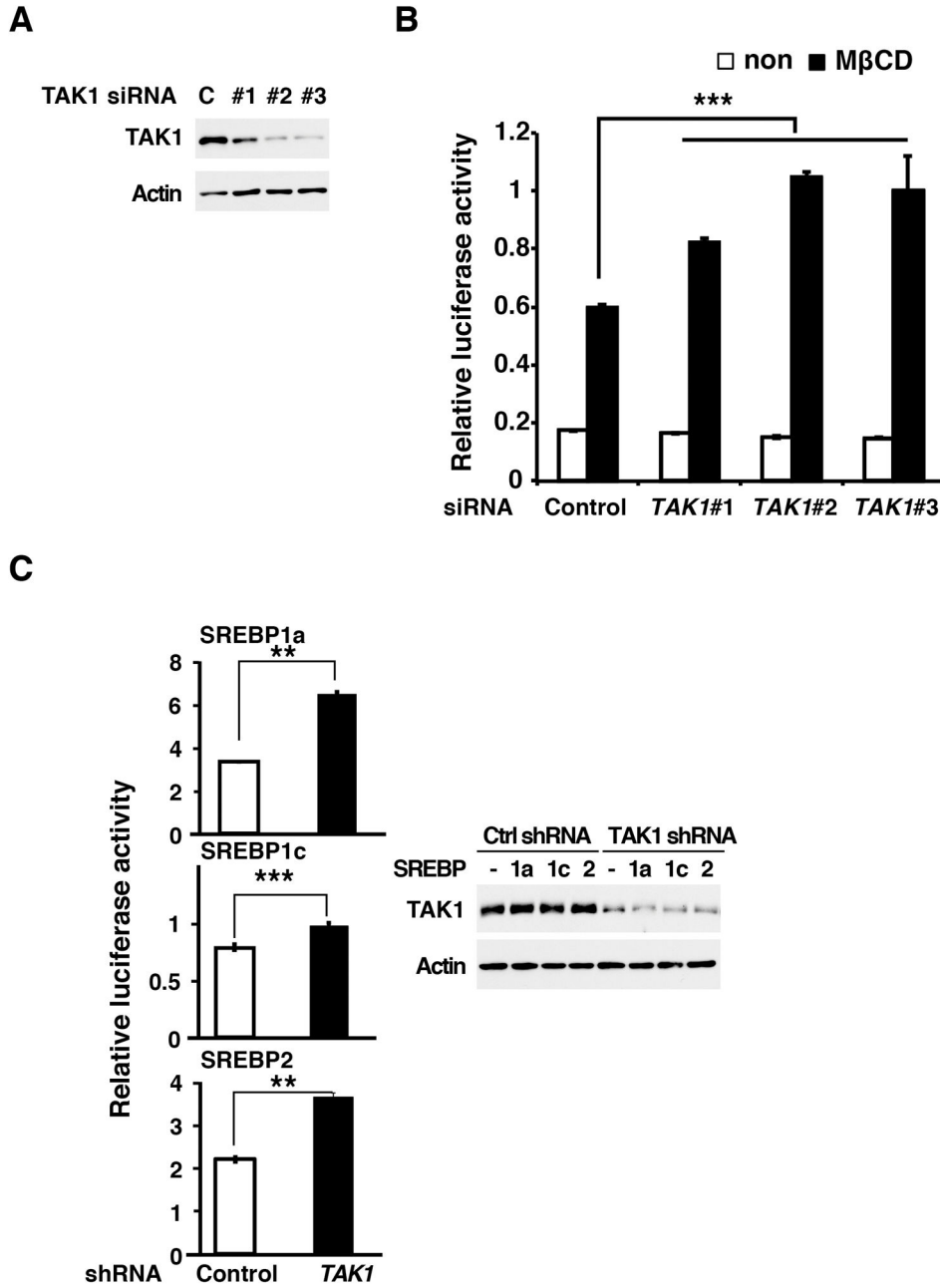
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**Figure 3. TAK1 inhibits transcription activity of SREBP**  
 HEK293 cells were transfected with the SREBP reporter (SRE-luciferase) and expression vectors for TAK1 (DsRed-TAK1) (A and C) or a kinase-dead form of TAK1 [TAK1(K63W)] (B and D) and mature forms of SREBP together with a constitutive promoter-driven renilla luciferase reporter. (A, B) The levels of transfected SREBPs and TAK1 were analyzed by immunoblotting. (C) SREBP-dependent transcriptional activity was determined by luciferase activity. Relative SREBP activity normalized to the protein levels of transfected SREBPs are shown. Means  $\pm$  SD.  $n = 3$ ; \*\*\*,  $p < 0.001$  (two-tailed unpaired Student's *t* test). (D) SREBP-dependent transcriptional activity normalized to renilla luciferase activity is shown. Means  $\pm$  SD ;  $n = 3$ ; \*\*\*,  $p < 0.001$  (one-way ANOVA).



**Figure 4. Ablation of TAK1 augments SREBP activity**  
 (A, B) HEK293 cells were treated with three independent siRNAs targeted against TAK1, and SRE-luciferase and control EF1 $\alpha$ -renilla luciferase vectors were subsequently transfected at one-day post siRNA introduction. Cells were left untreated or treated with methyl- $\beta$ -cyclodextrin 6 h before the cell harvesting. Proteins were analyzed by immunoblotting (A). Luciferase activity is normalized to renilla luciferase activity (B). Means  $\pm$ SD; n = 3; \*\*\*, p < 0.001 (one-way ANOVA). (C) A vector expressing small hairpin RNA targeted against TAK1 was transfected into HepG2 cells. Cells were subsequently transfected with the expression vectors for mature SREBPs and the same

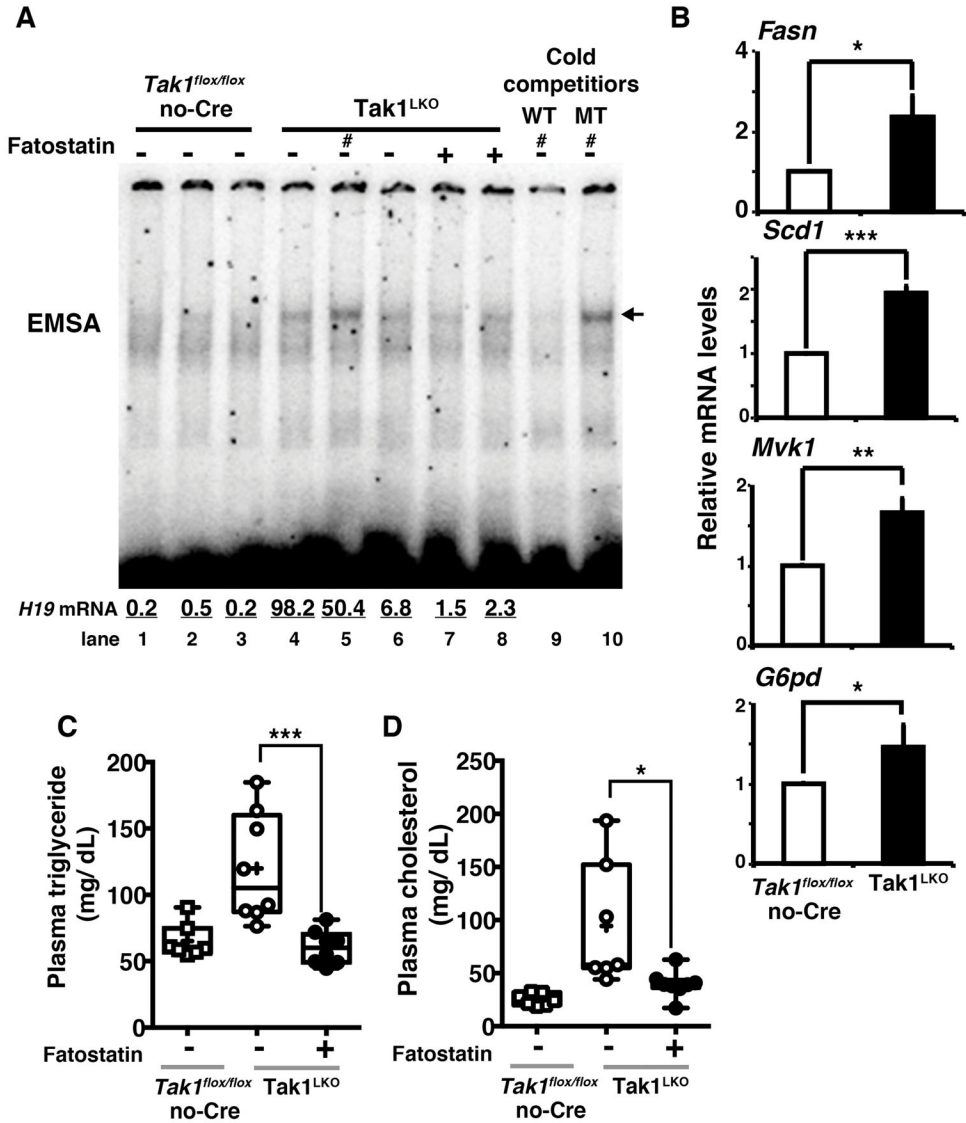
reporters used in (A, B). Proteins were analyzed by immunoblotting to determine the protein level of TAK1 (right panes). The level of TAK1 was not altered by the 2nd transfections. Luciferase activity is normalized to renilla luciferase activity. Means  $\pm$ SD; n = 3; \*\*, p < 0.01; \*\*\*, p < 0.001 (two-tailed unpaired Student's t test).

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**Figure 5. Deletion of *Tak1* upregulates SREBP in the liver**

(A) 4–6 weeks old control (*Tak1<sup>flox/flox</sup>* no-Cre) and *Tak1<sup>LKO</sup>* mice were treated with or without fatostatin for 30 days. Liver extracts were analyzed by electron mobility shift assay (EMSA). 100 fold excess cold wild type (WT) or mutant (MT) oligonucleotides described in Materials and Methods were added to the reaction mixture to examine the specific binding of the band (lane 9 and 10). Lanes indicated by a symbol # used the protein extract from the same mouse. The relative mRNA levels of *H19* relative to the levels of *Gapdh* are shown in the bottom of the panels. (B) Livers were isolated from 5–6 month old control (*Tak1<sup>flox/flox</sup>* no-Cre) and *Tak1<sup>LKO</sup>* mice, and the mRNA levels of SREBP target genes were analyzed. Means  $\pm$  SEM; n = 3; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 (two-tailed unpaired Student's t test). (C, D) Blood was collected from the control (*Tak1<sup>flox/flox</sup>* no-Cre) and *Tak1<sup>LKO</sup>* mice used in the experiment shown in (A). Blood triglyceride and cholesterol were determined. All data points are shown in the box and whisker plots: median and distribution of 50% of values are shown in the box: whiskers indicate distribution of minimum and

maximum values. Asterisks indicate means. Control (*Tak1<sup>flox/flox</sup> no-Cre*), n = 7; Tak1<sup>LKO</sup>, n = 7; Tak1<sup>LKO</sup> fatostatin treated, n = 8; \*, p < 0.05; \*\*\*, p < 0.001 (one-way ANOVA).

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