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Article

AMP as a Low-Energy Charge Signal Autonomously Initiates Assembly of AXIN-AMPK-LKB1 Complex for AMPK Activation

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SUMMARY

The AMP-activated protein kinase (AMPK) is a master regulator of metabolic homeostasis by sensing cellular energy status. AMPK is mainly activated via phosphorylation by LKB1 when cellular AMP/ADP levels are increased. However, how AMP/ADP brings about AMPK phosphorylation remains unclear. Here, we show that it is AMP, but not ADP, that drives AXIN to directly tether LKB1 to phosphorylate AMPK. The complex formation of AXIN-AMPK-LKB1 is greatly enhanced in glucose-starved or AICAR-treated cells and in cell-free systems supplemented with exogenous AMP. Depletion of *AXIN* abrogated starvation-induced AMPK-LKB1 colocalization. Importantly, adenovirus-based knockdown of *AXIN* in the mouse liver impaired AMPK activation and caused exacerbated fatty liver after starvation, underscoring an essential role of AXIN in AMPK activation. These findings demonstrate an initiating role of AMP and demonstrate that AXIN directly transmits AMP binding of AMPK to its activation by LKB1, uncovering the mechanistic route for AMP to elicit AMPK activation by LKB1.

INTRODUCTION

Levels of AMP and ADP, as a result of consuming ATP, will rise under nutrient-poor conditions or when exercise is heightened (Hardie et al., 2012b; Carling et al., 2012). To maintain energy homeostasis, regulatory proteins inside the cell must possess the ability to sense the falling energy status. The AMP-activated protein kinase (AMPK) has been shown to be the principal sensor, switching off anabolic pathways such as fatty acid, triglyceride, cholesterol, and protein synthesis, and switching on catabolic pathways such as fatty acid oxidation and glycolysis. By virtue of switching off the anabolic processes, at least in part, AMPK activation also suppresses cell proliferation, which may account for the tumor suppression of its upstream activating kinase liver kinase B1 (LKB1) (Hardie and Alessi, 2013; Hardie et al., 2012a; Motoshima et al., 2006). These properties have led to growing efforts to pharmacologically target AMPK for treating metabolic diseases including obesity, type 2 diabetes, cardiovascular diseases, and cancer (Steinberg and Kemp, 2009).

In mammals, AMPK exists as a heterotrimer, comprised of one catalytic subunit (α) and two regulatory subunits (β and γ). The catalytic α subunit contains an N-terminal Ser/Thr kinase domain, followed by an autoinhibitory domain (AID) that directly binds to and thus inhibits the kinase domain (Chen et al., 2009; Crute et al., 1998; Pang et al., 2007). The mechanism of AMPK activation requires the presence of LKB1 that phosphorylates the conserved threonine residue in the activation loop of AMPKα (Thr172 in human α1), leading to a more than 100-fold increase of its kinase activity (Hawley et al., 1996, 2003; Shaw et al., 2004; Woods et al., 2003). LKB1 is allosterically activated when forming a heterotrimeric complex with the pseudokinase STRAD and the adaptor protein MO25 (Baas et al., 2003; Boudeau et al., 2003; Hawley et al., 2003; Zeqiraj et al., 2009). Genetic depletion of *LKB1* leads to aberrant activation of AMPK (Shaw et al., 2005). AMPK can also be phosphorylated at Thr172 in response to calcium flux, through calmodulin-dependent protein kinase kinase β (CaMKKβ) (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). In addition to phosphorylation, acetylation/deacetylation has also been suggested to take part in the regulation of AMPK activity (Lin et al., 2012). The AMPKβ subunit plays a key role in heterotrimerization by tethering the α subunits and γ subunits (Hudson et al., 2003; Iseli et al., 2005). Recently, myristoylation on AMPKβ that facilitates membrane binding of AMPK is reported as an essential step for the initiation of AMPK signaling in response to rises in both AMP and ADP (Oakhill et al., 2010, 2011).

The γ subunit contains four tandem repeats (CBS1–CSB4) that form four putative binding sites for adenosyl-containing molecules (Bateman, 1997; Kemp, 2004; Scott et al., 2004), and the binding of different nucleotides can lead to significant changes of the γ subunit conformation (Chen et al., 2012), endowing

AMPK with the ability to sense physiological AMP or ADP elevation from stresses such as nutrient deprivation, hypoxia, or prolonged exercises. Several functional modes of AMP and ADP have been demonstrated: promoting Thr172 phosphorylation at AMPKα (Hawley et al., 1995, 2003; Oakhill et al., 2010, 2011) and preventing AMPK dephosphorylation (Davies et al., 1995; Oakhill et al., 2010, 2011; Sanders et al., 2007). Studies have also shown that AMP causes AMPK to undergo allosteric changes (Chen et al., 2009; Moore et al., 1991; Oakhill et al., 2010; Suter et al., 2006). However, although early studies employing crude AMPK and its upstream kinase reported a direct promoting effect of AMP on Thr172 phosphorylation (Davies et al., 1995; Moore et al., 1991), follow-up experiments demonstrated that recombinant AMPK expressed in *E. coli* was not stimulated by AMP (Sanders et al., 2007; Suter et al., 2006; Woods et al., 2003). Recently this discrepancy was reconciled by using COS-7-cell-expressed AMPK heterotrimer containing myristoylated AMPKβ (Oakhill et al., 2010), highlighting the significance of additional eukaryotic modification in AMPK activation. It is important to note that the effects of AMP and ADP on AMPK activation are elicited by direct binding to AMPK, but not by regulating the upstream activating kinase LKB1 or phosphatase (Hardie et al., 2012b). Thus, how AMP or ADP promotes AMPK activation is mechanistically unclear. Here, we show that AMP, but not ADP, directly triggers a signaling loop for AMPK activation, in that AMP binding drives AMPK to interact with AXIN-LKB1, resulting in the formation of the AXIN-AMPK-LKB1 complex to facilitate phosphorylation of LKB1 toward AMPK. Importantly, knockdown of *AXIN* renders AMPK unable to be activated, leading to exacerbated starvation-induced fatty liver as a result of dysregulated AMPK target proteins or genes such as acyl-CoA carboxylase (ACC) and sterol regulatory element-binding protein-1c (*SREBP-1C*). We have thus uncovered a necessary role of AXIN, which integrates the activating role of AMP, allosteric changes upon nucleotide binding, the requirement of LKB1, and myristoylation of the β subunit as a necessary element for AMPK activation in response to energy stress.

RESULTS

AXIN Is Essential for AMPK Activation and Regulation of Lipid Homeostasis

AXIN was originally discovered as a central regulator of Wnt signaling, and its deficiency leads to duplication of body axis (Zeng et al., 1997). It has also been shown to play critical roles in the crosstalk between Wnt signaling and regulation of mammalian target of rapamycin (mTOR) (Inoki et al., 2006), cell-fate determination upon DNA damage (Li et al., 2009), and insulin-stimulated glucose uptake (Guo et al., 2012). In the present study, we employed adenovirus-based gene silencing approach to specifically deplete AXIN in the mouse liver, the organ that plays a major role in metabolism on all levels. Severe starvation may lead to steatosis (Kneeman et al., 2012; van Ginneken et al., 2007). Knockdown of *AXIN* separately by two independent siRNAs significantly increased hepatic triglyceride contents in both fed and 16 hr-fasted mice, as measured by a colorimetric assay and oil red O staining (Figures 1A and 1B and see Figure S1 [available online]). The effect of *AXIN* knockdown was particularly marked in fasted mice, suggesting a defect in the control of lipid biosynthesis. As AMPK plays a key role in lipid homeostasis (Assifi et al., 2005; Steinberg and Kemp, 2009), we first investigated the activity of AMPK by determining levels of phosphorylation of Thr172 on AMPKα, and phosphorylation of its substrate ACC, a critical enzyme controlling the rate of the synthesis of fatty acids. ACC is inhibited upon phosphorylation by AMPK (Davies et al., 1990). As shown in Figures 1C and 1D, levels of phospho-AMPK and phospho-ACC were greatly reduced in both regularly fed (basal) and starved mice injected with adenovirus delivering *AXIN* siRNA compared to the respective mice injected with the control virus, indicating that AMPK is not properly activated when *AXIN* is knocked down. This conjecture is also supported by the observation that the mRNA levels of *SREBP-1C*, a target gene negatively regulated by AMPK (Li et al., 2011), and of fatty acid synthase (*FAS*) and stearoyl-CoA desaturase-1 (*SCD-1*), the target genes of *SREBP-1C*, were significantly increased in the mice with *AXIN* knockdown (Figure 1E). These data indicate that depletion of *AXIN* results in exacerbated fatty liver under prolonged starvation, which could be attributed to attenuated AMPK inhibition of lipogenesis and its upregulation of lipid consumption.

We also investigated the connection between AXIN and AMPK in regulating lipid metabolism in cell-culture systems. Human liver L02 cells were transfected with *AXIN* siRNA vector or control empty vector carrying a GFP reporter. Oleic acid was kept in the culture medium overnight to increase the basal lipid content. Knockdown of *AXIN* led to a much greater increase of lipid droplets compared to cells transfected with control vector (Figure 2A). The culture medium was then changed to glucose-free medium or supplemented with the cell-permeable AMP mimetic AICAR to speed up fat consumption. An obvious fall in the lipid content was observed in control vector transfected cells, which was partially blocked by depletion of *AXIN* (Figures 2A and S2A). We also investigated the lipid contents in *AMPKα1 and AMPKα2* double knockout (DKO) mouse embryonic fibroblasts (MEFs). Deficiency of AMPKα led to enhanced lipid accumulation in cells treated with oleic acid (Figures 2B and S2B). The content of lipids failed to decrease when these cells were deprived of glucose or treated with AICAR (Figure S2B), resembling the effect of knockdown of *AXIN* (Figure S2C). *AXIN* siRNA made no further accumulation of lipid droplets in *AMPKα* DKO MEFs (Figure 2B), indicating that AXIN and AMPK function in the same axis for controlling lipid homeostasis. In L02 cells, the hepatic cancer HepG2 cells, or MEF cells, knockdown of *AXIN* severely impaired AMPK activation induced by glucose starvation (Figures 2C and S2D), by treatment with 2-deoxy-D-glucose (2-DG) (Figures 2D and S2D), or AICAR (Figures 2E and S2D–S2F). These results indicate that AXIN is essential for AMPK activation in intact cells.

AXIN, AMPK, and LKB1 Form a Complex

We then explored the mechanism by which AXIN modulates AMPK activation, first by testing whether AXIN could physically interact with AMPK or the activating kinase LKB1 in the cells. We subjected lysates of MEF cells to immunoprecipitation (IP) by antibody against AMPK, followed by immunoblotting to detect AXIN and LKB1-STRAD-MO25 as well as AMPK. AXIN was coimmunoprecipitated with AMPK along with LKB1-STRAD-MO25, and their coimmunoprecipitation (coIP) was

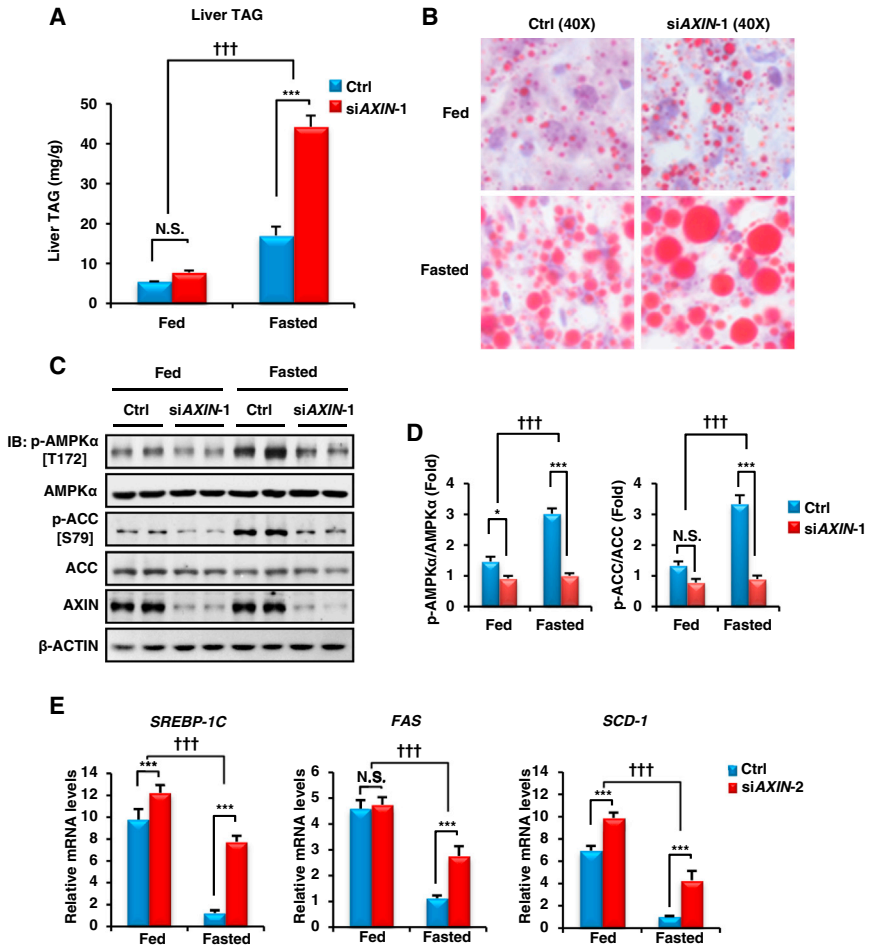

Figure 1. Exacerbated Fatty Liver in Mice Depleted of Hepatic AXIN

(A) Adenovirus expressing *AXIN* siRNA or control vector was injected into the tail veins of 3-month-old BALB/c mice. Five days later, hepatic triglyceride (TAG) contents in the liver of the fed or 16 hr-fasted mice were determined. The dark-light cycle is from 8 a.m. to 8 p.m. The food was withdrawn from the cage at 5 p.m., and the mice were sacrificed at 9 a.m. the next day. Values are presented as mean ± SEM, n = 5–7, ***p < 0.001, †††p < 0.001 (ANOVA followed by Tukey).

(B) Oil red O staining of sections of the livers from control and *AXIN* knockdown mice in fed or fasted state.

(C) Immunoblot analysis of phospho-AMPKα, phospho-ACC, total levels of AMPKα, ACC, and AXIN in the livers of the infected mice.

(D) Statistical analysis of p-AMPKα/AMPKα and p-ACC/ACC levels in the livers of infected mice. Values are presented as mean ± SEM, n = 8–10, *p < 0.05, ***p < 0.001, †††p < 0.001 (ANOVA followed by Tukey).

(E) Levels of *SREBP-1C*, *FAS*, and *SCD-1* mRNA in the liver of mice infected with *AXIN* siRNA-2. Control and Adeno-si*AXIN*-2-infected mice were sacrificed on day 5 after virus injection. Livers were quickly removed and fixed in Trizol reagent. The mRNA levels of *SREBP-1C*, *FAS*, and *SCD-1* were analyzed by real-time-RT-PCR. Values are presented as mean ± SEM, n = 4, **p < 0.01, ***p < 0.001, †††p < 0.001 (ANOVA followed by Tukey). See also Figure S1.

dramatically enhanced by glucose starvation (Figure 3A, left panel). Similarly, when IP was performed with antibody against AXIN, increased amounts of AMPK and LKB1-STRAD-MO25 were coimmunoprecipitated under starvation (Figure 3A, right panel; Figure S3A). Pull-down assays showed a direct interaction between AXIN and LKB1 as well as between AXIN and AMPK holoenzyme (Figures 3B and 3C). As STRAD and MO25 were coprecipitated with AXIN, we asked whether such association depends on the presence of LKB1. In LKB1-deficient HeLa cells transfected with HA-STRAD, FLAG-MO25, and Myc-AXIN as indicated, no interaction between AXIN and STRAD or MO25 was detected. LKB1 overexpression in these cells restored the interaction between these proteins (Figure S3B). However, knockdown of *AXIN* attenuated glucose starvation-enhanced LKB1-STRAD-MO25 interaction (Figure S3C). Domain mapping using a series of deletion mutants of AXIN revealed that the region encompassing residues aa 507–731 is critical for interacting with both LKB1 and AMPK (Figures S3D–S3F), coinciding with the sites for interacting with PP2c, RNF111, PIRH2, and TIP60 (Li et al., 2009; Liu et al., 2006; Strovel et al., 2000).

To unequivocally determine whether AXIN, AMPK, and LKB1 could coexist in the same complex, we employed a two-step coIP assay using lysates from cells coexpressing FLAG-AXIN, HA-AMPK, and Myc-LKB1, in that AXIN was immunoprecipitated with anti-FLAG, and the eluted proteins were subjected to a second round of IP by using anti-HA to precipitate AMPK. The result showed that the three components were all detected in the final immunoprecipitate (Figure S3G). We next explored the possibility that AXIN might facilitate the association of LKB1 with AMPK by ectopically expressing AXIN in HEK293T cells. It was found that increased expression of AXIN indeed facilitates the interaction between AMPK and LKB1 (Figure S3H). Immunofluorescent costaining in L02 cells revealed that knockdown of *AXIN* abrogates the colocalization of AMPK and HA-LKB1 in cells deprived of glucose (Figure 3D). It is observed that *AXIN* knockdown depressed the increase of LKB1 cytosol localization upon GS treatment (Figure 3D and Figure S3I).

We also tested for a possible role of AXIN in the CaMKK-β-AMPK axis. Knockdown of *AXIN* did not blunt AMPK activation in LKB1-deficient HeLa cells by the CaMKKβ activator calcium ionophore A23187 (Figure S3J). Consistently, no interaction between AXIN and CaMKKβ was detected (Figure S3K). These results indicate that AXIN specifically regulates the AMPK phosphorylation process catalyzed by LKB1 but not by CaMKKβ.

AMP Increases the Affinity of AMPK for AXIN

We then set out to investigate how the formation of the AXIN-AMPK-LKB1 complex is regulated. Nucleotide binding is

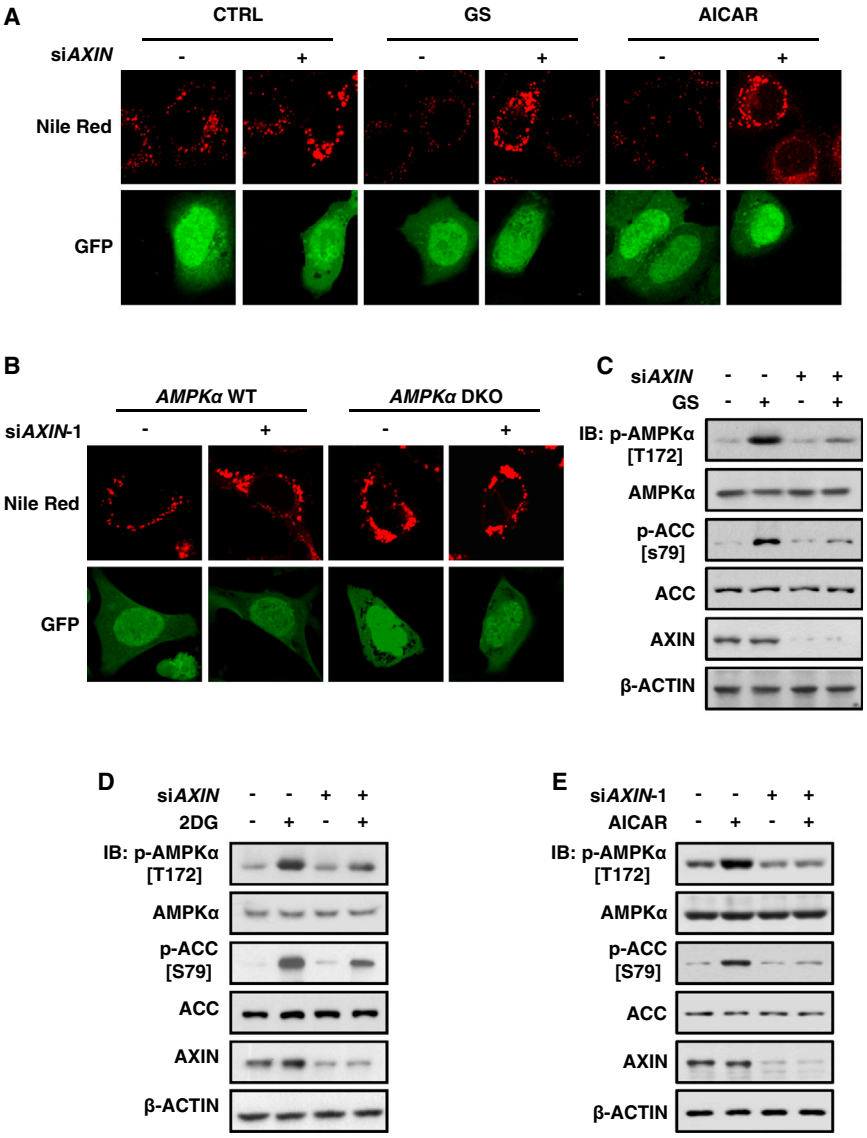

Figure 2. AXIN Is Essential in AMPK Activation

(A) Knockdown *AXIN* promotes lipid accumulation in L02 cells. Human liver L02 cells were transfected with pLL3.7-GFP or pLL3.7-GFP-si*AXIN* expressing siRNA against AXIN. Some 6 hr later, L02 cells were incubated in medium with 200 μM sodium oleate overnight. Then cells were washed once with PBS and cultured in medium with or without glucose (1 mM AICAR) for 4 hr. Lipid droplets were stained with Nile red (0.05 μg/ml) for 10 min. GS, glucose starvation.

(B) Depletion of AMPKα exhibits similar effect on lipid accumulation as *AXIN* knockdown. *AMPKα1* and *AMPKα2* double knockout MEFs (*AMPKα* DKO) and its wild-type (WT) counterparts were infected with Adeno-GFP-si*AXIN*-1 virus or Adeno-GFP virus as a control. Six hours later, cells were incubated in fresh medium supplemented with 200 μM sodium oleate overnight (20 hr). Lipid droplets were stained with Nile red (0.05 μg/ml) for 10 min.

(C–E) Impaired AMPK activation in *AXIN* knockdown cells. L02 cells (C), HepG2 cells (D), or MEF cells (E) were infected by lentivirus expressing control siRNA or *AXIN* siRNA. Cells were incubated with culture medium deprived of glucose for 1 hr (C) and treated with 2-DG (25 mM) (D) or AICAR (1 mM) (E) for 30 min. Cell lysates were then analyzed by immunoblotting with indicated antibodies.

See also Figure S2.

reported to be essential for promoting Thr172 phosphorylation on AMPKα (Hawley et al., 2003; Oakhill et al., 2010, 2011). We thus tested the effect of adenosyl nucleotides on the interaction of AXIN and AMPK at their endogenous levels in a cell-free system. Addition of AMP to L02 cell lysates robustly increased the coIP of AMPK and AXIN, whereas ATP decreased the interaction (Figures 4A and 4B). Surprisingly, ADP had no effect on the interaction between AXIN and AMPK (Figure 4A). We also analyzed the AXIN-AMPK interaction in cells treated with AICAR. It was found that AICAR significantly increased the affinity of AMPK for AXIN (Figure 4C). To further test the importance of AMP binding in enhancing the affinity of AMPK for AXIN, we expressed an AMPK holoenzyme with the γ-D317A mutation in CBS4 which is unable to bind AMP (Adams et al., 2004; Chen et al., 2012; Xiao et al., 2007). Exogenous AMP did not increase the affinity of the human AMPK mutant for AXIN in the lysates of HEK293T cells (Figure 4D).

AMPK Myristoylation Is Required for AMP-Induced AXIN-AMPK Interaction

When we tried to test the promoting effect of AMP on AXIN-AMPK interaction by in vitro reconstitution using purified proteins expressed in bacteria, it was found that AMP failed to, or only mildly, increase the affinity of AMPK for AXIN (Figures 5A and S4A). The discrepancy between the results obtained using bacterially purified AMPK and eukaryotically expressed native AMPK suggested that posttranslational modification is required for AMP to promote AXIN-AMPK interaction. As it was reported that myristoylation is necessary for AMP or ADP to promote AMPK activation (Oakhill et al., 2010, 2011), we tested whether the increased AMPK-AXIN interaction requires prior myristoylation of AMPK β subunit in the cell by ectopically expressing AMPKβ-G2A, a mutant with its myristoylation site Gly2 changed to Ala. As shown in Figure 5B, AMPKβ-G2A blunted the AMP-enhanced interaction between AMPK and AXIN. The effect of AMP on the affinity of myristoylated AMPK for AXIN was recapitulated by in vitro reconstitution, in that AMPK was purified from bacteria cotransformed with or without N-myristoyltransferase 1 (NMT1) (Figures 5C and S4B). Consistent with the result in cell-free systems, AMP increased the affinity of AMPK for AXIN, while addition of ADP has no discernible effect (Figures 5D and S4C). We also found that AMP promotes the

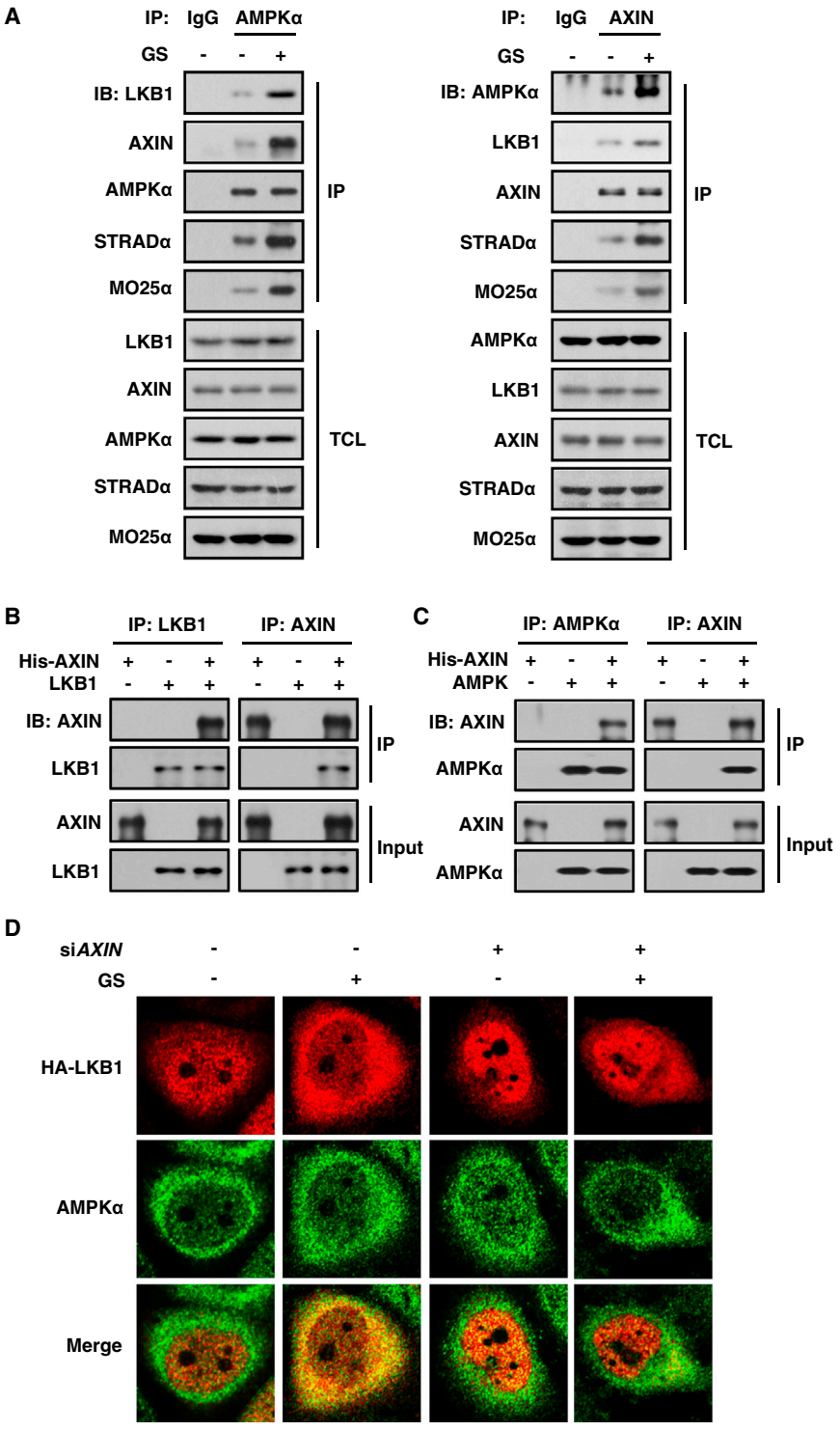

Figure 3. Complex Formation among AXIN, AMPK, and LKB1

(A) Coimmunoprecipitation (coIP) of endogenous AXIN, AMPK, and LKB1 in MEF cells deprived of glucose. Protein extracts were immunoprecipitated with antibody against AMPKα (left panel) or AXIN (right panel), followed by immunoblotting with antibodies indicated. TCL, total cell lysate.

(B) Direct interaction between AXIN and LKB1. AXIN and LKB1 were incubated in a cell lysis buffer described in the Experimental Procedures. Pull-down assays were performed with antibody against LKB1 (left panel) or AXIN (right panel), and the immunoprecipitates were subjected to immunoblotting with indicated antibodies.

(C) Direct interaction between AXIN and AMPK complex. Pull-down assays were performed with antibody against AMPKα (left panel) or AXIN (right panel), followed by immunoblotting with antibodies indicated.

(D) Glucose deprivation-induced colocalization between AMPK and LKB1 depends on AXIN. Knockdown of *AXIN* impaired the association of HA-LKB1 with AMPK in L02 cells glucose starved for 3 hr. Mouse anti-AMPKα antibody, donkey anti-mouse IgG Alexa Fluor 488 secondary antibody for AMPKα (green), and rabbit anti-HA antibody and donkey anti-rabbit IgG Alexa Fluor 594 secondary antibody for LKB1 (red) were used.

See also Figure S3.

interaction of AMPK and AXIN in a dose-dependent manner (Figure 5E). Such an increase was not seen with the AXIN mutant lacking the region of aa 507–731, a domain that was mapped responsible for binding to AMPK and LKB1 (Figure 5F). These results indicate that myristoylation on AMPKβ is a prerequisite modification for AMP to drive AMPK to interact with AXIN.

AXIN Promotes Phosphorylation of AMPK by LKB1

We then investigated the effects of AXIN and AMP on the phosphorylation of AMPK by LKB1, a crucial step for AMPK activation. We first tested whether the AMPK-LKB1 interaction is modulated by AMP in the presence or absence of AXIN using in vitro reconstitution. When AXIN was absent, AMP failed to increase the association of LKB1 with AMPK regardless of myristoylation. In the presence of AXIN, AMP enhanced the interaction of LKB1 with AMPK most dramatically when the AMPK protein is myristoylated (Figures 6A, S5A, and S5B). Consistent with its inability to promote AMPK-AXIN interaction, ADP did not enhance the interaction between LKB1 and myristoylated AMPK even when AXIN was present (Figure 6B). We also analyzed the effects of AXIN and AMP on AMPK-LKB1 interaction in a cell-free system using lysates from MEF cells. AMP addition increased the amount of LKB1 in the AMPK immunoprecipitate from the MEF cells, but not from *AXIN* siRNA-expressing cells (Figure 6C), indicating that AMP depends on AXIN to promote AMPK-LKB1 interaction. Similar results were obtained in AICAR-treated MEF cells (Figures 6D and S5C). These results demonstrate that the interaction

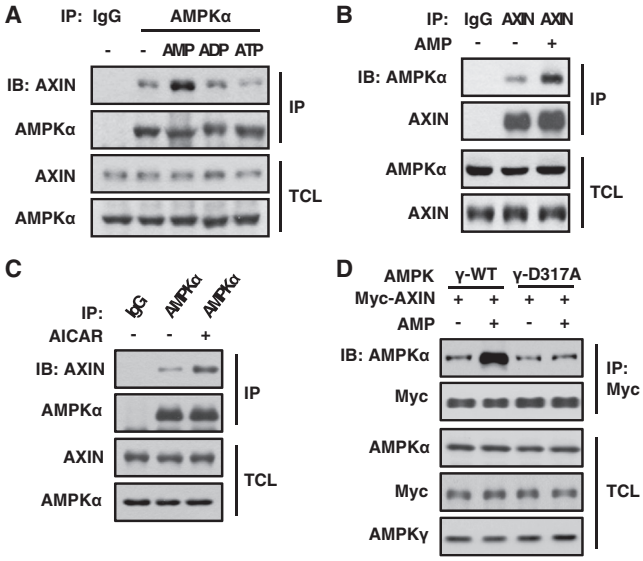

Figure 4. AMP Increases the Affinity of AMPK for AXIN

(A) Exogenous AMP promotes interaction between AXIN and AMPK. L02 liver cell lysates were incubated with 200 μM AMP, ADP, or ATP-γ-S together with 20 μM adenylate kinase inhibitor Ap_5A overnight at 4°C. IP was then performed against AMPKα using the lysates, followed by immunoblotting using the antibodies indicated.

(B) Exogenous AMP enhances the interaction between endogenous AMPK and AXIN. Lysates of L02 liver cells were incubated with AMP overnight at 4°C. IP was then performed against AXIN using the lysates, followed by immunoblotting using the antibodies indicated.

(C) AICAR promotes AXIN-AMPK interaction. L02 liver cells were treated with AICAR (1 mM) for 1 hr. IP was then performed against AMPKα using cell lysates, followed by immunoblotting using the antibodies indicated.

(D) Mutational analysis for the importance of AMP binding to AMPKγ in intrasteric regulation of the affinity of AMPK for AXIN. $AMPK\gamma_1$ or $AMPK\gamma_1$-D317A was cotransfected with Myc-AXIN, HA-AMPKα, and AMPKβ into HEK293T cells. The lysates of the transfected cells were added with or without AMP, followed by IP using anti-Myc antibody.

between AMPK and LKB1 is regulated and that AXIN is essential for AMP- or AICAR-enhanced LKB1-AMPK association.

We then explored the functional consequence of AXIN on AMPK activation by assessing Thr172 phosphorylation on AMPKα. AXIN and AMP synergistically enhanced the phosphorylation of Thr172 on myristoylated AMPKα, but not on nonmyristoylated AMPKα (Figure 6E). We also used AMPK immunoprecipitated from HEK293T cells and found again that AMP or purified AXIN protein alone only moderately increased Thr172 phosphorylation level, whereas in the presence of both AMP and AXIN, Thr172 phosphorylation was dramatically increased (Figure S5D). AXINΔ507-731, defective in binding to AMPK or LKB1, failed to promote Thr172 phosphorylation, indicating that the binding to both LKB1 and AMPK is required for AXIN to promote AMP-dependent phosphorylation of AMPK (Figure S5E). It has been reported that phosphatase PP2c is an AXIN-interacting protein (Strovel et al., 2000) and involves in the dephosphorylation of Thr172 on AMPK (Davies et al., 1995; Hawley et al., 1996; Wang and Unger, 2005; Wu et al., 2007). We thus also tested whether AXIN could regulate the dephosphorylation process of AMPK. As shown in Figure S5F, addition of AXIN has no influence on PP2c-catalyzed dephosphorylation of AMPK. These results indicate that AXIN regulates AMPK activity mainly through promoting its phosphorylation by LKB1.

DISCUSSION

We have clearly demonstrated here that AXIN forms a complex with AMPK and LKB1, which is initiated by AMP binding to the γ subunit of AMPK. Perhaps due to the lethality of its deficiency, little was known about the physiological roles of AXIN in tissues or at the organismal level. It is uncovered here that depletion of *AXIN* in the liver, separately by two independent adenovirus-based gene silencing vectors, engenders failure of AMPK activation, leading to dysregulation of the AMPK signaling axis. The affected AMPK targets include ACC and *SREBP-1C*, which converge to cause loss of lipid homeostasis as manifested by exacerbated starvation-induced fatty liver in the mouse infected by si*AXIN*-expressing adenoviruses (Figures 1A, 1B, and S1). The essential role of AXIN in AMPK activation and lipid homeostasis control can also be seen in cell-culture systems. Knockdown of *AXIN* dampens AMPK activation in response to glucose starvation or AICAR treatment in L02, HepG2, or MEF cells (Figures 2C–2E and S2D) and leads to accumulation of large lipid droplets in L02 cells (Figures 2A and S2A) and MEF cells (Figures 2B, S2B, and S2C) cultured with supplemented oleic acid.

We have provided multiple lines of evidence to unravel the mechanism for the role of AXIN in mediating AMP-triggered AMPK activation by LKB1. First of all, depletion of *AXIN* abrogates colocalization of AMPK with LKB1 under starvation (Figure 3D), suggesting that AXIN plays a critical role in tethering LKB1 to AMPK. We showed that AXIN directly interacts with AMPK and LKB1 as determined by two-step coIP and by reconstitution using purified proteins (Figure S3G). The two critical regulators of LKB1, STRAD and MO25, are copresent in the AXIN-LKB1-AMPK complex (Figure 3A), in an LKB1-dependent manner (Figure S3B). Importantly, the interaction of AXIN with AMPK depends on the presence of AMP (Figures 4A–4D). It is conceivable that AMP binding, as an initial step in sensing a low-energy state, increases the affinity of AMPK, likely by causing changes in AMPK conformation (Chen et al., 2009), for the scaffold protein AXIN that tethers the activating kinase LKB1 to phosphorylate AMPK at Thr172 (Figure 6F). In earlier studies, LKB1 was demonstrated to readily phosphorylate AMPK in enzymatic assays (Hawley et al., 2003; Hong et al., 2003; Shaw et al., 2004). Despite this, we were able to show that LKB1 phosphorylation of AMPK is synergistically enhanced in a reconstituted reaction that includes AXIN and AMP (Figures 6E and S5D). Consistently, mutations in the γ subunit of AMPK that fail to bind AMP cannot be stimulated by AXIN (Figure 4D). These data emphasize the importance of AMP as a triggering factor in initiating the activation of AMPK during starvation. Furthermore, prior cotranslational modification by myristoylation, which has been shown to be a prerequisite step for AMPK activation, is critical for AXIN to promote the activation of AMPK triggered by AMP binding, determined in cells as well as by in vitro reconstitution (Figures 5A–5F).

We found that ADP does not appear to play a role in promoting the AXIN-based complex formation. Unlike AMP, ADP does not promote interaction between AXIN and AMPK when added into cell-free systems, nor does it promote LKB1-AMPK interaction

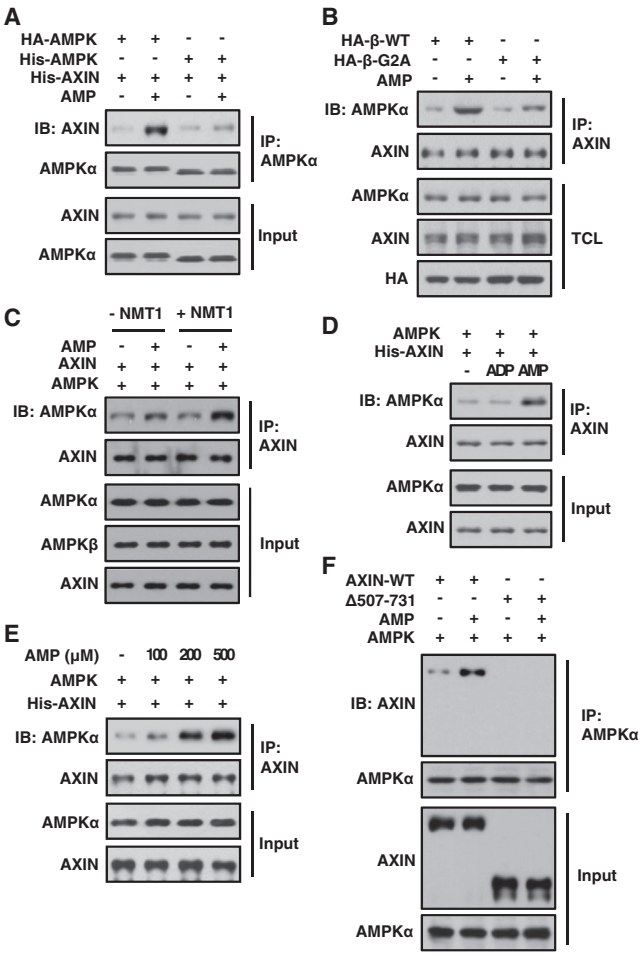

Figure 5. Myristoylation on AMPKβ Is Required for AMP-Induced AXIN-AMPK Interaction

(A) AMP only mildly increases the affinity of bacterially expressed unmyristoylated AMPK for AXIN. Bacterially expressed AXIN was incubated with HA-AMPK immunoprecipitated from HEK293T cells or His-AMPK purified from *E. coli*. The mixtures were supplemented with 200 μM AMP and then immunoprecipitated with antibody against AMPKα and subjected to immunoblotting with antibodies indicated.

(B) AMP-enhanced AMPK-AXIN interaction was blunted by overexpression of AMPKβ-G2A that cannot be myristoylated.

(C) Promotion of AXIN-AMPK interaction by AMP depends on myristoylation of AMPK. Purified AXIN and AMPK bacterially expressed with or without NMT1 were incubated with 200 μM AMP. The mixtures were immunoprecipitated with antibody against AXIN and then subjected to immunoblotting with antibodies indicated.

(D) AMP, but not ADP, enhances the interaction between AXIN and myristoylated AMPK. Purified AXIN and myristoylated AMPK purified from bacteria coexpressing NMT-1 were mixed with or without 200 μM AMP or ADP as indicated. The mixtures were immunoprecipitated with antibody against AXIN and then subjected to immunoblotting with antibodies indicated.

(E) AMP increases the binding affinity of AMPK for AXIN in a dose-dependent manner. Purified AXIN and myristoylated AMPK were incubated with AMP in the range between 0 and 500 μM. The mixtures were immunoprecipitated with antibody against AXIN and then subjected to immunoblotting with antibodies indicated.

(F) The region encompassing aa 507–731 of AXIN is essential for AMP-triggered AXIN-AMPK association. AXIN or its deletion mutants were incubated with myristoylated-AMPK with or without 200 μM AMP, followed by IP of AMPKα.

See also Figure S4.

in vitro (Figures 4A and 6B). As noted, ADP is accumulated during mild energy stress (Hardie et al., 2012b); when the ADP:ATP ratio rises further under more severe stresses, the adenylate kinase is displaced to convert ADP to AMP, giving rise to a significant increase of AMP. It therefore stands to reason that the AMP-driven AXIN-based activating loop may occur only during severe energy stress, and that ADP displaces ATP in AMPK to elicit an early response to energy shortage by sustaining AMPK at its phosphorylated state, although it is possible that ADP may also stimulate LKB1 phosphorylation of AMPK after the formation of the complex (Oakhill et al., 2011). Our experiments also conform to the finding that myristoylation is required for AMPK activation. We showed that AMP fails to promote interaction of AXIN with unmyristoylated AMPK (Figure 5A), and importantly that AMP or AXIN could not efficiently promote LKB1 phosphorylation of nonmyristoylated AMPK, as assessed by in vitro reconstitution experiments (Figure 6E).

In the regulation of the Wnt pathway, AXIN interacts with the tumor suppressor adenomatous polyposis coli (APC), β-catenin, casein kinases, and glycogen synthase kinase 3-β (GSK3-β), serving as a platform for the degradation complex for β-catenin, whose accumulation has been linked to several cancer types (Stamos and Weis, 2013). AXIN also assembles an activating complex for p53 in response to DNA damage (Li et al., 2009). The AXIN-GSK3 connection has also been shown to play a role in Wnt-stimulated mTOR signaling and phosphorylates and inhibits the tuberous sclerosis complex subunit 2 (TSC2) tumor suppressor, which leads to deinhibition of mTOR (Inoki et al., 2006), pointing to diverse roles of AXIN in regulating metabolic homeostasis. We have shown that knockdown of *AXIN* leads to severely compromised AMPK activation and ACC inhibition, which resulted in severer fatty liver in mice under starvation and lipid accumulation in cell-culture systems (Figures 1A–1C, 2A, and 2B). Whether or not AXIN plays a general role in coordinating the regulation of AMPK, mTOR, and lipid homeostasis remains to be further explored. In addition, as AXIN is a key player of p53 signaling that plays multiple functions in metabolism (Levine and Puzio-Kuter, 2010; Li et al., 2009; Vousden, 2010), including regulation of anabolic and catabolic pathways as well as its involvement in AMPK signaling, it remains to be seen whether the AXIN-p53 axis also converges to regulate AMPK or form an intricate circuit in the control of metabolic homeostasis. Moreover, it has recently been shown that GSK3, which strongly interacts with AXIN, also plays a part in regulation of AMPK activity (Suzuki et al., 2013). It would be of interest to explore how the AXIN-GSK3 connection would regulate AMPK signaling. Taken together, our current work has provided the evidence of, and mechanistic insights into, the initiating role of AMP in the activation of AMPK by LKB1, an attribute of its ancestral role as a bona fide energy sensor. We have also revealed that AXIN plays an essential role in AMPK activation and lipid homeostasis. Moreover, we have provided evidence to suggest that AMP and ADP may serve distinct functions in response to different degrees of energy stress.

EXPERIMENTAL PROCEDURES

Tail Vein Injection of Adenoviruses

Adenovirus was propagated in AD293 cells and purified by cesium chloride density gradient ultracentrifugation. Adenoviruses (1×10^9

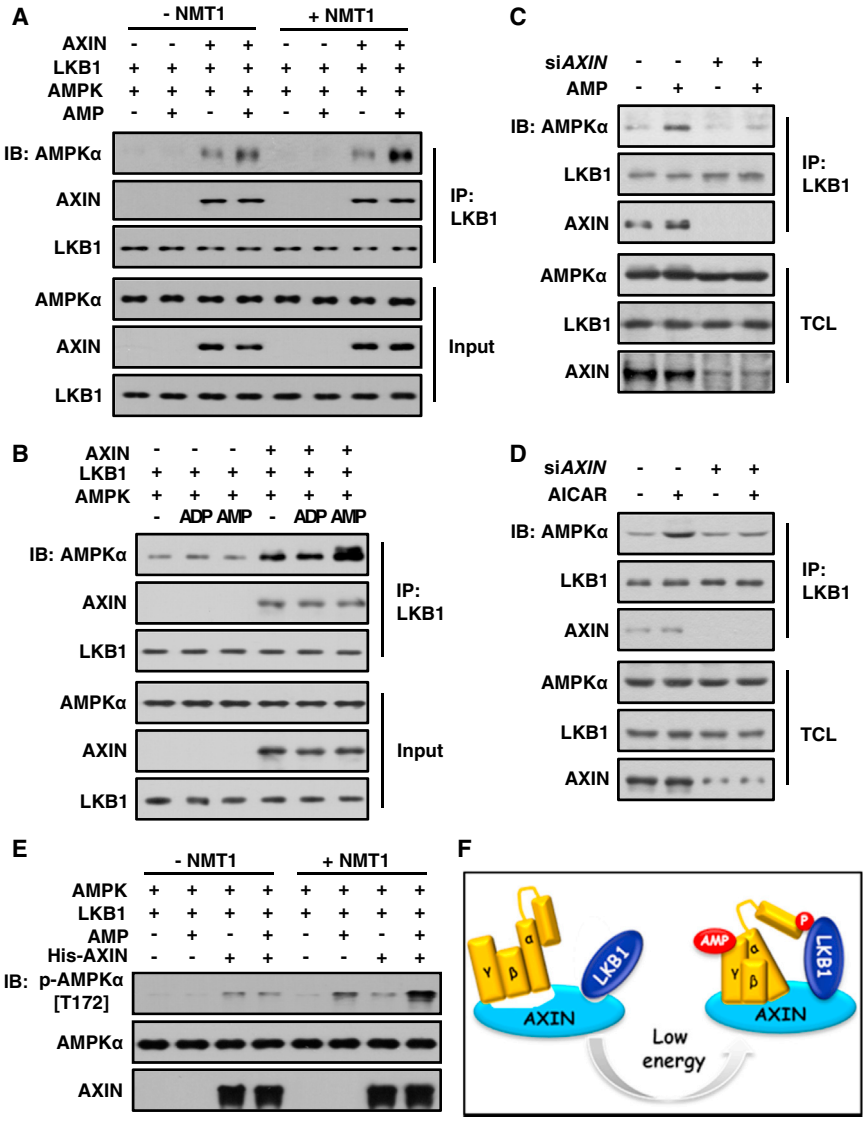

Figure 6. AXIN Promotes Phosphorylation of AMPK by LKB1

(A) AMP depends on AXIN to increase association of AMPK with LKB1. AXIN, myristoylated AMPK, and LKB1 complex were incubated with or without AMP. The mixtures were then immunoprecipitated with antibody against LKB1, followed by immunoblotting with antibodies indicated.

(B) AMP, but not ADP, enhances the interaction between LKB1 and myristoylated AMPK. LKB1 complex (active, containing MO25 and STRAD), myristoylated AMPK, and AXIN were incubated with or without 200 μM AMP or ADP. The mixtures were then immunoprecipitated with antibody against LKB1, followed by immunoblotting with antibodies indicated.

(C and D) Knockdown of *AXIN* in MEF cells, by using siRNA expressing lentivirus, in MEF cells diminished (C) AMP- or (D) AICAR-enhanced interaction between AMPK and LKB1. Cells were treated with 1 mM AICAR for 1 hr as indicated.

(E) AXIN and AMP synergistically facilitate the phosphorylation by LKB1 of AMPK that is myristoylated. AMPK holoenzyme was expressed and purified from bacteria cotransformed with or without NMT1. The conditions for kinase reactions are described in the Experimental Procedures.

(F) Simplified model demonstrating that AXIN plays an essential role for AMPK activation by orchestrating AMPK and LKB1. AMP exerts an initiating role for the assembly of the AMPK activating complex, by increasing the affinity of AMPK for AXIN that simultaneously binds LKB1. The resulting complex brings LKB1 to the vicinity of AMPK, promoting the phosphorylation of AMPK at Thr172.

See also Figure S5.

plaque-forming units) were injected via tail veins of 3-month-old BALB/c mice. Five days after injection, liver triglyceride contents were determined using Labassay triglyceride reagent (290-63701, Wako Pure Chemical Industries, Ltd.).

Oil Red O Staining

Liver frozen sections were fixed in 10% formalin and then stained for 10 min with Oil Red O. The red lipid droplets were visualized by microscopy.

Quantitative Real-Time PCR

Total RNA from liver was extracted with Trizol reagent. The cDNA was then synthesized using M-MLV RTase system. The analyses were performed using Step One Plus, the fast Real-Time PCR System from ABI (Applied Biosystems).

Cell Culture

AMPKα1/2 DKO MEFs were kindly provided by Drs. Benoit Viollet and Keith R. Laderoute. HEK293T and MEF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Human liver L02 and HepG2 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Lentivirus Infection

Lentiviruses for infection of L02, HepG2, and MEF cells were packaged in HEK293T cells using Lipofectamine 2000 transfection. Thirty hours after transfection, medium was collected and added to the target cells.

Immunoprecipitation and Western Blotting

Cell lysis and IP were carried out as previously described (Rui et al., 2004), with some modifications. Briefly, 50 mM NaCl was included in the lysis buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). ADP or AMP (200 μM) was added into the lysis buffer before IP where indicated. Cell lysates were incubated with respective antibodies overnight. The overnight protein aggregates were removed by centrifugation, and protein A/G beads were then added into the lysates and incubated for another 3 hr. Levels of total proteins and the levels of phosphorylation of the corresponding proteins were analyzed on separate gels.

Protein Production

Heterotrimeric AMPK (α1β1γ1 and mutants indicated) was expressed in the *E. coli* strain BL21 (DE3) as described previously (Neumann et al., 2003; Zhu et al., 2011). To obtain myristoylated AMPK heterotrimer, pET21b-AMPK was cotransformed with pET-28a-NMT1 and purified as described previously

(Oakhill et al., 2010). The proteins were then purified with Nickel affinity gel (catalog number P6611, Sigma) and followed by size exclusion chromatography as described previously (Oakhill et al., 2010).

The pET32a-AXIN vector was transformed into the *E. coli* strain BL21 (DE3). The transformed cells were induced with IPTG. After growing for 4 hr at 16°C, the cells were collected, homogenized, and sonicated. The supernatant was collected and applied to ultracentrifugation at 150,000 × *g* for 30 min, followed by purification of expressed protein with Nickel affinity gel. The eluted protein was then concentrated to about 3 mg/ml before further purification by gel filtration.

AMPKα-Thr172 Phosphorylation Assays

AMPK purified from *E. coli* (800 ng) was incubated with active LKB1/MO25/STRAD complex (200 ng, at 32°C, 15 min) in a kinase buffer (50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 2 mM $MgCl_2$, 200 μM ATP) and 200 μM AMP with or without AXIN (final reaction volume 50 μl).

Statistical Analyses

ANOVA with Tukey's post test was used to compare values among different experimental groups using the SPSS Statistics 17.0 program. Values are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2013.09.005.

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