Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway

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YAP (Yes-associated protein) is a transcription co-activator in the Hippo tumour suppressor pathway and controls cell growth, tissue homeostasis and organ size. YAP is inhibited by the kinase Lats, which phosphorylates YAP to induce its cytoplasmic localization and proteosomal degradation. YAP induces gene expression by binding to the TEAD family transcription factors. Dysregulation of the Hippo–YAP pathway is frequently observed in human cancers. Here we show that cellular energy stress induces YAP phosphorylation, in part due to AMPK-dependent Lats activation, thereby inhibiting YAP activity. Moreover, AMPK directly phosphorylates YAP Ser 94, a residue essential for the interaction with TEAD, thus disrupting the YAP–TEAD interaction. AMPK-induced YAP inhibition can suppress oncogenic transformation of Lats-null cells with high YAP activity. Our study establishes a molecular mechanism and functional significance of AMPK in linking cellular energy status to the Hippo–YAP pathway.

The Hippo pathway was originally discovered in *Drosophila* to regulate organ size and is highly conserved in higher eukaryotes. Core components of the mammalian Hippo pathway include MST1/2 and Lats1/2 kinases and their downstream effectors, YAP and TAZ transcription co-activators. In response to unfavourable growth conditions, MST1/2 phosphorylate and activate Lats1/2, which in turn phosphorylate and inhibit YAP/TAZ. The Lats-dependent phosphorylation of YAP/TAZ promotes their binding to 14-3-3, resulting in cytoplasmic localization and functional inactivation.

Moreover, phosphorylated YAP/TAZ are targeted for ubiquitylation and degradation. The Hippo pathway thus suppresses tumorigenesis by limiting the activity of YAP/TAZ (refs 9–11). YAP/TAZ activity must be strictly regulated for tissue/organisms to reach and maintain proper size. YAP/TAZ can promote cell proliferation and inhibit apoptosis; abnormal activation of YAP/TAZ is frequently found in human cancers. However, the function of YAP in human cancer is complex and could be cell-type-dependent. For instance, YAP could function as a tumour suppressor in some cell types, such as haematological cancers, by inducing apoptosis in response to DNA damage.

Recent studies have revealed a large number of extra- and intracellular signals that modulate YAP/TAZ activity. For example, cell density/cell contact strongly suppresses YAP activity concomitantly with increased Lats kinase activity, which may be mediated by tight junctions and adherent junctions. Diffusible hormones, such as lysophosphatidic acid and epinephrine, acutely regulate YAP activity (either stimulatory or inhibitory) via G-protein-coupled receptor (GPCR). Furthermore, mechanical stress has been shown to regulate YAP activity. These signals might all act through alteration of actin dynamics to modulate Lats kinase activity and YAP function.

In this study, we discovered that YAP is inhibited by cellular energy stress. AMPK is a key cellular energy sensor activated by increasing AMP levels and functions to coordinate cell growth with energy availability. We show that AMPK can inhibit YAP by two mechanisms: directly via phosphorylation of YAP Ser 94, and indirectly via activation of the Lats kinase. This work therefore reveals molecular insights of Hippo–YAP regulation by cellular energy status.

**RESULTS**

**Cellular energy starvation induces YAP phosphorylation and inactivation**

Cell growth is an energy-consuming process and must be coordinated with cellular energy status. Given the key function of YAP in cell growth regulation, we investigated whether YAP is regulated by energy stress. Glucose starvation strongly induced YAP phosphorylation, as determined by mobility shift on a phos-tag gel (Fig. 1a). Previous
Figure 1  Cellular energy starvation activates Lats and inhibits YAP. (a) Glucose starvation induces YAP phosphorylation. HEK293A cells were starved of glucose as indicated for 1 h. Cell lysates were prepared and subjected to immunoblotting for the indicated proteins and phosphorylation. Phos-tag denotes phos-tag gel used to resolve phosphorylated YAP based on mobility shift. Vin stands for vinculin as a loading control. Protein molecular markers are labelled on the right of each panel. (b) 2-DG induces YAP phosphorylation. HEK293A cells were treated with different doses of 2-DG in glucose-rich (25 mM) or glucose-free (0 mM) DMEM medium for 1 h. Cell lysates were immunoblotted with anti-AMP-activated protein kinase α (AMPKα), anti-pAMPKα (Thr 172), anti-acetyl-CoA carboxylase (ACC) and anti-pACC (Ser 79). Both pAMPKα and pACC were analysed as indicators of energy starvation. Experiments were similar to a. (c) 2-DG decreases YAP nuclear localization. HEK293A cells were treated with 25 mM 2-DG for 1 h. YAP subcellular localization was determined by immunofluorescence staining for endogenous YAP (green); DAPI (blue) was used to stain cell nuclei. Scale bars, 10 μm. (d) 2-DG disrupts the interaction between YAP and TEAD1. Cells were treated with 25 mM 2-DG for 2 h as indicated. Cell lysates were precipitated with YAP or IgG control antibody. Co-immunoprecipitation of TEAD was detected by western blot. (e) 2-DG inhibits YAP target gene expression. HEK293A cells were treated with 10 mM and 25 mM 2-DG for 4 h; mRNA levels of CTGF and CYR61 were measured by quantitative RT-PCR and normalized to HPRT control (error bars represent ± s.e.m. from n=3 independent experiments). (f) Energy stress increased Lats1 phosphorylation. HEK293A cells were treated with 25 mM 2-DG for 1 h. Endogenous Lats1 was immunoprecipitated and phosphorylation of the activation loop and hydrophobic motif was detected by western blot with phosospecific antibodies. (g) Glucose starvation activates Lats1 kinase activity. Cells were treated with glucose starvation for 1 h. Lats1 was immunoprecipitated and in vitro kinase assays were performed using recombinant GST–YAP as the substrate. Phosphorylation of GST–YAP was determined by immunoblotting with the phospho-YAP (Ser 127) antibody. All blots and immunofluorescence shown are representatives of three independent experiments. Uncropped blots are shown in Supplementary Fig. 5.

studies have shown that Lats phosphorylates YAP on five serine residues and phosphorylation of Ser 127 is critical for the interaction between YAP and 14-3-3, which sequesters YAP in the cytoplasm. Glucose starvation induced YAP Ser 127 phosphorylation (Fig. 1a), suggesting that cellular energy stress inhibits YAP. AMPK is a key cellular energy sensor. As expected, glucose starvation activated AMPK, as indicated by the increased phosphorylation of the activation loop Thr 172 in AMPKα (Fig. 1a). 2-deoxy-D-glucose (2-DG) is a non-metabolizable glucose analogue that can inhibit normal glucose metabolism, therefore inducing cellular energy stress. Addition of 2-DG activated AMPK, as indicated by the increased phosphorylation of acetyl-CoA carboxylase (ACC), an AMPK substrate, and also...
induced YAP phosphorylation (Fig. 1b). The effect of 2-DG on YAP phosphorylation was more robust in low-glucose conditions. We tested YAP phosphorylation using a similar concentration of sorbitol and found that the effect of 2-DG was specific, excluding a possible osmotic effect (Supplementary Fig. 1a). 2-DG increased the phosphorylation of AMPK\(\alpha\) and YAP in a dose- and time-dependent manner (Supplementary Fig. 1b,c). Notably, 2-DG also induced a TAZ mobility shift, indicating that 2-DG also increased TAZ phosphorylation. It is worth noting that ACC phosphorylation was more sensitive to 2-DG treatment than YAP phosphorylation (Fig. 1b), consistent with the distinct cellular functions of ACC (fatty acid synthesis and energy storage) and YAP (cell growth and survival). One may speculate that fatty acid synthesis should be inhibited before growth inhibition and apoptosis. Thus, ACC could be inhibited under mild energy stress whereas more severe energy stress is required to inhibit YAP. Energy-stress-induced YAP phosphorylation was also observed in myoblast C2C12, mammary epithelial MCF10A, and the cervical cancer HeLa cell lines (Supplementary Fig. 1df).

AMPK is required for energy-starvation-induced YAP phosphorylation

AMPK directly monitors cellular ATP and AMP levels and regulates cell metabolism and growth in response to cellular energy status\(^2^9\). We investigated the role of AMPK in YAP regulation by comparing AMPK\(\alpha\) wild-type (WT) and AMPK\(\alpha\)1/AMPK\(\alpha\)2 double-knockout (DKO) mouse embryonic fibroblasts (MEFs) that lack the two AMPK catalytic alpha subunits (Supplementary Fig. 3a). Notably, 2-DG treatment failed to induce YAP mobility shift in the AMPK\(\alpha\) DKO MEFs, indicating a critical role of AMPK\(\alpha\) in YAP regulation by energy stress (Fig. 2a). Treatment with lambda phosphatase (\(\lambda\)-PPase) abolished the 2-DG-induced YAP mobility shift (Supplementary Fig. 3b). These data indicate that cellular energy stress induces YAP phosphorylation in an AMPK-dependent manner.

We next determined whether AMPK is sufficient to induce YAP phosphorylation. Expression of WT AMPK\(\alpha\)1 or AMPK\(\alpha\)2, but not the DN mutant, induced significant YAP phosphorylation, as indicated by the altered mobility shift on a phos-tag gel (Fig. 2b), suggesting that AMPK can induce YAP phosphorylation. Moreover, 2-DG could activate Lats1 in AMPK\(\alpha\) WT MEFs, but 2-DG-induced Lats phosphorylation was partially compromised in the AMPK\(\alpha\) DKO MEFs (Fig. 2c). These data suggest that AMPK relays, at least partly, the energy stress signal to Lats activation. Consistent with a role in YAP inhibition, we confirmed that AMPK co-transfection disrupted the YAP–TEAD interaction (Fig. 2d) and suppressed TEAD–luciferase reporter activity (Fig. 2e). Furthermore, the AMPK-DN increased reporter activity, consistent with the notion that AMPK–DN interferes with endogenous AMPK function in a dominant-negative fashion.

Metformin, a widely prescribed drug for type 2 diabetes\(^3^5\), activates AMPK, inhibits cell growth and reduces tumour incidence\(^3^6,3^7\). Most in vitro studies use doses of metformin significantly higher than the therapeutic levels of plasma metformin\(^3^8\). We found that metformin treatment increased AMPK activity and YAP phosphorylation (Fig. 2f). Metformin also increased TAZ phosphorylation. Aminimidazole carboxamide ribonucleotide (AICAR) is a well-known AMPK activator\(^2^9\). Similar to metformin, AICAR induced YAP and TAZ phosphorylation (Supplementary Fig. 3c). YAP Ser 127 phosphorylation was decreased by AICAR treatment, indicating a possible disconnection between AMPK activation and Lats activation under this condition. A YAP–TEAD co-immunoprecipitation experiment revealed that AICAR reduced the interaction between YAP and Lats (Fig. 2g). Metformin and AICAR treatments also decreased the expression of YAP–TEAD target genes Ctgf and Cyr61 (Fig. 2h). The stronger inhibitory effects of AICAR on YAP target genes are consistent with the fact that AICAR induced a strong YAP phosphorylation (Supplementary Fig. 3c,d). Another AMPK activator, A769662 (ref. 39), also stimulated YAP phosphorylation in HepG2 cells (Supplementary Fig. 3e). Collectively, our data show that pharmacological activation of AMPK leads to YAP phosphorylation and inhibition, demonstrating a general role of AMPK in YAP regulation.

Energy stress induces both Lats-dependent and Lats-independent YAP phosphorylation

Lats is the main kinase responsible for YAP/TAZ inhibition, although other kinases have also been implicated in their regulation\(^4^0,4^1\). To confirm the role of Lats in energy-stress-induced YAP phosphorylation by cellular energy stress, we measured YAP target gene expression and TAZ phosphorylation. To further confirm the negative regulation of YAP by cellular energy stress, we measured YAP target gene expression and localization, 2-DG blocked the interaction between YAP and TEAD (Fig. 1d). Glucose starvation also diminished YAP–TEAD interaction (Supplementary Fig. 1g). These observations link cellular energy stress to YAP inhibition. To further confirm the negative regulation of YAP by cellular energy stress, we measured YAP target gene expression and found that 2-DG treatment significantly decreased the expression of TEAD target genes, Ctgf and Cyr61 (Fig. 1e). Collectively, our data suggest that cellular energy stress inhibits YAP and TAZ activity.

Energy stress activates Lats kinase

The increase of YAP Ser 127 phosphorylation indicates that Lats kinase activity is enhanced by energy stress (Fig. 1a). Phosphorylation of Ser 909 and Thr 1079 in Lats1 positively correlates with Lats activity\(^3^3,3^4\). We therefore monitored Ser 909 and Thr 1079 phosphorylation in endogenous Lats1 in response to energy stress. 2-DG treatment significantly increased phosphorylation of both residues in immunoprecipitated Lats1 (Fig. 1f). In addition, we directly measured Lats1 kinase activity through an in vitro kinase assay using recombinant YAP as a substrate. Lats1 immunoprecipitated from glucose-starved HEK293A cells exhibited a significantly higher kinase activity towards YAP (Fig. 1g). Next, we investigated the function of MST in energy-stress-induced YAP phosphorylation. Overexpression of wild-type MST2 had a minor effect on 2-DG-induced YAP phosphorylation whereas the MST2 kinase-inactive (MST2-KR) mutant partially blocked the YAP mobility shift (Supplementary Fig. 2a). However, 2-DG did not activate MST, as the activation loop phosphorylation of MST (Thr 183) was not increased (Supplementary Fig. 2b). Thus, we conclude that cellular energy stress activates Lats activity and promotes YAP phosphorylation and inhibition, which might have a role in cell growth suppression in response to energy starvation.

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AMPK is required for Hippo–YAP regulation by energy stress. (a) The 2-DG-induced YAP phosphorylation depends on AMPK. AMPKα WT and AMPKα DKO MEFs were treated with 25 mM 2-DG for the indicated durations. YAP phosphorylation is determined by phos-tag gel. (b) AMPK induces YAP phosphorylation. HEK293A cells were transiently co-transfected with the indicated plasmids and AMPKα (AMPKα WT), but not the kinase-inactive AMPKα (AMPKα DN), increased YAP phosphorylation. (c) AMPK is required for Lats1 activation by energy stress. AMPKα WT and AMPKα DKO MEFs were incubated with or without 25 mM 2-DG for 2 h. Endogenous Lats1 was immunoprecipitated and phosphorylation both activation loops and the hydrophobic motif was detected by western blot. (d) AMPK disrupts YAP and TEAD1 interaction. HEK293A cells were transiently co-transfected with the indicated plasmids. Flag–YAP was immunoprecipitated and the co-immunoprecipitated Myc–TEAD1 was detected by Myc western blot. (e) AMPK inhibits the TEAD reporter. A luciferase reporter controlled by multiple TEAD binding sequences was transfected into HEK293A cells together with AMPKα WT or AMPKα DN. After 48 h, the firefly luciferase activity was measured and normalized to the co-transfected Renilla luciferase internal control (error bars represent ± s.e.m. from n=6 biological replicates; r.l.u., relative luciferase units). (f) Metformin activates AMPK and increases YAP phosphorylation. Primary mouse hepatocytes were treated with different doses of metformin for 4 or 8 h. Phosphorylation of YAP and AMPK was measured by western blot. TAZ phosphorylation was indicated by a mobility shift. (g) AICAR disrupts YAP and TEAD interaction. Primary mouse hepatocytes were treated with 2 mM AICAR for 4 h. The endogenous YAP was immunoprecipitated and the co-precipitated TEAD was detected by western blot. (h) AICAR and metformin inhibits YAP target gene expression. Primary mouse hepatocytes were treated with 2 mM AICAR or 2 mM metformin for 4 h; mRNA levels of Ctgf and Cyr61 were measured by quantitative RT-PCR (error bars represent ± s.e.m. from n=3 independent experiments). All experiments are representatives of three independent experiments except e. ∗ ∗ ∗ P < 0.001, ∗ P < 0.01, two-tailed t-test. Uncropped images of blots/gels are shown in Supplementary Fig. 5.

phosphorylation, we compared the Lats1−/− Lats2fl/fl (Lats1 KO) MEFs with the Lats1/2−/− (Lats1/2 DKO) MEFs (ref. 42) generated by infection of the Cre virus in the Lats1 KO MEFs. As expected, deletion of Lats1/2 completely abolished YAP Ser 127 phosphorylation and increased YAP protein levels (Fig. 3a). Moreover, the 2-DG-induced YAP mobility shift was significantly diminished in Lats1/2
Figure 3  Energy stress induces YAP phosphorylation via both Lats-dependent and Lats-independent mechanisms. (a) Deletion of Lats1/2 diminishes, but does not completely abolish, YAP phosphorylation by 2-DG. Lats1−/− Lats2−/− (denoted as Lats1 KO) MEFs and Cre-transduced Lats1−/− Lats2fl/fl (denoted as Lst1/2 DKO) MEFs were treated with 10 and 25 mM 2-DG. YAP phosphorylation was detected by phospho-YAP (Ser 127) antibody and phospho-tag gel. Lats2 deletion was confirmed by western blot. All lysates were run on the same gel and the vertical line in the YAP phospho-tag blot indicates different exposures of the same western blot (the YAP phospho-tag blot of Lats1/2 DKO samples had a shorter exposure due to high YAP protein levels in these samples). (b) Overexpressed kinase-inactive Lats2 (Lats2-KR) abolishes 2-DG-induced Ser 127 phosphorylation, but not total phosphorylation, in YAP. Flag–YAP was co-transfected with Lats2-WT or Lats2-KR mutant. After transfection, cells were treated with 25 mM 2-DG for 1 h. (c) AMPK induces mobility shift of YAP-SSA. Flag–YAP-SSA was co-transfected with AMPKα WT or AMPKα DN in HEK293A as indicated. (d,e) 2-DG stimulates the interaction of YAP and AMPKα. HEK293A cells transfected with the indicated plasmids were immunoprecipitated with anti-FLAG (for Flag–YAP) or anti-HA (for HA–AMPK) and blotted with the indicated antibodies for co-immunoprecipitation. (f) AICAR or metformin promotes the association between endogenous AMPK and YAP. H2.35 cells were treated with AICAR or metformin. Cell lysates were immunoprecipitated with YAP or control IgG and western blotted with AMPKα. All experiments are representatives of three independent experiments, except e,f. The experiments in e,f were performed two times with similar results. Uncropped images of blots/gels are shown in Supplementary Fig. 5.

DKO MEFs, supporting a role of Lats in energy-stress-induced YAP phosphorylation. However, 2-DG still induced a visible YAP mobility shift in the Lats1/2 DKO cells (Fig. 3a), suggesting that 2-DG could induce YAP phosphorylation independent of Lats. Interestingly, AMPK activation was noticeably enhanced in the Lats1/2 DKO MEFs compared with Lats1 KO MEFs (Fig. 3a). The enhanced AMPK activation in the Lats1/2 DKO MEFs indicates that these cells are under more severe energy stress. This phenomenon can be explained by a model in which the high YAP activity in the Lats1/2 DKO cells promotes cell growth and increases energy expenditure, thereby leading to energy stress in these cells.

We further investigated the function of Lats in energy-stress-induced YAP phosphorylation. Co-transfection of Lats2 increased basal YAP phosphorylation, whereas the Lats2 kinase-inactive (Lats2-KR) mutant blocked YAP phosphorylation (Fig. 3b). Interestingly, Lats2-KR did not completely block 2-DG-induced YAP mobility shift, although it strongly reduced 2-DG-induced YAP Ser 127 phosphorylation, suggesting that 2-DG could also induce Lats-independent phosphorylation of YAP, consistent with the observation in Fig. 3a.

We used the YAP-SSA mutant, which lacks the five known Lats phosphorylation consensus serine residues (including Ser 61, Ser 109, Ser 127, Ser 164 and Ser 381; ref. 4) to investigate the AMPK and YAP interaction. The YAP-SSA mutant, induced a mobility shift in the YAP-SSA mutant, consistent with the observation in Fig. 3a.

AMPKα WT, but not the AMPKα DN mutant, induced a mobility shift of the YAP-SSA mutant (Fig. 3c), indicating that AMPK can promote Lats-independent YAP phosphorylation. Energy stress increases the interaction between AMPK and its physiological substrates43–45; accordingly, AMPK co-precipitated with YAP and this interaction was further enhanced by 2-DG treatment (Fig. 3d,e).
AMPK inhibits YAP by directly phosphorylating Ser 94

To gain mechanistic insight into AMPK-mediated regulation of YAP, we tested whether YAP is a direct substrate of AMPK. AMPK phosphorylated recombinant YAP WT in vitro (Fig. 4a). The YAP-S127A and YAP-SSA mutants were also phosphorylated by AMPK, although more weakly, indicating that AMPK can phosphorylate YAP on residues distinct from the Lats phosphorylation sites. In vitro phosphorylation by AMPK altered the migration of YAP-SSA (Supplementary Fig. 4a). Mass spectrometry experiments on several batches of in vitro-phosphorylated YAP-SSA identified several phosphorylation sites on YAP, including high- and low-confidence sites (Supplementary Fig. 4b). Further in vitro phosphorylation experiments confirmed that serine residues 94, 366 and 463 were direct AMPK targets (Fig. 4b and Supplementary Fig. 4c). We examined the robustness of YAP as an AMPK substrate by comparing with known physiological AMPK substrates TSC2 and ULK1. AMPK phosphorylated YAP as efficiently as ULK1 (Supplementary Fig. 4d–f); the efficiency of YAP (51–270) phosphorylation by AMPK was comparable to the TSC2 fragment (1300–1367). As energy starvation or AMPK co-transfection inhibits YAP TEAD-mediated transcriptional activity (Fig. 1d and Fig. 2d,e), we examined the effect of the putative AMPK phosphorylation sites on YAP activity. Mutation of YAP Ser 94 to glutamate or alanine diminished the ability of YAP to activate the TEAD1 reporter, whereas mutation of Ser 366 or Ser 463 did not significantly affect YAP activity (Fig. 5a,b). Therefore, we focused our further studies on Ser 94. Notably, substitution of Ser 94 by any residue, including alanine or glutamate, abolished YAP–TEAD interaction (Fig. 5c) and decreased TEAD reporter activity. We propose that phosphorylation of YAP Ser 94 by AMPK disrupts its interaction with TEAD.

Identification of YAP Ser 94 as an AMPK phosphorylation site is exciting because our previous studies had demonstrated that YAP Ser 94 is directly involved in and is essential for the interaction with TEAD (ref. 46). Based on the YAP–TEAD co-crystal structure, YAP Ser 94 forms a hydrogen bond with Tyr 406 in TEAD (refs 46, 47). Notably, Ser 94 is functionally conserved from Drosophila Yki to mammalian YAP/TAZ and is also predicted to be phosphorylated by PhosphositeDB (http://www.phosphosite.org). However, AMPK co-transfection did not inhibit the interaction between YAP and RUNX2 (Supplementary Fig. 4g), suggesting a specific role for Ser 94 phosphorylation in regulating the interaction with TEAD. These observations collectively suggest that AMPK could inhibit YAP function by phosphorylating Ser 94 and disrupting its interaction with TEAD.

We generated a phospho-YAP (Ser 94) specific antibody and confirmed that it selectively recognized in vitro phosphorylated YAP, but not unphosphorylated or the S94A mutant of YAP (Fig. 5d). As expected, AMPK transfection or activation of AMPK by AICAR increased YAP Ser 94 phosphorylation (Fig. 5e,f). Collectively, our data indicate that AMPK directly phosphorylates YAP on Ser 94 in vivo in response to energy stress.

Energy stress inhibits the oncogenic potential of Lats1/2 DKO cells

To further substantiate the Lats-independent regulation of YAP, we measured the expression of YAP target genes in Lats1/2 DKO MEFs. AICAR could still repress the YAP target genes Ctgf and Cyr61, supporting Lats-independent YAP inhibition by energy stress (Fig. 6a). We next examined the role of such Lats-independent YAP inhibition in oncogenic transformation. Lats1/2 DKO MEFs could form colonies in soft agar, an indication of oncogenic transformation (Fig. 6b). YAP/TAZ knockdown blocked the anchorage-independent growth.
AMPK inhibits YAP activity through phosphorylation of Ser 94. (a) The phosphomimetic mutant of YAP Ser 94 abolishes YAP activity. The indicated plasmids were co-transfected with a 5×UAS–luciferase reporter for Gal4–TEAD4 and Renilla luminescence activity was measured and normalized to Renilla luminescence activity levels (error bars represent ± s.e.m. from n = 6 biological replicates; r.l.u., relative luciferase units). (b) Ser 94 is important for YAP activity and inhibition by AMPK. All three AMPK phosphorylation sites in YAP were mutated to alanine. The YAP plasmids were co-transfected with the 5×UAS–luciferase reporter for Gal4–TEAD4 into HEK293T cells together with the control Renilla luciferase. After 48 h, the luciferase activity was measured and normalized to the co-transfected Renilla luciferase internal control (error bars represent ± s.e.m. from n = 6 biological replicates; r.l.u., relative luciferase units). (c) Ser 94 of YAP is essential for 2-DG-induced disruption of the YAP–TEAD complex. HEK293A cells were transiently co-transfected with the indicated plasmids followed by treatment with 2-DG. Interaction between Flag–YAP and Myc–TEAD4 was determined by co-immunoprecipitation. (d) Evaluation of phosphospecific antibodies for YAP Ser 94 phosphorylation. Phospho-antibody was prepared by immunizing rabbits with synthetic phosphopeptides containing phospho-YAP Ser 94. Recombinant GST–YAP (51–270) fragment was purified from bacteria and phosphorylated by AMPK in vitro. After the reaction, 5 ng of the GST–YAP protein was detected by phospho-YAP Ser 94 or GST antibody. The non-phosphorylatable mutant GST–YAP-S94A was used as a negative control. (e) AMPK increases YAP Ser 94 phosphorylation in transfected cells. Flag–YAP WT and YAP-S94A mutant were transfected into HEK293 cells with or without AMPK as indicated. Flag–YAP was immunoprecipitated and phosphorylation of Ser 94 was detected by the pYAP(Ser 94) phospho-antibody. (f) AICAR increases YAP Ser 94 phosphorylation in vivo. Primary hepatocytes were treated with 2 mM AICAR for 4 h. YAP was immunoprecipitated and phosphorylation of Ser 94 was detected by the pYAP(Ser 94) phospho-antibody. All blots shown are representatives from three independent experiments, except e. ** P < 0.01, *** P < 0.001, two-tailed t-test. Uncropped images of blots/gels are shown in Supplementary Fig. 5.

of the Lats1/2 DKO MEFs, suggesting that YAP/TAZ are required for the transforming potential of these cells. On the basis of our model, AMPK could inhibit YAP in the Lats1/2 DKO MEFs by phosphorylating Ser 94, and therefore should suppress the anchorage-independent growth of Lats1/2 DKO MEFs. Indeed, treatment with AICAR or metformin inhibited anchorage-independent growth of Lats1/2 DKO MEFs in vitro (Fig. 6c). In an immunocompromised nude mouse xenograft model, injection of Lats1/2 DKO MEFs resulted in tumour development (Fig. 6d). Consistent with the cell culture results, metformin injection significantly reduced xenografted tumour growth in these mice. Collectively, these data support a model in which AMPK could inhibit YAP in vivo in the absence of Lats1/2.

**AMPK is required for YAP inhibition by energy stress**

To demonstrate that AMPK mediates the effect of AICAR on YAP inhibition, AMPK was knockdown by short interfering RNA (siRNA) in Lats1/2 DKO MEFs. Knockdown efficiency was confirmed by western blot (Fig. 7a). Depletion of AMPK blocked the inhibitory effect of AICAR or A769662 on YAP target gene expression (Fig. 7a,b). These results further support that AMPK is involved in YAP inhibition in the Lats1/2 DKO MEFs on energy stress.

It is possible that AICAR reduced Ctgf/Cyr61 expression and anchorage-independent growth of Lats1/2 DKO MEFs by inhibiting factors other than YAP. Therefore, we tested whether AICAR- or metformin-induced YAP inhibition is important to achieve their tumour-suppressive effects in Lats1/2 DKO MEFs. We generated a YAP construct resistant to AMPK inhibition by fusing the carboxy-terminal transactivation domain of YAP(AD) to TEAD1 fragment residues 1–289, which contains only the DNA-binding domain. When the TEAD1AC–YAP(AD) plasmid was expressed in Lats1/2 DKO MEFs, messenger RNA levels of Ctgf and Cyr61 were not significantly increased, suggesting that activity of the ectopic
Figure 6 Energy stress attenuates tumorigenicity of Lats1/2 DKO MEFs. (a) AICAR inhibits YAP target gene expression in Lats1/2 DKO MEFs. Lats1/2 DKO MEFs were treated with 0.5 mM and 1 mM of AICAR for 5 h. mRNA levels of Ctgf and Cyr61 were measured by quantitative RT-PCR (error bars represent ± s.e.m. from n = 3 independent experiments). (b) Knockdown of YAP or TAZ attenuates anchorage-independent growth of Lats1/2 DKO MEFs. YAP or TAZ was knocked down by shRNAs in Lats1/2 DKO MEFs (the experiments were repeated twice). The Lats1/2 DKO MEFs (3 × 10^3 cells) were seeded onto a culture medium containing 0.3% agar and incubated at 37°C for two weeks. The colonies were stained with 0.005% crystal violet. (c) Energy stress attenuates anchorage-independent growth of Lats1/2 DKO MEFs. Lats1/2 DKO MEFs (3 × 10^3 cells) were seeded onto a culture medium containing 0.3% agar and incubated at 37°C for two weeks. The colonies were stained with 0.005% crystal violet and quantified. AICAR and metformin treatments are indicated (error bars represent ± s.e.m. from n = 3 independent experiments). (d) Metformin inhibits tumour growth of Lats1/2 DKO MEFs in xenografted mice. Mice were ectopically implanted with 1 × 10^6 cells on both sides of each mouse. Daily intraperitoneal injection of 250 mg kg^-1 dose of metformin or an equal volume (300 μl) of vehicle (PBS) for 15 days. At the end of the experiment, mice were euthanized 16 days after implantation and tumour weight was measured (error bars represent ± s.e.m. from eight tumours (four mice) per group). Scale bar, 1 cm. *P < 0.05, two-tailed t-test.

TEADΔC–YAP(AD) was not significantly higher than the fully activated endogenous YAP/TAZ in the Lats1/2 DKO MEFs (Fig. 7c). Interestingly, AICAR inhibited the expression of Ctgf and Cyr61 in the vector-transfected cells, but not the TEADΔC–YAP(AD)-expressing cells. Furthermore, AICAR, metformin and A769662 could not suppress the anchorage-independent growth of the TEADΔC–YAP(AD)-expressing cells, although they suppressed the control group Lats1/2 DKO MEFs (Fig. 7d). These data support a notion that activation of AMPK inhibits the anchorage-independent growth of the Lats1/2 DKO MEFs by suppressing YAP/TAZ activity.

DISCUSSION

Extensive studies have revealed that the Hippo–YAP signalling is regulated by a variety of extracellular signals. These include cell–cell contact signals (such as tight junctions and adherent junctions), physical signals (such as mechanical stress and matrix stiffness), and long-range hormonal signals (such as lysophosphatidic acid and epinephrine). In this study, we show that intracellular metabolic/energy status also regulates Lats and YAP activity. Therefore, the Hippo–YAP pathway can integrate and coordinate both extracellular and intracellular signals.

Cellular energy stress is detected by AMPK, which modulates cellular metabolism and limits cell growth. Our data show that energy stress inhibits YAP via two mechanisms, Lats dependent and Lats independent (Fig. 7e). In the former, AMPK may indirectly activate Lats in response to energy stress. Consistently, a recent report has shown that AMPK phosphorylates and stabilizes angiomotin-like 1 (AMOTL1) to stimulate Lats activity. The Lats-independent mechanism of YAP inhibition is achieved via direct AMPK-mediated phosphorylation of YAP. Ser 94 phosphorylation by AMPK plays a
Figure 7 AMPK is required for energy stress to inhibit YAP activity. (a) AMPK is required for inhibition of Ctgf and Cry61 by AICAR. Lats1/2 DKO MEFs were transfected with control or AMPK siRNAs. The knockdown efficiency of AMPK was confirmed by western blot (left panels). After treating with the AMPK activator AICAR (0.5 mM), mRNA levels of Ctgf and Cry61 were measured by quantitative RT–PCR. The mRNA relative unit values are normalized to 1 (controls without AICAR treatment; the experiments were repeated twice). (b) Lats1/2 DKO MEFs were transiently transfected with control (Con siRNA) or AMPK (AMPKα siRNA) siRNA. After treating with the AMPK activator A769662 (0.3 mM for 4 h), mRNA levels of Ctgf and Cry61 were measured by quantitative RT–PCR (the experiments were repeated twice). (c) AICAR cannot inhibit YAP target genes in cells expressing TEAD1ΔC–YAP(AD). HA–TEAD1ΔC–YAP(AD), which is a fusion of the TEAD DNA-binding domain with the YAP transactivation domain, was stably expressed in Lats1/2 DKO MEFs. Expression of HA–TEAD1ΔC–YAP(AD) was confirmed by western blot. Cells were treated in AICAR as indicated and mRNA levels of Ctgf and Cry61 were measured by quantitative RT–PCR (error bars represent ± s.e.m. from n = 5 independent experiments). (d) Expression of HA–TEAD1ΔC–YAP(AD) confers Lats1/2 DKO MEFs resistant to growth inhibition by AMPK activators. Soft-agar colony-formation assay was performed with Lats1/2 DKO MEFs expressed with vector (top row) or HA–TEAD1ΔC–YAP(AD) (bottom row). Treatments with PBS control, AICAR, metformin, or A769662 are indicated. (e) A model for regulation of the Hippo–YAP pathway by energy stress and AMPK. When the energy is sufficient, AMPK is inactive and YAP is active. When cellular energy level is low (energy stress), both AMPK and Lats are active. The active AMPK and Lats inhibit YAP by phosphorylation. Uncropped images of blots/gels are shown in Supplementary Fig. 5.

critical role in YAP inhibition by disrupting the YAP–TEAD complex, leading to repression of YAP target genes and inhibition of cell growth. Our previous biochemical and structural studies have defined an essential function of Ser 94 in YAP–TEAD complex formation by forming a hydrogen bond with Tyr 406 in TEAD1 (ref. 46). Ser 94 is conserved in TAZ and mutation of the corresponding Ser 51 in
TAZ also abolished TAZ–TEAD interaction60. Moreover, YAP Ser 94 is also conserved in Yki and mutation of the corresponding Ser 97 abolishes Yki–sd interaction11. Therefore, we propose that TAZ and Yki could be similarly regulated by AMPK in response to energy stress, suggesting an evolutionarily conserved mechanism of Hippo pathway regulation by cellular energy status. Our model also indicates that stress-induced AMPK activation can inhibit YAP even in the absence of Lats, providing an additional layer of YAP regulation. Consistent with this notion, AMPK activation inhibits tumorigenesis in Lats1/2 DKO MEFs.

It is worth noting that AMPK can phosphorylate YAP on additional residues, such as Ser 366 and Ser 463. In the accompanying paper, AMPK-mediated phosphorylation of YAP on Ser 61 inhibits YAP activity51. We did not identify Ser 61 as an AMPK phosphorylation site because the YAP-5SA mutant, in which Ser 61 was already replaced by an alanine, was used in our in vitro experiments to map the AMPK phosphorylation sites in YAP. However, consistent with the data in the accompanying paper, we noticed that energy starvation induced a much more marked mobility shift of YAP than the YAP-5SA mutant, supporting YAP Ser 61 as one of the AMPK phosphorylation sites. We propose that AMPK has multiple mechanisms to regulate YAP activity in response to varying degrees of energy stress (Fig. 7e). Further evidence supporting a physiological function of the Hippo pathway in cellular energy response is the observation that AMPK activity was markedly elevated in Lats1/2 DKO MEFs (Fig. 3a), indicating that the Lats1/2 DKO MEFs are under constant energy stress. Therefore, Lats is likely to play a role in cellular energy homeostasis. Energy-stress-induced Lats activation reduces energy expenditure and cell growth, possibly by inhibiting YAP. Our study provides a mechanism of signal integration and crosstalk at YAP through multiple mechanisms of regulation.

Considering the general role of YAP in promoting cell proliferation and inhibiting apoptosis, it is not surprising that YAP activity is negatively regulated by cellular energy stress. When the Hippo pathway is inhibited under favourable conditions of low cell density and high serum (or hormonal signals), such conditions would normally allow YAP activation and cell growth. However, cell proliferation should not proceed if cellular energy is limited. Therefore, the direct inhibition of YAP by AMPK provides a mechanism to ensure that cell proliferation occurs only when favourable growth conditions are available. YAP inhibition by AMPK adds new dimensions to both physiological regulation of YAP and the biology of AMPK in cell growth control. Inhibition of YAP may contribute to the growth inhibitory and tumour suppressing activity of AMPK. Indeed, metformin has the significant benefit of reducing tumour incidence and is the most prescribed among all drugs for this purpose67. Inhibition of YAP may contribute to the tumour inhibitory effect of metformin. Thus, pharmacological activation of AMPK may offer a potential therapeutic benefit for cancers with high YAP activity, even in tumours with diminished Lats activity.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
J-S.M. and K-L.G. designed the experiments, analysed data and wrote the paper. J-S.M. performed the experiments with assistance from Z.M., Y.C.K., H.W.P., C.G.H. and S.K.; D-S.L. established the Lats knockout MEFs. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Antibodies and reagents. Anti-CtG (sc-14939, 1:500), Cry61 (sc-13100, 1:500) and donkey anti-goat IgG HRP Santa Cruz (sc-2020, 1:5,000) antibodies were obtained from Santa Cruz Biotechnology. Anti-YAP (no. 4912, 1:1,000), phospho-YAP (Ser 127) (290488, 1:1,000), TAZ (no. 4883, 1:1,000), Lats1 (sc-3477, 1:1,000), phospho-Lats1/2 (Ser 909/822) (9157, 1:1,000), phospho-Lats1/2 (Thr 1079/999) (no. 9159, 1:1,000), MST1 (no. 3682, 1:1,000), phospho-MST1/2 (Thr 183/180) (no. 3681, 1:1,000), AMPKα (no. 5232, 1:1,000), phospho-AMPKθ (Thr 272) (no. 2535, 1:1,000), ACC (no. 3676, 1:1,000) and phospho-ACC (Ser 79) (no. 3661, 1:1,000) were obtained from Cell Signaling. Anti-Lats1 (A300-378A, 1:500) and YAP (A302-309A, 1:500) were obtained from Bethyl Laboratory. Anti-α-vinulin (V9131, 1:1,000) was obtained from Sigma-Aldrich. Anti-TEF (TEAD1) (A29012, 1:500) was obtained from BD Biosciences. Actin (ab3280, 1:5,000) was obtained from Abcam. Alexa Fluor 555-conjugated secondary antibody (A13172, 1:1,000) and Alexa Fluor 488 goat-anti-rabbit IgG (H+L) antibody (A11008, 1:1,000) was obtained from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies (NA931V and NA934V, 1:5,000) were obtained from GE Healthcare. Monoclonal anti-FLAG M2-peroxidase (HRP) antibody (A8592, 1:5,000) was obtained from Sigma-Aldrich. HA-Tag (6E2) mouse (HRP conjugate) (no. 2999, 1:5,000) antibody was obtained from Cell Signaling. Anti-phosphorylated Ser 94 antibody was generated by immunizing rabbits with phosphopeptides (Abbiotec and GeneTex, 1:500). The phosphospecific antibodies were affinity purified. Phos-tag-conjugated acrylamide was purchased from Wako Chemicals. The following chemicals were used in this study: AICAR (no. 2840), metformin hydrochloride (no. 2864) and A769662 (no. 3356) were purchased from Thermo Fisher Scientific and Toronto Research Chemicals. 2-Deoxy-2-phosphogluconate (D8375) was purchased from Sigma-Aldrich. Recombinant AMPK α1/β1/γ1 complex was purchased from GenScript. Mutagenesis was performed based on Quick-Change mutagenesis (Stratagene).

Cell culture and transfection. HEK293A, HEK293P, HEK293T, H2.35, NIH3T3, C2C12 and HeLa cells were cultured at 37 °C in Dulbecco’s modiﬁed Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen) and 50 μg ml−1 penicillin/streptomycin in a humidified incubator with 5% CO2. HEK293A, HEK293P, HEK293T, H2.35, NIH3T3, C2C12 and HeLa cells were cultured at 37 °C in Dulbecco’s modiﬁed Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen) and 50 μg ml−1 penicillin/streptomycin in a humidified incubator with 5% CO2. Phoenix retrovirus packaging cells with pPGS empty vector or TEAD1−ΔC−YAP(AD); TEAD1−ΔC (1–289) fused with the YAP C-terminal activation domain (290–488) construct. After transfection (48 h), retroviral supernatant was ﬁltered through a 0.45-μm syringe ﬁlter and used to infect Lats1/2 DKO MEFs in the presence of PolyBren (10 μg ml−1, Sigma-Aldrich). Infected cells were selected using G418 (VWR) or hygromycin (Invitrogen) in the culture medium.

Western blot and immunoprecipitation. Immunoblotting was performed using a bacterial expression construct (pGEX-KG) containing the indicated genes. The expression of the recombinant GST-fusion proteins within the transformed bacteria was induced by using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C. Cells were re-suspended in PBS containing 0.5% Triton X-100, 1 mM PMSF and 2 mM β-mercaptoethanol, followed by ultrasonication. The proteins were protamine. A single step using glutathione beads according to the manufacturer’s protocol (Amersham Bioscience). Purified proteins were dialysed against 20 mM Tris at pH 8.0, and 10% glycerol. Purified GST–YAP protein (0.5 μg) was used for each AMPK assay.

In vitro kinase assay. To analyse kinase activity, HEK293A cells were collected and lysed with lysis buffer (50 mM HEPES at pH 7.5, 130 mM NaCl, 1 mM EDTA, 1% NP-40, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM NaVO4, 1 mM phenylmethyl sulphonyl ﬂuoride (PMF), 1 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche)) and centrifuged at 12,000g for 15 min at 4 °C. The supernatants were incubated with 5 μl of 1 μg ml−1 GST fusion proteins as substrate. Phosphorylation of YAP was detected by phospho-YAP (Ser 127) antibody. For AMPK kinase assays, purified YAP proteins were incubated with recombinant AMPK α1/β1/γ1 (rabbit, 1:500) antibodies (GenScript). The reaction mixtures were incubated for 30 min at 30 °C, terminated with sample buffer, and subjected to SDS-PAGE. Phosphorylation of YAP was determined by phospho–YAP (Ser 94) antibody and 32P-autoradiography.

Primary hepatocyte isolation. Mouse primary hepatocytes were isolated according to a method described in ref. 46 with modifications. Briefly, under the perfusion, liver was washed in Ca2+- and Mg2+-free EBSS (Invitrogen) with 0.1 mM EGTA and 0.1 mM EDTA, and then digested with HBSS buffer (Invitrogen) containing 0.5 mg ml−1 collagenase IV and 0.5 mg ml−1 trypsin inhibitor (Roche). The perfused liver was dispersed and washed with DMEM. Hepatocytes were purified from the liver cells by gradient centrifugation in 50% Percoll (Invitrogen) and washed with DMEM. 1 × 107 hepatocytes were seeded onto each well of 6-well plates in DMEM supplemented with 10% FBS. William Medium E (Invitrogen) was used for the subsequent culture.

Retrovirus infection and stable cell lines. To generate TEAD1−ΔC−YAP(AD)-expressing stable cells, retrovirus infection was performed by transfecting 293 Phoenix retrovirus packaging cells with pPGS empty vector or TEAD1−ΔC−YAP(AD); TEAD1−ΔC (1–289) fused with the YAP C-terminal activation domain (290–488) construct. After transfection (48 h), retroviral supernatant was ﬁltered through a 0.45-μm syringe ﬁlter and used to infect Lats1/2 DKO MEFs in the presence of PolyBren (10 μg ml−1, Sigma-Aldrich). Infected cells were selected using G418 (VWR) or hygromycin (Invitrogen) in the culture medium.

Preparation of recombinant protein. Escherichia coli BL21 were transformed with bacterial expression constructs (pGEX-KG) containing the indicated genes. The expression of the recombinant GST-fusion proteins within the transformed bacteria was induced by using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C. Cells were re-suspended in PBS containing 0.5% Triton X-100, 1 mM PMSF and 2 mM β-mercaptoethanol, followed by ultrasonication. The proteins were purified as a single step using glutathione beads according to the manufacturer’s protocol (Amersham Bioscience). Purified proteins were dialysed against 20 mM Tris at pH 8.0, and 10% glycerol. Purified GST–YAP protein (0.5 μg) was used for each AMPK assay.

In vitro kinase assay. To analyse kinase activity, HEK293A cells were collected and lysed with lysis buffer (50 mM HEPES at pH 7.5, 130 mM NaCl, 1 mM EDTA, 1% NP-40, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM NaVO4, 1 mM phenylmethyl sulphonyl ﬂuoride (PMF), 1 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche)) and centrifuged at 12,000g for 15 min at 4 °C. The supernatants were incubated with the appropriate antibodies for 2 h at 4 °C and protein G- or protein A-conjugated beads were added in for additional 1 h. Immunoprecipitates were collected by centrifugation and washed four times with lysis buffer, and then proteins were eluted with SDS–PAGE sample buffer.

Phosphorylation of YAP was detected by phospho-YAP (Ser 127) antibody. For AMPK kinase assays, purified YAP proteins were incubated with recombinant AMPK α1/β1/γ1 (rabbit, 1:500) antibodies (GenScript). The reaction mixtures were incubated for 30 min at 30 °C, terminated with sample buffer, and subjected to SDS-PAGE. Phosphorylation of YAP was determined by phospho–YAP (Ser 94) antibody and 32P-autoradiography.

shRNA. YAP (TRCN0000303025), TAZ (TRCN0000319150) and control shRNA were from Sigma-Aldrich. pM2D.G and pPA2X were used to produce lentivirus in 293T cells.
siRNA. ON-TARGET plus SMARTpool siRNA oligonucleotides targeting mouse AMPKa1 and AMPKa2 were used (Dharmacon). A non-targeting scrambled siRNA duplex was used as a negative control (5'-CCAUUCCGAUCCUGAUCCG-3') and introduced into cells by transient transfection with RNAiMAX (Invitrogen), in accordance with the manufacturer's instructions.

Immunofluorescence staining. HEK293A cells were seeded on coverslips to the appropriate density. After 2-DG treatment, cells were fixed with 4% paraformaldehyde-phosphate buffered saline (PBS) for 15 min and permeabilized with 0.1% Triton X-100 in PBS. After blocking in 2% goat serum and 2% BSA in TBS-T for 30 min, cells were incubated with primary antibody diluted in 1% BSA for 1 h at room temperature. Slides were then washed three times and mounted with ProLong Gold antifade mountant with DAPI. The stained cells were detected using Olympus FV1000 confocal microscopy. The final images were obtained and analysed by using confocal microscopy with FLUOVIEW viewer software. Each image is a single Z section at the same cellular level.

Luciferase assay. For luciferase reporter assay, HEK293T cells were seeded in 12-well plates. Gal4-TEAD1, 5×UAS-Luc reporter, pGL3-CTGF-Luc reporter, pRL reporter and indicated plasmids were co-transfected. After transfection (48 h), cells were lysed and luciferase activity was assayed using the enhanced luciferase assay kit (Promega) following the manufacturer's instructions. The firefly luciferase activity levels were measured and normalized to Renilla luciferase activity.

Soft-agar assay. Each 6-well plate (ultralow attachment) was coated with 2 ml of bottom agar (DMEM containing 10% FBS and 0.7% Difco agar noble). Various Lats1/2 DKO MEFs (3×10⁶ cells) were suspended in 1.5 ml of top agar (DMEM containing 10% FBS and 0.4% Difco agar noble) into each well. Cells were incubated for three weeks and replaced with fresh medium every three days. Colonies were stained using 0.05% crystal violet.

RNA isolation and real-time PCR. Cells were washed with cold PBS and total RNA was extracted using an RNeasy kit (Qiagen) treated with RNA-free DNase. One microgram of RNA was used for reverse transcription with iScript reverse transcriptionase (Bio-Rad). cDNA was then diluted and used for real-time PCR with gene-specific primers using KAPA SYBR FAST qPCR master mix (Kapa Biosystems) and the 7300 real-time PCR system (Applied Biosystems). Relative abundance of mRNA was calculated by normalization to HPRT mRNA. Primers are described as HPRT, 5′-AGAATGTCTTGTATTGTGGAAAGAGCCTTCCTG/TTGGTGACGCCAGAAAGCCTTCCTG-3′; CTGF, 5′-CCAAATGACACGGCCCTCCTG/TGGTGCAGCCAGAAGCTC-3′; CYR61, 5′-CAAGGACCGCACAGCAGCT/AGAACAGGCGCTCCACTTG-3′; Cyr61, 5′-GCTCAGTCAGAGGACCAGACC/GTTCTTGGGGACAAGAGAG-3′.

Xenograft. Nude mice were provided by the UCSD animal core facility and housed in a pathogen-free room with a 12 h light/dark cycle. A total of 1×10⁶ cells were inoculated into eight-week-old male nude mice by subcutaneous injection. After the tumour size reached 2 mm in diameter, the mice were randomly divided into two groups with eight animals. One group was treated with metformin at a dose of 250 mg kg⁻¹ daily, and the other group was treated with an equal volume of PBS by intraperitoneal injection. Fifteen days after the first metformin treatment, the mice were euthanized and the tumours were weighed. All the procedures followed the UCSD animal care guidelines.

Statistical analysis. Each experiment was repeated two or three times as mentioned in each figure legend. Data are presented as mean ± s.e.m. All statistical tests were performed using a Student's t-test (unpaired, two-tailed), *P<0.05, **P<0.01, ***P<0.001. No statistical method was used to predetermine sample size. Investigators were blinded to allocation during experiments and outcome assessment.
Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway

Jung-SoonMo, Zhipeng Meng, Young Chul Kim, Hyun Woo Park, Carsten Gram Hansen, Soohyun Kim, Dae-Sik Lim and Kun-Liang Guan

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In the Methods section ‘Antibodies and reagents’ there was a typographical error in the company name GeneTex. The sentence should have read ‘Anti-phosphorylated Ser 94 antibody was generated by immunizing rabbits with phosphopeptides (Abbiotec and GeneTex, 1:500)’. This has now been corrected online.
Correction notice
Nature Cell Biology 17, 500–510 (2015).

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In the version of this Supplementary Information file originally published the labels on the fourth and fifth panels from the top in Fig. S1a were interchanged. This was corrected in this file on 14 April 2015.
Supplementary Figure S1  Energy stress induces YAP phosphorylation. (a) Osmotic stress does not affect YAP and TAZ mobility shift. HEK293A cells were treated with different concentrations of 2-DG or sorbitol for 1 hr. Cells were then lysed and subjected to immunoblotting using the indicated antibodies. (b) 2-DG induces YAP phosphorylation. HEK293A cells were treated with different concentrations of 2-DG for 30 min. Cells were then lysed and cell lysates were subjected to immunoblotting using the indicated antibodies. (c) Experiments are the same as in panel a except cells were treated with 25 mM 2-DG for various times. (d-f) Effect of 2-DG on YAP phosphorylation in different cell lines. C2C12 cells were treated with 10 mM and 25 mM 2-DG (d); MCF10A cells were treated with 10 and 25 mM 2-DG (e); HeLa cells were treated with 10 and 25 mM (f). (g) Glucose starvation inhibits YAP and TEAD1 interaction. HEK293A cells were starved with glucose for 2 hr as indicated. Endogenous YAP/TAZ and the co-immunoprecipitated TEAD were detected by Western blot.
Supplementary Figure S2 2-DG does not affect MST phosphorylation. (a) Effect of MST overexpression on 2-DG induced YAP phosphorylation. HEK293A cells were transiently co-transfected with indicated plasmids. After transfection, cells were treated with 25 mM 2-DG for 1 hr and YAP phosphorylation status was determined by phos-tag gel. (b) 2-DG does not affect MST1 phosphorylation. Endogenous MST1 was immunoprecipitated from control or 2-DG (25 mM, 2 hr) treated HEK293A cells. MST1 phosphorylation was detected by a phospho-MST (T183) specific antibody.
Supplementary Figure S3  Activation of AMPK increases YAP phosphorylation in various cell lines. (a) Genotyping of AMPKa1 and AMPKa2 wild-type (WT) and mutant (KO) alleles. DNA was isolated from AMPK +/+ or AMPK -/- cells as indicated. Genotyping was performed by PCR using primers specific to wild type AMPKa (left side of the dash line) or AMPKa KO (right side of the dash line). The PCR products were run on the same agarose gel. Samples in the left panel are PCR products using primer specific to AMPKa1 where samples in the right panel are PCR products using primers specific to AMPKa2. (b) Cells were treated with 25mM 2-DG for various times as indicated and lysed. Lysates were incubated with lambda phosphatase (λ PPase) as indicated for 1hr. Endogenous YAP mobility was examined by western blot. (c) AICAR increases YAP phosphorylation in hepatocytes. Primary mouse hepatocytes were treated with different doses of AICAR for 8 hr. Cell lysates were used for immunoblotting with indicated antibodies. (d) HepG2 cells were treated with different doses of AICAR for 2 hr. (e) HepG2 cells were treated with different doses of A769662 for 2 hr.
**Supplementary Figure S4** Identification of AMPK phosphorylation sites in YAP. (a) AMPK induces mobility shift of YAP-5SA. GST-YAP 5SA was used as substrates for in vitro AMPK phosphorylation. GST- YAP mobility was examined by western blot. (b) Mass spectrometry analyses of AMPK-phosphorylated YAP. GST-YAP-5SA was purified from E.coli and was used as a substrate for an in vitro AMPK assay. After electrophoresis, the phosphorylated GST-YAP 5SA band was cut and digested with trypsin/ thermolysine followed by mass spectrometry analyses. The green colored bars (top) and amino acid residues (lower part) are sequences that were detected by mass spectrometry. Phosphorylation sites identified by mass spectrometry are indicated on top of the amino acid sequence. (c) S94 is the major AMPK phosphorylation site in the YAP (51-121) fragment. Experiments were similar to panel a. (d) All three indicated residues of YAP were mutated to alanine. A luciferase reporter controlled by multiple TEAD binding sequences was transfected into HEK293T cells together with 5xUAS-luciferase reporter for Gal4-TEAD4 and Renilla constructs and indicated plasmids. After 48 hr, the firefly luciferase activity was measured and normalized to the co-transfected Renilla luciferase internal control. (d) Two different substrates of AMPK, TSC2 and ULK1, and YAP truncations were expressed, purified, and used as substrates for in vitro AMPK phosphorylation in the presence of 32P-ATP. Phosphorylation was detected by autoradiography. GST-ULK1 (279-425), TSC2 (1300-1367) and YAP fragment proteins were detected by Coomassie staining. (e) Experiments are the same as in panel c except that a GST-YAP truncated form and UKL1 were used as substrates for the indicated times. (g) AMPK has no effect on the YAP and RUNX2 interaction. HEK293A cells were transfected with the indicated plasmids. Flag-RUNX2 was immunoprecipitated and the co-precipitated HA-YAP was detected by Western blot.
Supplementary Figure S5 Full scans of original blots for data in Fig. 1, 2, 3, 4, 5, and 6. Panels corresponding to the figures in the paper are indicated.
Supplementary Figure S5 continued
Figure 2

Supplementary Figure S5 continued
Supplementary Figure S5 continued
Supplementary Figure S5 continued
Supplementary Figure S5 continued
Figure 4 h

Figure 6 a

Figure 6 c

Supplementary Figure S5 continued