AMPK modulates Hippo pathway activity to regulate energy homeostasis

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The Hippo pathway was discovered as a conserved tumour suppressor pathway restricting cell proliferation and apoptosis. However, the upstream signals that regulate the Hippo pathway in the context of organ size control and cancer prevention are largely unknown. Here, we report that glucose, the ubiquitous energy source used for ATP generation, regulates the Hippo pathway downstream effector YAP. We show that both the Hippo pathway and AMP-activated protein kinase (AMPK) were activated during glucose starvation, resulting in phosphorylation of YAP and contributing to its inactivation. We also identified glucose-transporter 3 (GLUT3) as a YAP-regulated gene involved in glucose metabolism. Together, these results demonstrate that glucose-mediated energy homeostasis is an upstream event involved in regulation of the Hippo pathway and, potentially, an oncogenic function of YAP in promoting glycolysis, thereby providing an exciting link between glucose metabolism and the Hippo pathway in tissue maintenance and cancer prevention.

The Hippo pathway was initially identified through genetic screening in Drosophila as having crucial roles in restricting tissue growth1–3. The evolutionarily conserved functions of this pathway in control of tissue and organ size were further demonstrated through genetically engineered mouse models. In mammalian systems, the Hippo pathway is composed of core kinase complexes (MST1/2 and LATS1/2), adaptor proteins (SAV1 for MST1/2 and MOB1 for LATS1/2), downstream effectors (YAP and TAZ) and nuclear transcription factors (TEAD1/2/3/4). MST1/2 kinase phosphorylates and activates LATS1/2 kinases. Active LATS1/2 phosphorylates YAP at Serine 127 (S127) and provides the docking site for the 14-3-3 protein, which sequesters YAP in cytoplasm. Moreover, LATS1/2 phosphorylates YAP at S381, which leads to YAP degradation through the β-TrCP E3 ligase complex4. Un-phosphorylated YAP translocates into the nucleus and functions as a transcriptional co-activator by binding to the TEAD family of transcription factors. The YAP–TEAD complex regulates transcription of genes that promote proliferation and inhibit apoptosis, two key events for organ size control. Nuclear protein VGLL4 directly competes with YAP for binding to TEAD transcription factors and consequently inhibits YAP’s transcriptional functions5,6.

Notably, ablation of Hippo pathway components leads to tumour formation7–9, which suggested that the Hippo pathway is a tumour suppressor pathway. As the major target of the Hippo pathway, YAP has been identified as an oncogene. Transgenic expression of YAP in mouse liver reversibly enlarged livers and eventually led to tumour formation10,11. Moreover, downregulation of Hippo pathway components and elevated activation of YAP/TAZ have been observed in various human cancers11,12, which further demonstrates the critical roles of the Hippo pathway in human cancer prevention.

Many studies in recent years have been devoted to identification of upstream regulators of the Hippo pathway to elucidate the mechanisms underlying organ size control. These studies uncovered many components of the cell adhesion junction and tight junction as Hippo pathway regulators, findings that agree with the known cell-density-dependent regulation of the Hippo pathway13. Cytoskeleton-mediated mechanical force also plays a key role in YAP regulation14–16. Moreover, G-protein-coupled receptors function upstream of the Hippo pathway through Rho GTPase and cytoskeleton remodelling17. However, the upstream signals that regulate the Hippo pathway in the context of organ size control and cancer prevention are still largely unknown.

In this study, we identified crosstalk between glucose metabolism and the Hippo pathway. The energy stress generated by a defect in glucose metabolism activated LATS kinase and AMPK kinase, leading to phosphorylation of YAP and inhibition of its cellular functions. On the other hand, YAP promoted glucose metabolism through upregulation of glucose-transporter 3 (GLUT3) expression at the transcriptional level. These findings revealed a critical crosstalk between energy homeostasis and the Hippo pathway, underlining...
Glucose homeostasis regulates YAP phosphorylation and localization

(a) The phosphorylation of YAP was regulated by glucose deprivation and stimulation. HEK293A cells were glucose starved for the indicated intervals and then stimulated with glucose (25 mM) for the indicated intervals. Cell lysates from each time point were subjected to western blotting. (b) Glucose but not 2-DG stimulated the dephosphorylation of YAP. Glucose-starved HEK293A cells were released into 25 mM glucose-containing Dulbecco modified essential medium (DMEM), glucose-free DMEM containing 25 mM D-glucose or glucose-free DMEM containing 25 mM 2-DG. Cell lysates from each time point were subjected to western blotting. (c) The localization of YAP was detected by immunostaining with YAP monoclonal antibody in cells released from energy stress (as described for Fig. 1b) for 1 h. Scale bar, 20 μm. The region inside each box is enlarged by a factor of 2.5 below. (d) 2-DG-induced YAP phosphorylation. HEK293A cells were treated with each indicated medium for 4 h. The D-glucose and 2-DG concentrations were 25 mM in each medium. (e) 2-DG-induced YAP phosphorylation was dose dependent. HEK293A cells were treated with glucose-free medium supplemented with 2-DG at the indicated concentrations for 4 h, and cell lysates representing each dose were subjected to western blotting. Phospho-tag-containing gel (p-tag) was used to detect YAP phosphorylation. Uncropped images of western blots are shown in Supplementary Fig. 7.

Results

Glucose homeostasis regulates YAP phosphorylation and localization

We explored whether any growth condition might control activation of the Hippo pathway and found that glucose starvation increased phosphorylation of YAP at S127 (Fig. 1a). The major phosphorylation site regulated by the Hippo pathway. When glucose was added back to glucose-deprived cells, the phosphorylation of YAP at S127 markedly decreased (Fig. 1a). This glucose-stimulated effect was transient, because the phosphorylation of YAP gradually recovered after 2 h or longer (Fig. 1a). The levels of YAP upstream kinases LATS1 and MST1 were not affected by glucose (Fig. 1a), whereas this glucose switch, as expected, regulated phosphorylation of ACC and activation of S6K, AKT and ERK (Fig. 1a).

To further validate these findings, glucose-starved cells were given different types of glucose-containing medium: regular glucose-rich medium (25 mM glucose), or glucose-free medium containing either D-glucose (25 mM) or 2-deoxy-D-glucose (2-DG, 25 mM). 2-DG is a glucose molecule that has the 2-hydroxyl group replaced by hydrogen, so that it fails to undergo further glycolysis and therefore leads to metabolic control of the Hippo pathway and a previously unknown function of the Hippo pathway in glucose metabolism.

Energy stress due to reduced ATP production. The phosphorylation of YAP decreased in cells stimulated by the regular glucose-rich medium or the D-glucose-containing medium, but not in cells stimulated by 2-DG-containing medium (Fig. 1b). The subcellular localization of YAP corresponded to its phosphorylation status: YAP localized mostly in cytoplasm in glucose-starved cells (Fig. 1c), and whereas glucose-rich medium and D-glucose-containing medium induced nuclear translocation of YAP, YAP was retained in the cytoplasm in cells cultured with 2-DG-containing medium (Fig. 1c). These data suggest that glucose status regulates YAP phosphorylation and subcellular localization.

To determine the role of 2-DG in YAP regulation, cells were treated transiently with glucose starvation, 2-DG or a combination. The combination increased YAP phosphorylation to a greater extent than 2-DG treatment alone (Fig. 1d). Moreover, the effect of 2-DG treatment on YAP was dose dependent (Fig. 1e). These data indicate that a 2-DG-induced defect in glucose metabolism could inhibit YAP function.

Release of energy stress activates YAP

Glucose is widely used as an energy source in various organisms, and defects in glucose metabolism create energy stress in the cell. To further address the role of energy stress in YAP function, we employed
three different methods to induce energy stress in cells: glucose starvation, treatment with 2-DG, and treatment with 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR). AICAR is phosphorylated by adenosine kinase to form ZMP, an analogue of AMP, thereby mimicking energy stress conditions with a high AMP level. Under all three of these conditions, YAP localized mostly in the cytoplasm in both HEK293A and MCF10A cells (Fig. 2a). Notably, however, YAP translocated into the nucleus when cells were released from energy stress (Fig. 2a). The decrease of YAP phosphorylation on release from energy stress was observed by both western blotting with YAP-phospho-specific antibody (S127) and the migration shift of YAP in phospho-tag gel (Fig. 2b). Consistent with these findings, YAP phosphorylation decreased in cells released from energy stress. Lysates of HEK293A cells under one of the energy stresses (S) described in a or released from energy stress (R) for 1 h were subjected to western blotting. YAP phosphorylation was detected by phospho-tag-containing gel (p-tag). (c) Release from energy stress for 1 h induced YAP–TEAD1 association but suppressed YAP-14-3-3 interaction. (d) Release of cells from energy stress increased YAP transcriptional activity. YAP-regulated gene transcripts (CTGF, Cyr61, and ANKRD1) were detected by quantitative PCR in cells under energy stress or released from energy stress for 2 h and normalized (mean ± s.d., n = 3 biological replicates). *P < 0.05, **P < 0.01 and ***P < 0.001 (Student t-test). (e) YAP phosphorylation was higher in livers of fasting mice than in livers of mice fed after fasting. Mice that had fasted for 16 h were then fed (n = 3) or fasted (n = 3) for another 5 h. Livers were collected and subjected to lysis and western blotting. The arrow indicates increasing YAP phosphorylation. (f) YAP translocated into the nucleus in the liver of a fasting mouse after feeding. A liver from a fasting mouse and a liver from a mouse fed after fasting from e were randomly chosen for immunohistochemical staining with YAP antibody, followed by haematoxylin staining to visualize the nucleus. Scale bar, 30 μm. The boxed region is enlarged below. (g) YAP transcriptional activity increased in fasting mouse liver after feeding. Transcription of YAP-regulated genes (Ctgf, Cyr61, Acta2 and Amotl2) was detected by quantitative PCR in livers randomly chosen from mice in e (mean ± s.d., n = 3 biological replicates). *P < 0.05 and **P < 0.01 (Student t-test). Statistics source data are shown in Supplementary Table 2. Uncropped images of western blots are shown in Supplementary Fig. 7.
were the observations that release from energy stress diminished YAP’s association with the 14-3-3 protein, while increasing its interaction with TEAD1 (Fig. 2c). Accordingly, release from energy stress also increased the transcription of YAP downstream target genes CTGF, CYR61 and ANKR1D (Fig. 2d), indicating that release from energy stress activates YAP.

We also investigated YAP activity in liver samples obtained from mice that had fasted for 16 h and then were fed or fasted for another 5 h. YAP was less phosphorylated in the liver samples from fed mice than in those from fasting mice (Fig. 2e). Moreover, in the livers of fed mice, YAP translocated into nucleus (Fig. 2f) and promoted transcription of its downstream target genes (Fig. 2g). These results indicate the physiological relevance of energy homeostasis in the regulation of YAP activity.

**AMPK associates with and phosphorylates YAP**

To identify the mechanisms underlying the regulation of YAP by energy stress, we isolated YAP-associated protein complexes in glucose-starved HEK293A cells through tandem affinity purification–mass spectrometry in glucose-starved HEK293A cells. Bait protein is marked in red. Identified AMPKα1 and γ1 subunits are marked in blue. The left column of numbers represents unique peptide number/total peptide number. The known YAP-associated proteins and the identified AMPK subunits are indicated. (b,c) YAP associated with AMPK. Indicated constructs were expressed in 293T cells for 24 h, and cell lysates were subjected to pulldown assays with S protein beads. (d) AMPK phosphorylated YAP in vivo. GST-tagged rat AMPK (amino acids 1–312) or its kinase-dead mutant (T172A) was co-expressed with SFB-tagged YAP in 293T cells. The phosphorylation of YAP was assessed by phospho-tag gel (p-tag). Uncropped images of western blots are shown in Supplementary Fig. 7.

**AMPK phosphorylates YAP on several residues including the S61 site**

In searching for AMPK phosphorylation sites on YAP, we again used mobility shift in phospho-tag gel to assess YAP phosphorylation. We found that the YAP-5SA mutant could not be phosphorylated as markedly as wild-type YAP by the active AMPK truncation mutant (Fig. 4a), which indicated that one or more of these mutated serine residues is the AMPK phosphorylation site(s). The YAP-5SA mutant was originally generated to mutate all of the putative LATS kinase phosphorylation sites on YAP (refs 4,13,22). We sequenced this mutant and confirmed that it comprises eight serine sites mutated to alanine (Fig. 4b). Mutation of each alanine back to serine generated six different YAP-4SA mutants, and of these only YAP-4SA-61S could be intensely phosphorylated by AMPK as evaluated by phospho-tag gel analysis (Fig. 4c). The YAP-S61A mutant could not be phosphorylated as much as wild-type YAP by the active AMPK truncation mutant (Fig. 4d), indicating that the YAP S61 site is an AMPK phosphorylation site.

We also performed in vitro kinase assays and analysed YAP phosphorylation site(s) by mass spectrometry as an independent method to identify AMPK phosphorylation sites. We again uncovered S61, as well as two other sites (S94 and T119), as AMPK phosphorylation sites (Supplementary Fig. 1 and Supplementary Table 1), suggesting that AMPK may phosphorylate S61 as well as other sites on YAP. These data agree with the observation that energy stress was capable of inducing a mild shift of the YAP-5SA mutant in phospho-tag gel (Supplementary Fig. 2A).
To further confirm that S61 is an AMPK phosphorylation site, we generated YAP S61 phospho-specific antibody and validated its specificity in vitro and in vivo (Supplementary Fig. 2B–D). Using this antibody, we showed that YAP S61 phosphorylation increased following 2-DG treatment in wild-type mouse embryonic fibroblasts (MEFs) but not in AMPKα-knockout MEFs (Fig. 4e), suggesting that AMPK phosphorylates YAP at the S61 site in response to energy stress. Notably, the S61 site is evolutionarily conserved (but not in Drosophila).

Figure 4 AMPK phosphorylates YAP at S61 and suppresses its transcriptional activity. (a) AMPK active truncation mutant was co-expressed with indicated plasmids in 293T cells. The protein phosphorylation was assessed by phospho-tag gel (p-tag). (b) Schematic illustration of YAP protein domains and reported LATS kinase phosphorylation sites. The mutated serine sites in the YAP-5SA mutant are listed. (c) AMPK phosphorylated the YAP-4SA-61S mutant. AMPK active truncation mutant was co-expressed with the indicated YAP-4SA mutants in 293T cells. (d) S61 was the AMPK phosphorylation site on YAP. AMPK active truncation mutant was co-expressed with YAP or YAP-S61A mutant in 293T cells. The phosphorylation of YAP was assessed by phospho-tag gel (p-tag). (e) 2-DG-induced AMPK-dependent YAP-S61 phosphorylation. Wild-type MEFs (WT) and AMPKα-knockout MEFs (AMPKα KO) were treated with 25 mM 2-DG in glucose-free medium for 4 h. Endogenous YAP was immunoprecipitated (IP) and immunoblotted with the indicated antibodies. (f) AMPK did not noticeably affect YAP phosphorylation at the S127 site. (g) 2-DG-induced LATS1 kinase activation was observed in both WT and AMPKα KO MEFs. MEF cells were treated with 25 mM 2-DG in glucose-free medium for 4 h. Endogenous LATS1 was immunoprecipitated and subjected to in vitro kinase assay with GST-YAP (2 μg) as substrate. (h) S61 was not the major phosphorylation site for LATS kinase. The Hippo pathway kinase complex (Myc-LATS2, HA-MOB1, HA-MST2 and HA-SAV1) was co-expressed with YAP-4SA-61S or YAP-4SA-127S mutant in 293T cells. (i) Phosphorylation of S61 suppressed YAP transcriptional activity. The transcripts of CTGF and CYR61 were detected in the indicated YAP-stable cells by quantitative PCR (mean ± s.d., n = 3 biological replicates). ***P < 0.001 (Student t-test). (j) AMPK suppressed YAP transcriptional activity. The transcription of YAP downstream genes was examined in WT and AMPKα KO MEF cells by quantitative PCR (mean, n = 2 biological replicates). (k) Loss of AMPK partially rescued YAP activity in 2-DG-treated MEF cells. WT and AMPKα KO MEF cells were mock-treated or treated with 25 mM 2-DG in glucose-free medium for 4 h. YAP downstream genes were examined by quantitative PCR (mean, n = 2 biological replicates) and normalized to untreated cells. Statistics source data are shown in Supplementary Table 2. Uncropped images of western blots are shown in Supplementary Fig. 7.
and shows some similarity to the consensus AMPK phosphorylation site (Supplementary Fig. 2E).

**AMPK phosphorylates YAP in parallel with the Hippo pathway**

Although YAP S127 phosphorylation increased following energy stress (Fig. 1), AMPK did not seem to be the major contributor to the phosphorylation of YAP at the S127 site (Fig. 4f). LATS1 phosphorylation and MST1 phosphorylation were not significantly increased with the phosphorylation of YAP at the S127 site (Fig. 4f). LATS1 kinase activation induced by 2-DG treatment was not markedly different in wild-type or AMPK-knockout MEFs (Fig. 4g and Supplementary Fig. 3C). These results indicate that energy stress activates the Hippo pathway in a manner that is largely independent of AMPK treatment was not markedly different in wild-type or AMPK-knockout MEFs (Fig. 4g and Supplementary Fig. 3C). These results indicate that energy stress activates the Hippo pathway in a manner that is largely independent of AMPK.

**AMPK-mediated YAP phosphorylation inhibits YAP transcriptional activity**

Previous studies suggested that phosphorylation of YAP increased its interaction with the 14-3-3 protein, which sequesters YAP in the cytoplasm. The S5A mutant of YAP blocked YAP’s association with the 14-3-3 protein, translocated YAP into the nucleus and increased YAP transcriptional functions. Since the YAP S61 site is among the putative phosphorylation sites mutated in the S5A mutant, we first examined whether AMPK would affect the interaction between YAP and the 14-3-3 protein. AMPK-mediated YAP phosphorylation did not increase the association of YAP with the 14-3-3 protein (Supplementary Fig. 4A). The S127 site, but not the S61 site, was the binding site for the 14-3-3 protein, which sequesters YAP in the cytoplasm. The active AMPK truncation mutant did not change YAP localization (Supplementary Fig. 4D), and the YAP S61 mutant did not regulate YAP nuclear localization (Supplementary Fig. 4E). However, we noticed that the S61A single mutation increased the transcription of YAP downstream target genes (CTGF and CYR61; Fig. 4i). These data suggest that YAP transcriptional activity was suppressed by S61 phosphorylation, although the binding between YAP and TEAD was...
Figure 6 YAP regulates GLUT3 transcription in the glucose metabolic pathway. (a,b) YAP activity increased the acidification of cell culture medium. The color of the medium varied in the same-time cultures of the indicated YAP-stable cell lines (a). The pH was measured in cultures of the indicated YAP-stable cell lines (mean ± s.d., n = 3 biological replicates) (b). *P < 0.05 and **P < 0.001 (Student t-test). (c,d) YAP-S9A mutation promotes glycolysis. 2-NBDG uptake (c) and lactate production (d) were examined in the indicated YAP-stable cells (mean ± s.d., n = 3 biological replicates) as described in Methods. *P < 0.05 and **P < 0.01 (Student t-test). (e) GLUT3 was identified as a YAP-regulated gene. The transcripts of glucose metabolism-related genes were detected by quantitative PCR in YAP-S9A cells and control vector-transfected cells. Fold increases are shown (mean, n = 2 biological replicates). (f-h) The control of GLUT3 transcription by YAP was confirmed in various cell lines by quantitative PCR (mean ± s.d., n = 3 biological replicates). **P < 0.01 and ***P < 0.001 (Student t-test). (i,j) YAP increased the level of GLUT3 protein in various cell lines. (k,l) Loss of YAP attenuated the increased GLUT3 expression in cells released from 16 h of glucose starvation. The downregulation of YAP protein was confirmed by western blotting (k). The transcription of GLUT3, AMOTL2 and CYR61 was detected by quantitative PCR in cells under glucose starvation or released from glucose starvation for 2 h and normalized (mean ± s.d., n = 3 biological replicates) (l). (m) GLUT3 was knockdown in YAP-S9A HEK293A cells by two different GLUT3 shRNAs. (n,o) GLUT3 knockdown suppressed the acidification of YAP-S9A cell medium. The color of the medium varied in the cultures of the indicated cell lines (n). The pH was measured in the indicated cells (mean ± s.d., n = 3 biological replicates) (o). ***P < 0.001 (Student t-test). (p,q) Knockdown of GLUT3 suppressed YAP-S9A-induced glycolysis. 2-NBDG uptake (p) and lactate production (q) were examined (mean ± s.d., n = 3 biological replicates) as described in Methods. **P < 0.01 and ***P < 0.001 (Student t-test). Statistics source data are shown in Supplementary Table 2. Individual data are plotted for b-d-o-q. Uncropped images of western blots are shown in Supplementary Fig. 7.
not noticeably affected by the phosho-mimetic mutant of the S61 site (S61D) (Supplementary Fig. 4F). Exactly how AMPK-dependent S61 phosphorylation inhibits YAP transcriptional activity remains unknown. It is possible that this AMPK-dependent phosphorylation at the YAP S61 site may affect the binding of YAP with some yet unknown proteins required for YAP activity, and we are now investigating this hypothesis.

On the other hand, the YAP S94 site was also identified as an AMPK phosphorylation site (Supplementary Fig. 1 and Supplementary Table 1), and the phosho-mimetic mutant of this site (S94D) greatly diminished YAP–TEAD interaction (Supplementary Fig. 4F). Moreover, a single phosho-mimetic mutation of either S61 or S94 suppressed YAP transcriptional activity, and the combined phosho-mimetic mutation of both sites further suppressed it (Supplementary Fig. 4G). These data indicate that AMPK may inhibit YAP transcriptional activities by phosphorylating multiple sites on YAP.

To further confirm the suppressive role of AMPK in YAP regulation, we examined YAP transcriptional activity in wild-type or AMPK-deficient MEFs. The transcription of YAP downstream genes was elevated in AMPK-knockout MEFs (Fig. 4j). Moreover, loss of AMPK attenuated the relative suppressive effect on YAP induced by 2-DG compared with that in control MEFs (Fig. 4k). Together, these results indicate that AMPK is a negative regulator of YAP in response to energy stress.

Energy stress activates LATS kinase but not MST kinase in the Hippo pathway

The observation that energy-stress-regulated YAP S127 phosphorylation (Fig. 1) indicates that the Hippo pathway was also activated by energy stress. Interestingly, 2-DG treatment increased LATS1 kinase phosphorylation (T1079) (Fig. 5a) but not MST1 kinase phosphorylation (T183) (Fig. 5b). Release from energy stress rapidly decreased the LATS1 phosphorylation but not MST1 phosphorylation (Fig. 5c). Moreover, YAP S127 phosphorylation was increased by 2-DG in MST1/2 double-knockout cells (Fig. 5d). These data indicate that energy stress induces YAP S127 phosphorylation through activation of LATS kinases but not MST kinases in the Hippo pathway.

Energy stress induces LATS kinase activation mainly through Rho GTPase and cytoskeleton

Previous studies suggested that Rho GTPase-mediated remoulding of actin cytoskeleton could inhibit LATS kinase and activate YAP independent of MST kinase[14,17]. We therefore examined the roles of actin cytoskeleton and Rho GTPase in energy-stress-regulated YAP phosphorylation. In cells released from energy stress, latrunculin B-mediated actin depolymerization reversed the decrease of YAP S127 phosphorylation (Fig. 5e). Rho GTPase inhibitor C3 also rescued the decrease of YAP S127 phosphorylation (Fig. 5e). Furthermore, release from energy stress increased the Rho GTPase activity (GTP-formed

Figure 7 YAP and GLUT3 expression positively correlate with each other in human colon and liver cancers. (a) Representative normal colon and colon carcinoma specimens and normal liver and liver carcinoma specimens in tissue arrays were subjected to immunohistochemical staining of YAP and GLUT3. Brown staining indicates positive immunoreactivity. The region in each box is enlarged below. Scale bar, 30 μm. (b) Correlations between YAP and GLUT3 protein levels in human colon and liver tumours were analysed. Statistical significance was determined by the chi-square test; R: correlation coefficient.
Rho; Fig. 5f). Although detailed mechanisms are still unclear, several studies have shown that the activity of Rho GTPase is regulated by glucose treatment, which is consistent with our findings presented in Fig. 5f. Thus, energy stress may activate LATS kinases by modulating Rho GTPase activity. It remains to be determined whether the actin cytoskeleton plays a direct and specific role in this process.

**Active YAP promotes glycolysis**

The results presented so far established a connection between glucose homeostasis and the regulation of YAP. The next question is the functional significance of this regulation. We noticed, interestingly, that the culture medium for YAP-5SA cells quickly turned yellow compared with the culture media for the vector-control cells, the wild-type YAP cells or the TEAD non-binding mutant YAP-S94A (ref. 26) cells (Fig. 6a), although the numbers of cells in each culture were similar. The yellow colour of the medium indicated that it had been acidified and the level of glycolysis increased in these cells (Fig. 6a). Indeed, the YAP-5SA medium showed a lower pH value but higher glucose uptake and lactate production than media from other cell lines (Fig. 6b–d). These data suggest that YAP is involved in the glucose metabolism pathway and that this involvement is probably mediated by its target genes.

**YAP upregulates GLUT3 in glucose metabolism pathway**

To identify YAP-regulated genes involved in glucose metabolism, we examined the transcription of a panel of glucose metabolism-related genes in cells expressing the YAP-5SA mutant and in vector-control cells. The analysis showed that GLUT3 transcription was much higher in YAP-5SA cells than in vector-control cells (Fig. 6e). The dependence on YAP transcriptional activity was confirmed in YAP-5SA-S94A cells, in which GLUT3 transcription decreased (Fig. 6f). Similar findings were obtained from other cell lines (Fig. 6g, h). Furthermore, a higher level of GLUT3 protein was detected in YAP-5SA cells than in other cells by western blotting (Fig. 6i, j). Downregulation of YAP (Fig. 6k) suppressed the increase of GLUT3 transcription in cells released from energy stress (Fig. 6l). We identified a conserved TEAD-binding site in the GLUT3 promoter, and ENCODE data showed that this site was bound by TEAD (Supplementary Fig. 5). These results indicate that YAP–TEAD may directly regulate the transcription of GLUT3. Moreover, results from two other studies suggested that the expression of GLUT3 was upregulated by active YAP, which support our findings that YAP regulates GLUT3 expression.

To determine the role of GLUT3 in YAP-mediated glycolysis, we knocked down GLUT3 in YAP-5SA cells (Fig. 6m) and found that this downregulation of GLUT3 partially reversed medium acidification, glucose uptake and lactate production in cells expressing the YAP-5SA mutant (Fig. 6n–q). These data suggest that GLUT3 is at least one of the downstream effectors involved in YAP-regulated glycolysis.

**GLUT3 expression correlates with YAP protein level in human liver and colon cancers**

Since high expression of GLUT3 has been identified in various types of cancers, we examined expression of YAP and GLUT3 in samples of human cancer, specifically colon and liver cancers. Indeed, the expression of YAP and GLUT3 was higher in tumour samples than in normal tissues (Fig. 7a), and their expressions were positively correlated in tumour samples (Fig. 7b). These data indicate a potential role for YAP in regulating GLUT3 expression in human cancers.

**DISCUSSION**

In this study, we unexpectedly discovered that AMPK, the master kinase that senses the cellular AMP/ATP ratio, directly phosphorylates YAP and inhibits YAP transcriptional activity. We demonstrated that AMPK directly phosphorylates YAP at multiple sites and thus suppresses YAP transcriptional activity. We primarily investigated phosphorylation at S61 by AMPK, as this phosphorylation is the major event contributing to YAP migration shift on phospho-tag gel. We also noticed a minor shift of the YAP-5SA mutant on phospho-tag gel, indicating that there are additional phosphorylation sites. Indeed, our mass spectrometry analysis supports these findings and revealed two more AMPK phosphorylation sites on YAP, S94 and T119. The estimated phosphorylation on these three sites indicated that S61 (11.27%) and S94 (2.39%) are relatively dominant phosphorylation sites compared with T119 (0.53%). The S94 site is known to be required for YAP’s binding to TEAD and mutation of this site (S94A) abolishes TEAD-dependent YAP transcriptional activity. The phospho-mimetic mutant of S94 (S94D) also inhibited the interaction between YAP and TEAD as well as transcription of YAP downstream target genes. In this issue, Guan and colleagues report similar findings about AMPK-dependent YAP phosphorylation on the S94 site, complementing our results. Together, with their findings, our results suggest that AMPK may phosphorylate multiple sites on YAP and inhibit YAP transcriptional activity.

We also revealed that energy stress increases S127 phosphorylation and cytoplasmic localization of YAP by activating the Hippo pathway. We explored the crosstalk between AMPK and the Hippo pathway, because both pathways are activated following energy stress. In LATS- or MST-deficient cells, AMPK activity, as indicated by ACC S79 phosphorylation, was still increased by 2-DG treatment (Fig. 5d), suggesting that AMPK is activated by energy stress independent of the Hippo pathway. On the other hand, 2-DG-induced LATS kinase activation was slightly decreased in AMPKtet-knockout MEF cells (Fig. 4g and Supplementary Fig. 3C), indicating the involvement of both AMPK-dependent and -independent pathways in the regulation of LATS kinase activity in response to energy stress. The AMPK-dependent regulation of the Hippo pathway in agreement with recent finding that AMPK may activate the Hippo pathway by increasing the levels of angiogenin family proteins under energy stress. Our findings showed that the AMPK-independent pathway may be regulated by Rho GTPase, although the detailed mechanisms remain to be elucidated. Similar findings are reported by Guan and colleagues in this issue. Therefore, both AMPK and the Hippo pathway are activated following energy stress, which act together to suppress YAP activity (Supplementary Fig. 6).

Our results demonstrated that the Hippo pathway is intimately associated with glucose homeostasis. This is highly significant as it establishes a connection between a pathway involved in organ size control and nutrient availability. The discovery of AMPK as a kinase for YAP, reported here, extends our understanding of YAP regulation outside of the classic Hippo pathway. The identification of GLUT3 as a YAP-regulated gene offers a potential link between the Hippo pathway and cancer metabolism. As hallmarks of cancer,
tumour cells are known to reprogram their signalling pathways and metabolism to support their uncontrolled proliferation and survival. Our study proposes yet another oncogenic function of YAP, via promotion of glucose uptake and glycolysis, which warrants further investigation.

METHODS

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

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AUTHOR CONTRIBUTIONS

W.W. performed all of the experiments with assistance from Z-D.X., X.L., K.E.A., B.G., R.L. and J.C. W.W. and J.C. designed the experiments. J.C. supervised the study. W.W. and J.C. wrote the manuscript. All authors commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**METHODS**

**Antibodies.** Anti-YAP antibody (1:1,000 dilution) was raised by immunizing rabbits with bacterially expressed and purified GST-fused human YAP protein. Anti-AMOTL2 antibody (1:1,000 dilution) was raised by immunizing rabbits with bacterially expressed and purified GST-fused human AMOTL2 protein (amino acids 1–675). Anti-serum was affinity purified by using the AminoLink Plus immobilization and purification kit ( Pierce). Anti-YAP S61 phospho-specific antibody (1:1,000 dilution) was raised against keyhole limpet hemocyanin-conjugated phospho-peptide Biotin-QHVHRGd(phospho-S)-JEDTLEALC, and antisera was affinity purified through SulfoLink peptide coupling gel. Additional anti-YAP (sc101199, 1:200 dilution for immunostaining) and anti-phospho-YAP (S127) (4911S, 1:2,000 dilution) antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling Technology respectively. Anti-α-tubulin (T6199-200UL, 1:5,000 dilution) and anti-flag (M2) (F3165-5MG, 1:5,000 dilution) monoclonal antibodies were obtained from Sigma-Aldrich. Anti-Myc (sc-40, 1:1,000 dilution), anti-GST (sc-138, 1:1,000 dilution) and anti-14-3-3 (sc-732, 1:1,000 dilution) monoclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-TEAD1 (610922, 1:1,000 dilution) monoclonal antibody was obtained from Millipore. Anti-phospho-AKT1 (Ser473) (9271S, 1:1,000 dilution), anti-phospho-ERK1/2 (Thr202/Tyr204) (9100S, 1:1,000 dilution), anti-phospho-ACC (Ser79) (3661S, 1:1,000 dilution), anti-ACC (3662S, 1:1,000 dilution), anti-phospho-p70 S6K (Thr389) (9205S, 1:1,000 dilution), anti-phospho-AMPKα (Thr172) (2531S, 1:1,000 dilution), anti-AMPKα (2325S, 1:1,000 dilution), anti-phospho-LAT1 (Thr1079) (6065S, 1:1,000 dilution), anti-LAT1 (9153S, 1:1,000 dilution and 3477S, 1:100 dilution for immunoprecipitation), anti-phospho-MST1 (Thr183/MST2 (Ser180) (3681S, 1:1,000 dilution) and anti-MST1 (3682S, 1:1,000 dilution) polyclonal antibodies were purchased from Cell Signaling Technology. Anti-GLUT3 (sc-30107, 1:1,000 dilution) monoclonal antibody was purchased from Santa Cruz Biotechnology. Anti-RhoA (ARH03-A, 1:1,000 dilution) monoclonal antibody and GST-Rhotekin-RBD protein (RT01-A) were obtained from Cytoskeleton.

**Glucose uptake assay.** Cells were seeded in 10-cm dishes. Twenty-four hours later, cells were washed and incubated with fluorescein isothiocyanate or rhodamine-conjugated secondary antibody against primary antibodies (1:3,000 dilution, Jackson Immunoresearch) for 1 h. Cells were counterstained with 100 ng ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) for 2 min to visualize nuclear DNA. The cover slips were mounted onto glass slides with anti-fade solution and visualized on a Nikon ECLIPSE E800 fluorescence microscope with a Nikon Plan Fluor 100× oil objective lens (NA 1.30).

**RNA extraction, reverse transcription and real-time PCR.** RNA samples were extracted with TRIZOL reagent (Invitrogen). Reverse transcription assay was performed by using the ProtoScript M-MuLV Taq RT-PCR Kit (New England Biolabs) according to the manufacturer’s instructions. Real-time PCR was performed by using Power SYBR Green PCR master mix (Applied Biosystems). For quantification of PCR data, the 2⁻ΔΔCt method was used. GAPDH expression was used for normalization. The sequence information for each primer used for gene expression analysis is as follows:

For human cell lines: CTGF-Forward: 5'-CCAAATTGACAAAGCCCTTGCTC-3', TGF-Reverse: 5'-GAGCTTCTTGCTGCTGACCA-3', CYR61-Forward: 5'-AGCCTGCGATTCTACACCC-3', CYR61-Reverse: 5'-GATGCCGGCTTTTGGAAAGA-3', ANKR1D1-Forward: 5'-CACCCTTGAACCTCTGGTG-3', ANKR1D1-Reverse: 5'-GCCCTTTGATCAGTCGGAACG-3', Ctgf-Forward: 5'-GAGTGCCGCCTTGTGAAAGA-3', Ctgf-Reverse: 5'-GGAGTGCCCTTGTGAAAGA-3'.

For mouse cell lines: 18S-Forward: 5'-TCTCTTGCTCTGGGCTTCATCC-3', 18S-Reverse: 5'-TGTGCTGACAGTCGGAACG-3'.

**Immunofluorescent staining.** Cells cultured on coverslips were fixed for 4% paraformaldehyde for 10 min at room temperature and then extracted with 0.5% Triton X-100 solution for 5 min. After blocking with Tris-buffered saline and Tween 20 solution containing 1% bovine serum albumin, cells were incubated with the indicated primary antibodies for 1 h at room temperature. After that, cells were washed and incubated with fluorescein isothiocyanate or rhodamine-conjugated secondary primary antibodies (1:3,000 dilution, Jackson Immunoresearch) for 1 h. Cells were counterstained with 100 ng ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI) for 2 min to visualize nuclear DNA. The cover slips were mounted onto glass slides with anti-fade solution and visualized on a Nikon ECLIPSE E800 fluorescence microscope with a Nikon Plan Fluor 100× oil objective lens (NA 1.30).

**Glucose uptake assay.** Cells were seeded in 10-cm dishes. Twenty-four hours later, cells were washed and incubated with fluorescein-dextran with D-glucose (1 mg ml⁻¹) for 1 h and
washed with ice-cold phosphate-buffered saline solution twice. Glucose uptake was quantified in the propidium iodide-negative population of cells using fluorescence-activated cell sorting analysis.

**Lactate production assay.** Cells were seeded in 6-well dishes. Forty-eight hours later, cell medium was removed and lactate concentration was determined by using Lactate Plus test strips and a Lactate Plus meter (Nova Biomedical). The cells remaining in the dish were harvested and counted by using a haemocytometer on a microscope. The rate of lactate production was calculated (lactate production = total lactate mole number/cell number).

**Immunohistochemical analysis.** Colon and liver tissue arrays were purchased from US Biomax. Samples were deparaffinized and rehydrated, and antigens were retrieved by applying Unmask Solution (Vector Laboratories) in a steamer for 30 min. To block endogenous peroxidase activity, the sections were treated with 1% hydrogen peroxide in methanol for 30 min. After 1 h pre-incubation in 10% goat serum to prevent nonspecific staining, the samples were incubated with an antibody to YAP (4912S, Cell Signaling Technology, 1:20 dilution) or GLUT3 (goat serum) to prevent nonspecific staining, the samples were incubated with SignalStain Boost Detection Reagent for 30 min at room temperature. Colour was developed with the SignalStain DAB Chromogen diluted solution (all reagents were obtained from Cell Signaling Technology). Sections were counterstained with Mayer haematoxylin. The correlation between YAP and GLUT3 and the correlation of YAP or GLUT3 with tissue type (normal versus cancer) were determined by the chi-square test.

**Statistical analysis.** Each experiment was repeated twice or more, unless otherwise noted. No samples or animals were excluded from the analysis. For the mouse fasting experiment, no statistical method was used to predetermine sample size. The samples or animals were randomly assigned to different groups. A laboratory technician who provided animal care and collected the livers was blinded to the group allocation during all animal experiments and outcome assessment. Differences between groups were analysed by the Student t-test and Pearson chi-square analysis. A P value <0.05 was considered statistically significant.

**Phosphorylation analysis of YAP by liquid chromatography–mass spectrometry.** Excised gel bands were cut into approximately 1 mm2 pieces. The samples were reduced with 1 mM dithiothreitol for 30 min at 60°C and then alkylated with 5 mM iodoacetamide for 15 min in the dark at room temperature. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure. Gel pieces were washed and dehydrated with acetonitrile for 10 min, after which acetonitrile was removed and gel pieces were completely dried in a SpeedVac. The gel pieces were rehydrated with 50 mM ammonium bicarbonate solution containing 12.5 ng ml⁻¹ modified sequencing-grade trypsin (Promega) at 4°C. Samples were then placed in a 37°C room overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a SpeedVac (~1 h) and the samples were stored at 4°C until analysis.

On the day of analysis the samples were reconstituted in 5–10 μl of high-performance liquid chromatography solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 2.6 μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter × ~25 cm length) with a flame-drawn tip. After the column was equilibrated, each sample was loaded via a Famos auto sampler (LC Packings) onto the column. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As each peptide was eluted, it was subjected to electrospray ionization and was then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific). Eluted peptides were detected, isolated and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein or translated nucleotide databases with the acquired fragmentation pattern with the software program Sequest (ThermoFinnigan). The modification of 79.9663 mass units to serine, threonine or tyrosine was included in the database searches to determine phospho-peptides. Phosphorylation assignments were determined by the Ascore algorithm. Phospho-peptide with Ascore >13 was considered a putative phosphorylation site according to the instructions from the Taplin Mass Spectrometry Facility, Harvard Medical School.

**In vitro kinase assay.** Bacteria-purified GST proteins (GST, GST-YAP, GST-YAP-S61A and GST-ACC [amino acids 1–130]) were subjected to dialysis against Tris-HCl (pH 8.0; 20 mM) and 10% glycerol at 4°C overnight. GST fusion proteins (2 μg) were used for the in vitro kinase assay in all the experiments. For the AMPK kinase assay, SFB-AMPKα1 or its kinase-dead mutant (K47R) was co-expressed with Flag-AMPKβ2 or Flag-AMPKβ2 plasmid in 293T cells for 48 h. AMPK complex was pulled down by S protein beads and subjected to the kinase assay in the presence of cold ATP (500 μM) and indicated GST fusion protein. The reaction mixture was incubated at 30°C for 30 min, terminated with SDS loading buffer and subjected to SDS–PAGE and autoradiography. For the LATS kinase assay, endogenous LATS kinase was immunoprecipitated from the indicated cell lysates and the assay performed similarly. Phosphorylation of YAP at the S127 site was determined by YAP S127 phospho-antibody.

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Supplementary Figure 1 Identification of AMPK phosphorylation sites on YAP by mass spectrometry. Bacteria-purified GST-YAP (2 mg) was used as substrate for AMPK in vitro kinase assay in the presence of cold ATP (500 μM) as described in the Supplementary Methods. Phosphorylation analysis of YAP was performed by the Taplin Mass Spectrometry Facility. Information on the identified phospho-peptides is shown.
**Supplementary Figure 2** Validation of YAP S61 phospho-antibody. (A) 2-DG induced YAP 5SA mutant phosphorylation. HEK293A cells were transfected with indicated plasmids and treated with glucose-free medium supplemented with 2-DG for 4 hours. Cell lysates were subjected to Western blotting. (B) *In vitro* kinase assay using purified AMPK kinase. SFB-AMPKa1 or its kinase-dead mutant (K47R) was co-expressed with Flag-AMPKb2 or Flag-AMPKg2 plasmid in 293T cells for 48 hours. AMPK complex was purified by S protein beads and subjected to the *in vitro* kinase assay, where GST-ACC (residues 1-130) (2 mg) was purified from bacterial and taken as the substrate. Reaction samples were subjected to Western blotting with indicated antibodies to verify the AMPK-dependent phosphorylation of ACC. (C-D) Validation of YAP S61 phospho-antibody. YAP S61 phospho-antibody was used to detect AMPK-mediated YAP phosphorylation by the *in vitro* kinase assay (C). Short exp and long exp represent short exposure and long exposure respectively. YAP S61 antibody was also used to detect AMPK active truncation mutant-mediated YAP phosphorylation *in vivo* (D). AMPK kinase-dead mutant (K47R) and active AMPK truncation dead mutant (T172A) were used as negative controls for AMPK. YAP-S61A mutant was used as the control for wild-type YAP. (E) Sequence alignment of the AMPK phosphorylation sites in YAP from different species and other known AMPK substrates. Uncropped images of Western blots are shown in Supplementary Figure 7.
Supplementary Figure 3 AMPK is not essential for Hippo pathway activation under energy stress. (A-B) AMPK did not noticeably affect LATS1 and MST1 activation. SFB tagged LATS1 (A) or MST1 (B) was co-expressed with active AMPK truncation mutant in 293T cells. Cell lysates were subjected to Western blotting with indicated antibodies. (C) LATS1 kinase activation was observed in both wild-type and AMPK-deficient cells. Wild-type MEFs (WT) and AMPKα-knockout MEFs (AMPKα KO) were treated with 2-DG (25 mM) in glucose-free medium and rapamycin (50 nM) for 4 hours. Western blotting analysis was performed using indicated antibodies. Uncropped images of Western blots are shown in Supplementary Figure 7.
Supplementary Figure 4  AMPK-regulated YAP phosphorylation does not affect 14-3-3 binding or subcellular localization of YAP, but suppresses its transcriptional activity. (A) Phosphorylation of YAP by AMPK did not affect the association of YAP with the 14-3-3 protein. Indicated plasmids were co-expressed in 293T cells. Cell lysates were used for pulldown assay with S protein beads and subjected to Western blotting. (B-C) Phosphorylation of YAP on the S61 site was not required for the binding of YAP to 14-3-3 (B). The active AMPK truncation mutant did not increase the association between the YAP-4SA-61S mutant and 14-3-3 (C). (D) AMPK did not affect cellular localization of YAP. HeLa cells with exogenously expressed active AMPK truncation were subjected to immunostaining with the GST and YAP antibodies. Nuclei were visualized by Dapi. Scale bar=20 mm. (E) S61 mutation did not affect YAP localization. HeLa cells with exogenously expressed indicated YAP mutants were subjected to immunostaining with Flag antibody. Nuclei were visualised by Dapi. Scale bar=20 mm. (F) Phospho-mimetic mutation of the S94 site (S94D), but not the 61 site (S61D), interfered with YAP-TEAD binding. Indicated plasmids were co-expressed in 293T cells. Cells lysates were used for pulldown assay with S protein beads and subjected to Western blotting. (G) Phospho-mimetic mutants of the S61 site (S61D) and the S94 site (S94D) suppressed the transcription of YAP downstream genes. The transcripts of YAP-regulated genes (CTGF and CYR61) were detected by quantitative PCR in indicated YAP stable cells and normalised (mean±s.d, n=3 biological replicates). *** p<0.001 (Student t-test). Statistics source data are shown in Supplementary Table 2. Uncropped images of Western blots are shown in Supplementary Figure 7.
Supplementary Figure 5 Predicted TEAD-binding site in the GLUT3 promoter region. A conserved TEAD binding site was identified in the GLUT3 promoter region. ENCODE data showed that this site was occupied by TEAD transcription factor.
Supplementary Figure 6 A proposed model of YAP regulation by AMPK and Hippo pathway under energy stress. YAP is phosphorylated and suppressed in response to energy stress. Both LATS kinase and AMPK are activated by energy stress and contribute to YAP inhibition via phosphorylating it on multiple sites. Energy stress induced-LATS kinase activation largely depends on the suppression of Rho GTPase and actin cytoskeleton dynamics, but it is also indirectly regulated by AMPK. With glucose is abundant, YAP translocates into nucleus and promotes glycolysis at least in part by upregulating GLUT3 at transcriptional level.
Table 1 Identification of YAP phosphorylation site by AMPK.
AMPK-mediated phosphorylation of YAP was conducted by in vitro kinase assay and the samples were subjected to analysis by the Taplin Mass Spectrometry Core Facility. Detailed information about phospho-peptides identified is shown in this table.
Table 2 Statistics source data.
This table includes the statistics source data for all of the indicated figures and supplementary figures.
Supplementary Figure 7  Uncropped images of key Western blots and gels.
Supplementary Figure 7 continued
Supplementary Figure 7 continued
Supplementary Figure 7 continued
