p53-mediated control of aspartate-asparagine homeostasis dictates LKB1 activity and modulates cell survival

Longfei Deng\textsuperscript{1,2}, Pengbo Yao\textsuperscript{1,2}, Le Li\textsuperscript{1,2}, Fansen Ji\textsuperscript{1,3}, Shuang Zhao\textsuperscript{1,2}, Chang Xu\textsuperscript{1,2}, Xun Lan\textsuperscript{1,3} & Peng Jiang\textsuperscript{1,2}✉

Asparagine synthetase (ASNS) catalyses the ATP-dependent conversion of aspartate to asparagine. However, both the regulation and biological functions of asparagine in tumour cells remain largely unknown. Here, we report that p53 suppresses asparagine synthesis through the transcriptional downregulation of ASNS expression and disrupts asparagine-aspartate homeostasis, leading to lymphoma and colon tumour growth inhibition in vivo and in vitro. Moreover, the removal of asparagine from culture medium or the inhibition of ASNS impairs cell proliferation and induces p53/p21-dependent senescence and cell cycle arrest. Mechanistically, asparagine and aspartate regulate AMPK-mediated p53 activation by physically binding to LKB1 and oppositely modulating LKB1 activity. Thus, we found that p53 regulates asparagine metabolism and dictates cell survival by generating an autoamplification loop via asparagine-aspartate-mediated LKB1-AMPK signalling. Our findings highlight a role for LKB1 in sensing asparagine and aspartate and connect asparagine metabolism to the cellular signalling transduction network that modulates cell survival.
The dysregulation of asparagine synthetase (ASNS) expression in childhood acute lymphoblastic leukaemia (ALL) cells is considered to increase cell susceptibility to the toxicity of L-asparaginase (ASNase), a first-line therapy for ALL that breaks down asparagine. ASNS inhibition also renders some types of tumour cells more susceptible to glutamine withdrawal-induced apoptosis, and asparagine addition sufficiently reverses this effect independent of TCA cycle anaplerosis. ASNS and asparagine may be crucial for tumour cell proliferation, as the depletion of either can arrest cell proliferation and/or induce apoptosis in some types of tumour cells. However, the mechanisms underlying these observations are poorly understood.

The tumour suppressor p53 is the most frequently mutated gene in human cancers. Consistent with this, p53-null mice are highly prone to the spontaneous development of different tumours. However, ~70% of spontaneous tumours arising in p53-deficient mice are lymphomas, with the underlying mechanisms unknown. The activation of p53 is able to induce a range of apoptotic responses, including cell apoptosis, senescence and differentiation, and metabolic regulation appears to be central to the tumour-suppressive function of p53.

In addition to the induction of permanent proliferation arrest or cell death, under some mild metabolic stresses, such as transient nutrient starvation, p53 activation confers adaptation to stress and helps cells survive. This survival-supporting ability of p53 is mostly implemented by a successful p21-mediated pause in cell cycle progression, which allows cells to repair DNA lesions and/or maintain metabolic homeostasis.

Here, we report that p53 plays a role in regulating asparagine metabolism by repressing the expression of ASNS. High levels of ASNS or asparagine maintain cell survival and promote tumour cell proliferation via stabilising AMPK-mediated p53 activation. To generalise how asparagine may affect tumours in a broader context, we extended our study into various human cell lines besides mouse lymphoma. Moreover, by studying these factors, we found that LKB1 is a natural sensor of cellular asparagine-aspartate homeostasis, uncovering a role for asparagine as a signalling molecule in tumour growth.

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Identification of ASNS as a target of p53. We next investigated how p53 affects asparaginase production. ASNS is critical for maintaining the physiological equilibrium between asparagine and aspartate (Fig. 2a). To study whether ASNS is a physiological target for p53, and also due to the limited transfection efficiency of lymphomas, we examined the effect of p53 on ASNS expression in lymphomas and various human tumour cell lines through different approaches. By comparing the gene expression of p53+/+ and p53−/− HCT116 cells, we found that ASNS expression was significantly augmented in p53−/− cells (Fig. 2b, c). Similar findings were observed in U2OS cells (Fig. 2b, c). Conversely, the forced expression of p53 reduced ASNS mRNA levels (Supplementary Fig. 3a). The pharmacological activation of p53 by nutlin-3 decreased ASNS expression (Fig. 2d and Supplementary Fig. 3b, c), and the suppression of p53 by using PFT-α elevated ASNS expression (Supplementary Fig. 3b). This p53-dependent repression was illustrated by the abrogation of ASNS expression following nutlin-3 treatment in p53-null cells (Fig. 2d and Supplementary Fig. 3c). DNA damage signals such as etoposide...
(ETO) and doxorubicin (DOX) can stabilise p53 protein. Treatment with ETO decreased ASNS expression in p53+/+ cells but not in p53−/− cells (Supplementary Fig. 3d). Similarly, supplying EL4 cells with Nutlin-3, ETO or DOX resulted in a reduction in ASNS expression, whereas PFT-a treatment increased it (Fig. 2e).

To both elucidate whether the p53-mediated regulation of ASNS is cell type specific and further confirm the specificity of the effect observed with p53 depletion at the basal level, we knocked down p53 in a variety of cell lines expressing endogenous wildtype p53 or mutant p53. The silencing of p53 increased ASNS expression in wildtype cell lines but not in mutant cells (Supplementary Fig. 3e, f). In keeping with this, nutlin-3 treatment failed to alter the expression of ASNS in p53-mutated cell lines (Supplementary Fig. 3g).

Table 1 summarises the expression levels of p53, p21, and ASNS in various cell lines treated with Nutlin-3, ETO, or DOX. The results indicate that PFT-a and ETO increase ASNS expression in a p53-dependent manner, while Nutlin-3 decreases it.

Table 1. Expression of p53, p21, and ASNS in various cell lines treated with Nutlin-3, ETO, or DOX.

- **Cell Line**: EL4, HCT116, U2OS, Brain, Liver, Pancreas
- **Treatment**: Nutlin-3, ETO, DOX, PFT-a
- **Protein Levels**: p53, p21, ASNS

The results were analysed using a t-test, and the significance levels are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

To further understand the mechanism behind the regulation of ASNS expression by p53, we performed ChIP-qPCR experiments. The results showed that p53 binding to the ASNS promoter is increased upon p53 depletion, supporting the hypothesis that p53 directly regulates ASNS transcription.

Figure 2f illustrates this finding, with a significant increase in p53 ChIP-qPCR signal in p53−/− cells compared to p53+/+ cells, indicating a direct transcriptional regulation of ASNS by p53.

In conclusion, our findings suggest that p53 plays a crucial role in the regulation of ASNS expression in various cell types, and that this regulation is critical for the maintenance of the normal cell cycle and the prevention of cancer development.
the results show that p53 mutant tumour cells lack the ability to suppress ASNS expression.

ASNS is expressed in various mouse tissues (Supplementary Fig. 3i). Consistent with the observations that plasma from p53−/− mice had higher levels of asparagine (Fig. 1c), multiple tissues from p53−/− mice had higher levels of ASNS expression than did those from p53+/+ mice (Fig. 2f and Supplementary Fig. 3j). Intriguingly, no significant enhancement in ASNS mRNA levels was observed in bone marrow from p53−/− mice, despite a significant increase in ASNS protein (Fig. 2f and Supplementary Fig. 3j), indicating the existence of tissue-specific and transcription-independent mechanism(s) for the regulation of ASNS expression by p53. Nevertheless, these data suggest that ASNS is a physiological target for p53, and its expression is suppressed by p53 at both the genotoxic stress and basal levels.

We next investigated the mechanism for the regulation of ASNS expression by p53. By analysing the ASNS gene sequence for potential p53 protein response elements, which share the consensus sequence of 5′-RRRCWWGGYYY-(0-13 base pair (bp) spacer)-RRRCWWG YYY-3′ (where R is a purine, Y a pyrimidine, and W an A or T; ref. 19), we identified three putative p53 response elements in the ASNS gene (Supplementary Fig. 3k). Chromatin immunoprecipitation (ChiP) assays revealed that p53 bound to all these response element regions (Fig. 2g). Moreover, p53 repressed the expression of a luciferase gene driven by a genomic fragment containing these response elements (ASNS-RE1, ASNS-RE2, or ASNS-RE3, Supplementary Fig. 3l). Together, these results suggest that p53 binds to the ASNS gene and suppresses ASNS expression.

p53 regulates Asn-Asp homeostasis. Next, we investigated whether p53 regulates asparagine metabolism by ASNS (Fig. 2a). Notably, the lack of p53 resulted in increased ASNS activities (Fig. 2h). Moreover, intracellular asparagine levels were significantly augmented in p53-depleted cells (Fig. 2i), consistent with the observations that p53 loss enhances extracellular asparagine levels (Fig. 1h and Supplementary Figs. 2i and 4a). In line with the expression data (Fig. 2e), pharmacological treatment of EL4 cells with p53 activators led to decreased intracellular asparagine (Fig. 2j). However, we found that extracellular asparagine levels varied, which could be attributed to the potential side effects of these compounds on asparagine transport (Supplementary Fig. 4b). Nevertheless, cells treated with PFT-a displayed enhanced asparagine production (Fig. 2j and Supplementary Fig. 4b). p53 loss increased intracellular and extracellular asparagine (Figs. 1h and 2i and Supplementary Figs. 2i and 4a). In contrast, asparagine both within and outside cells declined significantly when p53 was absent (Fig. 2k).

Next, we directly assessed the effect of p53 on natural asparagine synthesis derived from aspartate. Three cell lines (EL4, HCT116 and U2OS) were cultured in medium containing 15N-asparagine, and a portion of intracellular 15N-aspartate and 15N-asparagine was found, suggesting that aspartate could be taken up and converted to asparagine by these cells (Fig. 2l and Supplementary Fig. 4c, d). Remarkably, isotope tracing using 15N-asparagine showed that p53 deficiency enhanced asparagine synthesis from aspartate (Fig. 2m), further confirming the data that a lack of p53 enhances ASNS expression and activity (Fig. 2b–h and Supplementary Fig. 3a–i). Similar findings were obtained in cells cultured with 13C-labeled glutamine ([1-13C]Gln, which could support Asn synthesis by providing carbons through the TCA cycle. While p53 depletion caused an overall decline in aspartate levels (Fig. 2k), cellular [1-13C]Gln-derived asparagine increased when p53 was absent (Fig. 2n). Nevertheless, a significant increase in 13C-asparagine was found in p53-deficient cells (Fig. 2o).

To verify the cell culture findings in animals, we compared the asparagine levels in tissues from p53+/+ mice and p53−/− mice. Consistent with the plasma data (Fig. 1c, Supplementary Fig. 1c and h), higher levels of asparagine were found in liver and pancreas tissues from p53−/− mice than in those from p53+/+ mice (Fig. 2p). These data together demonstrate that p53 regulates ASNS-mediated asparagine-aspartate homeostasis.

ASNS promotes tumour cell proliferation through Asn. The expression of ASNS is ultimately associated with the resistance of leukaemia cells and leukaemic lymphoblasts to asparagine depletion. However, the physiological effect of ASNS on other somatic tumour cells remains largely unknown. As shown in Supplementary Fig. 5a–c, HCT116 cell proliferation was blocked when ASNS was knocked down. In addition, ASNS depletion exerted a more profound effect on p53−/− cells than on wildtype control cells. Likewise, the depletion of ASNS impeded the growth of tumours derived from p53+/+ and p53−/− HCT116 cells (Fig. 3a, b), suggesting that ASNS is important for tumour growth. Similarly, the removal of asparagine from the cell medium by adding ANase remarkably reduced the proliferation of both p53 knockdown HCT116 cells and their control counterparts (Fig. 3c). Analogously, siRNA-mediated silencing of ASNS led to proliferation arrest (Fig. 3d). The underlying mechanism seems to be mediated by asparagine because the readdition of asparagine restored cell proliferation (Fig. 3d, e).

Similar results were obtained using 1-albizzine (1-Alb), a competitive inhibitor of ASNS. Treatment with 1-Alb led to a dose-dependent inhibition of the proliferation of p53+/+ and p53−/− cells. Asparagine addition was sufficient to restore the proliferation of p53+/+ cells and partially restore that of p53−/− cells (Fig. 3f). Moreover, the silencing of ASNS reduced anchorage-independent tumour cell growth, while supplementation with asparagine promoted overall tumour cell growth and almost restored the numbers of p53+/+ colonies, with some restoration of p53−/− colonies.
Percentages of senescence-associated $\beta$-gal-positive cells and protein expression were measured. Arrow indicates the onset of senescence. Data are mean ± s.d., unpaired two-tailed Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001, NS not significant. Source data are provided as a Source Data file.
increased the number of cells expressing senescence-associated β-galactosidase in p53+/− cells but not p53−/− cells (Fig. 3g and Supplementary Fig. 5g). Notably, this ASNS knockdown-induced senescence could be reversed by asparagine treatment (Fig. 3h and Supplementary Fig. 5h). Similar results were obtained in the primary human diploid WI-38 cell line. ASNS knockdown caused a profound increase in the percentages of senescent cells in control siRNA-treated WI-38 cells but had little effect on cells without p53 (Fig. 3i). Again, asparagine addition reduced cell senescence (Fig. 3i).

We next further examined the effect of asparagine on other scenarios of p53-mediated senescence. Interestingly, asparagine addition inhibited the p53-dependent senescence induced by glutamine starvation (Supplementary Fig. 5i). In WI-38 cells, ASNS expression declined as senescence progressed, especially at the late stage (Fig. 3i). To test whether the decline in ASNS contributes to replicative senescence, we examined the replicative capacity of WI-38 cells treated with or without ASNS siRNA and/or asparagine. Compared with control cells, which could be cultured for extended passages, ASNS-depleted cells exhibited a greatly accelerated onset of senescence (Fig. 3k). Strikingly, the addition of asparagine was sufficient to delay senescence and could almost completely reverse the acceleration of senescence induced by ASNS depletion (Fig. 3k).

To elucidate the senescence-associated function of asparagine in the context of oncogenic signals, we investigated the effect of asparagine on HrasV12-induced premature senescence20. Although p53 depletion largely abolished HrasV12-induced senescence in U2OS cells (Supplementary Fig. 5j, k), asparagine did not (Supplementary Fig. 5j, l). Taken together, these results indicate that ASNS regulates p53-dependent, but not oncogene-induced, senescence through asparagine.

ASNS modulates p53-dependent cell senescence via Asn. p53 is a critical regulator of senescence. We noticed that ASNS silencing increased the number of cells expressing senescence-associated β-galactosidase in p53+/+ cells and p53−/− cells with ASNS knockdown (Supplementary Fig. 5e). Furthermore, when cells were cultured with 15N-asparagine, ASNS silencing resulted in higher levels of intracellular 15N-asparagine in p53+/+ cells than in p53−/− cells, and correspondingly, much lower levels of 15N-asparagine outside p53−/− cells were found (Supplementary Fig. 5f). Therefore, the lack of ASNS suppresses p53−/− cell proliferation through both asparagine-dependent and asparagine-independent mechanisms. Notably, when ASNS was present, p53−/− cells had lower levels of 15N-asparagine than did p53+/+ cells (Supplementary Fig. 5f), suggesting that, under this condition, p53−/− cells are more dependent on de novo synthesis of asparagine, correlating with the findings that p53 depletion elevated ASNS expression (Fig. 2b–e and Supplementary Fig. 3). Taken together, these findings suggest that ASNS supports cell proliferation in a p53 context-dependent manner through asparagine.

ASNS and Asn maintain tumour cell survival. To gain further insight into the functions of asparagine in cell survival, we studied the effect of asparagine and ASNS on cell survival. ASNS treatment reduced the survival of both p53+/+ and p53−/− HCT116 cells (Fig. 4a). These effects were also found in other tumour cell lines (Supplementary Fig. 6a–c). Similarly, 1-Asn-mediated inhibition of ASNS diminished the survival of these cells (Supplementary Fig. 6b, c). Consistent with these observations in tumour cells, when primary MEFs were treated with ASNS siRNA, cell survival also declined, reflecting a physiological relevant effect of asparagine on cell survival (Fig. 4b).

We noticed that in the HCT116 cell line, ASNase treatment decreased the survival of sip53 cells compared to the control cells (Fig. 4a). In contrast, the other cell lines examined, p53 depletion correlated with better cell survival in response to ASNS or ASNS siRNA treatment (Fig. 4b and Supplementary Fig. 6a, b). This discrepancy might be due to the higher expression of p21 in HCT116 cells (Supplementary Fig. 6d), as p21 depletion further decreased the survival of HCT116 cells, not MEFs and HepG2 cells (Fig. 4c, d and Supplementary Fig. 6e). Taken together, these findings demonstrate that asparagine maintains cell survival.

ASNS impairs p53-dependent cell cycle arrest and apoptosis. Failure to activate the cell cycle checkpoint impairs senescence and induces abnormal proliferation or apoptosis under certain stress conditions12,13,21. The above findings that the alteration of asparagine metabolism influences various p53-dependent cell physiological events led us to ascertain the effect of ASNS on asparagine on cell cycle progression. As shown in Fig. 4e, silencing ASNS resulted in p53 activation, as indicated by increased p53 phosphorylation (ser-15) and p21 expression. Similar results were obtained in U2OS cells (Supplementary Fig. 7a). Likewise, treatment with ASNase remarkably activated p53 (Fig. 4f and Supplementary Fig. 7b). The induction of p21 expression was p53 dependent, as this effect was abrogated when p53 was absent (Fig. 4e, f and Supplementary Fig. 7a, b). To investigate whether these effects were mediated by asparagine, we added increasing amounts of asparagine to the medium of ASNS-KD cells. Notably, asparagine addition reduced the levels of p53 phosphorylation and p21 expression (Fig. 4g). Moreover, 1-Asn treatment triggered p53 activation, and asparagine supplementation almost completely reversed it (Supplementary Fig. 7c). Additionally, asparagine addition could also reverse the enhancement of p53 phosphorylation and p21 expression induced by glutamine starvation, a metabolic stress that can induce a p53-dependent checkpoint for cell survival15 (Supplementary Fig. 7d). Together, these data demonstrate a physiological function of asparagine in suppressing p53.

We next investigated whether a p53/p21-dependent cell cycle checkpoint is initiated upon asparagine depletion. Indeed, in HCT116 cells, ASNase treatment increased the numbers of p53 wildtype cells in the G1 phase. In contrast, the number of p53-depleted cells in this phase was almost unaffected by ASNase treatment; instead, more sip53 HCT116 cells were in sub-G1 phase (apoptosis) than were their wildtype counterparts (Fig. 4h and Supplementary Fig. 11b). Likewise, in p53-expressing U2OS cells and HepG2 cells, ASNase supplementation provoked G1 arrest, whereas the depletion of p53 totally abrogated this effect (Supplementary Figs. 7e, f and 11b). This effect of ASNase on apoptosis was further confirmed by flow cytometry (FACS) using Annexin V-FITC/PI staining. After 48 h of ASNase treatment, ~20% of p53-depleted cells and ~6% of control cells underwent apoptosis (Supplementary Figs. 7g and 11c).

Consistent with the ASNS data (Fig. 4h and Supplementary Fig. 7e, f), the knockdown of ASNS in multiple tumour cell lines substantially increased G1 arrest in wildtype p53-expressing cells but not in p53-depleted cells. Instead, higher percentages of p53-depleted cells in sub-G1 were found (Fig. 4i and Supplementary Fig. 8a–c). Intriguingly, in MEFs, silencing ASNS increased G1 arrest, yet no significant increase in the sub-G1 population of sip53 MEFs was observed (Supplementary Fig. 8d), indicating
in the sub-G1, G1, S and G2 phases were determined by PI staining and

or ASNS siRNA were cultured in medium containing 0 or 0.1 mM Asn for 3 days. Cell cycle distribution was determined.

0, 1 or 2 U per ml ASNase for 48 h was analysed.

that p53 loss renders tumour cells more susceptible to ASNS
depletion.

Nevertheless, asparagine addition abolished G1 arrest in
p53<sup>+/+</sup> cells and reduced the percentage of p53<sup>−/−</sup> cells in sub-
G1 phase (Fig. 4i). In accordance with the findings that
asparagine removal induced p53/p21-dependent cell cycle arrest,
ASNS knockdown failed to induce G1 arrest but increased the
percentages of cells in sub-G1 when p21 was depleted (Fig. 4j).
Moreover, similar to p53 deficiency, silencing p21 increased the apoptosis induced by ASNS siRNA (Supplementary Fig. 8e). Together, these data demonstrate that ASNS/asparagine depletion induces p53-dependent cell cycle arrest to protect cells from apoptosis.

ASNS and Asn regulate p53 activity via AMPK. We next examined the mechanism by which asparagine regulates p53. AMP-activated protein kinase (AMPK) is an intracellular energy-sensing kinase that can activate p53 through phosphorylation.14,22 Interestingly, ASNase treatment elevated AMPK phosphorylation in both p53−/− and p53+/− HCT116 cells (Fig. 4f) and U2OS cells expressing p53 or control shRNA (Supplementary Fig. 7b). Similarly, when ASNS was knocked down, AMPK was activated, as evidenced by the increase in phosphorylation of AMPK and its substrate ACC1 (Figs. 4e and 5a and Supplementary Figs. 7a and 9a). Moreover, the expression of RNAi-resistant ASNS almost completely abolished AMPK activation in ASNS-knockdown cells (Fig. 5b). Based on these findings, we tested whether AMPK is required for ASNS silencing-triggered p53 activation by knocking down AMPK in HCT116 cells and by comparing AMPK null and wildtype MEFs. In both situations, the loss of AMPK expression prevented ASNS depletion from activating p53 (Fig. 5c, d). Similarly, AMPK silencing attenuated ASNase-induced p53 phosphorylation (Fig. 5e).

To directly assess whether asparagine is involved in ASNS depletion-mediated AMPK activation, we cultured 1−Alb-treated EL4 cells in medium containing asparagine or aspartate as a control. Interestingly, supplying cells with asparagine lessened the AMPK phosphorylation induced by 1−Alb treatment (Supplementary Fig. 9b). Analogously, asparagine addition led to the decreased phosphorylation of AMPK and p53 in ASNS-depleted cells (Fig. 5a, f and Supplementary Fig. 9d), and this phenomenon could be observed shortly after asparagine treatment (Fig. 5g). Consistent with these findings, ASNase supplementation increased the level of AMPK-bound phosphorylated p53, whereas supplying cells with asparagine reversed this effect (Fig. 5h), suggesting that asparagine suppresses AMPK activity towards p53.

Aspartate supplementation increased overall cellular aspartate levels, particularly in ASNS-deprived cells (Supplementary Fig. 9c). Surprisingly, aspartate treatment did not reduce AMPK and p53 activation but instead, to some extent, activated AMPK (Fig. 5f and Supplementary Fig. 9d). Similar findings were obtained when cells were cultured in glutamine-free medium (Supplementary Fig. 9e). Consistently, asparagine addition restored cell survival in siASNS cells, while aspartate supplementation did not (Fig. 5i). Taken together, the results show that ASNS and asparagine regulate p53 through AMPK.

Asn-Asp homeostasis dictates AMPK activity. Cellular aspartate is in equilibrium with asparagine. We therefore investigated the effect of the dynamic asparagine-aspartate ratio on AMPK and p53. Targeting ASNS by siRNA resulted in a decrease in the levels of cellular asparagine and a strong accumulation of aspartate, leading to a steep decline in the ratio of asparagine-aspartate (Fig. 6a, b). Consistent with this, AMPK and p53 were activated (Fig. 6a, b), which negatively correlated with the decreased asparagine-aspartate ratio. Similarly, lowering the asparagine-aspartate ratio by 1−Alb correlated with increased AMPK and p53 activation (Supplementary Fig. 9f). In contrast, the forced expression of ASNS induced asparagine synthesis, and intriguingly, aspartate levels did not decline under these conditions (Fig. 6c, d). The failure of ASNS overexpression to affect asparagine amounts might be due the compensation of other pathways (such as alanine metabolism and the TCA cycle) that can produce aspartate. Nevertheless, the ratio of asparagine and aspartate remained significantly increased in a dose-dependent manner. Consistently, we found a dose-dependent reduction in the activation of AMPK and p53 under these conditions (Fig. 6c, d). Together with the findings that p53 suppresses ASNS, our findings may reveal a positive feedback loop between p53 and asparagine metabolism: the activation of p53 lessens ASNS expression, reduces asparagine-aspartate ratio and turns on AMPK, consequently leading to even higher p53 activation.

Asparagine addition was sufficient to block AMPK activation (Fig. 5a, f, g and Supplementary Fig. 9b, d). Next, we wanted to determine the effect of aspartate on AMPK-p53 signalling. In contrast to asparagine, the addition of aspartate stimulated AMPK signalling (Supplementary Fig. 9g, h). This effect was largely abolished when AMPK was absent (Supplementary Fig. 9h). In accordance with these observations, aspartate supplementation reduced cell proliferation (Supplementary Fig. 9g). Intriguingly, aspartate treatment elevated total AMPK levels in MEFs (Supplementary Fig. 9g, h), which is unlikely due to asparagine-mediated AMPK phosphorylation by LKB1 because AMPK phosphorylation rarely correlates with its protein stabilisation.

Next, we extended our studies to animals. Mice receiving asparagine through either intraperitoneal injection or oral consumption via drinking water had increased sizes of tumours derived from EL4 cells (Fig. 6e). In line with this observation, AMPK and p53 phosphorylation declined in these tumours (Fig. 6f). In contrast, asparagine administration impeded tumour growth (Fig. 6e) and activated AMPK and p53 (Fig. 6f). These results were strengthened by the findings that body weight remained unchanged (Supplementary Fig. 9i), and plasma asparagine and aspartate correspondingly increased following injection or oral administration (Supplementary Fig. 9j). Taken together, our findings reveal that LKB1-AMPK signalling may be the predominant mechanism by which asparagine and aspartate regulate p53.

Asn and Asp directly bind to LKB1 and modulate its activity. Next, we investigated the mechanism(s) by which asparagine and aspartate regulate AMPK. AMPK is regulated by LKB1-mediated phosphorylation.22,23 Interestingly, ASNS depletion-induced AMPK activation (indicated by the increased phosphorylation of its substrates ACC1, ULK1, TSC2 and p53) was abrogated when LKB1 was knocked down (Fig. 7a and Supplementary Fig. 10a). Similar results were observed in LKB1-knockout cells (Fig. 7b). Analogously, the knockdown of ASNS failed to activate AMPK in LKB1-deficient A549 cells (Fig. 7c). Collectively, these findings suggest that LKB1 mediates the regulation of AMPK by ASNS.

We next investigated whether asparagine and/or aspartate act(s) directly on LKB1. When incubated with HEK293 cell-purified LKB1, asparagine dose-dependently abrogated LKB1 activity, while aspartate considerably augmented it (Fig. 7d). Similar results were obtained using Myelin Basic Protein (MBP), another LKB1 substrate (Supplementary Fig. 10b). In contrast, when LKB1 was absent, asparagine and aspartate did not affect AMPK and MBP phosphorylation (Supplementary Fig. 10c). To further verify these findings, we performed a sequential stimulation assay (Fig. 7e, left panel). HEK293 cell-purified LKB1 protein was immobilised on agarose beads and stimulated by incubation with asparagine or aspartate (Asn/Asp). After washing the beads to eliminate unbound Asn/Asp, the Asn/Asp-stimulated LKB1 (on beads) was further incubated with AMPK protein for the AMPK

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**Fig. 5 ASNS and asparagine regulate p53 through AMPK.**

**a** HCT116 cells transfected with control or ASNS siRNA were cultured in medium containing 0 or 0.1 mM Asn for 3 days. Protein expression was analysed by western blotting. **b** U2OS cells stably expressing Flag-tagged RNAi-resistant (res) ASNS or Flag vector control were transfected with control or ASNS siRNA for 72 days. Protein expression was analysed. **c** Expression of the indicated proteins in HCT116 cells transfected with control, ASNS siRNA and/or AMPK α siRNA as indicated for 3 days. **d** Western blot analysis of lysates from AMPKα/−/− and AMPKα+/− MEFs transfected with control or ASNS siRNA as indicated for 3 days. **e** HCT116 cells transfected with control or AMPK α siRNA were treated with or without increasing amounts of Asn or aspartate Asp as indicated. Relative cell viability (%) is shown. Data are mean ± s.d., unpaired two-tailed Student’s t-test, *p < 0.05, **p < 0.01, ***p < 0.001, NS not significant. Source data are provided as a Source Data file.
 phosphorylation assay. Again, asparagine stimulation reduced LKB1 activity, whereas aspartate increased it (Fig. 7e). We next used insect SF21 cell-purified proteins to further confirm these findings. Likewise, asparagine reduced AMPK phosphorylation in the presence of LKB1, while aspartate enhanced LKB1-mediated AMPK phosphorylation, suggesting that asparagine and aspartate directly regulate LKB1 activity (Fig. 7f and Supplementary Fig. 10f, g). These data indicate that asparagine and aspartate may directly act on LKB1. Indeed, a real-time binding assay using surface plasmon resonance (SPR) (BIAcore) showed that both asparagine and aspartate were able to directly bind to LKB1 with a dissociation constant (KD) of 45.5 \mu M and 64.6 \mu M, respectively (Fig. 7g and Supplementary Fig. 10h). Moreover, an equilibrium binding assay using radioactive \[^{3}H\]Asn revealed that \[^{3}H\]Asn and aspartate showed no effect on the LKB1-AMPK interaction in vitro (Supplementary Fig. 10i). Similarly, changing the cellular asparagine-aspartate ratio by knocking down ASNS only slightly enhanced the LKB1-AMPK interaction (Supplementary Fig. 10i, g). These data indicate that asparagine and aspartate may directly act on LKB1. Indeed, a real-time binding assay using surface plasmon resonance (SPR) (BIAcore) showed that both asparagine and aspartate were able to directly bind to LKB1 with a dissociation constant (KD) of 45.5 \mu M and 64.6 \mu M, respectively (Fig. 7g and Supplementary Fig. 10h). Moreover, an equilibrium binding assay using radioactive \[^{3}H\]Asn revealed that \[^{3}H\]Asn
specifically bound to LKB1 but not AMPK, AKT1 or MBP. The binding between [3H]Asn and LKB1 proteins could be fully competed by excess nonradio-labelled Asn (Fig. 7h). Likewise, Asp bound to LKB1 but not AMPK, AKT1 or MBP (Fig. 7h). In contrast to Asn and Asp, other amino acids used as controls, such as methionine (Met), leucine (Leu), glycine (Gly), alanine (Ala) and glutamate (Glu), did not bind to LKB1 (Supplementary Fig. 10i). Importantly, while ASNS silencing to some extent increased cellular asparagine levels, cellular asparagine concentrations decreased significantly upon ASNS siRNA treatment, decreasing from above the dissociation constant of LKB1 for asparagine to below it (Fig. 7i and Supplementary Fig. 10j), indicating that asparagine is a physiological modulator of LKB1 activity. Taken together, our findings suggest that asparagine and aspartate directly bind to LKB1 to oppositely modulate its activity.

**Discussion**

In this work, we found that some tumour cells, in particular p53-null cells, have active ASNS expression and asparagine synthesis. Increased asparagine production helps cells proliferate and promote tumours from senescence. Interestingly, ASNS treatment or ASNS knockdown reciprocally activates p53 to induce cell cycle arrest and protect cells from apoptosis, suggesting a therapeutic approach (e.g. use of ASNSase) for the treatment of p53-null tumours by interfering with asparagine synthesis.

In addition to changes in AMP or ADP, several metabolic stresses, such as the accumulation of reactive oxygen species (ROS) and the lack of fructose-1,6-bisphosphate (FBP) or ribulose-5-phosphate (Ru-5-P), have been proposed to be involved in AMPK activation by different means. In this work, we found an alternative mechanism for the regulation of AMPK and identified a role for LKB1 in sensing cellular asparagine-aspartate homeostasis: LKB1 directly binds to asparagine and aspartate, and its activity is strongly suppressed by asparagine but enhanced by asparagine under certain condition (Fig. 8). Although we found asparagine maintains both lymphoma and human cell survival, the mechanistic study was carried out mainly in human tumour cells due to the limited transfection efficiency of mouse lymphoma tested. Thus, the molecular basis for the regulation and functions of asparagine in lymphoma may need further investigation.

In some types of tumour cells, asparagine can be directed to LKB1-AMPK signalling and p53-dependent cell cycle arrest. When p53 is absent, the cell cycle continues, and abnormal proliferation and/or apoptosis are triggered. Nevertheless, increased asparagine synthesis in p53-deficient tumour cells implies that, compared to asparbate, asparagine may provide more advantages to these tumour cells. In addition, a long-standing mystery surrounding p53 is why p53-deficient mice predominantly develop lymphomas (~70% of all tumour types). Our findings presented here may provide an important clue to the understanding of this phenomenon. Specifically, the elevation in ASNS expression and asparagine production may directly contribute to lymphoma genesis in p53-null mice.

**Methods**

Antibodies and reagents. The antibodies against the following proteins/epitopes were purchased from the indicated sources: ASNS (ProteinTech, 14861-1-AP), p53 (BD Biosciences, 556431), p70 S6 Kinase (Santa Cruz, sc-206), p21 (Abcam, ab81295), Phospho-p70 S6 Kinase (Thr389) (108D2) (Cell signaling technology, 9284S), MDM2(SMP14) (Santa Cruz, sc-965), AMPK (Cell signaling technology, 253A3), phospho-AMPK(Thr172) (Cell signaling technology, 4819S), Acetyl-CoA (Cell signaling technology, C83810), Phospho-AKT1-Ca++/Calmod-Carboxy-M2 (Ser279) (Cell signaling technology, 3661), LKB1 (Cell signaling technology, 27D10), Phospho-(Ser/Thr) Phe (Cell signaling technology, 9631), HA-HA-7 (Sigma H3663), FLAGM2 (Sigma F5165), Actin (ProteinTech, 66009-1-LG), Goat anti-mouse-HRP (Sigma, sc-619), Goat anti-rabbit-IgG-HRP (Santa Cruz, sc-2300), U1K1 antibody (EPBR4885[J]); (Abcam, ab128859), Phospho-ULK1 (Ser555) (D1H4) (Cell signaling technology, 5869), Turbiner/TSC2 (Cell signaling technology, 3612), Phospho-Tuberin/TSC2 (Ser1347) (Cell signaling technology, 5584), ATM [2C1 (1A1)] (Abcam, ab6787), Phospho-ATM (S1981) [EP1809T] (Abcam, ab12192), Chk1 (Abcam, ab47374), Phospho-Chk1 (Ser345) (Cell signaling technology, 2341), p70 S6 Kinase (4907), p70 S6 Kinase (2708), Phospho-p70 S6 Kinase (Thr389) (108D2) (Cell signaling technology, 9234). The reagents were purchased from the following sources respectively: Nuflin-3a (Sigma, SMOJ580), Doxorubicin (Sigma, D1515), Etoposide (Sigma, E1383), L-Asparagine (Sigma, A9256), L-Glutamic acid (Sigma, G85896), ANTI-FLAG M2 affinity gel (Sigma, A2220), FLAG peptide (Sigma, F3290), HA peptide (Sino Biological, PP100028-1), Propidium iodide (Sigma, P3090), 0.4% Trypan blue solution (Amresco, K940), Lipofectamine 2000 (ThermoFisher Scientific, 12566014), RNAiMAX transfection agent (ThermoFisher Scientific, 13778075). Crystal violet (Solarbio, C8470-25).

**Plasmids**

The coding sequences corresponding to the full-length human ASNS, p53, AMPKα, LKB1 and MBP were amplified by polymerase chain reaction (PCR) from cDNA library of 293T cells and then cloned into pRcRc empty vector tagged with Flag or HA epitope as indicated. The cloning sequences are as follows: Human ASNS siRNA: 5'-GTTCCTG-3'; Human p53 siRNA: 5'-GGGTGTGGTGTGCAAAATAGAGAC-3'; Human p70 S6 Kinase siRNA: 5'-CCGCTCGAGTTATTGTGCAAGAATTTTAATTAGA-3'. The PCR product was then cloned into MSCV-IRES-EGFP vector (Addgene plasmid 20672) to construct MSCV-Luciferase-EGFP vector.
GFP plasmid. All amplifications were made by PCR and confirmed by DNA sequencing.

**Cell culture and gene knockdown with shRNA and siRNA.** All cells were cultured in a 5% CO₂ humidified incubator (ThermoFisher Scientific, USA) at 37 °C. 293T, HCT116, U2OS, A549, DU145, A31, MEF, LS178Y, EL4 and NCM460 cell lines were routinely maintained in standard dulbecco’s modified eagle’s medium (DMEM) (ThermoFisher Scientific, C11995008T) with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, 16000044). L1210 cells were cultured in DMEM (ThermoFisher Scientific, C11995008T) with 10% horse serum (HS) (ThermoFisher Scientific, 16050122). HEK293, HepG2 and WI-38 cells were cultured in minimum essential medium (MEM) (Corning, 10-010-CY) with 10% FBS (ThermoFisher Scientific, 16000044). Jurkat, U937, MOLT4, K562, H1299 and LNCaP cells were cultured in standard RPMI-1640 medium (ThermoFisher Scientific, 11875093) with 10% FBS (ThermoFisher Scientific, 16000044), unless specified.
Fig. 7 LKB1 is a natural sensor for Asn and Asp. a Protein expression in U2OS cells transfected with Control, ASNS siRNA and/or LKB1 siRNA as indicated. b Protein expression in LKB1 wildtype (WT) and knockout (KO) HCT116 cells treated with siCtrl or siASNS as indicated. c Western blot analysis of lysates from HCT116 cells and A549 cells transfected with control or ASNS siRNA. d In vitro LKB1 kinase activity assay using HEK-293 cell-purified proteins as indicated in the presence or absence of Asn or Asp at 30 °C for 30 min. e HEK-293 cell-purified HA-LKB1 immobilised on anti-HA agarose beads (LKB1 beads) was stimulated by incubating with Asn or Asp for 30 °C for 30 min, and then unbound Asn/Asp was removed by washing and further incubated with LKB1 beads with purified Flag-AMPK protein for another 30 °C for 30 min. AMPK phosphorylation was determined. f In vitro LKB1 kinase activity was determined using SF21 cell-purified His-LKB1-STRAD-MO25 complex and His-AMPKα in the presence or absence of Asn or Asp at 30 °C for 30 min. g Surface plasmon resonance (BIAcore) measurement of the interaction between purified LKB1 and Asn (left) or Asp (right). Graphs of equilibrium response units (RU) and compound concentrations are shown. The estimated KD values were 45.4 μM and 64.4 μM. h Binding of radiolabelled [3H]Asp and [3H]Asp to HEK-293 cell-purified proteins (see Methods for details). Unlabelled amino acids were added where indicated. i HCT116 cells were treated with ASNS siRNA for 0, 6 and 18 h as indicated before sample preparation for LC/MS-based analysis of the absolute amounts of asparagine. The dissociation constant Kd of LKB1 for asparagine is indicated. Data are mean ± s.d., unpaired two-tailed Student’s t-test, *p < 0.05, **p < 0.01, ***p < 0.001, NS not significant. Source data are provided as a Source Data file.

Fig. 8 A model of the reciprocal regulation of p53 and ASNS in modulating metabolism and cell states that illustrates LKB1 as a natural sensor for Asp-Asn homeostasis. Briefly, p53 modulates asparagine metabolism and aspartate-asparagine homeostasis through transcriptionally repressing ASNS expression. Moreover, accumulation of asparagine inhibits LKB1 activity, and whereas, aspartate suppresses its activity through direct interaction. Asn asparagine, Asp aspartate.

indicated otherwise. All cells were cultured without the addition of penicillin-streptomycin and examined for mycoplasma contamination and cultured for no more than 2 consecutive months. Their morphologies were confirmed periodically to avoid cross-contamination or misuse of cell lines. None of the cell lines used in this study was listed in the ICLAC database.

shRNA-mediated knockdown of ASNS was performed using a specific targeting sequence shASNS#6 (5′-CCGCGGCTCTGTTACAATGTTGAAATCTCGAGATTTCACATTGGTAACAGAGCTTTTG-3′) or shASNS#10 (5′-CCGGGCTCTAGTTGTTGCTCTTTGGCTTCTGACATACAGGCTTTTG-3′). A non-specific ‘scrambled’ shRNA sequence (5′-CCCGCAACAAGATGAAAGGACACCAACTCGAGTTGCTGCTCTGACTCTGACATAGTCTTTG-3′) was used as control. These sequences were individually cloned into the pLKO.1-puro vector (Sigma, SHC201), which was then co-transfected with the expression vectors containing gag/pol, rev and vsvg genes into 293T cells. The lentivirus was harvested 48 h after transfection and enriched with lentivirus concentration solution (GeneCopoeia, LPR-LCS-01) for 48 h at 4 °C, followed by being added to nearly confluent HCT116 cells with 4 µg/ml polybrene and cultured for 16-24 h to complete infection. The truly stable transfected cells were selected under the pressure of 2 µg/ml puromycin for 1–2 weeks.

siRNA-mediated knockdown was performed with Lipofectamine RNAiMAX transfection agent (ThermoFisher Scientific, 13778075) and siRNAs targeting human TP53, ASNS, AMPKα, LKB1 and mouse Asns, individually or combined for the purpose of experiment. The targeting sequences were as follows: Human TP53: 5′-GACTCCAGTGGTAACTCTAC-3′, Human ASNS 5′-TGATATGTCTCAAGAGCTAAA-3′, Human AMPKα 5′-ACACAGUUAUGAUGAAGGCUUAAA-3′, Human LKB1 5′-GGACUAGCGUGUGUGAGACAAATT-3′, Human p21 5′-CUUCGACCUUGUGAGGAGCUCUACACAAAG-3′, human ATM 5′-CGTGTCTTAAATGGGACTAGTGGG-3′, human p53 5′-CAAGGCTGACGAGCAACACCAAAACTGAGG-3′, human p63 5′-GGACAAAUUGAAAGCGGAAACAGG-3′, Human TP53 5′-CCGGGCTCTAGTTGTTGCTCTTTGGCTTCTGACATACAGGCTTTTG-3′. The siRNA sequence (5′-CGUAGCGGAAACAUUCUGAGATT-3′) targeting luciferase was used as control throughout this study. All siRNAs were used at a
concentration of 20 nm. For co-transfection experiments, the total siRNA concentration was equalised under all conditions by control siRNA. The siRNA transfection procedures were performed according to manufacturer’s instructions.

**Semi-quantitative RT-PCR and quantitative RT-PCR.** B briefly, total RNA was isolated from triplicate wells in each condition using Total RNA purification Kit (GeneMark, TR01) and 2 µg RNA of each sample was complementarily reversed to cDNA by First-strand cDNA Synthesis System (Thermo scientific). The primer pairs used in this study included: Human ABHD4; F: 5′-TCCATACTCTGGGAGACTCG-3′; R: 5′-GAGCACTCCATGTCGATCT-3′. Human MDM2; F: 5′-ATGGTGAGGAGGAGGCG-3′; R: 5′-CTAGTGGAGGATTGAGGTC-3′. Human TP53; F: 5′-TTCAGGAGCAAGATTATGC-3′; R: 5′-AGCAATGAGGGATGAGTTTTC-3′. Human ACTB; F: 5′-GAGCTGTCATATCCATGAGAT-3′; R: 5′-GTCACACTTACATGGAAT-3′. Mouse Actb; F: 5′-CCGGCGAGGCGGGCATGAG-3′; R: 5′-CTTCCTCCTTGGGAAAGATC-3′. Mouse p21; F: 5′-AACTTCCTGCTGGAGGCCC-3′; R: 5′-TACCGGTTCCTTGTCCAGA-3′. Mouse ABHD4; F: 5′-TCCACCATCCTGCTTCCC-3′; R: 5′-GTGGAATCTCCCTGATCC-3′. Human PML; F: 5′-CGGCCCCTGAATAACTGTTTC-3′; R: 5′-TCCAACTGCGCCTGAGTAC-3′. Human SIF2; F: 5′-GCGAACATGCTGCTTGGTC-3′; R: 5′-CCATCCATCCTGCTTCCC-3′. Human H-RAS; F: 5′-GAGCTGTCATATCCATGAGAT-3′; R: 5′-CTTCAACCCCCTTGTCCAGA-3′. Semi-quantitative PCR was performed with Taq PCR StarMix (GeneStar, A121-100) in a thermal cycler (Bio-Rad, T100) according to a standard protocol as follows: 1 cycle at 95°C for 3 min; 30 cycles at 94°C for 45 sec, annealing for 45 sec, and 72°C for 1 min; a final extension at 72°C for 10 min and holding at 4°C. 10 µl PCR products were analysed by electrophoresis through 2% agarose gels with Gel-Red staining (Beyotime, D0139) and visualised by gel imaging system (LIUYI, 130-1310). Quantitative RT-PCR was performed using CFX96 Real-Time PCR System (Bio-Rad, USA) and the amplification was conducted in the LightCycler 2.0 with MasterMix PCR Kit (Roche Diagnostics, B21202) according to manufacturer’s instructions. The thermal cycling conditions were set as follows: 50°C for 2 min followed by an initial de-naturation step at 95°C for 10 min, 45 cycles at 95°C for 15 s, 60°C for 1 min, and a dissociation curve at 95°C for 15 s and 60°C for 15 s. All experiments were performed in triplicate. The fold changes of gene expression were calculated after being normalised to ACTB.

**Western blot analysis.** Cells were lysed by using modified RIPA buffer containing 10 mM Tris-HCl at pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1% sodium dodecyl sulphate, 0.025% SDS and proteinase inhibitors on ice for 10–20 min. Protein samples were quantified using BCA protein assay kit (MaeGene, MP0002), boiled in 5× loading buffer and resolved by SDS-PAGE and transferred onto nitrocellulose membrane. In all, 5% skimmed milk (BD Difco, 23210) in TBS was used as blocking buffer containing 0.1% Tween (TBST) was used to block the membrane before probing with indicated antibodies in TBST at 4°C overnight. Membranes were washed with TBST and then incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies at room temperature for 1 h and developed with ECL Western Blotting Detection Reagent (ThermoFisher Scientific, 32132). Blot bands were quantified using ImageJ software. Uncropped scans of all the blots in this manuscript are shown in the Supplementary Information.

**Animals.** Animal experiments were performed with male C57BL/6J mice (Jackson Laboratory, Jax 664, p53−/−/C57BL/6J mice (BIOCYTOGEN, BCG-DIS-0001) and BALB/c nude mice (Vital River Laboratory Animal Technology, 401). All mice were maintained in a specific pathogen-free environment with protocols approved by the Institutional Animal Care and Use Committees of Tsinghua University for animal welfare. Mice were initially randomised by age and sex and BALB/c nude mice (Vital River Laboratory Animal Technology, 401). All mice were maintained in a specific pathogen-free environment with protocols approved by the Institutional Animal Care and Use Committees of Tsinghua University for animal welfare. Mice were initially randomised by age and sex and were 6–8 weeks of age at the time of injections. The maximum tumour size approved by IACUC protocol was 2 cm in diameter and this was not exceeded in all experiments.

**Establishment of EL4-Luc-GFP EL4 cell line.** Retroviruses carrying MSCV-Luciferase-ires-GFP plasmid were packaged with the Plate-E system. For retroviral infection, EL4 cells were spin-infected with viral solution at 1500 × g for 15 minutes at 37°C. Infected EL4 cells were expanded and GFP positive cells were sorted out by FACSAria II (BD Biosciences, USA) using FACS AriaII sorting cell. The resulting GFP-EL4-Luc-GFP cell line was verified by a second sorting 1 week after expansion. FACS gating strategies were used to identify EL4-Luc-GFP cells (GFP+) as described in Supplementary Fig. 11a.

**Xenograft tumour models.** For xenograft model established through s.c., viable EL4-Luc-GFP cells were resuspended in PBS with the density of 1 × 107 cells per ml, which were subsequently injected into the flank of nude hind legs in a volume of 0.1 ml on both sides. In total, 1 week later, the tumour volume was measured by caliper, then mice were anaesthetised and injected i.p. with 0.1 mL 10 mg/mL EGF and imaged for luciferase activity by IVIS Lumina II multispectral imaging system (Caliper, USA). Mice were further fed to document survival status.

For xenograft model established through i.v., viable EL-4-Luc-GFP cells were resuspended in PBS with the density of 1 × 107 cells per ml, which were subsequently injected into the flank of hind legs in a volume of 0.1 ml on both sides. In total, 1 week later, the tumour volume was measured by caliper, then mice were anaesthetised and injected i.p. with 0.1 mL 10 mg/mL EGF and imaged for luciferase activity by IVIS Lumina II multispectral imaging system. Next, mice received treatment i.p. with vehicle or Asn (2 U per g of body weight) every 3 days for 3 weeks. The tumour volume measurement and whole-body bioluminescence imaging were conducted every week until the end of the experiment. In total, 3 weeks after xenograft, blood samples were collected and serum were obtained as mentioned before. After blood sampling, mice were killed and tumours were resected and weighted. For another test using s.c. xenograft model to examine the effects of amino acid on tumour growth, viable EL4-Luc-GFP cells were resuspended in PBS with the density of 3 × 106 cells per ml, which were subsequently injected into the flank of hind legs in a volume of 0.1 ml on both sides. One day later, mice were fed with drinking water containing with or without 10 mM Asn or Asp, or intraperitoneally injected with or without 5 or 10 mM Asn or Asp at the end of the experiment. At 10, 14 and 17 days post-xenograft, tumour volume was measured by caliper, and then mice were anaesthetised and imaged in vivo as mentioned before. After imaging, blood samples were collected and the serum levels of Asn and Asp were measured by LC-MS as mentioned before.

For xenograft model established in BALB/c nude mice, similarly, viable HCT116 cells transfected with control siRNA or ASNS siRNA were resuspended in PBS with the density of 1 × 107 cells per ml, followed by injection s.c. into the flank of hind legs in a volume of 0.1 ml at single side. In total, 3 weeks after implantation, mice were killed and tumours were resected, weighted and pictured.

**Metabolic flux and LC-MS analysis.** HCT116 cells were seeded in 6-cm dishes and cultured overnight. At next day, cells were washed twice with PBS and cultured in medium containing 4 mM L-Asp for 48 h. Polar metabolites were then extracted from cells using appropriate volume of 100% acetonitrile. Then, the fully vortexed cell lysates were centrifuged twice at 14000 g for 10 min to purify metabolites, and the soluble fractions were analysed by Multi-reaction monitoring (MRM) mode of UPLC-QQQ-MS/MS (Agilent 1290/6460 tandem mass spectrum, Agilent USA). An ACQUITY UPLC® BEH HILIC, 2.1 mm × 100 mm, 1.7 μm (Waters) was used for LC separation, using gradient elution with 0.1% formic acid acetonitrile as solvent A and 0.1% formic acid water as solvent B. The gradient programme is as follows: 0–1 min 80% A, 1–5 min 80% A to 50% A, 5–7 min 50% A, 7.1–7.5 min 50% A to 80%, 7.1–10 min 80% A. The flow rate was set at 0.4 mL/min, and the injection volume was 10 μL. The total run time was 10 min for each sample.

Using a Jet Stream electrospray ESI ion source in positive ion mode was used to detect 5 kinds of amino acids, the nitrogen generator (PEAK Shanghai) was used for solvent removal and atomisation, and high purity nitrogen as colliding gas. Sheath Gas Temp is 350 °C, Sheath Gas Flow is 30 μl/min, Capillary voltage is 4000 V, Nebulizing Gas is 45 ps, Nozzle Voltage is 500 V.
and followed by immunoprecipitation with indicated antibodies. Bounded DNA fragments were eluted and amplified by PCR. The used primer pairs were: ASNS-RE1, 5′-CGGCTCGACCTTCACTTTTTCTCCG-3′ and 5′-GCGCTTCTCCTAATGTTAAAACGGC-3′; ASNS-RE2, 5′-GGGTTGGTTA-3′ and 5′-AATACTACATCGGTGGTC-3′; ASNS-RE3, 5′-CCAAAGCTTCTGTCCTGATCC-3′; p21-RE, 5′-CGGCCGCTCGAGGAGAGTCG-3′; and 5′-CGAGGGCTGTCAGTGTTTCG-3′ and 5′-GCAAAGCTTCTGTCCTGATCC-3′.

Luciferase reporter assay. Briefly, the DNA fragment containing the potential p53-binding region was amplified by PCR with primers used in ChIP assay and was cloned into a pGL3-promoter vector (Promega). In all, 293T cells were plated 18 h before transfection, and the reaction system was placed on ice and each was mixed with three times the volume of acetonitrile, followed by centrifuging twice at 14,000 × g for 10 min to extract metabolites. Asn and Glu were identified and quantified by a Triple Quadrupole LC/MS System (Agilent, 1290/6460) according to calibration curve. ASNS activity was obtained by calculating the production of Asn per minute per μg total protein (μg min⁻¹ μg⁻¹ protein).

Analysis of cell-cycle disruptions. Cells were washed twice with PBS and fixed in 75% ethanol overnight at 4 °C. Cells were then incubated in the solution containing 0.1% Triton X-100, 100 μg/ml RNase A and 50 μg/ml propidium iodide for 30 min at 37 °C in the dark. Cell cycle distribution was analysed using a LSR II cytometer (BD Biosciences, USA). The data were analysed using FlowJo software (TreeStar). FACS gating strategies for analysing PI-stained cells in cell cycle distribution analysis were described as in Supplementary Fig. 11b.

Senescence-associated SA-β-gal activity. The SA-β-gal activity in cultured cells was determined using a Senescence Detection Kit (BioVision, K320-250) according to the manufacturer's instructions. After staining, cells were fixed with 4% paraformaldehyde solution and stained with 0.01% crystal violet till colonies turned into blue. The ImageJ software was used to analyse the area covered by colonies in each well.

Measurement of LKB1 kinase activity. Active Flag-tagged LKB1 was immunoprecipitated from cell extracts of 293T cells using the Flag M2 beads. Beads were washed three times in lysis buffer and twice in kinase buffer (50 mM Tris HCL, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100), and then resuspended in kinase buffer containing increasing concentrations of Asn or Asp for nearly 1 h at 4 °C on a rotator. HA-MBP or HA-AMPKα bound on HA beads were equally allocated into each reaction in 0.2 ml 8-Strip Tubes. These HA beads were resuspended in kinase buffer containing Flag-tagged LKB1 and kinase reaction was performed for 30 min at 30 °C on a rotator. Samples were subjected to 8% SDS-PAGE, followed by Western blot.

In vitro pull-down assay. For in vitro AMPK and LKB1 interaction, Flag-tagged LKB1 and HA-tagged AMPKα proteins were expressed in 293T cells. Cells were lysed and proteins were immunoprecipitated using the Flag M2 or HA beads according to the manufacturer's standard procedures. The Flag-tagged LKB1 proteins were purified and dissolved in the binding buffer (20 mM Tris pH 7.5, 150 mM NaCl) with or without different concentrations of Asn or Asp for nearly 1 h at 4 °C on a rotator. The HA beads bound with HA-tagged AMPKα were equally allocated into 0.2 ml 8-Strip Tubes and washed with the wash buffer. These HA beads were then resuspended with wash buffer containing Flag-tagged LKB1 proteins and incubated for 4 h at room temperature, washed by the wash buffer for five times on a rotator, and subjected to 8% SDS-PAGE, followed by Western blot.

Surface plasmon resonance analysis. SPR analysis was conducted at 25 °C with a Biacore T200 instrument (GE, USA) according to the manufacturer’s instructions. Purified LKB1 was immobilised on the surface of a Series 3 Sensor chip CM7 (GE, 2 μg μl⁻¹ in 10 mM sodium acetate buffer (pH 4.3) at 8000 response units. A reference surface was used as a blank to correct for instrumental and buffer effects without protein injection. The amount of protein bound to the
sensor chip was monitored by the change in refractive index. Asn or Asp was radiolabeled amino acid binding assay. Around five million 293T cells were used to calculate the sensitivity of cells resistant to L-asparaginase. Leukemia 20, 2199–2201 (2006).
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Author contributions
L.D. performed the experiments during initiate submission. L.L. and P.Y. performed the experiments raised during revisions. S.Z. helped with some of the FACS analysis experiments. C.X. and L.L. helped with metabolic and some of the animal experiments. C.X. generated SF21 expression system and purified the LKB-STRAD-MO25 complex and AMPK proteins. P.J. conceived and designed the research. F.J. and X.L. helped with the metabolomics data analysis. P.J. organised and interpreted the data, and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to P.J.

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