





CARS senses cysteine deprivation to activate AMPK for cell survival

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Abstract

Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is an important cellular metabolite-sensing enzyme that can directly sense changes not only in ATP but also in metabolites associated with carbohydrates and fatty acids. However, less is known about whether and how AMPK senses variations in cellular amino acids. Here, we show that cysteine deficiency significantly triggers calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2)-mediated activation of AMPK. In addition, we found that CaMKK2 directly associates with cysteinyl-tRNA synthetase (CARS), which then binds to AMPK γ 2 under cysteine deficiency to activate AMPK. Interestingly, we discovered that cysteine inhibits the binding of CARS to AMPK γ 2, and thus, under cysteine deficiency conditions wherein the inhibitory effect of cysteine is abrogated, CARS mediates the binding of AMPK to CaMKK2, resulting in the phosphorylation and activation of AMPK by CaMKK2. Importantly, we demonstrate that blocking AMPK activation leads to cell death under cysteine-deficient conditions. In summary, our study is the first to show that CARS senses the absence of cysteine and activates AMPK through the cysteine–CARS–CaMKK2–AMPK γ 2 axis, a novel adaptation strategy for cell survival under nutrient deprivation conditions.

Keywords AMPK; CARS; cell survival; cyst(e)ine

Subject Categories Metabolism; Signal Transduction

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Introduction

Sensing changes in external nutrient levels is essential for cellular activities and survival (Efeyan *et al*, 2015). (AMP)-activated protein kinase (AMPK) is one of the most important energy-sensing enzymes in cells and is activated when energy levels are deficient

(Hardie *et al*, 2012; Jeon, 2016; Herzig & Shaw, 2018). AMPK is a heterotrimer composed of α , β and γ subunits, and when activated, AMPK can promote cell survival by inhibiting anabolism, promoting catabolism, and reducing reactive oxygen species (ROS) levels (Jeon, 2016; Hayes *et al*, 2020). When the nutrient supply in the cells is restored, the ATP/AMP ratio increases to inhibit the activity of AMPK. With the identification of AMP-binding sites on the AMPK γ subunit isoforms, researchers realized that AMPK activation is achieved upon the changes in the level of ATP, the energy currency of cells (Xiao *et al*, 2007; Gu *et al*, 2017). However, this simple model cannot explain how AMPK responds instantaneously to complex cellular metabolic processes and precisely regulates downstream genes. A recent study showed that glycogen inhibits AMPK activity by directly binding to the glycogen-binding site on the AMPK β subunit isoforms (McBride *et al*, 2009). Zhang *et al* found that AMPK can be activated by sensing a decrease in the glycolytic intermediate fructose-1,6 diphosphate before the AMP/ATP ratio is changed upon induced glucose deficiency (Zhang *et al*, 2017a; Li *et al*, 2019). Long-chain fatty acids bind to the ADaM site of the AMPK β 1 subunit, thereby allosterically activating AMPK (Pinkosky *et al*, 2020). Thus, in addition to sensing the AMP/ATP ratio, AMPK also has fine-tuned functions for sensing variations in glucose- and fatty acid-derived metabolites. It is currently unclear whether and how AMPK responds to variations in other nutrients or metabolites, such as amino acids. Hence, further identification of the molecular basis with respect to these factors is vitally important to help us gradually discover the precise regulatory mechanism of AMPK involved in different cellular metabolic processes.

Amino acids not only participate in the translation of proteins but also support various other metabolic processes, such as nucleotide synthesis and redox homeostasis (Wu, 2009). Therefore, cellular sensing of different amino acid levels is important for cell growth, and many intracellular amino acid sensors have been identified. For example, GCN2 indirectly senses all twenty kinds of amino acids by combining with free tRNA (Dong *et al*, 2000). SLC38A9 and CASTOR1 were identified as arginine sensors; Sestrin2 and LARS were identified as leucine sensors; and all these sensors

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eventually activate mTORC1 (Han *et al*, 2012; Wang *et al*, 2015; Chantranupong *et al*, 2016; Wolfson *et al*, 2016; Wyant *et al*, 2017). It has been reported that 20 aminoacyl tRNA synthase (AARS) enzymes can sense the concentration of all 20 amino acids in cells by recognizing aminoacylation modifications of lysine residues on the corresponding protein (He *et al*, 2018). Although amino acid-sensing pathways have been described in general, for many amino acids, only some universal sensing pathways have been identified, which obviously cannot explain the specific metabolic activities of different amino acids. Therefore, the identification of different amino acid-specific sensing molecules will help us understand complex amino acid metabolism pathways.

In this study, by subjecting cells to conditions in which a single amino acid is absent, we discovered that cystine starvation significantly activated AMPK. By promoting the binding of AMPK γ 2 to CaMKK2 upon cystine deficiency, the cysteine-specific sensor CARS is important for AMPK activation. These findings not only provide a better understanding of the regulatory role of AMPK in amino acid sensing but also suggest a combination strategy of cystine starvation and CaMKK2 inhibitor and/or AMPK inhibitor treatment for potential cancer therapy.

Results

Cystine deprivation activates AMPK through CaMKK2

The AMPK pathway is activated by intermediate products of carbohydrate or fatty acid metabolism to promote cell survival (McBride *et al*, 2009; Zhang *et al*, 2017a; Pinkosky *et al*, 2020); however, it remains largely unclear whether AMPK directly senses changes in amino acid metabolites. To investigate the connection between the AMPK pathway and amino acid metabolism, we cultured cells in DMEM without selected individual amino acids. The results showed that, of the 15 amino acids tested, cystine deprivation markedly activated AMPK, as indicated by its phosphorylation, in both 293T cells and RCC4 cells, although tryptophan, tyrosine, isoleucine or leucine deprivation also activated AMPK (Figs 1A and EV1A). Thus, we focused on cystine deprivation and AMPK activation. It is worth mentioning that cysteine, a component of the culture medium, is

easily oxidized, so cystine is added to form complete culture medium. After entering the cells, cystine will be decomposed into cysteine and participate in cell metabolism. Further experiments showed that cystine deprivation activated AMPK and its substrates including ACC and Raptor in a time-dependent manner, starting 30 min after cystine was removed from the culture medium (Fig 1B). A cystine concentration gradient assay also showed that the gradual activation of AMPK and its substrates in 293T cells was correlated with cystine concentration in the culture medium (Fig 1C), confirming that cystine deprivation activates AMPK and AMPK-mediated phosphorylation of its substrates including ACC and Raptor. Similar results were observed in other cell lines, including RCC4, SK-MES, SK-Hep1 and Hep3B cells (Fig 1D).

Next, we sought to investigate the molecular mechanism by which cystine deprivation activates AMPK. We hypothesized that there might be at least two steps required to activate AMPK during cystine deprivation. First, cystine deficiency is sensed via a specific sensing mechanism, such as the known GCN2-eIF4 pathway, aminoacylation pathway (Dong *et al*, 2000; He *et al*, 2018) or another unknown pathway(s), and second, the sensor protein transmits this deprivation signal to AMPK kinases or phosphatases, leading to the phosphorylation of AMPK (Fig 1E). To test this hypothesis, we first knocked down three AMPK kinases, LKB1, CaMKK2 and TAK1, by specific shRNAs. Interestingly, we observed that knocking down CaMKK2, but not LKB1 or TAK1, significantly inhibited the AMPK phosphorylation induced by cystine deprivation, and AMPK-mediated ACC and Raptor phosphorylation (Fig 1F). Consistently, cystine deprivation-induced AMPK phosphorylation and subsequent AMPK-mediated ACC and Raptor phosphorylation were suppressed by a CaMKK2 inhibitor (STO-609) but not by inhibitors of LKB1 (Pim1) or TAK1 (Takinib) (Figs 1G and EV1B), suggesting that CaMKK2 is involved in cystine deprivation-induced AMPK activation. Since AMPK kinases directly bind to and phosphorylate the α subunit of AMPK, we first tested the binding of these three kinases to AMPK α 1 during cystine starvation. Our results of the coimmunoprecipitation assay demonstrated that cystine deprivation enhanced the binding of CaMKK2 to AMPK α 1 without affecting the association of LKB1 or TAK1 with AMPK α 1 (Figs 1H and EV1EV1C and D). More importantly, our results also revealed that endogenous AMPK α 1 interacted with CaMKK2 during

Figure 1. Cystine deprivation activates AMPK through CaMKK2.

- A 293T cells were cultured in complete medium made of dialysed serum for 24 h which was then replaced with medium in which one amino acid was eliminated and cultured for 8 h. Then, WB was used to measure p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression. "Nor" = normal medium. Actin served as the loading control. The red text "-Cys" indicates that cystine deficiency most significantly activates AMPK and its substrates.
- B–D 293T cells were treated with cystine-deficient medium for 0, 0.5, 1, 2, 4 or 8 h (B) or treated with medium containing 200, 100, 50, 25 or 0 μ M cystine for 8 h (C); RCC4, SK-MES, SK-hep-1 or Hep3B cells were treated with cystine-deficient medium for indicated time (D). Protein levels of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor were measured by WB, and actin served as the loading control.
- E A schematic diagram of the hypothetical mechanism showing how cystine deprivation induces AMPK activation.
- F WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein in 293T cells transfected with shRNAs targeting LKB1, CaMKK2, TAK1 or a non-targeting control (NTC) that were treated with cystine-deficient medium for 8 h. Actin served as the loading control.
- G WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein in 293T cells and RCC4 cells treated with 1 μ g/ml STO-609 or DMSO for 8 h during cystine deprivation. Actin served as the loading control.
- H 293T cells were transfected with HA-AMPK α 1 alone or together with Flag-CaMKK2 for 48 h and then cultured with cystine-deficient medium or complete medium for 8 h. Cell lysates were immunoprecipitated with anti-Flag, and then, WB analysis was performed.
- I 293T cells cultured with cystine-deficient medium or complete medium for 8 h were harvested and subjected to immunoprecipitation with anti-AMPK α , followed by WB analysis with anti-AMPK α and anti-CaMKK2.

Source data are available online for this figure.

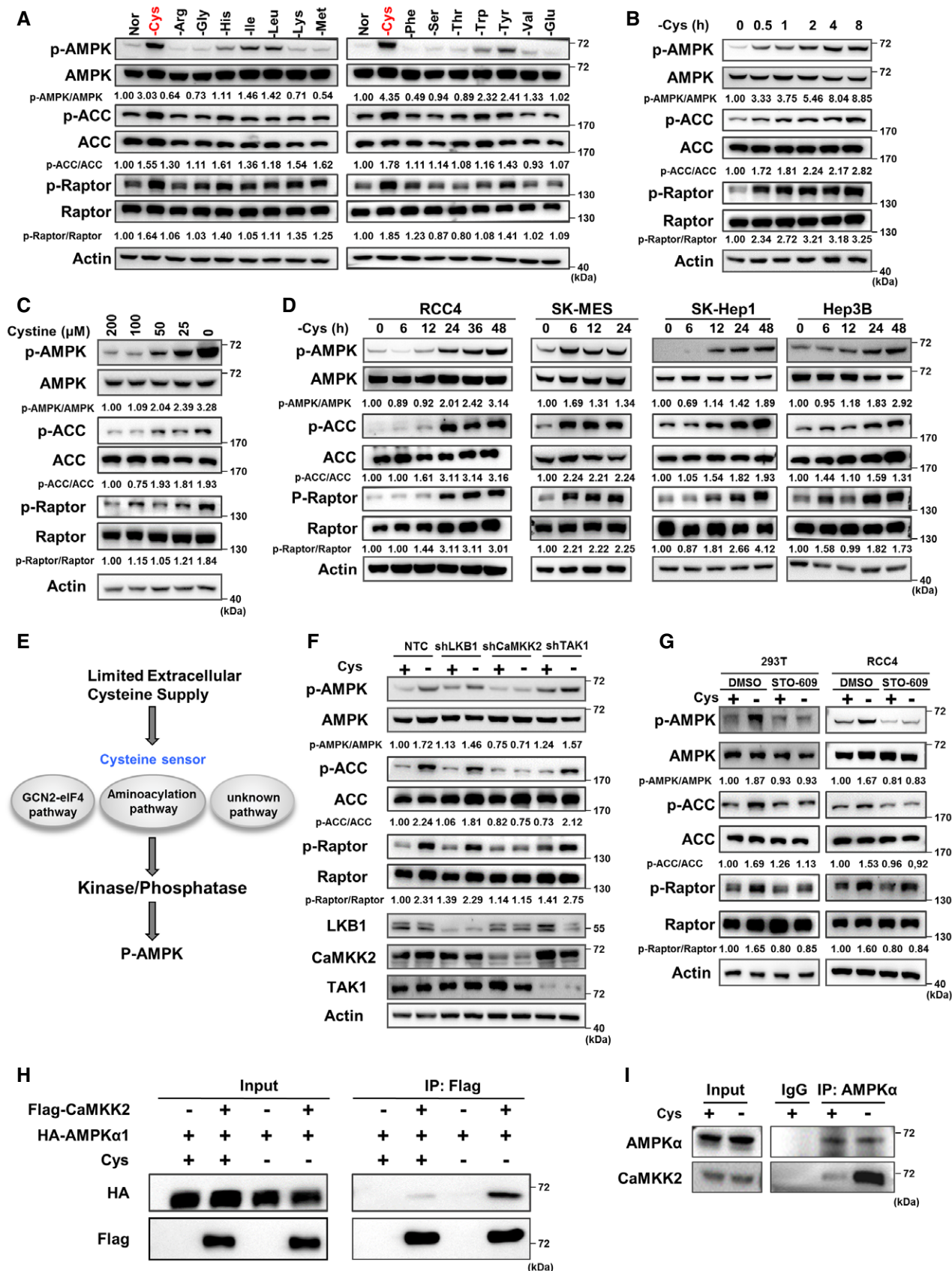


Figure 1.

cystine starvation (Fig 1I). These results indicate that the binding of CaMKK2 and AMPK α 1 is enhanced under cystine deprivation conditions to facilitate AMPK and its substrate activation.

CARS is critical for CaMKK2-mediated AMPK activation under cystine deprivation conditions

To explore whether the two known pathways are involved in the sensing of cystine starvation (Fig 1E), we next independently knocked down GCN2 and SIRT3 using specific shRNAs to block the GCN2-eIF4 and aminoacylation pathways, respectively (Dong *et al*, 2000; He *et al*, 2018). The results showed that the suppression of GCN2 or SIRT3 led to no significant changes in AMPK phosphorylation (Fig EV2A and B), suggesting that neither the GCN2-eIF4 nor aminoacylation pathways are involved in cystine deprivation-induced AMPK activation. To identify the molecule that senses cystine changes, we speculated that the sensor is likely an upstream factor that binds to CaMKK2. Thus, we performed an immunoprecipitation assay with an anti-CaMKK2 antibody followed by mass spectrometry analysis (IP-MS) to identify the CaMKK2-binding proteins under cystine deprivation conditions. By analysing the mass spectrometry results, we found that 238 proteins are potentially associated with CaMKK2, of which two proteins, AHCY and CARS, are known to be involved in cyst(e)ine metabolism (Fig 2A). Coimmunoprecipitation experiments revealed that CARS, but not AHCY, interacted with CaMKK2 in the presence or absence of cystine (Figs 2B and EV2C). Our further endogenous immunoprecipitation experiments revealed that CaMKK2 interacted with CARS independent of cystine concentration (Fig 2C). In addition, Flag pull-down assays using prokaryotically expressed Flag-CaMKK2 and His-tagged CARS proteins revealed that CARS directly binds to CaMKK2 (Fig 2D). These results demonstrate the binding of CARS to CaMKK2, suggesting a novel function in the activation of AMPK during cystine deprivation.

To directly test the roles of CARS in AMPK activation induced by cystine deprivation, we knocked down CARS by shRNAs, and our

results showed that AMPK and its substrate activation was dramatically abrogated by shCARS (Fig 2E). However, knocking down AHCY had no effect on cystine deprivation-induced AMPK activation (Fig EV2D), which was consistent with our early observation that AHCY did not interact with CaMKK2 (Fig EV2C). Moreover, knockdown of CARS attenuated the binding of CaMKK2 to AMPK α 1 under cystine deprivation conditions (Fig 2F), similar results were observed by endogenous immunoprecipitation experiments (Fig 2G). These results indicate that CARS is important for CaMKK2-mediated AMPK and its substrate activation. Notably, the suppression of CARS had no effect on glucose deprivation-induced AMPK activation (Fig EV2E), suggesting that CARS may specifically sense cystine deprivation to activate AMPK.

Moreover, we observed that overexpression of CARS significantly activated AMPK and its substrates in both 293T and RCC4 cells, and AMPK activation was attenuated by the suppression of CaMKK2 but not by suppression of the AMPK kinase LKB1 (Pim1) or TAK1 (Takinib) (Fig 2H and I). Consistently, CARS overexpression-induced AMPK activation was abolished by the CaMKK2 inhibitor STO-609 but not by inhibitors of LKB1 or TAK1 (Figs 2J and EV2F). We therefore concluded that, similar to cystine deprivation, overexpression of CARS activates AMPK and its substrates through CaMKK2. However, our Western blot results showed that cystine starvation had no effect on CARS protein expression, indicating that cystine deprivation-induced AMPK activation is not caused by upregulation of the CARS protein (Fig EV2G). Taken together, these results provide evidence that CARS mediates AMPK activation induced by cystine deficiency.

CARS senses cystine starvation to activate AMPK by binding to AMPK γ 2

At this point in our study, the data showed that CARS constitutively interacted with CaMKK2, regardless of cystine concentration (Fig 2B and C), while the binding of CaMKK2 and AMPK α 1 was enhanced by cystine starvation and was dependent on CARS (Figs 1H and I,

Figure 2. CARS is critical for AMPK activation under cystine deprivation conditions.

- Venn diagram showing the overlap of CaMKK2-binding proteins during cystine deprivation and cyst(e)ine metabolism-related proteins. The schematic diagram shows the peptides detected in the results of IP-MS assays of the AHCY and CARS proteins. The CaMKK2-binding proteins are listed in Table EV3, and the cyst(e)ine metabolism-related proteins are listed in Table EV4.
- 293T cells were transfected with HA-CaMKK2 alone or with Flag-CARS for 48 h and then cultured with cystine-deficient medium or complete medium for 8 h. Cell lysates were immunoprecipitated with anti-Flag, and a WB analysis was performed.
- 293T cells cultured with cystine-deficient medium or complete medium for 8 h were harvested and subjected to immunoprecipitation with anti-CaMKK2, followed by WB analysis with anti-CaMKK2 and anti-CARS.
- Flag pull-down assays were performed with Flag-EV or Flag-fused CaMKK2 protein and His-CARS purified from *E. coli*.
- WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor protein expression in 293T cells transfected with shRNAs targeting CARS that were further treated with cystine-deficient medium for 8 h. Actin served as the loading control.
- 293T cells were first transfected with shRNAs targeting CARS or NTC, then transfected with HA-AMPK α 1 or HA-AMPK α 1 and Flag-CaMKK2 for 48 h, and then, they were cultured with cystine-deficient medium for 8 h. Cell lysates were immunoprecipitated with anti-Flag and subjected to Western blot analysis.
- 293T cells were transfected with shRNAs targeting CARS or NTC; then, they were cultured with cystine-deficient medium for 8 h. Cell lysates were immunoprecipitated with anti-AMPK α and subjected to WB analysis with anti-AMPK α and anti-CaMKK2.
- WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, CARS protein expression in 293T cells and RCC4 cells overexpressing Flag-CARS. Actin served as the loading control.
- WB analysis of p-AMPK and total AMPK protein expression in CARS-overexpressing 293T cells were further transfected with shRNAs targeting LKB1, CaMKK2 or TAK1. Actin served as the loading control.
- WB analysis of p-AMPK and total AMPK protein expression in CARS-overexpressing 293T cells and RCC4 cells that were treated with 1 μ g/ml STO-609 or DMSO for 8 h. Actin served as the loading control.

Source data are available online for this figure.

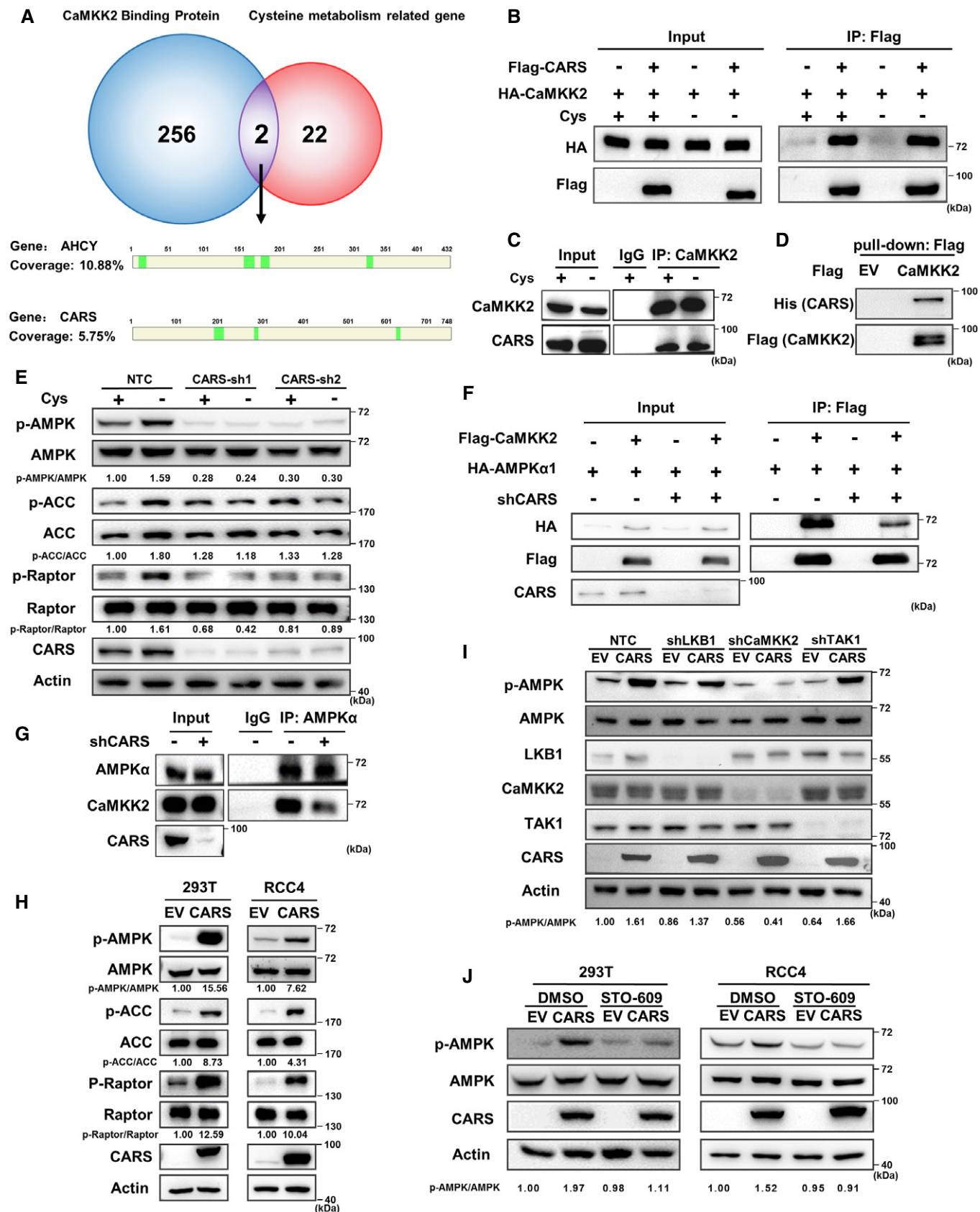


Figure 2.

and 2F and G). Next, we sought to determine how CARS transmits the cystine deficiency signal to CaMKK2. Since the AMPK complex is composed of three subunits, α , β and γ , we first performed pull-down assays using purified prokaryotically expressed His-tagged CARS (Fig EV3A) and HA-tagged AMPK subunit proteins. Our results indicated that only the HA-tagged AMPK γ 2 subunit was pulled down by the His-tagged CARS protein (Fig 3A), suggesting that CARS directly binds to the AMPK γ 2 subunit. Additional pull-down experiments using GST-tagged AMPK γ 1 or AMPK γ 2 protein confirmed the direct interaction of AMPK γ 2 and CARS (Fig 3B). Since previous reports have suggested the functional implications of the homology differences in AMPK γ subunits and the tissue expression profile in organisms (Mahlpuu *et al*, 2004; Willows *et al*, 2017), we further compared the amino acid sequences of AMPK γ 2 and AMPK γ 1 (Fig EV3B) and found that the C-terminus of AMPK γ 2 (265–569 amino acids) is basically the same as the full-length sequence of AMPK γ 1, with the only difference in individual amino acids, while the N-terminus (1–264 amino acids) of AMPK γ 2 is unique. Therefore, we hypothesized that AMPK γ 2 binds to CARS primarily through its N-terminus. Pull-down experiments with truncated AMPK γ 2 protein and CARS protein purified from *Escherichia coli* revealed that AMPK γ 2 binds to CARS via its N-terminus (Fig 3C). On the other hand, based on the CARS protein structure analysis via the online website UniProt (<http://www.ebi.ac.uk/intelpro/>), we constructed three vectors expressing the N-terminus (N), middle fragment (M) and C-terminus (C) of the CARS proteins, respectively, in which the N-terminus fragment contains cysteine-binding and catalytic domains, and the middle fragment contains tRNA-binding domain. (Fig EV3C, upper panel). The results of Co-IP experiments demonstrated that CARS binds to AMPK γ 2 through its N-terminus (Fig EV3C, bottom panel). Our data also showed that the AMP-binding site mutant AMPK γ 2-R531G (Xiao *et al*, 2007) exhibited a similar effect as wild-type AMPK γ 2 on AMPK activation under cystine starvation conditions, suggesting that cystine starvation-induced activation of AMPK is independent of intracellular ATP/AMP (Fig EV3D).

Moreover, we found that suppression of AMPK γ 2 by shRNAs abolished cystine deprivation- or CARS overexpression-induced AMPK and its substrate activation (Fig 3D and E), demonstrating that AMPK γ 2 is essential for cystine deprivation- or CARS overexpression-induced AMPK activation. More interestingly, we found that cystine deficiency promoted the binding of CARS and AMPK γ 2 (Fig 3F and G), and with increasing cystine concentration,

the binding of CARS and AMPK γ 2 decreased gradually (Fig 3H). These results suggest that cysteine competitively inhibits the binding of CARS and AMPK γ 2. In the presence of cyst(e)ine, cysteine occupied CARS at the N-terminus, which blocked the interaction of CARS with AMPK γ 2. However, when cystine was eliminated from the medium, CARS proteins were able to sense intracellular cyst(e)ine deficiency and bind to AMPK γ 2, thus recruiting CaMKK2 to the AMPK complex and leading to phosphorylation and activation of AMPK (Fig 3I).

The CARS–CaMKK2–AMPK axis promotes cell survival when cystine is eliminated

To investigate the effect of AMPK activation on cell survival under cystine deprivation conditions, we added the CaMKK2 inhibitor STO-609 to block AMPK activation induced by cystine deficiency in 293T, RCC4 and SK-MES cells and evaluated cell survival with crystal violet staining experiments. Our results showed that cystine starvation alone had a marginal effect on cell survival; however, when cystine-starved cells were treated with STO-609, the majority of the cells died (Fig 4A). Flow cytometry analysis confirmed that the combination of cystine deprivation and STO-609 treatment triggered dramatic cell death compared with cystine deprivation alone (Figs 4B and EV4A and B). Moreover, knocking down CARS, CaMKK2 or AMPK γ 2 markedly induced cell death under cystine deprivation conditions, which was attenuated by adding the AMPK activator AICAR, PT1 and GSK621 (Figs 4C and EV4C), although the mechanism of PT1's role in AMPK activation remains controversial (Jensen *et al*, 2015). Similar results were observed when cell death was detected by flow cytometry (Figs 4D and EV4D). These results demonstrate that AMPK activation by the CARS–CaMKK2 axis is important for cell survival when cystine is absent.

In addition to 293T, RCC4 and SK-MES cells that can activate AMPK when cystine is deficient, we also identified several other cell lines, such as 786-O, NCI-1650 and HT1080, wherein AMPK was not activated even when cystine was eliminated from the culture system (Fig EV4E). Consistent with these results, cystine deprivation alone resulted in extensive cell death, which was attenuated by the addition of AICAR (Fig EV4F). However, our Western blot data further showed that AMPK γ 2 and CaMKK2 were significantly lower in cystine-nonresponsive 786-O, NCI-1650 and HT1080 cells than in cystine-responsive 293T, RCC4 and SK-MES cells (Fig 4E). These results further underline the importance of the CARS–CaMKK2–

Figure 3. CARS activates AMPK by sensing cystine deficiency upon binding to AMPK γ 2.

- A His pull-down assay was performed with His-EV- or His-fused CARS protein and HA-tagged AMPK subunit proteins overexpressed in 293T cells.
 B, C GST pull-down assay was performed with GST-EV, GST-AMPK γ 1, GST-AMPK γ 2 (B) or GST-EV, GST-AMPK γ 2, GST-AMPK γ 2-N, GST-AMPK γ 2-C (C) proteins and His-tagged CARS protein purified from *E. coli*. The segments of AMPK γ 2 are shown in the bar graph above (C). The red arrows indicate the target bands.
 D, E WB analysis of p-AMPK, p-ACC, p-Raptor and total AMPK, ACC, Raptor, AMPK γ 2 protein expression in 293T cells transfected with shRNAs targeting AMPK γ 2 that were further treated with cystine-deficient medium for 8 h (D) or transfected with Flag-CARS for 48 h (E). Actin served as the loading control.
 F 293T cells were transfected with HA-AMPK γ 2 alone or with Flag-CARS for 48 h and then cultured with cystine-deficient medium or complete medium for 8 h. Cell lysates were immunoprecipitated with anti-Flag, and a WB analysis was performed.
 G 293T cells were transfected with HA-AMPK γ 2 for 48 h and then cultured with cystine-deficient medium or complete medium for 8 h. Cell lysates were immunoprecipitated with anti-CARS, followed by WB analysis with anti-HA and anti-CARS.
 H GST pull-down of His-CARS by GST-AMPK γ 2 using protein purified from *E. coli*, followed by WB analysis. Cysteine (0, 2, 10, 50 and 250 μ M) was added to the experimental buffer.
 I The schematic diagram shows that cystine deficiency-induced AMPK activation is mediated by CaMKK2, which is recruited by CARS and the AMPK γ 2 complex.

Source data are available online for this figure.

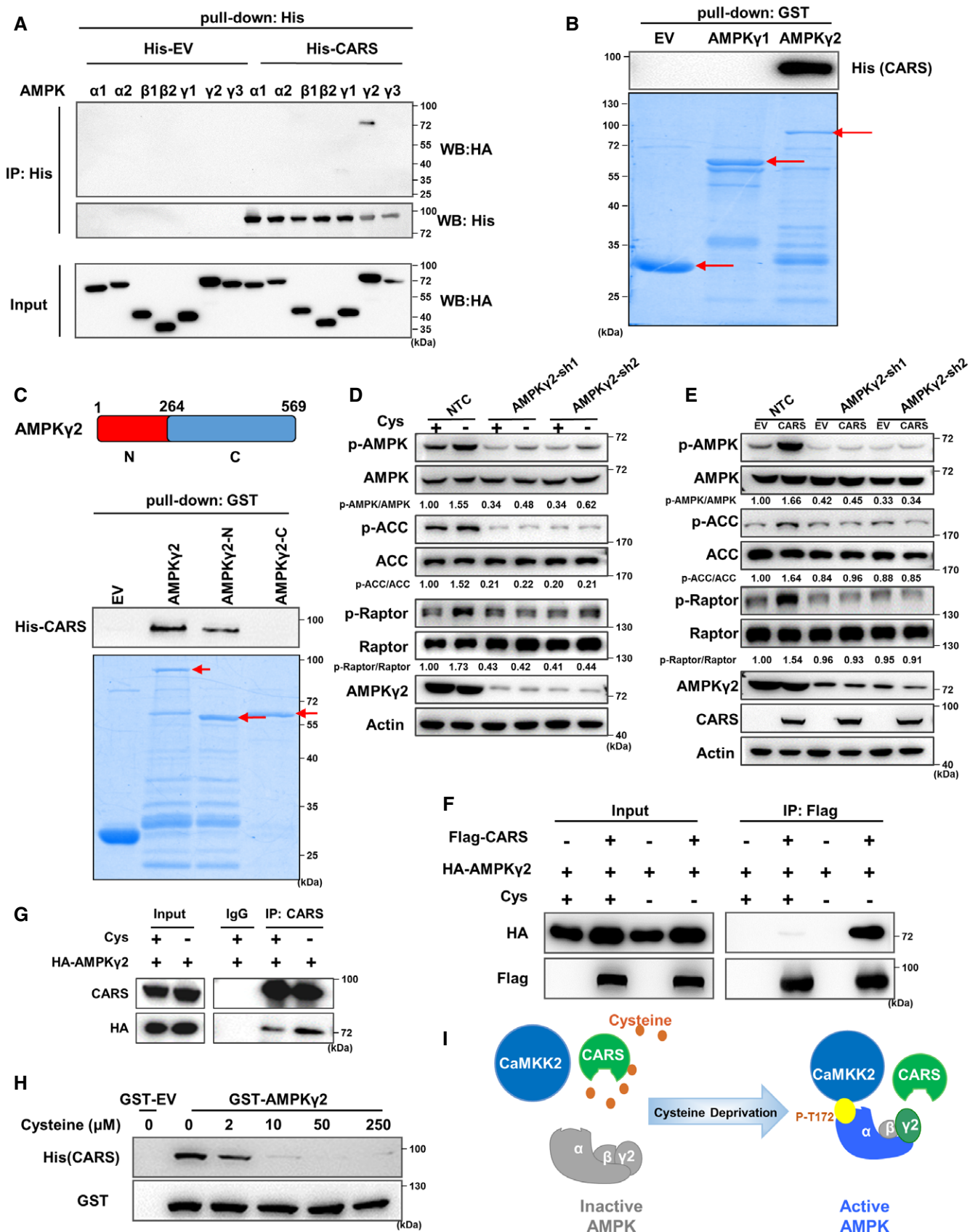


Figure 3.

AMPK axis in protecting cells under cystine-deficient conditions. By overexpressing CARS, AMPK γ 2 and CaMKK2 in 786-O cells, we found that AMPK was more notably activated when cystine was deficient (Fig 4F). Further, crystal violet staining experiments showed that overexpression of AMPK γ 2 and CaMKK2 significantly suppressed the cell death of 786-O cells induced by cystine deprivation (Fig 4G). These results indicate that the differential expression of AMPK γ 2 and CaMKK2 in different cells may determine their sensitivity to cystine deficiency and the subsequent AMPK activation and cell survival.

In summary, our results demonstrate that cystine deficiency promotes the binding of CARS and AMPK γ 2, whereby CARS recruits its partner CaMKK2 to activate AMPK, leading to cell survival under nutrient stress conditions (Fig 5).

Discussion

(AMP)-activated protein kinase directly senses metabolic intermediates, which has recently attracted increased attention (Steinberg & Kemp, 2009; Hardie *et al*, 2012; Hardie, 2014; Zhang *et al*, 2014). In this study, we report, for the first time, that CARS senses cysteine deprivation conditions and activates AMPK to promote cell survival. We discovered a fine-tuned sensing mechanism of AMPK to cyst(e)ine variation. Under cystine deficiency conditions, the CARS protein fails to bind cysteine, and this dissociation can release the inhibitory effect of cysteine on the binding of CARS to AMPK γ 2. CARS then recruits the AMPK complex to the vicinity of CARS-bound CaMKK2, causing CaMKK2-induced AMPK phosphorylation and activation, thereby promoting the survival of cells under cystine deprivation stress. Hence, we identified a novel mechanism whereby AMPK is activated to trigger cell survival when the amino acid cystine is depleted.

(AMP)-activated protein kinase can sense intracellular glucose and lipids (McBride *et al*, 2009; Zhang *et al*, 2017a; Li *et al*, 2019; Pinkosky *et al*, 2020), but surprisingly, little is known about the relationship between AMPK and its ability to sense amino acids. A previous study showed that withdrawal of essential amino acids induced the activation of AMPK through CaMKK2 (Ghislat *et al*, 2012), but Dalle Pezze *et al* found that AMPK activation by amino acid replenishment is mediated by CaMKK2 (Dalle Pezze *et al*, 2016). These reports indicate that there is still considerable confusion in this field, and that, to date, no data linking AMPK to the

deficiency of a single specific amino acid have been documented. In this study, we explored, for the first time, the connection between the availability of each amino acid and AMPK activation and found that cystine deficiency alone can notably activate AMPK (Figs 1A–D and EV1A). As a heterotrimer, AMPK has two α subunits, two β subunits and three γ subunit forms, suggesting 12 possible combinations, which clearly indicate functional redundancy (Ross *et al*, 2016). Therefore, different combinations of AMPK complexes may correspond to different functions under different physiological conditions, and the three kinases of AMPK may also be potentially critical for sensing differences in nutrient levels (Hawley *et al*, 2003; Hong *et al*, 2003; Woods *et al*, 2003, 2005; Mahlapuu *et al*, 2004; Momcilovic *et al*, 2006; Koay *et al*, 2010; Oakhill *et al*, 2010; Kim *et al*, 2012; Ross *et al*, 2016). In this regard, intriguingly, we discovered that AMPK activation induced by cystine deprivation is specifically dependent on the AMPK γ 2 subunit (Figs 3D and E, and EV3D), and this activation can be mediated only by CaMKK2 (Fig 1F and G).

In recent years, aminoacyl tRNA synthase (AARS), a sensor of amino acid sensors, has increasingly piqued the interest of scientists (Hountondji *et al*, 2000; Yanagisawa *et al*, 2010; Han *et al*, 2012; He *et al*, 2018). The traditional function of AARS is linking amino acids to corresponding tRNAs to form aminoacyl tRNAs during protein translation. AARS in most eukaryotes has a novel domain that does not participate in aminoacylation, suggesting that AARS may be involved in other biological processes (Guo & Schimmel, 2013). Recently, many nontranslational functions of AARS have been discovered, most notably the sensing of metabolites (Ko *et al*, 2000, 2001; Yakobov *et al*, 2017; Gupta & Laxman, 2019). As a metabolic sensing molecule, AARS also conforms to the principle of making the best use of biological evolution. For instance, AARS must first be able to bind directly to a sensed metabolite; second, the molecule then must transmit a signal. Our results show that CARS, an AARS, can sense cyst(e)ine deprivation and enhance its binding with AMPK γ 2 (Fig 3F–G), ultimately activating AMPK by binding its kinase CaMKK2 (Fig 2A–D). These results substantially extend our understanding of the metabolic sensing function of AARS.

As cysteine is the only reducing amino acid in cells, an increasing number of reports in recent years have shown that cysteine plays an important role in the occurrence and development of tumours. Ioannis *et al* found that lung cancer with EGFR mutations is particularly sensitive to cysteine loss. Eliminating cysteine in mice can inhibit

Figure 4. The CARS-CaMKK2-AMPK axis promotes cell survival when cystine is eliminated.

- A The crystal violet assay was performed in cystine-deficient medium-cultured 293T, RCC4 and SK-MES cells with or without 1 μ g/ml STO-609 for 24 h.
- B The cell death level was determined in 293T cells with the same treatment conditions as in Fig 4A. Data are presented as the mean (\pm SD) of three independent experiments. NS, not significant; * P < 0.05 [two-tailed Student's t -test], compared with the indicated groups.
- C, D The crystal violet assay (C) and apoptosis rate assay (D) were performed in 293T cells transfected with shRNAs targeting NTC, CARS, CaMKK2 or AMPK γ 2 and further treated with cystine-deficient medium for 24 h with or without 1 mM AICAR. Data are presented as the mean (\pm SD) of three independent experiments. NS, not significant; * P < 0.05 [two-tailed Student's t -test], compared with the indicated groups.
- E WB analysis of CARS, CaMKK2 and AMPK γ 2 protein expression in RCC4, SK-MES, 293T, 786-O, NCI-1650 and HT1080 cells. Actin served as the loading control.
- F, G WB analysis of p-AMPK, total AMPK, CARS, AMPK γ 2 and CaMKK2 protein expression (F) and a crystal violet assay were performed (G, left) with 786-O cells transfected with Flag-EV, Flag-CARS, Flag-CaMKK2 or Flag-AMPK γ 2 and further treated with cystine-deficient medium for 8 h. Actin served as the loading control. After scanning, 20% glacial acetic acid was added to dissolve the crystal violet, and the absorbance of each well was measured at 570 nm (OD570) to calculate relative cell viability (G, right). Data are presented as the mean (\pm SD) of three independent experiments. NS, not significant; * P < 0.05 [two-tailed Student's t -test], compared with the indicated groups.

Source data are available online for this figure.

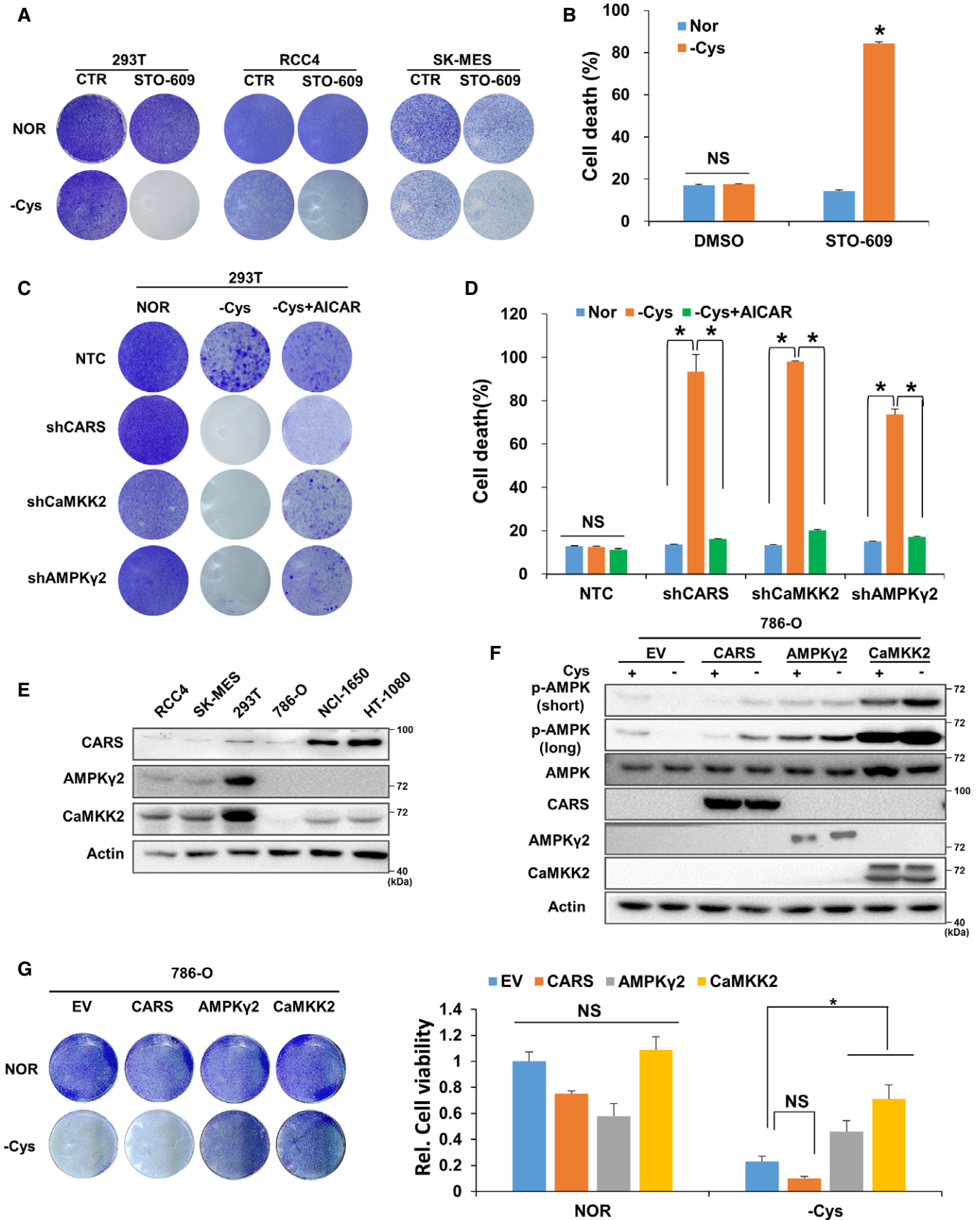


Figure 4.

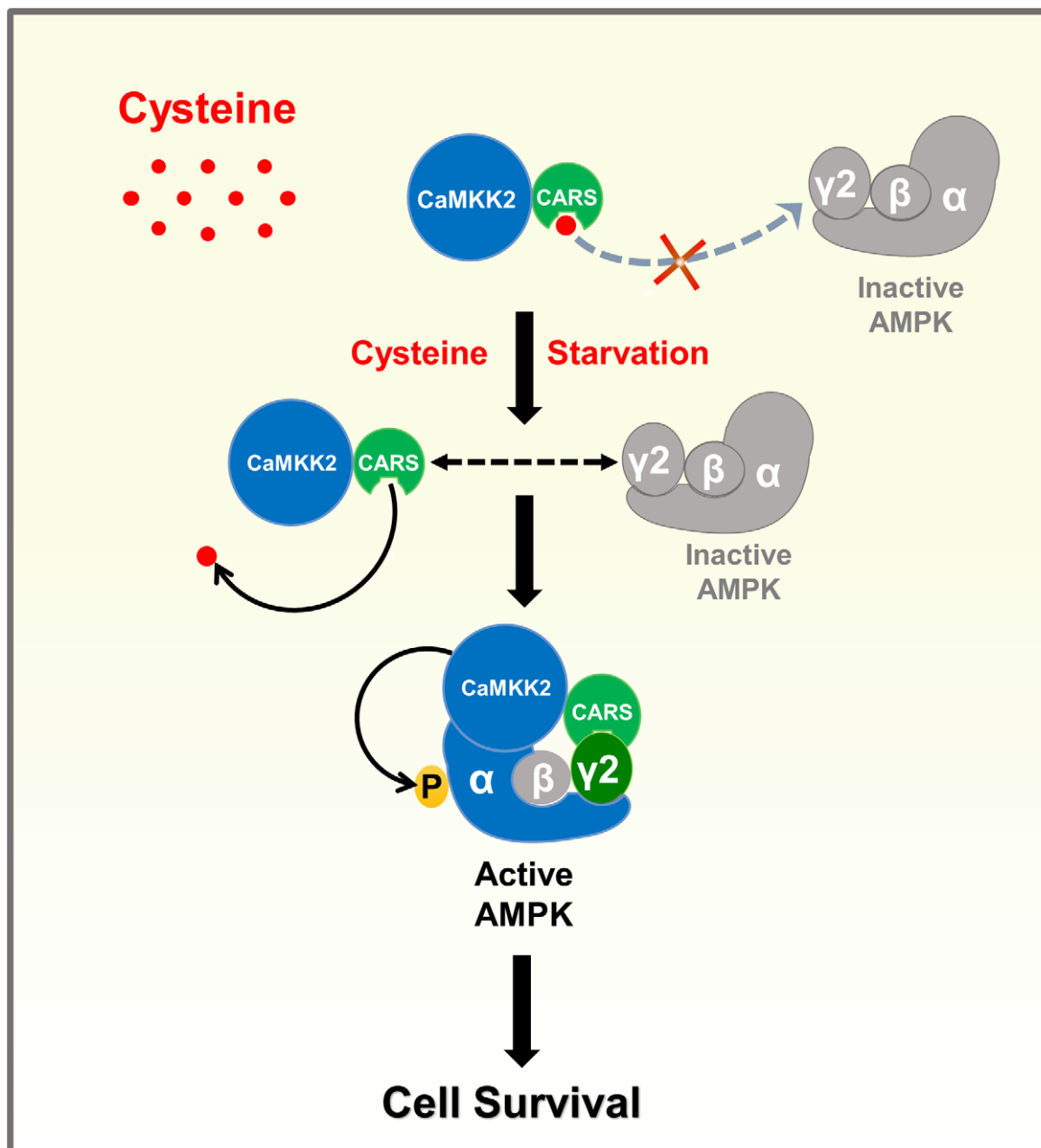


Figure 5. Working model: CARS-CaMKK2-AMPK γ 2 pathway activates AMPK to promote cell survival under cystine starvation condition.

In the presence of cyst(e)ine, cysteine blocked the interaction of CARS with AMPK γ 2, the AMPK complex is in an inactive state. In the deficiency of cystine, CARS is able to bind to AMPK γ 2, thus recruiting CaMKK2 to the AMPK complex and leading to phosphorylation and activation of AMPK, which ultimately promotes cell survival.

the growth of lung cancer cell-transplanted tumours (Poursaitidis *et al*, 2017); Shira *et al* found that clearing cysteine from mouse serum can significantly inhibit the development of prostate cancer and breast cancer, as well as increases the average survival time of lymphoma mice by twofold (Cramer *et al*, 2016); Michael A *et al* found cysteine deprivation can induce the specific ferroptosis of mouse pancreatic cancer cells and then inhibit the development of pancreatic cancer (Badgley *et al*, 2020). Collectively, these studies showed that the elimination of cysteine to inhibit the occurrence

and development of tumours has broad application prospects. However, any therapy for tumour treatment must consider the scope of the application and possible cell resistance. In our study, we found that some tumour cells prevent cell death by activating AMPK under cystine deprivation, but the addition of the CaMKK2 inhibitor STO-609 inhibited this phenomenon, suggesting that blocking AMPK activation may be a possible therapeutic option when cells are insensitive to cystine elimination. Additionally, considering the importance of AMPK γ for cell survival under cysteine

deprivation conditions (Fig 4D), and the differences in the expression of AMPK γ subunits in different tissues (Mahlapuu *et al*, 2004), treating cancer cells with low AMPK γ 2 expression in combination with cysteine starvation might be a potential cancer targeting strategy.

In this study, we show that AMPK is activated by cysteine deprivation. In addition, we found that CARS, the sensor of cysteine, can bind to AMPK γ 2 when cysteine is deficient and then recruit the AMPK complex to CARS-bound CaMKK2, causing AMPK phosphorylation and activation by CaMKK2. In addition, we observed that AMPK activation in the absence of cysteine is closely related to the survival of cells. Hyemin *et al* (Lee *et al*, 2020) found that AMPK activation can inhibit ferroptosis, which again illustrates the importance of AMPK activation when cells face oxidative stress. In addition to cysteine, there are several amino acids whose deficiency activates AMPK. Determination of the amino acids accurately sensed to activate AMPK, the sensors involved and the physiological significance of this activation on cellular fate and function warrants further study. We found that CARS functions as a sensor of cysteine, but whether other AARS can also sense specific amino acid content to regulate molecular pathways in cells or whether they can sense the corresponding tRNA instead of amino acids remains to be further explored.

Materials and Methods

Cell culture and reagents

All cells were purchased from ATCC and cultured in Dulbecco's modified essential medium (DMEM) containing 10% FBS and 1% penicillin–streptomycin. All cells were cultured in a humidified incubator at 37°C with 5% CO₂. Cysteine and other amino acid deprivation experiments were carried out as described previously (Zhang *et al*, 2017b). In brief, cells were cultured in DMEM deprived of a selected nutrient. Amino acid deprivation studies of Figs 1A and I, 2C and G and 3G used dialysed serum (Biological Industries; Cat# O4-011-1), while normal serum was used for the rest of the experiments. Takinib (HY-103490), Pim1 (HY-10371) and GSK621 (HY-100548) were purchased from MCE, and AICAR (A8184), ATO-609 (B6787) and PT1 (B7572) were purchased from APEXBIO.

Plasmids and established stable cells

Lentiviral shRNAs targeting the LKB1, CaMKK2, TAK1, CARS, AMPK γ 2, GCN2 and Sirt3 plasmids were purchased from Sigma. The sequences of shRNAs used in this study are listed in Table EV1. pSin-3XFlag-CaMKK2, pSin-3XFlag-LKB1, pSin-3XFlag-TAK1, pSin-3XFlag-AHCY, pSin-3XFlag-CARS-wt, pSin-3XFlag-CARS-mut1, pSin-3XFlag-mut2, pSin-3XFlag-AMPK γ 2-wt and pSin-3XFlag-AMPK γ 2-R531G vectors were used to construct a Flag-overexpressing plasmid, and pSin-HA-AMPK α 1, pSin-HA-AMPK α 2, pSin-HA-AMPK β 1, pSin-HA-AMPK β 2, pSin-HA-AMPK γ 1, pSin-HA-AMPK γ 2, pSin-HA-AMPK γ 3 and pSin-HA-CaMKK2 vectors were used to construct a HA-expressing plasmid. All plasmids targeting specific genes were cotransfected with plasmids carrying Δ 8.9 and VSVG into HEK293T cells using PEI (Invitrogen) for 48 h, and then, the viruses were

collected by filtering the supernatant of the HEK293T cells. The collected viruses were used to infect cells in the presence of 8 μ g/ml polybrene (Sigma-Aldrich). The transduced cells were selected by puromycin.

Western blot analysis

Cells were harvested as described previously (Zhang *et al*, 2017b). Protein concentration was measured using a Bradford Assay Kit (Sangon Biotech). Equal amounts of proteins were fractionated by 6–10% SDS–PAGE. The following primary antibodies were used: anti-p-AMPK (CST; 2535; 1:1,000), anti-AMPK (CST; 253; 1:750), anti-p-ACC-S79 (CST; 3661; 1:1,000), anti-p-Raptor-S792 (CST; 2083; 1:1,000), anti-AMPK γ 2 (CST; 2536s; 1:1,000), anti-ACC (PTG; 67373-1-Ig; 1:1,000), anti-Raptor (PTG; 20984-1-AP; 1:1,000), anti-CARS (PTG; 15296-1-AP; 1:1,000), anti-CaMKK2 (PTG; 11549-1-AP; 1:1,000), anti-LKB1 (PTG; 10746-1-AP; 1:1,000), anti-TAK1 (PTG; 12330-2-AP; 1:1,000), anti-HA-HRP (CST; 2999; 1:5,000), anti-His-Tag (Biotechnology; D110002; 1:2,000) and anti-GST-Tag (PTG; 10000-0-AP; 1:1,000). HRP-conjugated anti-rabbit and anti-mouse (Bio-Rad) secondary antibodies were used. Signals were detected using a Western ECL substrate (Bio-Rad). Detailed information for all antibodies used is provided in Table EV2.

Immunoprecipitation

Immunoprecipitation was carried out as described previously (Wu *et al*, 2017). In brief, 293T cells were collected and lysed in IP buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1.5 mM MgCl₂, 0.5% NP-40) for 1–2 h, and the supernatant was transferred to a 20- μ l solution containing protein A/G beads and precleared for 1 h. After protein quantification, the corresponding primary antibody was added and incubated overnight. Next, the protein A/G beads were incubated for 2 h and washed four times with IP buffer. Protein loading was added, and the beads were placed in a 100°C metal bath to fully denature the protein. Western blot analysis was used to detect protein binding.

LC-MS and data analysis

The sample was prepared according to a preparation kit (Thermo: 90057), and LC-MS was carried out as described previously (Jiang *et al*, 2019). A Q-Exactive Plus mass spectrometer (Thermo, USA) coupled to an EASY-nLC 1200 HPLC system (Thermo, USA) with a nano-electrospray ion source and operated in data-dependent mode was used to analyse the samples. Raw LC-MS data were further analysed using proteomics discovery software (version 2.1, Thermo Fisher Scientific) against the human UniProt database (version 20140922, 20,193 sequences). A result is considered positive when the peptide of detected constitutes more than 5% of the total peptides in the protein.

Fusion protein pull-down experiment

The PET-22b-CARS plasmid was transfected into *Escherichia coli* (BL21), and the expression of the His-CARS tag protein was induced with IPTG. The bacterial cells were collected, and the proteins were subjected to by ultrasonic lysis. The His-CARS protein was selected

on a nickel column, which was rinsed with imidazole, by elution with solutions of imidazole at different concentrations, which was used to obtain purified His protein. The PET-22b-Flag-CaMKK2 plasmid was transfected into *E. coli* (BL21), and the expression of the Flag-CaMKK2 tag protein was induced using IPTG. Pierce™ anti-DYKDDDDK magnetic agarose (Thermo; A36797) was used to purify Flag-tagged protein. PGEX-4T1-EV, PGEX-4T1-AMPK γ 1, PGEX-4T1-AMPK γ 2, PGEX-4T1-AMPK γ 2-N and PGEX-4T1-AMPK γ 2-C plasmids were transfected into *E. coli* (BL21). IPTG was used to induce the expression of GST-tagged proteins, and then, GSH beads were used to adsorb the GST-tagged proteins. Next, we used PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.1% Tween 20) buffer to rinse and purify the GST-tagged proteins. Finally, the purified Flag-tagged protein or GST-tagged protein and His-CARS protein were subjected to a pull-down experiment. The proteins were detected by WB and Coomassie Blue staining.

Crystal violet staining

After cells were treated as indicated, the culture medium was discarded, and the cells were carefully washed twice with PBS to remove the residual culture medium. The cells were fixed with methanol for 30 min and stained with 0.5% crystal violet for 30 min, which was washed away with PBS. After natural drying, the plates were scanned with a scanner. To quantify the cells stained with crystal violet, 20% glacial acetic acid was added to each well and mixed by pipetting. The absorbance at 570 nm (OD₅₇₀) of each well was measured by a microplate reader, and relative cell viability was calculated according to the following formula: [OD₅₇₀ (treated group) – OD₅₇₀ (blank)]/[OD₅₇₀ (control) – OD₅₇₀ (blank)] × 100%.

Flow cytometry

Cultured cells were collected from the 6-well plate and washed twice with PBS to remove the residual culture medium. For the cell death analysis, the collected cells were stained with 5 μ g/ml propidium iodide (PI), and the percentage of PI-positive dead cells was recorded by flow cytometry (BD FACS Celesta). The data were further analysed by FlowJo software.

Statistical analysis

Data are presented as the mean (\pm SD) of three independent experiments unless otherwise noted. Group differences ($P < 0.05$) were analysed by two-tailed Student's *t*-test.

Data availability

The immunoprecipitation mass spectrometry data of CaMKK2-binding proteins relating to Fig 2A have been deposited at PRIDE (Perez-Riverol *et al*, 2019) hosted at the EBI (<https://www.ebi.ac.uk/pride/archive/projects/PXD027017/>). Data are available via ProteomeXchange with identifier PXD027017.

Expanded View for this article is available online.

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Author contributions

PG, HZ and LS conceived and supervised this study. PG, HZ, LS and MY designed the experiments. MY, RY, YZ, YQ, ZJ, HL, LS and YW executed the experiments. PG, HZ, LS, MY, RY and YZ wrote the manuscript. All the authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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