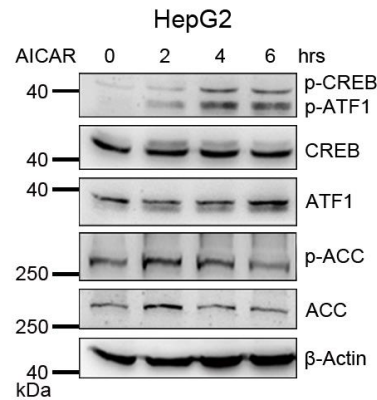
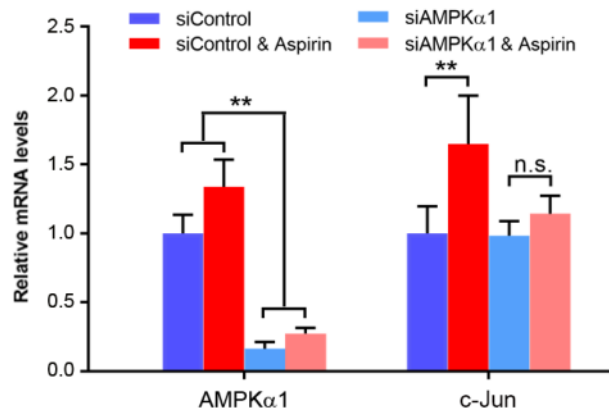


# Blockade of AMPK-Mediated cAMP–PKA–CREB/ATF1 Signaling Synergizes with Aspirin to Inhibit Hepatocellular Carcinoma

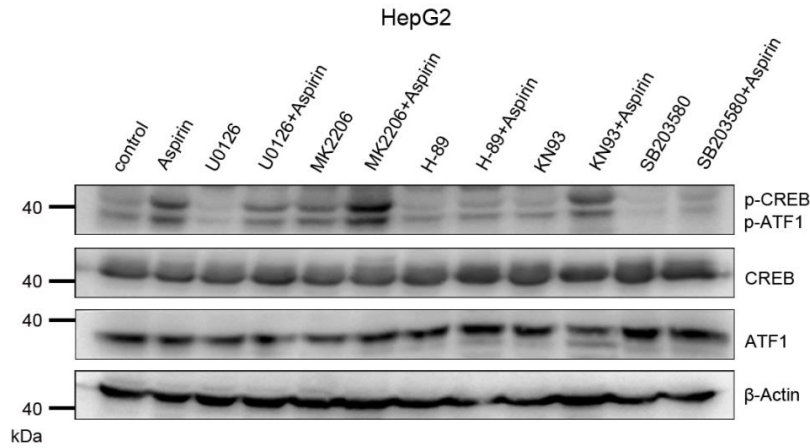
Hongying Zhang, Songpeng Yang, Jiao Wang, Yangfu Jiang



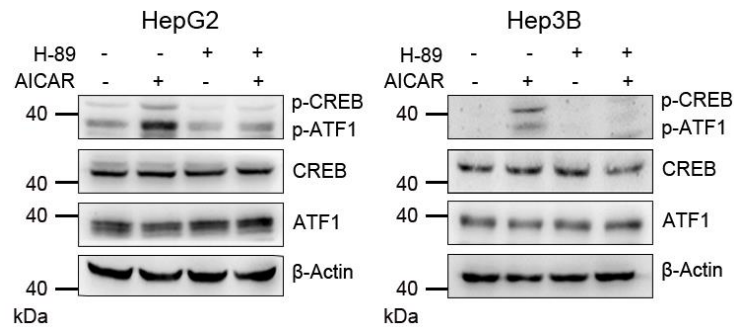
**Figure S1.** The AMPK agonist AICAR induces CREB/ATF1 phosphorylation in HepG2 cells. HepG2 cells were treated with 0.5 mM AICAR for indicated periods, followed by western blot analysis of indicated proteins.



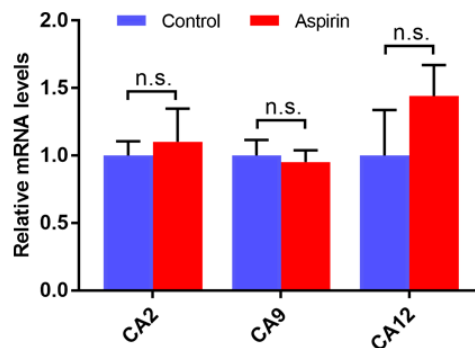
**Figure S2.** AMPK $\alpha$ 1 knockdown abrogates the induction of c-Jun transcription by aspirin. Hep3B cells were transfected with siControl or siAMPK $\alpha$ 1, and treated with or without 5 mM aspirin, followed by real-time RT-PCR analysis of indicated genes. The relative mRNA levels were plotted. Values represent mean  $\pm$  SD ( $n = 3$ ). \*\*,  $p < 0.01$ . *n.s.*, not significant.



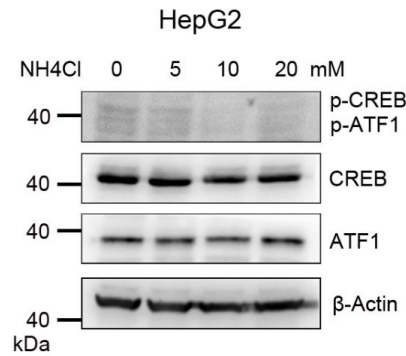
**Figure S3.** The effects of MEK inhibitor U0126, Akt inhibitor MK2206, PKA inhibitor H-89, CAMK inhibitor KN93 and p38 inhibitor SB203580 on the induction of CREB/ATF1 phosphorylation by aspirin in HepG2 cells. HepG2 cells were treated with 5 mM aspirin individually or in combination with indicated inhibitors for 32 hours, followed by western blot analysis of indicated proteins.



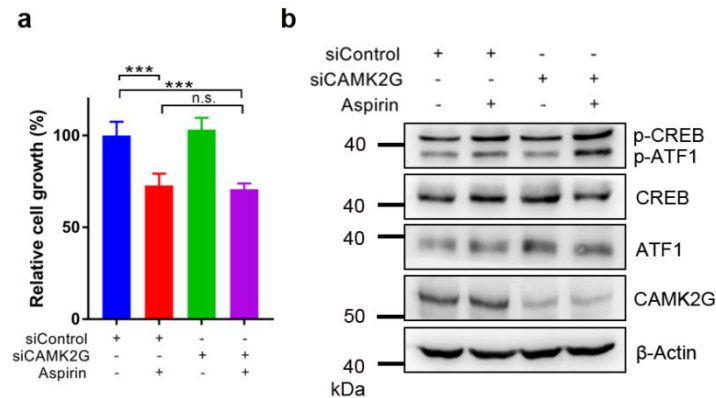
**Figure S4.** The effects of PKA inhibitor H-89 on the induction of CREB/ATF1 phosphorylation by AMPK agonist AICAR. HepG2 and Hep3B cells were incubated with 20 μM H-89 for 24 hours, and treated with 0.5 mM AICAR for further 6 hours, followed by western blot analysis of indicated proteins.



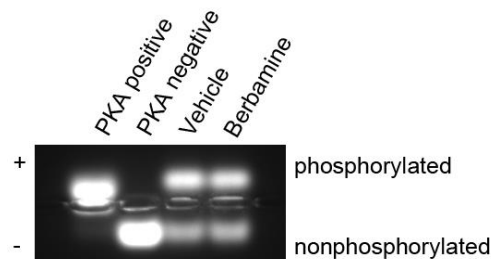
**Figure S5.** The effects of aspirin on CA2, CA9 and CA12 expression in HepG2 cells. HepG2 cells were treated with 5 mM aspirin for 48 hours, followed by quantitative RT-PCR analysis of CA2, CA9, and CA12 transcription. The relative mRNA levels were plotted. Values represent mean ± SD (n = 3). n.s., not significant.



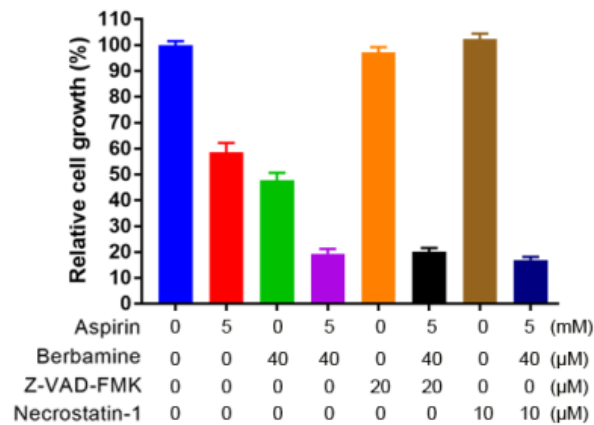
**Figure S6.** NH<sub>4</sub>Cl does not induce CREB/ATF1 phosphorylation in HepG2 cells. HepG2 cells were treated with indicated concentration of NH<sub>4</sub>Cl for 48 hours, followed by western blot analysis of indicated proteins.



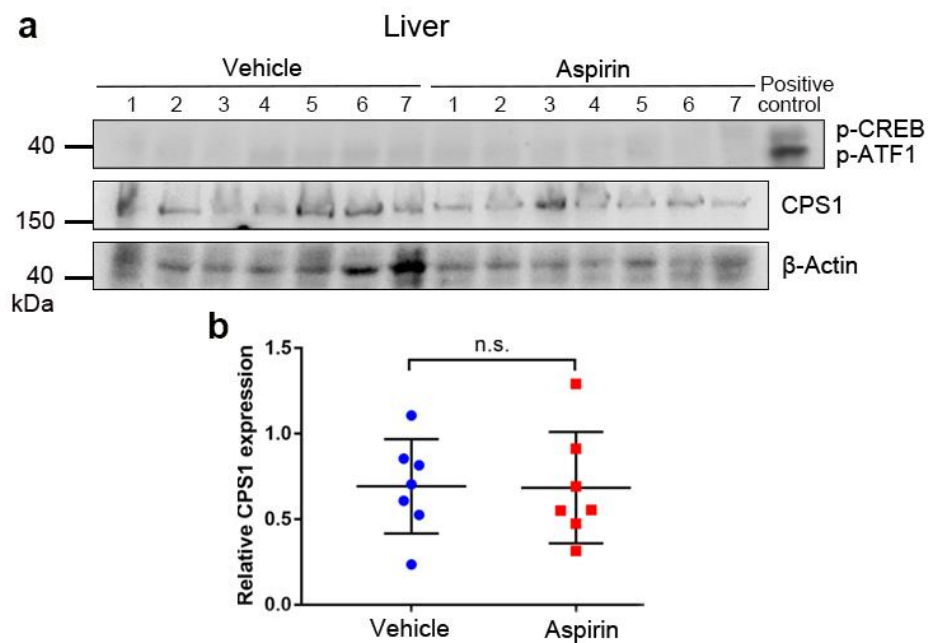
**Figure S7.** CAMK2G knockdown does not sensitize Hep3B cells to aspirin. **(a)** Hep3B cells were transfected with siControl or siCAMK2G, and treated with or without 5 mM aspirin for 48 hours, followed by CCK-8 assays. The relative cell growth was plotted. Values represent mean  $\pm$  SD ( $n = 5$ ). \*\*\*,  $p < 0.001$ . n.s., not significant. **(b)** Hep3B cells were transfected with siControl or siCAMK2G, and treated with or without 5 mM aspirin for 48 hours, followed by western blot analysis of indicated proteins.



**Figure S8.** Berbamine does not inhibit PKA activity. HepG2 cells were treated with or without 40  $\mu$ M berbamine, followed by PKA kinase assay. PKA-phosphorylated substrate migrated toward the anode (+), whereas the unphosphorylated substrate migrated toward the cathode (-).



**Figure S9.** The caspase inhibitor Z-VAD-FMK and necroptosis inhibitor necrostatin-1 do not affect the inhibition of HepG2 cells growth by aspirin and berbamine. HepG2 cells were incubated with indicated inhibitors for 48 hours, followed by CCK-8 assays. The relative cell growth was plotted. Values represent mean  $\pm$  SD ( $n = 5$ ).



**Figure S10.** Aspirin does not induce CREB/ATF1 phosphorylation and inhibit CPS1 expression in mouse liver tissues. **(a)** BALB/c nude mice were treated with or without aspirin (100 mg/kg/day) for 26 days. The expression of CPS1 and the phosphorylation of CREB/ATF1 in liver tissues were detected by western blotting. **(b)** The levels of CPS1 expression were plotted. *n.s.*, not significant.

**Table S1.** List of the chemicals used in this study

| Chemicals      | Source          | Cat. number |
|----------------|-----------------|-------------|
| Aspirin        | Sigma Aldrich   | A2093       |
| AICAR          | Selleck         | S1802       |
| U0126          | Selleck         | S1102       |
| MK2206         | Selleck         | S1078       |
| H-89           | Targetmol       | T6250       |
| KN93           | Targetmol       | T2606       |
| Berbamine      | Targetmol       | T2920       |
| SB203580       | MedChem Express | HY-10256    |
| Etomoxir       | MedChem Express | HY-50202    |
| Compound C     | MedChem Express | HY-13418    |
| Lactic acid    | Sangon Biotech  | A504045     |
| Gefitinib      | Targetmol       | T1181       |
| Sorafenib      | Targetmol       | T0093L      |
| Liproxstatin-1 | MedChem Express | HY-12726    |
| Z-VAD-FMK      | Targetmol       | T6013       |
| Okadaic acid   | Beyotime        | S1786       |

**Table S2.** List of the antibodies used in this study

| Antibodies             | Source                    | Cat. number |
|------------------------|---------------------------|-------------|
| p-CREB(S133)/ATF1(S63) | Cell Signaling Technology | 9198        |
| p-AMPK $\alpha$ (T172) | Cell Signaling Technology | 2535        |
| AMPK $\alpha$ 1        | Cell Signaling Technology | 2795        |
| p-ACC(S79)             | Cell Signaling Technology | 11818       |
| ACC                    | Cell Signaling Technology | 3676        |
| CREB                   | Proteintech               | 12208-1-AP  |
| $\beta$ -Actin         | Proteintech               | 60008-1-AP  |
| CPS1                   | Proteintech               | 18703-1-AP  |
| CAMK2G                 | Proteintech               | 12666-2-AP  |
| ATF1                   | Abcam                     | ab181569    |
| sAC                    | Abcam                     | ab203204    |
| cyclin D1              | Abcam                     | ab40754     |
| PCNA                   | Abcam                     | ab92552     |

**Table S3.** List of the siRNA sequences for RNA interference

| siRNA             | sequences (5'-3')     |
|-------------------|-----------------------|
| siControl         | UUCUCCGAACGUGUCACGU   |
| siCREB            | GUCUCCACAAGUCCAAACA   |
| siATF1            | CGAACUACACCUUCAGCUATT |
| siAMPK $\alpha$ 1 | CCUCAAGCUUUUCAGGCAU   |
| si-sAC            | GAAUAUCUAGGUACAUGGA   |
| siCPS1#1          | CAAGGAATATGGTGTGAAA   |
| siCPS1#2          | GGCUUAAAGCAAGCGGAUA   |
| siCAMK2G          | CGAAAACAGGAGAUCAUUA   |

**Table S4.** List of the primer sequences for quantitative reverse-transcription polymerase chain reaction

| Gene names        | Primer sequences (5'-3') |
|-------------------|--------------------------|
| GAPDH-F           | ATGGGCAGCCGTTAGGAAAG     |
| GAPDH-R           | ATCACCCGGAGGAGAAATCG     |
| c-Jun-F           | ACTCGGACCTCCTCACCTCG     |
| c-Jun-R           | CTCCTGCTCATCTGTCACGTTCT  |
| c-Fos-F           | GTCTCCAGTGCCAACTTCATT    |
| c-Fos-R           | TCCTTTCCCTTCGGATTCTC     |
| CREB-F            | CTGCTCCCACCGTAACTCT      |
| CREB-R            | CTTCAATCCTTGGCACTCC      |
| ATF1-F            | TCCTCACAGAAAGCCACG       |
| ATF1-R            | CAGCAGCAGAAACTCCAGAAT    |
| sAC-F             | GGCACTGAAGCTCCTCAACCG    |
| sAC-R             | CCTGGAGGTGGGCTCTCTTGG    |
| CPS1-F            | TGGACGCTGTTGGCAAAGATGG   |
| CPS1-R            | TGGTTTGTGTGGGCAGCATCAG   |
| CA2-F             | TCGTGGCCTCCTTCCTGAATCC   |
| CA2-R             | GCTCGCTGCTGACGCTGATG     |
| CA9-F             | GCTGCTTCTGGTGCCTGTC      |
| CA9-R             | GGGAGCCCTCTTCTTCTGATT    |
| CA12-F            | AACTCAGACCTTTATCCTGACG   |
| CA12-R            | TCCTGGCCTTTGTACTTTACAT   |
| AMPK $\alpha$ 1-F | ACCCATATTATTTGCGTGTA     |
| AMPK $\alpha$ 1-R | CTGTGGAGTAGCAGTCCCT      |