



www.sciencemag.org/cgi/content/full/science.1215327/DC1

## Supplementary Materials for

### **The Ancient Drug Salicylate Directly Activates AMP-Activated Protein Kinase**

Simon A. Hawley, Morgan D. Fullerton, Fiona A. Ross, Jonathan D. Schertzer, Cyrille Chevtzoff, Katherine J. Walker, Mark W. Peggie, Darya Zibrova, Kevin A. Green, Kirsty J. Mustard, Bruce E. Kemp, Kei Sakamoto, Gregory R. Steinberg, D. Grahame Hardie\*

\*To whom correspondence should be addressed: E-mail: [d.g.hardie@dundee.ac.uk](mailto:d.g.hardie@dundee.ac.uk)

Published 19 April 2012 on *Science Express*  
DOI: 10.1126/science.1215327

**This PDF file includes:**

Materials and Methods  
Figs. S1 to S7  
References

## Experimental Procedures

### *Proteins, antibodies and other materials*

Rat liver AMPK was purified to the gel filtration step (5). Polycistronic plasmids for expression of the human  $\alpha1\beta1\gamma1$  and  $\alpha1\beta2\gamma1$  complexes were constructed as described (6). All DNA amplifications were carried out using KOD Hot Start DNA Polymerase (Novagen). Mutagenesis was performed using the QuickChange method (Agilent) but with KOD Hot Start DNA Polymerase. AMPK- $\alpha1$  (NCBI NP\_006242.5) was amplified from IMAGE EST 4839033, - $\beta1$  (NCBI NP\_006244.2) from IMAGE EST 2961281, - $\beta2$  (NCBI NP\_005390.1) from IMAGE EST 6091476, and - $\gamma1$  (NCBI AAH00358.1) from IMAGE EST 2964434. AMPK- $\beta1$  and - $\beta2$  were cloned into the BamH1 and Not1 sites of pcDNA5FRTTO (Life Technologies). The polycistronic plasmids were expressed in *E. coli*, purified using a HisTrap column (GE Healthcare) and eluted using an imidazole gradient. The eluted protein was dialysed into 50 mM Na HEPES pH 8.0, 150 mM NaCl, and incubated with bacterially expressed CaMKK $\beta$  in the presence of MgATP for 30 min at 30 °C. CaMKK $\beta$  and unreacted MgATP were removed by size exclusion chromatography and an in-line Glutathione-Sepharose column (GE Healthcare). Recombinant PP2C $\alpha$  was expressed and purified as described previously (7). Antibodies against AMPK- $\alpha1$  and - $\alpha2$  were as described (8). PKA was expressed and purified as described (9). Antibody against pACC were as described (10), against pAMPK were from Cell Signalling, against GAPDH and AMPK - $\gamma1$  were from Abcam, and against the FLAG epitope were from Sigma. The pan- $\beta$  antibody was a monoclonal antibody developed by immunizing mice with the bacterially expressed human AMPK ( $\alpha2\beta2\gamma1$ ) complex and selecting clones that recognized  $\beta1$  and  $\beta2$ . Salicylate, aspirin, A23817 and quercetin were from Sigma, Poole, UK. STO-609 was from Tocris Bioscience, Bristol, UK. A-769662 was synthesized as described previously (11).

### *Animal experiments*

$\beta1$ -KO mice were described previously (12); all protocols complied with the McMaster University Animal Ethics Research Board. Hepatocytes were prepared from  $\beta1$ -KO or WT mice and incubated in culture as described (13). Hepatic fatty acid oxidation was assessed by measuring the oxidation of

[<sup>14</sup>C]palmitate (Perkin Elmer) as previously described (12). Activation of AMPK *in vivo* was assessed after intraperitoneal injection of salicylate (250 mg/kg) or A-769662 (30 mg/kg) in  $\beta$ 1-KO or WT mice. These doses are comparable to those used in previous studies that have observed improvements in insulin sensitivity following chronic treatment in obese rodent models (120 mg/kg salicylate by continuous infusion (14); 30 mg/kg intraperitoneal A-769662 (15). Tissues were harvested (16) after 90 min and stored at -80°C until analysis. Analysis of respiratory exchange ratio was performed using the Oxymax Columbus Instruments Comprehensive Lab Animal Monitoring System; mice were acclimatized to the system for 24 hr prior to data collection.  $\beta$ 1-KO and WT mice were fasted overnight and allowed access to food at 07:00. After a re-feeding period, mice were injected with salicylate (250 mg/kg), A-769662 (30 mg/kg) or vehicle (saline for salicylate, 5% dimethyl sulfoxide in phosphate-buffered saline for A-769662) at 09:00. After injection, mice were denied access to food, and respiratory measurements were continued until 19:00. The same cohort was used for all treatments, with one week separating salicylate and A-769662 injections. Rates of carbohydrate ( $4.58 \cdot VCO_2 - 3.23 \cdot VO_2$ ) and fat ( $1.70 \cdot VO_2 - 1.69 \cdot VCO_2$ ) utilization were calculated as previously described (17). Analyses of *in vivo* phosphorylation and expression of AMPK and ACC, and serum non-esterified fatty acids (NEFAs), were performed as described (4).

### ***Cell culture***

HEK-293 cells stably expressing WT or R531G mutant  $\gamma$ 2 have been described (1). HEK-293 cells expressing FLAG-tagged human  $\beta$ 1 (WT or S108A mutant) or WT  $\beta$ 2 from a tetracycline-inducible promoter were made using the Flp-In™ system (Invitrogen) according to manufacturers' protocols. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% (v/v) foetal bovine serum, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin and 200  $\mu$ g/ml hygromycin B. Expression of  $\beta$ 1/ $\beta$ 2 subunit was induced, 24 hr prior to treatment, by addition of 1  $\mu$ g/ml tetracycline (Invitrogen). After various treatments as indicated in figure legends, cells were lysed using the rapid lysis procedure (2) and lysates stored at -80°C until use.

### ***Kinase and phosphatase assays***

AMPK was assayed using the *SAMS* peptide (Figs. 2A-E) or *AMARA* peptide (all other Figures) as previously described (2, 18), substrate except that assays were at 2 mM rather than 200  $\mu$ M ATP. In pilot experiments performed at the sub-physiological ATP concentration of 200  $\mu$ M, we found that although salicylate activated AMPK at concentrations of 0.3 - 3 mM, at concentrations above 3 mM it began to inhibit (not shown). The inhibitory effect could be eliminated by carrying out the assays at 2 mM ATP (Fig. 2A), suggesting that it was due to competition between salicylate and ATP at the catalytic site on AMPK. AMPK from cell lysates (60  $\mu$ g protein) was assayed in immunoprecipitates made using anti- $\alpha$ 1/ $\alpha$ 2 antibodies or EZview Red anti-FLAG M2 affinity gel (Sigma) as specified. PP2C $\alpha$  was assayed using a generic kinase peptide substrate (19) after phosphorylation using PKA. Briefly, 12  $\mu$ g of GST-[GGGGRRATV]<sub>3</sub>, bound to glutathione-Sepharose, was incubated with PKA (2 units) for 30 min at 30°C in the presence of 5 mM MgCl<sub>2</sub> and [ $\gamma$ -<sup>32</sup>P]ATP (200  $\mu$ M; 2500 cpm/pmole). After extensive washing to remove the PKA and unreacted ATP the incorporation was determined to be 2.8 moles phosphate per mole substrate. Phosphorylated GST-[GGGGRRATV]<sub>3</sub> (50 ng) was incubated with 200 ng of PP2C $\alpha$  and 5 mM MgCl<sub>2</sub> for 10 min at 30°C in the presence or absence of salicylate at the concentrations indicated (Fig. S1) and the release of <sup>32</sup>P measured.

#### ***Analysis of dephosphorylation of AMPK in cell-free assays***

Bacterially expressed human AMPK (either  $\alpha$ 1 $\beta$ 1 $\gamma$ 1,  $\alpha$ 1 $\beta$ 1[S108A] $\gamma$ 1 or  $\alpha$ 1 $\beta$ 2 $\gamma$ 1) was incubated with protein PP2C $\alpha$  (200 ng)  $\pm$  5 mM MgCl<sub>2</sub>, along with either AMP, A-769662 or salicylate as indicated. After 10 min pT172 phosphorylation was determined by Western blotting, and samples were also diluted 75-fold into AMPK assays to determine kinase activity.

#### ***SDS-PAGE and Western blotting***

SDS/PAGE was performed using precast Bis-Tris 4%–12% gradient polyacrylamide gels in the MOPS buffer system (Invitrogen). Proteins were transferred to nitrocellulose membranes (Bio-Rad) using the Xcell II Blot Module (Invitrogen). Membranes were blocked for 1 hr in Odyssey blocking buffer (Li-Cor Biosciences, Cambridge, UK). The membranes were probed with appropriate antibody (0.1–1  $\mu$ g/ml) in Odyssey blocking buffer. Detection was performed using secondary antibody (0.2  $\mu$ g/ml) coupled to IR 680 or IR 800 dye and the membranes scanned using the Li-Cor Odyssey IR imager.

Protein concentrations were determined by Coomassie Blue binding (20) with bovine serum albumin as standard.

***Other analytical procedures***

ADP/ATP ratios and cellular O<sub>2</sub> uptake were measured as described previously (1).

***Presentation of data and tests for statistical significance***

Where error bars are not visible in X-Y plots, they are smaller than the symbol used to represent the data point. Assays of effects in cultured HEK-293 cells were of replicate dishes of cells, with each value being the mean of duplicate immunoprecipitate kinase assays. Statistical significances of differences were assessed using GraphPad Prism 5 for Mac OSX, with the specific tests used being given in Figure legends.

## Legends to Supplementary Figures

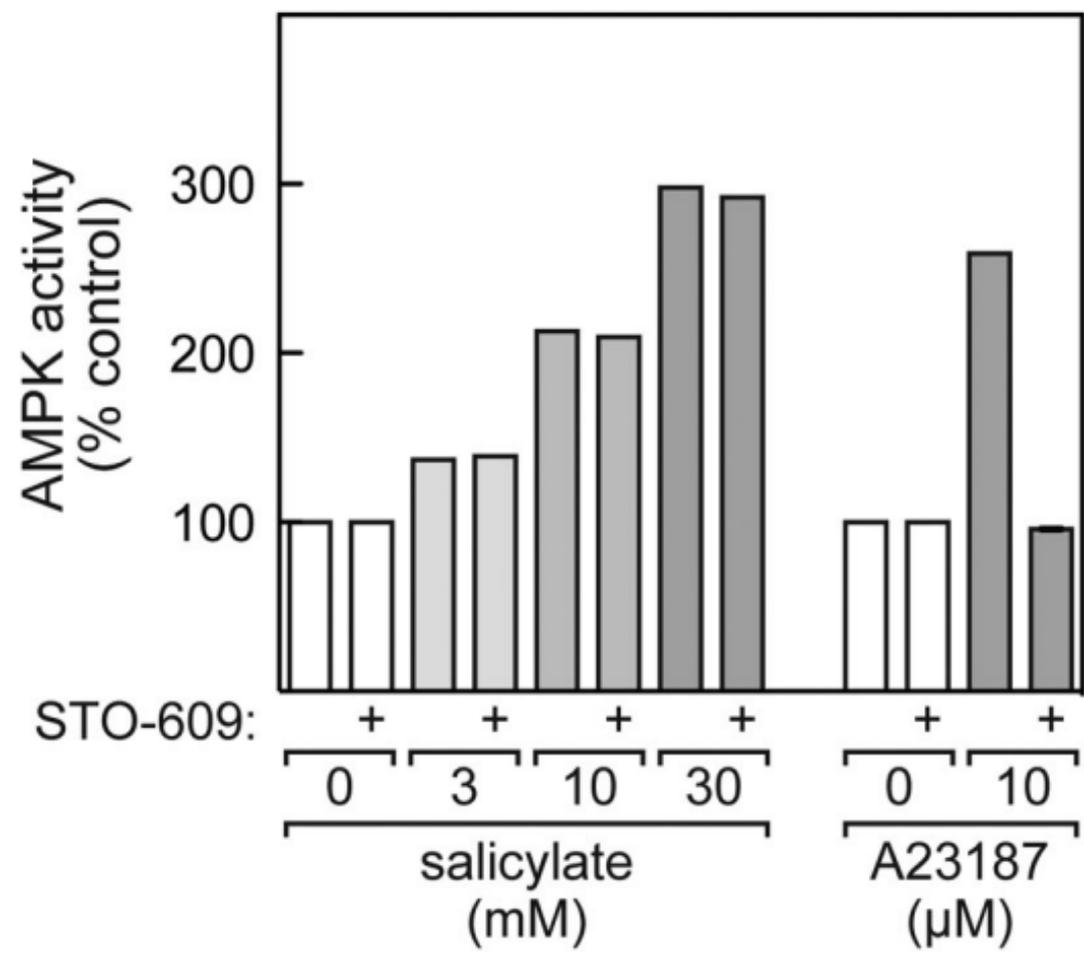
- Figure S1:** Evidence that the effect of salicylate is not mediated by the  $\text{Ca}^{2+}$ -CaMKK pathway. (A) Effect of salicylate or A23187  $\pm$  STO-609. (B) Effect of salicylate or A23187  $\pm$  STO-609 on AMPK phosphorylation. HEK-293 cells were incubated in duplicate dishes with or without 25  $\mu\text{M}$  STO-609; results in (A) show means of duplicates that agreed to within 10%.
- Figure S2:** Salicylate has no effect on the activity of PP2C $\alpha$  measured using a  $^{32}\text{P}$ -labelled peptide substrate. Results are means of duplicate assays. Pilot experiments were performed to ensure that the release of  $^{32}\text{P}$  at a fixed time was proportional to the amount of PP2C $\alpha$  added.
- Figure S3:** Salicylate and A-769662 cause increasing phosphorylation of (A) AMPK and (B) ACC in hepatocytes isolated from WT but not  $\beta 1$ -KO mice. Results are mean  $\pm$  SEM (n = 3) and are expressed as ratios of absorbance (determined by densitometry) of the pAMPK or pACC bands relative to a glyceraldehyde phosphate dehydrogenase (GAPDH) loading control. Significance of differences was determined by 2-way ANOVA with Bonferroni's test of each treatment versus control (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).
- Figure S4:** (A) Treatment of isolated hepatocytes from WT mice with salicylate up to 10 mM; and (B) treatment of WT mice with salicylate (250 mg/kg) or A-769662 (30 mg/kg), does not change adenine nucleotide ratios. (A) Hepatocytes from WT mice were incubated for 1 hr with the concentrations of salicylate shown, extracted with 0.5 M perchloric acid and analyzed by capillary electrophoresis as described previously (1). (B) Liver samples were snap frozen and ground to a powder in liquid  $\text{N}_2$ , and the powder extracted with 0.5 M perchloric acid and analyzed by capillary electrophoresis (1). Results are expressed (mean  $\pm$  SEM, n = 4 to 8 for hepatocytes, n = 6 or 7 for livers) as AMP/ATP or ADP/ATP ratios (the AMP peak was too small to reliably quantify from hepatocytes). Neither salicylate nor A-769662 caused significant changes in these ratios by 1-way ANOVA.

**Figure S5:** Treatment of mice with salicylate (250 mg/kg) *in vivo* causes activation and phosphorylation of AMPK in soleus muscle and adipose tissue. Kinase activities (top) were determined using immunoprecipitate kinase assays (2) and results expressed as mean  $\pm$  SEM (n = 6 or 7). Statistical significance of differences from controls without salicylate was assessed using two-tailed t tests: \*\*\*p<0.001. Duplicate Western blots of equal protein loadings from two animals are shown at the bottom. Note that the  $\alpha$ 1 and  $\alpha$ 2 subunit isoforms of AMPK in mice differ in mass (64 and 62 kDa respectively) and can be resolved by SDS-PAGE; soleus muscle expresses both isoforms whereas adipose tissue expresses mainly  $\alpha$ 1.

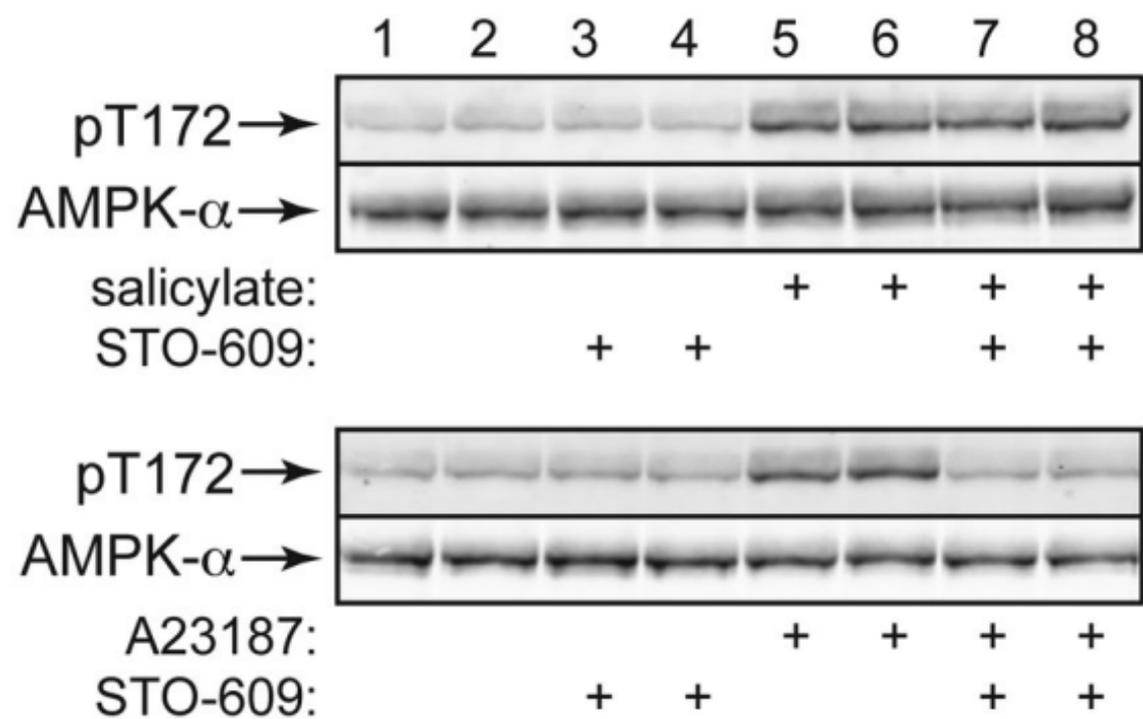
**Figure S6:** Differences in: (A/B) area under curve of respiratory exchange ratio; and (C/D) carbohydrate utilization, calculated from the experiments shown in Figs. 4C-F. Results are mean  $\pm$  SEM, n = 6 to 8; significance of differences from controls without drug were determined by 1-way ANOVA, with Bonferroni's test comparing drug treatment with control for each genotype (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

**Figure S7:** Parameters of glucose homeostasis measured in high fat-fed  $\beta$ 1-KO mice and controls. WT and  $\beta$ 1-KO mice were fed a high-fat diet starting at 7 weeks of age (45% fat by kcal, Research Diets). After 12 weeks, mice were injected daily with salicylate (250 mg/kg) or an equal volume of saline. After 12-14 days of this treatment, (A) fasting serum glucose and (B) insulin were measured, (C) glucose tolerance tests performed, and (D) homogeneous model assessment-insulin resistance (HOMA-IR) calculated as described (3, 4). Results are mean  $\pm$  SEM, n = 5 to 7; significance of differences was determined by 1-way ANOVA, with Bonferroni's test comparing drug treatment with control for each genotype (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001). After salicylate treatment, whole-body and epididymal fat pad weights were 44.2  $\pm$  1.6/45.8  $\pm$  1.7, and 2.53  $\pm$  0.16/2.78  $\pm$  0.10 (mean  $\pm$  SEM in gram, WT/ $\beta$ 1-KO mice respectively). These differences were not significant by t test.

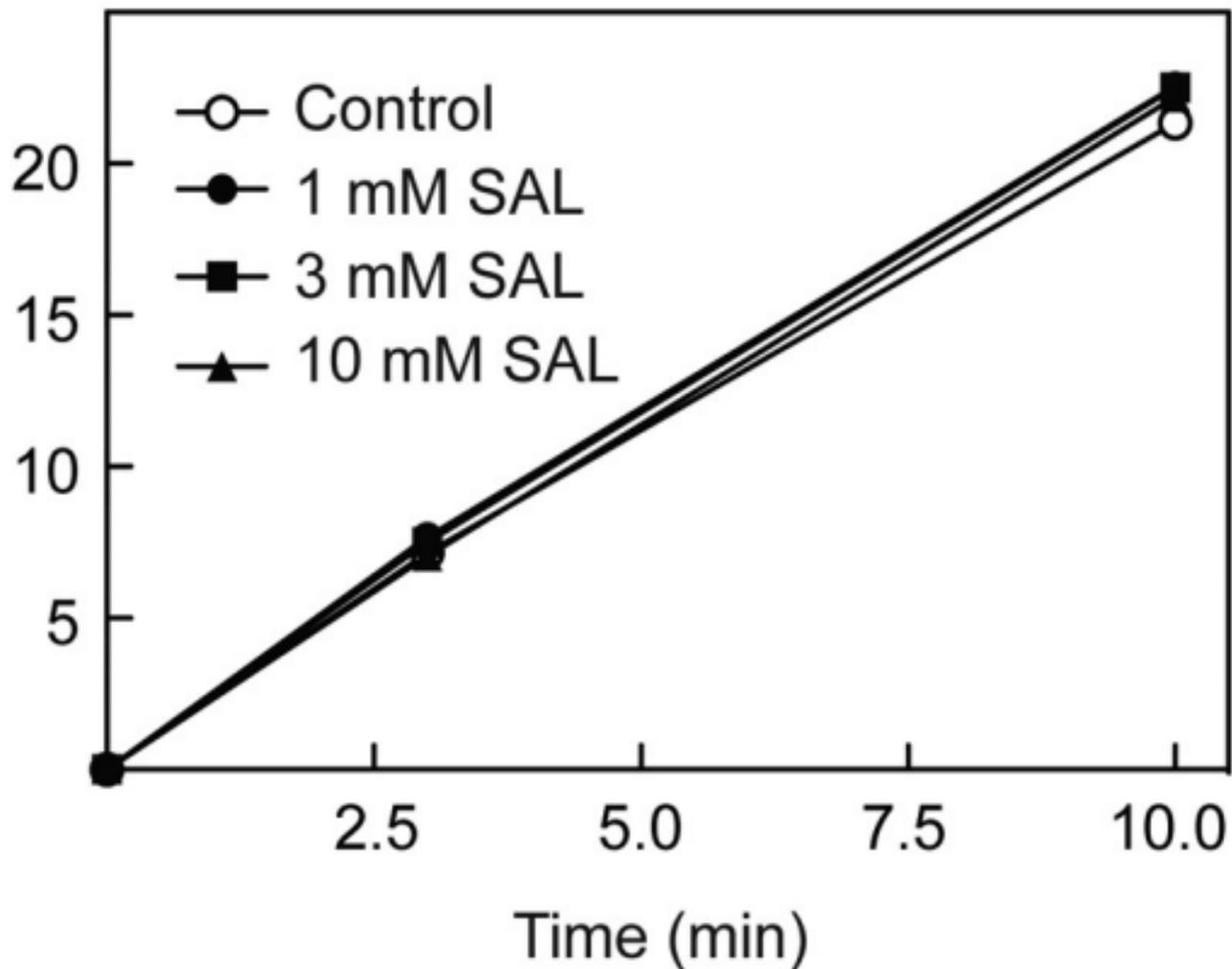
A) Effect of STO-609 on AMPK activation



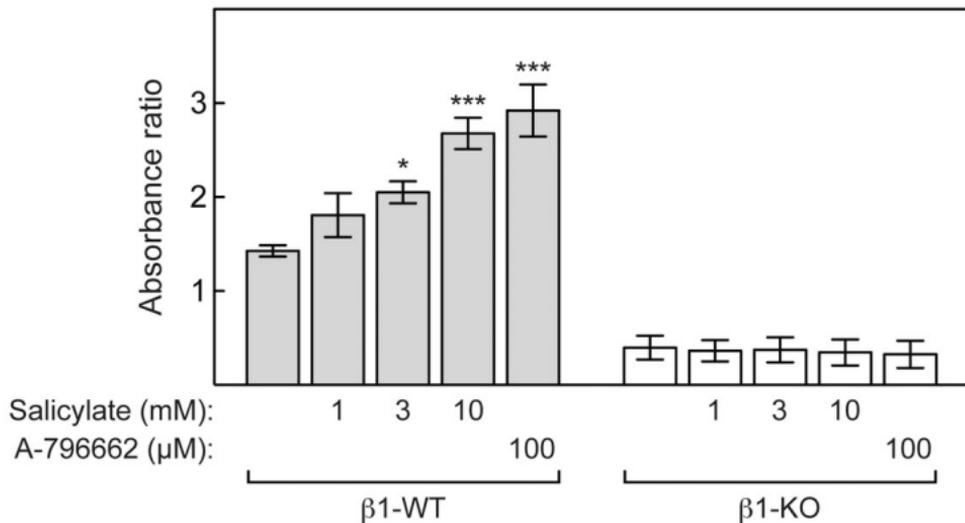
B) Effect of STO-609 on AMPK phosphorylation



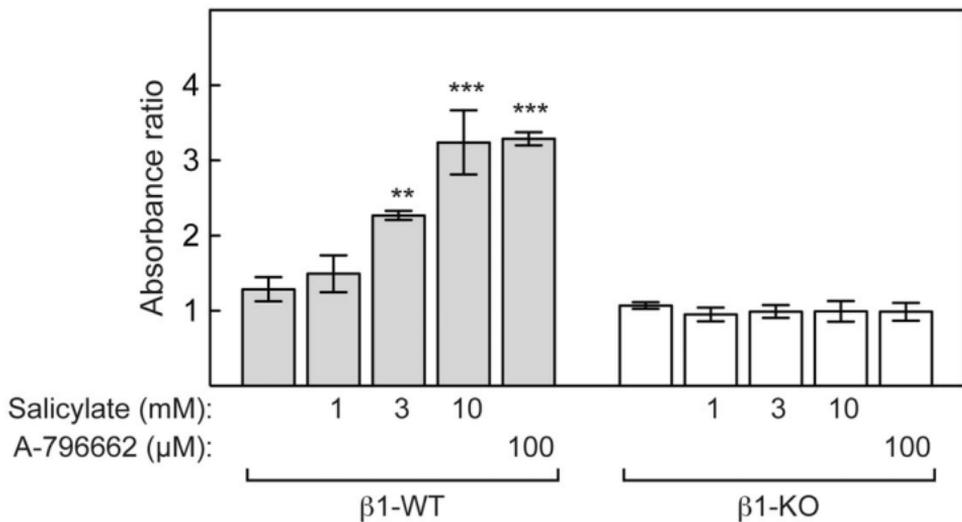
$^{32}\text{P}$  release (% total)



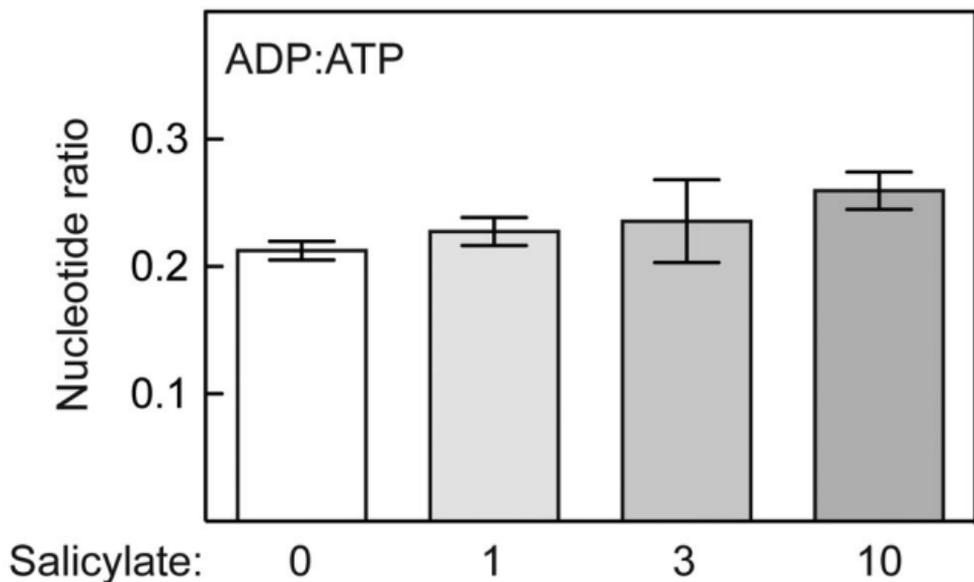
A) pAMPK:GAPDH ratio



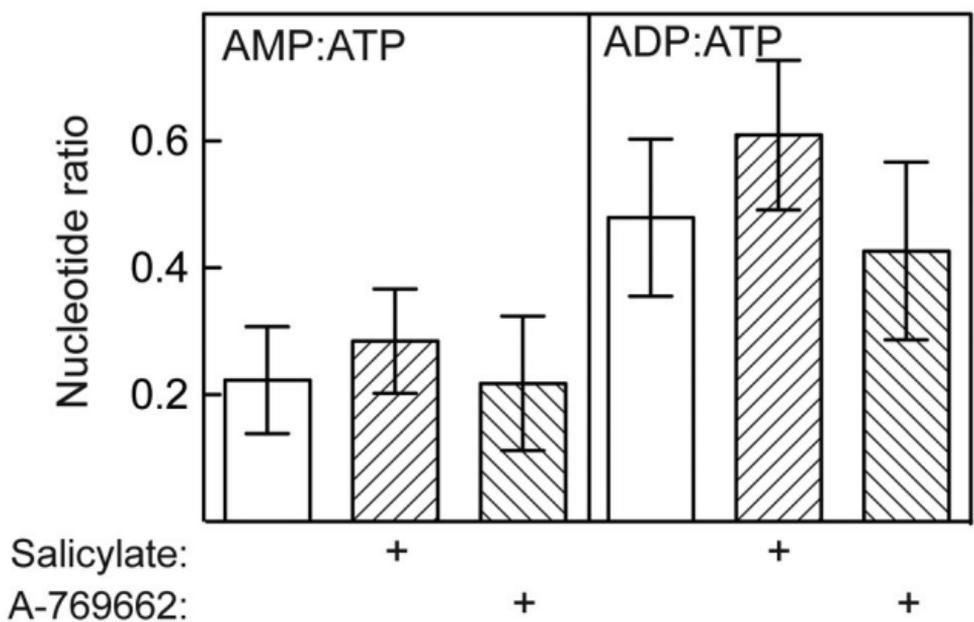
B) pACC:GAPDH ratio

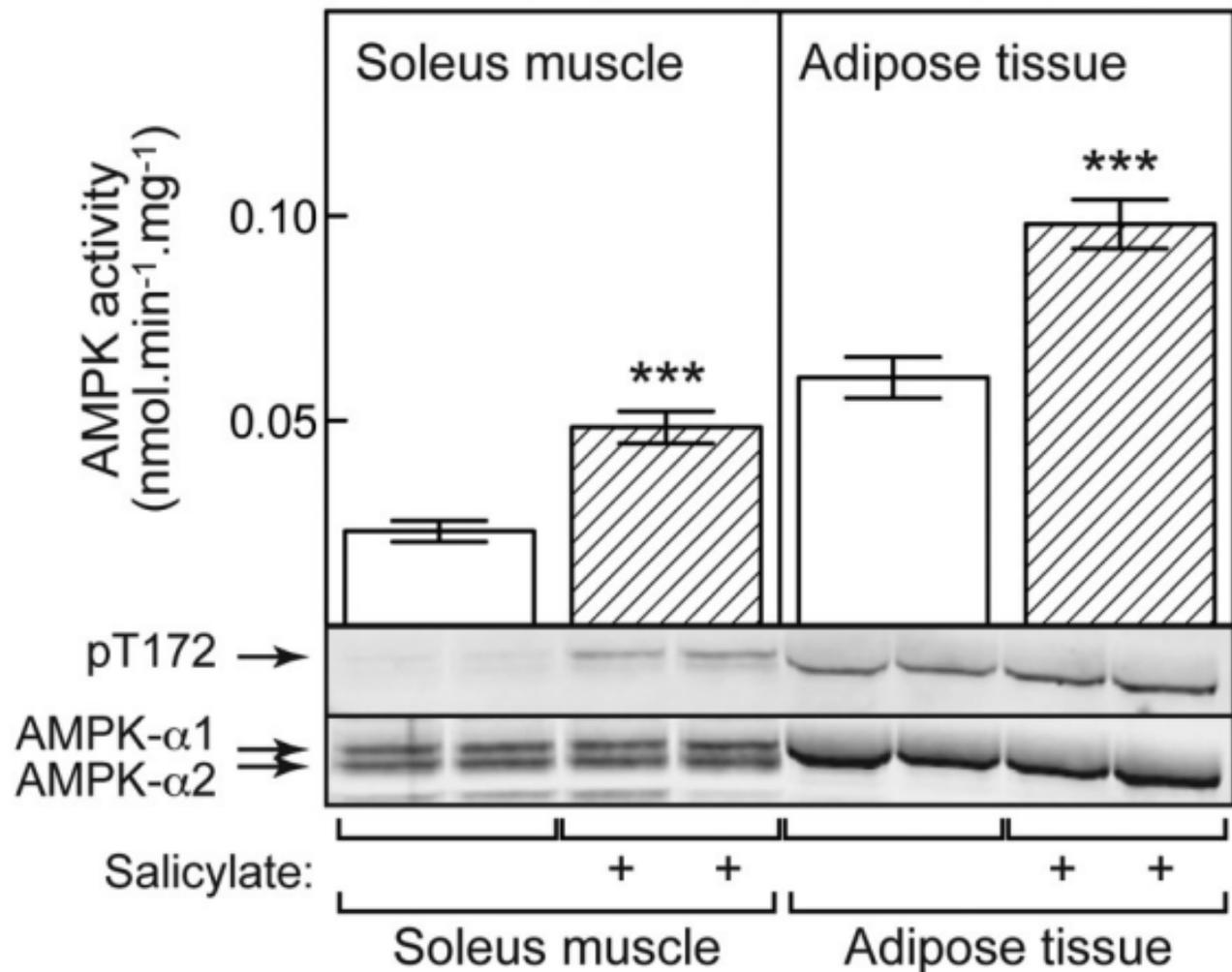


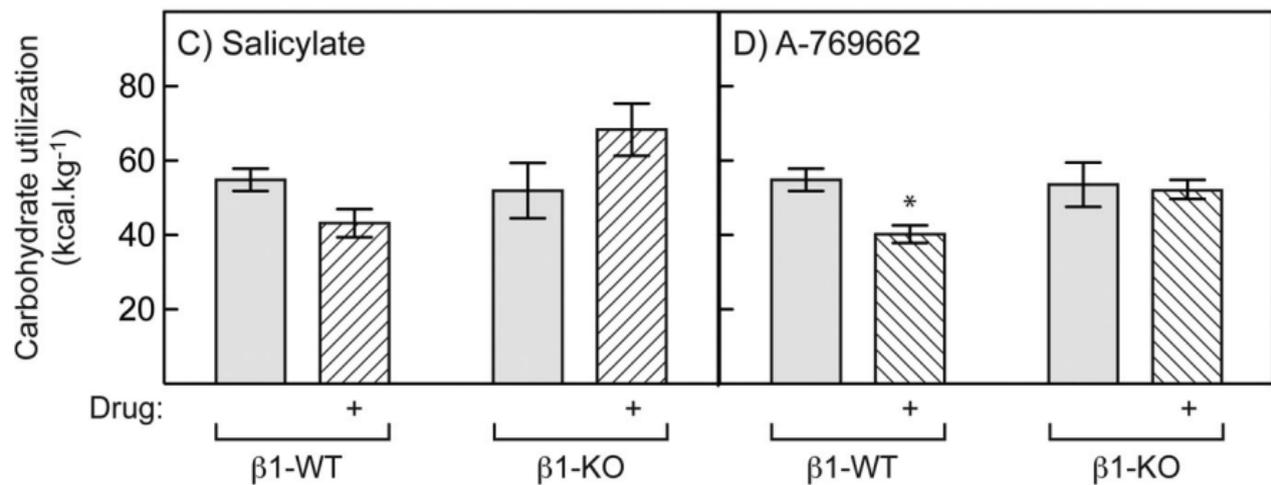
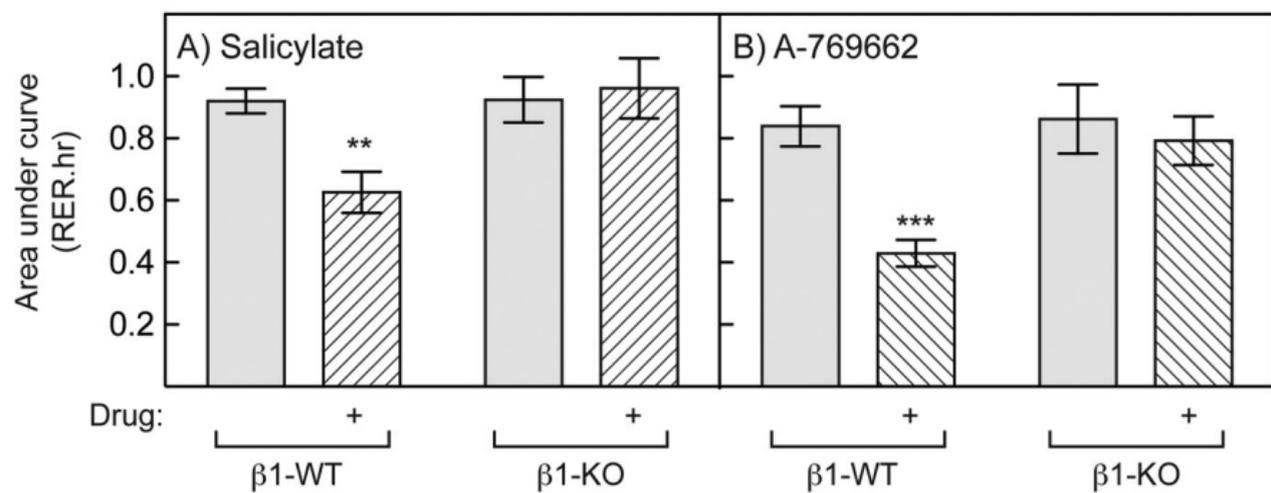
### A) Isolated $\beta$ 1-WT hepatocytes



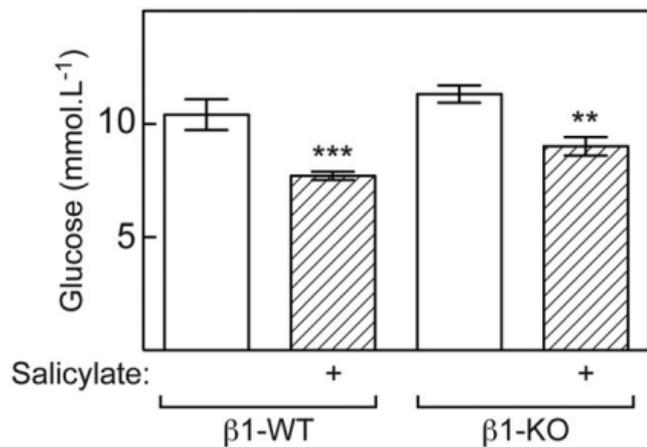
### B) Liver of $\beta$ 1-WT mice in vivo



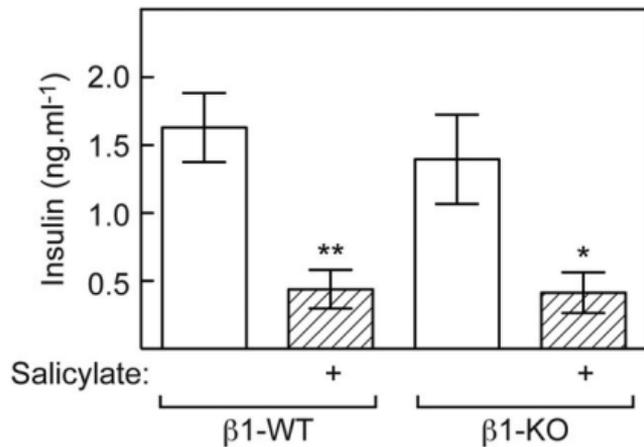




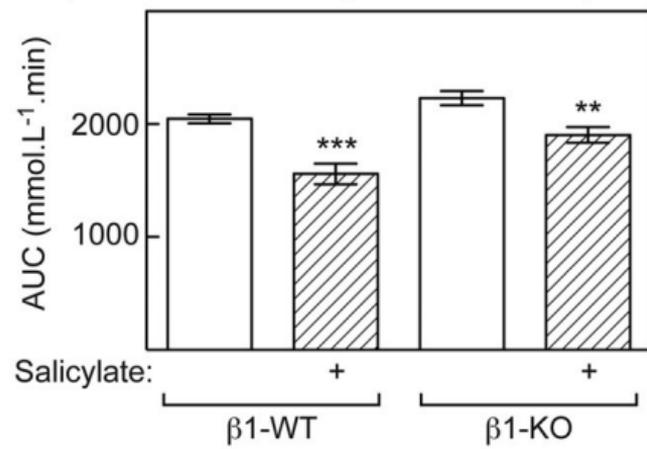
A) Fasting glucose



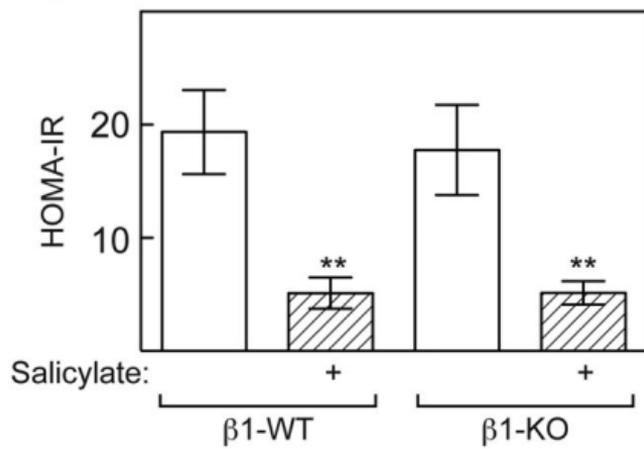
B) Fasting insulin



C) Glucose tolerance (area under curve)



D) HOMA-IR



## References

1. S. A. Hawley *et al.*, Use of cells expressing gamma subunit variants to Identify diverse mechanisms of AMPK activation. *Cell Metab.* **11**, 554 (Jun 9, 2010).
2. D. G. Hardie, I. P. Salt, S. P. Davies, Analysis of the role of the AMP-activated protein kinase in the response to cellular stress. *Methods Mol. Biol.* **99**, 63 (2000).
3. D. R. Matthews *et al.*, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**, 412 (Jul, 1985).
4. G. R. Steinberg *et al.*, Whole body deletion of AMP-activated protein kinase  $\beta$ 2 reduces muscle AMPK activity and exercise capacity. *J Biol Chem* **285**, 37198 (Nov 26, 2010).
5. S. A. Hawley *et al.*, Characterization of the AMP-activated protein kinase kinase from rat liver, and identification of threonine-172 as the major site at which it phosphorylates and activates AMP-activated protein kinase. *J. Biol. Chem.* **271**, 27879 (1996).
6. S. Tan, A modular polycistronic expression system for overexpressing protein complexes in *Escherichia coli*. *Protein. Expr. Purif.* **21**, 224 (Feb, 2001).
7. S. P. Davies, N. R. Helps, P. T. W. Cohen, D. G. Hardie, 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C $\alpha$  and native bovine protein phosphatase-2A $c$ . *FEBS Lett.* **377**, 421 (1995).
8. A. Woods, I. Salt, J. Scott, D. G. Hardie, D. Carling, The  $\alpha$ 1 and  $\alpha$ 2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity *in vitro*. *FEBS Lett.* **397**, 347 (1996).
9. S. P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95 (2000).
10. S. A. Hawley *et al.*, Complexes between the LKB1 tumor suppressor, STRAD $\alpha/\beta$  and MO25 $\alpha/\beta$  are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* **2**, 28 (2003).
11. R. R. Iyengar *et al.*, Patent number (2005).
12. N. Dzamko *et al.*, AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *J. Biol. Chem.* **285**, 115 (Jan 1, 2010).

13. M. D. Fullerton, F. Hakimuddin, A. Bonen, M. Bakovic, The development of a metabolic disease phenotype in CTP:phosphoethanolamine cytidylyltransferase-deficient mice. *J. Biol. Chem.* **284**, 25704 (Sep 18, 2009).
14. M. Yuan *et al.*, Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* **293**, 1673 (Aug 31, 2001).
15. B. Cool *et al.*, Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* **3**, 403 (Jun, 2006).
16. S. P. Davies, D. Carling, M. R. Munday, D. G. Hardie, Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. *Eur. J. Biochem.* **203**, 615 (1992).
17. S. Couture, D. Massicotte, C. Lavoie, C. Hillaire-Marcel, F. Peronnet, Oral [(13)C]glucose and endogenous energy substrate oxidation during prolonged treadmill running. *J. Appl. Physiol.* **92**, 1255 (Mar, 2002).
18. S. P. Davies, D. Carling, D. G. Hardie, Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.* **186**, 123 (1989).
19. P. T. W. Cohen, World Intellectual Property Organization, Patent number WO 2008/0653897 A2 (2008).
20. M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248 (1976).