SUPPLEMENTAL INFORMATION

Chemical genetic screen for AMPKα2 substrates uncovers a network of proteins involved in mitosis and cytokinesis

Max R. Banko, Jasmina J. Allen, Bethany E. Schaffer, Erik W. Wilker, Peiling Tsou, Jamie L. White, Judit Villén, Beatrice Wang, Sara R. Kim, Kei Sakamoto, Steven P. Gygi, Lewis C. Cantley, Michael B. Yaffe, Kevan M. Shokat, and Anne Brunet

EXTENDED DISCUSSION

Chemical genetics approach to identify substrates of a protein kinase in cells

The chemical genetic approach performed in cells represents a powerful strategy for identifying direct substrates of a kinase of interest in vivo. Prior to this study, identification of direct substrates by chemical genetics was mostly done in vitro (Polson et al., 2001; Ubersax et al., 2003). Using a different approach, a previous study identified a number of potential AMPK substrates by incubating murine brain extracts with purified AMPK in vitro and using tandem mass spectrometry to identify the phosphorylated peptides (Tuerk et al., 2007). These screens, while important, were performed in vitro using cell lysates, limiting their ability to identify bona fide direct substrates of a protein kinase within cells. In addition, some of the substrates may have been phosphorylated by other kinases that were themselves phosphorylated and activated by the protein kinase of interest. A cell-based method was also previously used with mouse fibroblasts in which the analog-specific form of the ERK2 protein kinase was expressed from the endogenous locus (Allen et al., 2007). This approach allowed the labeling of known direct substrates of ERK2 within cells (B.W. and K.M.S., unpublished data). However, only one substrate of ERK2 was identified using this approach, probably due to limiting levels of expression of the analog-specific kinase. A related approach was recently developed to identify in vivo substrates of a protein kinase. In this approach, an analog-sensitive version of Cdk1 in *S. cerevisiae* was inhibited by selective pyrimidine-based inhibitors and quantitative

2

mass spectrometry was performed (Holt et al., 2009). However, this approach does not allow the identification of direct substrates of a protein kinase. Thus, the method we present in this study provides a rapid and physiological means of identifying the direct in vivo substrates of a protein kinase in cells, and could be applied to other kinases.

It is possible that the mutation to generate an analog-specific kinase alters the substrate specificity of the kinase. While it is difficult to completely rule out that AS-AMPKα2 has different substrate specificity than WT-AMPKα2, this possibility is unlikely for several reasons. First, analog-specific mutations in Src do not dramatically alter the specificity constants of the protein kinase (Liu et al., 2000). Furthermore, the labeling pattern of lysates from cells expressing WT ZAP-70 and AS-ZAP-70 is similar (Levin et al., 2008), suggesting that the substrates are not dramatically changed by the generation of the analog-specific kinase. Finally, in the case of AS-AMPKα2, we confirmed the phosphorylation of two of the identified substrates in vivo by independent means, thereby corroborating that specificity of the AS-AMPKα2 is not drastically altered.

Phosphorylation of AMPK substrates by other AMPK family members
Some of the substrates identified in our screen may be targeted not only by AMPK, but also by other AMPK family members (Alessi et al., 2006). For example, PPP1R12A, which we identified in the screen as being phosphorylated by AMPK, has recently been shown to be phosphorylated by the AMPK-related

3

kinase NUAK1 in response to cell detachment (Zagorska et al., 2010). However, the sites phosphorylated by NUAK1 on PPP1R12A (Zagorska et al., 2010) were not phosphorylated by AMPK (data not shown), suggesting that AMPK family members may have overlapping substrates, but may phosphorylate different sites in these substrates, perhaps to coordinate their regulation in response to different stimuli.

Role of AMPK in mitosis in mammalian cells

While our results point to a role of AMPK in mitotic cells, more work will still be needed to definitively address the role of AMPK in mitosis. Compound C (also known as Dorsomorphin) is not a highly specific inhibitor of AMPK. Compound C specificity has been tested in vitro on a panel of 119 protein kinases (Vogt et al., 2011) or 72 protein kinases (Bain et al., 2007), and it inhibits other kinases in addition to AMPK, including MELK, PHK, DYRK1, ERK8, and MINK1. Nevertheless, most kinases inhibited by compound C (apart from other AMPK family members, such as MELK) have consensus phosphorylation motifs that are very different from the AMPK consensus phosphorylation motif. Thus, these other kinases would not be able to phosphorylate S452 of PPP1R12C. Together with A769662 and the cell lines expressing S452A PPP1R12C, the compound C experiments help support a role for AMPK in mitosis.

SV40-transformed mouse embryonic fibroblasts (MEFs) from the AMPKα1/α2 double knock-out mice (Laderoute et al., 2006) do not display major growth

4

defects (M.R.B and A.B, data not shown), indicating that AMPK is unlikely to be absolutely necessary for mitosis in these cells. However, this could also be due to the capacity of cells chronically lacking AMPKα1/α2 to compensate for this defect by upregulating other kinases (probably of the large AMPK family). More generally, using a loss of function approach for testing the importance of a protein in mitosis is not trivial for several reasons. First, depletion of some proteins (e.g. abundant proteins, enzymes, etc.) by RNAi is difficult whereas chemical inhibition is more straightforward because it is based on the activity of the protein. For example, Myosin II, cannot be easily depleted by RNAi, but it can be easily inhibited by a chemical inhibitor, Blebbistatin, which targets it ATPase activity, thereby allowing the identification of its role in cytokinesis (Straight et al., 2003). Second, many of the mitotic kinases act at multiple points in the cell cycle (e.g., PLK or CDK), so RNAi or knock-out lead to very pleiotropic effects, which render the interpretation of the protein's role at a specific stage of the cell cycle more difficult. Third, RNAi may even cause different phenotypes from chemicals because some of the mitotic kinases (e.g. Aurora kinase) also play structural roles (Weiss et al., 2007). Thus, chemical activators and inhibitors often provide a better "scalpel" to study the importance of a protein kinase activity in a process as precisely controlled as mitosis because they can be added at the desired point of the cell cycle.

EXTENDED EXPERIMENTAL PROCEDURES

Antibodies

The phospho-specific antibody to S452 of PPP1R12C (P-S452 PPP1R12C) was generated by injecting the phosphopeptide LQRSA(pS)SSWLEG-C coupled to KLH into rabbits and purified by using a phospho-peptide affinity column (Abmart). The antibodies to the M2 and T7 epitope tags were obtained from Sigma-Aldrich and Novus Biologicals respectively. The β-actin antibody was obtained from Novus Biologicals Inc. The HA 12CA5 antibody was obtained from Roche. The antibodies to AMPKα1 and AMPKα2 were obtained from Bethyl Laboratories, Inc. The antibodies to P-S19 MRLC, P-T18/S19 MRLC, total MRLC, P-S79 ACC1, total ACC1, P-S20 PAK2, total PAK2, and GST were obtained from Cell Signaling Technology. The DM1A tubulin antibodywas obtained from Abcam. The thioP antibody has been previously described (Allen et al., 2005; Allen et al., 2007). The antibody to immunoprecipitate endogenous FOXO3 has been previously described (Greer et al., 2007).

Constructs

The constructs encoding M2-AMPKα2, M2-14-3-3ζ, GST-FOXO3, and M2-FOXO3 (WT and mutants at the AMPK phosphorylation sites) have been previously described (Brunet et al., 1999; Brunet et al., 2004; Greer et al., 2007). The construct encoding M2-SIRT2 was obtained from Eric Verdin (North et al., 2003). The plasmids encoding M2-CRTC2 (Conkright et al., 2003) and M2-TSC2 (Manning et al., 2002) were generous gifts from M. Montminy and L. Cantley,

respectively. The construct encoding the AMPKAR reporter for AMPK activity has been previously described (Tsou et al., 2011).

The constructs encoding HA-AMPKα2, HA-AMPKβ1, and HA-AMPKγ1 were generated by subcloning the respective human cDNAs (Origene) into the pECE mammalian expression vector in frame with the M2 or HA-epitope tag. The constructs encoding M2-PPP1R12A, M2-PPP1R12C, T7-PPP1R12C, M2-PAK2, T7-PAK2, M2-BAIAP2, M2-CDC27, M2-CARM1, M2-PP1Cβ, M2-MRLC were generated by cloning the respective cDNAs into the pECE mammalian expression vector, in frame with the M2(Flag)- or T7-epitope tag. The constructs encoding GST-PPP1R12A, GST-PPP1R12C, GST-PAK2, GST-BAIAP2, GST-CDC27, GST-CARM1 were generated by cloning the respective cDNAs into pGEX-4T3 (Promega) in frame with GST. CARM1 human cDNA was a generous gift from Mark Bedford (Cheng et al., 2007). All the other human cDNAs were obtained from the Orfeome library of cDNA (Open Biosystems). The 5' end of PPP1R12C cDNA was generated de novo by Genscript and was cloned in frame with the 3' end of PPP1R12C (Orfeome library, Open Biosystems) to reconstitute the full-length cDNA. WT and AS-HA-AMPKα2 were also subcloned into the retroviral vector pQXCIP (Clontech).

HA-AMPKα2 M93G mutant (AS-AMPKα2), PPP1R12C S344A and S452A mutants, BAIAP2 S263A, S329A, and S366A mutants, CDC27 S222A, S379A, and T409A mutants, PAK2 S20A, S36A, S55A, S141A, S197A, T238A, and

S507A mutants were generated by site-directed mutagenesis. The presence of

the mutation was verified by sequencing and the entire region was sequenced to

verify that other mutations were not introduced by PCR. Lentivirus constructs

expressing M2-PPP1R12C WT and S452A mutant and M2-PAK2 WT and S20A

mutant were generated using the lentiviral vector FUW (Lois et al., 2002). shRNA

constructs to PPP1R12C and AMPKα1/α2 were generated by cloning the hairpin

shRNAs targeting the gene of interest into the PSR vector (Ventura et al., 2004).

The region of PPP1R12C targeted by the PPP1R12C shRNA hairpin is in the 5'

end of PPP1R12C that was optimized by Genscript, thereby changing the

nucleotide sequence from that of endogenous PPP1R12C, and allowing

overexpression of either WT- or S452A-PPP1R12C in the context of endogenous

PPP1R12C knock-down. siRNAs to AMPKα1, AMPKα2, PPP1R12C, and

negative control siRNAs were ON-TARGET*plus* siRNAs containing a mixture of 4

siRNAs directed against the target gene (Dharmacon RNAi Technologies).

Primers used for cloning and site directed mutagenesis

Primers for cloning genes into pECE:

M2/T7-PPP1R12C forward:

ATAGAATTCGGAGGGGCTGCTGAAGGCGGAGATCG

M2/T7-PPP1R12C reverse:

ATATCTAGATCACTTGGAGAGTTTGCTGATGACGCG

M2/T7-PAK2 forward:

ATACCCGGGAATTCGTCTGATAACGGAGAACTGGAAGATAAGC

M2/T7-PAK2 reverse: ATATCTAGATTAACGGTTACTCTTCATTGCTTCTTTAGC

M2-PPP1R12A forward:

ATAGAATTCGCAGATGGCGGACGCGAAGCAGAAGCGGAACGAGC

M2-PPP1R12A reverse:

ATAGCTAGCTTATTTGGAAAGTTTGCTTATAACTCTGATCAAGG

M2-BAIAP2 forward: ATAGAATTCGTCTCTGTCTCGCTCAGAGGAGATGC

M2-BAIAP2 reverse: ATATCTAGATTACCTGCTCATGGACCGCGCTCC

M2-CDC27 forward: ATAGAATTCGACGGTGCTGCAGGAACCCGTCC

M2-CDC27 reverse: ATATCTAGATTAAAATTCATCACTTTCAGCTGC

M2-CARM1 forward: ATAGAATTCGGCAGCGGCGGCGGCGGCGGTGGGGC

M2-CARM1 reverse:

ATATCTAGATTAGCTCCCGTAGTGCATGGTGTTGGTCGG

M2-PP1Cβ forward: ATAGAATTCGACAATGGATAAAAGTGAGCTGG

M2-PP1Cβ reverse: ATATCTAGATTAGTTCTCTCCCTCCCCAGCGTCTCC

M2-MRLC forward: ATAGAATTCCAGCAAGCGGGCCAAAGCCAAGACC

M2-MRLC reverse: ATATCTAGATTAGTCGTCTTTATCCTTGGCGCC

Primers for cloning into FUW:

FUW-M2-PPP1R12C forward:

ATAGAATTCGGCCACCATGTCTGGTGAGGATGGACCAGCTGC

FUW-M2-PPP1R12C reverse:

ATAGAATTCTCTAGATTACTGGGAGCCGGAGTGGCGGGC

FUW-M2-PAK2 reverse:

ATAATAGGCGCGCCTTAACGGTTACTCTTCATTGCTTCTTTAGC

Primers for cloning shRNAs into PSR:

AMPKα1/α2 shRNA forward:

TGATGTCAGATGGTGAATTTTTCAAGAGAAAATTCACCATCTGACATCTT

TTTTC

AMPKα1/α2 shRNA reverse:

TCGAGAAAAAAGATGTCAGATGGTGAATTTTCTCTTGAAAAATTCACCATCT

GACATCA

PPP1R12C shRNA forward:

TGAATTGCTCCTTCATGACATTCAAGAGATGTCATGAAGGAGCAATTCTTTTT

TC

PPP1R12C shRNA reverse:

TCGAGAAAAAAGAATTGCTCCTTCATGACATCTCTTGAATGTCATGAAGGAG

CAATTCA

Primers for cloning into pGEX-4T3:

GST-PPP1R12C forward: ATTCCAGAAGTAGTGAGGAGGC

GST-PPP1R12C reverse:

ATACGGCCGTTACTTGGAGAGTTTGCTGATGACGCGG

GST-PAK2 forward: ATAGGATCCTCTGATAACGGAGAACTGGAAGATAAGC

GST-PAK2 reverse: GTTCAGGGGGAGGTGTGGGAGG

GST-PPP1R12A forward: ATTCCAGAAGTAGTGAGGAGGC

GST-PPP1R12A reverse:

ATACGGCCGTTATTTGGAAAGTTTGCTTATAACTCTG

GST-MRLC forward: ATAGGATCCTCCAGCAAGCGGGCCAAAGCCAAGACC

GST-MRLC reverse: ATATCTAGATTAGTCGTCTTTATCCTTGGCGCC

GST-BAIAP2, GST-CDC27, GST-CARM1 were cloned into pGEX-4T3 using the same primers as described above for cloning the M2-tagged versions of these constructs.

Primers for site-directed mutagenesis:

HA-AMPKα2 M93G forward:

CCAACAGATTTTTTTATGGTAGGGGAATATGTGTCTGGAGG

HA-AMPKα2 M93G reverse:

CCTCCAGACACATATTCCCCTACCATAAAAAAATCTGTTGG

M2-PPP1R12C S344A forward:

AGCAGCAAACACAGAAGGAGCGCTGTGTGTCGTCTGAGCAGTCGC

M2-PPP1R12C S344A reverse:

GCGACTGCTCAGACGACACACAGCGCTCCTTCTGTGTTTGCTGCT

M2-PPP1R12C S452A forward:

GCTGGGCTGCAGCGCTCGGCTGCCTCCTCCTGGCTGGAAGGGACC

M2-PPP1R12C S452A reverse:

GGTCCCTTCCAGCCAGGAGGAGGCAGCCGAGCGCTGCAGCCCAGC

M2-PAK2 S20A forward:

GCACCTCCTGTGCGAATGAGCGCCACCATCTTTAGCACTGGAGGC

M2-PAK2 S20A reverse:

GCCTCCAGTGCTAAAGATGGTGGCGCTCATTCGCACAGGAGGTGC

M2-PAK2 S36A forward:

GACCCTTTGTCAGCCAATCACGCTTTGAAACCTTTGCCCTCTGTT

M2-PAK2 S36A reverse:

AACAGAGGGCAAAGGTTTCAAAGCGTGATTGGCTGACAAAGGGTC

M2-PAK2 S55A forward:

AAGCCCAGGCATAAAATCATCGCCATATTCTCAGGCACAGAGAAA

M2-PAK2 S55A reverse:

TTTCTCTGTGCCTGAGAATATGGCGATGATTTTATGCCTGGGCTT

M2-PAK2 S141A forward:

ACAGTGAAGCAGAAATATCTGGCCTTTACTCCTCCTGAGAAAGAT

M2-PAK2 S141A reverse:

ATCTTTCTCAGGAGGAGTAAAGGCCAGATATTTCTGCTTCACTGT

M2-PAK2 S197A forward:

ACGAAATCAATTTACACACGGGCTGTAATTGACCCTGTTCCTGCA

M2-PAK2 S197A reverse:

TGCAGGAACAGGGTCAATTACAGCCCGTGTGTAAATTGATTTCGT

M2-PAK2 T238A forward:

GAGATTATGGAGAAATTAAGAGCTATCGTGAGCATAGGTGACCCT

M2-PAK2 T238A reverse:

AGGGTCACCTATGCTCACGATAGCTCTTAATTTCTCCATAATCTC

M2-PAK2 S507A forward:

CTGAAACTGGCCAAACCGTTAGCTAGCTTGACACCACTGATCATG

M2-PAK2 S507A reverse:

CATGATCAGTGGTGTCAAGCTAGCTAACGGTTTGGCCAGTTTCAG

M2-BAIAP2 S263A forward:

AGCGCCCTGTCGGCCTCCAAGGCCAACCTGGTCATTTCCGACCCC

M2-BAIAP2 S263A reverse:

GGGGTCGGAAATGACCAGGTTGGCCTTGGAGGCCGACAGGGCGCT

M2-BAIAP2 S329A forward:

AAATCCCTGTCTCCTCCGCAGGCTCAGAGCAAGCTCAGCGACTCC

M2-BAIAP2 S329A reverse:

GGAGTCGCTGAGCTTGCTCTGAGCCTGCGGAGGAGACAGGGATTT

M2-BAIAP2 S366A forward:

AAGACTCTGCCTCGCTCGAGCGCCATGGCAGCCGGCCTGGAGCGC

M2-BAIAP2 S366A reverse:

GCGCTCCAGGCCGGCTGCCATGGCGCTCGAGCGAGGCAGAGTCTT

M2-CDC27 S222A forward:

TTGAATTTAGAATCTTCCAATGCAAAGTACTCCTTGAATACAGAT

M2-CDC27 S222A reverse:

ATCTGTATTCAAGGAGTACTTTGCATTGGAAGATTCTAAATTCAA

M2-CDC27 S379A forward:

AACGCACTGCCTCGAAGAAGTGCACGACTCTTTACTAGTGACAGC

M2-CDC27 S379A reverse:

GCTGTCACTAGTAAAGAGTCGTGCACTTCTTCGAGGCAGTGCGTT

M2-CDC27 T409A forward:

CCTAAAATCCCAAACAGAAAAGCAAAAGTAAAACTAATAAAGGA

M2-CDC27 T409A reverse:

TCCTTTATTAGTTTTACTTTTTGCTTTTCTGTTTGGGATTTTAGG

Cell culture

The HEK293T (293T) human epithelial kidney and U2OS human osteosarcoma
cell lines were cultured in DMEM and McCoy's 5a Medium respectively
supplemented with 10% fetal bovine serum (FBS, Gibco) and 1%
Penicillin/Streptomycin/Glutamine (P/S/Q, Invitrogen) at 37°C in 5% CO_2 and
95% humidity.

Generation of stable cell lines

293T cells were seeded at 3.5 x 10^6 cells per 10 cm plate and transfected with 10
μg of the shRNA PSR constructs together with 5 μg of the VSVg and 5 μg of the
Δ8.2 helper plasmids (Ventura et al., 2004) using a calcium phosphate
procedure. The medium was changed 12 hours after transfection. U2OS cells (1
x 10^6 cells/10 cm plate) were infected by 0.45 μm-filtered supernatent from virus-
producing cells in the presence of 8 μg/ml polybrene (Sigma). The cells were
infected 2 times over 24 hours. After the second infection, the cells were treated
with 5 μg/ml of puromycin (Sigma) and maintained in puromycin-containing

medium. U2OS cells ectopically expressing M2-WT- or S452A-PPP1R12C were generated as above, with the exception of the selection with puromycin. Immunocytochemistry with an antibody to the M2 tag was performed to ensure that the cell lines were stably expressing M2-PPP1R12C (WT or S452A) in most cells.

To generate cell lines stably expressing the pQXCIP-WT- or AS-AMPKα2 retrovirus, Amphopack 293 cells (Clontech) were seeded at 3.5 x 10^6 cells per 0.2% gelatin-coated 10 cm plate and trasfected with 20 μg of the retroviral vector using a calcium phosphate procedure. The medium was changed 12 hours after transfection. 293T cells (2 x 10^6 cells/10 cm plate) were infected by 0.45 μm-filtered supernatent from virus-producing cells in the presence of 8 µg/ml polybrene (Sigma). The cells were infected 2 times over 24 hours. After the second infection, the cells were treated with 5 μg/ml of puromycin (Sigma) and maintained in puromycin-containing medium.

Stimulation of AMPK in mammalian cells

To examine the phosphorylation of ectopically expressed PPP1R12C, PAK2, and MRLC, 293T cells were seeded at 4 x 10^5 cells/35 mm dish and were transfected with 2.5 μg of each of the indicated constructs using the calcium phosphate method. Forty-eight hours after transfection, cell were stimulated and lysed. To examine the phosphorylation of endogenous PPP1R12C, PAK2, and MRLC, 293T cells were seeded at 3 x 10^7 cells/10 cm plate and U2OS were seeded at 2

x 10^7 cells/10 cm plate. Cells were stimulated and lysed two or three days after seeding. To activate endogenous AMPK, cells were incubated in serum-free medium for two hours and were then incubated with 100 mM 2DG (Sigma, 100 mM) for 5 min or with 100 μM A-769662 (generously provided by K. Sakamoto) for the final hour of serum starvation. For nutrient deprivation, cells were plated at 2 x 10^6 cells/10 cm plate in McCoy's 5a supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% glutamine (Invitrogen). After three days, cells were incubated in PBS with $CaCl_2$ and $MgCl_2$ (Gibco) for the indicated times before lysing in SDS lysis buffer (2% SDS, 50 mM Tris pH 6.8, 50 mM NaF, 40 mM β-glycerolphosphate, 2 mM Na_3VO_4, 1 mM PMSF, 1% aprotinin, and 1 μM microcystin LR). For glucose starvation, cells were plated at 1.2 x 10^6 cells/10 cm plate in DMEM containing 10% FBS and 1% PSQ. Two days after plating, cells were washed twice with PBS and incubated in glucose-free DMEM containing 1% glutamine and 10% dialyzed FBS for the indicated times. Cells were then lysed in SDS lysis buffer.

To inhibit endogenous AMPK activity, compound C (Calbiochem, 40 μM) was added for one hour. Cells were then lysed and processed for western blot or immunoprecipitation experiments as described above.

Western blots and immunoprecipitation

Cellular extracts were obtained by lysing the cells in lysis buffer (50 mM Tris HCl [pH 8.0], 100 mM NaCl, 2 mM EGTA, 10 mM NaF, 40 mM β-glycerophosphate,

0.4% Triton X-100, 1 mM PMSF, and 12 μg/ml aprotinin,) in the presence of 1

μM microcystin LR (Elimbio). After centrifugation to remove cellular debris, SDS

sample buffer (0.0945 M Tris-HCl [pH 6.8], 9.43% glycerol, 2.36% w/v SDS, and

5% β-mercaptomethanol) was added. For immunoprecipitation, protein extracts

were incubated with agarose beads coupled to M2 antibody (Sigma).

Immunoprecipitated proteins were released from the beads by a 1-hour

incubation with Flag peptide (Sigma, 0.5 mg/ml 1:1 bead volume), and SDS

sample buffer was added. Samples were resolved on SDS-PAGE gels (10%)

and transferred to nitrocellulose membranes. The membranes were incubated

with the indicated primary antibodies and the primary antibody was visualized

using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and

enhanced chemiluminescence (ECL, Amersham).

In vivo phosphorylation and alkylation of candidate substrates

293T cells were seeded at 4 x 10^5 cells/35 mm dish and were transfected with

1.25 μg of HA-WT- or AS-AMPKα2, 1.25 μg HA-AMPKβ1, 1.25 μg HA-AMPKγ1,

and 1.25 μg of the indicated candidate substrate using the calcium phosphate

method. Forty-eight hours after transfection, the cells were washed twice with

serum-free DMEM and incubated for 2 hours prior to stimulation with 100 mM 2-

deoxyglucose (2DG, Sigma) for 5 min. Following stimulation, 200 μl of

phosphorylation buffer (20 mM HEPES [pH 7.3], 100 mM KOAc, 5 mM NaOAc, 2

mM MgOAc$_2$, 1 mM EGTA, 10 mM MgCl$_2$, 0.5 mM DTT, 5mM creatine phosphate

(Calbiochem), 57 μg/ml creatine kinase (Calbiochem), 30 μg/ml digitonin, 5 mM

17

GTP, 0.1 mM ATP, 0.1 mM N^6-(phenethyl) ATPγS, 0.45 mM AMP, 1X

phosphatase inhibitor cocktail I and II (Sigma), and 1X complete protease

inhibitors, EDTA-Free [Roche]) was added. After 20 min at room temperature,

200 μl of 2X RIPA (100 mM Tris pH8, 300 mM NaCl, 2% NP-40, 0.2% SDS, and

20 mM EDTA) with 2.5 mM p-*nitrobenzyl mesylate* (PNBM) (Epitomics) and 5%

DMSO was added and cells were incubated for an additional hour at room

temperature. The cell lysates were then subjected to immunoprecipitation using

agarose beads coupled to M2 antibody.

Immunoprecipitation of endogenous FOXO3 for AS-AMPKα2 assay

293T cells were seeded at 5 x 10^5 cells/35 mm dish and were transfected with

1.67 μg of HA-WT- or AS-AMPKα2, 1.67 μg HA-AMPKβ1, and 1.67 μg HA-

AMPKγ1, using the calcium phosphate method. Forty-eight hours after

transfection, the cells were washed twice with serum-free DMEM and incubated

for 2 hours prior to stimulation with 100 mM 2-deoxyglucose (2DG, Sigma) for 5

min. The in vivo phosphorylation and alkylation was then carried out as

described above for candidate substrates. The labeled lysates from 3 wells was

then pooled for each condition and 7.5 µg of FOXO3 or IgG control antibody

were added to each sample. The lysates and antibodies were then incubated for

2 hours at 4°C and then subjected to immunoprecipitation using agarose beads

coupled to Protein A antibody (Sigma). Immunoprecipitated proteins were

released from the beads by the addition of SDS sample buffer. Samples were

resolved on SDS-PAGE gels (10%) and transferred to nitrocellulose membranes.

The membranes were incubated with the indicated primary antibodies and the primary antibody was visualized using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

AMPK in vitro kinase assay

Purified AMPK (Upstate Biotechnology) was incubated with the indicated substrate (2.5 µg) in kinase reaction buffer (62.5 mM HEPES [pH 7.5], 1.625 mM DTT, 46.9 mM $MgCl_2$, 15.6 mM β-glycerophosphate, 3.125 mM EGTA, 312.5 µM ATP) and 12.5 µCi radiolabeled γ-^{32}P ATP and 750 µM AMP, for 20 min at 30°C. SDS sample buffer was added to stop the reactions. The samples were resolved on SDS PAGE and phosphorylation was detected by incorporation of radiolabeled γ-^{32}P-ATP.

In vitro kinase assays using ATPγS analogs were performed using M2-tagged WT or AS-AMPKα2 expressed and purified by immunoprecipitation from 293T cells as described above. The immunoprecipitated kinases were incubated at 30°C for 20 min with kinase reaction buffer, 1 mM GTP, and 2.5 µg of GST-FOXO3 as a substrate in the presence of 0.1 mM ATPγS, N^6-(benzyl) ATPγS, N^6-(phenethyl) ATPγS, or N^6-(cyclopentyl) ATPγS as indicated. After the phosphorylation reaction, EDTA, PNBM, and DMSO were added to final concentrations of 20 mM, 2.5 mM, and 5% respectively to each reaction and the reactions were incubated for 1 hour at room temperature. SDS sample buffer was then added to each reaction and the samples were analyzed by western blot

analysis as described above using the indicated antibodies.

Tandem mass spectrometry to identify AS-AMPKα2 substrates

All gel-handling steps were performed in a laminar flow hood to minimize

contamination. Methanol-washed siliconized tubes (PGC Scientifics) and HPLC

quality solvents were used. Gel lanes from WT and AS-AMPKα2 kinase

reactions were sectioned into 1 mm slices, chopped into 1 mm cubes, and

washed twice with 200 µl aliquots of 25 mM ammonium bicarbonate, 50%

acetonitrile. After desiccation using vacuum centrifugation, the gel pieces were

rehydrated with 20 µl of 5 ng/µl TPCK-modified sequencing grade trypsin

(Promega) and incubated at 37°C overnight. Peptides were extracted by addition

of 30 µl of 5% formic acid in 50% acetonitrile. The samples were concentrated

using vacuum centrifugation and analyzed by nano-liquid chromatography-

electrospray ionization tandem mass spectrometry as follows. Peptides were

separated using a 75 µm × 15 cm reverse phase C-18 column (LC Packings) at a

flow rate of 350 nl/min, running a 3–32% acetonitrile gradient in 0.1% formic acid

on an Eksigent nano 1D HPLC (Eksigent) equipped with an auto sampler (Agilent

Technologies). The liquid chromatography (LC) eluent was coupled to a

microionspray source attached to a LTQ-FT mass spectrometer (ThermoFisher).

Peptides were analyzed in positive ion mode. From each MS spectrum, the most

intense multiple charged peak was selected for generation of subsequent

collision-induced dissociation (CID) mass spectra. Proteins unique to AS-AMPK

samples were identified using Protein Prospector software, queried against the
Uniprot Database.

Tandem mass spectrometry to identify phosphorylation sites

A Coomassie-stained band corresponding to GST-PAK2 or GST-PPP1R12A
phosphorylated by AMPK in vitro was excised from an SDS-PAGE gel, divided in
half, reduced with DTT, alkylated with iodoacetamide, and digested with either
trypsin or chymotrypsin. Peptide mixtures were separated by microcapillary
reverse-phase chromatography and online analyzed in a hybrid linear ion trap-
orbitrap (LTQOrbitrap, Thermo Electron) mass spectrometer using data
dependent acquisition methods with both collision-induced dissociation (CID) and
electron-induced dissociation (ETD) fragmentation mechanisms. MS/MS spectra
were database-searched using the SEQUEST algorithm. All peptide matches
were initially filtered based on enzyme specificity, mass measurement error,
Xcorr and ΔCorr' scores and further manually validated for peptide identification
and site localization.

Double thymidine/monastrol block for isolation of protein samples U2OS
cells were seeded at 1 x 10^6 cells/flask in T-75 flasks. Twelve hours after
seeding, the cells were arrested in S phase by incubation with 2.5 mM thymidine
(Sigma) for 19 hours. The cells were then washed 3 times in McCoy's 5A
containing 17 μM deoxycytidine (Sigma) and allowed to recover for 3 hours in
this medium. After 3 hours, the medium was replaced with McCoy's 5A without

deoxycytidine for 6 hours. McCoy's 5A containing 100 µM monastrol (Sigma) was then added for 6 hours. Mitotic cells were then collected by mechanical agitation and washed 3 times in McCoy's 5A and replated for the indicated period of time. Cells were then lysed in SDS lysis buffer.

Nocodazole block for isolation of protein samples

U2OS cells were seeded at 1.2 x 10^6/10 cm plate in McCoy's 5A medium with 10% FBS and 1% glutamine. The following day, the medium was removed and replaced with medium containing 100 ng/ml nocodazole. Following a 16-18 hour incubation, the mitotic cells were collected by mechanical agitation, washed 2x in 25 ml nocodazole free medium, and replated for the indicated period of time. Cells were then lysed in SDS lysis buffer.

Fluorescence resonance energy transfer (FRET) in cell lines expressing the AMPK activity reporter AMPKAR

U2OS cells and A-431 cells stably expressing the AMPKAR reporter were generated using lentiviral infection (Tsou et al., 2011). COS-7 cells expressing the AMPKAR reporter were transiently transfected using FuGENE 6 (Roche Diagnostics), and imaging was carried out 18 to 36 hr after transfection. Live cell imaging was carried out as described (Tsou et al., 2011). Briefly, cells were imaged in 37°C in a 5% CO2 incubation chamber on a Nikon TE 2000E and a Nikon and Ti motorized inverted microscope with Plan Apo 20x/0.75 NA objective lens. Dual emission ratio imaging was performed with a 430/24 filter, a

diachromic mirror (Chroma #89002 dual), and two emission filters (470/24 for cyan and 535/40 for yellow). Images were collected every 10 minutes with a Hamamatsu ORCA-AG cooled CCD camera controlled with Elements software (Nikon).

Mitosis assay in response to chemical inhibitors or activators of AMPK

U2OS cells were seeded at 1 x 10^6 cells/flask in T-75 flasks. Eight hours after seeding, the cells were arrested in S phase by incubation with 2.5 mM thymidine (Sigma) for 19 hours. The cells were then washed 3 times in McCoy's 5A containing 17 µM deoxycytidine (Sigma) and allowed to recover for 3 hours in this medium. After 3 hours, the medium was replaced with McCoy's 5A without deoxycytidine for 6 hours. McCoy's 5A containing 100 µM monastrol (Sigma) was then added for 6 hours. Mitotic cells were collected by mechanical agitation and washed 3 times in McCoy's 5A prior to replating on coverslips coated with poly-L-lysine (Sigma) at 10,000 cells/12 mm slip. Cells were allowed to reattach for 1.5 hours prior to addition of 20 µM BTO-1 (Sigma), 40 µM compound C (VWR), 20 µM LY294002 (EMD chemicals), or 100 µM A-769662 for 4.5 hours. Cells were fixed in 4% paraformaldehyde and stained with DAPI and wheat germ agglutinin, Alexa Fluor 594 conjugate. The percent of multinucleated cells was determined for each condition using fluorescence microscopy by counting at least 300 cells per condition per replicate.

SUPPLEMENTAL REFERENCES

Alessi, D.R., Sakamoto, K., and Bayascas, J.R. (2006). LKB1-dependent signaling pathways. Annu Rev Biochem *75*, 137-163.

Allen, J.J., Lazerwith, S.E., and Shokat, K.M. (2005). Bio-orthogonal affinity purification of direct kinase substrates. J Am Chem Soc *127*, 5288-5289.

Allen, J.J., Li, M., Brinkworth, C.S., Paulson, J.L., Wang, D., Hubner, A., Chou, W.H., Davis, R.J., Burlingame, A.L., Messing, R.O.*, et al.* (2007). A semisynthetic epitope for kinase substrates. Nat Methods *4*, 511-516.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J *408*, 297-315.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y.*, et al.* (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Cheng, D., Cote, J., Shaaban, S., and Bedford, M.T. (2007). The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. Mol Cell *25*, 71-83.

Conkright, M.D., Guzman, E., Flechner, L., Su, A.I., Hogenesch, J.B., and Montminy, M. (2003). Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness. Mol Cell *11*, 1101-1108.

Greer, E.L., Oskoui, P.R., Banko, M.R., Maniar, J.M., Gygi, M.P., Gygi, S.P., and Brunet, A. (2007). The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem *282*, 30107-30119.

Holt, L.J., Tuch, B.B., Villen, J., Johnson, A.D., Gygi, S.P., and Morgan, D.O. (2009). Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. Science *325*, 1682-1686.

Laderoute, K.R., Amin, K., Calaoagan, J.M., Knapp, M., Le, T., Orduna, J., Foretz, M., and Viollet, B. (2006). 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. Mol Cell Biol *26*, 5336-5347.

Levin, S.E., Zhang, C., Kadlecek, T.A., Shokat, K.M., and Weiss, A. (2008). Inhibition of ZAP-70 kinase activity via an analog-sensitive allele blocks T cell receptor and CD28 superagonist signaling. J Biol Chem *283*, 15419-15430.

Liu, Y., Witucki, L.A., Shah, K., Bishop, A.C., and Shokat, K.M. (2000). Src-Abl tyrosine kinase chimeras: replacement of the adenine binding pocket of c-Abl with v-Src to swap nucleotide and inhibitor specificities. Biochemistry *39*, 14400-14408.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.

North, B.J., Marshall, B.L., Borra, M.T., Denu, J.M., and Verdin, E. (2003). The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell *11*, 437-444.

Polson, A.G., Huang, L., Lukac, D.M., Blethrow, J.D., Morgan, D.O., Burlingame, A.L., and Ganem, D. (2001). Kaposi's sarcoma-associated herpesvirus K-bZIP protein is phosphorylated by cyclin-dependent kinases. J Virol *75*, 3175-3184.

Straight, A.F., Cheung, A., Limouze, J., Chen, I., Westwood, N.J., Sellers, J.R., and Mitchison, T.J. (2003). Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science *299*, 1743-1747.

Tsou, P., Zheng, B., Hsu, C.H., Sasaki, A.T., and Cantley, L.C. (2011). A fluorescent reporter of AMPK activity and cellular energy stress. Cell Metab *13*, 476-486.

Tuerk, R.D., Thali, R.F., Auchli, Y., Rechsteiner, H., Brunisholz, R.A., Schlattner, U., Wallimann, T., and Neumann, D. (2007). New candidate targets of AMP-activated protein kinase in murine brain revealed by a novel multidimensional substrate-screen for protein kinases. J Proteome Res *6*, 3266-3277.

Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859-864.

Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Van Parijs, L., Jaenisch, R., and Jacks, T. (2004). Cre-lox-regulated conditional RNA interference from transgenes. Proc Natl Acad Sci U S A *101*, 10380-10385.

Vogt, J., Traynor, R., and Sapkota, G.P. (2011). The specificities of small molecule inhibitors of the TGFβ and BMP pathways. Cell Signal *23*, 1831-1842.

Weiss, W.A., Taylor, S.S., and Shokat, K.M. (2007). Recognizing and exploiting differences between RNAi and small-molecule inhibitors. Nat Chem Biol *3*, 739-744.

Zagorska, A., Deak, M., Campbell, D.G., Banerjee, S., Hirano, M., Aizawa, S., Prescott, A.R., and Alessi, D.R. (2010). New roles for the LKB1-NUAK pathway in controlling myosin phosphatase complexes and cell adhesion. Sci Signal *3*, ra25.
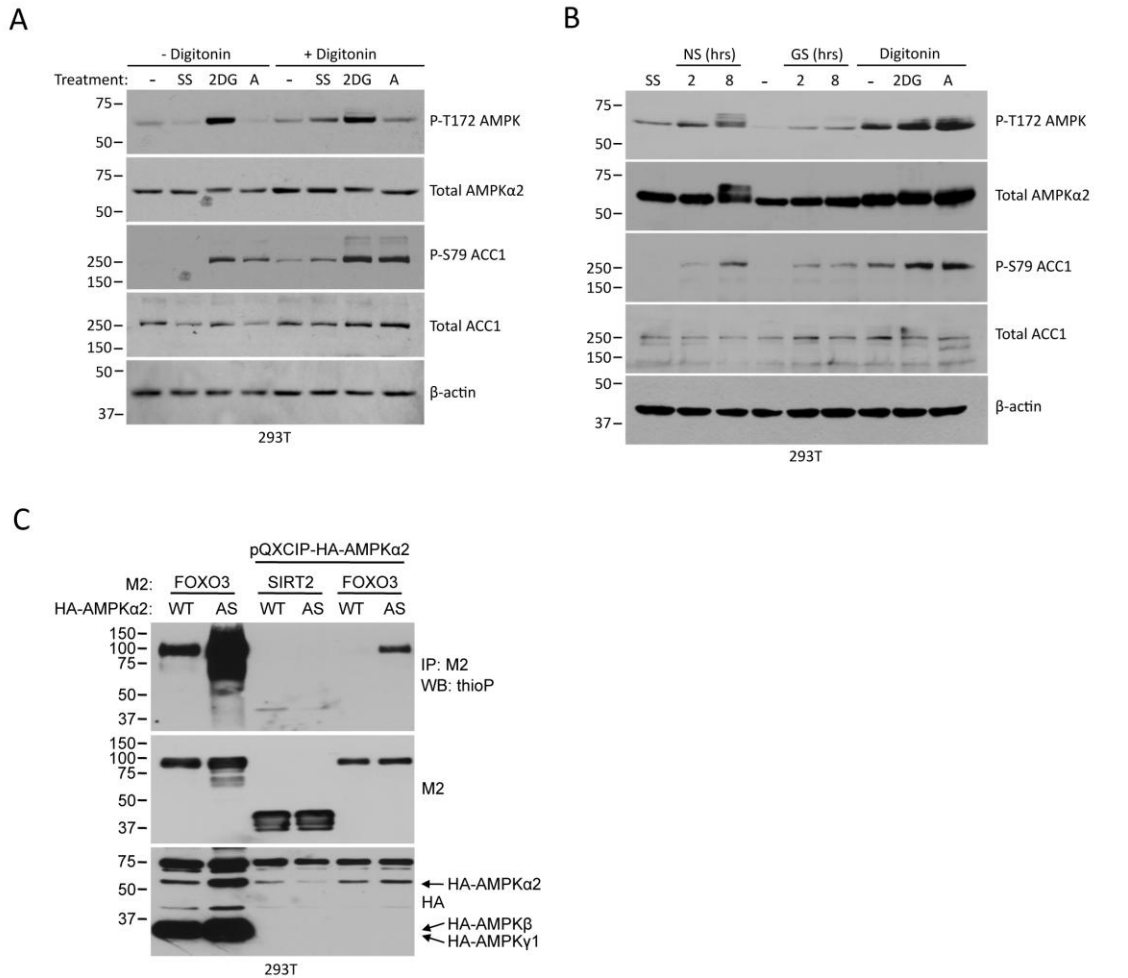
Figure S1. Endogenous AMPK signaling is functional in the conditions of analog-specific labeling and AS-AMPKα2 phosphorylates FOXO3 in vivo in stable cell lines with more physiological levels of AS-AMPKα2 and endogenous levels of AMPKβ and AMPKγ, Related to Figure 1

(A) 293T cells were incubated in the absence of serum (serum starvation, SS for 2 hours), in the absence of serum and in the presence of 2-deoxyglucose (2DG, 100 mM for 5 minutes), or in the absence of serum and in the presence of A-769662 compound (A, 100 μM for 1 hour), either in the absence or in the presence of digitonin (30 μg/ml). Cell extracts were analyzed by western blot using the indicated antibodies.

(B) 293T cells were incubated in the absence of serum (serum starvation, SS) for 2 hours, in PBS containing Ca^{2+} and Mg^{2+} (nutrient starvation, NS) for the indicated lengths of time (in hours), in glucose-free medium (glucose starvation, GS) with dialysed serum for the indicated lengths of time (in hours), or with 2 deoxyglucose (2DG, 100 mM for 5 minutes) or A-769662 compound (A, 100 μM for 1 hour) in the presence of digitonin (30 μg/ml). Cell extracts were analyzed by western blot using the indicated antibodies.

(C) 293T cells were stably infected by retroviruses expressing WT or AS-AMPKα2. In vivo labeling was performed as in Figure 1D with M2-FOXO3 as a substrate and M2-SIRT2 as a protein that is not phosphorylated by AMPK. As a positive control (2 left lanes), 293T cells were transfected by plasmids expressing WT or AS-AMPKα2 with HA-AMPKβ1 and HA-AMPKγ1. Molecular weights (in kDa) are indicated to the left of western blots.

26
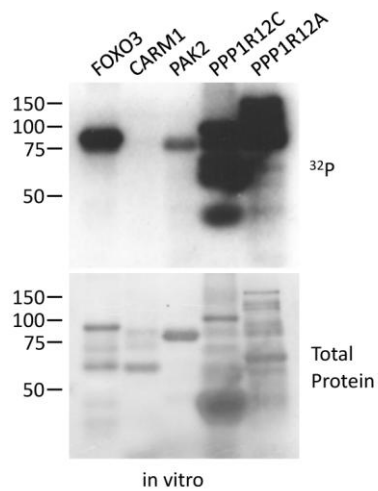
Figure S2. AMPK does not phosphorylate GST-CARM1 in vitro, Related to Figure 2
In vitro kinase assay of substrates identified as specific substrates of AS-AMPKα2. Purified WT-AMPK was incubated with recombinant GST-tagged substrates in the presence of radiolabeled γ^{32}P-ATP. Total protein levels were visualized using Coomassie brilliant blue stain. Molecular weights (in kDa) are indicated to the left of the gels. Representative of 3 independent experiments.
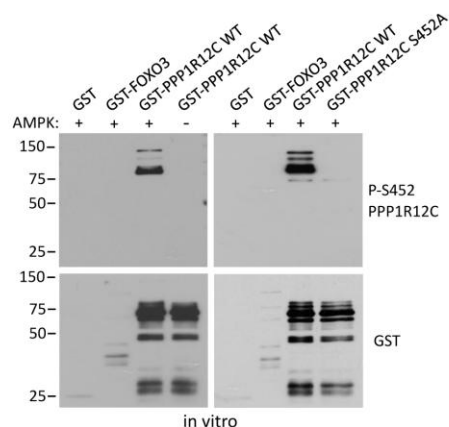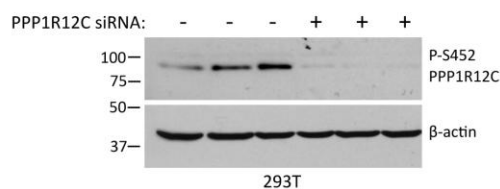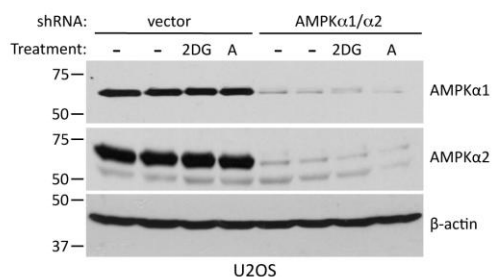
Figure S3. Specificity of the phospho-specific antibody to S452 PPP1R12C and efficiency of the shRNA to both AMPKα1 and α2 in human cells, Related to Figure 4

(A) Purified WT-AMPK was incubated with recombinant GST-tagged PPP1R12C (WT or S452A mutant) in the presence of radiolabeled γ^{32}P-ATP. Total protein levels were visualized using an antibody to GST.

(B) 293T cells were transiently transfected with control scramble siRNA or PPP1R12C siRNA. Cell extracts were analyzed by western blot using the indicated antibodies.

(C) U2OS cells were stably transduced by control lentiviruses (vector) or lentiviruses expressing an shRNA to both AMPKα1 and α2 (AMPKα1/α2). Cell lysates were analyzed by western blot using the indicated antibodies. Molecular weights (in kDa) are indicated to the left of western blots.
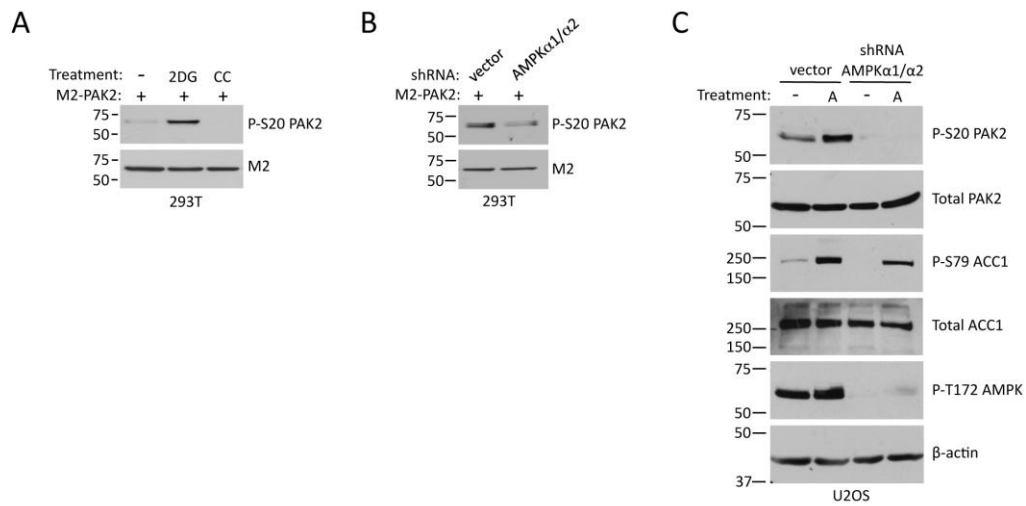
Figure S4. AMPK promotes the phosphorylation of PAK2 at S20 in human cells, Related to Figure 4

(A) Phosphorylation of M2-PAK2 at S20 is stimulated by 2DG and inhibited by compound C (CC) in 293T cells. Western blots representative of 2 independent experiments.

(B) Phosphorylation of M2-PAK2 at S20 is diminished in 293T cells expressing an shRNA to both AMPKα1 and α2 (AMPKα1/α2) compared to control cells (vector). Western blots representative of 2 independent experiments.

(C) Phosphorylation of endogenous PAK2 at S20 induced by A-769662 (A) is decreased in cells expressing an shRNA to both AMPKα1 and α2 (AMPKα1/α2) compared to control cells (vector). Cell extracts were analyzed by western blot. The panels for P-S79 ACC1, Total ACC1, P-T172 AMPK and β-actin are also shown in Figure 4D.
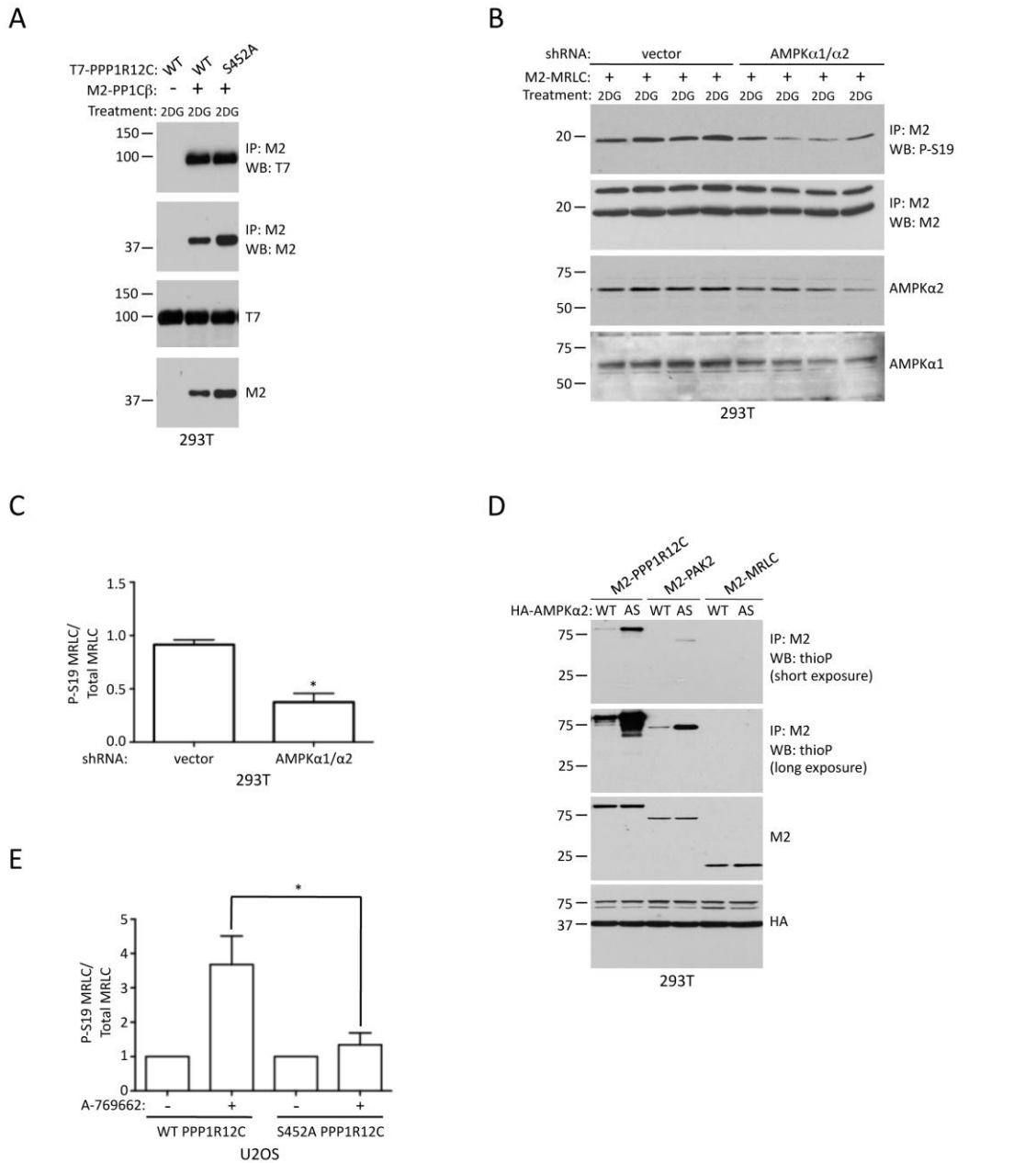
Figure S5. AMPK is important for the phosphorylation of MRLC in cells, but MRLC is not directly phosphorylated by AMPK in cells, Related to Figure 5

(A) PPP1R12C S452 is not required for the interaction between PPP1R12C and PP1Cβ. M2-PP1Cβ was co-expressed with WT or S452A T7-PPP1R12C in 293T cells. Cells were stimulated with 2-deoxyglucose (2DG) for 5 minutes, lysed, and M2-PP1Cβ was immunoprecipitated. Cell extracts and immune-complexes were analyzed by western blot with the indicated antibodies. Representative of 2 independent experiments.

(B) Knock-down of AMPKα1 and α2 results in a decrease in the phosphorylation of MRLC at S19. 293T cells were transiently transfected with PSR empty vector or PSR encoding an shRNA targeting both AMPKα1 and α2. Cells were then lysed and analyzed by western blot with the

indicated antibodies. The knock-down of endogenous AMPKα1 and α2 was not complete with transient transfection of PSR encoding the AMPKα1/α2 shRNA, likely because transfected cells were not selected. The efficiency of the AMPKα1/α2 shRNA was further tested in cells stably expressing the AMPKα1/α2 shRNA (Figure S3C).

(C) Quantification of the effect of knock-down of AMPKα1 and α2 on the phosphorylation of MRLC at S19. Phosphorylation levels were normalized to total M2-MRLC levels and 4 data points were combined for each condition. *p<0.05 by t-test.

(D) AS-AMPKα2 directly phosphorylates PPP1R12C and PAK2, but not MRLC, in cells. In vivo labeling with AS-AMPKα2 was carried out as described in Figure 1D. Overexpressed MRLC is likely to be in a conformation that allows phsophorylation under these conditions, because overexpressed MRLC can be phosphorylated in vivo in response to AMPK activation (see Figures 5F and 5G). Molecular weights (in kDa) are indicated to the left of western blots. Representative of 2 independent experiments.

(E) Quantification of the effect of PPP1R12C S452A expression on AMPK-induced phosphorylation of MRLC at S19 shown in Figure 5F. Phosphorylation levels were normalized to untreated cells expressing WT or S452A PPP1R12C for each experiment. The fold-increase in P-S19 MRLC in response to A-769662 was then measured and the results of 4 independent experiments were combined. *p<0.05 by one-way ANOVA.
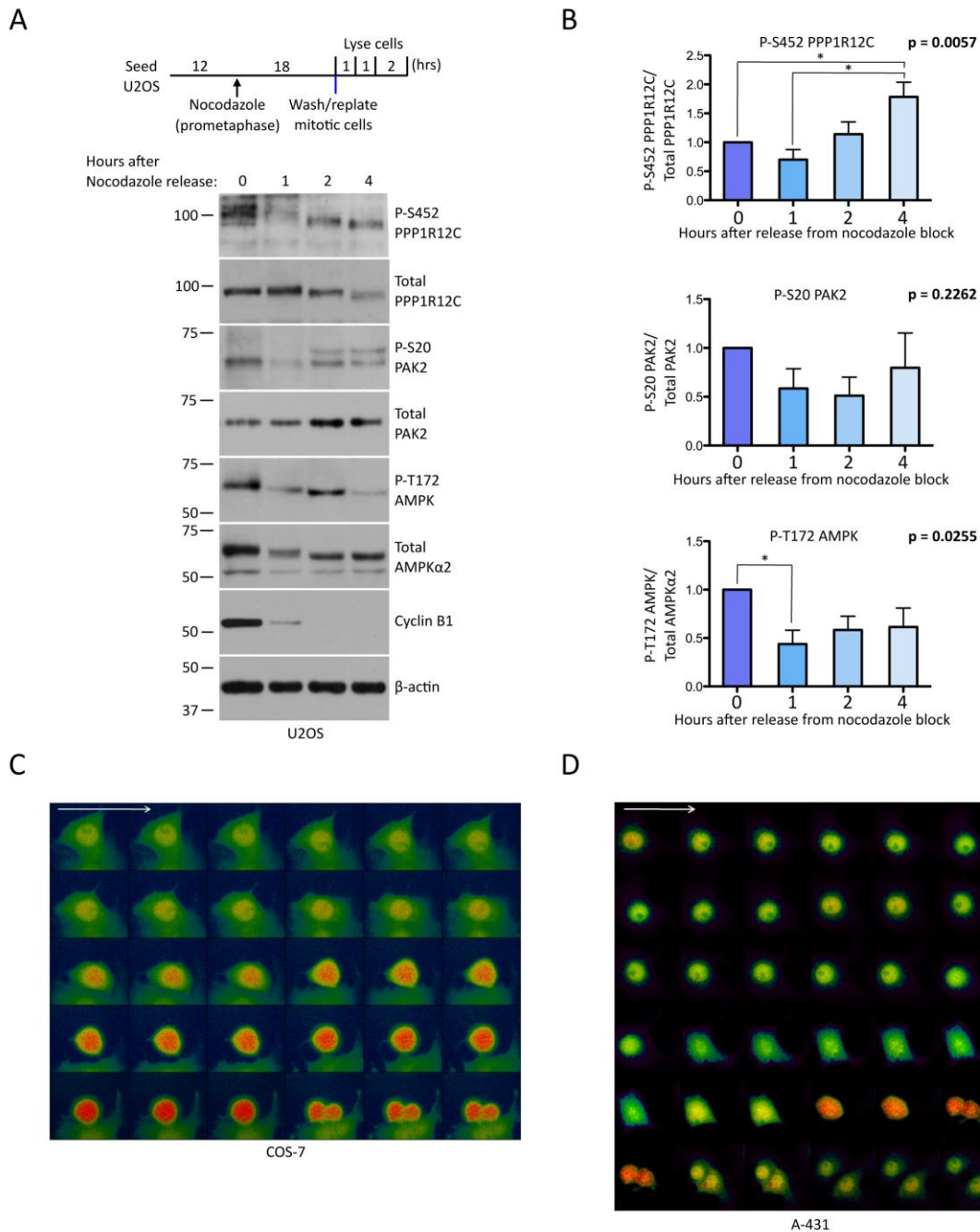
Figure S6. Phosphorylation and activity of endogenous AMPK and phosphorylation of endogenous AMPK substrates in cells undergoing mitosis, Related to Figure 6
(A-B) Phosphorylation of endogenous AMPK, PPP1R12C, and PAK2 in U2OS cells synchronized in prometaphase using nocodazole and collected at different time points after the release from the nocodazole block (blue bar). (A) Western blots representative of 5 independent experiments. (B) Quantification of phosphorylation levels relative to total levels. Data were normalized with respect to the first time point. Means +/- SEM of 5 experiments are shown. *p<0.05 by one-way ANOVA and Tukey post-test. Overall p<0.05 indicates significant phosphorylation differences during mitotic progression.
(C-D) An increase in AMPK FRET signal was observed during mitosis in COS-7 (C) and A-431 (D) cells. Representative live cell images from cells expressing AMPKAR. The emission ratio for AMPKAR was measured at 10 min intervals. Red: increased AMPK activity.
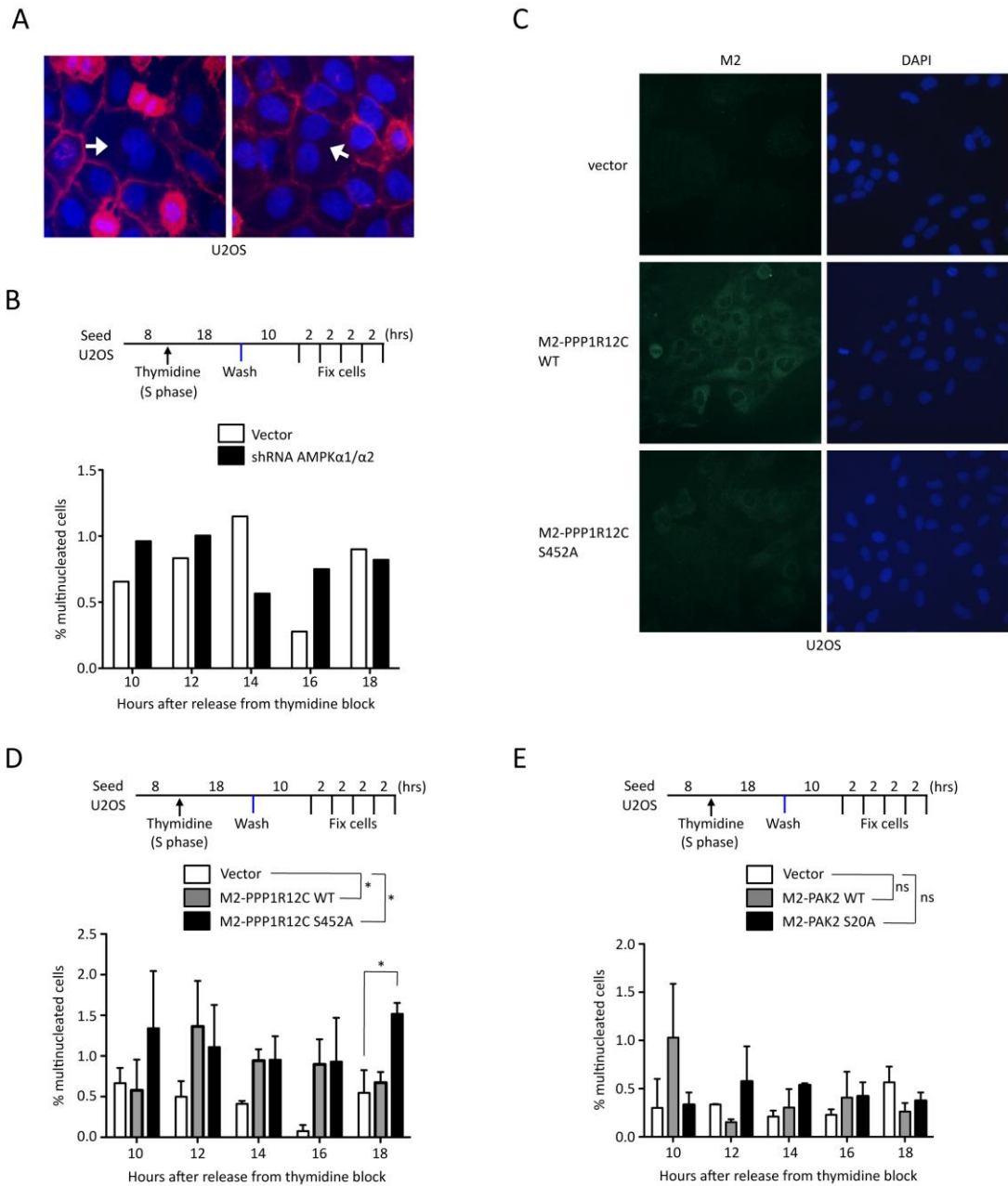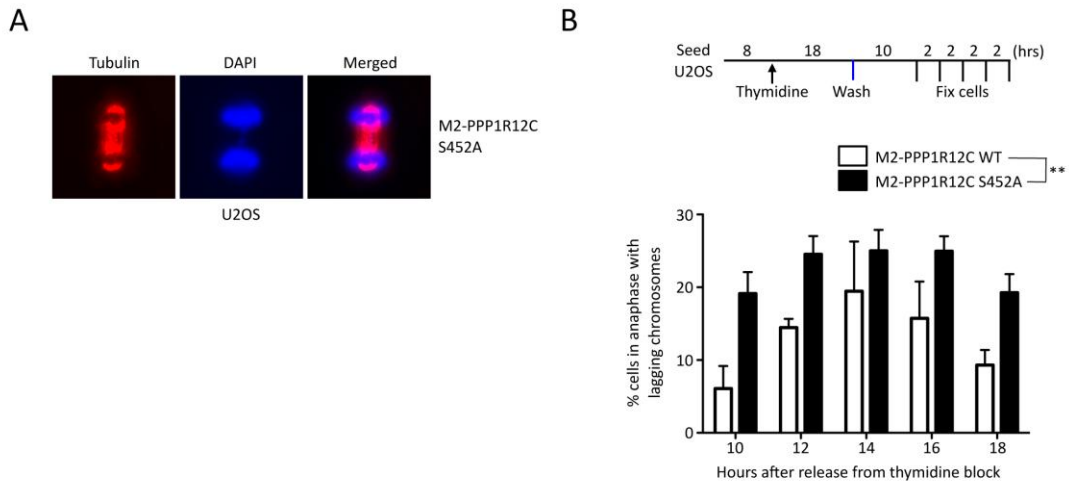
Figure S7. Generation of stable U2OS cell lines expressing WT or S452A PPP1R12C and expression of PPP1R12C S452A results in lagging chromosomes, Related to Figure 6
(A) Representative images of multinucleated U2OS cells. White arrows: multinucleated cells.
(B) U2OS cells expressing PSR empty vector (vector) or an shRNA to AMPKα1 and α2 (AMPKα1/α2) were synchronized by thymidine block. Cells were fixed post-release from the thymidine block (blue bar), and stained with DAPI and wheat germ agglutinin. Percent of multinucleated cells in each condition are shown.
(C) U2OS cells were infected with lentiviruses encoding M2-WT or M2-S452A PPP1R12C. Expression of the M2-PPP1R12C proteins was assessed by immunocytochemistry with an antibody to the M2 epitope and nuclei were visualized using DAPI.

(D-E) U2OS cells expressing FUW (vector), M2-PPP1R12C (D), and M2-PAK2 (E) were synchronized by thymidine block. Cells were fixed post-release from the block (blue bar). Percent of multinucleated cells in each condition are shown. Means +/- SEM of 3 (D) or 2 (E) independent experiments. ns: not statistically significant. *p<0.05 by one-way ANOVA analysis.

Figure S8. Expression of PPP1R12C S452A results in lagging chromosomes, Related to Figure 6

(A-B) Cells were treated as described in Figure 6D. Representative images of an anaphase cell expressing S452A PPP1R12C with lagging chromosomes (A) and percent of anaphase cells with lagging chromosomes (B) are shown.

Means +/- SEM of three independent staining and counting for a synchronization experiment are shown. **$p<0.01$ in 2-way ANOVA.