Supporting Online Material for

Signaling Kinase AMPK Activates Stress-Promoted Transcription via Histone H2B Phosphorylation

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This PDF file includes:

Supplementary Methods

Cell lines, DNA Constructs, and Antibodies

AMPKα-deficient MEFs (lacking both AMPKα1 and AMPKα2), LKB1-deficient MEFs, and LKB1-deficient MEFs re-expressing LKB1 have been previously described(*1-2*). HCT116 cells were a gift from S. McMahon (Thomas Jefferson University). 293T cells were obtained from the ATCC. Expression constructs for myc-AMPK, myc-AMPK-DN, FLAG-LKB1, FLAG-LKB-KDM, and FLAG-H2B have been previously described(*2-3*). The S36A mutation was into the coding sequence of FLAG-H2B by QuikChange mutagenesis (Stratagene). Silencing of mouse or human AMPKα was achieved by retroviral transduction of cells with LMP shRNA vectors co-expressing GFP or hCD8 markers(*4*). Transduced cells were selected with 20 μg/ml puromycin, and GFP/hCD8 double-positive cells enriched by MoFlo sorting. The shRNA sequences used are as follows: AMPKα1: GAGGAGAGCTATTTGATTA, AMPKα2: GCCATAAAGTGGCAGTTAA, Firefly luciferase (control): GAGCTGTTTCTGAGGAGCC. The H2B S36ph antibody was generated by Open Biosystems. Antibodies for AMPKα (total and T172ph) and myc-tag were from Cell Signaling Technologies, anti-AMPKα2 was from R&D technologies, anti-histone H3, H3 K9Me, and H3 S10ph were from Abcam, anti-H2B and ACC1 S79ph were from Upstate Biotechnologies, purified and agarose-conjugated anti-FLAG (M2) was from Sigma, and anti-actin and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnologies.

Cell Culture and Apoptosis Measurements

For glucose withdrawal, cells were washed with PBS, then cultured for 24 hours in glucose-free DMEM (10% dialyzed FBS) containing 0 mM or 25 mM glucose. For UV treatments, medium containing (25 mM glucose) was removed and cells irradiated with 5 – 20 J/m^2 UVC. Following irradiation, the original medium was replaced and cells cultured for an additional 6 – 8 hours. For 2-DG treatment, cells were washed with PBS, then incubated for 5 – 15 minutes with 25 mM 2-DG in glucose-free DMEM. Apoptosis was detected by 7-aminoactinomycin D (7AAD) exclusion using a FACScan flow cytometer. For other cellular stresses exposure times were: UV, IR, CPT, Adr - 6 hours, MNNG, H_2O_2 - 30 minutes.

Kinase Assays

AMPK kinase assays were performed as previously described(*3*). In brief, myc-AMPK or FLAG-LKB1 immunprecipitated from 2-DG-treated cells were used in a kinase reaction in HEPES-Brij buffer containing 5 mM $MgCl_2$, 0.2 mM ATP with 0.5 Ci/ml [γ-32P]ATP, 0.3 mM AMP, and target protein substrate. Histones were obtained from Roche. H2B peptides containing S32A, S36A, and S38A substitutions were generated by Open Biosystems.

Histone Extraction

Histones were isolated by acid extraction(*5*). In brief, cells were collected from a 10 cm plate, washed twice with PBS, and then 1mL of 0.4N H_2SO_4 was added to the cell pellet. Cells were sonicated and incubated 30 minutes on ice. Lysates

were cleared by centrifugation for 15 minutes at 14 000 rpm at 4^{o}C. Trichloroacetic Acid (TCA) was added to a final concentration of 20% to precipitate protein. Precipitates were incubated 30 minutes on ice, and then pelleted by centrifugation. The pellet containing histones were washed with acidified acetone (acetone + 0.05% HCl), followed by acetone, and then dried. Pellets were resuspended in water and used in immunoblotting assays.

Chromatin Immunoprecipitation and Real-Time PCR

Relative gene expression was determined by quantitative real-time PCR. RNA was isolated using an RNeasy Mini kit (Qiagen). First-strand cDNA synthesis was conducted using SuperScript II reverse transcriptase (Invitrogen) and 50 ng of random hexamers (Roche), or using TaqMan Reverse Transcription reagents (Applied Biosystems). cDNA samples were then used as templates for quantitative real-time PCR using a ABI 7900HT detection system and SYBR Green (Applied Biosystems). Primer sets for mouse p21 (QT00137053), human p21(00062090), mouse cpt1c (QT00146468), mouse hprt1 (QT00166768) , and human HPRT (QT00059066) were obtained from Qiagen.

Chromatin immunoprecipitation was carried out as previously described(*6*). In brief, wild-type MEFs were grown to 85% confluence in DMEM (10% FBS), followed by treatment for 24 hours in glucose-free DMEM, UVC or 2-DG (see above for conditions). Cells were fixed with 1% formaldehyde (for AMPK and LKB1 ChIPs, cells are first fixed with 0.5mM Ethylene Glycol-bis Succinimidylsuccinate (EGS), Pierce #21565) and lysed with lysis buffer (50 mM

HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% sodium deoxycholate, with protease inhibitors). The cell lysate was sonicated with a Bioruptor (Gendiode) for a total of 20 min with 30-s 'on' and 1-min 'off' cycles, to shear the DNA to a final size of 200–500 base pairs. After preclearing with Protein A or G beads (Upstate), protein bound to chromatin was immunoprecipitated using: anti-mouse p53 (Pab 246) (sc-100, Santa Cruz Biotechnologies), anti-FLAG M2 (F3165, Sigma), anti-c-Myc (9E10) (sc-40, Santa Cruz), anti-human/mouse/rat AMPKα2 (AF2850, R&D Systems) or affinity-purified anti H2B S36ph (Open Biosystems). The next day, Protein A-agarose or Protein G-agarose beads were added and incubated for a further 1–2 h. The complex was washed twice with lysis buffer, once with high salt buffer (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% sodium deoxycholate), twice with LiCl buffer (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate, 1 mM EDTA) and once with TE buffer, followed by elution in TE buffer containing 1% SDS. Following reversal of crosslinking, the DNA was purified and subjected to analysis by quantitative real-time PCR. Primers were designed along the mouse Cpt1c promoter, transcribed region and 3’ UTR. Primer sets 1-7: (1 - sense – GGCATGCCTGAGGCTTATCA, antisense – TCTGCCCTGTTCCCGAGTAG); (2 - sense – GTCCTGGAGCTCACTCTAGACCA, antisense – AGGCGAATTTCTGAGTTTGAGG); (3 - sense – CCTGCCACCTCCTTAGTGCA, antisense – TCTGTGAAGGTCCTCGGGC); (4 - sense – AAAGAGCAGGGACAGAACGC, antisense – AGGTTTTATCCCCGCCTCC); (5

– sense - GCGTGTCTGGGTGAGGAGAA, antisense – GAAGCCCCGAAATCTGAATTC); (6 – sense - GACGGGTAACAGGCAACGG antisense – GGTTCGCTGTCAGGCTCTCT); (7 – sense – ACCCCACCTTGACTTCCCAG, antisense – GTAGTGCGGGCTGTGGTTAAG). Primers were designed along the mouse p21 promoter, transcribed region and 3' UTR: (1 - sense – GGGATCCAACTCCTGGCTTC, antisense – TCCGTTAATATAGCGCTTGCC); (2 - sense – AAATATTTGTTGAGTACTTTTGTGGTG, antisense – TGGGAAGCCAGAAGTTGTTTAA), (3 - sense – GCGCAGATCCACAGCGATAT, antisense – TGCCACGAAGCTCTCACCT); (4 - sense – GGGACAAGGGCTACACTTGC, antisense – AAGCCCAAAGTATTTCCCGC); (5 – sense - GCAACTGCTCACTGTCCACG, antisense – GGAGCAAAGTGTGCCGTTGT); (6 – sense – CACTCCAAGCGCAGATTGGT, antisense – GTGGGCACTTCAGGGTTTTC); (7 – sense – TACAAGGAGCCAGGCCAAGA,
antisense – CCTGTGAGCTCCCTTAGCCC). Primers for human p21 promoter (sense – GGCTGGTGGCTATTTTGTCC; antisense – TCCCCTTCCTCCCTGAAAAC). Primers were designed along the mouse GAPDH promoter, transcribed region and 3' UTR. Primer sets 1-7: (1 - sense – TGCTCCTCCCTGTTCCAGAG, antisense – CACCTGGCACTGCACAAGAA); (2 - sense – GGGCCACGCTAATCTCATTTT, antisense – TTTGTCTACGGGACGAGGCT); (3 - sense - GCTTGGGCTTCCTTTAGGGT antisense – CTTCCCGTTTGCAACATGG); (4 - sense –

GGAGCGAGACCCCACTAACA, antisense – ACATACTCAGCACCGGCCTC); (5 – sense - TCATCCCTGCATCCACTGGT, antisense – CTGGGATGACCTTGCCCAC); (6 – sense - ACGGCTACAGCAACAGGGTG antisense – CCTTGGAGGCCATGTAGGC); (7 – sense – GGGCGCCCAGTACAGTAAAA antisense – GCTACAGCCCCTCTTCATGG). Data are displayed for triplicate samples as the fold increase of IP/input over control IgG-IP/input for AMPK and IP/input over H4/input for pS36 H2B, and expressed as mean ±SEM.

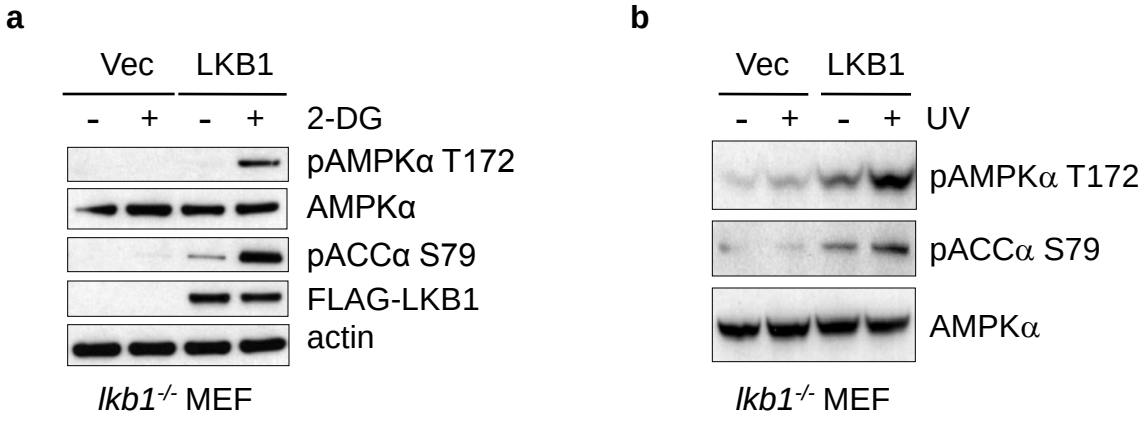

Fig. S1. LKB1-deficient MEFs are defective for AMPKα activation. LKB1-deficient MEFs expressing empty vector (Vec) or FLAG-tagged LKB1 (LKB1) were treated with 25 mM 2-deoxyglucose (2-DG) for 10 minutes (a), or 20 J/m^2 UV radiation and then cultured for an additional 6 hours (b). Whole cell lysates were processed, and phosphorylated AMPKα (pT172) and ACCα (pS79) were detected by Western Blot following SDS-PAGE and transfer to nitrocellulose.

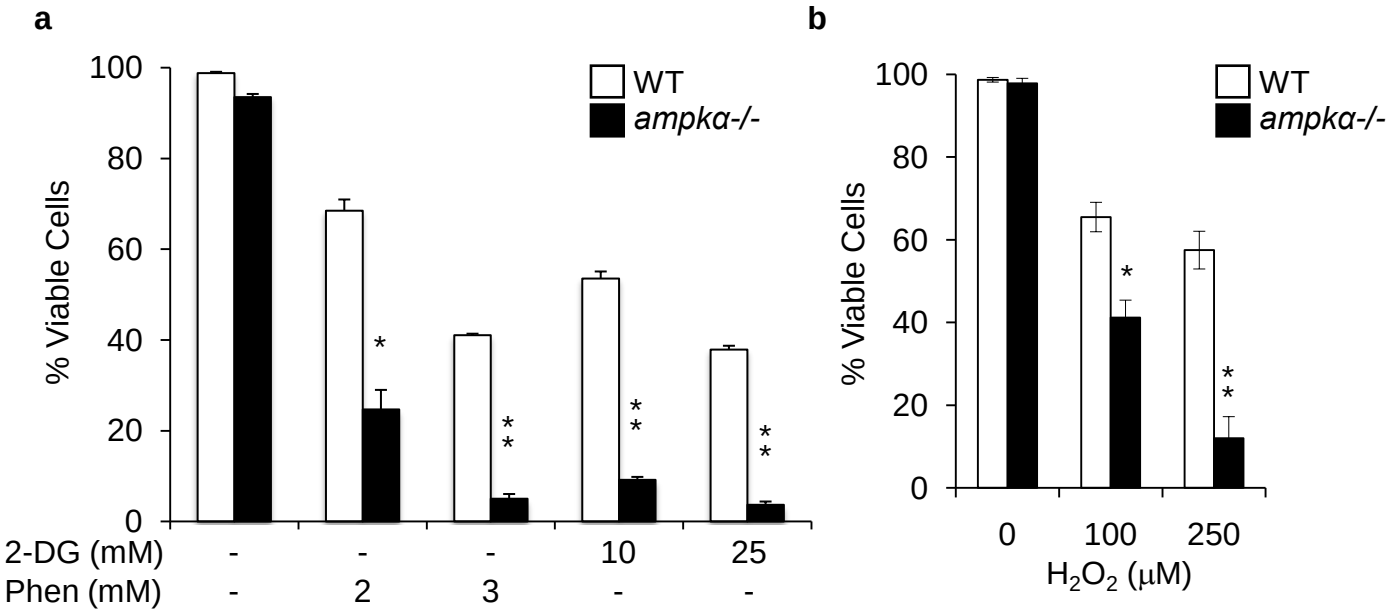

Fig. S2. AMPKα-deficient MEFs display increased rates of apoptosis. Wild-type (WT, black bars) or AMPKα1-/-, α2-/- (*ampkα-/-*, white bars) MEFs were treated with various concentrations of 2-deoxyglucose (2-DG) or phenformin (Phen) (a), or hydrogen peroxide (H_2O_2) (b), and cell viability was determined by viability dye exclusion. Cell viability was determined 48 hours post-treatment for 2-DG and phenformin, and 24 hours for H_2O_2. Statistical significance was determined by paired Student's t-test (*$p<0.05$, **$p<0.02$).

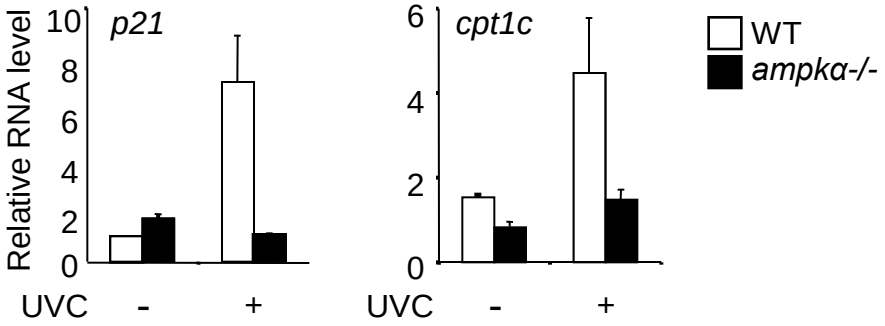

Fig. S3. AMPK-dependent transcription of p53-regulated genes in response to UV irradiation. Relative expression of p21 and cpt1c mRNA (normalized to *hprt*) in wild-type (WT, white bars) or AMPKα-deficient (*ampkα-/-*, black bars) MEFs 6 hours following treatment with UVC radiation (20 J/m^2) by quantitative real-time PCR.

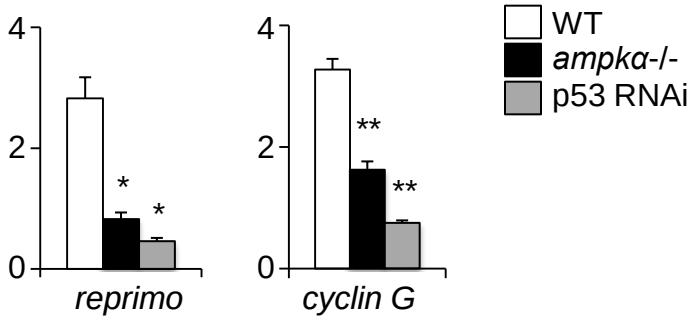

Fig. S4. AMPK is required for stress-dependent transcription. Relative expression of *reprimo* and *cyclinG* mRNA in wild-type (white bars), AMPKα-deficient (black bars), or p53 shRNA–expressing MEFs following glucose withdrawal by quantitative real-time PCR. Data are expressed relative to cell grown under glucose-replete conditions. Statistical significance: *$p<0.05$, **$p<0.01$,

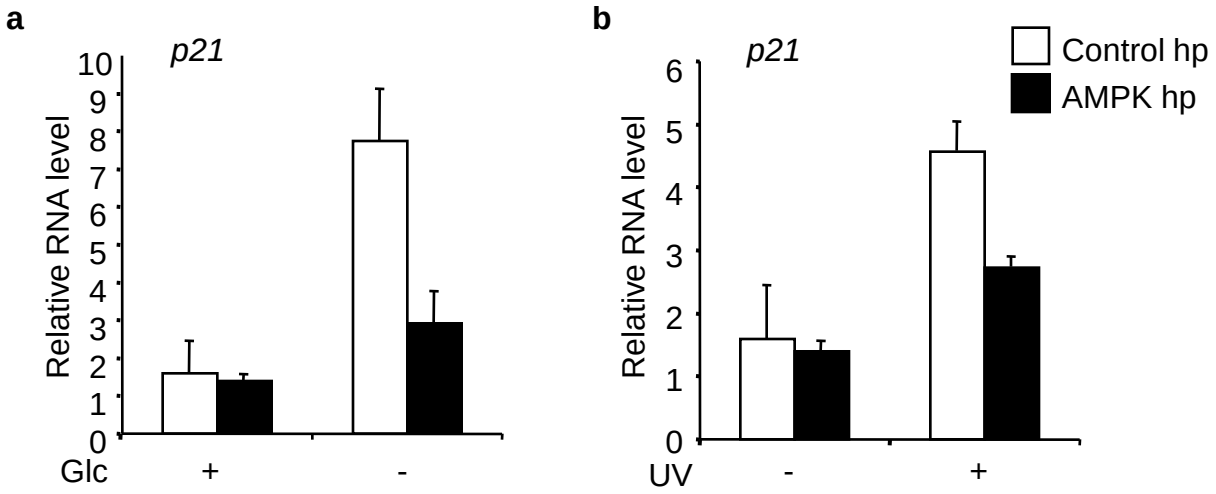

Fig. S5. AMPK-dependent induction of p21 mRNA in human cells. HCT116 colon carcinoma cells stably expressing control shRNA (Control hp, white bars) or shRNA directed against AMPKα1 and AMPKα2 (AMPK hp, black bars) were subjected to glucose withdrawal (a) or UV (b), and p21 mRNA was measured using real-time PCR. The values shown are the relative expression of p21 to HPRT message (mean $\pm$ s.d.) for samples in triplicate.

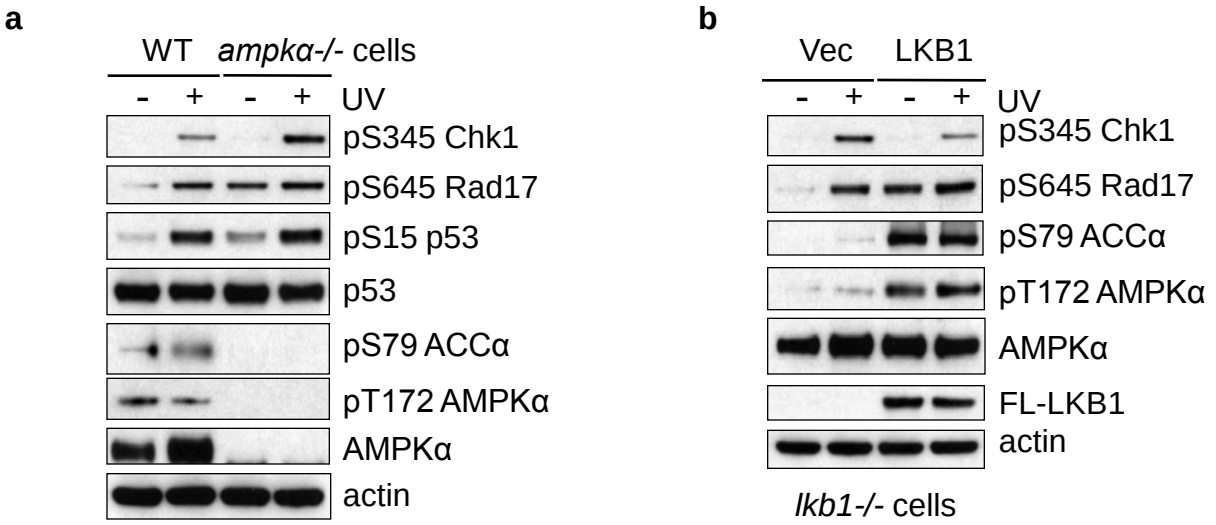

Fig. S6. Analysis of DNA damage pathways in AMPK- and LKB1-deficient mouse embryonic fibroblasts (MEFs) following UV radiation. Wild-type (WT) or AMPKα1-/-, α2-/- MEFs (a) or LKB1-deficient MEFs expressing empty vector (Vec) or FLAG-tagged LKB1 (LKB1) (b) were subjected to UV radiation (20 J/m^2). Whole cell lysates were harvested 6 hours post-treatment, and the indicated proteins detected by Western Blot following SDS-PAGE and transfer to nitrocellulose membrane.

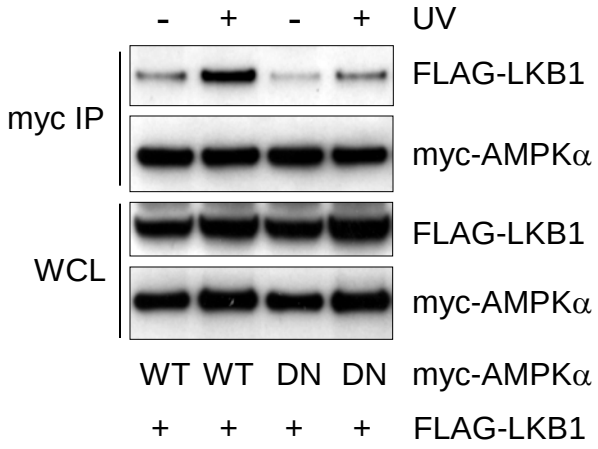

Fig. S7. Co-immunoprecipitation of FLAG-LKB1 and myc-AMPKα following UV treatment in 293T cells. 293T cells were transiently transfected with myc-tagged AMPKα and FLAG-tagged LKB1 expression constructs, and then treated with UV radiation (20 J/m^2). AMPK was immunoprecipitated from whole cell lysates (WCL) using anti-myc tag antibody, and the immunoprecipitates resolved by SDS-PAGE and probed for FLAG-LKB1 or myc-AMPK by Western Blot. WT, wild-type AMPKα; DN, dominant-negative AMPKα .

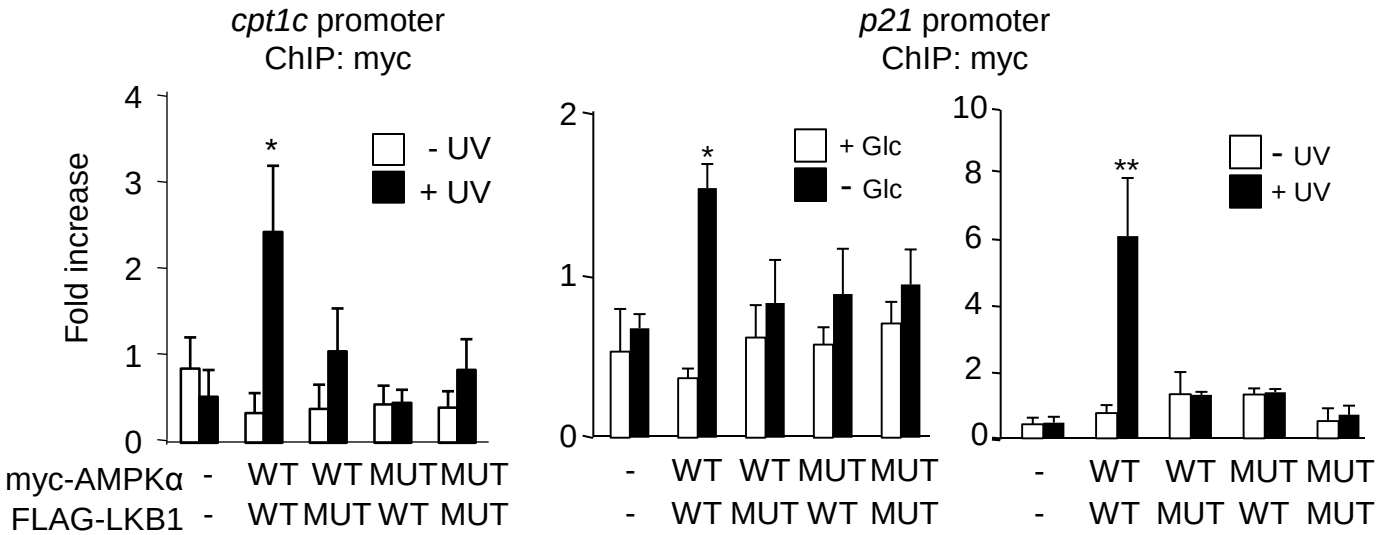

Fig. S8. AMPK is recruited promoters in an LKB-dependent manner. AMPK-deficient MEFs expressing wild-type (WT) or dominant-negative (MUT) AMPK and either wild-type or kinase-dead mutant (MUT) LKB1 were treated with glucose withdrawal (-Glc) or UV, and the presence of AMPK at the cpt1c (left panel) or p21 (middle and right panels) promoter was measured by ChIP and real-time PCR using an anti-myc tag antibody. Data are displayed as fold increase of myc-AMPK-IP/input over control IgG-IP/input for each condition in triplicate. Shown is the mean ± SEM for values from three independent experiments. Statistical significance: *$p<0.04$, **$p<0.05$.

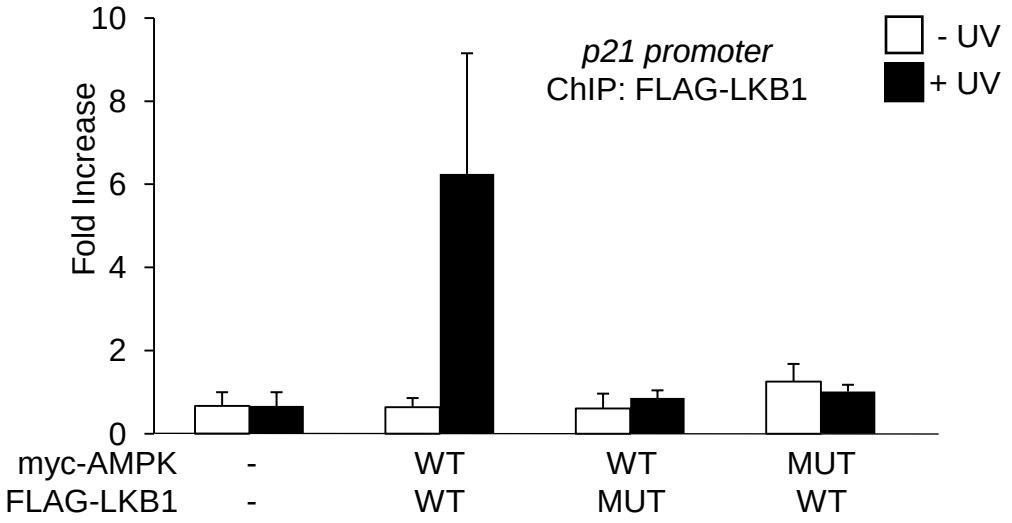

Fig. S9. LKB1 is recruited to the p21 promoter in an AMPK-dependent manner. AMPK-deficient MEFs expressing wild-type (WT) or dominant-negative (MUT) AMPK and either wild-type or kinase-dead mutant (MUT) LKB1 were treated with UV, and the presence of LKB1 at the p21 promoter was measured by ChIP and real-time PCR using an anti-FLAG antibody. Data are displayed as fold increase of LKB1-IP/input over control IgG-IP/input for each condition in triplicate. Shown is the mean ± SEM for values from three independent experiments.

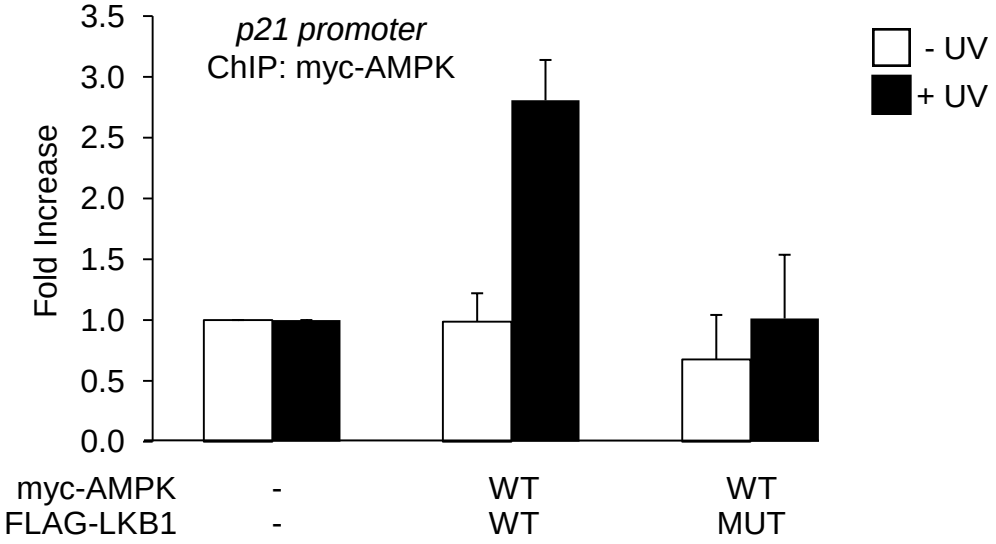

Fig. S10. AMPK is recruited to the p21 promoter in response to UV in human cells. HCT116 colon carcinoma cells expressing wild-type (WT) AMPK and either wild-type or kinase-dead mutant (MUT) LKB1 were treated with UV, and the presence of myc-AMPK at the p21 promoter was measured by ChIP and real-time PCR using an anti-myc antibody. Data are displayed as fold increase of AMPK-IP/input over control IgG-IP/input for each condition in triplicate. Shown is the mean ± SEM for values from two independent experiments.

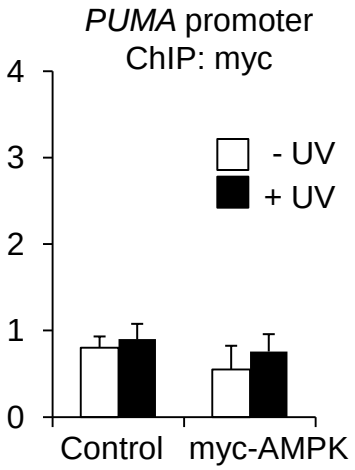

Fig. S11. AMPK is not recruited to the pro-apoptotic PUMA promoter in response to DNA damage. Chromatin immunoprecipitation (ChIP) of wild type (WT) or dominant negative (MUT) myc-AMPK to the *PUMA* promoter following treatment with 20J/m^2 UV in *ampk-/-* MEFs. Data represent the mean ± SEM for three independent experiments.

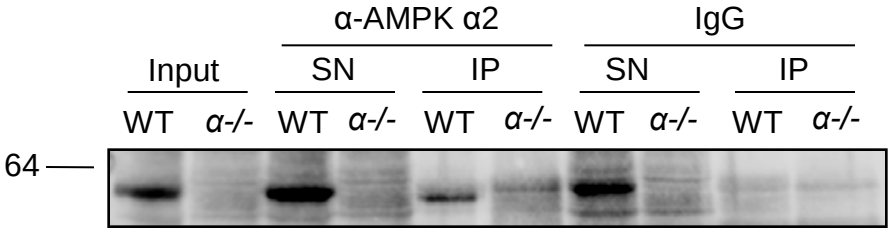

Fig. S12. Immunoprecipitation of AMPKα2. Whole cell extracts were prepared from wild-type (WT) and *ampkα-/-* (*α-/-*) MEFs. Anti-AMPKα2 antibody or IgG control was used for IP. Input, supernantant (SN) and eluted (IP) material was used for western blot. A pan anti-AMPKα antibody was used for detection.

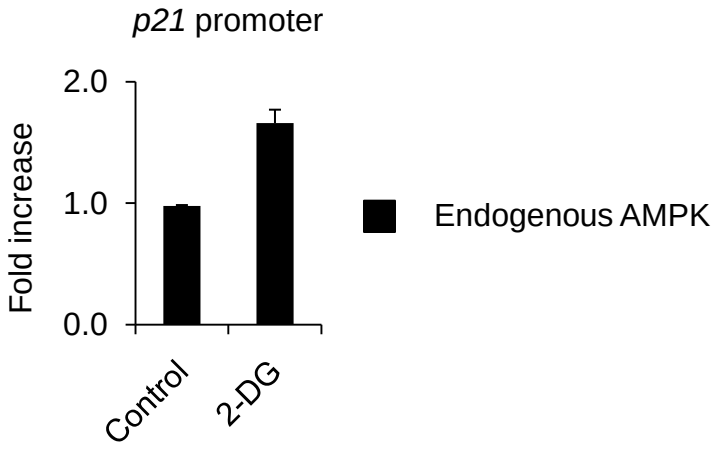

Fig. S13. Endogenous AMPK localizes to the p21 promoter in response to 2-DG. WT MEFs were treated with 2-DG, and the presence of endogenous AMPK at the p21 promoter was measured by ChIP and real-time PCR using an anti-AMPKα2 antibody. Data are displayed as fold increase of AMPK-IP/input over control IgG-IP/input for each condition in triplicate. Shown is the mean ± SEM for values from two independent experiments.

Optimal AMPK Motif	L	R	R	V	x	S	x	x	N	L
Secondary substitutions	M	K	K	S		S			D	V
Additional substitutions	I		H	R					E	I
ACC1 (Ser79)	I	R	S	S	M	S	G	L	H	L
TSC2 (Ser1387)	L	S	K	S	S	S	S	P	E	L
eNOS (Ser1177)	R	I	R	T	Q	S	F	S	L	Q
H. Sapiens H2BE (Ser36)	R	S	R	K	E	S	Y	S	I	Y

Fig. S14. Alignment of histone H2B Ser-36 relative to an optimal AMPK motif. Amino acid sequence of human H2BE relative to the optimal AMPK motif derived by positional scanning peptide library screening. The established motifs for three AMPK substrates, ACC1, TSC2 and eNOS, are also displayed. Green, phosphoacceptor site (Ser/Thr); Yellow, hydrophobic residues; Red, basic residues; Grey, acidic residues; Blue, additional consensus residues.

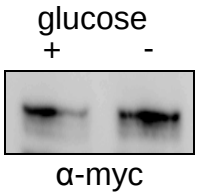

Fig. S15. Expression of ectopic myc-AMPK. Western blot of myc-AMPK immunmoprecipitated from *ampk-/-* MEFs treated with (+) or without (-) glucose.

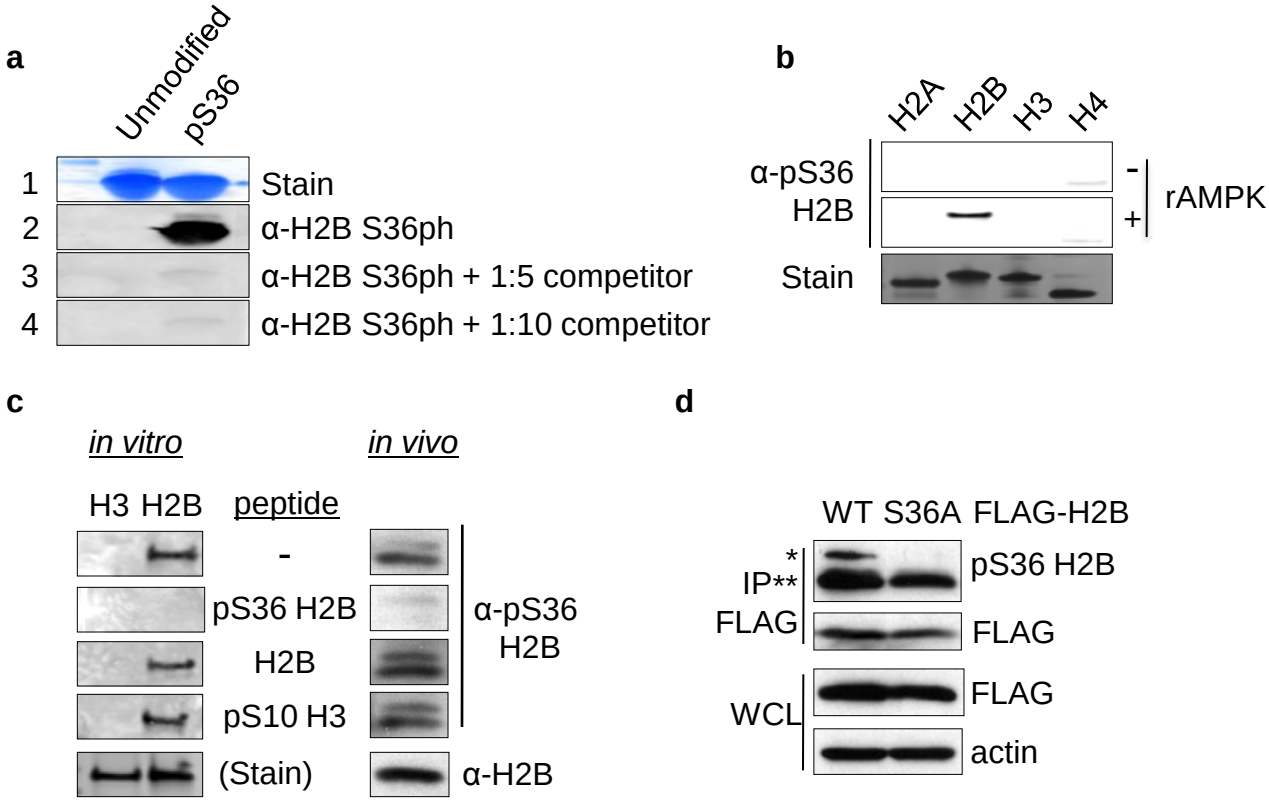

Fig. S16. Characterization of rabbit αH2B pS36 antibody. a) An unmodified or S36-phosphorylated (pS36) form of an H2B N-terminal tail peptide (RKESYSIYVYKVLKQC) was used to test the specificity of the H2B pS36 antibody. 20μg of peptide were loaded per lane. Top panel: Coomassie stain of peptides; Panels 2-4: Western blots with H2B pS36 antibody with no competitor peptide, 1:5 ab:competitor (wt:wt), 1:10 ab:competitor, respectively. **b)** Western blot with H2B pS36 antibody probing histones either left untreated (top panel) or treated with recombinant AMPK (middle panel). **c)** Competition assay for anti-pS36 H2B antibody binding on AMPK-treated H3 and H2B histones (left panel), or *in vivo* histones extracted from cells treated with AICAR (right panel) using pS36 H2B, H2B, or pS10 H3 peptides. **d)** Western blot for pS36 H2B in FLAG-immunoprecipitated nuclear fractions from FLAG-H2B WT or S36A cell lines (* FLAG-H2B; ** endogenous H2B).

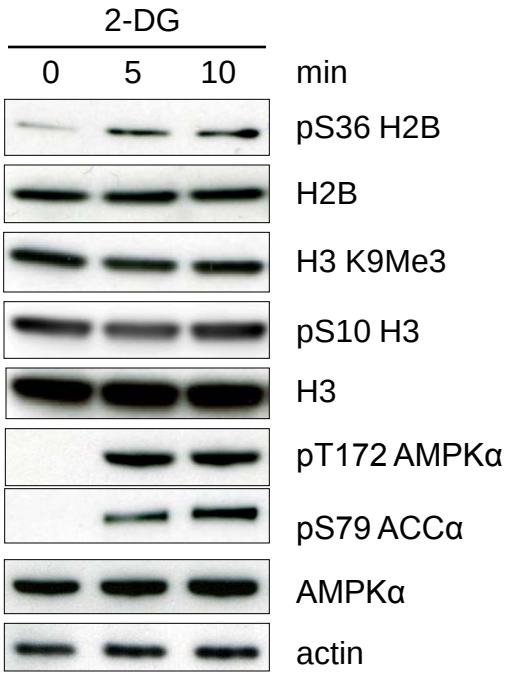

Fig. S17. H2B is an AMPK target. Western blot of H2B pS36 induction in MEFs following 2-DG treatment (25 mM) for the indicated times.

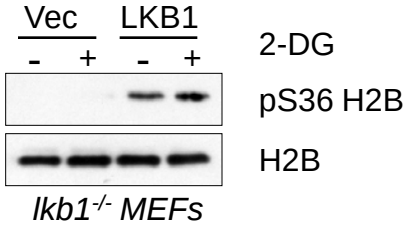

Fig. S18. LKB1 is required for pS36 H2B. Western blot for pS36 H2B in *lkb1*-/- MEFs following 2-DG treatment. Vec, vector control; LKB1, LKB1 re-expressing cells.

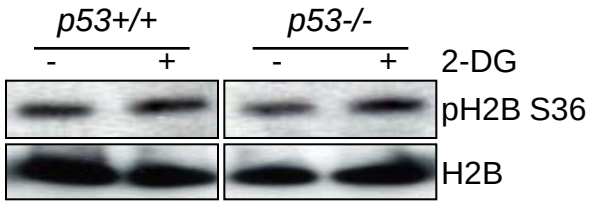

Fig. S19. H2B S36 phosphorylation in p53-deficient MEFs. Wild-type (*p53+/+*) or p53-deficient (*p53-/-*) MEFs were left untreated or treated with 25 mM 2-deoxyglucose (2-DG) for 10 minutes. Histones were isolated by acid extraction, and resolved by SDS-PAGE. Levels of total H2B and phosphorylated H2B (pS36) were determined by Western Blotting using the indicated antibodies. Note: Although we have previously shown that AMPK phosphorylates p53 on Ser-15, experiments to examine the effect of this site on H2B S36 phosphorylation could not be accurately addressed because ectopically expressed p53 S15A in p53-null cells is unstable due to excessive apoptosis.

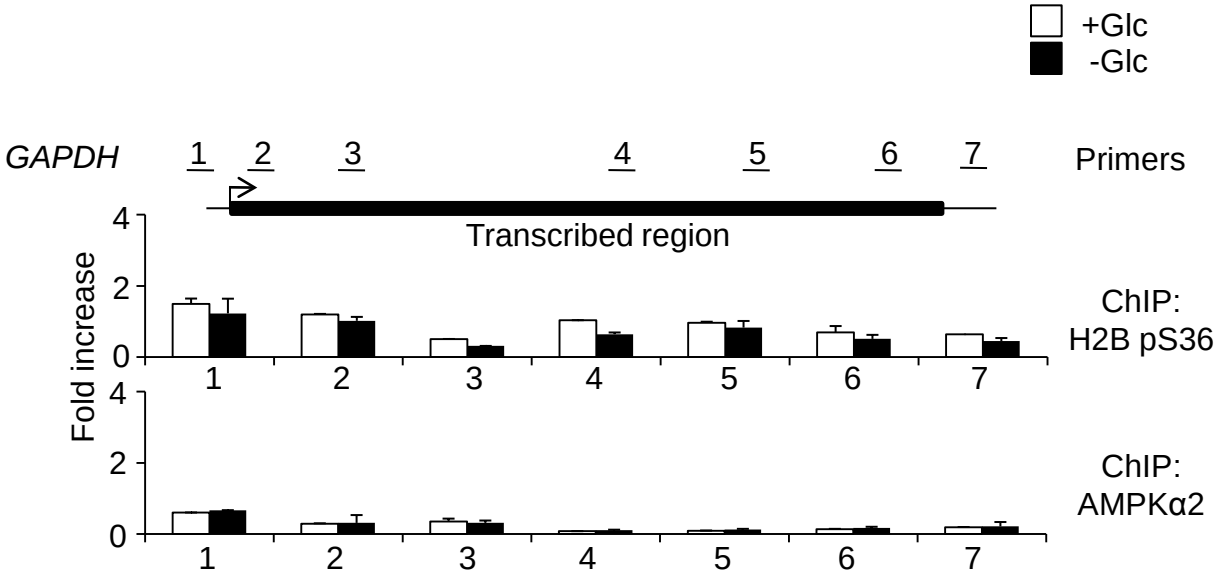

Fig. S20. AMPK and H2B S36p show no specific localization across the GAPDH transcribed region in response to low glucose. WT MEFs were treated with low glucose media for 24 hrs. and the presence of endogenous AMPK and H2B pS36 along the *GAPDH* gene was measured by ChIP and real-time PCR. Data are displayed as fold increase of IP/input over control IgG-IP/input for AMPK and IP/input over H4-IP/input for H2B pS36. Shown is the mean ± SEM for values from three independent experiments.

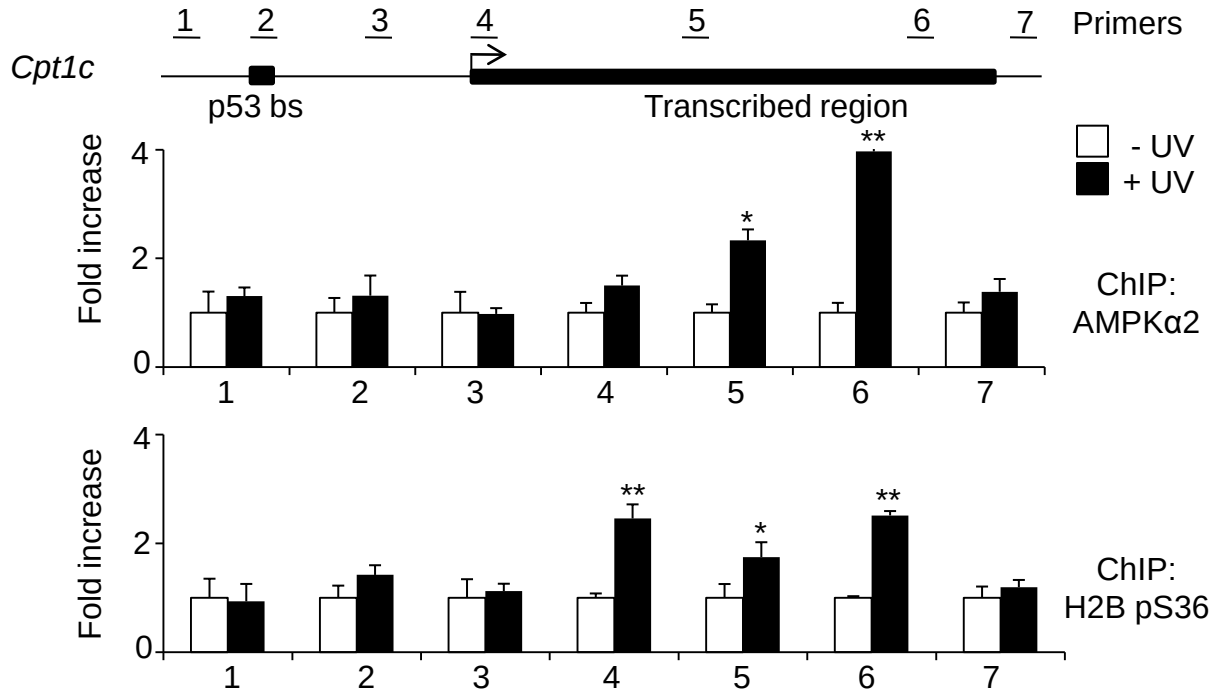

Fig. S21. AMPK and H2B S36p localize across the Cpt1c transcribed region in response to UV. WT MEFs were treated with UV, and the presence of endogenous AMPK and H2B pS36 along the *cpt1c* gene was measured by ChIP and real-time PCR. Data are displayed as fold increase of IP/input over control IgG-IP/input for AMPK and IP/input over H4-IP/input for H2B pS36. Shown is the mean ± SEM for values from three independent experiments. *p<0.05, **p<0.01

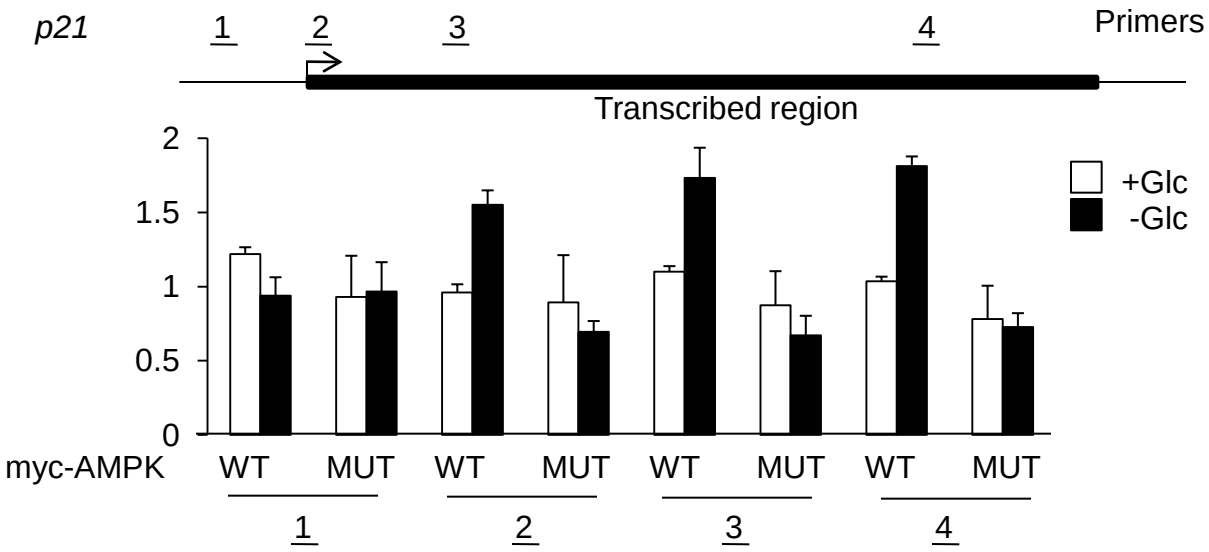

Fig. S22. myc-AMPK localization across the *p21* transcribed region is dependent on its catalytic activity. *ampkα-/-* MEFs were transiently transfected with either wild type (WT) or dominant negative (MUT) myc-AMPK and treated with low glucose. The presence of myc-AMPK along the p21 gene was measured by ChIP and real-time PCR. Data are displayed as fold increase of IP/input over control IgG-IP/input. Shown is the mean ± SEM for values from three independent experiments.

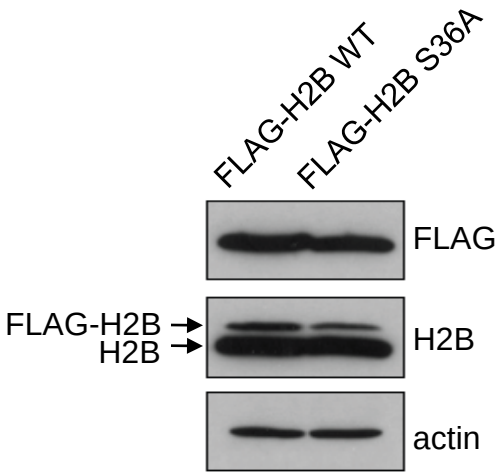

Fig. S23. Characterization of ectopic H2B expression in MEFs by Western Blot. Wild-type MEFs were transduced with retrovirus (MSCV-hygro) expressing either wild-type FLAG-H2B (FLAG-H2B WT) or mutant H2B (FLAG-H2B S36A), and then selected for 14 days in the presence of Hygromycin B. Expression of FLAG-tagged H2B in the indicated whole cell lysates was measured by Western blot using anti-FLAG and anti-H2B antibodies. Both endogenous and ectopically-expressed H2B proteins are detected by anti-H2B antibodies (middle panel).

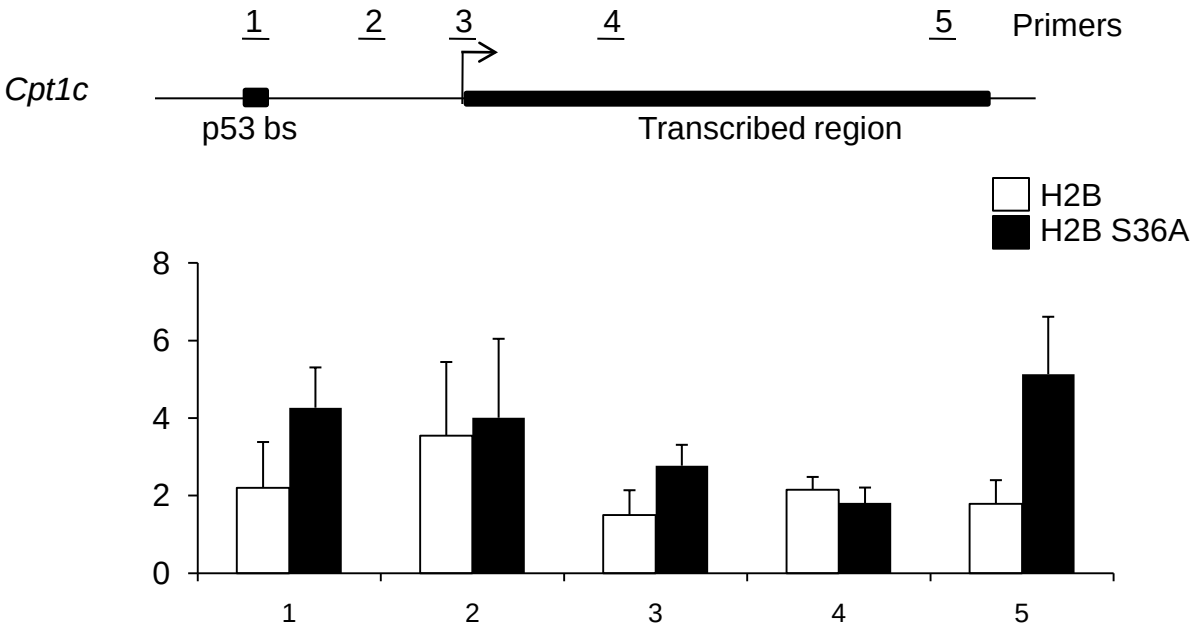

Fig. S24. FLAG-H2B WT and S36A incorporate into chromatin. Wild-type MEFs were transduced with retrovirus (MSCV-hygro) expressing either wild-type FLAG-H2B (H2B) or mutant H2B (H2B S36A). The presence of FLAG-H2B along the *cpt1c* gene was measured by ChIP and real-time PCR. Data are displayed as fold increase of IP/input over control IgG-IP/input. Shown is the mean ± SEM for values from three independent experiments.

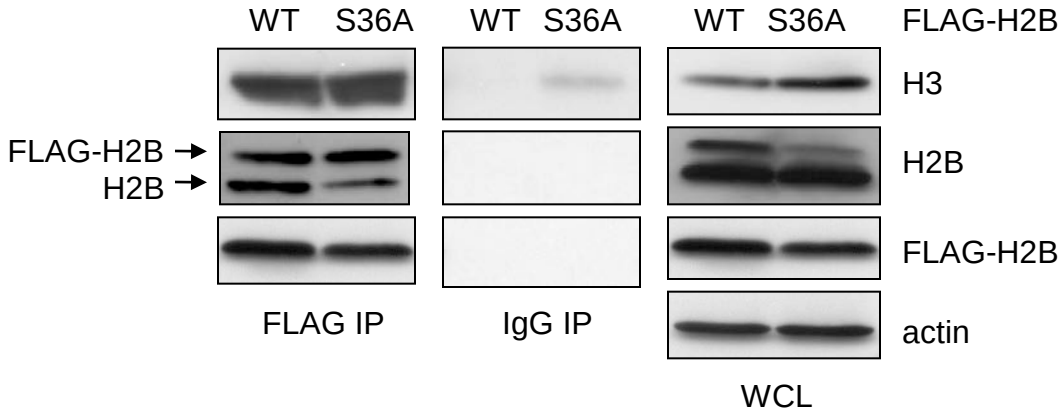

Fig. S25. Incorporation of ectopically-expressed FLAG-tagged histone H2B into chromatin. Actively growing wild-type MEFs expressing either wild-type FLAG-H2B (WT) or S36A mutant H2B (S36A) were lysed in AMPK lysis buffer, and sonicated. Ectopically expressed FLAG-H2B and FLAG-H2B S36A were immunoprecipitated from whole cell lysates using anti-FLAG antibodies or non-specific IgG, and the immunoprecipitates resolved by SDS-PAGE and transfer to nitrocellulose. Association of ectopically-expressed FLAG-tagged H2B histones with endogenous histones was determined by Western blotting using antibodies against histones H3 and H2B. Levels of H3, H2B, FLAG-tagged H2B and actin in whole cell lysates (WCL) are indicated.

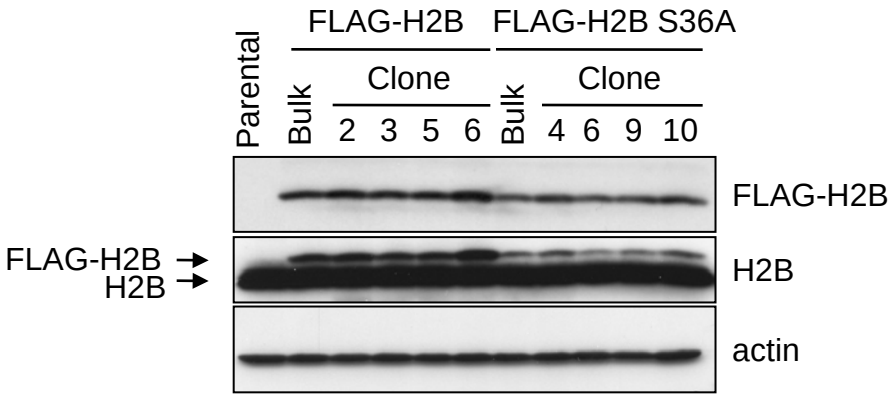

Fig. S26. Characterization of ectopic H2B expression in MEF cell clones. Wild-type MEFs stably expressing wild-type FLAG-H2B or mutant H2B (FLAG-H2B S36A) were subcloned by limiting dilution in the presence of Hygromycin B. Cell clones derived from a single initiating cell were expanded, and expression of FLAG-tagged H2B (top panel), endogenous and ectopically-expressed H2B (middle panel), and actin were measured by Western blot using the indicated antibodies. “Parental” indicates the original wild-type MEF line, “Bulk” indicates the bulk Hygromycin B-selected population for FLAG-H2B or FLAG-H2B S36A cell lines.

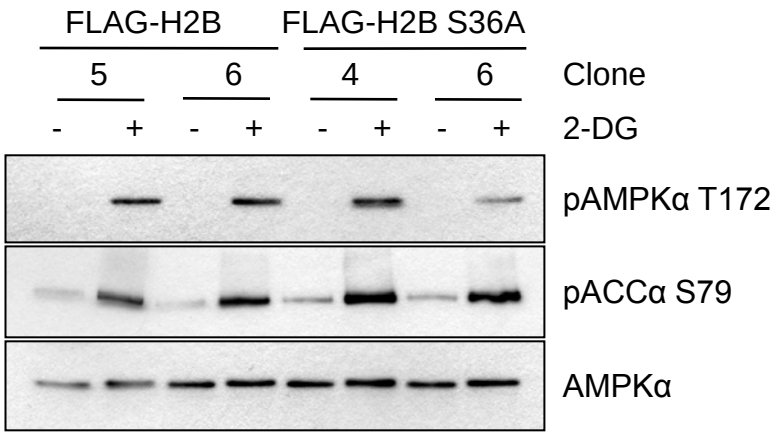

Fig. S27. AMPK activation in FL-H2B and FL-H2B S36A MEF cell clones. FLAG-H2B clones (5 and 6) and FLAG-H2B S36A clones (4 and 6) were left untreated or treated with 25 mM 2-deoxyglucose (2-DG) for 10 minutes. Whole cell lysates were processed and transferred to nitrocellulose, and the levels of total and activated AMPKα (pT172) and phosphorylated ACCα (pS79) were detected by Western Blot.

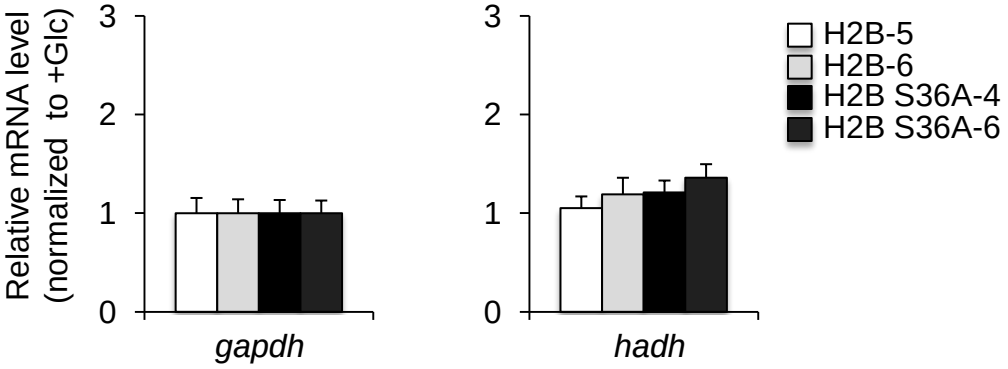

Fig. S28. Relative expression of *gapdh* and *hadh* mRNA in FLAG-H2B and FLAG-H2B S36A expressing clones. MEF clones expressing either wild-type FLAG-H2B (H2B) or mutant H2B (H2B S36A) were grown in the presence or absence of glucose for 24 hours, and total RNA extracted from cells. Relative expression of *gapdh* or *hadh* mRNA (normalized to *hprt*) was determined for each cell clone by quantitative PCR. Data are expressed as the mean fold increase of mRNA levels in –Glc conditions over +Glc conditions for samples in triplicate.

Supplementary References

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