Figures

Fig. S1 Activation of AMPK induces mobility shift in TXNIP in various cell lines, related to Fig. 1.

Western blots of total cell lysate probed with indicated antibodies after treatment of various AMPK activators of ACHN (A), MCF7 (B) and T47D (C) cells: 25mM 2DG for 10', 2mM AICAR for 60', 1mM A769662 for 30'.

Fig. S2 TXNIP S308 phosphorylation by AMPK, related to Fig. 2.

(A). HepG2 cells without and with 20' pretreatment of MG132 to stop protein degradation were then stimulated with A769662. The total cell lysates were analyzed with Western blots using the indicated markers. MG132 treatment alone accumulates the faster migrating TXNIP form while the additional AMPK activation accumulates the slower migrating form of TXNIP. (B). The MS/MS spectrum for the Glu-C digested phosphorylated peptide sequence TSpSMASRTSSE acquired using a hybrid ion trap-orbitrap mass spectrometer via CID. The **b-** and **y-** fragment ion series suggest that the single phosphorylation site is localized to Ser3 in the above sequence (Ser308 in TXNIP). (C). *In vitro* AMPK kinase assay using ^{32}P-ATP with GST-TXNIP WT and S308A mutant fusion protein purified from *E. coli*. (D). *In vitro* AMPK kinase assay on optimal AMPK peptide SAMS, TXNIP WT peptide and TXNIP S308A mutant peptide. (E). 2DG induced degradation of HA-TXNIP WT and PPxY1/2 mutant in Hela cells stably reconstituted with LKB1. Cells were pretreated with cyclohexamide for 20min prior to addition of 25mM 2DG, and harvested at indicated times. MG132 was added as a control to observe the protein species prior to degradation. The HA-tag blot was quantified as

shown in the bottom graph. (PPxY1/2 mutant contains 4 Ala mutations: P332, Y334, P376, Y378.)

Fig. S3 Effect of TXNIP on Glut1, related to Fig. 4.

(A). co-IP of Myc-Glut1 and HA-TXNIP transiently expressed in 293T cells. (B). HepG2 cells stably expressing TXNIP knock-down shRNA 1 and shRNA 1 resistant HA-WT and HA-S308A constructs were tested for the effect of phenformin. Cells were treated with 2mM phenformin for 0', 30' and 60'. Whole cell lysates were blotted with various antibodies as indicated. Quantification of HA-TXNIP blot is shown on the bottom. (C). Glucose uptake was measured on the same stable cells using trace ^{3}H-2DG in media with 1mM glucose and 2mM pyruvate. 2mM phenformin was added to the media at the beginning of the assay and cells harvested by addition of NaOH after each indicated time point. ^{3}H-2DG was added 15min prior to each harvest. The relative radioactivity was normalized to 0' time point for both cells. Experiments were performed in triplicates and the mean ± STDV was reported.

Fig. S4 Our model of TXNIP regulation of Glut1 with AMPK modulation, related to Fig. 4.

Video S1 Confocal time-lapse of GFP-TXNIP on plasma membrane in HepG2 cells

Video S2 TIRF time-lapse of GFP-TXNIP on plasma membrane in HepG2 cells

Video S3 Confocal time-lapse of GFP-TXNIP, mCherry clathrin light chain and Alexa647-transferrin on plasma membrane in HepG2 cells

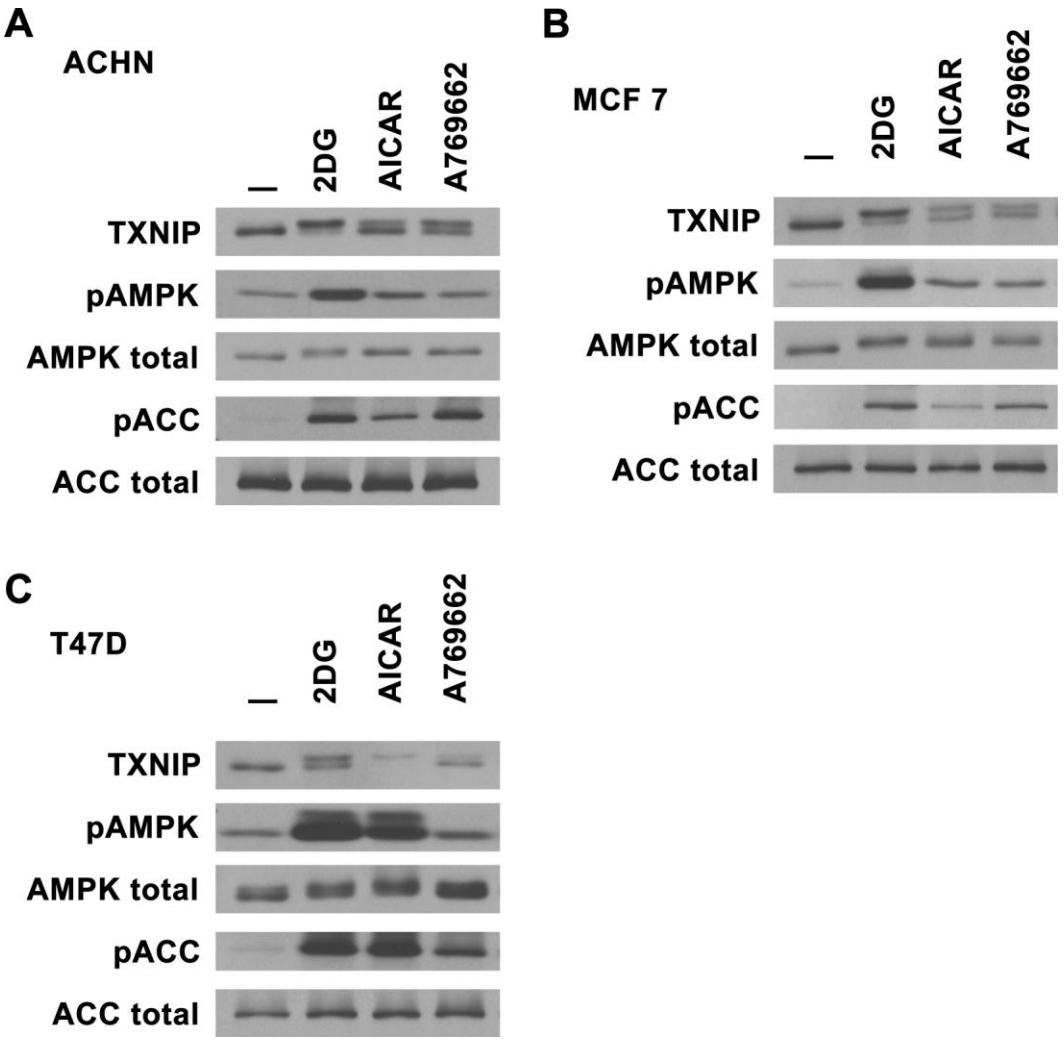
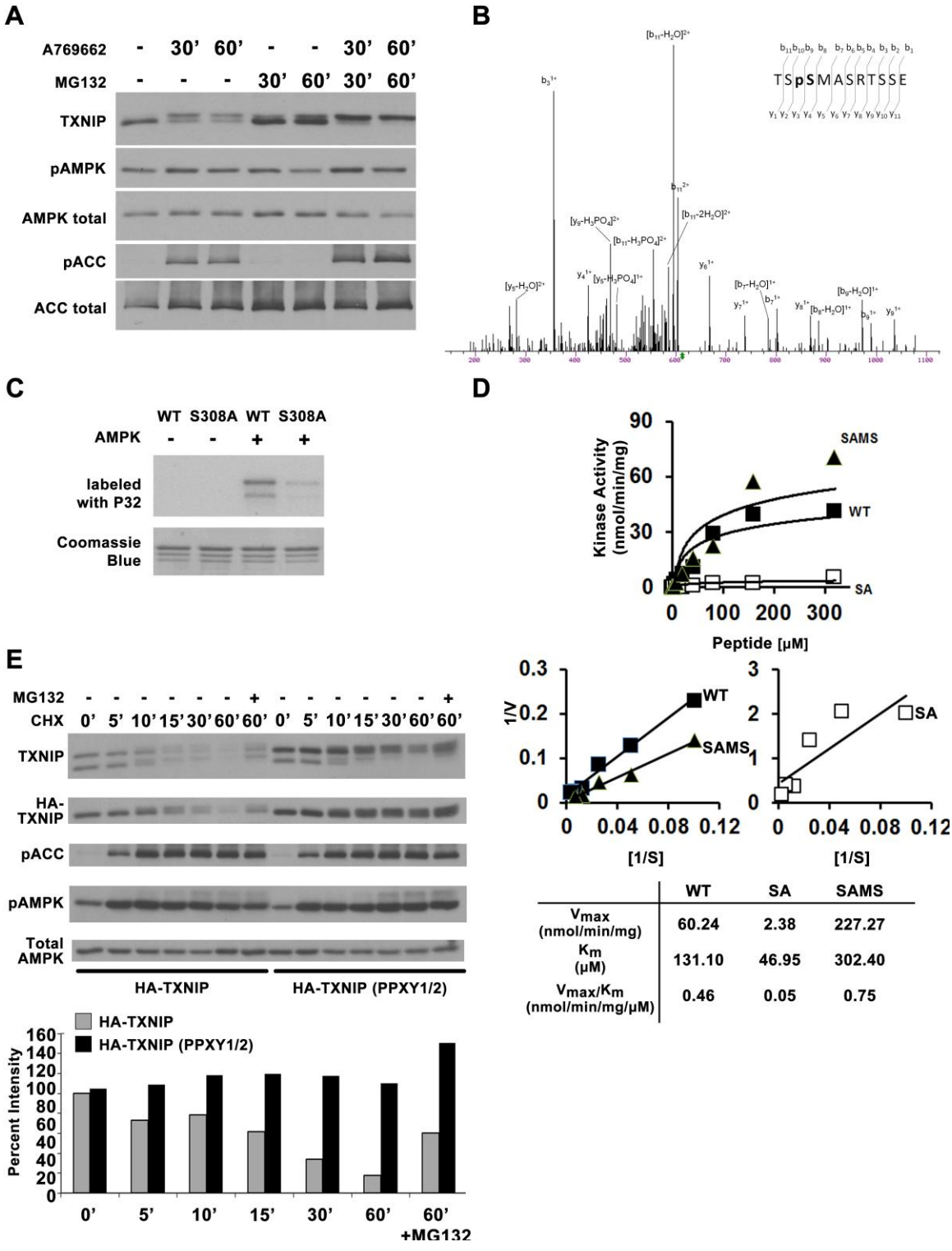

A
ACHN
—
2DG
AICAR
A769662
TXNIP
pAMPK
AMPK total
pACC
ACC total
B
MCF 7
—
2DG
AICAR
A769662
TXNIP
pAMPK
AMPK total
pACC
ACC total
C
T47D
—
2DG
AICAR
A769662
TXNIP
pAMPK
AMPK total
pACC
ACC total

Fig. S1



	WT	SA	SAMS
V_{max} (nmol/min/mg)	60.24	2.38	227.27
K_m (μM)	131.10	46.95	302.40
V_{max}/K_m (nmol/min/mg/μM)	0.46	0.05	0.75

Fig. S2

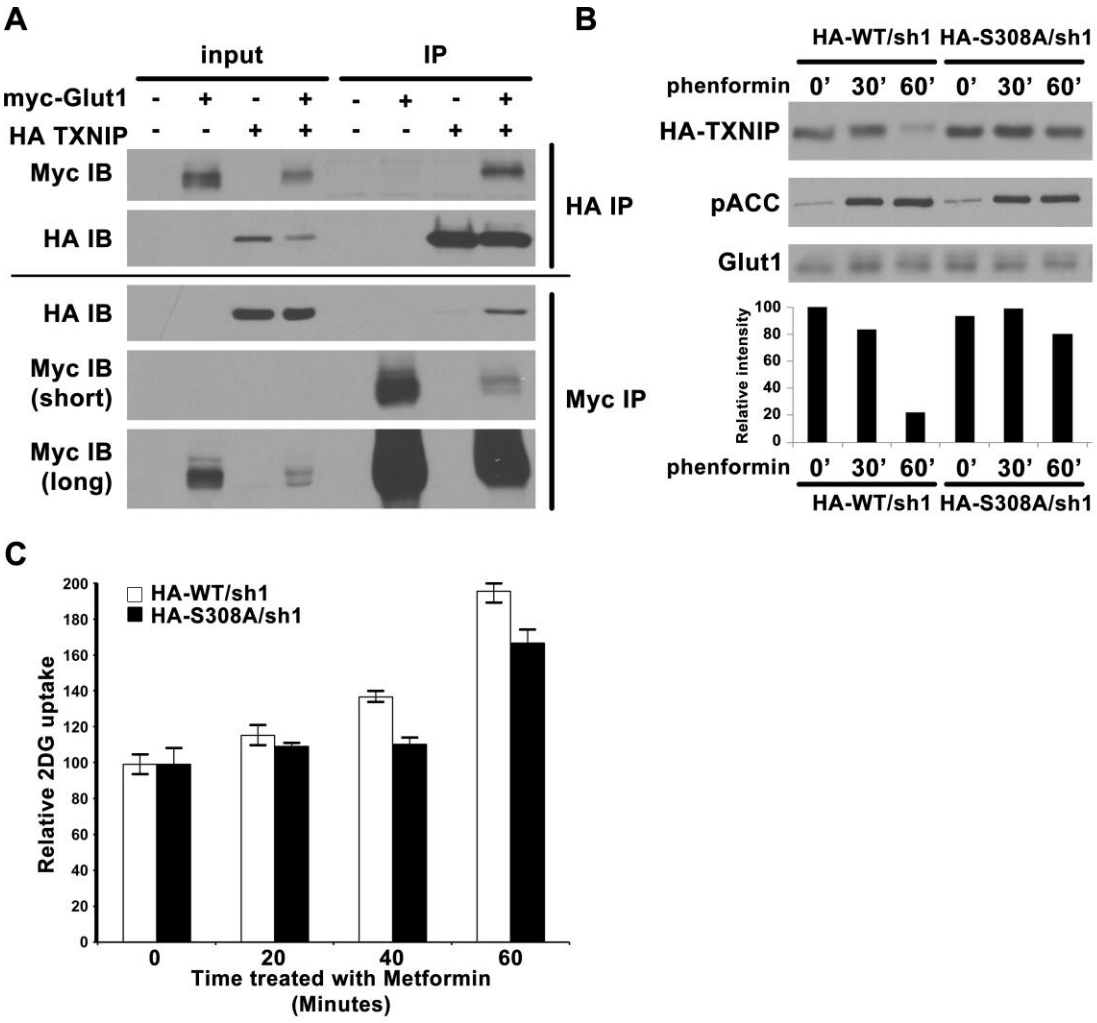

A
input
IP
myc-Glut1 - + - + - + - +
HA TXNIP - - + + - - + +
Myc IB
HA IB
HA IP
HA IB
Myc IB (short)
Myc IB (long)
Myc IP
B
HA-WT/sh1
HA-S308A/sh1
phenformin 0' 30' 60' 0' 30' 60'
HA-TXNIP
pACC
Glut1
Relative intensity
100
80
60
40
20
0
phenformin 0' 30' 60' 0' 30' 60'
HA-WT/sh1
HA-S308A/sh1
C
HA-WT/sh1
HA-S308A/sh1
Relative 2DG uptake
200
180
160
140
120
100
80
60
40
20
0
0
20
40
60
Time treated with Metformin (Minutes)

Fig. S3

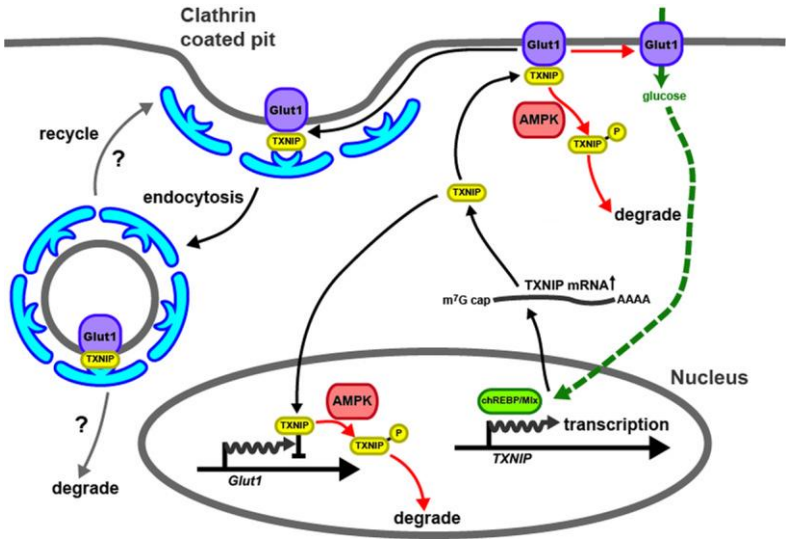

Clathrin
coated pit
Glut1
TXNIP
recycle
?
endocytosis
Glut1
TXNIP
?
degrade
Glut1
TXNIP
Glut1
glucose
AMPK
TXNIP
P
TXNIP
degrade
TXNIP
TXNIP mRNA↑
m7G cap
AAAA
Nucleus
AMPK
TXNIP
TXNIP
P
Glut1
degrade
chREBP/Mlx
transcription
TXNIP

Fig. S4

Experimental procedures

Cloning

Human TXNIP was cloned into a homemade pcDNA3.1+HA vector at HindIII and XhoI sites. The HA-TXNIP fragment was PCR amplified using primers 5'-ggggacaagtttgtacaaaaaagcaggcttcggcaccatggcctacccatacgacgtcccagactacgc and 5'-ggggaccactttgtacaagaaagctgggtctcgagtcactgcacattgttgttgaggatg, recombined into pDONR221, then transferred into pLenti6/UbC/V5-DEST vector (Invitrogen) using the gateway system. To generate the S308A and L351AL352A mutants, site-directed mutagenesis was carried out on pDONR-HATXNIP using primer sets: 5'-ggtctaagcagcagaacatccGCGatggccagccgaaccagctctg and 5'-cagagctggttcggctggccatCGCggatgttctgctgcttagacc for S308A; and primer sets 5'-caccgattggagagcccaaccactcctGCCGCGgatgacatggatggctctcaagac and 5'-gtcttgagagccatccatgtcatcCGCGGCaggagtggttgggctctccaatcggtg for L351AL352A. To generate PPxY1/2 mutant, all 4 residues: P332, Y334, P376 and Y378 are mutated to alanine by site directed mutagenesis. pLentiGFP-TXNIP was made from pDONR-HATXNIP and a homemade pLentiGFP-DEST vector from Wade Harper lab. Similarly, human Glut1 was cloned into pDONR221 and subsequently a modified pLenti6. Double Flag tag, Myc tag or HA tag was inserted after Gly53.

The sense sequences for TXNIP shRNA 1 and shRNA 2 are: 5'-AGGAAGATGAAAAGGGAAA and 5'-GAGCTGATTTGATGGACAA.

Live cell imaging and TIRF

For confocal images, cells were grown on 1.5mm glass cover slips and mounted on a heated stage. Images were collected with a Yokogawa spinning disk confocal on a Nikon Ti inverted microscope equipped with 60x Plan Apo objective lens and the Perfect Focus System. For the transferrin uptake, Alexa647 conjugated transferrin was purchased from Invitrogen. Cells were washed with ice-cold media and incubated with media containing 25μg/ml labeled transferrin at 4^{o}C for 1h. The heated stage was cooled before mounting the glass cover slip and the imaging was carried out at room temperature.

For TIRF images, cells were grown in glass bottom wells. The whole imaging stage was temperature and CO_2 controlled. A system equipped with Nikon Ti-E motorized inverted microscope with the Perfect Focus System, Hamamatsu ORCA-R2 cooled CCD camera (widefield) and 100x objective oil lens was used. All images were collected and analyzed with MetaMorph software.

In vitro kinase assay

GST-tagged WT and S308A TXNIP proteins were incubated with purified AMPK proteins (α1/β1/γ1, Millipore,14-840) in kinase buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 1 mM DTT, 100 μM AMP and 50 μM ATP) in the presence of 1 μCi γ-^{32}P-ATP at 30°C for 30 min, followed by SDS-PAGE and autoradiography.

AMPK substrate peptide SAMS (HMRSAMSGLHLVKRR), TXNIP-WT (RSGLSSRTSSMASRTS) and TXNIP-SA (RSGLSSRTSAMASRTS) peptides were synthesized from Thermo Scientific. Various concentrations of TXNIP-WT or TXNIP-SA peptides were incubated with 50 ng AMPK in 25 μl reaction buffer (25 mM MOPS

pH 7.4, 25 mM $MgCl_2$, 12.5 mM β-glycerol-phosphate, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT, 100 μM AMP and 50 μM ATP solution containing [γ-^{32}P] ATP (0.167 μCi/μl)). Reactions were started by adding the ATP solution and incubated for 20 minutes at 30^{o}C. To terminate the reactions, 20 μl of reaction mixture were spotted onto phosphocellulose P81 paper. Air-dried P81 paper was sequentially washed 4 times for 10 minutes in 1% phosphoric acid. The radioactivity that had been incorporated in the peptides was determined by liquid scintillation counting of the P81 paper.

Tandem mass spectrometry

Coomassie-stained SDS-PAGE gel bands containing TXNIP were excised and subjected to reduction with DTT and alkylation with iodoacetamide, then in-gel digestion with endoproteinase Glu-C overnight at pH 8.3. Reversed-phase microcapillary liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify phosphorylated and non-phosphorylated TXNIP sequences using a EASY-nLC nano HPLC (Thermo Fisher Scientific, San Jose, CA) with a self-packed 75μm-id × 15-cm C_{18} column connected to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) in Top 6 (1 FT-MS scan followed by 6 IT-MS/MS scans) data-dependant acquisition and positive ion mode at 300 nL/min. MS/MS spectra collected via collision-induced dissociation (CID) in the ion trap were searched against the concatenated target and decoy (reversed) Swiss-Prot protein database (v. 2011_07) and the single entry TXNIP protein database using Sequest [Proteomics Browser Software (PBS), Thermo Scientific] with differential modifications for Ser/Thr/Tyr phosphorylation (+79.97) and the sample processing artifacts Met oxidation (+15.99), Cys alkylation (+57.02) and

deamidation of Asn and Gln (+0.984). Phosphorylated and unphosphorylated peptide sequences were identified if they initially passed the following Sequest scoring thresholds: 1+ ions, Xcorr ≥ 2.0 Sf ≥ 0.4, p ≥ 5; 2+ ions, Xcorr ≥ 2.0, Sf ≥ 0.4, p ≥ 5; and 3+ ions, Xcorr ≥ 2.60, Sf ≥ 0.4, p ≥ 5 against the target protein database. Passing MS/MS spectra were manually inspected to be sure that all **b-** and **y**-fragment ions aligned with the assigned sequence and modification sites. Determination of the exact sites of phosphorylation was aided using *FuzzyIons* and *GraphMod* software (PBS, Thermo Fisher Scientific). Peptide false discovery rates (FDR) were calculated to be less than 1.5 %.

2DG uptake assay

^{3}H-2DG was purchased from PerkinElmer (NET549A250UC). Cells were washed and kept in DMEM with 10% dialyzed serum and various amount of glucose (see text) depending on the assay. 0.05μM ^{3}H-2DG was added for 30' (Fig. 4C) or 15' (Fig. S6) before the cells were washed and lysed with 0.2N NaOH, neutralized and spun down. Clear lysate was counted in a scintillation counter.