Cell Reports

Supplemental Information

AMP-activated Protein Kinase Directly Phosphorylates and Destabilizes Hedgehog Pathway Transcription Factor GLI1 in Medulloblastoma

Yen-Hsing Li, Jia Luo, Yung-Yi C. Mosley, Victoria E. Hedrick, Lake N. Paul, Julia Chang, GuangJun Zhang, Yu-Kuo Wang, Max R. Banko, Anne Brunet, Shihuan Kuang, Jen-Leih Wu, Chun-Ju Chang, Matthew P. Scott and Jer-Yen Yang

Supplementary Experimental Procedures

Reagents and plasmids

AMPK activators 2DG and metformin were purchased from Sigma and AICAR from Calbiochem and A-769662 were purchase from Selleckchem. Cycloheximide (CHX) and glucose were purchased from Sigma. The dual-luciferase assay kit was purchased from Promega. The Gli1-luciferase reporter contained eight directly repeated copies of the consensus Gli1 binding site (Sasaki et al., 1997). GLI1 HA-tagged, Flag-tagged and pCDH-CMV-MCS-EF1-Puro constructs were gifts from Dr. Mien-Chie Hung (Wang et al., 2012) (UTMDACC, Houston, TX). LKB1 shRNA plasmids were gifts from Dr. Hui-Kuan Lin (UTMDACC, Houston, TX). Mutated constructs derived from the control HA-tagged and Flag-tagged $GLI1^{3A}$ and $GLI1^{3E}$ were generated using the Quick Change multisite-directed mutagenesis kit from Stratagene. NheI-forward primer: 5' GGCGAGCTAGCATGGACTACAAAGACCATGAC 3' and BstBI-reverse primer: 5' AGTATTCGACACCCCGGATCCTC 3' were used to subclone the $GLI1^{WT}$, $GLI1^{3A}$ and $GLI1^{3E}$ into pCDH-CMV-MCS-EF1-Puro.

Cell Lines

NIH 3T3 cells were obtained from ATCC. Shh-Light2 cells containing stably transfected Gli-dependent firefly luciferase and constitutively active Renilla luciferase constructs, and 293T cells for producing Shh-conditioned media, have been described previously (Rohatgi et al., 2007). The latter two cell lines were kindly provided by Drs. James Chen and Phil Beachy. $PZp53^{MED}$ cells were obtained from Dr. Phil Beachy (Corcoran and Scott, 2006). AMPKα wild-type and null MEFs were gifts from Dr. Keith R. Laderoute (Laderoute et al., 2006).

Phospho-GLI1 antibody preparation

Phospho-antibodies against GLI1 S102, S408, and T1074 were generated by the Biosmart company and tested in our laboratory but only T1074 worked fine (Taiwan, ROC). Ser/Thr-phosphorylated and non-phosphorylated peptides corresponding to GLI1 amino acid sequences (S102: CTVIRT(pS)PSSLV, S408: CLPRAP(pS)ISTVE, T1074: CGSSGH(pT)PPPSG) were synthesized. Two rabbits for each phospho-peptide were immunized with keyhole limpet hemocyanin (KLH)–phospho-peptide conjugate mixed with Freund's complete adjuvant, and bled 7days after the last immunization. Phospho-peptide reactive antibodies were captured using a column containing phosphopeptide-conjugated sepharose. The antibodies were eluted, and those reactive to sequences other than phospho-serine/threonine were removed using a column containing non-phosphorylated peptides. Specific reactivity with the targeted phospho-serine/threonine sequence was confirmed by an ELISA in which phosphorylated and non-phosphorylated peptides were attached to the surface. The phospho-GLI1 antibodies isolated from one of the three rabbits that had responded to inoculation were further verified by immunoprecipitation and western blots to test their specificity (Fig 3G, S3G).

Nanoelectrospray mass spectrometry

A nanoscale capillary LC-MS/MS analysis was performed using an ultimate capillary LC system (LC Packings) coupled to a $QSTAR_{XL}$ quadrupole (Q)–time-of-flight (TOF) mass spectrometer (Applied Biosystems). The nanoscale capillary LC separation was performed on an RP C18 column (15 cm × 75 m i.d.) with a flow rate of 200 nl min^{-1} and a 70-minute linear gradient of 5–50% buffer B. Buffer A contained 0.1% formic acid in 2% aqueous ACN, and buffer B contained 0.1% formic acid in 98% aqueous ACN. The nanoscale capillary LC tip used for online LC-MS was a PicoTip (FS360-20-10-D-20; New Objective). Data were acquired using an automatic information-dependent acquisition system (Applied Biosystems), which locates the most intense ions in a TOF

MS spectrum and performs an optimized MS/MS analysis on the selected ions. The product ion spectra generated by nanoscale capillary LC-MS/MS were searched against National Center for Biotechnology Information databases for exact matches using the ProID (Applied Biosystems) and MASCOT search programs (Hirosawa et al., 1993). A *Homo sapiens* taxonomy restriction was used, and the mass tolerance of both precursor ions and fragment ions was set to ± 0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, and serine, threonine, and tyrosine phosphorylation were set as variable modifications. All phosphopeptides identified were confirmed by manual interpretation of the spectra.

Lentivirus infection

pCDH-CMV-MCS-EF1-Puro GLI1-lentivirus packaging and infection was done according to the manual from SBI System Bioscience (Cat. #sCD500–CD700). Infected NIH-3T3 cells were treated with 2.5 ug/ml puromycin for two weeks to eliminate non-infected cells.

In vitro kinase assay

Immunoprecipitated GLI1-flag wild-type and mutant proteins were incubated with active recombinant AMPK protein (GenScript, NJ, USA) in the presence of 50 mM ATP in a kinase buffer containing 5 μCi γ-^{32}P-ATP for 30 min at 30 °C. Reaction products were resolved by SDS–PAGE, and products labeled with ^{32}P were visualized by autoradiography.

Animal studies

10^7 3T3 cells that stably produced one of the GLI1 protein variants were subcutaneously injected into nude mice (*n* = 10 per group). Tumor size was measured twice per week

with a caliper, and tumor volume was determined using the formula: $l \times w^2 \times 0.52$, where l is the longest diameter and w is the shortest diameter.

Zebrafish treatment

5mM 2DG was directly incubated with the embryos for indicated hours. Embryos were dechorionated in an Eppendorf tube using a plastic pipette, and placed on ice for sedation. Deyolking buffer was prepared as follows: 10 ml of cold PBS with 0.3 mM PMSF and 1 mM EDTA). Water bathing the embryos was removed as much as possible before addition of the deyolking buffer. The yolks were then removed by triturating with a 200 µl pipette tip. Deyolking fluid was then removed, as much as possible, from the tube and samples were then rinsed twice with ice-cold PBS. After removal of excess liquid, samples in the Eppendorf tubes were frozen in liquid nitrogen before storage at -80C.

Zebrafish MO injection

The 0.01 uM of ampka1 antisense Morpholino (MO) (5′ CAGATCATGTTTACTCACAGTTTGA′) and MO with five base pair mismatches (5′-CACATCATCTTTAGTCAGACTTTGA-3′) (Gene Tools, LLC) were administered by microinjection was performed at the one-cell stage as previously described (Li et al., 2013)

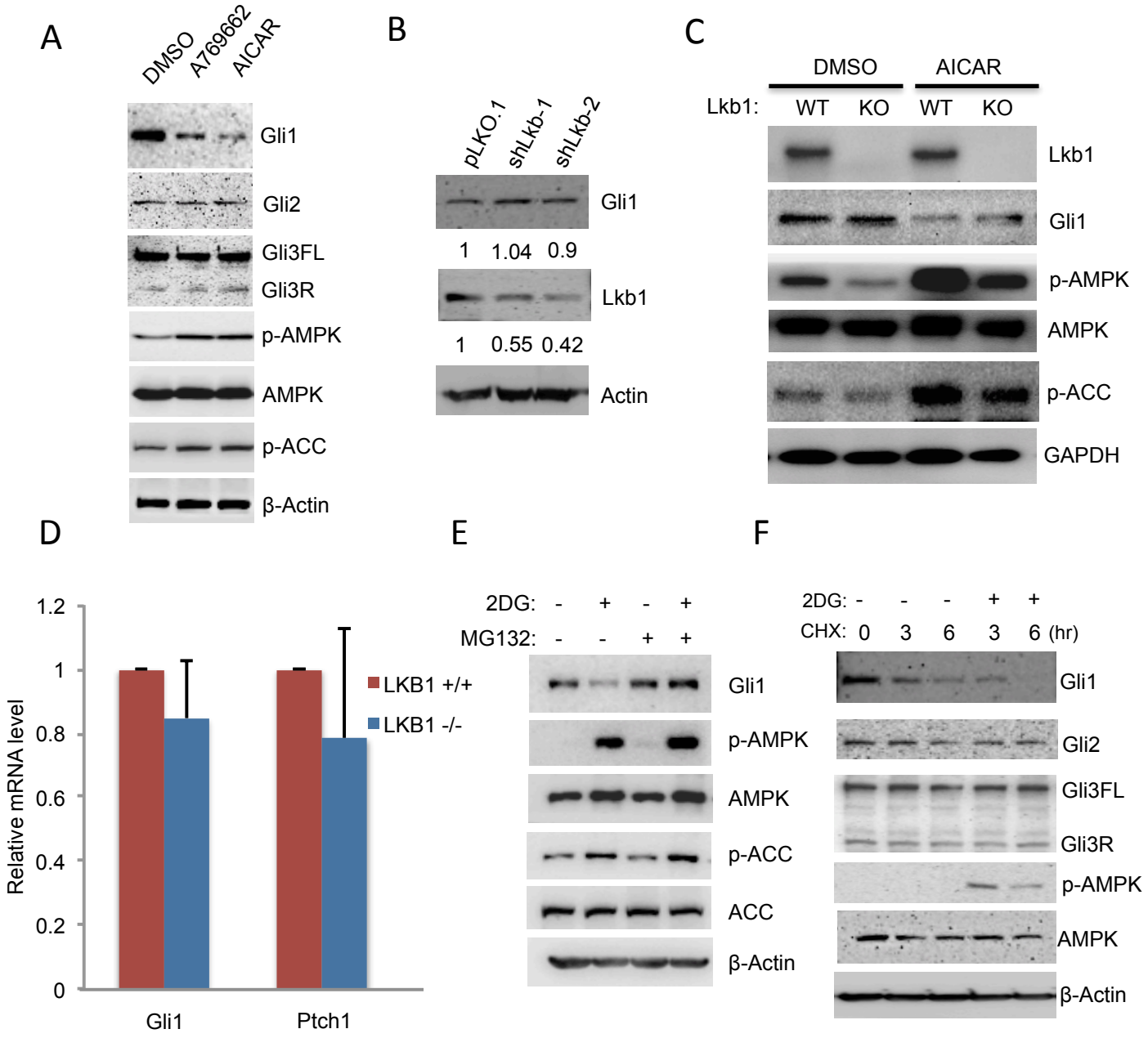


Figure S1 (refers to Figure 1). Activation of AMPK inhibits Gli1 but not Gli2 and Gli3 protein levels and Stability, and it is not through Lkb1. (A) Med1 cells were treated with 25mM A769662 and 0.75mM AICAR for 4 hours. Harvested cells lysates were subject to immunoblotting with the indicated antibodies. To compare Gli1, 2 and 3 in the same blotting, Gli1, p-AMPK, p-ACC were from figure 1B. (Gli3FL: Full length of Gli3; Gli3R: repressor form of Gli3) **(B)** Knocking down Lkb1 was not affected Gli1 protein level in Med1 cell. **(C)** *Lkb1*$^{+/+}$ and *Lkb1* $^{-/-}$ myoblast cells were maintained for 6 hours with or without 0.75 mM AICAR. Harvested cells lysates were subject to immunoblotting with the indicated antibodies. **(D)** Amount of *Gli1* or *Ptch1* RNA was analyzed by RT-qPCR from *Lkb1*$^{+/+}$ and *Lkb1* $^{-/-}$ myoblast cells. Three replicate experiments were done with standard deviations. **(E)** Med1 cells were treated with 25mM 2DG for 4 hours with or without 10μM MG132. Harvested cells lysates were subject to immunoblotting with the indicated antibodies. **(F)** Med1 cells were harvested at different times after treatment with cycloheximide (CHX, 1 μg ml^{-1}) and with or without 25mM 2DG, and analyzed by immunoblot with the indicated antibodies.

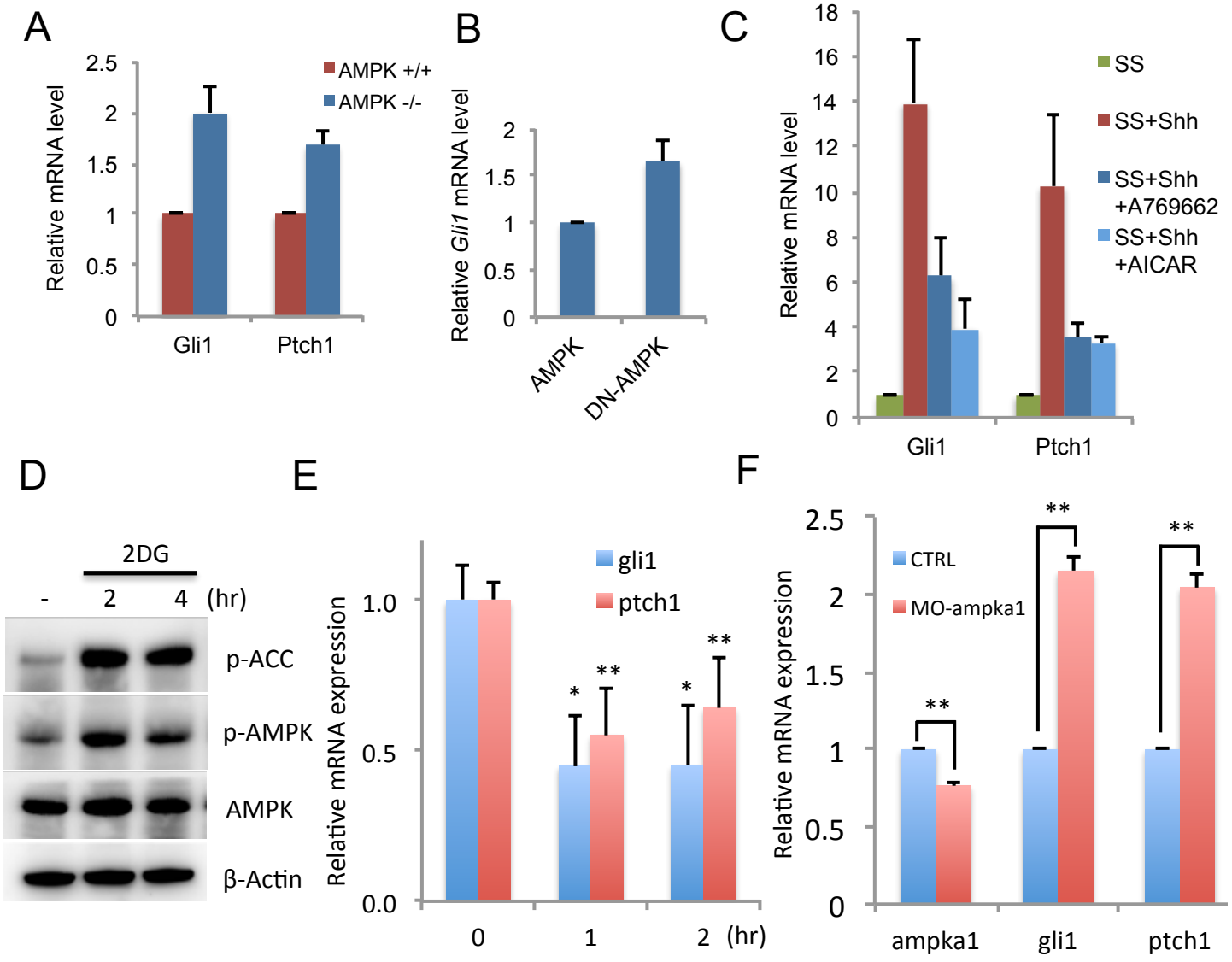


Figure S2 (refers to Figure 2). AMPK suppresses Hh/Gli1 transcriptional activity in cells and zebrafish embryos. (A) *AMPK*$^{+/+}$ and *AMPK* $^{-/-}$ MEFs were culture in 10% FBS and amount of *Gli1* or *Ptch1* RNA was analyzed by qRT-PCR and normalized with AMPK$^{+/+}$ MEFs. **(B)** 293 Cells were transfected with WT and DN-AMPK, and amount of RNA was analyzed by qRT-PCR and normalized with WT AMPK. **(C)** NIH3T3 cells were serum-starved (SS) in DMEM (0.5% bovine calf serum) overnight, stimulated with Shh with or without A769662 (150μM) and AICAR (0.75mM) for 6 hours, and the amount of *Gli1* or *Ptch1* RNA was analyzed by qRT-PCR and normalized to *Gli1* and *Ptch1* mRNA levels in serum-starved cells. All above are three replicate experiments and with standard deviations. **(D)** 2 hpf-zebrafish embryos were treated with 5 mM 2DG for 2 and 4 hours. After deyolking, protein was extracted for Western blotting analysis. **(E)** 2 hpf-zebrafish embryos were treated with 5 mM 2DG for 1 and 2 hour. RNA was extracted and reverse-transcribed for qPCR analysis. The *gli1* and *ptch1* mRNA expression were suppressed compared with control at 22 hpf. Relative expression is normalized with beta-actin. *, p<0.05; **, p<0.01. N=3 **(F)** The 0.01 uM of ampka1 antisense Morpholino (MO) and MO with five base pair mismatches were administered by microinjection at the one-cell stage. Total RNA was extracted and reverse-transcribed for qPCR analysis. The ampka1, gli1 and ptch1 mRNA expression were compared with control MO administration. Relative expression is normalized with beta-actin. *, p<0.05; **, p<0.01. N=3

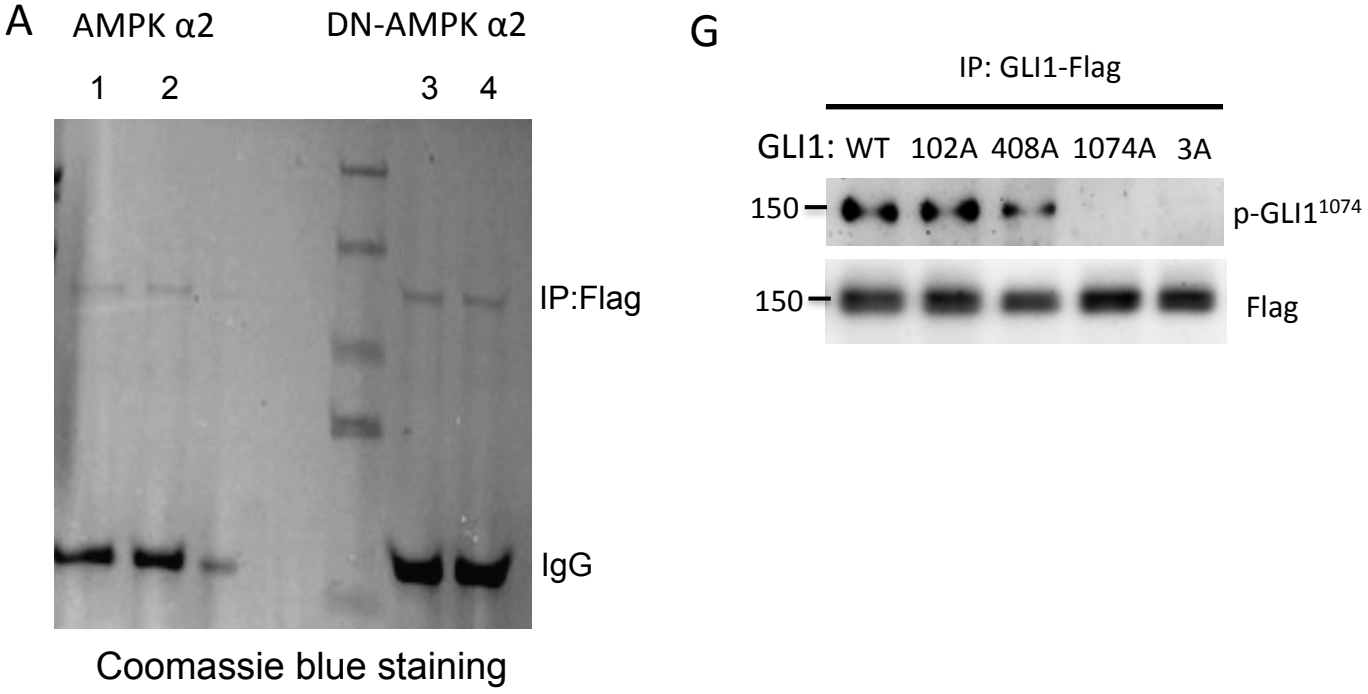
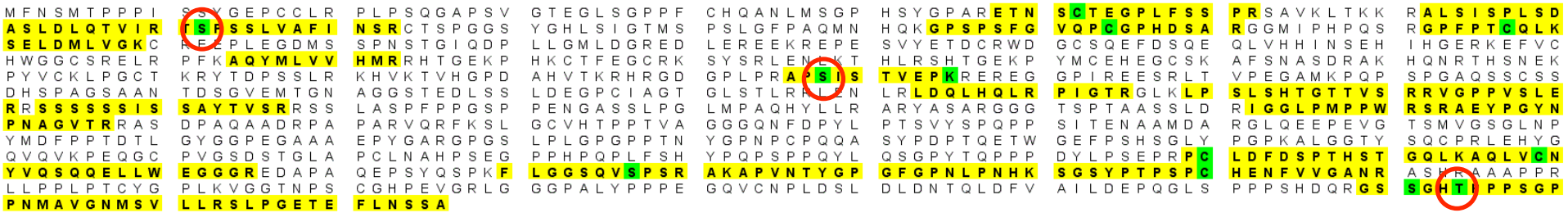


Figure S3 (refers to Figure 3). Identify AMPK-mediated GLI1 phosphorylation sites by mass spectrometry. HEK293 cells were co-transfected with Flag-tagged GLI1 and AMPKα2 (Lane 1 and 2), β1, γ1 or DN- AMPKα2 (Land 3 and 4), β1, γ1. Cells were lysed in NP40 lysis buffer. The lysates were subjected to immunoprecipitation using the antibody to Flag-tag, and after SDS-PAGE and Coomassie blue staining the GLI1-Flag band was isolated and subjected to mass spectrometry **(A)**. The data were further analyzed using Scaffold 3 software. The yellow-labeled amino acids were detected and analyzed by mass spectrometry; green labeling indicates the modified amino acids. Ser 102, Ser 408, and Thr 1074 are labeled with red circles; these were phosphorylated when AMPK was present **(B, C)** but not if DN-AMPK was expressed **(D)**. Mouse embryonic fibroblast (MEF) cells were infected with lenti-virus containing Flag-tagged GLI1 gene and selected with puromycin for a week. Cells were treated with **(E)** or without **(F)** 2DG (25mM) for 30 mins and lysed in NP40 lysis buffer. The lysates were subjected to immunoprecipitation using antibody to Flag-tag, and the GLI1-Flag band was isolated and subjected to mass spectrometry. Peak areas indicated the peptide was phosphorylated. (n/d : Non detected) **(G)** HEK293 cells were transfected with Flag-tagged $GLI1^{WT}$, $GLI1^{102A}$, $GLI1^{408A}$, $GLI1^{1074A}$ and $GLI1^{3A}$. Cells were lysed in NP40 lysis buffer. The lysates were subjected to immunoprecipitation using the antibody to Flag-tag and immunoblotting with p-$Gli1^{1074}$ antibody.

Figure S3 Continue

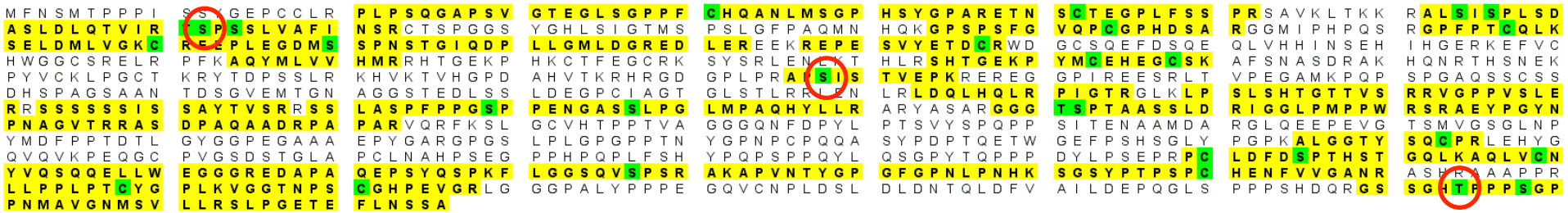
B AMPK α2

IPI00027451 (100%), 117,904.4 Da
RecName: Full=Zinc finger protein GLI1; AltName:
24 unique peptides, 37 unique spectra, 141 total spectra, 306/1106 amino acids (28% coverage)



C AMPK α2

IPI00027451 (100%), 117,904.4 Da
RecName: Full=Zinc finger protein GLI1; AltName:
40 unique peptides, 61 unique spectra, 212 total spectra, 521/1106 amino acids (47% coverage)



D DN-AMPK α2

IPI00027451 (100%), 117,904.4 Da
RecName: Full=Zinc finger protein GLI1; AltName:
23 unique peptides, 25 unique spectra, 48 total spectra, 337/1106 amino acids (30% coverage)

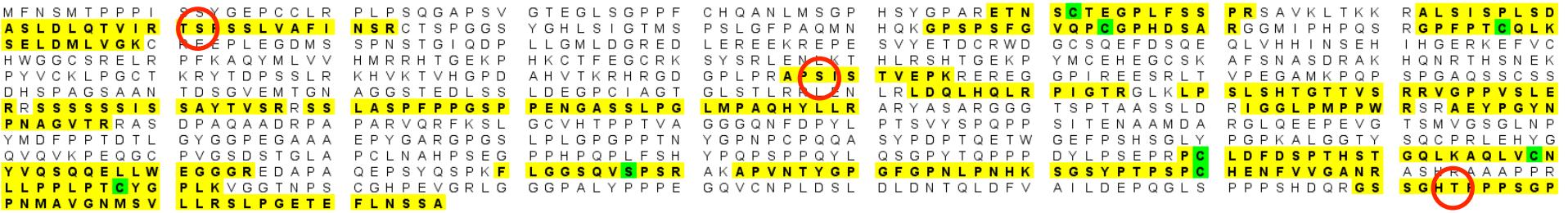


Figure S3 Continue

E Peak areas of GLI1 with 2DG treatment

Peptide	m/z	Phosphorylation Position	Peak Areas
TSPSSLVAFINSR	689.86719^{2+}	S102	3342000
TpSPSSLVAFINSR	729.85846^{2+}	pS102	859300
		Ratio (S/pS)	**3.889212149**

Peptide	m/z	Phosphorylation Position	Peak Areas
APSISTVEPK	514.78497^{2+}	S408	2820000
APpSISTVEPK	554.78019^{2+}	pS408	12051.77
		Ratio (S/pS)	**233.9905259**

Peptide	m/z	Phosphorylation Position	Peak Areas
GSSGHTPPPSGPPNMoxAVGNMoxSVLLR	831.40359^{3+}	T1074	814900
GSSGHpTPPPSGPPNMoxAVGNMoxSVLLR	858.06103^{3+}	pT1074	138600
		Ratio (T/pT)	**5.87950938**

F Peak areas of GLI1 without 2DG treatment

Peptide	m/z	Phosphorylation Position	Peak Areas
TSPSSLVAFINSR	689.86719^{2+}	S102	184000
TpSPSSLVAFINSR	n/d	pS102	n/d
		Ratio (S/pS)	**n/d**

Peptide	m/z	Phosphorylation Position	Peak Areas
APSISTVEPK	514.7848^{2+}	S408	480600
APpSISTVEPK	n/d	pS408	n/d
		Ratio (S/pS)	**n/d**

Peptide	m/z	Phosphorylation Position	Peak Areas
GSSGHTPPPSGPPNMoxAVGNMoxSVLLR	n/d	T1074	n/d
GSSGHpTPPPSGPPNMoxAVGNMoxSVLLR	n/d	pT1074	n/d

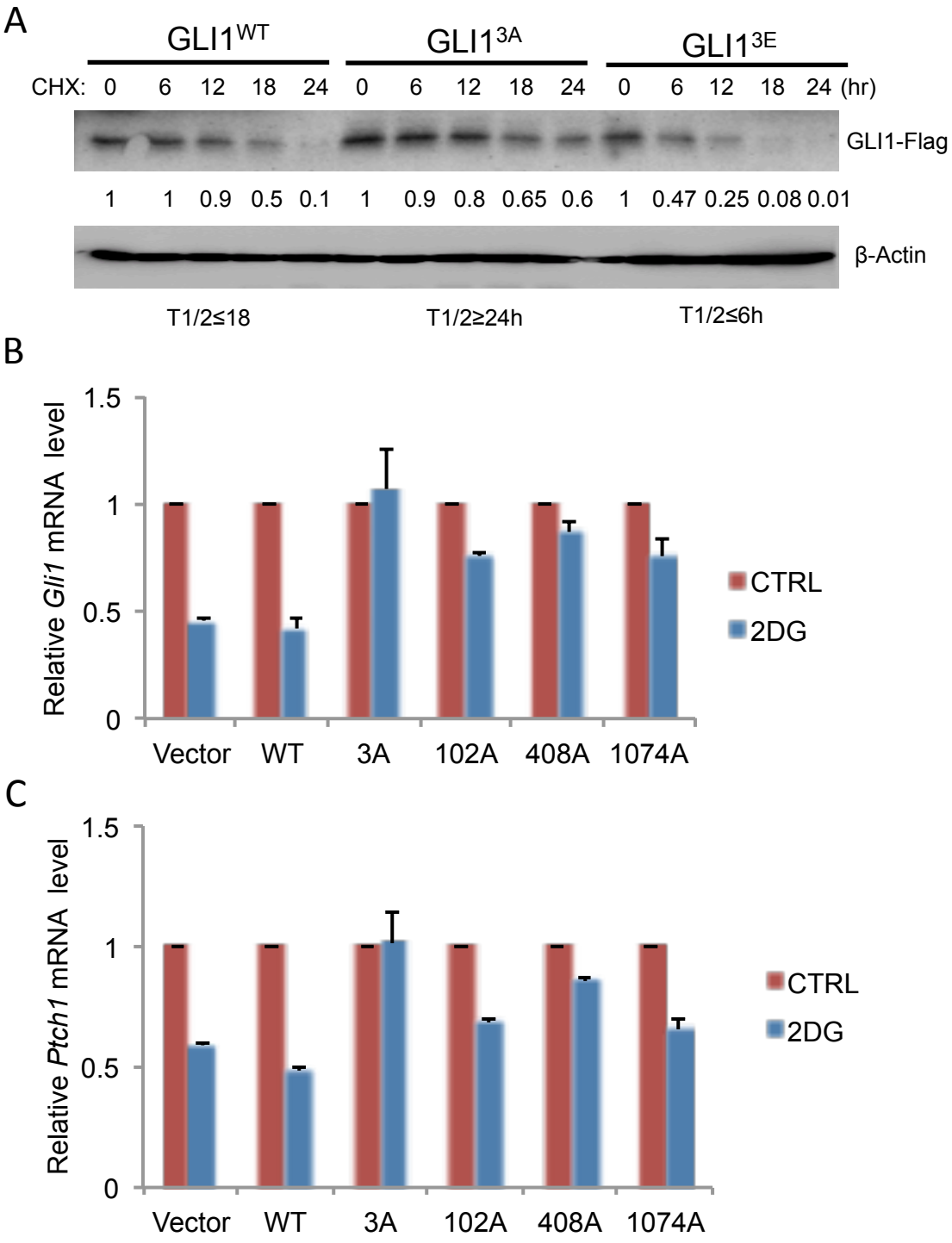


Figure S4 (refers to Figure 4). GLI1^{3A} has longer protein stability and resistant to AMPK-mediated inhibition of Gli1 transcriptional activity. **(A)** Lysates of NIH 3T3 GLI1 stable cell lines were harvested at different times after treatment with cycloheximide (CHX, 1 μg ml^{-1}) and analyzed by immunoblot. NIH3T3 cells were transfected with vector control, *GLI1^{WT}*, *GLI1^{3A}*, *GLI1^{102A}*, *GLI1^{408A}*, or *GLI1^{1074A}* for 36 hours, then treated with or without 2DG (25mM) for 4 hours. RT-PCR was used to measure **(B)** *Gli1* mRNA and **(C)** *Ptch1* mRNA. The experiment was repeated three times.

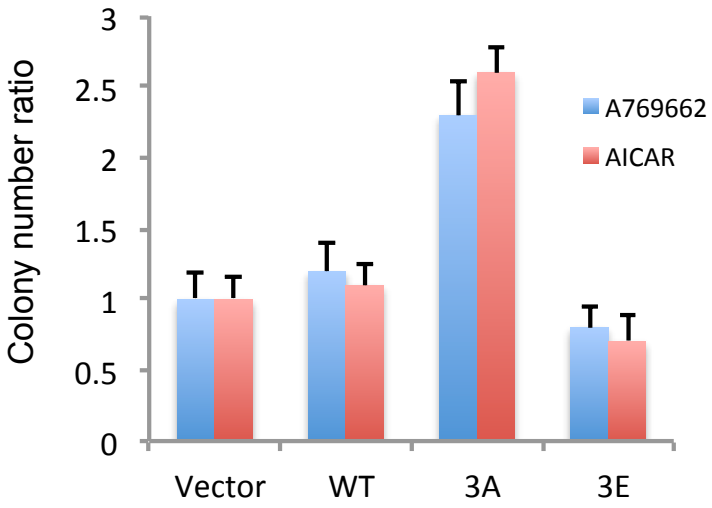


Figure S5 (refers to Figure 5). GLI1^{3A} was resistant to growth suppression of AMPK activators. NIH3T3 cells with wild-type GLI1 or GLI1 3A and 3E stably expressed were seeded into 6-well plates for colony formation assays for 2 weeks. Colonies larger than 1.5 mm were counted. The cells were treated with A769662 (150μM) and AICAR (0.75mM) containing medium for each A769662 and AICAR-treated wells were changed every three days and colony numbers were counted two weeks later. Each cell line was seeded in duplicate, with N=3.

	Scansite	-5	-4	-3	-2	-1	0	1	2	3	4
	Optimal Motif	L	R	R	V	X	S/T	X	X	N	L
	Alternative 1	M	K	K	S	X	S/T	X	X	D	V
	Alternative 2	I/R	P	H/S	R/N	X	S/T	X	X	X	I/F

Protein	Site	-5	-4	-3	-2	-1	0	1	2	3	4
ACC1	S79	I	R	S	S	M	S	G	L	H	L
FOXO3a	S413	M	Q	R	S	S	S	F	P	Y	T
TSC2	S1387	L	S	K	S	S	S	S	P	E	L
Raptor	S792	M	R	R	A	S	S	Y	S	S	L
ULK1	S467	I	R	R	S	G	S	T	S	P	L
ULK1	S556	G	C	R	L	H	S	A	P	N	L
PPP1R12C	S452	L	Q	R	S	A	S	S	S	W	L
GLI1	S102	T	V	I	R	T	S	P	S	S	L
GLI1	S408	L	P	R	A	P	S	I	S	T	V
GLI1	T1074	G	S	S	G	H	T	P	P	P	S

Table S1 (refers to Figure 3). Scansite prediction of potential AMPK phosphorylation sites in GLI1 protein. The AMPK optimal consensus phosphorylation motif, derived from Gwinn et al. (2008), is shown in the second row in single letter amino acid code, with less ideal sequences shown in the third and fourth rows**.** Positions denoted as ‘‘X’’ can provide additional modest selectivity but are not important discriminating positions compared to the others. Three matches to the consensus sequences are shown in the bottom three rows Known AMPK phosphorylation sites in ACC1, FOXO3, TSC2, Raptor, ULK1 and PPP1R12C are shown for comparison.