SUPPLEMENTAL DATA

Figure S1

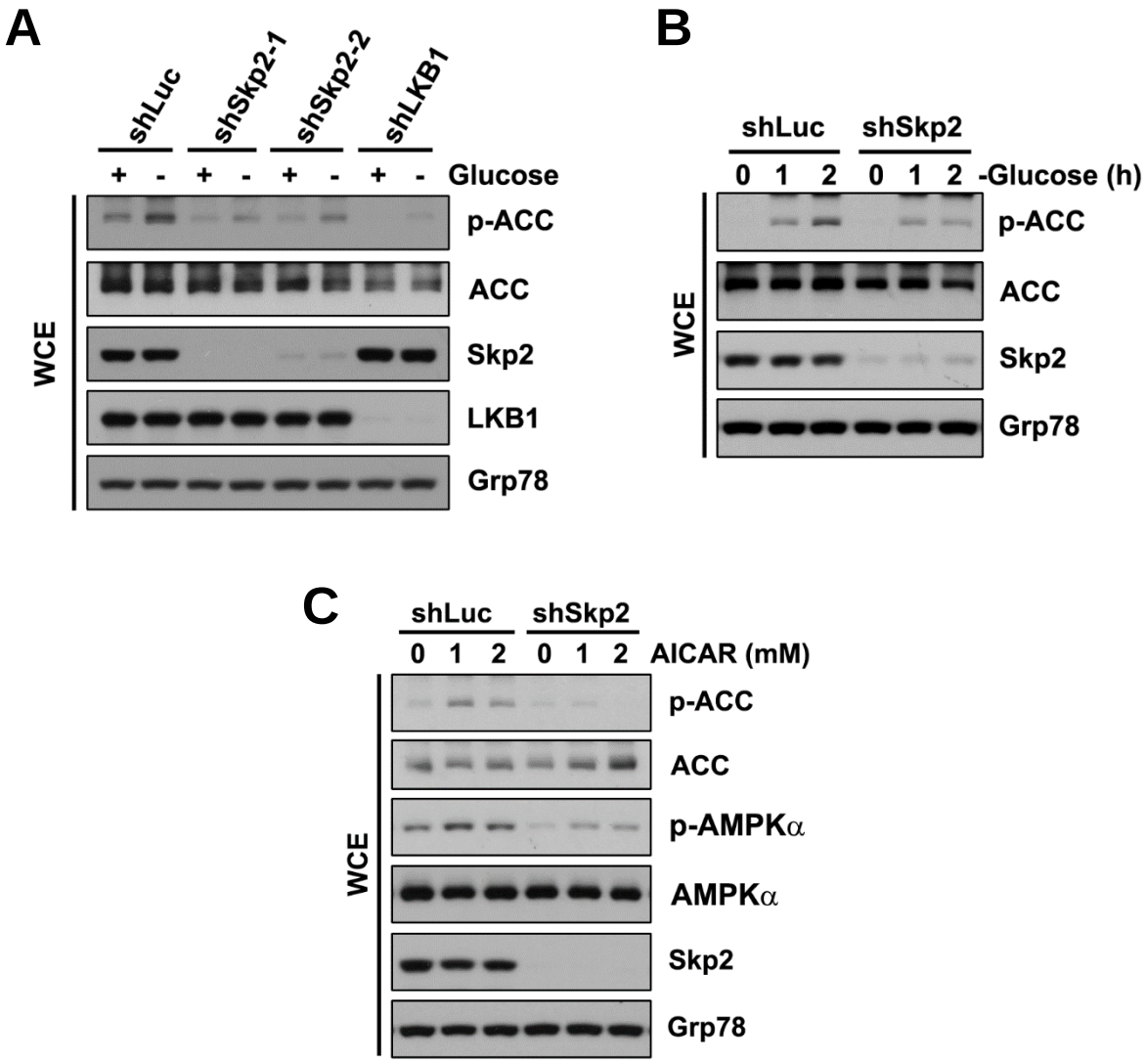

Figure S1, related to Figure 1. Skp2 knockdown attenuates the LKB1/AMPK signaling.
(A) BT-474 cells with control (shLuc), Skp2 or LKB1 knockdown cultured in the presence (+) or absence (-) of glucose were subjected to immunoblotting. WCE, whole cell extracts.
(B) MCF-7 cells with control (shLuc) or Skp2 knockdown cultured in the absence of glucose for the indicated time points were subjected to immunoblotting.
(C) HEK293T cells with control (shLuc) or Skp2 knockdown after treatment with AICAR at the indicated concentrations for 1 hour were subjected to immunoblotting.

Figure S2

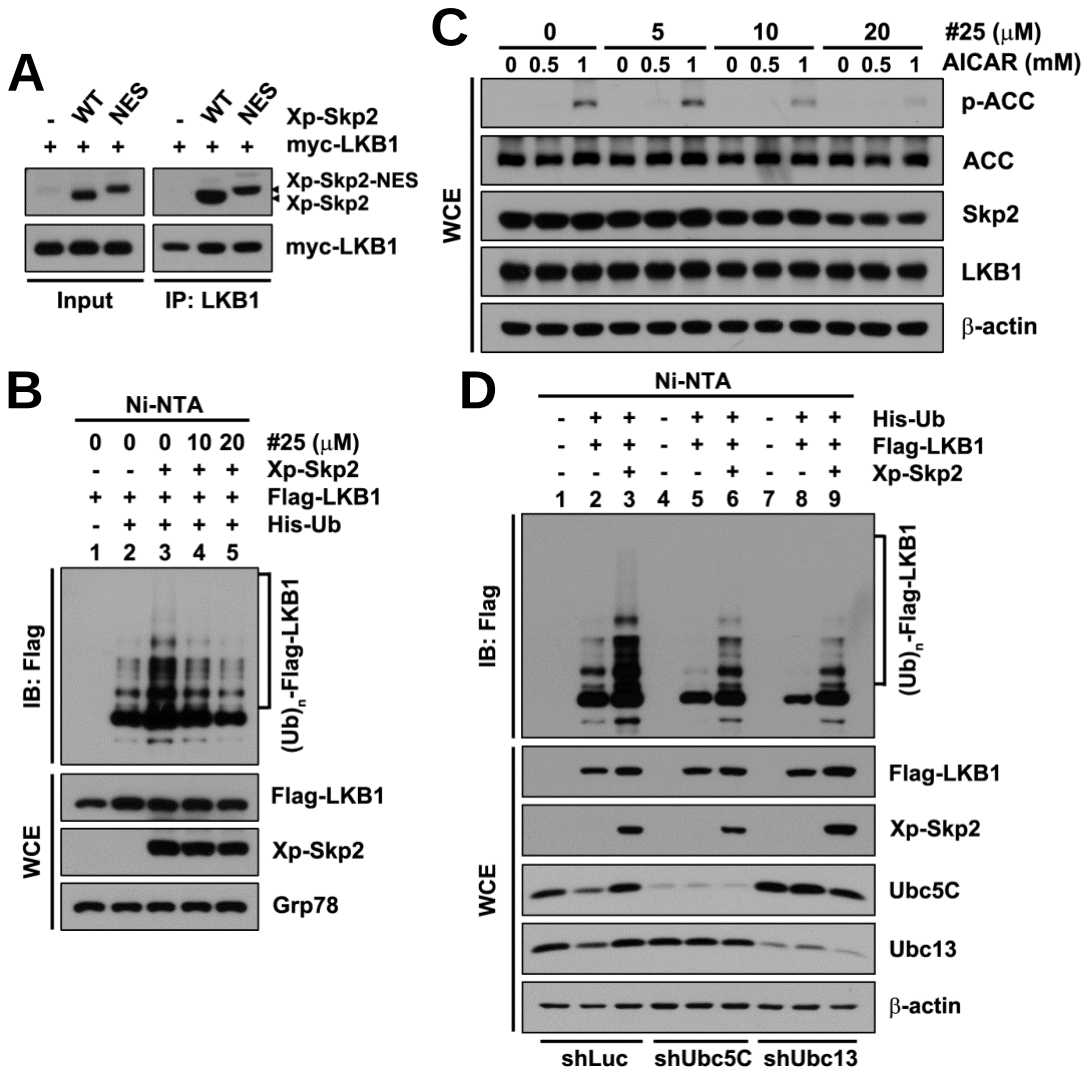
A
- WT NES - WT NES
Xp-Skp2
+ + + + + + myc-LKB1
Xp-Skp2-NES
Xp-Skp2
myc-LKB1
Input
IP: LKB1
B
Ni-NTA
0 0 0 10 20 #25 (μM)
- - + + + Xp-Skp2
+ + + + + Flag-LKB1
- + + + + His-Ub
1 2 3 4 5
IB: Flag
(Ub)n-Flag-LKB1
WCE
Flag-LKB1
Xp-Skp2
Grp78
C
0 5 10 20 #25 (μM)
0 0.5 1 0 0.5 1 0 0.5 1 0 0.5 1 AICAR (mM)
WCE
p-ACC
ACC
Skp2
LKB1
β-actin
D
Ni-NTA
- + + - + + - + + His-Ub
- + + - + + - + + Flag-LKB1
- - + - - + - - + Xp-Skp2
1 2 3 4 5 6 7 8 9
IB: Flag
(Ub)n-Flag-LKB1
WCE
Flag-LKB1
Xp-Skp2
Ubc5C
Ubc13
β-actin
shLuc shUbc5C shUbc13

Figure S2, related to Figure 2. Analysis of Skp2-mediated LKB1 polyubiquitination.

(A) Immunoprecipitates by anti-LKB1 antibody from HEK293T cells transfected with the indicated plasmids were subjected to immunoblotting.

(B) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids and treated with the Skp2 inhibitor (compound #25) at the indicated concentrations was followed by immunoblotting. Ni-NTA, nickel bead precipitates.

(C) Hep3B cells pretreated with the Skp2 inhibitor (compound #25) at the indicated concentrations for 24 hours were subjected to AICAR treatment at the indicated concentrations for 2 hours, followed by immunoblotting.

(D) *In vivo* ubiquitination assay in HEK293 cells with control (shLuc), Ubc5C or Ubc13 knockdown transfected with the indicated plasmids was followed by immunoblotting.

Figure S3

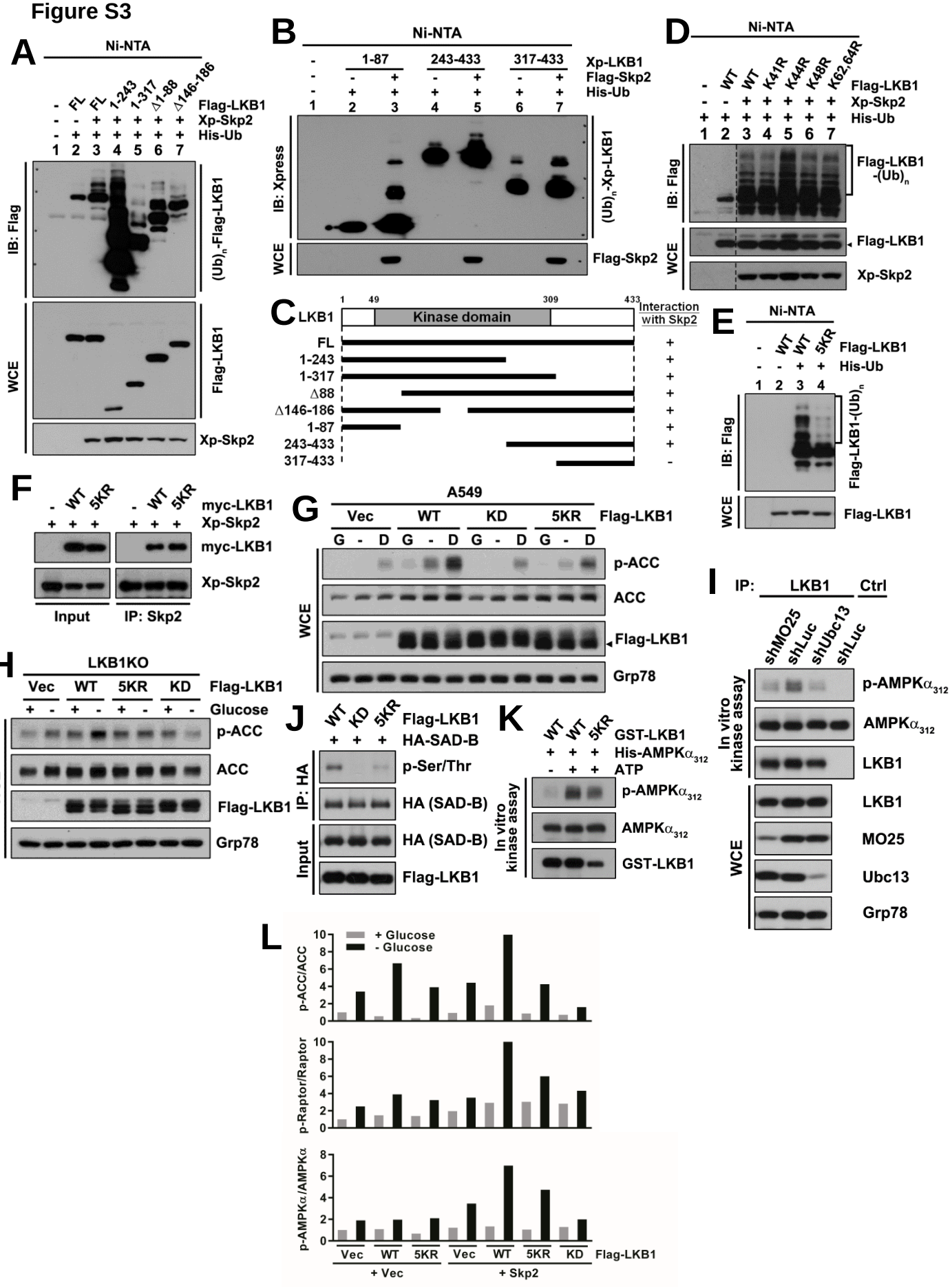
A
Ni-NTA
Flag-LKB1
Xp-Skp2
His-Ub
IB: Flag
(Ub)n-Flag-LKB1
WCE
Flag-LKB1
Xp-Skp2
B
Ni-NTA
1-87
243-433
317-433
Xp-LKB1
Flag-Skp2
His-Ub
IB: Xpress
(Ub)n-Xp-LKB1
WCE
Flag-Skp2
C
LKB1
Kinase domain
Interaction with Skp2
FL
1-243
1-317
Δ88
Δ146-186
1-87
243-433
317-433
D
Ni-NTA
WT
K41R
K44R
K48R
K62,64R
Flag-LKB1
Xp-Skp2
His-Ub
IB: Flag
Flag-LKB1 -(Ub)n
WCE
Flag-LKB1
Xp-Skp2
E
Ni-NTA
WT
5KR
Flag-LKB1
His-Ub
IB: Flag
Flag-LKB1-(Ub)n
WCE
Flag-LKB1
F
WT
5KR
myc-LKB1
Xp-Skp2
Input
IP: Skp2
G
A549
Vec
WT
KD
5KR
Flag-LKB1
G - D
p-ACC
ACC
Flag-LKB1
Grp78
WCE
H
LKB1KO
Vec
WT
5KR
KD
Flag-LKB1
Glucose
p-ACC
ACC
Flag-LKB1
Grp78
WCE
I
IP: LKB1 Ctrl
shMO25
shLuc
shUbc13
shLuc
In vitro kinase assay
p-AMPKα312
AMPKα312
LKB1
WCE
LKB1
MO25
Ubc13
Grp78
J
WT
KD
5KR
Flag-LKB1
HA-SAD-B
IP: HA
p-Ser/Thr
HA (SAD-B)
Input
HA (SAD-B)
Flag-LKB1
K
WT
WT
5KR
GST-LKB1
His-AMPKα312
ATP
In vitro kinase assay
p-AMPKα312
AMPKα312
GST-LKB1
L
+ Glucose
- Glucose
p-ACC/ACC
p-Raptor/Raptor
p-AMPKα/AMPKα
Vec
WT
5KR
Vec
WT
5KR
KD
Flag-LKB1
+ Vec
+ Skp2

Figure S3, related to Figure 3. Identification of LKB1 polyubiquitination-deficient mutant.

(A, B) *In vivo* ubiquitination assays in HEK293T cells transfected with the constructs expressing the indicated fragments of tagged-LKB1 and the other indicated plasmids were followed by immunoblotting. FL, full length.

(C) A diagram summary of the interaction between Skp2 and the different fragments of LKB1 in (A) and (B) based on the results of co-immunoprecipitation assays.

(D, E) *In vivo* ubiquitination assays in HEK293T cells transfected with the constructs expressing the indicated mutant forms of Flag-LKB1 and the other indicated plasmids were followed by immunoblotting.

(F) Immunoprecipitates by anti-Skp2 antibody from HEK293T cells transfected with the indicated plasmids were subjected to immunoblotting.

(G) LKB1-deficient A549 cells with stable reconstitution of the indicated Flag-LKB1 cultured with or without glucose, or treated with 5mM 2-DG for 2 hours were subjected to immunoblotting. G, with glucose; -, without glucose; D, with 2-DG.

(H) *LKB1*-KO MEFs with stable reconstitution of the indicated Flag-LKB1 cultured in the presence (+) or absence (-) of glucose for 2 hours were subjected to immunoblotting.

(I) Immunoprecipitates by anti-IgG control or anti-LKB1 antibody from HEK293T cells with control (shLuc), Ubc13 or MO25 knockdown were subjected to *in vitro* LKB1 kinase assay followed by immunoblotting.

(J) Immunoprecipitates by anti-HA antibody from HEK293T cells transfected with HA-SAD-B and the indicated Flag-LKB1 were subjected to immunoblotting.

(K) *In vitro* kinase assay, in which the indicated recombinant GST-LKB1 was incubated with recombinant His-AMPK$\alpha_{1\text{-}312}$ (substrate) in the presence (+) or absence (-) of ATP for reaction, was followed by immunoblotting.

(L) Semi-quantification analysis of Figure 3E. Hep3B cells with stable transduction of vector (Vec) or Skp2 along with the indicated Flag-LKB1 cultured in the presence (+) or absence (-) of glucose were subjected to immunoblotting. The band intensity of phospho-proteins was normalized to that of total proteins. The relative ratios are shown in bar graphs, and the ratios in cells transduced with vector only in the presence of glucose (the first lane) were set as 1.

Figure S4

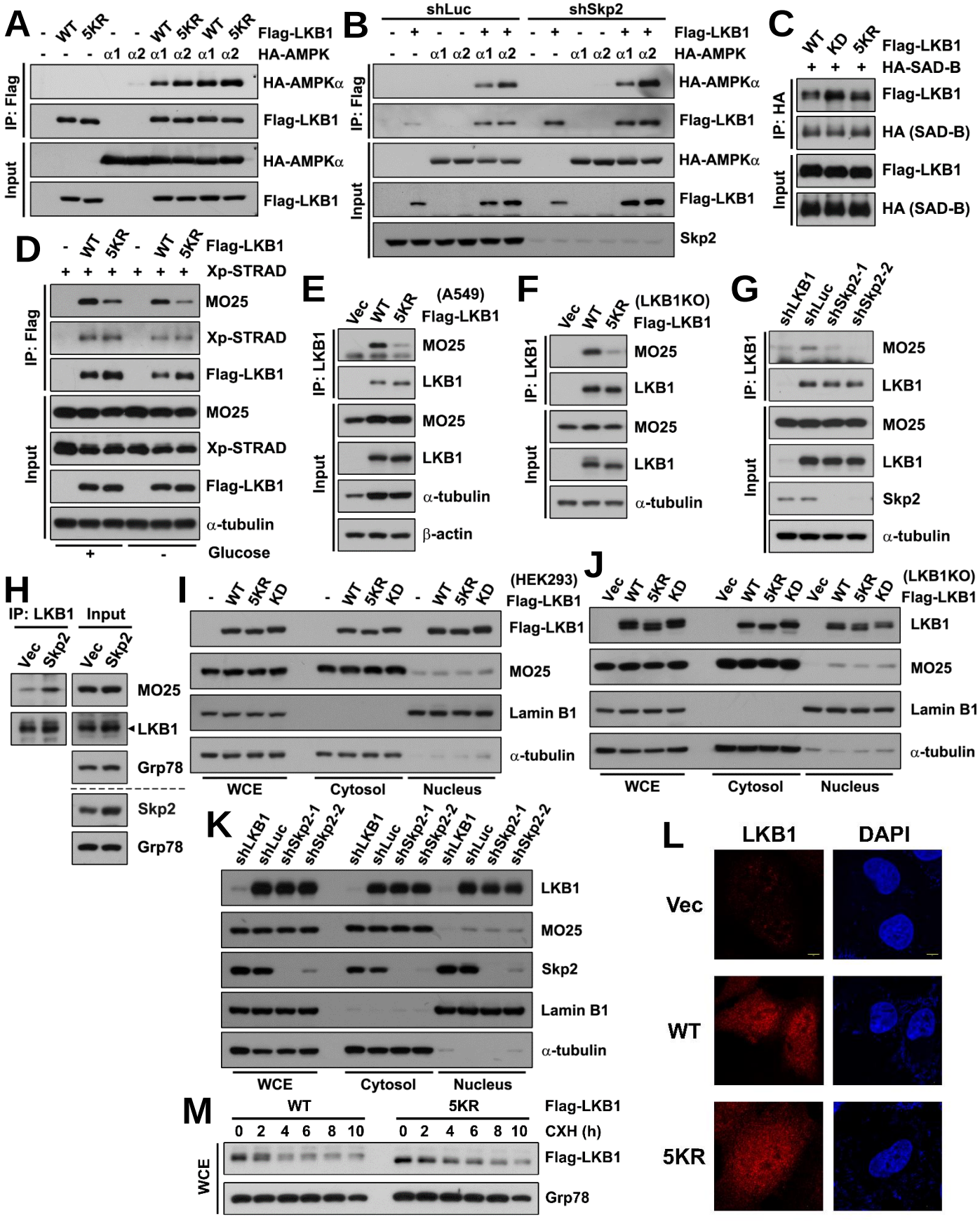
A
B
C
D
E
F
G
H
I
J
K
L
M
Flag-LKB1
HA-AMPK
HA-AMPKα
IP: Flag
Input
shLuc
shSkp2
HA-SAD-B
IP: HA
HA (SAD-B)
Xp-STRAD
MO25
α-tubulin
Glucose
(A549)
(LKB1KO)
IP: LKB1
LKB1
β-actin
shLKB1
shSkp2-1
shSkp2-2
IP: LKB1 Input
Vec
Skp2
Grp78
(HEK293)
Lamin B1
WCE
Cytosol
Nucleus
CXH (h)
LKB1
DAPI
Vec
WT
5KR
KD

Figure S4, related to Figure 4. Characterization of Skp2-mediated LKB1 polyubiquitination.

(A) HEK293T cells transfected with the indicated Flag-LKB1 and HA-AMPKα constructs were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblotting.

(B) HEK293 cells with control (shLuc) or Skp2 knockdown transfected with Flag-LKB1 and the indicated HA-AMPKα constructs were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblotting.

(C) Immunoprecipitates by anti-HA antibody from HEK293T cells transfected with HA-SAD-B and the indicated Flag-LKB1 were subjected to immunoblotting.

(D) Immunoprecipitates by anti-Flag antibody from cytosolic fractions of HEK293T cells transfected with Xp-STRAD and the indicated Flag-LKB1 cultured in the presence (+) or absence (-) of glucose for 2 hours were subjected to immunoblotting.

(E, F) Immunoprecipitates by anti-LKB1 antibody from cytosolic fractions of LKB1-deficient A549 cells (E) or *LKB1*-KO MEFs (F) with stable reconstitution of the indicated Flag-LKB1 were subjected to immunoblotting.

(G) Immunoprecipitates by anti-LKB1 antibody from cytosolic fractions of BT-474 cells with control (shLuc), Skp2 or LKB1 knockdown were subjected to immunoblotting.

(H) Immunoprecipitates by anti-LKB1 antibody from Hep3B cells with stable transduction of vector (Vec) or Skp2 were subjected to immunoblotting.

(I–K) HEK293 cells transfected with the indicated Flag-LKB1 (I), *LKB1*-KO MEFs with stable reconstitution of the indicated Flag-LKB1 (J), or HEK293 cells with control (shLuc), LKB1 or Skp2 knockdown (K) were subjected to nuclear/cytoplasmic fractionation followed by immunoblotting. Lamin B1 serves as a nuclear marker, and α-tubulin serves as a cytoplasmic marker. WCE, whole cell extracts.

(L) LKB1-deficient A549 cells with stable reconstitution of vector (Vec) or the indicated Flag-LKB1 were subjected to immunofluorescence assay with anti-LKB1 antibody. Scale bar, 10μm.

(M) LKB1-deficient A549 cells with stable reconstitution of the indicated LKB1 treated with cycloheximide (CHX) for the indicated time points were subjected to immunoblotting.

Figure S5

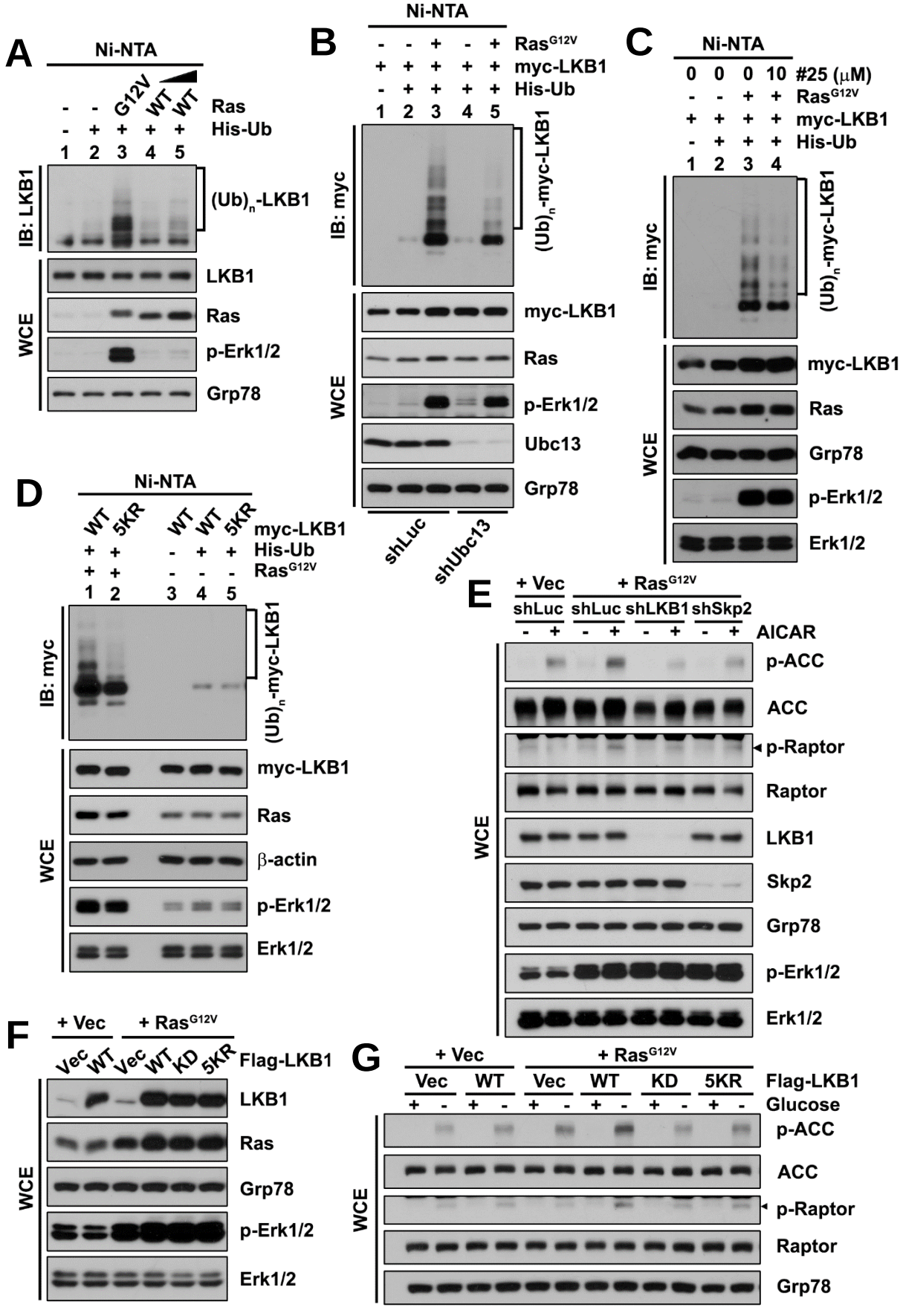
A
Ni-NTA
- - G12V WT WT Ras
- + + + + His-Ub
1 2 3 4 5
IB: LKB1
(Ub)n-LKB1
WCE
LKB1
Ras
p-Erk1/2
Grp78
B
Ni-NTA
- - + - + RasG12V
+ + + + + myc-LKB1
- + + + + His-Ub
1 2 3 4 5
IB: myc
(Ub)n-myc-LKB1
WCE
myc-LKB1
Ras
p-Erk1/2
Ubc13
Grp78
shLuc
shUbc13
C
Ni-NTA
0 0 0 10 #25 (μM)
- - + + RasG12V
+ + + + myc-LKB1
- + + + His-Ub
1 2 3 4
IB: myc
(Ub)n-myc-LKB1
WCE
myc-LKB1
Ras
Grp78
p-Erk1/2
Erk1/2
D
Ni-NTA
WT 5KR WT WT 5KR myc-LKB1
+ + - + + His-Ub
+ + - - - RasG12V
1 2 3 4 5
IB: myc
(Ub)n-myc-LKB1
WCE
myc-LKB1
Ras
β-actin
p-Erk1/2
Erk1/2
E
+ Vec + RasG12V
shLuc shLuc shLKB1 shSkp2
- + - + - + - + AICAR
WCE
p-ACC
ACC
p-Raptor
Raptor
LKB1
Skp2
Grp78
p-Erk1/2
Erk1/2
F
+ Vec + RasG12V
Vec WT Vec WT KD 5KR Flag-LKB1
WCE
LKB1
Ras
Grp78
p-Erk1/2
Erk1/2
G
+ Vec + RasG12V
Vec WT Vec WT KD 5KR Flag-LKB1
+ - + - + - + - + - + - Glucose
WCE
p-ACC
ACC
p-Raptor
Raptor
Grp78

Figure S5, related to Figure 5. Oncogenic Ras induces polyubiquitination of LKB1 and activation of the LKB1/AMPK signaling via Skp2.

(A, B, D) *In vivo* ubiquitination assays in HEK293T cells (A and D) or HEK293T cells with control (shLuc) or Ubc13 knockdown (B) transfected with the indicated plasmids were followed by immunoblotting.

(C) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids after treatment with the Skp2 inhibitor (compound #25) at the indicated concentrations was followed by immunoblotting.

(E) Hep3B cells with stable transduction of vector (Vec) or H-Ras along with the indicated stable knockdown were subjected to treatment with 1mM AICAR for 2 hours, followed by immunoblotting.

(F) Hep3B cells with stable transduction of vector (Vec) or H-Ras together with the indicated Flag-LKB1 were subjected to immunoblotting.

(G) Hep3B cells as in (F) cultured in the presence (+) or absence (-) of glucose were subjected to immunoblotting.

Figure S6

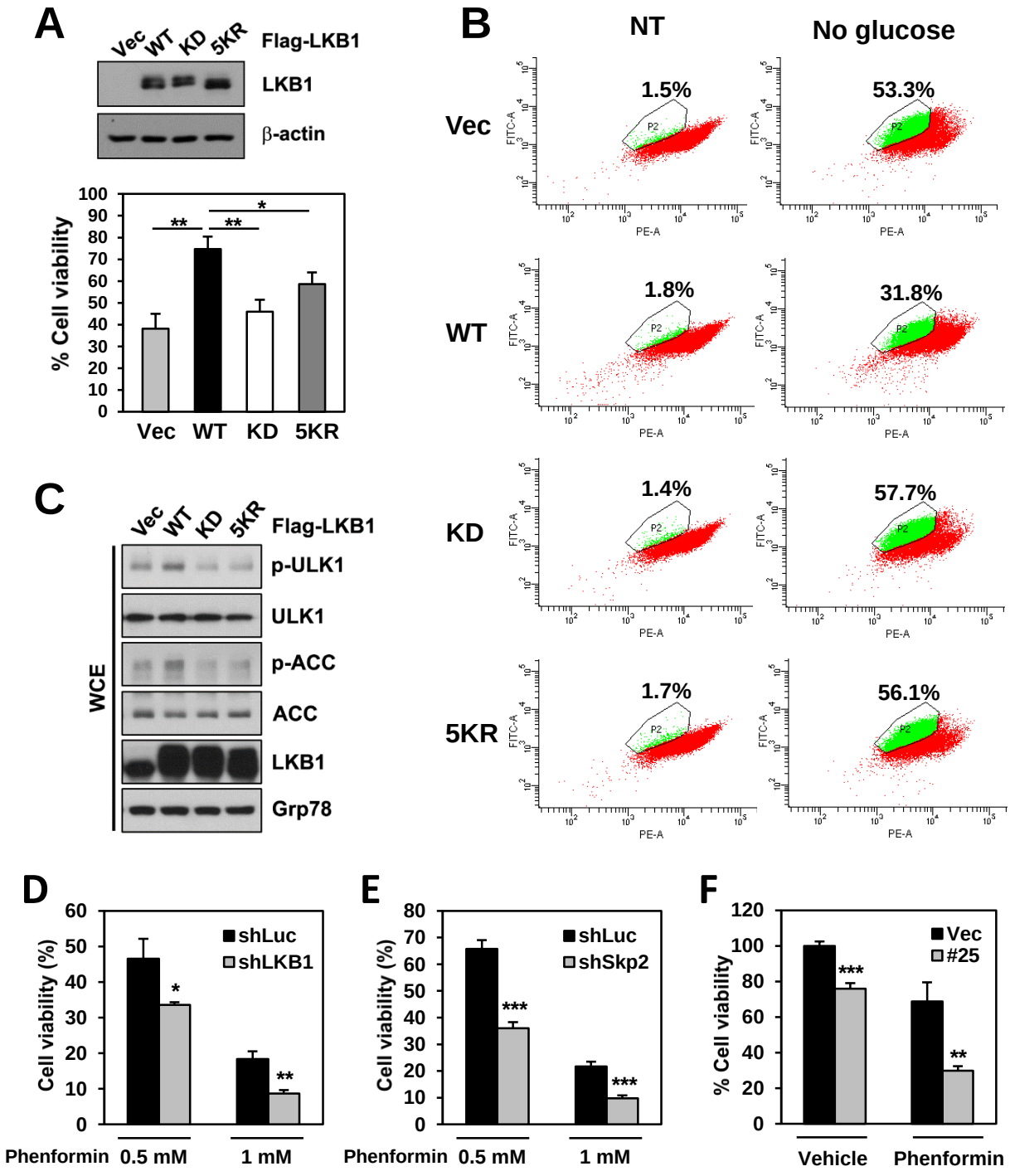
A
Vec
WT
KD
5KR
Flag-LKB1
LKB1
β-actin
% Cell viability
Vec
WT
KD
5KR
B
NT
No glucose
Vec
WT
KD
5KR
1.5%
53.3%
1.8%
31.8%
1.4%
57.7%
1.7%
56.1%
FITC-A
PE-A
P2
C
Vec
WT
KD
5KR
Flag-LKB1
WCE
p-ULK1
ULK1
p-ACC
ACC
LKB1
Grp78
D
Cell viability (%)
shLuc
shLKB1
Phenformin
0.5 mM
1 mM
E
Cell viability (%)
shLuc
shSkp2
Phenformin
0.5 mM
1 mM
F
% Cell viability
Vec
#25
Vehicle
Phenformin

Figure S6, related to Figure 6. Skp2-mediated polyubiquitination of LKB1 is critical for LKB1's function in cell survival under metabolic stress.

(A) *LKB1*-KO MEFs stably restored with the indicated Flag-LKB1 were subjected to treatment with 2mM AICAR for 24 hours. Cell viability was determined by trypan blue exclusion assay, and is expressed as a percentage of the vehicle-treated controls. Whole cell extracts of the untreated MEFs were subjected to immunoblotting (upper panel).

(B) Representative flow cytometric dot plots of Figure 6B. Mitochondria of Hep3B cells with stable transduction of the indicated Flag-LKB1 cultured in the presence or absence of glucose were co-stained with MitoTracker Green (FITC) and MitoTracker Red (PE), followed by flow cytometric analysis. The damaged mitochondria (FITC-positive and PE-negative or low; P2 fraction) were quantitated, and the percentages are shown. NT, non-treated.

(C) Hep3B cells with stable transduction of the indicated Flag-LKB1 culture in the absence of glucose for 10 hours as in (B) were subjected to immunoblotting.

(D, E) Cell viability of Hep3B cells with control (shLuc), LKB1 (D) or Skp2 knockdown (E) after treatment with phenformin at the indicated concentrations for 3 days was determined by trypan blue exclusion assay, and is expressed as a percentage of the untreated controls.

(F) Cell viability of Hep3B cells after combined treatment with Skp2 inhibitor (compound #25) and phenformin for 3 days was determined by trypan blue exclusion assay, and is expressed as a percentage of the vehicle-treated controls.

Results are shown as means ± s.d. (n=3) (A and D–F). *P<0.05; **P<0.01; ***P<0.005.

Figure S7

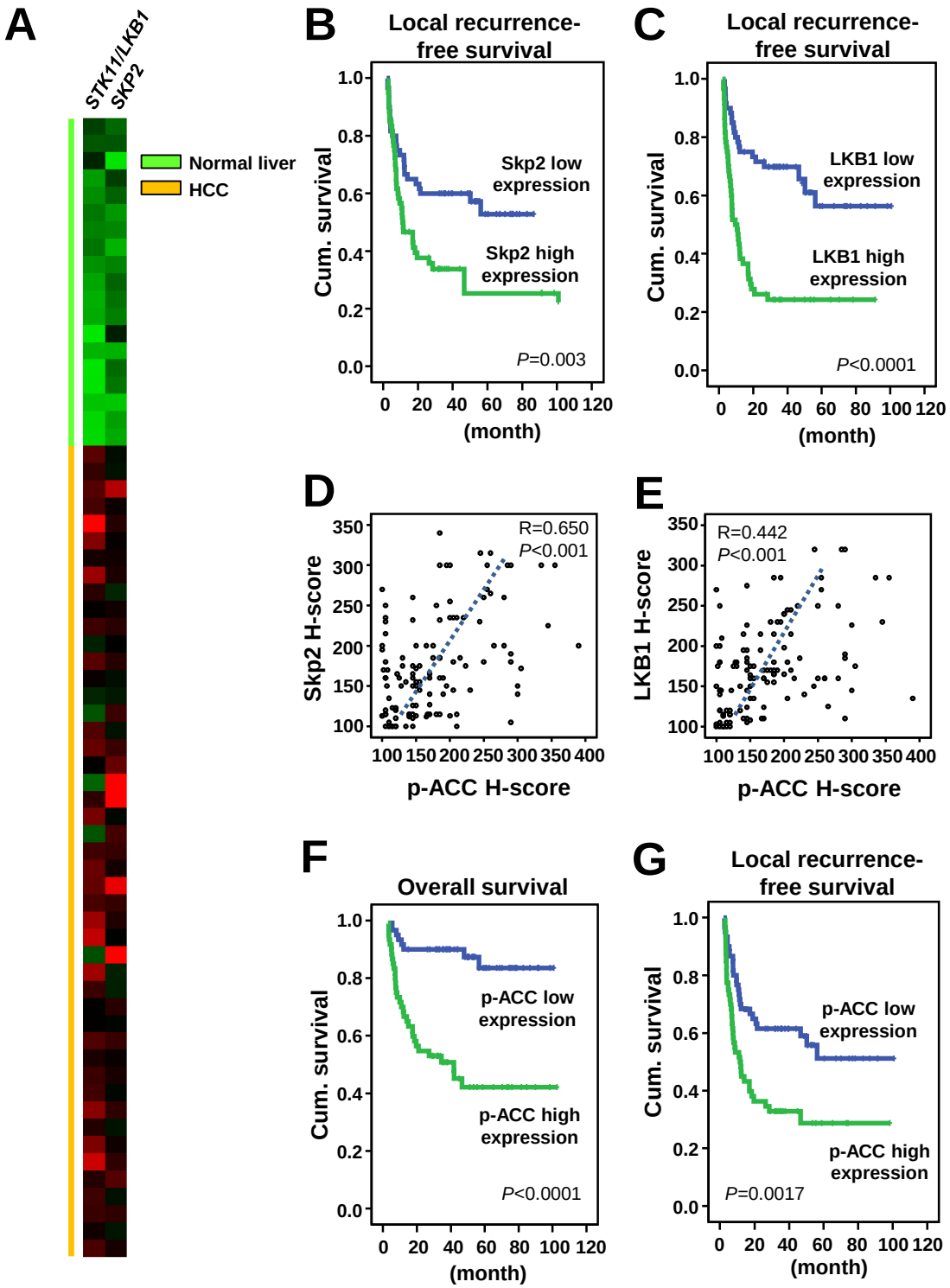
A
STK11/LKB1
SKP2
Normal liver
HCC
B
Local recurrence-free survival
Cum. survival
Skp2 low expression
Skp2 high expression
P=0.003
(month)
C
Local recurrence-free survival
Cum. survival
LKB1 low expression
LKB1 high expression
P<0.0001
(month)
D
Skp2 H-score
R=0.650
P<0.001
p-ACC H-score
E
LKB1 H-score
R=0.442
P<0.001
p-ACC H-score
F
Overall survival
Cum. survival
p-ACC low expression
p-ACC high expression
P<0.0001
(month)
G
Local recurrence-free survival
Cum. survival
p-ACC low expression
p-ACC high expression
P=0.0017
(month)

Figure S7, related to Figure 7. Both LKB1 and Skp2 are overexpressed in HCC and their overexpression predicts poor survival outcomes.

(A) The expression-profiling dataset of HCC tissues (*n*=47) versus normal liver tissue (*n*=19) from GSE14323 deposited in the Gene Expression Omnibus was analyzed. Both *LKB1* (*STK11*) and *SKP2* transcripts were significantly upregulated in HCC.

(B, C) Kaplan-Meier plots show that high expression of Skp2 (B) or LKB1 (C) significantly predicts poor local recurrence-free survival.

(D, E) Scatter plots of p-ACC levels versus Skp2 (D) or LKB1 (E) expression in HCC samples.

(F, G) Kaplan-Meier plots show that high p-ACC levels are significantly predictive for poor overall (F) or local recurrence-free (G) survival.

Table S1. Correlations between SKP2 and STK11 expressions to various clinicopathologic parameters, related to Figures 7 and S7

	No.	SKP2 H-score	*P*-value	STK11 H-score	*P*-value	pACC H-score	*P*-value
Sex			0.063		0.725		0.291
Male	93	169.18+/-56.94		172.03+/-51.59		172.48+/-63.27	
Female	27	200.74+/-74.70		183.41+/-66.55		190.00+/-73.78	
Age (years)			0.914		0.882		0.707
<60	53	172.06+/-49.43		170.68+/-46.54		178.09+/-63.94	
≧60	67	179.63+/-71.29		177.69+/-61.39		175.10+/-67.80	
Hepatitis			0.286		0.619		0.100
HBV	57	167.28+/-53.11		173.75+/-53.70		177.14+/-63.66	
HCV	47	181.15+/-69.95		175.89+/-60.63		181.53+/-72.388	
Both	6	230.00+/-82.219		200.00+/-48.89		210.83+/-40.67	
None	10	172.50+/-51.87		158.00+/-38.74		127.70+/-29.55	
Pugh-Child's Classification			**<0.001***		**0.005***		**0.011***
A	109	167.28+/-56.75		169.28+/-51.68		170.06+/-62.57	
B	8	290.00+/-186.51		250.00+/-55.36		256.88+/-63.86	
C	3	200.00+/-0.00		166.67+/-45.37		193.33+/-62.52	
AFP			**0.028***		**0.144**		0.411
<400	83	163.59+/-57.30		165.34+/-50.78		167.16+/-56.99	
≧400	31	186.32+/-53.75		181.87+/-52.13		186.19+/-76.52	
Tumor Multiplicity			**<0.001***		**<0.001***		**<0.001***
Solitary	68	153.26+/-43.43		156.38+/-42.64		159.51+/-57.48	
Multiple	52	206.38+/-70.67		198.40+/-60.86		198.54+/-70.02	
Differentiation			**0.003***		**0.031***		**0.041***
Well/Moderately differentiated	93	166.94+/-58.22		168.41+/-53.66		169.63+/-62.57	
Poorly differentiated	27	208.48+/-66.85		195.89+/-56.18		199.81+/-72.58	
Primary Tumor (pT)			**0.003***		0.083		0.335
pT1	46	156.59+/-54.73		163.52+/-55.99		165.30+/-59.14	
pT2	39	174.05+/-59.92		174.00+/-53.17		174.03+/-59.29	
pT3-4	35	204.66+/-65.70		189.80+/-54.37		193.71+/-78.39	
AJCC Stage			**<0.001***		**0.012***		0.162
Stage I	44	150.07+/-46.20		156.41+/-45.72		159.75+/-54.17	
Stage II	39	174.05+/-59.92		174.00+/-53.17		174.03+/-59.29	
Stage III-V	37	209.81+/-67.49		196.84+/-60.69		198.78+/-79.16	
CLIP score			**0.005***		0.097		**0.001***
0-1	85	166.27+/-59.25		169.73+/-56.27		165.07+/-61.91	
2-3	35	200.60+/-64.20		186.40+/-51.41		204.00+/-67.90	
Okuda stage			**0.004***		0.076		**0.013***
I	85	163.80+/-53.43		167.38+/-49.95		167.07+/-62.15	
II-III	35	206.60+/-72.56		192.11+/-63.72		199.14+/-69.93	
SKP2 Expression		-		r=0.675	**<0.001***	**r=0.460**	**<0.001***
STK11 H-score						**r=0.442**	**<0.001***

*****, statistically significant.

Table S2. Univariate survival analyses, related to Figures 7 and S7

		OS		LRFS	
Parameter	**No. of Case**	**No. Event**	***P*-value**	**No. Event**	***P*-value**
Sex					
Male	93	29	0.5670	53	0.3622
Female	27	11		14	
Age (years)					
<60	53	16	0.4994	25	0.1622
≧60	67	24		42	
Pugh-Child's Classification					
A	109	29	**<0.0001***	56	**<0.0001***
B	8	8		8	
C	3	3		3	
AFP					
<400	83	**20**	**0.0148***	41	**0.0820***
≧400	31	14		20	
Tumor Multiplicity					
Solitary	68	9	**<0.0001***	26	**<0.0001***
Multiple	52	31		41	
Differentiation					
Well/Moderately differentiated	93	25	**0.0010***	47	**0.0003***
Poorly differentiated	27	15		20	
Primary Tumor (pT)					
pT1	46	7	**0.0001***	18	**0.0003***
pT2	39	14		24	
pT3-4	35	19		25	
AJCC Stage					
Stage I	44	5	**<0.0001***	16	**0.0001***
Stage II	39	14		24	
Stage III-V	37	21		27	
CLIP score					
0-1	85	21	**0.0001***	41	**<0.0001***
2-3	35	19		26	
Okuda stage					
I	85	21	**0.0002***	44	**0.0356***
II-III	35	19		23	
SKP2 Expression					
Low expression (<median)	60	8	**<0.0001***	26	**0.0030***
High expression (>=median)	60	32		41	
STK11 Expression					
Low expression (<median)	60	13	**0.0040***	22	**<0.0001***
High expression (>=median)	60	27		45	
pACC Expression					
Low expression (<median)	60	8	**<0.0001***	26	**0.0017***
High expression (>=median)	60	32		41	

OS, overall survival; LRFS, local recurrence-free survival; *****, statistically significant.

Table S3. Multivariate survival analyses, related to Figures 7 and S7

Parameter	Category	OS			LRFS		
		RR	**95% CI**	***P*-value**	**RR**	**95% CI**	***P*-value**
SKP2	Low expression (<median)	1	-	**<0.001***	1	-	0.148
	High expression (>=median)	5.592	2.308-13.548		1.499	0.866-2.594	
STK11	Low expression (<median)	1	-	0.052	1	-	**<0.001***
	High expression (>=median)	2.017	0.955-4.087		3.001	1.7738-5.181	
pACC	Low expression (<median)	1		**0.036***	1	-	**0.716**
	Low expression (<median)	2.451	1.060-5.671		1.109	0.636-1.932	
CLIP score	0-1	1	-	0.060	1	-	**0.015***
	2-3	2.612	0.959-7.111		2.683	1.210-5.950	
Okuda stage	I	1	-	0.646	1	-	0.259
	II-III	1.234	0.503-3.029		1.575	0.716-3.460	
AJCC Stage	Stage I	1	-	**0.009***	1	-	**0.006***
	Stage II	4.028	1.426-11.381		2.455	1.264-1.767	
	Stage III-V	4.985	1.429-14.375		2.959	1.422-6.156	
Differentiation	Well/Moderately differentiated	1	-	0.845	1	-	0.249
	Poorly differentiated	1.085	0.483-2.433		1.453	0.769-2.746	

OS, overall survival; LRFS, local recurrence-free survival; *****, statistically significant.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture and reagents

WT and *Skp2*-KO MEFs were prepared from mice as previously described (Lin et al., 2010). All manipulations were performed under Institutional Animal Care and Use Committee approval protocol. *LKB1*-KO MEFs were a kind gift from Dr. Nabeel Bardeesy (Harvard Medical School). Hep3B, BT-474, MCF-7, HEK293, HEK293T, HeLa, and A549 cells were obtained from American Type Culture Collection. BT-474 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), and all the other cells were cultured in DMEM supplemented with 10% FBS. For glucose deprivation, cells were washed with PBS once and then incubated in glucose-free DMEM (Invitrogen) supplemented with 10% dialyzed FBS (Sigma) for the indicated times. 2-deoxyglucose (2-DG), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and phenformin were purchased from Sigma.

Plasmids

His_6-ubiquitin (His-Ub), His_6-ubiquitin-K48R (His-Ub-K48R), His_6-ubiquitin-K63R (His-Ub-K63R), Xpress-Skp2 (Xp-Skp2), Xpress-Skp2-NES (Xp-Skp2-NES), Flag-Skp2 and pBabe-H-RasG12V constructs were described previously (Chan et al., 2012; Lin et al., 2010; Lin et al., 2009). pcDNA3-Flag-LKB1, pcDNA3-Flag-LKB1-KD (kinase-dead; K78I), pBabe-Flag-LKB1, pBabe-Flag-LKB1-KD ,and pcDNA4-Xpress-STRAD (Xp-STRAD) constructs were purchased from Addgene. pET30a-AMPK$\alpha_{1\text{-}312}$ (His-AMPK$\alpha_{1\text{-}312}$) construct was a kind gift from Dr. Gary D. Lopaschuk (University of Alberta, Canada) (Altarejos et al., 2005). pSG-Flag-LKB1 constructs expressing various LKB1 fragments were kindly provided by Dr. Christelle Forcet (Institut de Génomique Fonctionnelle de Lyon, France) (Nony et al., 2003). pCIG2-HA-SAD-B was a kind gift from Dr. Franck Polleux (Columbia University Medical Center, New York) (Courchet et al., 2013). pcDNA3-HA-AMPKα1 and α2 constructs were a kind gift from Dr. Kun-Liang Guan (University of California, San Diego) (Inoki et al., 2003). The LKB1 constructs including pCMV-myc-LKB1, pcDNA4-Xpress-LKB1 (Xp-LKB1 with various LKB1 fragments), pWZL-myc-LKB1 and pGEX-5X-1-LKB1 (GST-LKB1) were subcloned from pcDNA3-Flag-LKB1. All LKB1-K-to-R mutant constructs were generated from the WT LKB1 constructs by using a PCR-based site-directed mutagenesis method. To re-express LKB1, which cannot be targeted by LKB1 shRNA, in cells with stable LKB1 knockdown, silent mutations were generated on LKB1 constructs by using a PCR-based site-directed mutagenesis method.

Transfection and viral infection

For transfection of HEK293 or HEK293T cells with exogenous plasmids, calcium phosphate transfection method was used. For control, Skp2, LKB1, Ubc13, Ubc5C, or MO25 knockdown,

the indicated cell lines were infected with lentiviruses packaged from HEK293T cells transfected with pLKO.1-puro-shRNA constructs (Sigma) and packaging plasmids (Chan et al., 2012). For transduction of vector, LKB1, LKB1-KD, LKB1-5KR or H-Ras, the indicated cell lines were infected with retroviruses packaged from HEK293T cells transfected with pBabe-puro constructs and packaging plasmids (Chen et al., 2011). The stably infected cells were selected by 1, 1.5 or 2 μg/ml puromycin for 3–7 days. The following lentiviral shRNAs (Sigma) were used: Skp2-lentivivral shRNA-1 (5'-GATAGTGTCATGCTAAAGAAT-3'), Skp2-lentivivral shRNA-2 (5'-GCCTAAGCTAAATCGAGAGAA-3'), LKB1-lentiviral shRNA (5'-CATCTACACTCAGGACTTCAC-3'), UbcH5c-lentiviral shRNA (5'-CCTGCATTATAGCTGGAATAA-3'), Ubc13-lentiviral shRNA (5'-CCTTCCAGAAGAATACCCAAT-3'), MO25-lentiviral shRNA (5'-GAGAAGTTACTTCATTCAGAA-3'), and Luciferase shRNA (as the control).

Immunoblotting, immunoprecipitation and antibodies

For immunoblotting analysis, cells were lysed by direct resuspension in RIPA buffer (50mM Tris-HCl [pH 8.0], 150mM NaCl, 5mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% (v/v) NP-40, 50mM NaF, 10mM sodium pyrophosphate, 10mM disodium glycerophosphate, protease inhibitor cocktail), and then clarified cell lysates were used. For immunoprecipitation, cells were lysed in RIPA buffer directly, or E1A buffer (50mM HEPES [pH 7.5], 250mM NaCl, 5mM EDTA, 0.1% (v/v) NP-40, protease inhibitor cocktail) followed by sonication. Clarified cell lysates were incubated with the indicated antibodies overnight, and then protein A/G beads (Santa Cruz Biotechnology) were added for 3–5 hours. Beads were washed four times with RIPA or E1A buffer. Proteins were eluted in SDS-sample buffer and subjected to immunoblotting analysis. For immunoprecipitation by anti-Lys63-specific ubiquitin antibody, cells were lysed in 6M urea lysis buffer (50mM Tris-HCl [pH 8.0], 300mM NaCl, 6M urea, 0.5% (v/v) NP-40, protease inhibitor cocktail) followed by sonication, and the concentration of urea in clarified cell lysates was diluted to 4M before addition of the antibody. Antibodies used in this study include: anti-LKB1, anti-ubiquitin, anti-Myc and anti-His from Santa Cruz Biotechnology; anti-Skp2, anti-Ubc13, anti-Xpress and anti-Cullin1 from Invitrogen; anti-phospho-ACC (Ser79), anti-ACC, anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-Ubc5C, anti-MO25, anti-phospho-Erk1/2 (Thr202/Tyr204), anti-Erk1/2, anti-phospho-Raptor (Ser792), anti-Raptor, anti-phospho-ULK1 (Ser555) and anti-ULK1 from Cell Signaling; anti-Flag, anti-α-tubulin and anti-β-actin from Sigma; anti-Skp1 and anti-Grp78 from BD Transduction Laboratories; anti-pan-Ras from Calbiochem; anti-HA from Convance; anti-Lys63-specific ubiquitin (K63-Ub) from Millipore; anti-phospho-serine/threonine and anti-Lamin B1 from Abcam.

In vitro LKB1 kinase assay

Recombinant His-AMPKα$_{1-312}$ protein was expressed in BL21 bacteria and purified from the bacterial lysates using a nickel-agarose column to serve as LKB1's substrate. Endogenous LKB1 or exogenous Flag-LKB1 was immunoprecipitated from cells by antibodies against LKB1 or Flag, and then immunoprecipitates were incubated with recombinant His-AMPKα$_{1-312}$ for 30 minutes at 30^{o}C in 20 μl of reaction buffer (25mM Tris-HCl [pH 7.5], 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na3VO4, 10mM $MgCl_2$, 0.5mM ATP). After incubation, proteins were eluted in SDS-sample buffer and subjected to immunoblotting analysis. LKB1 kinase activity was directly determined by measuring Thr172 phosphorylation of recombinant AMPKα$_{1-312}$ using anti-phospho-AMPKα (Thr172) antibody.

In vivo and *in vitro* ubiquitination assay

In vivo and *in vitro* ubiquitination assays were performed as described previously (Chan et al., 2012; Yang et al., 2009). For the *in vivo* ubiquitination assay, HEK293T or HEK293 cells were transfected with His-Ub and the indicated plasmids for 48 hours and lysed using high-stringency denaturing buffer (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, and 10mM imidazole at pH 8.0). The cell extracts were then incubated with nickel-agarose beads for 3 hours, washed, and subjected to immunoblotting analysis. For the *in vitro* ubiquitination assay, recombinant GST-LKB1 protein was expressed in BL21 bacteria and purified from the bacterial lysates using glutathione beads. SCF/Flag-Skp2 complex was expressed in HEK293T cells, immunoprecipitated using anti-Flag antibody, and eluted from protein A/G beads using Flag peptides according to the manufacturer's standard procedures (Sigma). Purified GST-LKB1 and SCF/Flag-Skp2 proteins were incubated for 3 hours at 37^{o}C in 20 μl of reaction buffer (20mM HEPES [pH 7.4], 10mM $MgCl_2$, 1mM DTT, 59mM ubiquitin, 50nM E1, 850nM Ubc13/Uev1a, and 1mM ATP). After incubation, protein mixtures were diluted in RIPA buffer and the supernatant fluid was immunoprecipitated overnight with anti-LKB1 antibody, after which protein A/G beads were added for an additional 3 hours. Beads were washed four times with RIPA buffer. Proteins were eluted in SDS-sample buffer and subjected to immunoblotting analysis.

Preparation of cytosolic and nuclear fractions

Cells were harvested and resuspended in hypotonic buffer (10mM Tris-HCl [pH 7.6], 10mM $MgCl_2$, 0.1% (v/v) NP-40, protease inhibitor cocktail) followed by Dounce homogenization. The suspensions were centrifuged at 1,300 x g for 5 minutes at 4^{o}C, and then the resulting supernatants and nuclear pellets were further processed for cytosolic and nuclear fractions, respectively. For cytosolic fractions, the supernatants were clarified by centrifugation at 13,500 rpm for 15 minutes at 4^{o}C. For further immunoprecipitation, the concentration of NP-40 in the

cytosolic fractions was adjusted from 0.1% to 1% (comparable to the NP-40 concentration in RIPA buffer). For nuclear fractions, the nuclear pellets were washed once with hypotonic buffer and then resuspended in RIPA buffer followed by sonication and centrifugation.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde/PBS for 10–15 minutes and then permeabilized with 0.2% Triton X/PBS for 15 minutes at room temperature. The primary antibody against LKB1 (Ley 37D/G6; Abcam) was diluted in 1% BSA/PBS. The fluorescent-conjugated secondary antibody was anti-mouse Alexa Fluor 594. Coverslips were washed three times with PBS between antibody incubations. The nuclei were stained with DAPI contained in a mounting solution.

Cell viability assay

For *Skp2*-KO and *LKB1*-KO MEFs, viable and dead cells were counted directly under the microscope using the trypan blue exclusion assay. For glucose-starved A549 and Ras-overexpressing Hep3B cells, cell death was measured using DAPI staining as previously described (Jeon et al., 2012). Briefly, cells were seeded at a low density in 12-well plates in triplicates overnight and then subjected to glucose deprivation. The treated cells were fixed by directly adding formaldehyde (final concentration 12%) to the culture medium. After overnight fixation at 4^{o}C, the cells were stained with DAPI (1 μg/ml) for 5 minutes, and then washed and left in PBS. Under the fluorescence microscope, compared with the untreated cells, the treated cells with condensed or fragmented nuclei were counted as dead cells. For administration of phenformin or/and the Skp2 inhibitor (compound #25), 1.2–1.5x10^{4} of cell were seeded in 12-well plates in triplicates for 24 hours and then treated with the indicated drug(s) at the indicated concentrations for 3 days. Drugs were refreshed once after treatment for 2 days. Viable cells were counted under the microscope using the trypan blue exclusion assay.

Detection of damaged mitochondria

Mitochondria were analyzed by MitoTracker staining followed by flow cytometry analysis as described previously (Tal et al., 2009). Cells were co-stained with MitoTracker Green (for total mitochondria) and MitoTracker Red (for live or respiring mitochondria) at 50 nM and 100 nM, respectively, in the culture medium for 20–30 minutes at 37^{o}C. After staining, the cells were washed, harvested and then resuspended in PBS for flow cytometry analysis. The FITC-positive and PE-negative/low populations were gated as defective mitochondria. The MitoTracker probes were purchased from Invitrogen.

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