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AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization

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Figure S1

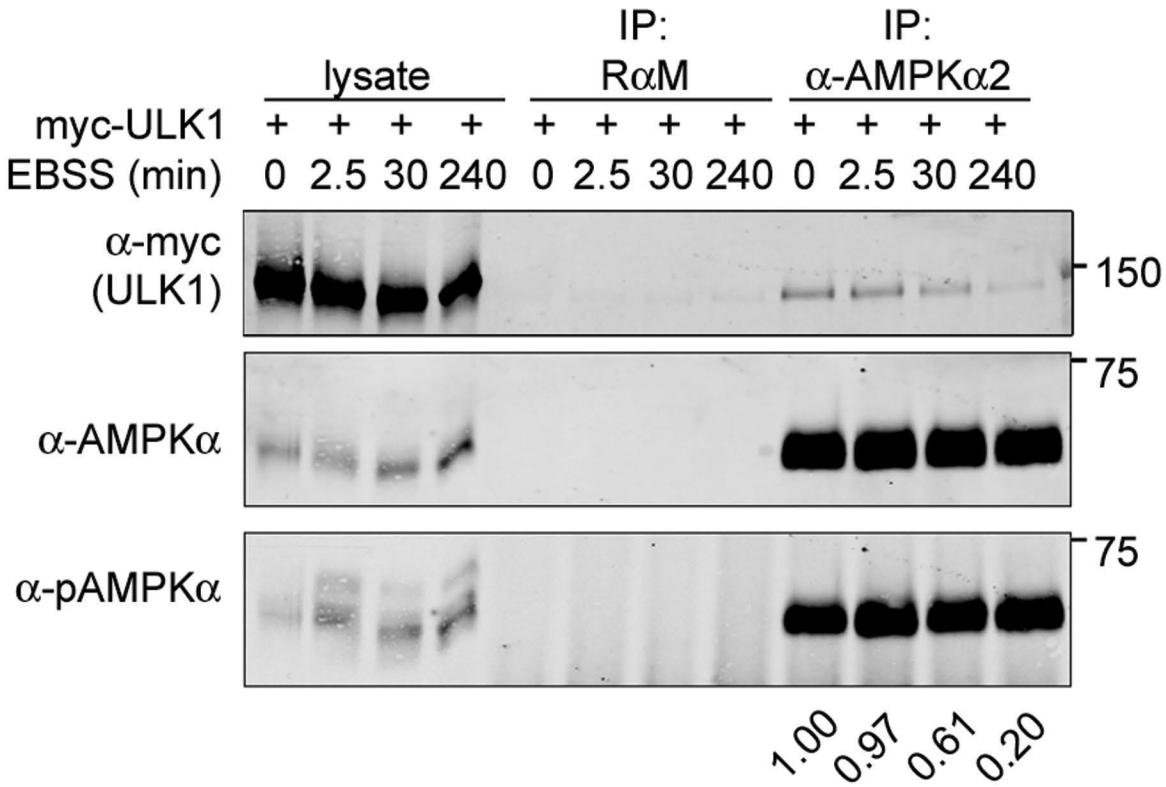

lysate
IP: RαM
IP: α-AMPKα2
myc-ULK1 + + + + + + + + + + + +
EBSS (min) 0 2.5 30 240 0 2.5 30 240 0 2.5 30 240
α-myc (ULK1)
α-AMPKα
α-pAMPKα
150
75
75
1.00 0.97 0.61 0.20

Figure S2

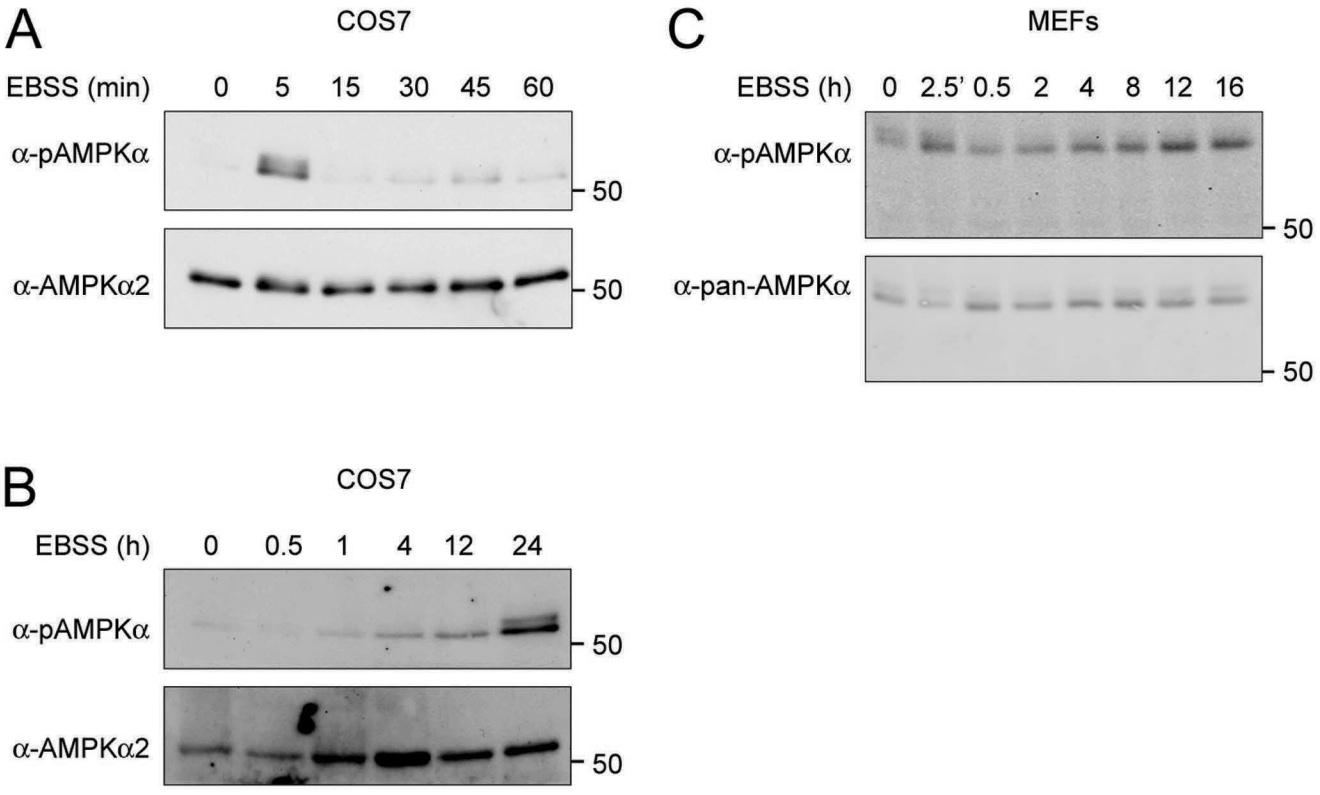

A
COS7
EBSS (min) 0 5 15 30 45 60
α-pAMPKα
α-AMPKα2
50
50
B
COS7
EBSS (h) 0 0.5 1 4 12 24
α-pAMPKα
α-AMPKα2
50
50
C
MEFs
EBSS (h) 0 2.5' 0.5 2 4 8 12 16
α-pAMPKα
α-pan-AMPKα
50
50

Figure S3

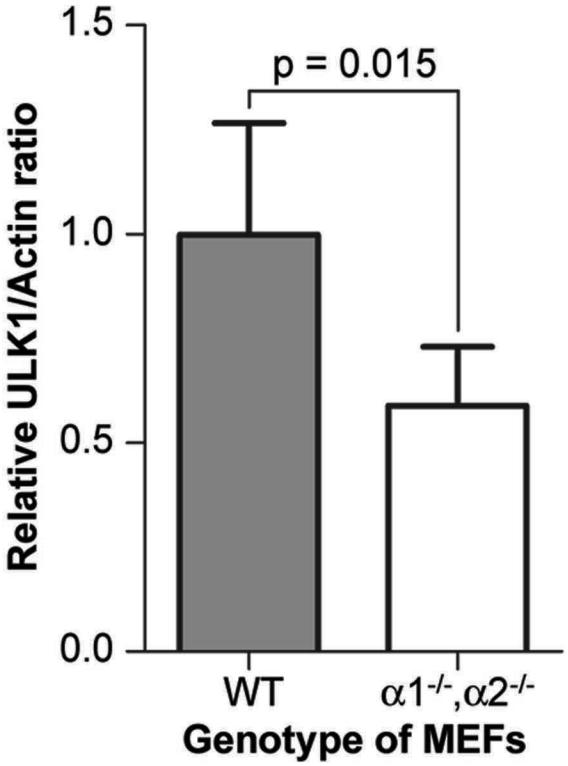
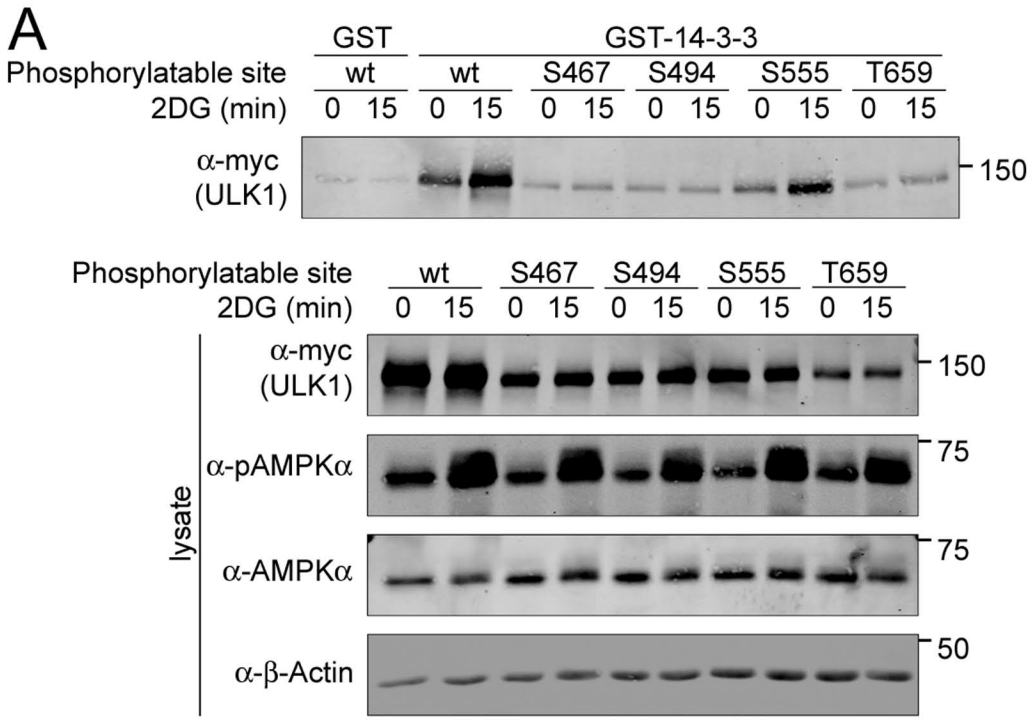

1.5
1.0
0.5
0.0
p = 0.015
Relative ULK1/Actin ratio
WT
α1-/-,α2-/-
Genotype of MEFs

Figure S4


A
GST
GST-14-3-3
Phosphorylatable site
wt
wt
S467
S494
S555
T659
2DG (min)
0 15 0 15 0 15 0 15 0 15 0 15
α-myc
(ULK1)
150
Phosphorylatable site
wt
S467
S494
S555
T659
2DG (min)
0 15 0 15 0 15 0 15 0 15
lysate
α-myc
(ULK1)
150
α-pAMPKα
75
α-AMPKα
75
α-β-Actin
50

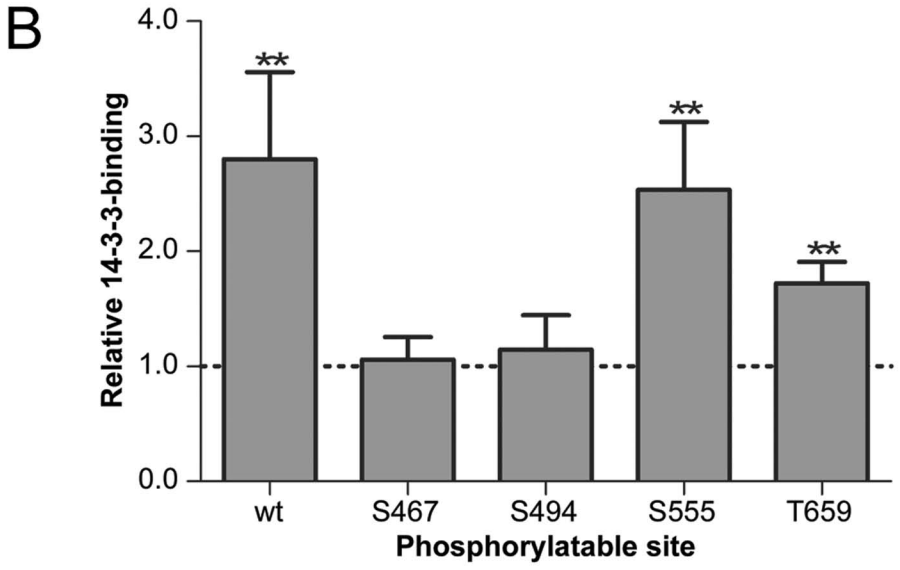

B
Relative 14-3-3-binding
4.0
3.0
2.0
1.0
0.0
**
**
**
wt
S467
S494
S555
T659
Phosphorylatable site

Figure S5

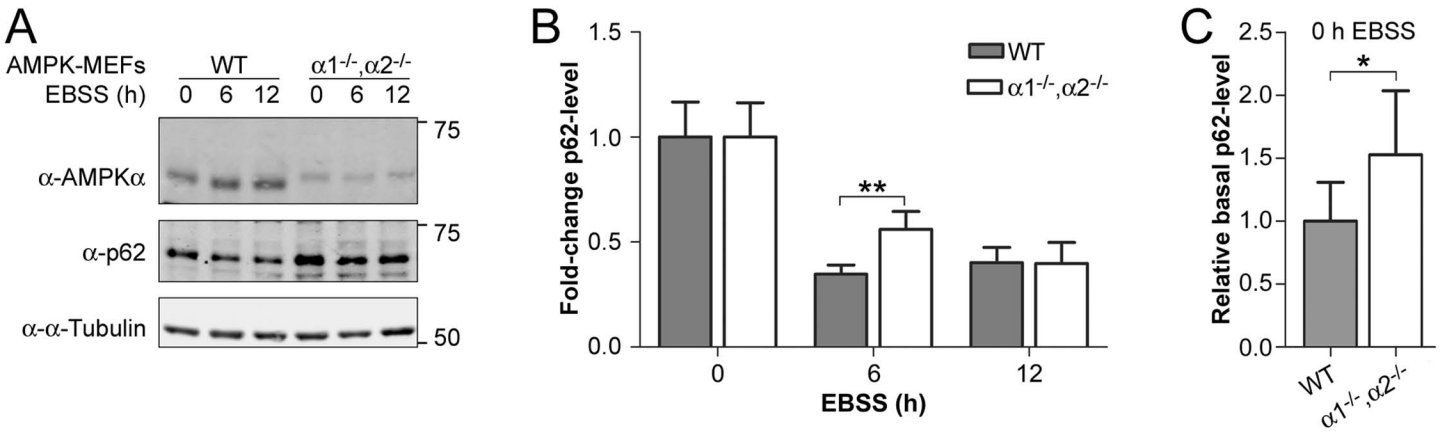

A
AMPK-MEFs
WT
α1-/-,α2-/-
EBSS (h)
0 6 12 0 6 12
75
α-AMPKα
75
α-p62
α-α-Tubulin
50
B
Fold-change p62-level
1.5
1.0
0.5
0.0
WT
α1-/-,α2-/-
**
0
6
12
EBSS (h)
C
0 h EBSS
Relative basal p62-level
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
α1-/-,α2-/-

Figure S6

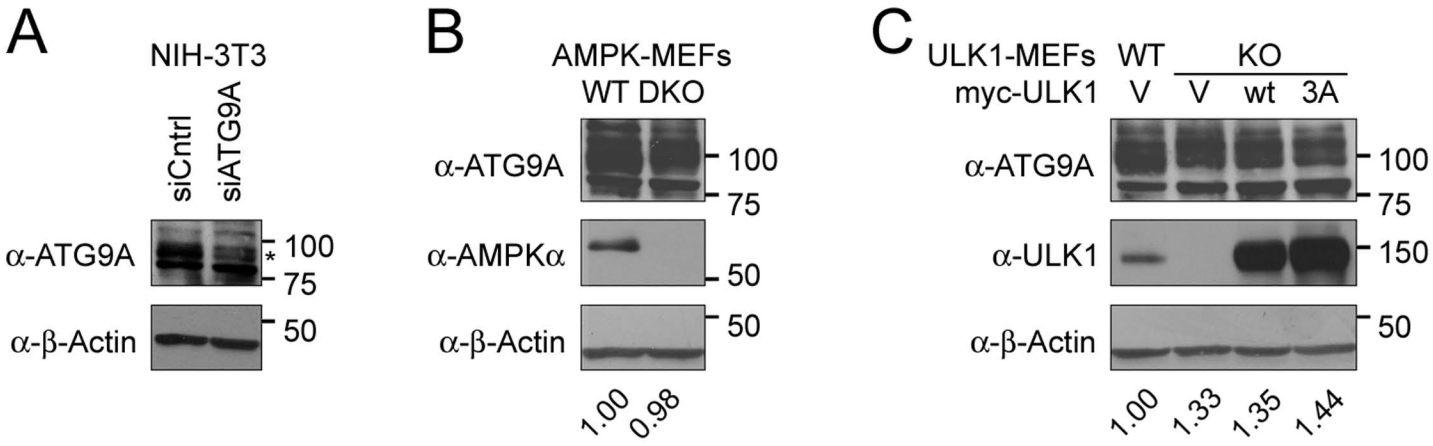

A
NIH-3T3
siCntrl
siATG9A
α-ATG9A
*
100
75
α-β-Actin
50
B
AMPK-MEFs
WT DKO
α-ATG9A
100
75
α-AMPKα
50
α-β-Actin
50
1.00
0.98
C
ULK1-MEFs
WT
KO
myc-ULK1
V
V
wt
3A
α-ATG9A
100
75
α-ULK1
150
α-β-Actin
50
1.00
1.33
1.35
1.44

Figure S1. Starvation induces dissociation of the ULK1-AMPK complex in HEK293T cells. HEK293T cells stably overexpressing myc-ULK1 were starved with EBSS for the times indicated and lysates incubated with the indicated antibodies. Lysates and immunoprecipitates were analyzed by western blot with the antibodies indicated. Western blot signals were quantified and analyzed as described for Figure 3. The result shown is representative of 4 independent experiments.

Figure S2. AMPK is activated in very early and in late stages of starvation. (A, B) COS7 cells or (C) MEFs were subjected to complete starvation with EBSS for the times indicated. Note that the shortest starvation time in (C) is 2.5 min. AMPK activation was assessed by western blot for phosphorylation of Thr172 (pAMPK). In panels (A) and (C), the unstarved control cells were subjected to a mock treatment with fresh media for the shortest starvation time.

Figure S3. AMPK-deficient MEFs display reduced ULK1 protein levels. Quantitation of western blot signals for endogenous ULK1 and actin under basal conditions in 12 independent experiments analyzing these two proteins in total cell lysates. For a representative experiment, cf. Fig. 6D, lanes 1 and 3 in the western blot analysis of the lysates. In each sample, the ULK1 signal was normalized to the actin signal. The graph shows the mean ULK1/actin ratios from the 12 experiments relative to the mean ULK1/actin ratio in wild-type cells. Error bars indicate standard errors of the mean. Statistical significance was determined by paired T-test.

Figure S4. S555 and T659 are AMPK-dependent YWHAZ binding sites in ULK1. (A) 2DG-treatment and GST-YWHAZ pulldown as described in Fig. 6D were performed on quadruple-

mutants of myc-tagged ULK1 in which the AMPK-site detected by mass spectrometry, S637, was mutated along with three out of the four candidate YWHAZ-binding sites examined in Fig. 6F; consequently, each mutant would contain only one site for potential phosphorylation by AMPK and YWHAZ-binding. These quadruple mutants were stably overexpressed in COS7 cells and, following treatment with 25 mM 2DG for 0 or 15 min, pulled down from the cell lysates with GST-YWHAZ. GST served as a negative control. Western blot analysis was performed with the antibodies indicated. (B) Quantitation of the experiment described in (A). Western blot signals were detected on a LI-COR Odyssey infrared scanner and quantified. The bar graph shows mean fold-change in YWHAZ-binding to each ULK1-mutant obtained in five independent repetitions of the experiment. Error bars indicate standard errors of the mean, the dotted line indicates the baseline association at 0 min 2DG, which was set to "1". ** indicates $p < 0.01$ in a one-sample t-test comparing the mean to "1".

Figure S5. Loss of AMPK delays starvation-induced degradation of SQSTM1. (A) Wild-type MEFs or MEFs lacking the genes for both PRKAA isoforms (*PRKAA1*$^{-/-}$,*PRKAA2*$^{-/-}$) were starved with EBSS for the times indicated and cell lysates were analyzed by western blot with the antibodies indicated. (B) Quantitation of six independent repetitions of the experiment described in (A). SQSTM1signals were normalized to corresponding Tubulin signals. The graph shows the mean SQSTM1/tubulin ratios ("SQSTM1-level") from the 6 experiments relative to the mean SQSTM1/tubulin ratio under basal conditions ("0 h EBSS") of the same cell type. (C) Experiments similar to the experiment described in (A), but cells were starved with EBSS for 3 h, i.e. for the same time as in the experiments to analyze ATG9-localization (cf. Fig. 8). The experiment in the left panel was conducted in wild-type and *PRKAA1*$^{-/-}$,*PRKAA2*$^{-/-}$ MEFs, the

experiment in the right panel in wild-type and *ULK1*$^{-/-}$ MEFs stably overexpressing an empty vector or myc-tagged wild type ULK1 or the mutants indicated: 2A – S555A-T659A, 3A - S555A-S637A-T659A, 6A – S467A-S494A-T574A-S555A-S637A-T659A, K46A – kinase dead. (D-G) Quantitative western blot analysis of SQSTM1-levels under (D, E) basal conditions ("0 h EBSS") or (F, G) upon 3 h of EBSS starvation in (D, F) wild-type and *PRKAA1*$^{-/-}$,*PRKAA2*$^{-/-}$ MEFs or (E, G) wild-type and *ULK1*$^{-/-}$ MEFs stably overexpressing an empty vector or myc-tagged ULK1-constructs as in (C). The SQSTM1-level is defined as the ratio of the SQSTM1 signal to the corresponding signal of a loading control (actin or tubulin). (D) Mean of the SQSTM1-levels determined in 19 independent experiments (including the experiments shown in [A and C]) relative to the mean SQSTM1-level in wild-type MEFs. (E) Mean of the SQSTM1-levels determined in 8 independent experiments (including the experiment shown in [C]) relative to the mean SQSTM1-level in wild-type MEFs expressing an empty vector. (F, G) Mean of the SQSTM1-levels from 4 independent experiments each (including the experiments shown in [C]) relative to the mean SQSTM1-level of the same cell type under basal conditions (not shown).

In (B, D-F), error bars indicate standard errors of the mean. Statistical significance was determined by paired T-test, with * indicating a nominal p-value < 0.05 and ** indicating a nominal p-value < 0.01. For reasons of clarity, nonsignificant nominal p-values > 0.05 were omitted. In (E, G), the dashed-dotted horizontal line indicates the mean relative SQSTM1-level of WT+V, the dotted line the mean *of ULK1*$^{-/-}$+V and the dashed line the mean of *ULK1*$^{-/-}$+wt.

Figure S6. Analysis of ATG9A protein levels in MEFs. (A) Validation of the ATG9 antibody used in these studies in mouse cells. NIH3T3 cells were transfected with 50 nM siRNA targeting murine ATG9A (ON-TARGETplus SMARTpool, Thermo Fisher Scientific, L-043531-01-0005)

or control siRNA (ON-TARGETplus nontargeting pool D-001810-10-05) for 72 h. Cells were lysed with modified RIPA and western blot analysis was performed with the antibodies indicated. The asterisk denotes specific band recognized by the anti-ATG9 antibody. (B) and (C) western blot analysis was performed as in (A) on cell lysates from (B) littermate matched AMPK wild-type and *PRKAA1*$^{-/-}$;*PRKAA2*$^{-/-}$ (DKO) MEFs or (C) littermate matched ULK1 wild-type and *ULK1*$^{-/-}$ MEFs. Western blot signals for ATG9A were quantified and normalized to the respective actin signal. Numbers below the panels indicate ATG9A/actin ratios relative to the respective wild-type control. The experiments shown are representative for three independent experiments each.

Site	Phosphorylated at 0 h	0.5 h	2 h	Sequence	Predicted kinase	Other studies reporting the site
S317	x		x	SHLASPP**s**LGEMPQL	PKCα	
S403		x		ESHGRTP**s**PSPTCSS	GSK3β	
S411			x	PSPTCSS**s**PSPSGRP	GSK3β ERK1	
S450		x	x	RIEQNLQ**s**PTQQQTA	p38	(Daub, Olsen et al. 2008; Dephoure, Zhou et al. 2008; Brill, Xiong et al. 2009; Oppermann, Gnad et al. 2009; Shang, Chen et al. 2011)
S477	x	x		PLGFGRA**s**PsPPsHT	ERK1	(Daub, Olsen et al. 2008)
S479		x		GFGRASP**s**PPSHTDG	CDC2 CDK5	(Daub, Olsen et al. 2008; Dorsey, Rose et al. 2009; Shang, Chen et al. 2011)
S494	x	x		AMLARKL**s**LGGGRPY	AMPK AKT PKA PKCδ PKCμ 14-3-3	
S521	x	x	x	PSWSRVP**s**PQGADVR		*(Dorsey, Rose et al. 2009)*
S532	x			ADVRVGR**s**PRPGSSV	CDK5	(Shang, Chen et al. 2011)(starvation-induced)
S543	x	x		GSSVPEH**s**PRTTGLG	CDC2 CDK5	
S555	x	x		GLGCRLH**s**APNLSDF	AMPK 14-3-3	Egan et al, (AMPK-induced) (Dephoure, Zhou et al. 2008; Pan, Gnad et al. 2008; Egan, Shackelford et al. 2011; Shang, Chen et al. 2011)
S614	x	x	x	PDFLQR**s**PLPPILGS		
S622	x	x	x	PLPPILG**s**PTKAGPS	CDC2 CDK5	(Daub, Olsen et al. 2008; Dephoure, Zhou et al. 2008; Trinidad, Thalhammer et al. 2008; Brill, Xiong et al. 2009; Chen, Yang et al. 2009; Dorsey, Rose et al. 2009; Shang, Chen et al. 2011)Shang et a (starvation-induced)l
T624	x			PPILGsP**t**KAGPSFD		(Brill, Xiong et al. 2009)
T635			x	PSFDFPK**t**PSSQNLL	CDK5	
S637	x	x	x	FDFPKTP**s**SQNLLTL	AMPK	(Cantin, Yi et al. 2008; Dephoure, Zhou et al. 2008; Pan, Gnad et al. 2008; Chen, Yang et al. 2009), Egan et al (Phenformin-induced)
T659	x		x	MTPPRNR**t**LPDLSEA	AKT AMPK 14-3-3	
S693		x	x	GPFGRSF**s**TSRITDL	AKT 14-3-3	
T694		x	x	PFGRSFS**t**SRITDLL		
S695	x			FGRSFST**s**RITDLLL		
S715	x	x	x	SDSGSTD**s**LQKPMEI	CK1	
S718		x	x	SDSGSTD**s**LQEKPME	CK1	
S747	x			ARGGGAS**s**PAPVVFT		(Beausoleil, Villen et al. 2006)
T754	x			SPAPVVF**t**VGSPPSG		
S757	x		x	PVVFTVG**s**PPsGATP		(Beausoleil, Villen et al. 2006; Dorsey, Rose et al. 2009)
T763	x			GSPPSGA**t**PPQSTRT	CDK5 GSK3β	(Beausoleil, Villen et al. 2006)

S774			x	STRTRMF**s**VGSSSSL	AKT CamK2 PKC∂ 14-3-3	(Shang, Chen et al. 2011)
S777		x		TRMFSVG**s**SSSLGSTS		
S780	x	x	x	FSVGSSS**s**LGSTGSS	PKC∂	
S1043	x		x	LCIERRL**s**ALLSGVY	AMPK PKA	(Dorsey, Rose et al. 2009)

Table S1. Phosphorylation sites on ULK1 identified by LC/MS/MS in this study under nutrient-rich and starvation conditions. Myc-ULK1 was stably overexpressed in MEFs, isolated from cells subjected to either 0 h, 0.5 h or 2 h of EBSS-starvation, and analyszed by tandem mass spectrometry. Conditions under which a site was detected are marked with "x". The sequences surrounding the detected sites are shown, with the phosphorylated Serine or Threonine highlighted in bold font, lower case. Kinases potentially phosphorylating these sites and binding sites for the regulatory protein 14-3-3 were predicted by Scansite. Also included are references to previous studies that identified some of the sites listed. Note: In the study by Egan *et al.*, Phenformin-treatment was used to activate AMPK. In the study by Shang et *al.*, SILAG was performed on ULK1 isolated from fed or EBSS-starved cells. Sites that showed higher phosphorylation upon starvation are denoted "starvation induced in this table. Also, Shang et *al.* number the sites according to their position in the human ULK1-protein sequence. This table gives the corresponding sequence postion in murine ULK1.

	Phosphorylated at						
Site	0.5 h EBSS	2 h EBSS	2.5 min EBSS	15 min 2DG	**Sequence**	**Predicted kinase**	**Other studies reporting the site**
S450	**x**	**x**	**x**		RIEQNLQ**s**PTQQQTA	p38	(Daub, Olsen et al. 2008; Dephoure, Zhou et al. 2008; Brill, Xiong et al. 2009; Dorsey, Rose et al. 2009; Oppermann, Gnad et al. 2009)
S460					QQQTARS**s**AIRRSGS		
S494	**x**		**x**	**x**	AMLARKL**s**LGGGRPY	AMPK AKT PKA PKCδ PKCμ 14-3-3	
S532			**x**	**x**	ADVRVGR**s**PRPGSSV	CDK5	(Shang, Chen et al. 2011) (starvation-induced)
S537			**x**		GRSPRPG**s**SVPEHSP		
S543	**x**		**x**	**x**	GSSVPEH**s**PRTTGLG	CDC2 CDK5	
S637	**x**	**x**		**x**	FDFPKTP**s**SQNLLTL	AMPK	(Cantin, Yi et al. 2008; Dephoure, Zhou et al. 2008; Pan, Gnad et al. 2008; Chen, Yang et al. 2009; Egan, Shackelford et al. 2011), Egan et al (Phenformin-induced)
S638				**x**	DFPKTPS**s**QNLLTLL		(Wissing, Jansch et al. 2007; Dephoure, Zhou et al. 2008)
T653	**x**				ARQGVVM**t**PPRNRTL	CDC2 CDK5	
T659		**x**			MTPPRNR**t**LPDLSEA	AKT AMPK 14-3-3	

Table S2. Phosphorylation sites on ULK1 identified by LC/MS/MS in this study upon EBSS-starvation and AMPK-activation. Myc-ULK1 was stably overexpressed in COS7-cells, isolated following treatment with EBSS for 2.5 min or with 25 mM 2DG for 15 min to activate AMPK, and analysed by tandem mass spectrometry. Some of the detected sites were also identified with

prolonged EBSS-treatment (0.5 h, 2 h; cf. Table S1). Conditions under which a site was detected are marked with "x". The sequences surrounding the detected sites are shown, with the phosphorylated Serine or Threonine highlighted in bold font, lower case. Kinases potentially phosphorylating these sites and binding sites for the regulatory protein 14-3-3 were predicted by Scansite. Also included are references to previous studies that identified some of the sites listed (cf. also legend to Table S1)

Site	Sequence	Predicted kinase	Studies reporting the site
S87	DFQEMAN**s**VYLVMEY		(Dorsey, Rose et al. 2009)
S195	MAPEVIM**s**QHYDGKA		(Dorsey, Rose et al. 2009)
S224	GKAPFQA**s**SPQDLRL		(Dorsey, Rose et al. 2009)
S341	AAGFLQG**s**RDSGGSS	PKCδ	(Dorsey, Rose et al. 2009), Shang et al
S405	HGRTPSP**s**PTCSSSP	***GSK3β***	Shang et al., 2011 (starvation-induced)
T456	QSPTQQQ**t**ARSSAIR		(Dephoure, Zhou et al. 2008)
S465	RSSAIRR**s**GSTTPLG		(Beausoleil, Villen et al. 2006)
S467	SAIRRSG**s**TTPLGFG	AMPK,PKCδ, 14-3-3	(Beausoleil, Villen et al. 2006; Dephoure, Zhou et al. 2008; Dorsey, Rose et al. 2009)
T468	AIRRSGS**t**TPLGFGR		(Brill, Xiong et al. 2009), Shang et al
S469	IRRSGST**s**PLGFGRA	ERK1	(Beausoleil, Villen et al. 2006; Dephoure, Zhou et al. 2008; Brill, Xiong et al. 2009; Dorsey, Rose et al. 2009), Shang et al
S405	HGRTPSP**s**PTCSSSP	***GSK3β***	Shang *et al.*, 2011
S504	GGRPYTP**s**PQVGTIPP		Shang *et al.*, 2011
S482	RASPSPP**s**HTDGAML		(Dorsey, Rose et al. 2009)
S760	FTVGSPP**s**GATPPQS		(Beausoleil, Villen et al. 2006)
T574	PKLPKPP**t**DPLGATQ		Egan et al., (Phenformin-induced)
S867	EIAALKG**s**ASEAAGG		(Dorsey, Rose et al. 2009)
S913	KVAELLS**s**GLQTAID		(Dorsey, Rose et al. 2009)
S1047	RRLSALL**s**GVYA		(Dorsey, Rose et al. 2009)

Table S3. Additional phosphorylation sites on ULK1 identified in previous studies. The sequences surrounding the detected sites are shown, with the phosphorylated Serine or Threonine highlighted in bold font, lower case. Kinases potentially phosphorylating these sites and binding sites for the regulatory protein 14-3-3 were predicted by Scansite. Cf. also legend to table S1.

Beausoleil, S. A., J. Villen, et al. (2006). "A probability-based approach for high-throughput protein phosphorylation analysis and site localization." Nat Biotechnol **24**(10): 1285-1292.

Brill, L. M., W. Xiong, et al. (2009). "Phosphoproteomic analysis of human embryonic stem cells." Cell Stem Cell **5**(2): 204-213.

Cantin, G. T., W. Yi, et al. (2008). "Combining protein-based IMAC, peptide-based IMAC, and MudPIT for efficient phosphoproteomic analysis." J Proteome Res **7**(3): 1346-1351.

Chen, R. Q., Q. K. Yang, et al. (2009). "CDC25B mediates rapamycin-induced oncogenic responses in cancer cells." Cancer Res **69**(6): 2663-2668.

Daub, H., J. V. Olsen, et al. (2008). "Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle." Mol Cell **31**(3): 438-448.

Dephoure, N., C. Zhou, et al. (2008). "A quantitative atlas of mitotic phosphorylation." Proc Natl Acad Sci U S A **105**(31): 10762-10767.

Dorsey, F. C., K. L. Rose, et al. (2009). "Mapping the phosphorylation sites of Ulk1." J Proteome Res **8**(11): 5253-5263.

Egan, D. F., D. B. Shackelford, et al. (2011). "Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy." Science **331**(6016): 456-461.

Oppermann, F. S., F. Gnad, et al. (2009). "Large-scale proteomics analysis of the human kinome." Mol Cell Proteomics **8**(7): 1751-1764.

Pan, C., F. Gnad, et al. (2008). "Quantitative phosphoproteome analysis of a mouse liver cell line reveals specificity of phosphatase inhibitors." Proteomics **8**(21): 4534-4546.

Shang, L., S. Chen, et al. (2011). "Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK." Proc Natl Acad Sci U S A **108**(12): 4788-4793.

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