Supplemental Material to:

Chang Wook Park, Sun Mi Hong, Eung-Sam Kim, Jung Hee Kwon, Kyong-Tai Kim, Hong Gil Nam and Kwan Yong Choi

BNIP3 is degraded by ULK1-dependent autophagy via MTORC1 and AMPK

Autophagy 2012; 9(3)
http://dx.doi.org/10.4161/auto.23072

www.landesbioscience.com/journals/autophagy/article/23072

Supplementary information

Supplementary methods

Plasmid Construction. Full-length *BNIP3* and *BNIP3*$^{\Delta TM}$ (aa ^{1}-^{163}) were amplified from human cDNA pool by PCR using the following primers: *BNIP3* (FL) : Fw=5'-GAACAAGCTTATGTCGCAGAACGGAGCGC-3', Rv=5'-CAGGGAATTCACCCCAGGATCTAACAGCTC-3' and *BNIP3*$^{\Delta TM}$: Fw=5'-GAACAAGCTTATGTCGCAGAACGGAGCGC-3', Rv=5'-GAGGGAATTCTCATTTCAGAAATTCTGCAG-3' . The PCR products were cloned into HindIII and EcoRI sites of pFLAG-CMV-2 Expression Vector (Sigma, E7033). The construction was verified by DNA sequence analysis.

Real-time quantitative PCR (qPCR). RNA was isolated by RNAeasy kit (Qiagen, 74104) using the manufacturer's instructions. For cDNA synthesis, M-MLV Reverse Transcriptase (Ambion, AM2043) and SUPERase-In RNase Inhibitor (Ambion, AM2694) were used. qPCR was performed on Light Cycler 2.0 (Roche, 03531414001) using SYBR® *Premix Ex Taq*™ (Takara, PR041A), and data were analyzed with the Light Cycler software 4.05 (Roche). Primers were designed using Primer3 software. The primers for human *BNIP3* were: (FW) 5'-GATACCAACAGGGCTTCTGAAACAG-3' and (RV) 5'-CAGAGAATATGCCCCCTTTCTTCA-3'. The *GAPDH* gene was used as control. The primer sequences of *GAPDH* were : (FW)5'-CCTGGTATGACAACGAATTT-3' and (RV)5'-GTACATGACAAGGTGCGGCT-3'. Copy number of target genes (relative to *GAPDH*) was determined by the $2^{-\Delta\Delta Ct}$ method.

Autophagy analysis. Quantitative GFP-LC3 assay was performed in SK-Hep-1 cells. SK-Hep-1 cells were transfected with a GFP-LC3-expressing plasmid (a gift from Dr. J. Jung, USC, USA) and then stable cells were selected with 800 ug/ml G418 (Calbiochem, 345810) for 4 weeks. The cells were plated on coverslip and cultured in normoxia, hypoxia and hypoxia plus starvation, respectively. The cells were then fixed and mounted on glass slides, and visualized using a confocal microscope (Olympus, Fluoview FV 1000, Japan). To quantify GFP-LC3 puncta, at least 4 random fields were imaged and the average number of puncta/cell was calculated using the ImageJ software (NIH). For the LC3 flux assay, SK-Hep-1 cells pretreated with hypoxia for 24 h followed by DMEM or EBSS in the presence or absence of 10 nM BafA1. Cells were harvested and lysed, the LC3 band was analyzed by western blotting. We quantified MAP1LC3B-II/MAP1LC3B-I ratios by densitometry as described above.

Immunoprecipitation. SK-Hep-1 cells were transfected with Flag-*BNIP3* or Flag-*BNIP3*$^{\Delta TM}$ expression vectors followed by the amino acid starvation in the presence or absence of Baf A1 or MG132. Cells were lysed in 1 ml ice-cold lysis buffer containing 40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.3% CHAPS or 0.1% NP-40 and one tablet of EDTA-free protease inhibitors (Roche, 04 693 124 001) per 10 ml. Cell extracts were incubated with anti-Flag antibody overnight at 4°C. Then, 30 µl of Protein G-Sepharose was added. After incubation for 2 hrs, the immunoprecipitates were washed three times with the lysis buffer and subjected to immunoblotting.

Colony formation assay. Scrambled shRNA and *BNIP3* shRNA was transfected into SK-Hep-1 cells and selected for one month. The selected stable cell lines were plated at low

density (1000 cells/well) in 6 well plates and then grown in hypoxic conditions for 7 d. Colonies are fixed and stained with crystal violet, and then colony numbers were counted.

Supplementary Table S1 : Primary antibodies used in the present study

Target	Host	Type	Titer	Manufacturer	Cat. #
Flag	Mouse	monoclonal	1:1000	Sigma	F3165
BNIP3L	Mouse	monoclonal	1:1000	Sigma	WH0000665 M1
BNIP3	Mouse	monoclonal	1:1000	Novus	NB100–1543
NBR1	Mouse	monoclonal	1:1000	Novus	H00004077-M05
LDHA	Rabbit	monoclonal	1:1000	Cell Signaling Technology	2012
RPS6KB1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	9202
MAP1LC3B	Rabbit	monoclonal	1:1000	Cell Signaling Technology	3868
ATG7	Rabbit	polyclonal	1:1000	Cell Signaling Technology	2631
MTOR	Rabbit	polyclonal	1:1000	Cell Signaling Technology	2972
RPS6	Rabbit	monoclonal	1:4000	Cell Signaling Technology	2217
p-RPS6	Rabbit	monoclonal	1:4000	Cell Signaling Technology	2211
EIF4EBP1	Rabbit	monoclonal	1:4000	Cell Signaling Technology	9644
p-EIF4EBP1	Rabbit	monoclonal	1:4000	Cell Signaling Technology	2855
BAK1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	6947
BID	Rabbit	polyclonal	1:1000	Cell Signaling Technology	2002
BAD	Rabbit	polyclonal	1:1000	Cell Signaling Technology	9239
BCL2L11	Rabbit	monoclonal	1:1000	Cell Signaling Technology	2933
BAX	Rabbit	monoclonal	1:1000	Cell Signaling Technology	5023
Actin	Goat	polyclonal	1:4000	Santa Cruz Biotechnology	sc-1615
SQSTM1	Mouse	monoclonal	1:1000	Santa Cruz Biotechnology	sc-28359
BECN1	Rabbit	polyclonal	1:1000	Santa Cruz Biotechnology	sc-11427

TP53	Mouse	monoclonal	1:1000	Santa Cruz Biotechnology	sc-126
HIF1A	Rabbit	polyclonal	1:1000	Bethyl Laboratories	A300–286A
AMPKα/ PRKAA1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	5832
p-AMPKα/ PRKAA1 Rabbit	monoclonal	1:1000	Cell Signaling Technology	2535	
ULK1	Rabbit	polyclonal	1:1000	Cell Signaling Technology	4773
p-ULK1 (S555)	Rabbit	monoclonal	1:500	Cell Signaling Technology	5869
p-ULK1 (S757)	Rabbit	polyclonal	1:500	Cell Signaling Technology	6888
PIK3C3	Rabbit	monoclonal	1:500	Cell Signaling Technology	4263
MAP1LC3A	Rabbit	monoclonal	1:500	Cell Signaling Technology	4599
MAP1LC3C	Rabbit	polyclonal	1:2000	Millipore	AB15414
TOMM20	Mouse	monoclonal	1:500	Santacruz	sc-17764
COX4I1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	4850
LAMP2	Rabbit	monoclonal	1:1000	Epitomics	3660–1

Supplementary Table S2 : siRNA sequences

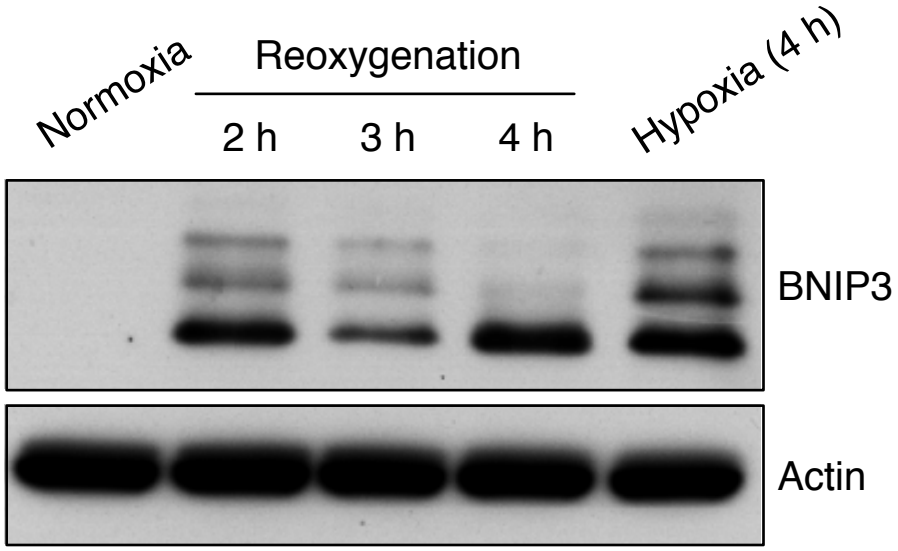
Target	Accession Number	Cat.#	Sequence
BECN1	NM_003766	D-010552–02	GGAUGACAGUGAACAGUUA
		D-010552–03	UAAGAUGGGUCUGAAAUUU
		D-010552–04	GCCAACAGCUUCACUCUGA
		D-010552–17	UUGAAAACCAGAUGCGUUA
MAP1LC3B	NM_022818	D-012846–01	CAAAGUUCCUUGUACCUGA
		D-012846–02	GAUAAUAGAACGAUACAAG
		D-012846–03	GUAGAAGAUGUCCGACUUA
		D-012846–04	AGGAGACGUUCGGGAUGAA
RPS6K	NM_003161	J-003616–13	CAUGGAACAUUGUGAGAAA
		J-003616–14	GGAAUGGGCAUAAGUUGUA
		J-003616–15	GUAAAUGGCUUGUGAUACU
		J-003616–16	CAAAUUAGCAUGCAAGCUU
LAMP2	NM_002294	D-011715–01	AAUGAGUUCCUGUGUGAUA
		D-011715–02	CUACUUAGACUCAAUAGCA
		D-011715–03	ACAGUACGCUAUGAAACUA
		D-011715–04	GAUAAGGUUGCUUCAGUUA
ULK1	NM_003565	D-005049–01	CCUAAAACGUGUCUUAUUU
		D-005049–02	ACUUGUAGGUGUUUAAGAA
		D-005049–03	GGUUAGCCCUGCCUGAAUC
		D-005049–04	UGUAGGUGUUUAAGAAUUG

MTOR	NM_004958	J-003008–11	GGCCAUAGCUAGCCUCAUA
		J-003008–12	CAAAGGACUUCGCCCAUAA
		J-003008–13	GCAGAAUUGUCAAGGGAUA
		J-003008–14	CCAAAGCACUACACUACAA
RPTOR	NM_020761	J-004107–05	GGCCAUAGCUAGCCUCAUA
		J-004107–06	CAAAGGACUUCGCCCAUAA
		J-004107–07	GCAGAAUUGUCAAGGGAUA
		J-004107–08	CCAAAGCACUACACUACAA
PIK3C3	NM_002647	J-005250–09	CACCAAAGCUCAUCGACAA
		J-005250–10	AUAGAUAGCUCCCAAAUUA
		J-005250–11	GAACAACGGUUUCGCUCUU
		J-005250–12	GAGAUGUACUUGAACGUAA
MAP1LC3A	NM_032514, NM_181509	J-013579–05	GGACGGCUUCCUCUAUAUG
		J-013579–06	CGGUGAUCAUCGAGCGCUA
		J-013579–07	UCGCGGACAUCUACGAGCA
		J-013579–08	UGAGCGAGUUGGUCAAGAU
MAP1LC3C	NM_001004343	J-032399–09	CUGGUCAGCAUGAGCGCAA
		J-032399–10	CGGUGGUAGUGGAGCGCUA
		J-032399–11	CAGAAAAUCCCAAGCGUCA
		J-032399–12	GUGUAAUGAGCUAGAGAUA
ATG7	NM_001136031; NM_006395	J-020112–05	CCAACACACUCGAGUCUUU
		J-020112–06	GAUCUAAAUCUCAAACUGA
		J-020112–07	GCCCACAGAUGGAGUAGCA
		J-020112–08	GCCAGAGGAUUCAACAUGA

Supplementary Table S3 : shRNA sequences

Target	Accession Number	Cat.#	Sequence
ATG7	NM_006395.1	TRCN0000007587	CCGGCCCAGCTATTGGAACACTGTACTCGAGTACAGTGTTCCAATAGCTGGGTTTTT
		TRCN0000007585	CCGGCCAGAGAGTTTACCTCTCATTCTCGAGAATGAGAGGTAAACTCTCTGGTTTTT
BNIP3	NM_004052.2	TRCN0000007832	CCGGGCTTCTGAAACAGATACCCATCTCGAGATGGGTATCTGTTTCAGAAGCTTTTT
		TRCN0000007833	CCGGGAACTGCACTTCAGCAATAATCTCGAGATTATTGCTGAAGTGCAGTTCTTTTT
Non-Targeting		SHC002V	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT

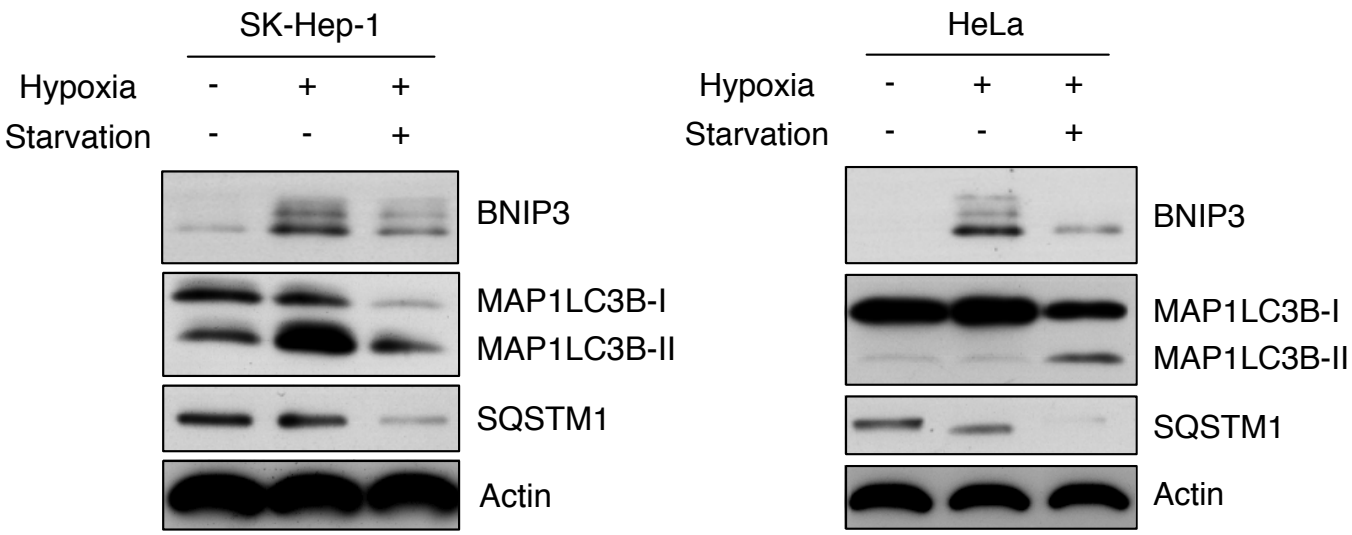
Sup. Fig. 1



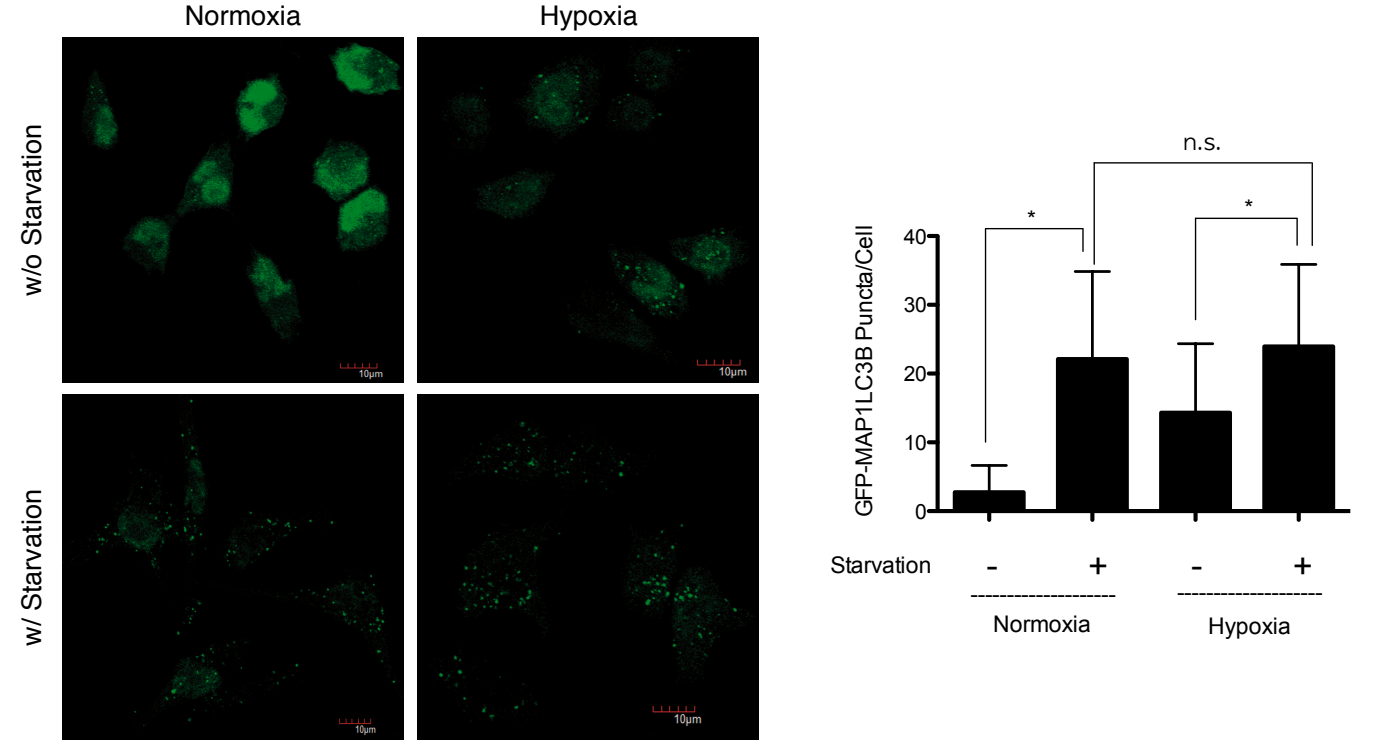
Supplementary figure 1. Short-term reoxygenation has little effect on the BNIP3 degradation. SK-Hep-1 cells were incubated under hypoxic conditions for 24 h and exposed to normoxia for indicated time points. For comparison, SK-Hep-1 cells were incubated under hypoxia and exposed to hypoxia for another 4 h. The BNIP3 level was assessed by western blot analysis.

Sup. Fig. 2

A

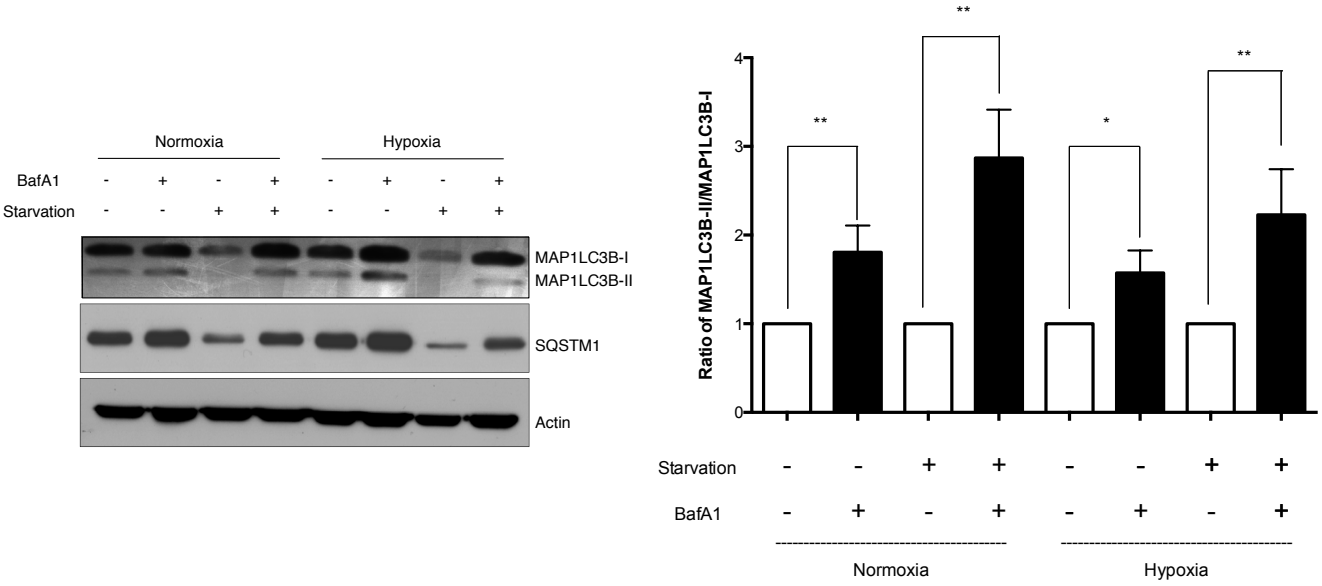

SK-Hep-1
Hypoxia - + +
Starvation - - +
BNIP3
MAP1LC3B-I
MAP1LC3B-II
SQSTM1
Actin
HeLa
Hypoxia - + +
Starvation - - +
BNIP3
MAP1LC3B-I
MAP1LC3B-II
SQSTM1
Actin

B


Normoxia
Hypoxia
w/o Starvation
w/ Starvation
10μm
n.s.
*
*
GFP-MAP1LC3B Puncta/Cell
40
30
20
10
0
Starvation - + - +
Normoxia
Hypoxia

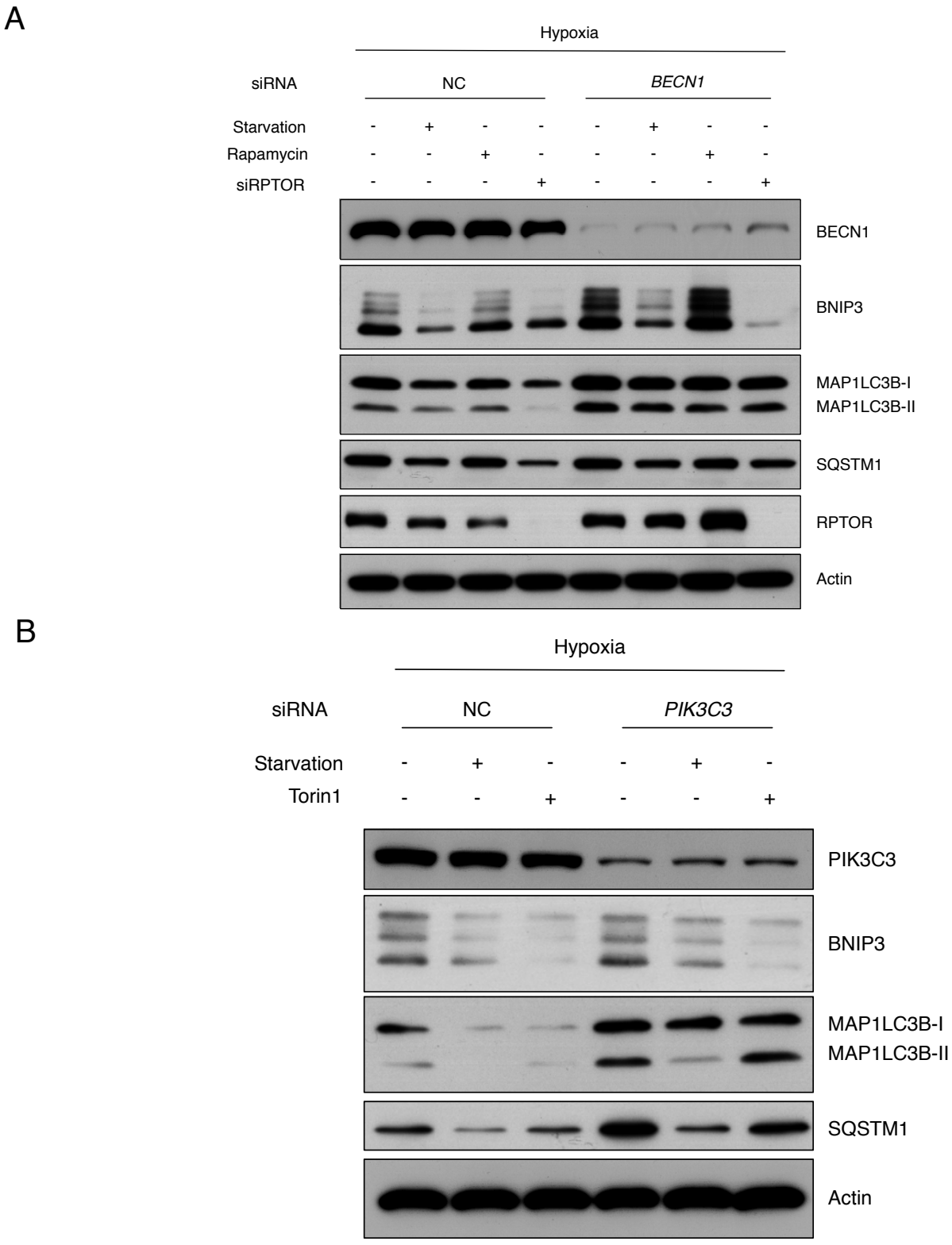
Sup. Fig. 2

C



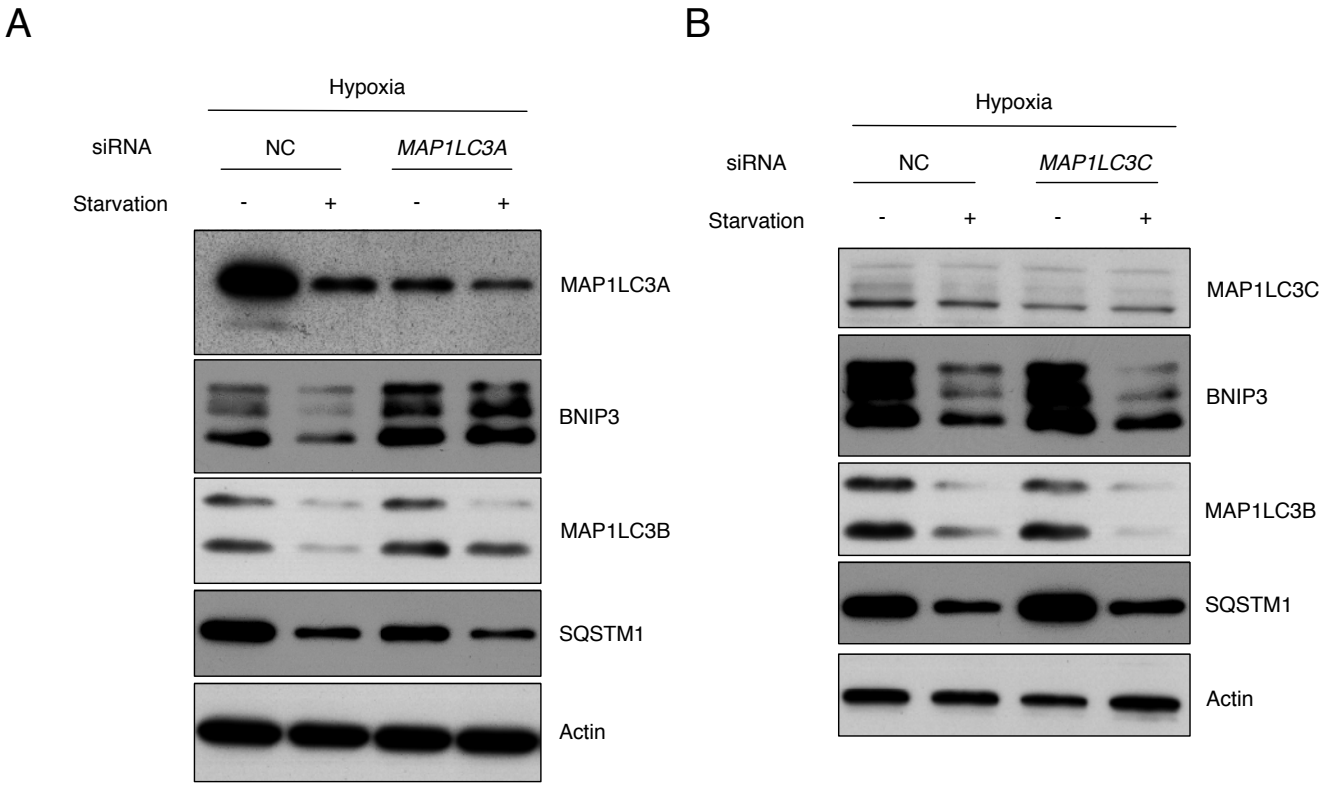
Supplementary figure 2. Autophagy occurs under hypoxic-starved condition inducing BNIP3 degradation. (A) SK-Hep-1 and HeLa cells were incubated under normoxic or hypoxic condition for 24 h followed by starvation under hypoxia for additional 4 h. The BNIP3 level was analyzed by western blotting. MAP1LC3B and SQSTM1 expression were used as autophagy markers. (B) SK-Hep-1 cells were transfected with *GFP-MAP1LC3B* and selected with G418. SK-Hep-1 cells stably expressing GFP-MAP1LC3B were exposed to normoxia and hypoxia for 24 h respectively or hypoxia (24 h) plus starvation (4 h) and then visualized by fluorescence microscopy (600 x). The number of GFP-MAP1LC3B dots was quantified using the ImageJ software. (C) SK-Hep-1 cells were exposed to normoxia or hypoxia in the presence or absence of amino acids and/or BafA1. The conversion of MAP1LC3B-I to MAP1LC3B-II, indicating the autophagy flux, was assessed by western blot analysis. Densitometric quantification of MAP1LC3B-II/MAP1LC3B-I levels were analyzed using the ImageJ software. The relative ratio of MAP1LCB-II/MAP1LC3B-I with the BafA1 treatment was compared with that without the treatment. Data are shown as means ± S.D. for three separate experiments. *P<0.05, **P<0.0005, t test; n.s., not significant.

Sup. Fig. 3



Supplementary figure 3. Degradation of BNIP3 has little dependency on the BECN1 complex. (A) SK-Hep-1 cells were transfected with *BECN1* siRNA for 48 h and incubated under hypoxia for 24 h. Amino acid starvation or rapamycin treatment were performed for an additional 4 h. For the knockdown of RPTOR, SK-Hep-1 cells were transfected with *RPTOR* siRNA for 48 h following the transfection of *BECN1* siRNA. Subsequently, SK-Hep-1 cells were exposed to hypoxia for 24 h followed by western blot analysis. (B) SK-Hep-1 cells were transfected with *PIK3C3* siRNA for 48 h and exposed to hypoxia for 24 h. Amino acid starvation or Torin1 treatment were carried out for an additional 4 h followed by western blot analysis.

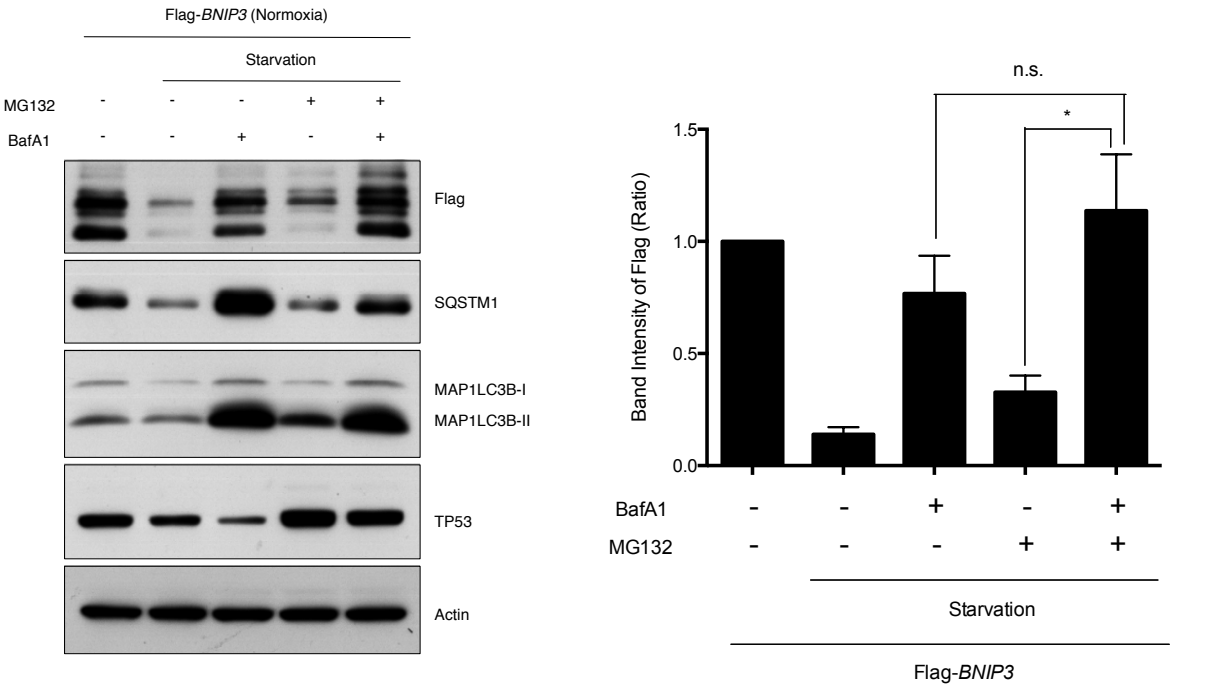
Sup. Fig. 4



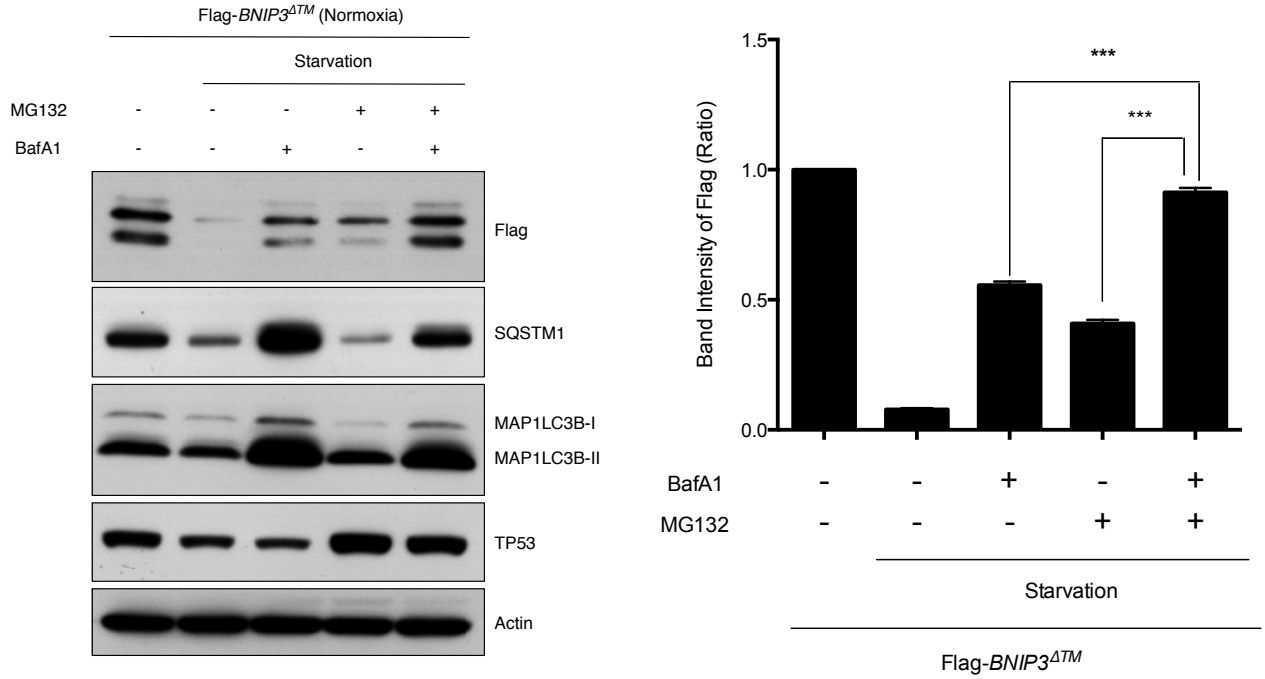
Supplementary figure 4. Autophagic degradation of BNIP3 is dependent on MAP1LC3A, not MAP1LC3C. SK-Hep-1 cells were transfected with *MAP1LC3A* (A) and *MAP1LC3C* (B) siRNAs for 48 h and exposed to hypoxia for 24 h. This was followed by a period of amino acid starvation for an additional 4 h and subsequent western blot analysis.

Sup. Fig. 5

A

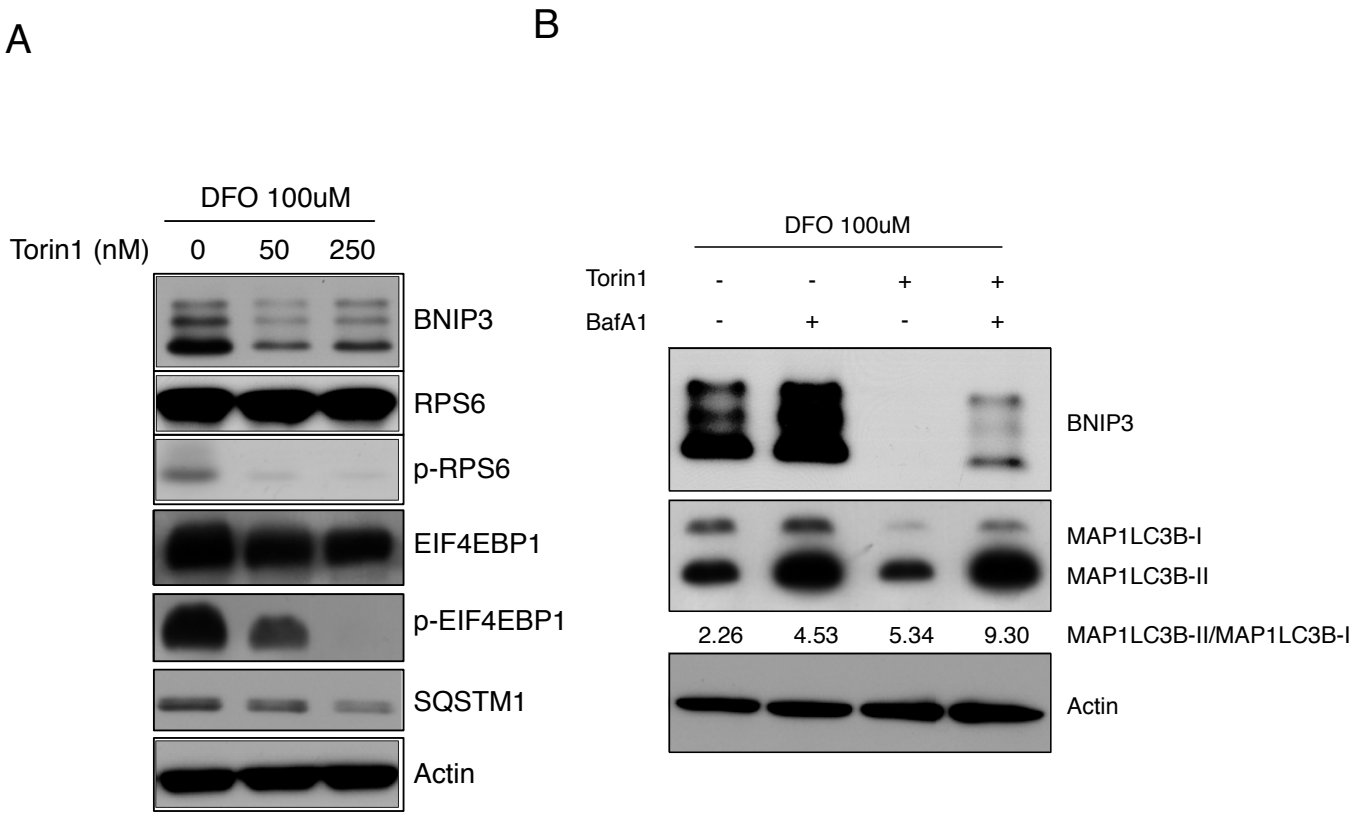


B



Supplementary figure 5. BNIP3 is degraded by autophagy as well as UPS. SK-Hep-1 cells were transfected with Flag-*BNIP3* (A) or Flag-*BNIP3*$^{\Delta TM}$ (B) under normoxia for 24 h and challenged with amino acid starvation in the presence or absence of BafA1 and/or MG132. SQSTM1 and MAP1LC3B were used as autophagy markers. TP53 expression was assessed for inhibition of UPS. BNIP3 expression levels were quantified by densitometry using the ImageJ software. Band intensities of BNIP3 protein were normalized by actin and compared to that of the first column. Data are shown as means ± S.D. for three separate experiments. *P<0.05, ***P<0.0005, t test.

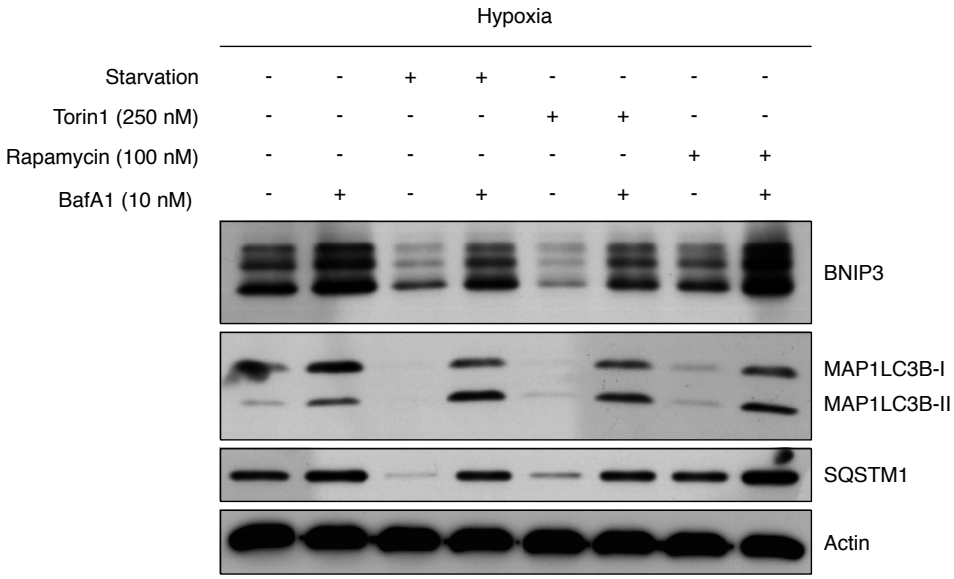
Sup. Fig. 6



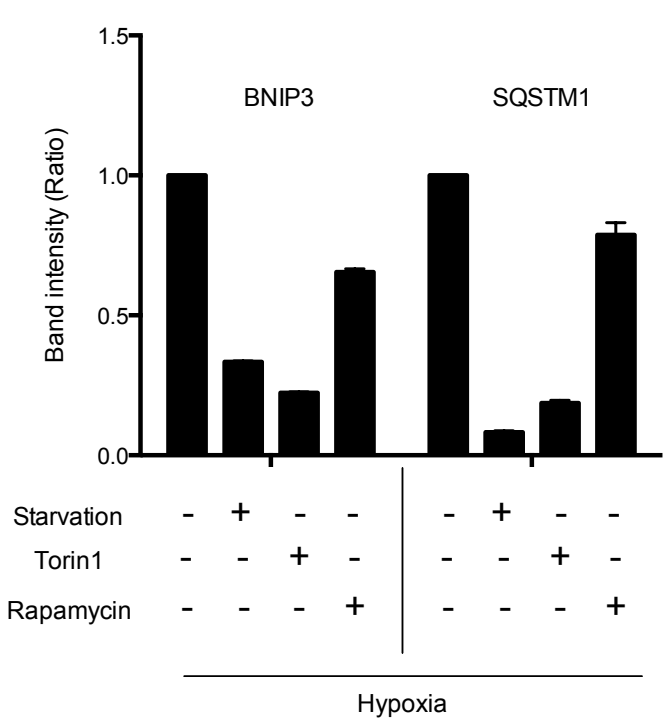
Supplementary figure 6. BNIP3 degradation is mediated by the inhibition of MTORC1 under a hypoxia-mimicking condition. (A) SK-Hep-1 cells were cultured under a hypoxia-mimicking condition (100 μM DFO) for 24 h. Torin1 was added for an additional 4 h with the indicated dose followed by western blot analysis. (B) SK-Hep-1 cells were exposed to hypoxia or DFO for 24 h. After hypoxia, the cells were treated with 250 nM Torin1 in the presence or absence of BafA1. The ratio of MAP1LC3B-II to MAP1LC3B-I was assessed by densitometry.

Sup. Fig. 7

A

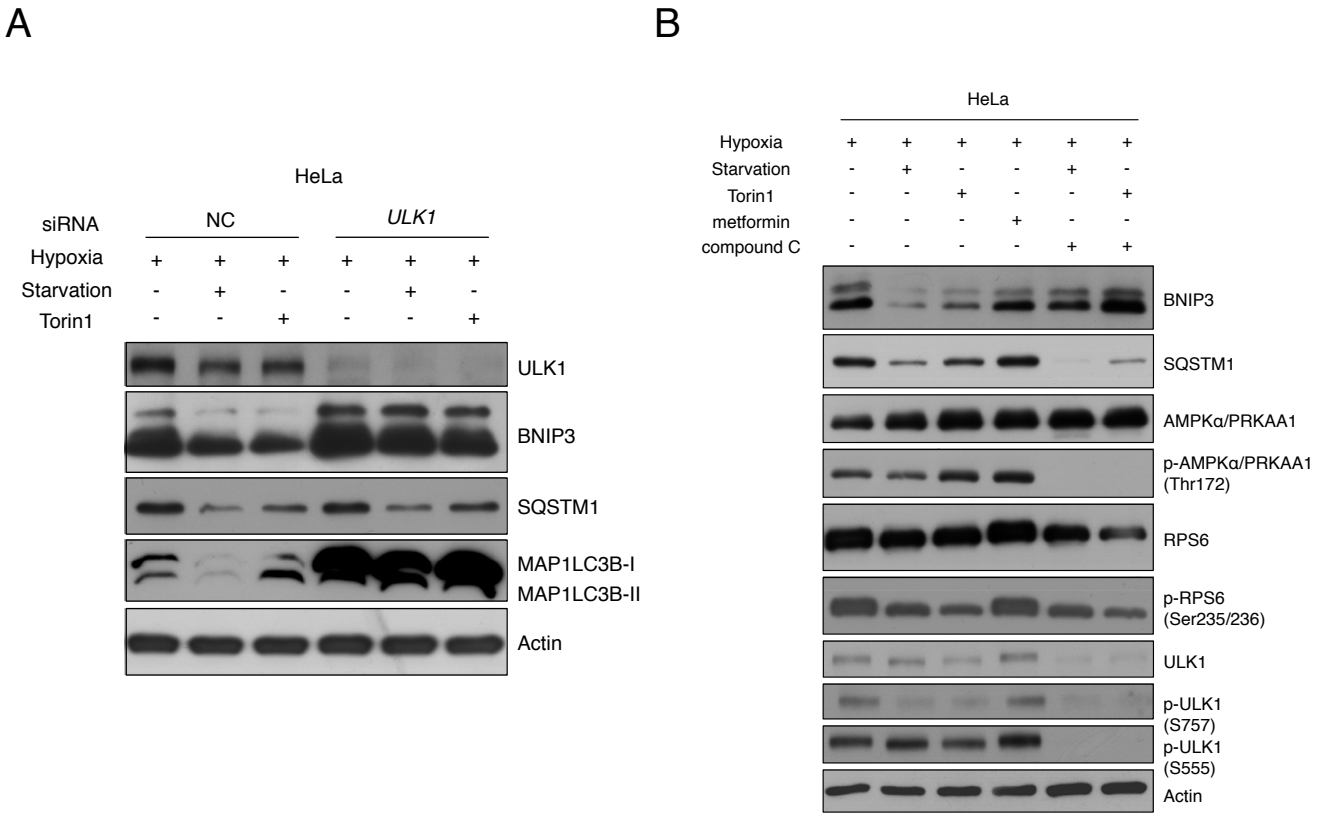


B



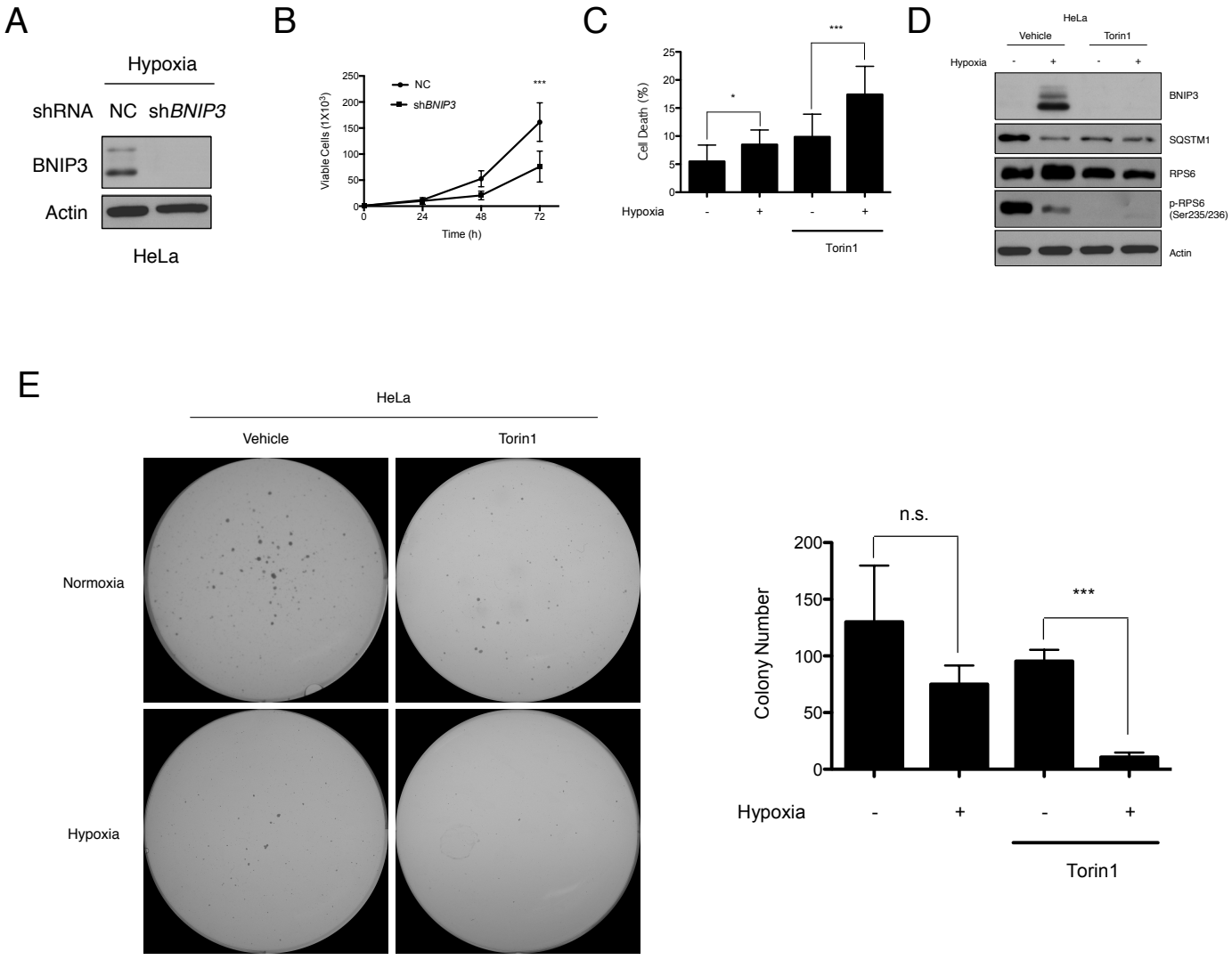
Supplementary figure 7. Rapamycin is less effective than Torin1 for BNIP3 degradation . (A) SK-Hep-1 cells were cultured under hypoxia for 24 h. Amino acid starvation, Torin1 treatment or rapamycin treatment was performed in the presence or absence of BafA1 for an additional 4 h with the indicated dose, followed by western blot analysis. (B) BNIP3 degradation and autophagy induction were analyzed by BNIP3 and SQSTM1 expression, respectively.

Sup. Fig. 8



Supplementary figure 8. Degradation of BNIP3 is regulated by ULK1 via MTORC1 and AMPK. (A) HeLa cells were transfected with *ULK1* siRNA for 48 h and exposed to hypoxia for 24 h. Amino acid starvation or Torin1 treatment were included as challenges for an additional 4 h followed by western blot analysis. (B) HeLa cells were incubated under hypoxic condition for 24 h and then treated with 250 nM Torin1, 5 mM metformin or 20 μM Compound C for 4 h. MTORC1 inhibition, AMPK activation or inhibition were confirmed by western blotting. ULK1 inhibition was assessed by the level of p-ULK1 (S757) and ULK1 activation by that of p-ULK1 (S555).

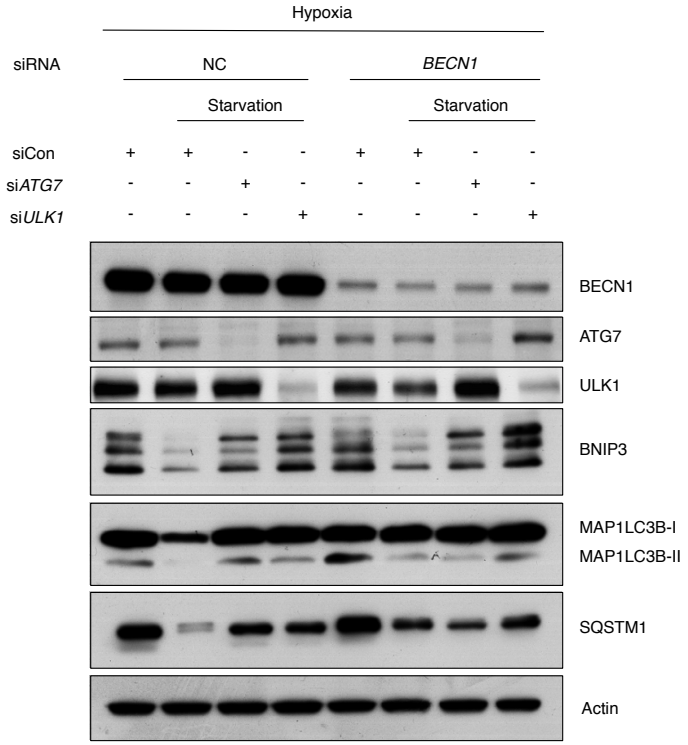
Sup. Fig. 9



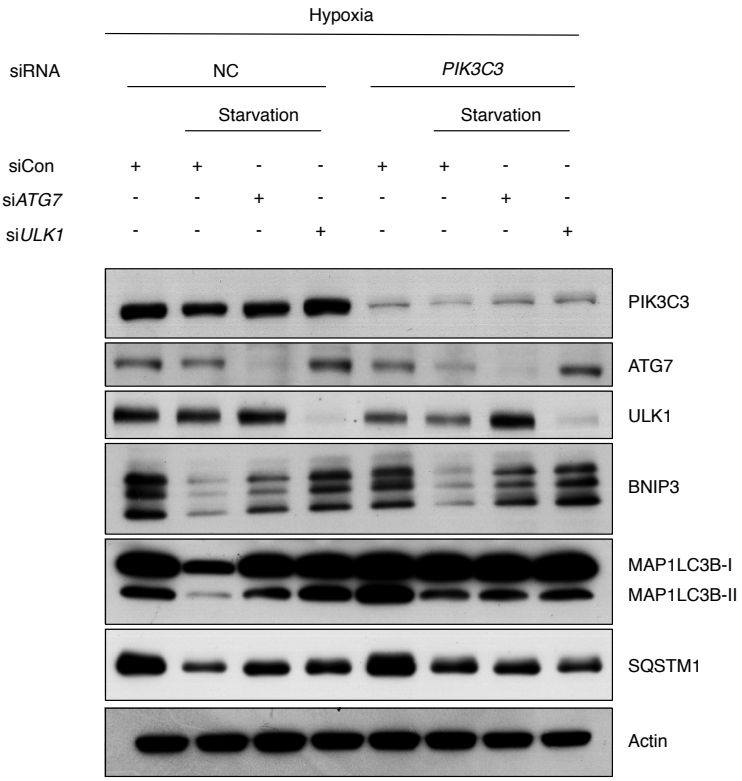
Supplementary figure 9. Loss of BNIP3 limits the survival of HeLa cells under hypoxic conditions. (A) Scrambled shRNA and *BNIP3* shRNA was transfected into HeLa cells. Knockdown of BNIP3 was confirmed by western blot analysis. (B) The selected stable cell lines were incubated under hypoxic conditions for up to 72 h. Proliferation was assessed by counting viable cells at the indicated time points. (C) HeLa cells were cultured under normoxia or hypoxia for 24 h followed by the Torin1 treatment for an additional 24 h. Cell death was assessed by the trypan blue exclusion assay. (D) The degradation of BNIP3, MTORC1 inhibition and the autophagy induction were confirmed by western blot analysis. (E) HeLa cells were incubated under normoxia or hypoxia for 24 h and then treated with Torin1 for 24 h. The long-term viability was analyzed by the soft agar assay. Data are shown as means ± S.D. for three independent experiments performed in triplicate. t test; *P<0.05, ***P<0.0005, n.s., not significant.

Sup. Fig. 10

A



B



Supplementary figure 10. BNIP3 is degraded in BECN1- or PIK3C3-depleted conditions and reversed by knockdown of ATG7 or ULK1. SK-Hep-1 cells were transfected with siRNA for *BECN1* (A) or *PIK3C3* (B) for 24 h followed by transfection with siRNA for *ATG7* or *ULK1* for 48 h. The transfected cells were incubated under hypoxia for 24 h and then amino acid starvation was performed for an additional 4 h followed by western blot analysis.

Supplementary information

Supplementary methods

Plasmid Construction. Full-length *BNIP3* and *BNIP3*$^{\Delta TM}$ (aa ^{1}-^{163}) were amplified from human cDNA pool by PCR using the following primers: *BNIP3* (FL) : Fw=5'-GAACAAGCTTATGTCGCAGAACGGAGCGC-3', Rv=5'-CAGGGAATTCACCCCAGGATCTAACAGCTC-3' and *BNIP3*$^{\Delta TM}$: Fw=5'-GAACAAGCTTATGTCGCAGAACGGAGCGC-3', Rv=5'-GAGGGAATTCTCATTTCAGAAATTCTGCAG-3' . The PCR products were cloned into HindIII and EcoRI sites of pFLAG-CMV-2 Expression Vector (Sigma, E7033). The construction was verified by DNA sequence analysis.

Real-time quantitative PCR (qPCR). RNA was isolated by RNAeasy kit (Qiagen, 74104) using the manufacturer's instructions. For cDNA synthesis, M-MLV Reverse Transcriptase (Ambion, AM2043) and SUPERase-In RNase Inhibitor (Ambion, AM2694) were used. qPCR was performed on Light Cycler 2.0 (Roche, 03531414001) using SYBR® *Premix Ex Taq*™ (Takara, PR041A), and data were analyzed with the Light Cycler software 4.05 (Roche). Primers were designed using Primer3 software. The primers for human *BNIP3* were: (FW) 5'-GATACCAACAGGGCTTCTGAAACAG-3' and (RV) 5'-CAGAGAATATGCCCCCTTTCTTCA-3'. The *GAPDH* gene was used as control. The primer sequences of *GAPDH* were : (FW)5'-CCTGGTATGACAACGAATTT-3' and (RV)5'-GTACATGACAAGGTGCGGCT-3'. Copy number of target genes (relative to *GAPDH*) was determined by the $2^{-\Delta\Delta Ct}$ method.

Autophagy analysis. Quantitative GFP-LC3 assay was performed in SK-Hep-1 cells. SK-Hep-1 cells were transfected with a GFP-LC3-expressing plasmid (a gift from Dr. J. Jung, USC, USA) and then stable cells were selected with 800 ug/ml G418 (Calbiochem, 345810) for 4 weeks. The cells were plated on coverslip and cultured in normoxia, hypoxia and hypoxia plus starvation, respectively. The cells were then fixed and mounted on glass slides, and visualized using a confocal microscope (Olympus, Fluoview FV 1000, Japan). To quantify GFP-LC3 puncta, at least 4 random fields were imaged and the average number of puncta/cell was calculated using the ImageJ software (NIH). For the LC3 flux assay, SK-Hep-1 cells pretreated with hypoxia for 24 h followed by DMEM or EBSS in the presence or absence of 10 nM BafA1. Cells were harvested and lysed, the LC3 band was analyzed by western blotting. We quantified MAP1LC3B-II/MAP1LC3B-I ratios by densitometry as described above.

Immunoprecipitation. SK-Hep-1 cells were transfected with Flag-*BNIP3* or Flag-*BNIP3*$^{\Delta TM}$ expression vectors followed by the amino acid starvation in the presence or absence of Baf A1 or MG132. Cells were lysed in 1 ml ice-cold lysis buffer containing 40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.3% CHAPS or 0.1% NP-40 and one tablet of EDTA-free protease inhibitors (Roche, 04 693 124 001) per 10 ml. Cell extracts were incubated with anti-Flag antibody overnight at 4°C. Then, 30 µl of Protein G-Sepharose was added. After incubation for 2 hrs, the immunoprecipitates were washed three times with the lysis buffer and subjected to immunoblotting.

Colony formation assay. Scrambled shRNA and *BNIP3* shRNA was transfected into SK-Hep-1 cells and selected for one month. The selected stable cell lines were plated at low

density (1000 cells/well) in 6 well plates and then grown in hypoxic conditions for 7 d. Colonies are fixed and stained with crystal violet, and then colony numbers were counted.

Supplementary Table S1 : Primary antibodies used in the present study

Target	Host	Type	Titer	Manufacturer	Cat. #
Flag	Mouse	monoclonal	1:1000	Sigma	F3165
BNIP3L	Mouse	monoclonal	1:1000	Sigma	WH0000665 M1
BNIP3	Mouse	monoclonal	1:1000	Novus	NB100–1543
NBR1	Mouse	monoclonal	1:1000	Novus	H00004077-M05
LDHA	Rabbit	monoclonal	1:1000	Cell Signaling Technology	2012
RPS6KB1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	9202
MAP1LC3B	Rabbit	monoclonal	1:1000	Cell Signaling Technology	3868
ATG7	Rabbit	polyclonal	1:1000	Cell Signaling Technology	2631
MTOR	Rabbit	polyclonal	1:1000	Cell Signaling Technology	2972
RPS6	Rabbit	monoclonal	1:4000	Cell Signaling Technology	2217
p-RPS6	Rabbit	monoclonal	1:4000	Cell Signaling Technology	2211
EIF4EBP1	Rabbit	monoclonal	1:4000	Cell Signaling Technology	9644
p-EIF4EBP1	Rabbit	monoclonal	1:4000	Cell Signaling Technology	2855
BAK1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	6947
BID	Rabbit	polyclonal	1:1000	Cell Signaling Technology	2002
BAD	Rabbit	polyclonal	1:1000	Cell Signaling Technology	9239
BCL2L11	Rabbit	monoclonal	1:1000	Cell Signaling Technology	2933
BAX	Rabbit	monoclonal	1:1000	Cell Signaling Technology	5023
Actin	Goat	polyclonal	1:4000	Santa Cruz Biotechnology	sc-1615
SQSTM1	Mouse	monoclonal	1:1000	Santa Cruz Biotechnology	sc-28359
BECN1	Rabbit	polyclonal	1:1000	Santa Cruz Biotechnology	sc-11427

TP53	Mouse	monoclonal	1:1000	Santa Cruz Biotechnology	sc-126
HIF1A	Rabbit	polyclonal	1:1000	Bethyl Laboratories	A300–286A
AMPKα/ PRKAA1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	5832
p-AMPKα/ PRKAA1 Rabbit	monoclonal	1:1000	Cell Signaling Technology	2535	
ULK1	Rabbit	polyclonal	1:1000	Cell Signaling Technology	4773
p-ULK1 (S555)	Rabbit	monoclonal	1:500	Cell Signaling Technology	5869
p-ULK1 (S757)	Rabbit	polyclonal	1:500	Cell Signaling Technology	6888
PIK3C3	Rabbit	monoclonal	1:500	Cell Signaling Technology	4263
MAP1LC3A	Rabbit	monoclonal	1:500	Cell Signaling Technology	4599
MAP1LC3C	Rabbit	polyclonal	1:2000	Millipore	AB15414
TOMM20	Mouse	monoclonal	1:500	Santacruz	sc-17764
COX4I1	Rabbit	monoclonal	1:1000	Cell Signaling Technology	4850
LAMP2	Rabbit	monoclonal	1:1000	Epitomics	3660–1

Supplementary Table S2 : siRNA sequences

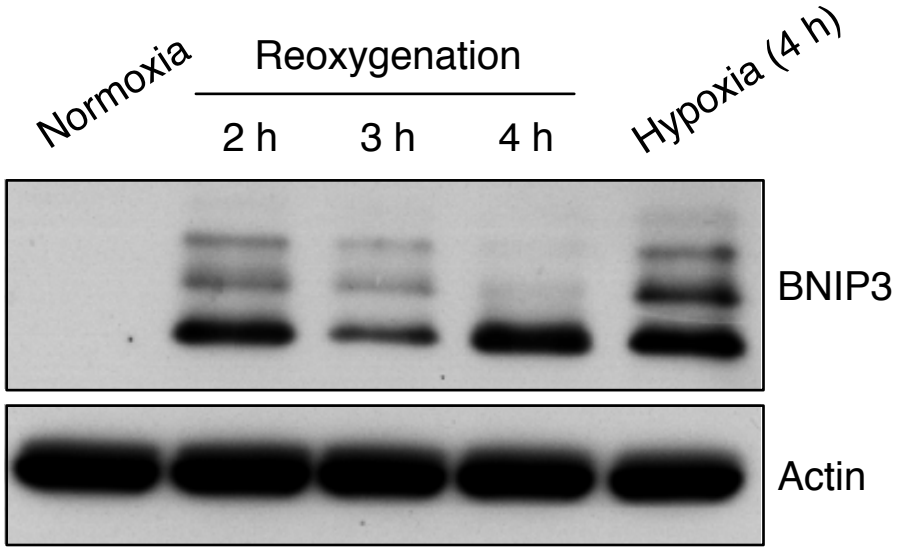
Target	Accession Number	Cat.#	Sequence
BECN1	NM_003766	D-010552–02	GGAUGACAGUGAACAGUUA
		D-010552–03	UAAGAUGGGUCUGAAAUUU
		D-010552–04	GCCAACAGCUUCACUCUGA
		D-010552–17	UUGAAAACCAGAUGCGUUA
MAP1LC3B	NM_022818	D-012846–01	CAAAGUUCCUUGUACCUGA
		D-012846–02	GAUAAUAGAACGAUACAAG
		D-012846–03	GUAGAAGAUGUCCGACUUA
		D-012846–04	AGGAGACGUUCGGGAUGAA
RPS6K	NM_003161	J-003616–13	CAUGGAACAUUGUGAGAAA
		J-003616–14	GGAAUGGGCAUAAGUUGUA
		J-003616–15	GUAAAUGGCUUGUGAUACU
		J-003616–16	CAAAUUAGCAUGCAAGCUU
LAMP2	NM_002294	D-011715–01	AAUGAGUUCCUGUGUGAUA
		D-011715–02	CUACUUAGACUCAAUAGCA
		D-011715–03	ACAGUACGCUAUGAAACUA
		D-011715–04	GAUAAGGUUGCUUCAGUUA
ULK1	NM_003565	D-005049–01	CCUAAAACGUGUCUUAUUU
		D-005049–02	ACUUGUAGGUGUUUAAGAA
		D-005049–03	GGUUAGCCCUGCCUGAAUC
		D-005049–04	UGUAGGUGUUUAAGAAUUG

MTOR	NM_004958	J-003008–11	GGCCAUAGCUAGCCUCAUA
		J-003008–12	CAAAGGACUUCGCCCAUAA
		J-003008–13	GCAGAAUUGUCAAGGGAUA
		J-003008–14	CCAAAGCACUACACUACAA
RPTOR	NM_020761	J-004107–05	GGCCAUAGCUAGCCUCAUA
		J-004107–06	CAAAGGACUUCGCCCAUAA
		J-004107–07	GCAGAAUUGUCAAGGGAUA
		J-004107–08	CCAAAGCACUACACUACAA
PIK3C3	NM_002647	J-005250–09	CACCAAAGCUCAUCGACAA
		J-005250–10	AUAGAUAGCUCCCAAAUUA
		J-005250–11	GAACAACGGUUUCGCUCUU
		J-005250–12	GAGAUGUACUUGAACGUAA
MAP1LC3A	NM_032514, NM_181509	J-013579–05	GGACGGCUUCCUCUAUAUG
		J-013579–06	CGGUGAUCAUCGAGCGCUA
		J-013579–07	UCGCGGACAUCUACGAGCA
		J-013579–08	UGAGCGAGUUGGUCAAGAU
MAP1LC3C	NM_001004343	J-032399–09	CUGGUCAGCAUGAGCGCAA
		J-032399–10	CGGUGGUAGUGGAGCGCUA
		J-032399–11	CAGAAAAUCCCAAGCGUCA
		J-032399–12	GUGUAAUGAGCUAGAGAUA
ATG7	NM_001136031; NM_006395	J-020112–05	CCAACACACUCGAGUCUUU
		J-020112–06	GAUCUAAAUCUCAAACUGA
		J-020112–07	GCCCACAGAUGGAGUAGCA
		J-020112–08	GCCAGAGGAUUCAACAUGA

Supplementary Table S3 : shRNA sequences

Target	Accession Number	Cat.#	Sequence
ATG7	NM_006395.1	TRCN0000007587	CCGGCCCAGCTATTGGAACACTGTACTCGAGTACAGTGTTCCAATAGCTGGGTTTTT
		TRCN0000007585	CCGGCCAGAGAGTTTACCTCTCATTCTCGAGAATGAGAGGTAAACTCTCTGGTTTTT
BNIP3	NM_004052.2	TRCN0000007832	CCGGGCTTCTGAAACAGATACCCATCTCGAGATGGGTATCTGTTTCAGAAGCTTTTT
		TRCN0000007833	CCGGGAACTGCACTTCAGCAATAATCTCGAGATTATTGCTGAAGTGCAGTTCTTTTT
Non-Targeting		SHC002V	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT

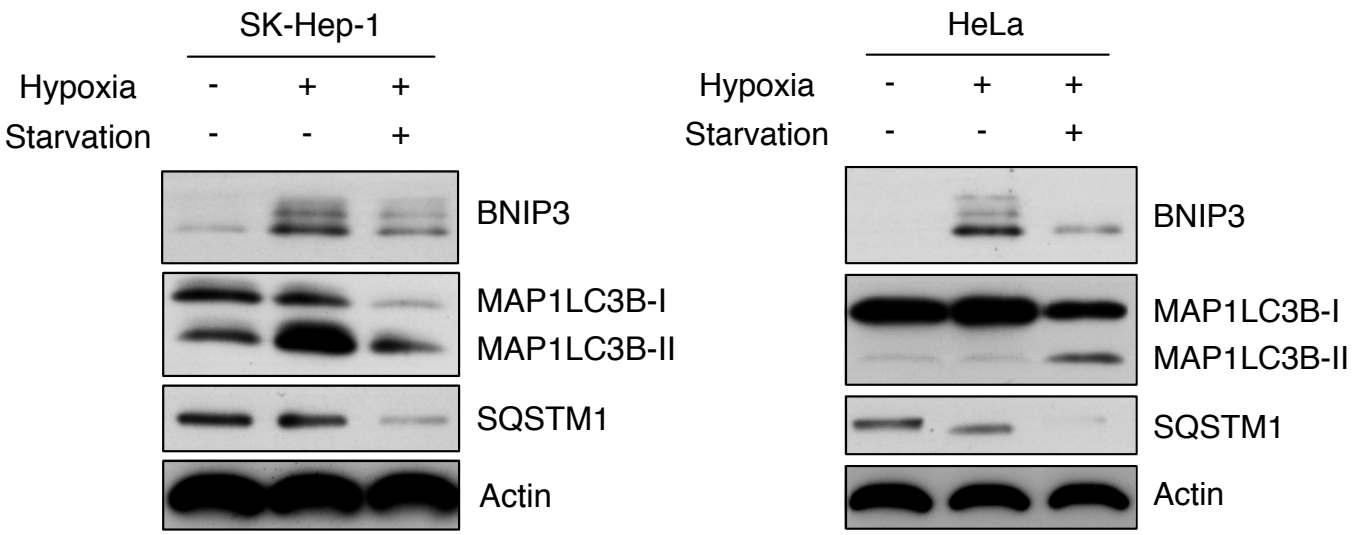
Sup. Fig. 1



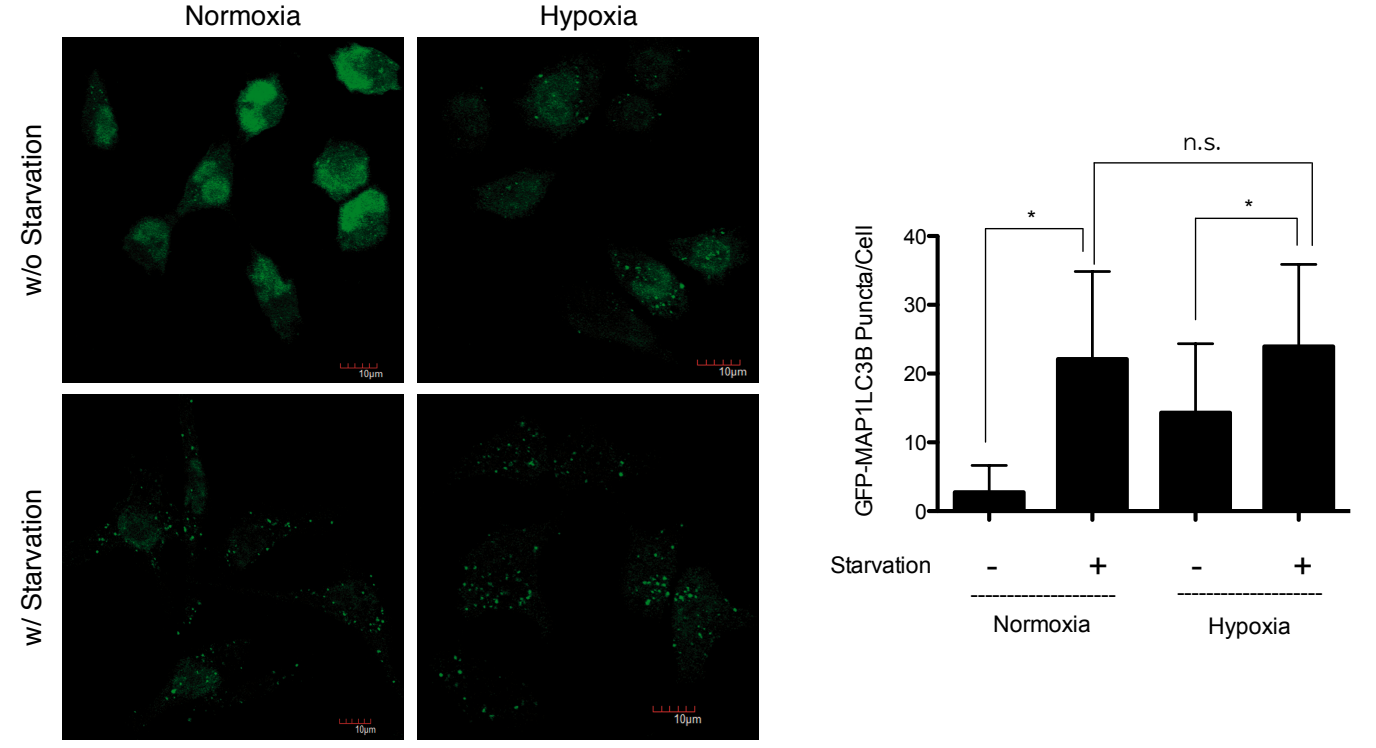
Supplementary figure 1. Short-term reoxygenation has little effect on the BNIP3 degradation. SK-Hep-1 cells were incubated under hypoxic conditions for 24 h and exposed to normoxia for indicated time points. For comparison, SK-Hep-1 cells were incubated under hypoxia and exposed to hypoxia for another 4 h. The BNIP3 level was assessed by western blot analysis.

Sup. Fig. 2

A

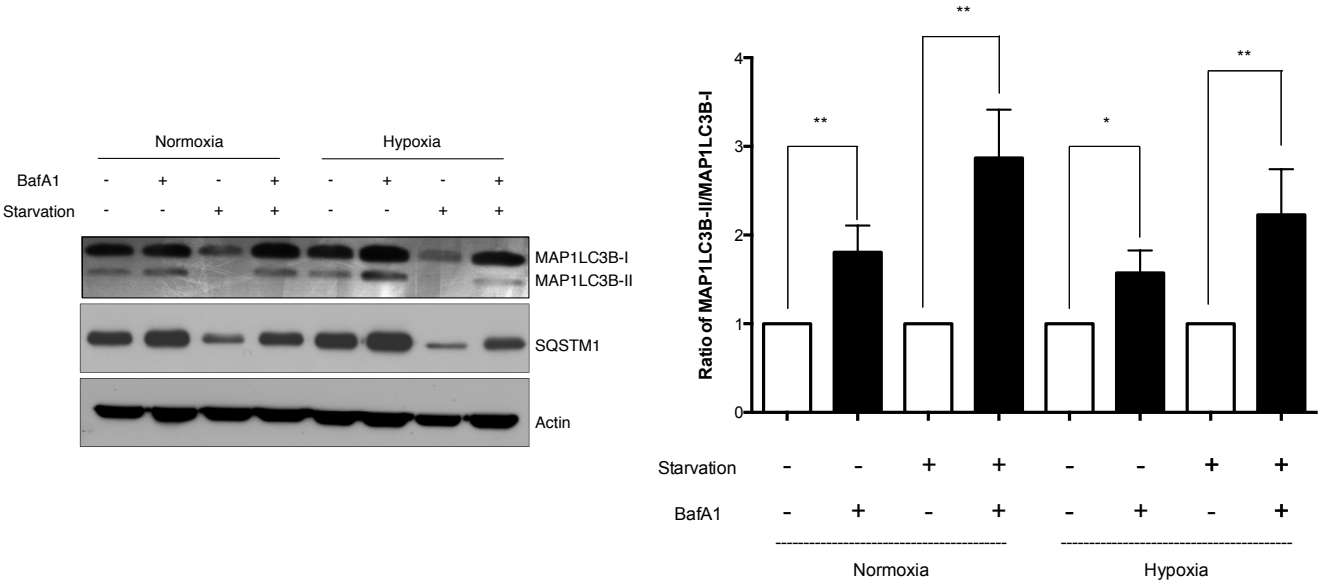

SK-Hep-1
Hypoxia - + +
Starvation - - +
BNIP3
MAP1LC3B-I
MAP1LC3B-II
SQSTM1
Actin
HeLa
Hypoxia - + +
Starvation - - +
BNIP3
MAP1LC3B-I
MAP1LC3B-II
SQSTM1
Actin

B


Normoxia
Hypoxia
w/o Starvation
w/ Starvation
10μm
n.s.
*
*
GFP-MAP1LC3B Puncta/Cell
40
30
20
10
0
Starvation - + - +
Normoxia
Hypoxia

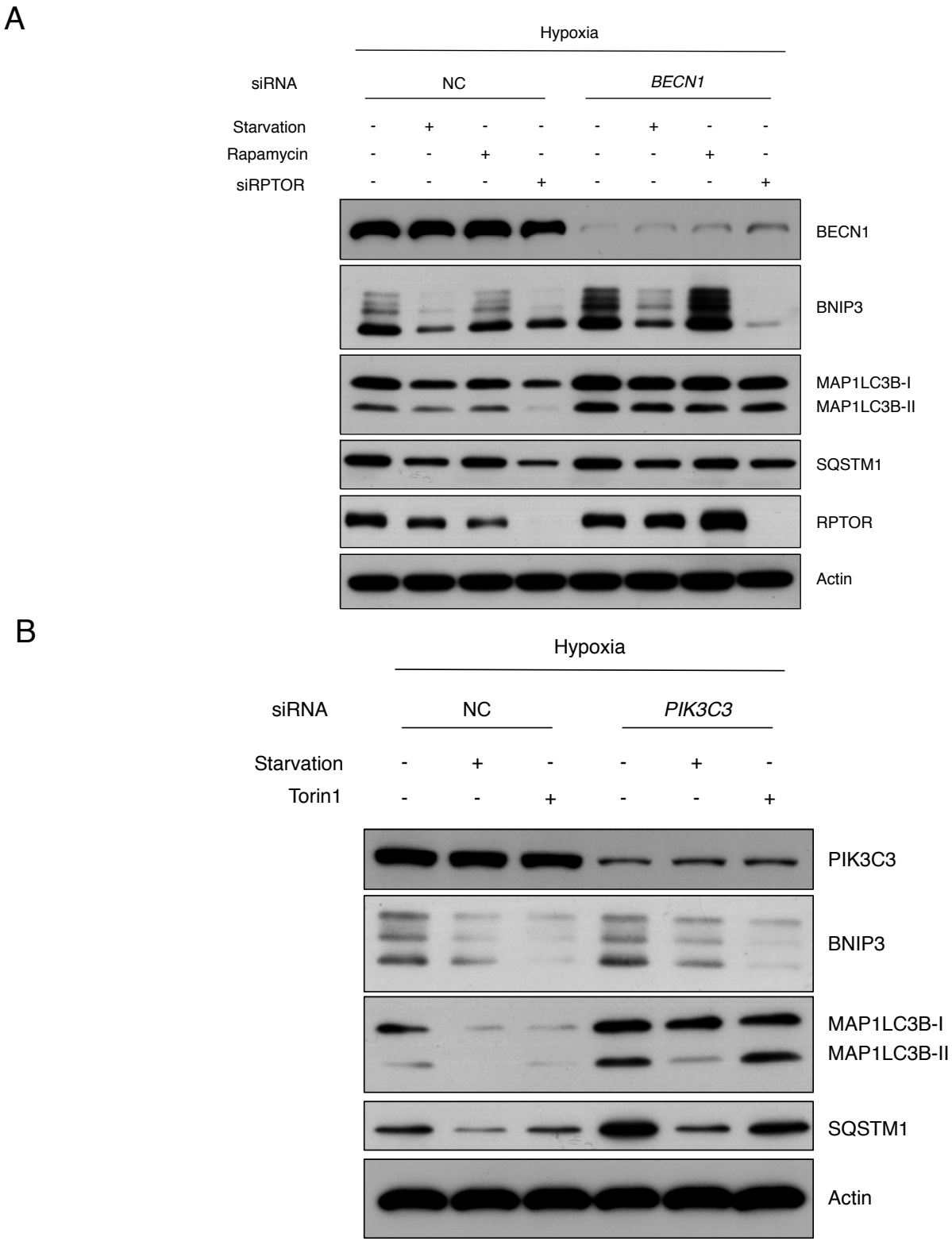
Sup. Fig. 2

C



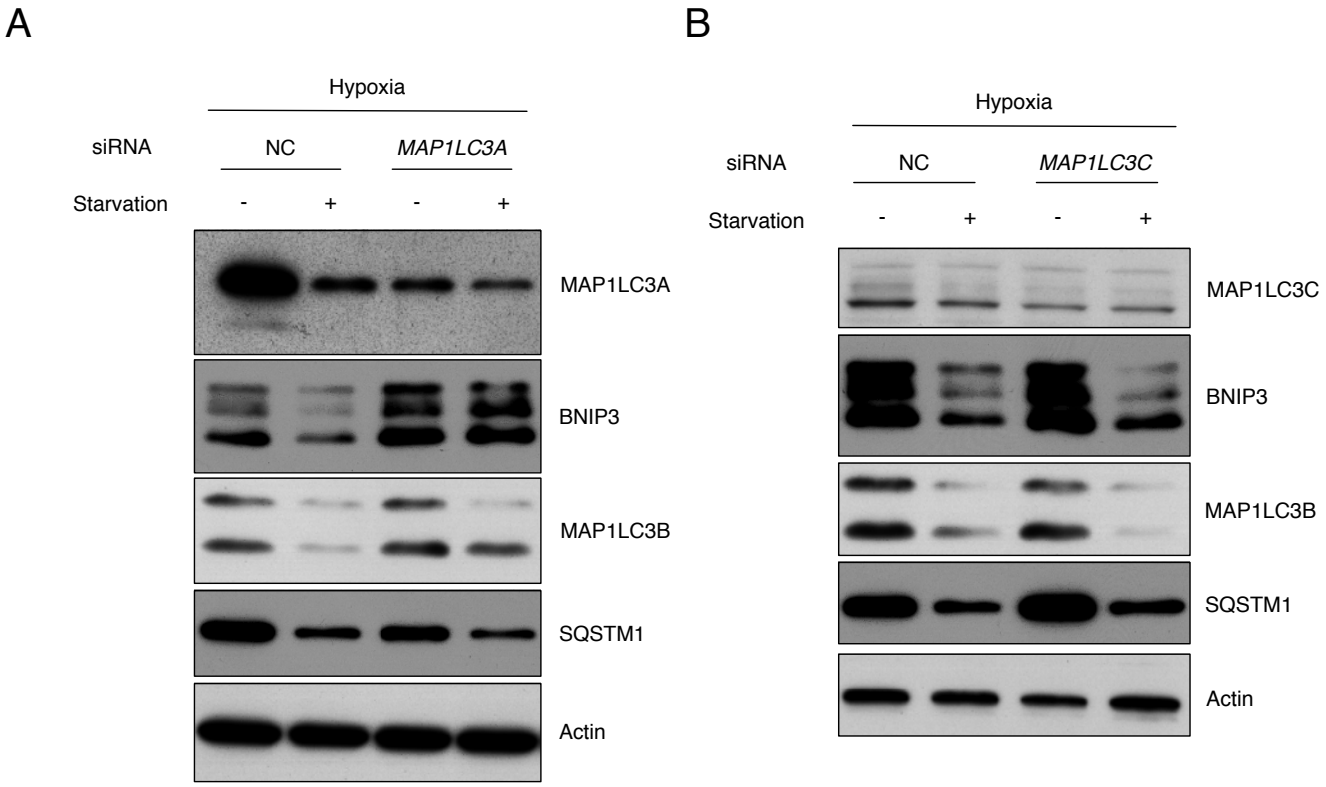
Supplementary figure 2. Autophagy occurs under hypoxic-starved condition inducing BNIP3 degradation. (A) SK-Hep-1 and HeLa cells were incubated under normoxic or hypoxic condition for 24 h followed by starvation under hypoxia for additional 4 h. The BNIP3 level was analyzed by western blotting. MAP1LC3B and SQSTM1 expression were used as autophagy markers. (B) SK-Hep-1 cells were transfected with *GFP-MAP1LC3B* and selected with G418. SK-Hep-1 cells stably expressing GFP-MAP1LC3B were exposed to normoxia and hypoxia for 24 h respectively or hypoxia (24 h) plus starvation (4 h) and then visualized by fluorescence microscopy (600 x). The number of GFP-MAP1LC3B dots was quantified using the ImageJ software. (C) SK-Hep-1 cells were exposed to normoxia or hypoxia in the presence or absence of amino acids and/or BafA1. The conversion of MAP1LC3B-I to MAP1LC3B-II, indicating the autophagy flux, was assessed by western blot analysis. Densitometric quantification of MAP1LC3B-II/MAP1LC3B-I levels were analyzed using the ImageJ software. The relative ratio of MAP1LCB-II/MAP1LC3B-I with the BafA1 treatment was compared with that without the treatment. Data are shown as means ± S.D. for three separate experiments. *P<0.05, **P<0.0005, t test; n.s., not significant.

Sup. Fig. 3



Supplementary figure 3. Degradation of BNIP3 has little dependency on the BECN1 complex. (A) SK-Hep-1 cells were transfected with *BECN1* siRNA for 48 h and incubated under hypoxia for 24 h. Amino acid starvation or rapamycin treatment were performed for an additional 4 h. For the knockdown of RPTOR, SK-Hep-1 cells were transfected with *RPTOR* siRNA for 48 h following the transfection of *BECN1* siRNA. Subsequently, SK-Hep-1 cells were exposed to hypoxia for 24 h followed by western blot analysis. (B) SK-Hep-1 cells were transfected with *PIK3C3* siRNA for 48 h and exposed to hypoxia for 24 h. Amino acid starvation or Torin1 treatment were carried out for an additional 4 h followed by western blot analysis.

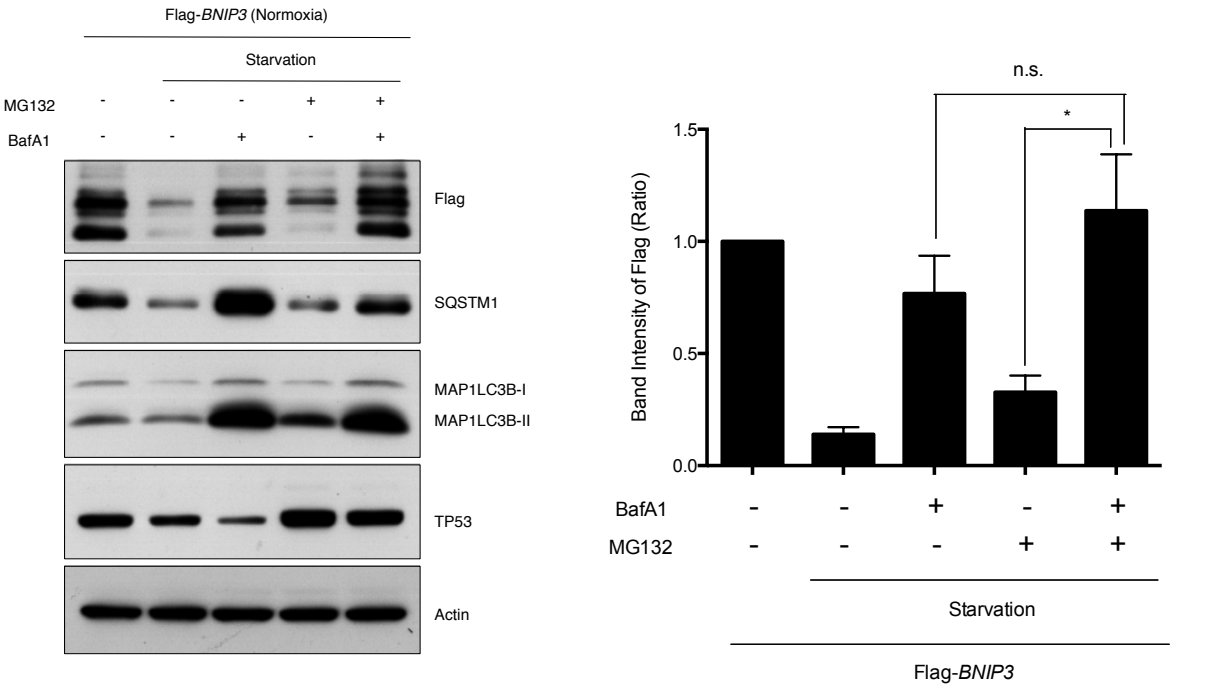
Sup. Fig. 4



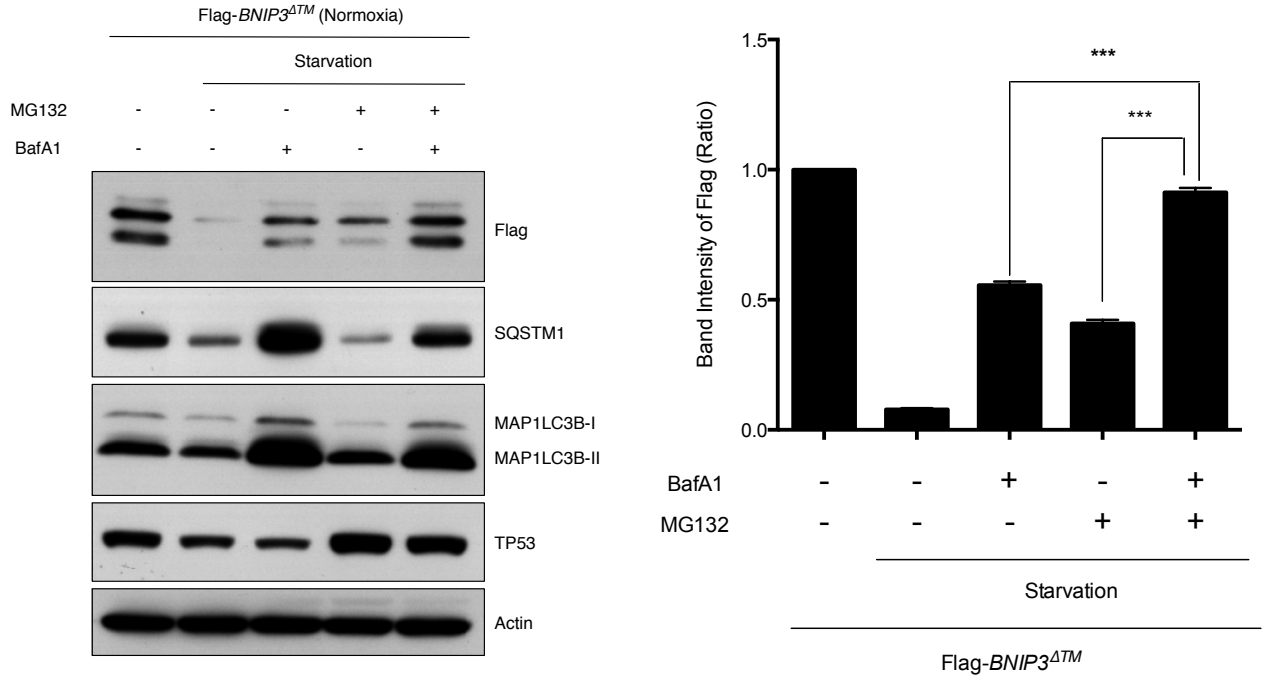
Supplementary figure 4. Autophagic degradation of BNIP3 is dependent on MAP1LC3A, not MAP1LC3C. SK-Hep-1 cells were transfected with *MAP1LC3A* (A) and *MAP1LC3C* (B) siRNAs for 48 h and exposed to hypoxia for 24 h. This was followed by a period of amino acid starvation for an additional 4 h and subsequent western blot analysis.

Sup. Fig. 5

A

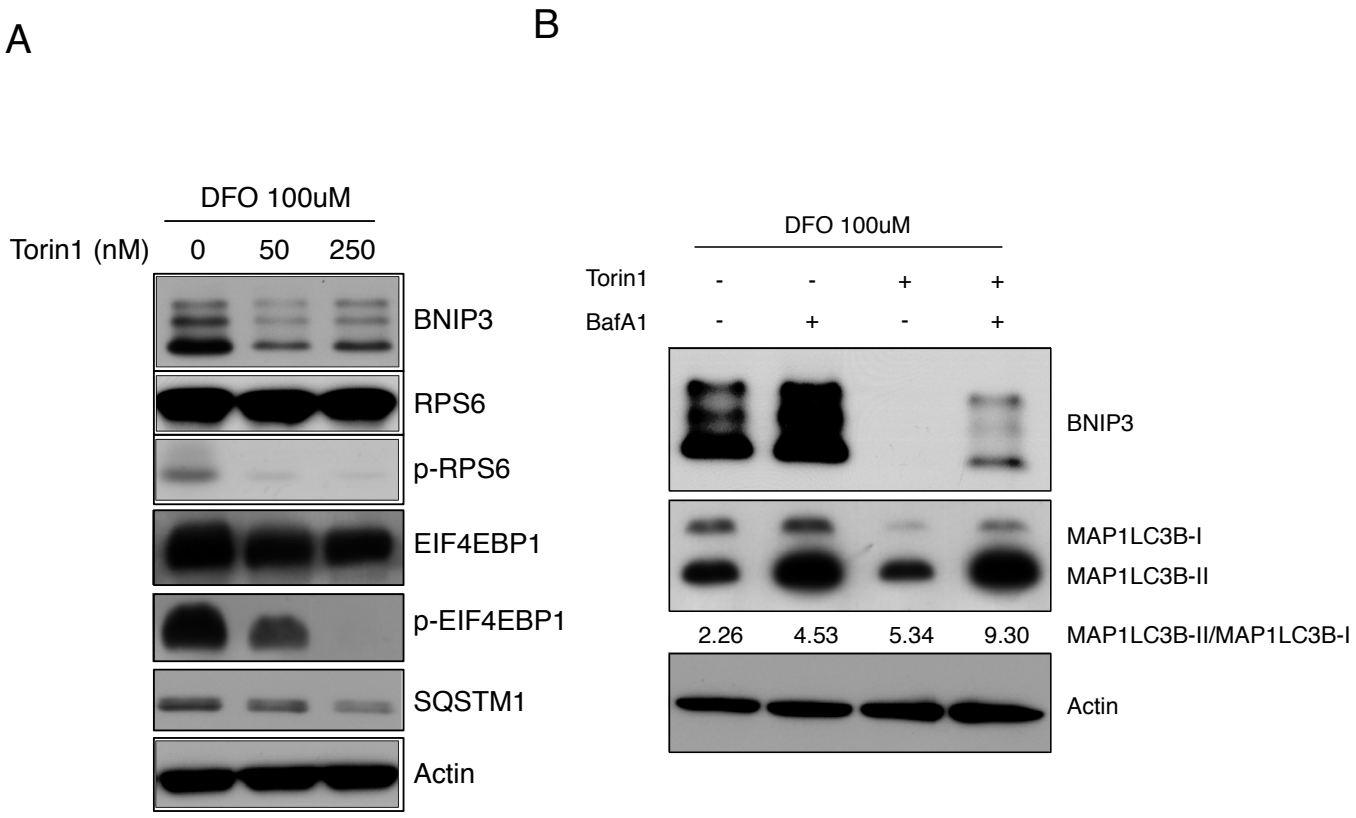


B



Supplementary figure 5. BNIP3 is degraded by autophagy as well as UPS. SK-Hep-1 cells were transfected with Flag-*BNIP3* (A) or Flag-*BNIP3*$^{\Delta TM}$ (B) under normoxia for 24 h and challenged with amino acid starvation in the presence or absence of BafA1 and/or MG132. SQSTM1 and MAP1LC3B were used as autophagy markers. TP53 expression was assessed for inhibition of UPS. BNIP3 expression levels were quantified by densitometry using the ImageJ software. Band intensities of BNIP3 protein were normalized by actin and compared to that of the first column. Data are shown as means ± S.D. for three separate experiments. *P<0.05, ***P<0.0005, t test.

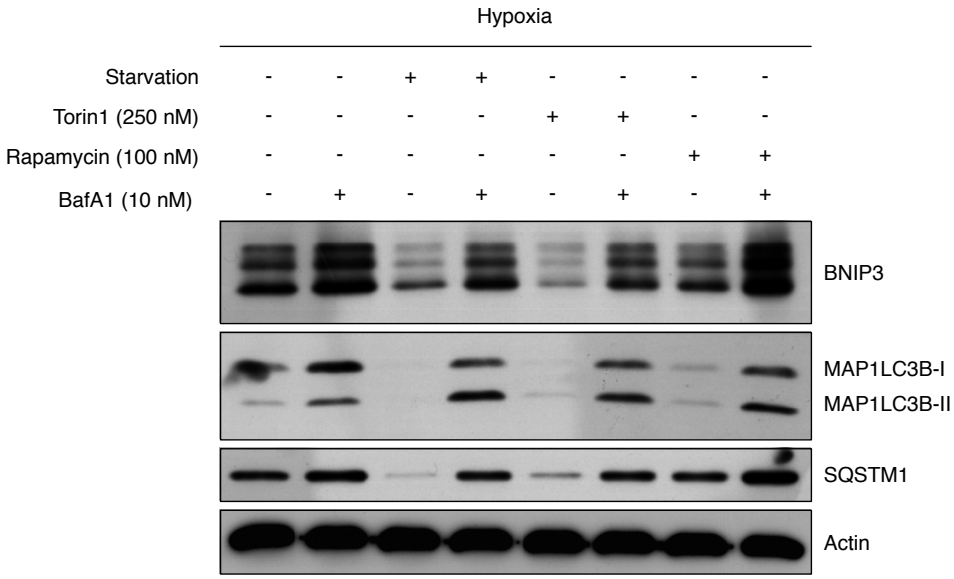
Sup. Fig. 6



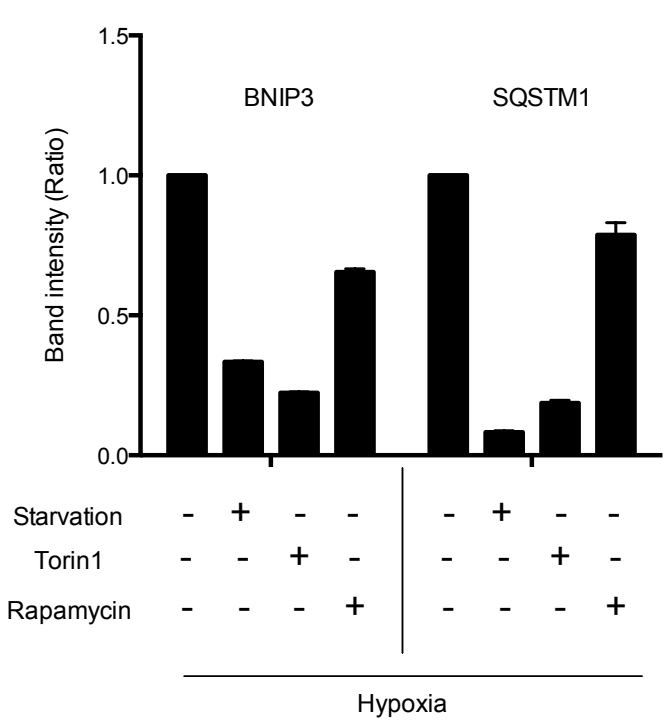
Supplementary figure 6. BNIP3 degradation is mediated by the inhibition of MTORC1 under a hypoxia-mimicking condition. (A) SK-Hep-1 cells were cultured under a hypoxia-mimicking condition (100 μM DFO) for 24 h. Torin1 was added for an additional 4 h with the indicated dose followed by western blot analysis. (B) SK-Hep-1 cells were exposed to hypoxia or DFO for 24 h. After hypoxia, the cells were treated with 250 nM Torin1 in the presence or absence of BafA1. The ratio of MAP1LC3B-II to MAP1LC3B-I was assessed by densitometry.

Sup. Fig. 7

A

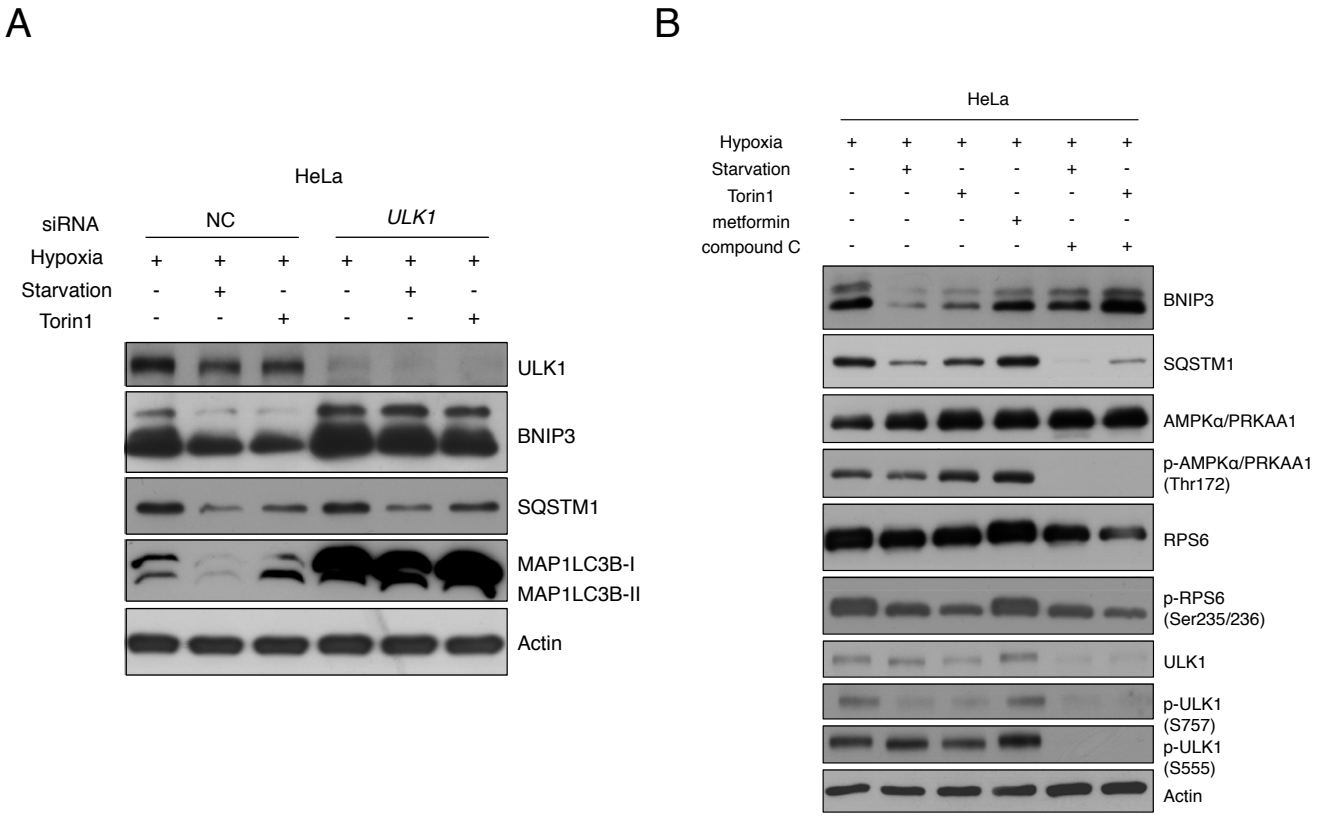


B



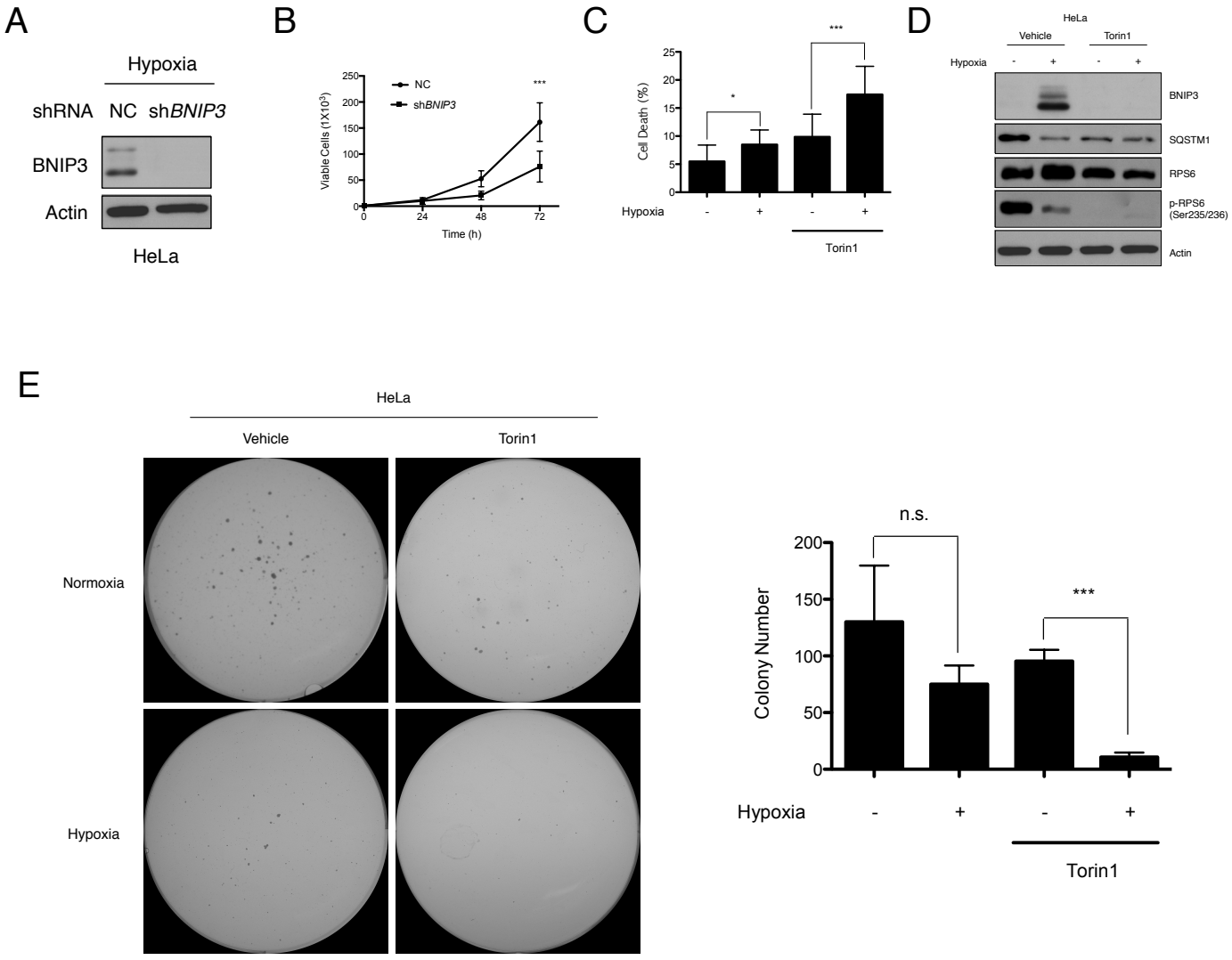
Supplementary figure 7. Rapamycin is less effective than Torin1 for BNIP3 degradation . (A) SK-Hep-1 cells were cultured under hypoxia for 24 h. Amino acid starvation, Torin1 treatment or rapamycin treatment was performed in the presence or absence of BafA1 for an additional 4 h with the indicated dose, followed by western blot analysis. (B) BNIP3 degradation and autophagy induction were analyzed by BNIP3 and SQSTM1 expression, respectively.

Sup. Fig. 8



Supplementary figure 8. Degradation of BNIP3 is regulated by ULK1 via MTORC1 and AMPK. (A) HeLa cells were transfected with *ULK1* siRNA for 48 h and exposed to hypoxia for 24 h. Amino acid starvation or Torin1 treatment were included as challenges for an additional 4 h followed by western blot analysis. (B) HeLa cells were incubated under hypoxic condition for 24 h and then treated with 250 nM Torin1, 5 mM metformin or 20 μM Compound C for 4 h. MTORC1 inhibition, AMPK activation or inhibition were confirmed by western blotting. ULK1 inhibition was assessed by the level of p-ULK1 (S757) and ULK1 activation by that of p-ULK1 (S555).

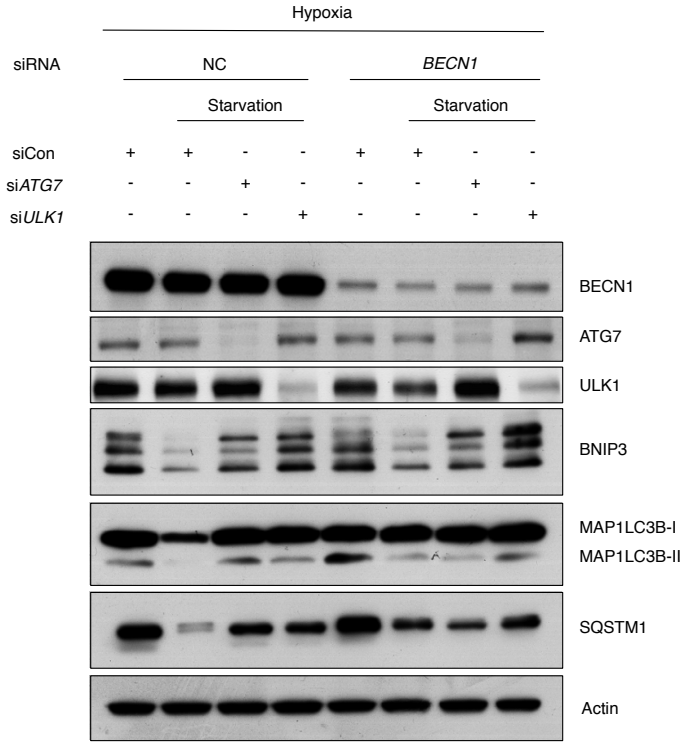
Sup. Fig. 9



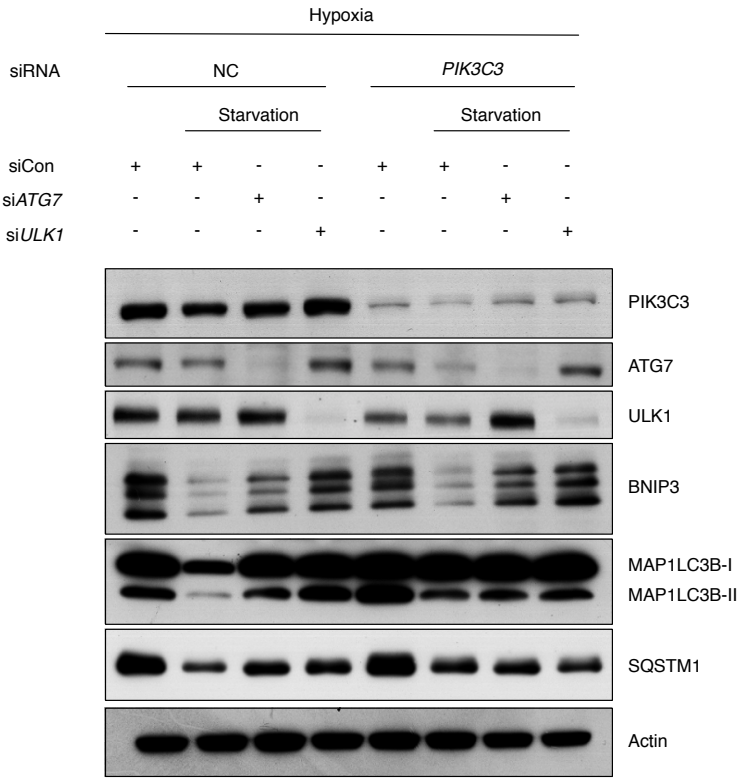
Supplementary figure 9. Loss of BNIP3 limits the survival of HeLa cells under hypoxic conditions. (A) Scrambled shRNA and *BNIP3* shRNA was transfected into HeLa cells. Knockdown of BNIP3 was confirmed by western blot analysis. (B) The selected stable cell lines were incubated under hypoxic conditions for up to 72 h. Proliferation was assessed by counting viable cells at the indicated time points. (C) HeLa cells were cultured under normoxia or hypoxia for 24 h followed by the Torin1 treatment for an additional 24 h. Cell death was assessed by the trypan blue exclusion assay. (D) The degradation of BNIP3, MTORC1 inhibition and the autophagy induction were confirmed by western blot analysis. (E) HeLa cells were incubated under normoxia or hypoxia for 24 h and then treated with Torin1 for 24 h. The long-term viability was analyzed by the soft agar assay. Data are shown as means ± S.D. for three independent experiments performed in triplicate. t test; *P<0.05, ***P<0.0005, n.s., not significant.

Sup. Fig. 10

A



B



Supplementary figure 10. BNIP3 is degraded in BECN1- or PIK3C3-depleted conditions and reversed by knockdown of ATG7 or ULK1. SK-Hep-1 cells were transfected with siRNA for *BECN1* (A) or *PIK3C3* (B) for 24 h followed by transfection with siRNA for *ATG7* or *ULK1* for 48 h. The transfected cells were incubated under hypoxia for 24 h and then amino acid starvation was performed for an additional 4 h followed by western blot analysis.