Inhibition of autophagy by caspase 8 cleavage of Beclin 1 following cytochrome *c* release in chemotherapy-induced apoptosis

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Supplementary Figure Legend

Fig. S1. Autophagy induction and Beclin 1 cleavage in *Cyt c*-KI HCT116 cells. (A) WT and *Cyt c*-KI HCT116 cells were treated with 500 nM camptothecin (CPT) for 24 hr. Cytosolic fractions were isolated and probed for Cyt *c* by Western blotting. Cytochrome oxidase subunit IV (Cox IV) and α-tubulin, which are expressed in mitochondria and cytosol, respectively, were analyzed as controls for loading and fractionation. **(B)** WT and *Cyt c*-KI HCT116 cells were treated with 500 nM camptothecin (CPT) for 24 hr, in the absence (Control) or presence of the lysosomal inhibitor chloroquine (40 μM) or E64d (10 μg/ml). LC3 was analyzed by Western blotting. **(C)** WT and *Cyt c*-KI HCT116 cells were transfected with V5-tagged Beclin 1 for 20 hr, and then treated with 500 nM of CPT for 24 hr. V5 expression was analyzed by Western blotting. Arrowheads indicate Beclin 1 cleavage fragments. **(D)** WT and *Cyt c*-KI HCT116 cells were treated with staurosporine (STS, 100 nM) or infected with an adenovirus expressing PUMA (Ad-PUMA, 10 MOI) for 48 hr. Beclin 1 expression was analyzed by Western blotting following treatment. Arrowheads indicate Beclin 1 cleavage fragments. **(E)** Western blot analysis of Beclin 1 in Caco2, RKO, and LoVo colon cancer cells treated with 500 nM CPT for 24 hr, in the presence or absence of the pan-caspase inhibitor zVAD-fmk at 20 μM.

Fig. S2. Caspase 8-mediated cleavage of Beclin 1 during apoptosis induced by anticancer agents. (A) Purified GST-Beclin 1 was incubated with active caspase 3, 8, or 9 at 37°C for 1 hr, and then analyzed by SDS-PAGE and Western blotting. *Left,* protein staining of SDS-PAGE.

Middle, Western blot with anti-Beclin 1 (C-terminal) antibody. *Right,* Western blot with anti-GST antibody. Arrowheads indicate Beclin 1 cleavage fragments. **(B)** HCT116 cells were transduced with a lentivirus expressing human *caspase 8*-specific shRNA and stable cell lines were isolated. Caspase 8 expression in the parental and *caspase 8*-knockdown **(***Casp 8*-KD) HCT116 cells was analyzed by Western blotting. **(C)** Parental and *Casp 8*-KD HCT116 cells were transfected with V5-tagged Beclin 1, and then treated with 500 nM CPT for 24 hr. V5 expression was analyzed by Western blotting. Arrowheads indicate Beclin 1 cleavage fragments. **(D)** Parental and *Casp 8*-KD HCT116 cells were treated with staurosporine (STS, 100 nM). Beclin 1 expression was analyzed by Western blotting following the treatment. Arrowheads indicate Beclin 1 cleavage fragments. **(E)** Parental and *Casp 8*-KD HCT116 transfected with GFP-LC3 were treated with 500 nM CPT for 24 hr. GFP-LC3 punctuate signals were quantified. *$p<0.05$. Values were means ± SD of three independent experiments, with 300 cells counted in each experiment.

Fig. S3. Identifying caspase 8 cleavage sites of Beclin 1. (A) HCT116 cells were transfected with V5-tagged WT or deletion mutant of Beclin 1, and then treated with 500 nM CPT for 24 hr. Transfected protein was analyzed by V5 Western blotting. Arrowheads indicate Beclin 1 cleavage fragments. **(B)** HCT116 cells were transfected with V5-tagged WT or indicated point mutants of Beclin 1, and then treated with 500 nM CPT for 24 hr. Transfected protein was analyzed by V5 Western blotting. **(C)** Purified GST-tagged WT and double mutant (DM, D133A/D146A) Beclin 1 were incubated with active caspase 3, 8, or 9 at 37°C for 1 hr, followed by SDS-PAGE analysis. A gel code blue staining picture of SDS PAGE is shown.

Fig. S4. Beclin 1 cleavage fragments lose autophagy function. (A) HCT116 cells were transfected with full-length Beclin 1 (WT Beclin 1), D133A/D146A double mutant (DM Beclin

1), N-terminal cleavage fragment of Beclin 1 (N-Beclin 1), or C-terminal cleavage fragment of Beclin 1 (C-Beclin 1). After transfection, cells were treated with 500 nM CPT for 24 hr in the presence of E64d (10 μg/ml) and pepstatin A (10 μg/ml). LC3 was analyzed by Western blotting. **(B)** Following transfection of HCT116 cells with WT Beclin 1, N-Beclin 1, or C-Beclin 1 and CPT treatment as in (A), apoptosis was analyzed by counting cells with condensed and fragmented nuclei following nuclear staining with Hoechst 33258. Values were means ± SD of three independent experiments, with 300 cells counted in each experiment. **(C)** Following transfection and CPT treatment as in (A), mitochondrial and cytosolic fractions were isolated and probed for transfected Beclin 1 by V5 Western blotting. Cox IV and α-tubulin were analyzed as the controls for loading and fractionation.

Fig. S5. Induction of autophagy in *Beclin 1*-KI HCT116 cells. (A) WT and *Beclin 1*-KI HCT116 cells were treated with 500 nM CPT for 24 hr, in the absence (Control) or presence of the lysosomal inhibitor chloroquine (40 μM) or E64d (10 μg/ml). LC3 was analyzed by Western blotting. **(B)** WT and *Beclin 1*-KI HCT116 cells transfected with GFP-LC3 were serum starved for 24 hr, exposed to 10 μM rapamycin for 24 hr, or treated with amino acid starvation for 3 hr. For amino acid starvation, cells were cultured in EBSS medium in the presence of E64d (2 μg/ml) and pepstatinA (2 μg/ml). GFP-LC3 punca signals in the treated cells were quantified. ** $p<0.01$. Values were means ± SD of three independent experiments.

Figure S1

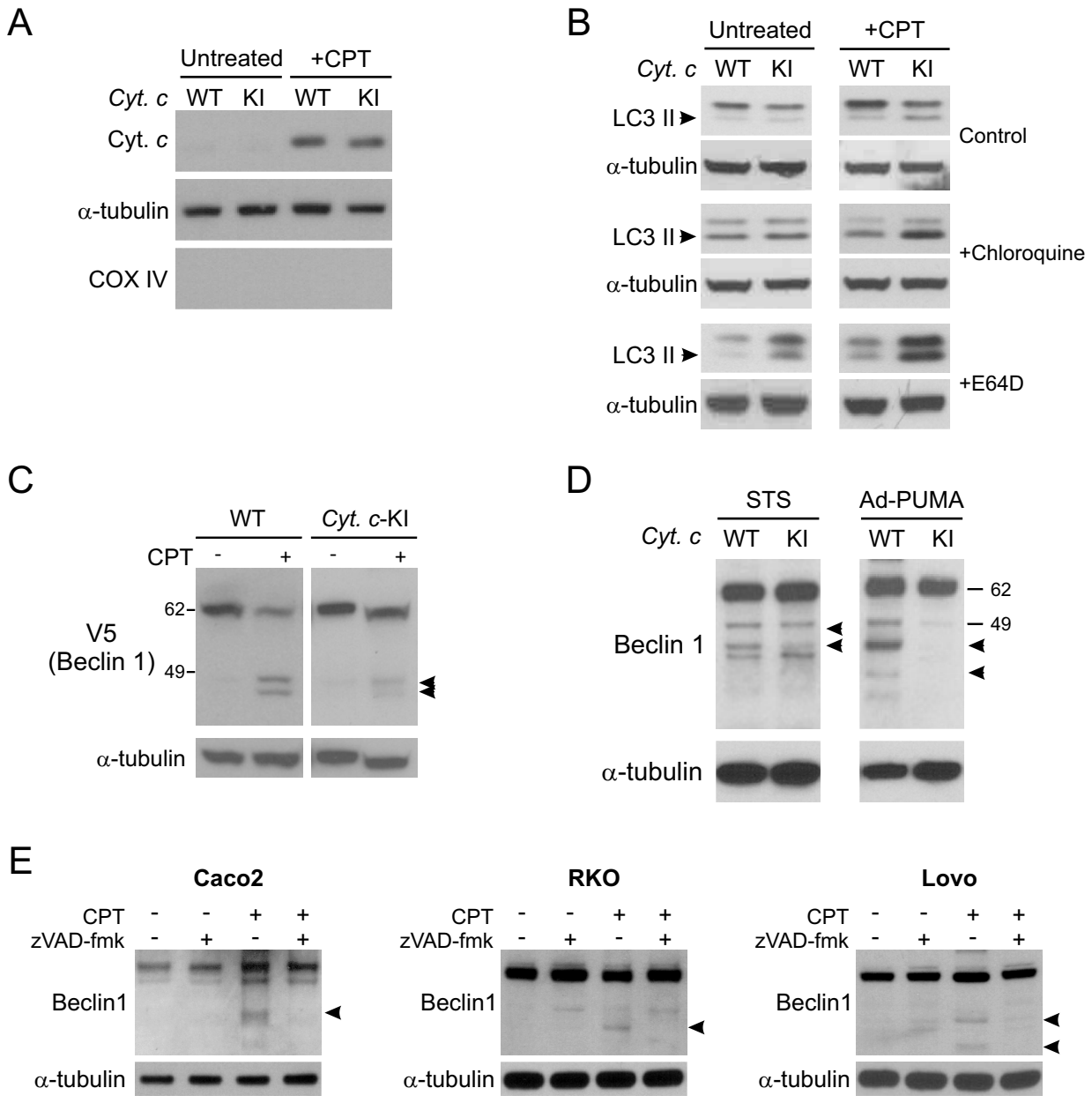
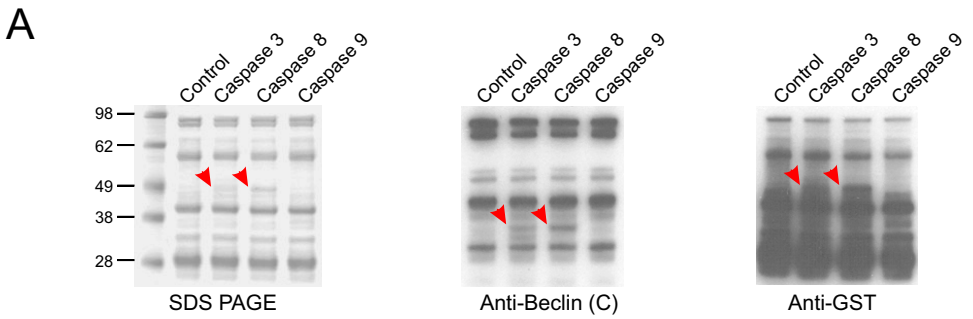
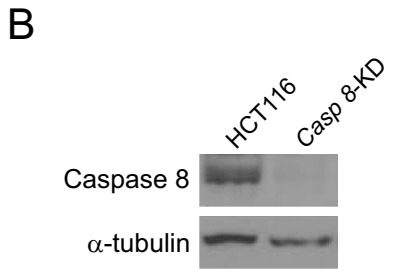
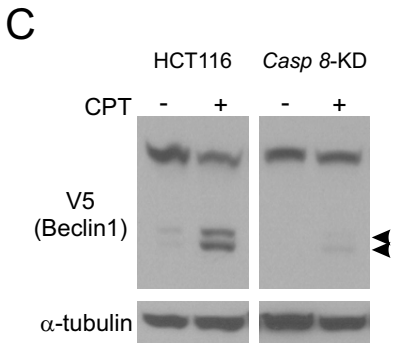
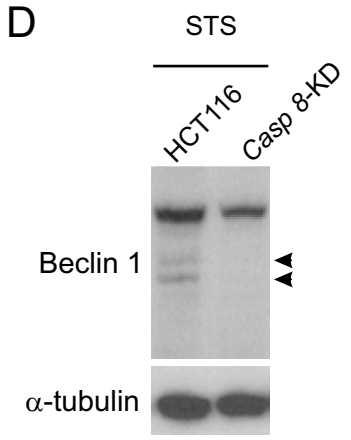

A
Untreated
+CPT
Cyt. c
WT
KI
WT
KI
Cyt. c
α-tubulin
COX IV
B
Untreated
+CPT
Cyt. c
WT
KI
WT
KI
LC3 II
α-tubulin
Control
LC3 II
α-tubulin
+Chloroquine
LC3 II
α-tubulin
+E64D
C
WT
Cyt. c-KI
CPT
-
+
-
+
62
49
V5
(Beclin 1)
α-tubulin
D
STS
Ad-PUMA
Cyt. c
WT
KI
WT
KI
62
49
Beclin 1
α-tubulin
E
Caco2
RKO
Lovo
CPT
zVAD-fmk
Beclin1
α-tubulin

Figure S2


A
Control
Caspase 3
Caspase 8
Caspase 9
98
62
49
38
28
SDS PAGE
Anti-Beclin (C)
Anti-GST


B
HCT116
Casp 8-KD
Caspase 8
α-tubulin


C
HCT116
Casp 8-KD
CPT
-
+
-
+
V5
(Beclin1)
α-tubulin


D
STS
HCT116
Casp 8-KD
Beclin 1
α-tubulin

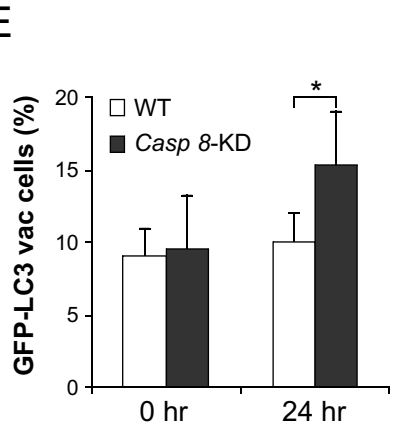

E
GFP-LC3 vac cells (%)
20
15
10
5
0
WT
Casp 8-KD
*
0 hr
24 hr

Figure S3

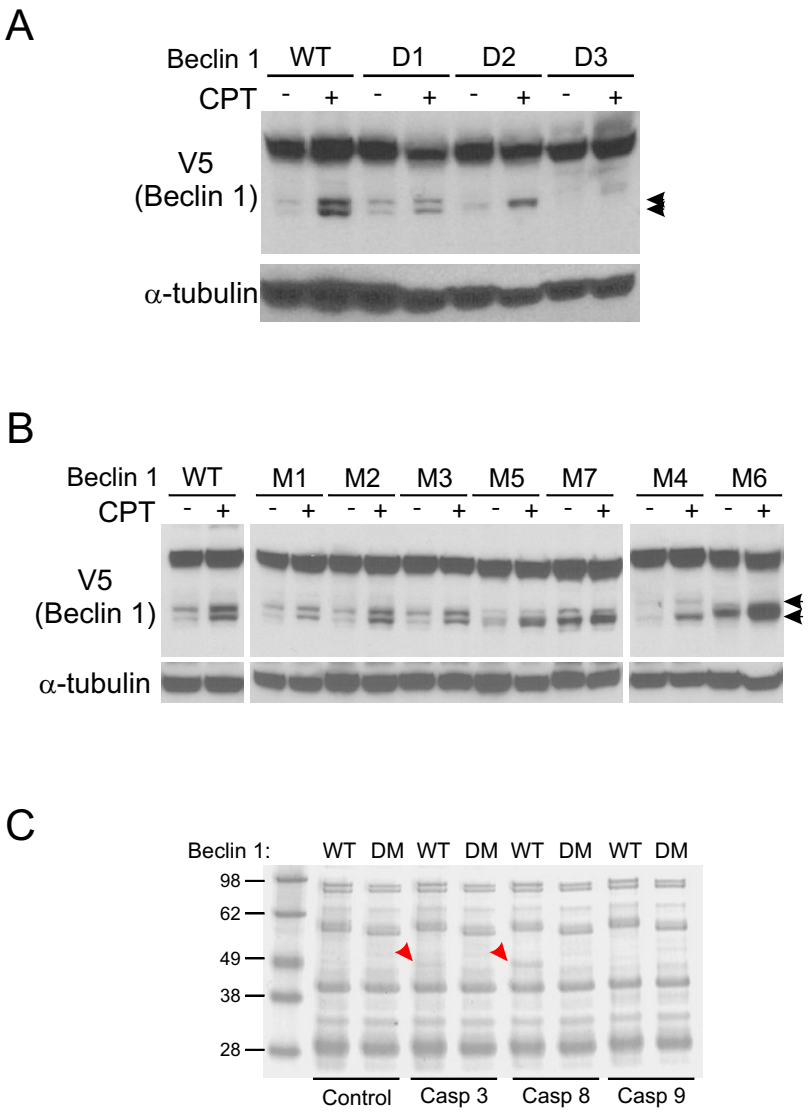

A
Beclin 1
WT
D1
D2
D3
CPT
- + - + - + - +
V5
(Beclin 1)
α-tubulin
B
Beclin 1
WT
M1
M2
M3
M5
M7
M4
M6
CPT
- + - + - + - + - + - + - + - +
V5
(Beclin 1)
α-tubulin
C
Beclin 1:
WT DM WT DM WT DM WT DM
98
62
49
38
28
Control
Casp 3
Casp 8
Casp 9

Figure S4

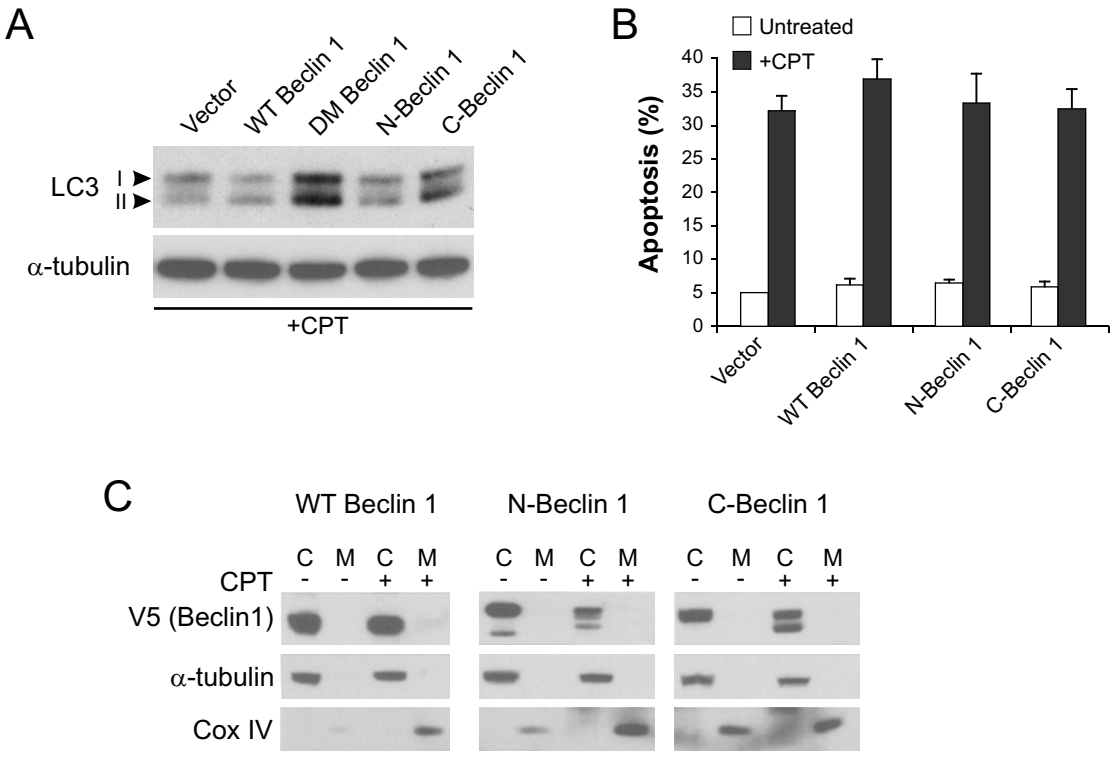
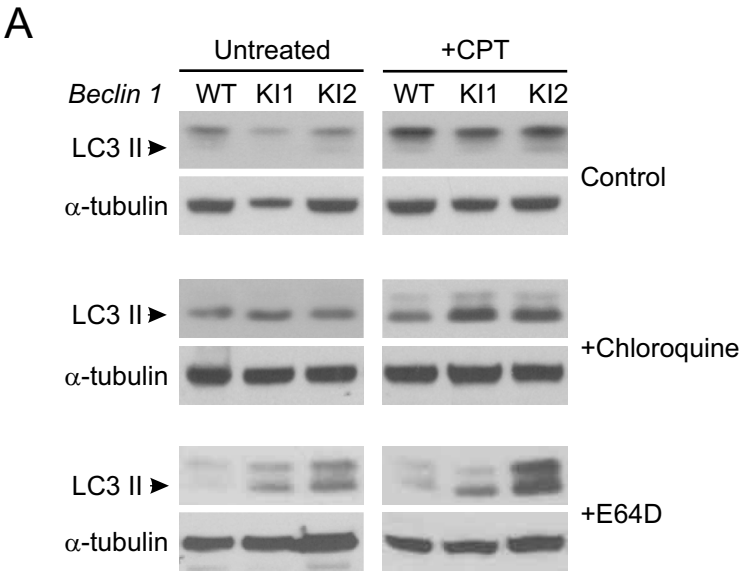

A
Vector
WT Beclin 1
DM Beclin 1
N-Beclin 1
C-Beclin 1
LC3 I
II
α-tubulin
+CPT
B
Untreated
+CPT
Apoptosis (%)
40
35
30
25
20
15
10
5
0
Vector
WT Beclin 1
N-Beclin 1
C-Beclin 1
C
WT Beclin 1
N-Beclin 1
C-Beclin 1
C M C M
- - + +
CPT
V5 (Beclin1)
α-tubulin
Cox IV

Figure S5


A
Untreated
+CPT
Beclin 1
WT
KI1
KI2
WT
KI1
KI2
LC3 II
α-tubulin
Control
+Chloroquine
+E64D

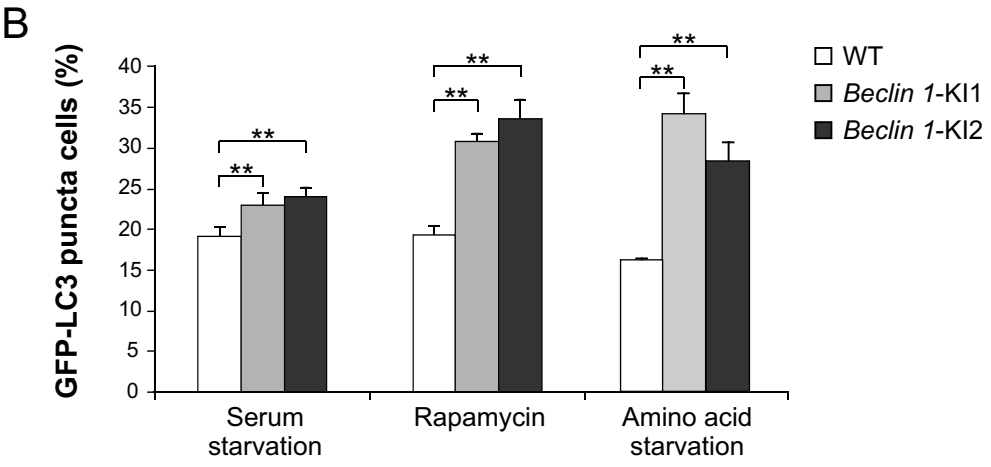

B
GFP-LC3 puncta cells (%)
0
5
10
15
20
25
30
35
40
**
Serum starvation
Rapamycin
Amino acid starvation
WT
Beclin 1-KI1
Beclin 1-KI2

Table S1. Primers used for *Beclin 1* knock-in vector construction and PCR screen

Purpose	Orientation	Sequence
Vector construction		
Left homologous arm	Forward	5'-GGG AAA GUg cag gca gtg aag agt cca gga gcc-3'
	Reverse	5'-GGA GAC AUg acc tcc caa ggg tac ctc tct ccc-3'
Right homologous arm	Forward	5'- GGTCCCAUct tgagagtctc tgccactggc-3'
	Reverse	5'- GGCATAGUtgctaggact acaggtctac cac-3'
First round screen		
Left arm	Forward	5'-gggatcctgt ggagcaacat cctg-3'
	Neo reverse	5'- ttg tgc cca gtc ata gcc g-3'
Right arm	*Neo* forward	5'- tct tga cga gtt ctt ctt ag-3'
	Reverse	5'-cca tga act ggc cat aat tgg cct-3'
Second round screen		
Right arm	Reverse	5'-gtgc attcctcaca gagtgggtg tg -3'
	Reverse	5'-cg ttgagctgag tgtccagctg tg-3'

Capital letters: incorporated restriction enzyme site sequences