Supplemental Material to:

Robert G, Gastaldi C, Puissant A, Hamouda A, Jacquel A, Dufies M, Belhacene N, Colosetti P, Reed JC, Auberger P, Luciano F. The anti-apoptotic Bcl-B protein inhibits Beclin 1-dependent autophagic cell death

Autophagy 2012; 8(4); http://dx.doi.org/10.4161/auto.8.4.19084

www.landesbioscience.com/journals/autophagy/article/19084

Supplemental Information

Supplemental figure 1. Bcl-B and *Beclin 1 co-localize mostly at the ER compartment.* (A), HeLa cells were co-transfected with plasmids encoding Myc-Bcl-B (top left panels), Flag-Beclin 1 (top right panels) or Flag-Beclin 1 and Myc-Bcl-B (bottom left panels). After 24 h, the cells were fixed, permeabilized, and successively incubated with anti-Myc, anti-Beclin 1 and anti-Hsp60 (mitochondrial marker) or anti-calreticulin (ER marker) antibody followed by secondary antibodies conjugated to blue, green and red fluorochromes. The antibodies localization was visualized by confocal UV microscopy. **(B),** The histograms represent the percentage of cells with ER or mitochondrial protein localization in the different conditions (Beclin 1, Bcl-B or Beclin 1 + Bcl-B).

Supplemental figure 2. ***The Bcl-B BH1 domain contributes to binding to Beclin 1.*** Plasmids encoding the different Bcl-B mutants were co-transfected with a plasmid encoding GFP-Beclin 1 protein in Hela cells. After 24 h, the cells were fixed, permeabilized, and successively incubated with an anti-Myc antibody followed by a secondary antibody conjugated to a red fluorochrome. The antibody localization was visualized by confocal UV microscopy to determine the co-localization of Bcl-B mutants with Beclin 1.

Supplemental figure 3. Bcl-B G95A still retains its capacity to interact with BimEL and to inhibit apoptosis. (A), 293T cells were co-transfected with plasmids encoding GFP, GFP-BimEL or GFP-Beclin 1 in combination with plasmids encoding Myc-tagged versions of either Bcl-B or Bcl-B G95A. After 24 h, IPs were performed using an anti-Myc antibody and the immune-complexes were analyzed by immunoblotting using anti-GFP (top panel) or anti-Myc antibody (middle panel). The cell lysates (50 μg) were analyzed directly by SDS-PAGE to confirm the GFP fusion protein expression (bottom panel). **(B)**, HeLa cells were treated with increasing concentrations of staurosporine (30 to 1000 nM) for 24 h (top panel) or etoposide (3 to 100 μM) for 48 h (bottom panel). Then, the cells were stained with propidium iodide, and the percentage of dead cells was assessed by flow cytometry.

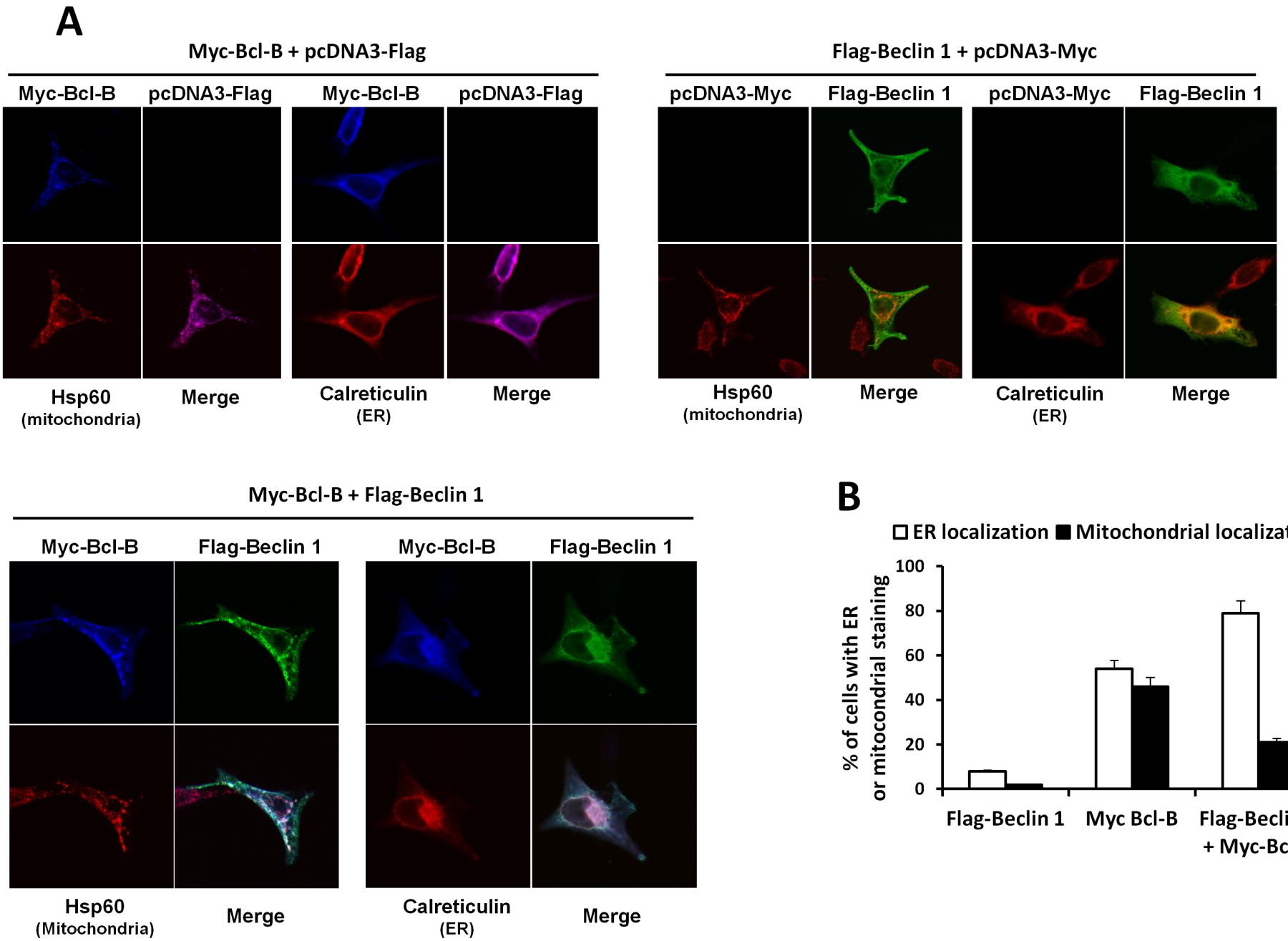
Supplemental figure 4. Bcl-B over-expression blocks the autophagic flux induced by AA starvation. (A), HeLa TET-Bcl-B cells were incubated with or without 1 μg/ml of doxycycline to induce Bcl-B expression and then maintained in DMEM media for 48 h. **(C)**, 293T cells were transfected with plasmids encoding either Myc-tag or

Myc-Bcl-B, and the cells were maintained in DMEM media for 24 h. Both of the cell lines were further incubated in DMEM or EBSS medium for 2 h in the presence or absence of BafA1 (20 nM), and the cell lysates (30 μg) were subjected to SDS-PAGE immunoblot using LC3, Bcl-B or Hsp60 antibody. **(A, C)**, LC3-II protein detection from three independent experiments was quantified and represented by histograms **(B, D)**. HeLa TET-Bcl-B cells were incubated with or without 1 μg/ml of doxycycline to induce Bcl-B expression, transfected with a plasmid encoding GFP-LC3 and maintained in DMEM media for 48 h. The cells were further incubated in DMEM or EBSS medium for 8 h in the presence or absence of BafA1 (20 nM). Confocal microscopy quantification of GFP-LC3 puncta was performed **(E)**. Where indicated, the cumulative data ± SD from three independent experiments are shown. **P*<0.05, ***P<0.005* in two-sided Student's *t*-test.

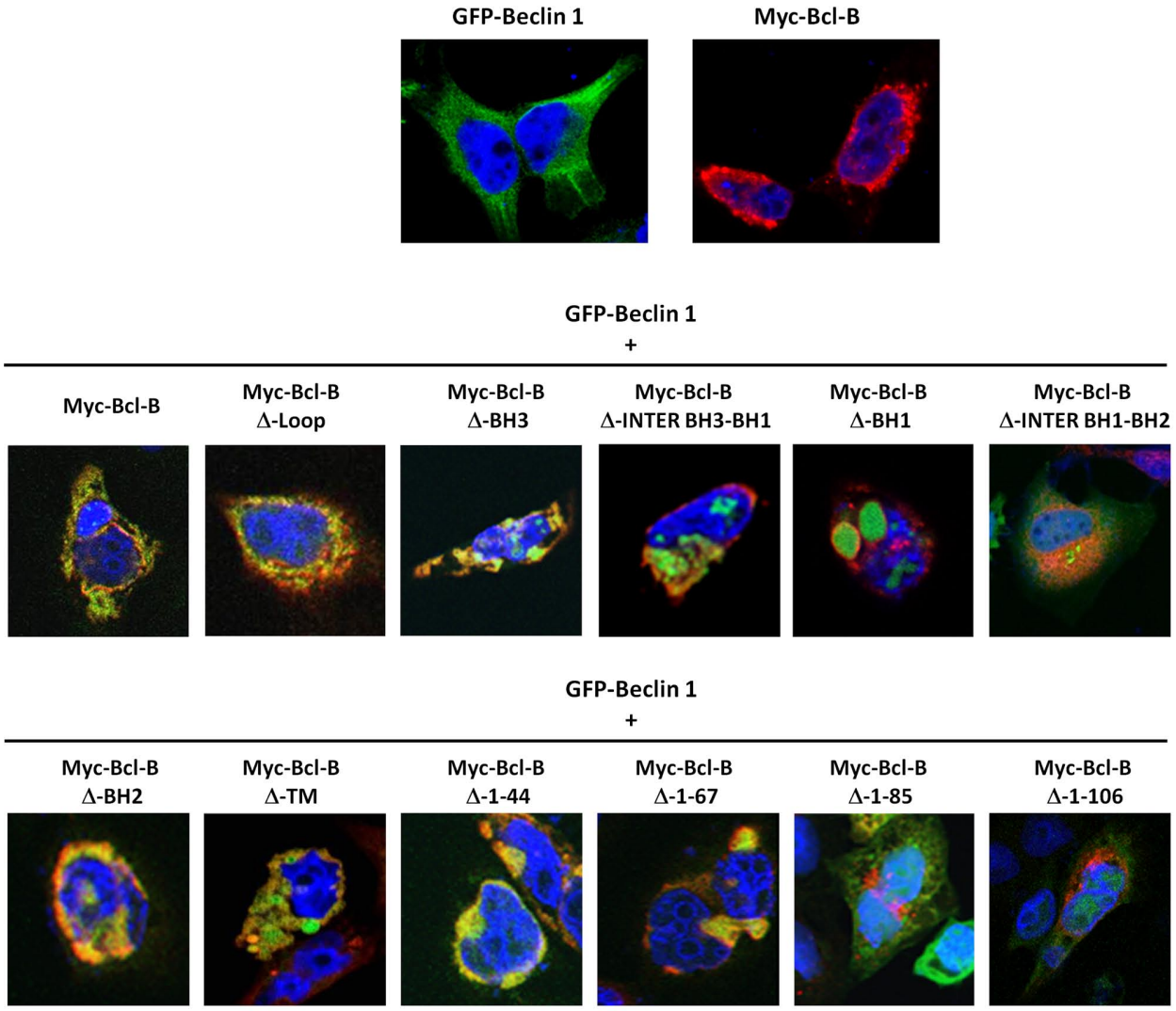
Supplemental figure 5. Bcl-B increases the capacity of cells to form colonies under AA starvation condition. (A), 10^3 HeLa TET-Bcl-B cells per well were treated or not with 1 μg/ml of doxycycline every 2 days during the course of the experiment to induce Bcl-B expression. After 72 h, the cells were incubated with DMEM or EBSS medium. After an additional 48 h, the cells were washed and the medium was replaced with DMEM complete media. Then, 5 days later, the cells were fixed, and the colonies were detected by adding crystal violet. **(B)**, The numbers of colonies were scored with ImageJ quantification software. The cumulative data ± SD from three independent experiments are shown. **P*<0.05 in two-sided Student's *t*-test.

Supplemental figure 6. ***In myeloma cells, an endogenous Bcl-B/Beclin 1 complex is formed, and Bcl-B silencing induces both apoptosis and autophagic cell death*****. (A)**, A co-immunoprecipitation experiment was performed using myeloma U266 cells with either mouse Ig (second lane) or mouse anti-Beclin 1 (third lane) antibodies, and the endogenous immune-complexes were analyzed by immunoblotting using anti-Beclin 1 or anti-Bcl-B antibody. The cell lysates (50 μg) were analyzed directly (Lane 1). **(B)**, U266 myeloma cells were transfected with either control siRNA or with 2 different Bcl-B siRNAs for 72 h. Then, one portion of the cells was incubated with propidium iodide, and the percentage of dead cells was assessed by flow cytometry. **(C)** The remainder of the cells were lysed, and the protein extracts were subjected to SDS-PAGE immunoblot using Bcl-B, LC3, PARP or Hsp60 antibody.

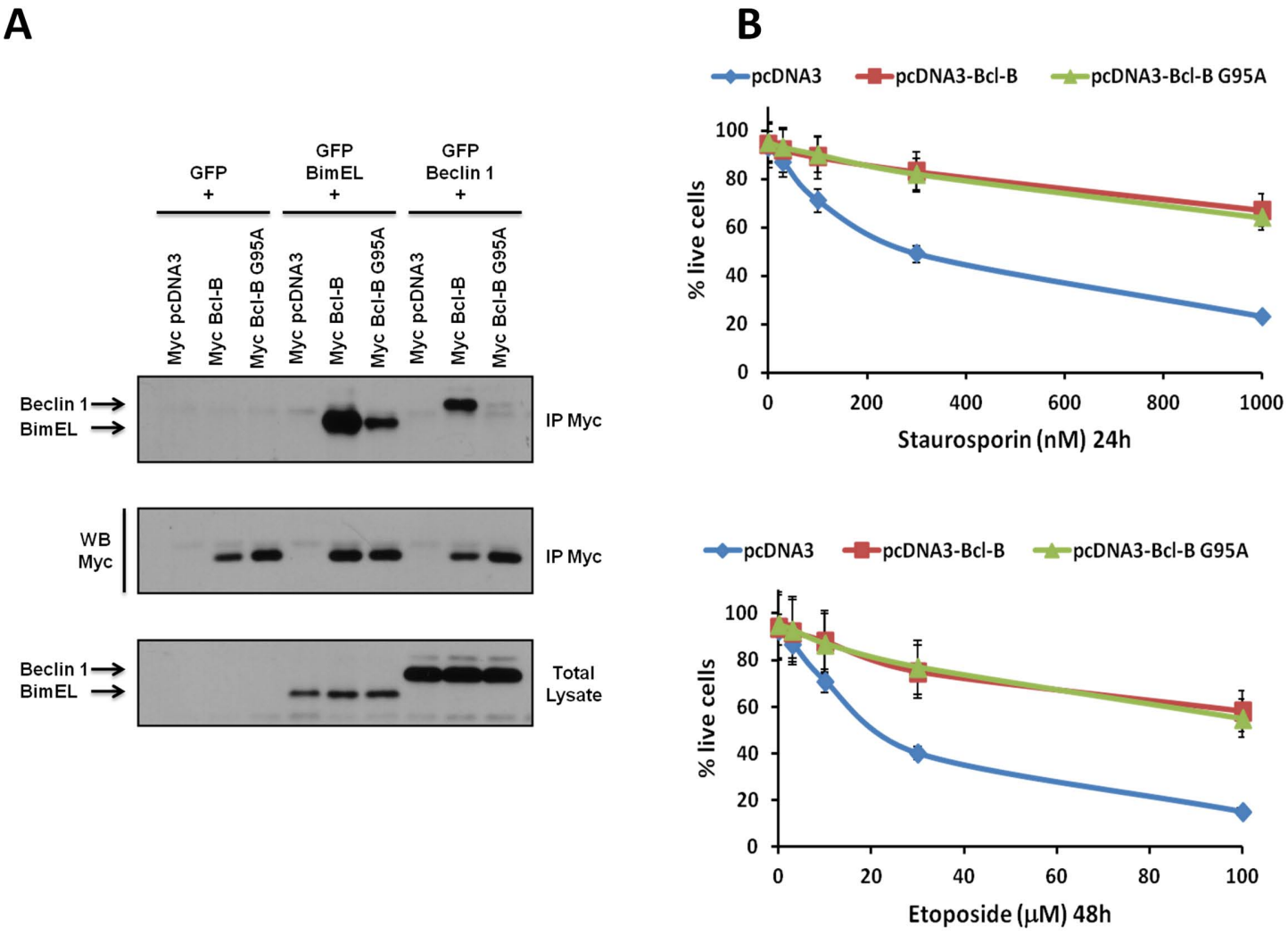
Supplemental figure 7. Beclin 1 contributes to the autophagic cell death induced by Bcl-B silencing. (A), HeLa cells were transfected with control, Beclin 1 or Bcl-B siRNA. The protein expression was verified by SDS-PAGE immunoblot using the indicated antibodies. **(B)**, The percentage of dead cells assessed by PI staining was analyzed by flow cytometry. **(C)**, Phase contrast microscopy showing the representative cell morphology after 48 h of siRNA transfection. **(D)**, The induction of autophagy was monitored by cathepsin B and L in cells transfected with control, Beclin 1, Bcl-B or Beclin 1 + Bcl-B siRNAs. Where indicated, the cumulative data ± SD from three independent experiments are shown. *P<0.05 in two-sided Student's t-test.



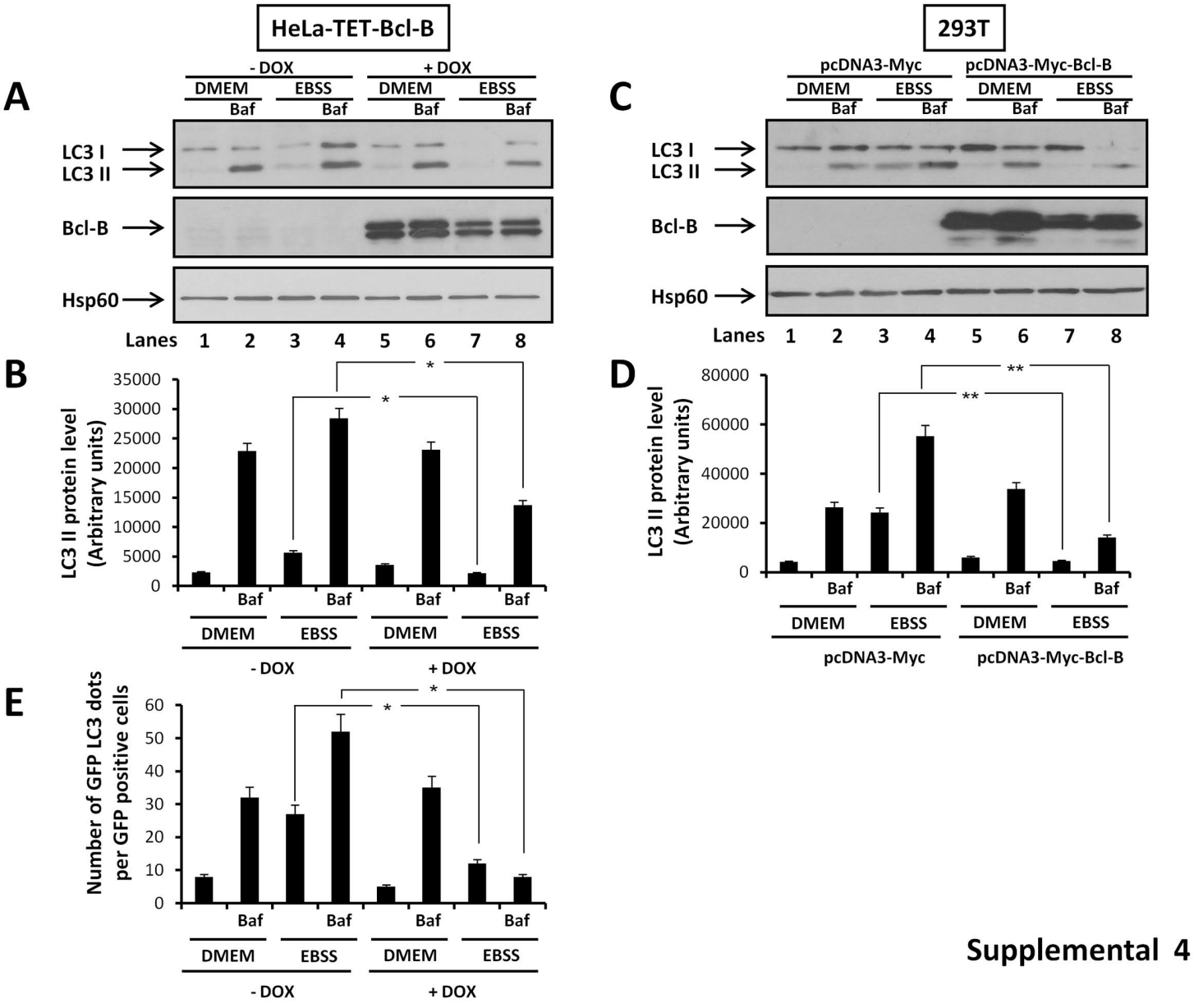
Supplemental 1



Supplemental 2

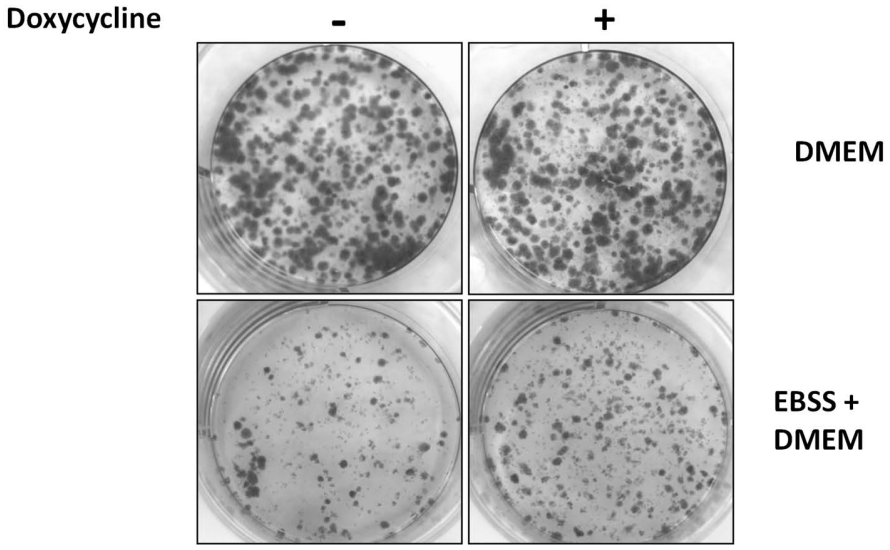


Supplemental 3

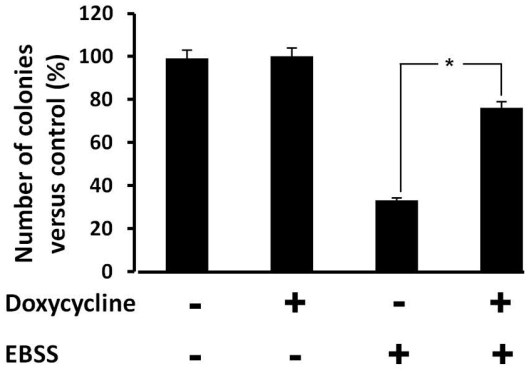


Supplemental 4

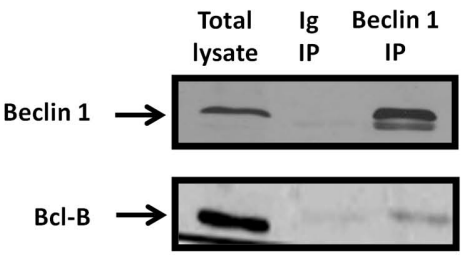
A

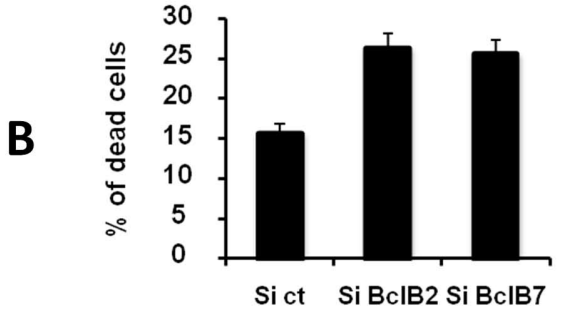


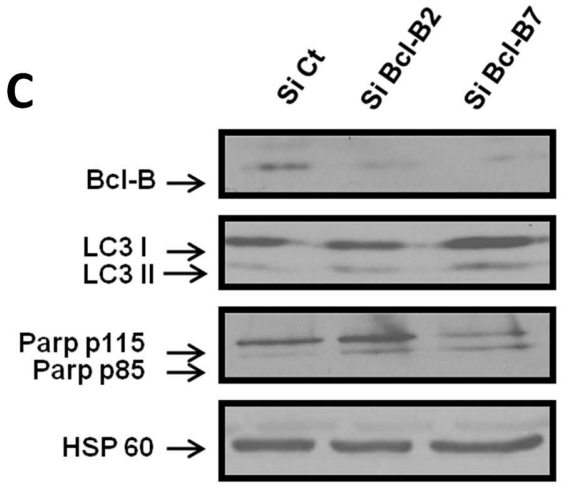
B



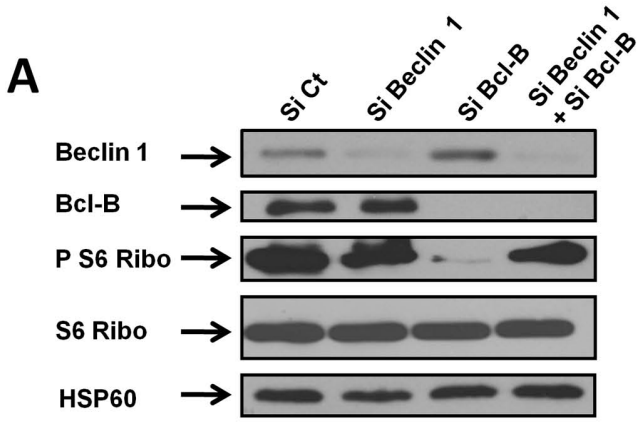
Supplemental 5

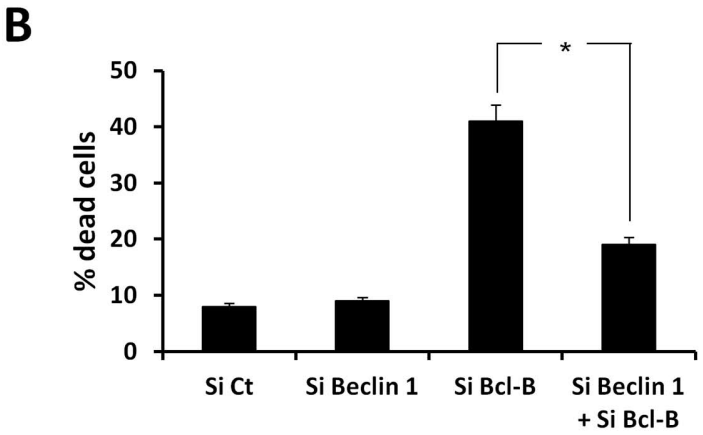


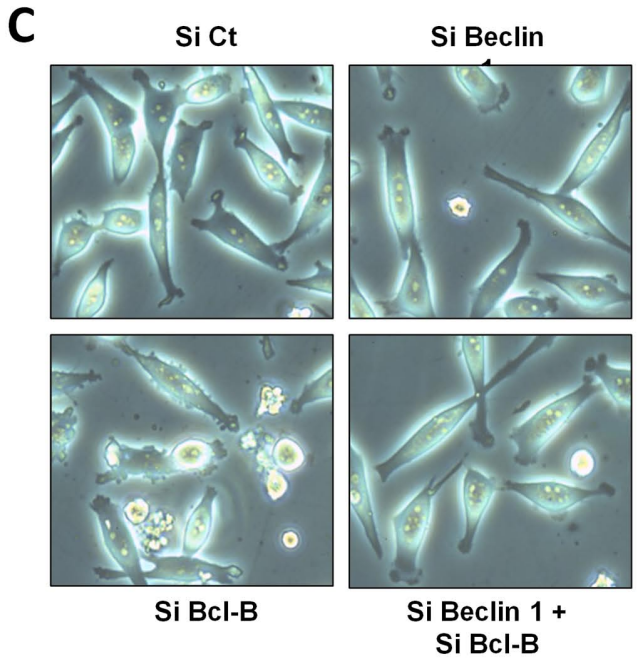


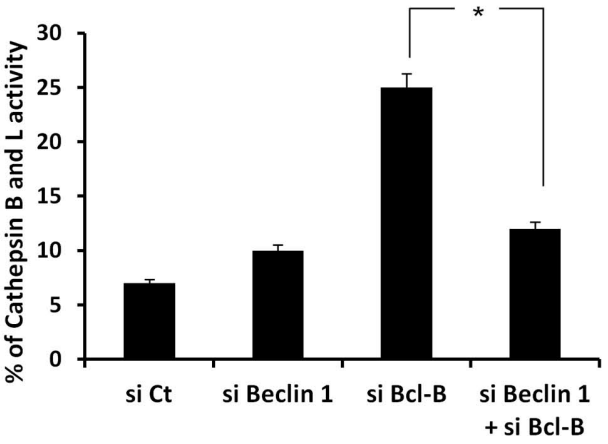


Supplemental 6









Supplemental 7