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

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Supplementary Materials and Methods

Cell culture and transfection. All parental and derivative cell lines were cultured at 37°C with 5% CO_2 in McCoy's 5A modified medium (Invitrogen), except for amino acid starvation experiment, in which cells were cultured using EBSS medium (Invitrogen) as previously described (1). Cell culture media were supplemented with 10% defined fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Drug treatment. Cells were plated in 12-well plates at 20-30% density 24 hr before treatment. DMSO stock solutions of anticancer agents, including camptothecin (Sigma-Aldrich), sulindac sulfide (Merck), staurosporine (EMD Biosciences), and rapamycin (LC Laboratories), were diluted into appropriate concentrations with cell culture medium, and added to the cells. Adenovirus expressing PUMA (Ad-PUMA) was previously described (2). In some experiments, cells were treated along with the lysosomal inhibitors E64d (EMD Biosciences) and pepstatin A (Sigma), or chloroquine (Sigma).

Image acquisition. Confocal imaging analyses were performed at 22°C, with McCoy's 5A modified medium (Invitrogen) as the imaging medium, and GFP as the fluorochrome. All confocal images were acquired using the same parameters: scaling: X, 0.04 μm, Y, 0.04 μm; stack size: X, 36.6 μm, Y, 36.6 μm; pixel dimensions: 1024×1024 pixels; and scan bit depth: 8 bit/channel.

Beclin 1 expression constructs. Expression constructs of full-length Beclin 1 and deletion mutants were generated by cloning the corresponding PCR-amplified human *Beclin 1* cDNA fragments into the pCDNA3.1/V5-His vector (Invitrogen). Beclin 1 point mutants were generated using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies), following the manufacturer's instructions. All constructs were verified by digestion with restriction enzymes and DNA sequencing.

siRNA and shRNA knockdown. For siRNA knockdown of *Beclin 1*, cells were transfected 24 hr before drug treatment with 200 pmoles of *Beclin 1* ON-TARGETplus siRNA (J-010552-05; Dharmacon), or scrambled siRNA (Dharmacon) as a control. Stable *caspase 8* knockdown cells were generated by transducing HCT116 cells with a lentiviral vector expressing shRNA specific for human *caspase 8* (Sigma; Cat #SH1911). Briefly, lentiviral particles were generated by co-transfecting 293-FT cells with pMD2.g(VSVG), pVSV-REV, PMDLg/pRRE, and pLKO.1-puro-shRNAcaspase 8 as previously described (3). Forty-eight hr after transfection, lentivirus-containing supernatant was collected and passed through 0.45 μM filters to isolate the viral particles, in accordance with procedures optimized by the UPCI Lentiviral facility. Following lentiviral transduction as described (3), cells were plated in 96-well plates in the presence of puromycin (2 μg/ml, Invitrogen). Caspase 8 expression of the puromycin-resistant clones was then analyzed by Western blotting.

Recombinant proteins. WT and mutant Beclin 1 proteins were generated by *in vitro* translation using TNT Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer's protocol. To generate GST fusion proteins, WT and mutant *Beclin 1* cDNA fragments were sub-cloned into pGEX-2TK vector (GE Healthcare). GST-fused Beclin 1 was expressed in BL21 (DE3) E. *coli* cells by induction with 0.1 mM IPTG (isopropyl β-D-1-

thiogalactopyranoside) for 3 hr at 37°C. GST fusion proteins were affinity purified with glutathione-Sepharose resin (GE Healthcare) according to the manufacturer's protocol. Protein samples were analyzed by NuPAGE (Invitrogen) gel electrophoresis, and visualized by staining the gels with Gel Code Blue Stain Reagent (Pierce).

***In vitro* caspase cleavage assays.** WT and mutant Beclin 1 proteins were incubated with 4 units of caspase 3, caspase 8, or caspase 9 (Sigma) in caspase buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% sucrose, 1 mM EDTA, 0.1% CHAPS, and 10 mM DTT) at 37°C for 1 hr. The reaction was stopped by adding 2× Laemmli sample buffer. Samples were heated at 95°C for 10 min and subsequently analyzed by NuPage gel electrophoresis, followed by Western blotting.

References

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