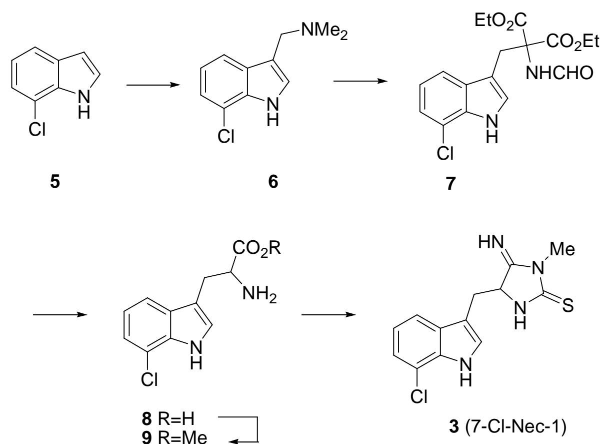
Supplementary Methods

Preparation of Nec-1 and its derivatives. Nec-1(**1**) is commercially available from Sigma-Aldrich. We prepared Nec-1i(**2**) as previously described [1].

7-Cl-Nec-1(3):

We prepared 7-Cl-Nec-1 (**3**) according to the following scheme:



We added dimethyl amine (2.05 mL, 16.3 mmol., 40% solution) to a mixture of acetic acid (13.6 mL) and formaldehyde (0.340 mL, 4.5 mmol, 37% solution) under argon. We stirred the reaction mixture for 10 min and then treated it with 7-chloroindole (**5**) (604 mg, 4.0 mmol.). We stirred the resulting mixture at room temperature for ~ 16 h. We first neutralized the reaction mixture with K_2CO_3, then basified it with NaOH (2N), and then extracted it in ethyl acetate, washed with water, dried, and concentrated. We recrystallized obtained solid from ethyl acetate and hexane to give **6**: yield 86%, mp 136 - 138 ^{o}C, ^{1}H NMR (500 MHz, $CDCl_3$): 2.27(s, 6H), 3.61 (s, 2H), 7.04 (dd, J = 8.0 and 8.0 Hz, 1H), 7.15 (d, J = 2.5 Hz, 1H), 7.18 (d, J = 7.5 Hz, 1H), 7.60 (d, J = 7.5 Hz, 1H), 8.53 (s, 1H).

We refluxed suspension of **6** (2.8 mmol), 2-formylamino-malonic acid diethyl ester (3.1 mmol), and NaOH (30 mg) in toluene (20 mL) under argon for 3 days. We concentrated the reaction mixture and purified it by column chromatography on silica gel using 40% ethyl acetate – hexane to give **7**: yield 65%, mp 170 - 174 ^oC, ^1H NMR (500 MHz, $CDCl_3$): 1.28 (t, J = 7.5 Hz, 6H), 3.87 (s, 2H), 4.17 – 4.31 (m, 4H), 6.80 (s, 1H), 7.00 (d, J = 2.5 Hz, 1H), 7.02 (dd, J = 7.5 and 8.0 Hz, 1H), 7.17 (d, J = 7.5 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 8.18 (d, J = 1.0 Hz, 1H), 8.32 (s, 1H).

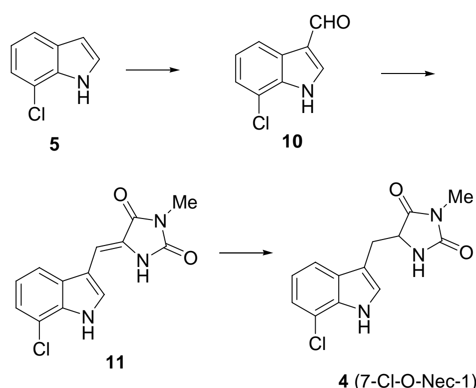
We treated solution of **7** in THF with NaOH (300 mg in 10 mL water) at room temperature for 24 h. We slowly acidified the mixture with acetic acid (5 mL) and then refluxed it for 24 h. We concentrated the reaction mixture under vacuum, and treated it with diluted HCl (10 mL, 3M) and then again refluxed for ~ 16 h. We allowed the reaction to cool to room temperature and adjusted pH to 6.0 with 2M KOH. We filtered the white solid that formed, washed it with water, and dried it under vacuum to give **8**: yield 83%, mp 236 - 239 ^oC, ^1H NMR (500 MHz, DMSO-d_6): 2.95 – 3.00 (dd, J = 8.5 and 15 Hz, 1H), 3.24 – 3.28 (dd, J = 4.0 and 15 Hz, 1H), 3.41 – 3.43 (dd, J = 4.0 and 8.5 Hz, 1H), 6.95 (dd, J = 7.5 and 7.5 Hz, 1H), 7.10 (d, J = 7.5 Hz, 1H), 7.22 (s, 1H), 7.52 (d, J = 7.5 Hz, 1H), 11.18 (s, 1H).

We dissolved thionyl chloride (0.09 mL, 1.2 mmol) in 3 mL of anhydrous methanol at 0 °C and then added this solution to a flask containing crude **8** (200 mg, 0.5 mmol). After stirring at –5 °C for 4 h, we allowed the reaction mixture to warm to room temperature and stirred it overnight before being concentrated. We collected the white solid, washed it with ethyl acetate and dried it in vacuo. We directly used the product (**9**) without further purification.

We added triethyl amine (0.1 mL) to a solution of **9** (1.0 mmol) in dichloromethane (10 mL) followed by methylisothiocyanate (7.4 mg, 0.1 mmol). We stirred the reaction mixture at room temperature for 1 h and then concentrated it. We purified the obtained residue by column chromatography on silica gel using 30% ethyl acetate in hexane to give 7-Cl-Nec-1(**3**): mp 249 – 253 °C; ^{1}H NMR (d_6-DMSO-$CDCl_3$, 500 MHz) δ 3.02 (s, 3H), 3.18 – 3.22 (dd, 1H, J1=14.5 Hz, J2 = 5.5 Hz); 3.30 – 3.34 (dd, 1H, J1=14.5 Hz, J2 = 5.5 Hz); 4.35 (dd, 1H, J1=5.5 Hz, J2 = 4.5 Hz); 7.00 (t, 1H, J=7.5 Hz); 7.13 (d, 1H, J=7.5 Hz); 7.19 (d, 1H, J=2.0 Hz); 7.52 (d, 1H, J=7.5 Hz); 9.92 (s, 1H), 10.45 (s, 1H); ^{13}C NMR, (d_6-DMSO, 100 MHz) δ 25.92, 26.61, 59.45, 108.84, 115.64, 117.56, 119.45, 120.46, 125.44, 129.19, 132.59, 174.43, 182.83; Analysis calculated for $C_{13}H_{12}ClN_3OS$: C, 53.15; H, 4.12; N, 14.30. Found: C, 53.16; H, 4.21; N, 14.01.

7-Cl-O-Nec-1(4):

Oxygen-containing derivative of 7-Cl-Nec-1(**3**), 7-Cl-O-Nec-1(**4**), was prepared according to the following scheme:



We added phosphorus oxychloride (0.66 mL, 7 mmol) dropwise to anhydrous DMF (5 mL) at 0 °C under argon. Next, we added a solution of **5** (1g, 6.6 mmol) in anhydrous

DMF (15 mL) dropwise at room temperature and stirred the resulting mixture for 2 h. We poured the reaction mixture into ice and saturated $NaHCO_3$ and extracted it with ethyl acetate. We washed the combined organic solutions with saturated NaCl (10mL × 3), dried it over anhydrous $MgSO_4$, filtered and concentrated it to give 990 mg of product, **10**, as a yellow-orange solid (83 %). ^{1}NMR (500 MHz, DMSO-d_6) δ 12.22 (1H, br s), 9.93 (1H, s), 8.34 (1H, s), 8.07 (1H, d, *J* = 9.0 Hz), 7.57 (1H, d, *J* = 1.5), 7.25 (1H, dd, *J* = 1.8, 7.8 Hz).

We heated a mixture of **10** (1 mmol) and 1-methylimidazol-2,5(1,3*H*)-dione (which we synthesized according to the method used by Janin et al. [2]) (250mg, 2.5 mmol) in piperidine (2 mL) at 110°C for 4h under an argon atmosphere. Then, we allowed the reaction mixture to cool in a refrigerator (~ 5°C) with the addition of ether (2 mL). We filtered the precipitate and washed it with ether to give **11**. ^{1}NMR (500 MHz, DMSO-d_6): δ 12.15 (1H, br s), 10.26 (1H, br s), 8.23 (1H, s), 7.79 (1H, d, *J* = 8.0 Hz), 7.27 (1H, d, *J* = 8.0 Hz,), 7.13 (1H, t, *J* = 7.8 Hz), 6.82 (1H, s), 2.97 (3H, s).

We added $CoCl_2$ (1.0 mmol) and $NaBH_4$ (10 mmol) portion wise to a solution of **11** (0.5 mmol) in a mixed solvent of anhydrous MeOH/THF (1:1, 40 mL). We stirred the mixture at room temperature overnight and then diluted it with ethyl acetate (100 mL). We washed the mixture sequentially with saturated $NaHCO_3$ (30 mL),1N HCl (30 mL), saturated NaCl (30 mL) and then dried it over anhydrous $MgSO_4$, filtered and concentrated it. We purified the crude product by column chromatography on silica gel to give 7-Cl-O-Nec-1 **(4)** : mp 173 – 175 °C; 1H NMR (CDCl3, 500 MHz) δ 8.43 (1H, br s), 7.50 (1H, d, J = 8.0 Hz), 7.22 (1H, d, J = 7.5 Hz), 7.13 (1H, d, J = 2.0 Hz), 7.06 (1H, t, J = 7.8 Hz), 5.69 (1H, br s), 4.27 (1H, ddd, J = 1.0, 3.5, 8.8 Hz,), 3.43 (1H, dd, J = 3.5,

14.5 Hz), 3.01 (1H, dd, J = 9.3, 14.8 Hz), 2.95 (3H, s); 13C NMR, (CDCl3, 100 MHz) δ 173.4, 157.2, 133.6, 128.4, 123.6, 122.0, 120.8, 117.2, 116.9, 111.0, 58.0, 28.1, 24.5; Elemental analysis calculated for C13H21ClN3O2: C, 56.22; H, 4.36; N, 15.13. Found: C, 56.19; H, 4.41; N, 15.10.

Cell Culture. We obtained all cell lines from ATCC, except FADD-deficient Jurkat cells (a generous gift of J. Blenis, Harvard Medical School). We prepared mouse embryonic fibroblasts as in [3] and immortalized through infection with SV-40-encoding retrovirus. Atg5$^{-/-}$ MEF cells have been previously described [4].

DNA chip analyses. We double purified mRNA from the cells using Poly(A)Purist kit (Ambion). Agilent DNA chip analysis was performed by Harvard Center for Genomics Research.

Immunofluorescence. We washed Balbc 3T3 cells in PBS, fixed the cells in 4% formaldehyde for 15 min at 25°C, rinsed them twice in PBS and permeabilized/blocked in 0.4% Triton X-100, 10% normal goat or donkey serum (Jackson Immunoresearch) in PBS for 30 min at 25°C. We incubated the samples with the appropriate primary antibodies, diluted according to the manufacturer's instructions in 0.1% Triton, 1% serum in PBS, for 16 h at 4°C, followed by three washes with PBS and incubation with fluorophore-conjugated secondary antibodies diluted 1:200 in the same buffer as primary antibodies for 30 min at 25°C. Following 2 washes with PBS, we stained the cells with TO-PRO-3 or phalloidin-TRITC, diluted in PBS according to manufacturer's instructions, for 10 min at 25°C, washed them once with PBS and mounted the samples using ProLong Antifade kit (Molecular Probes). We acquired the images using Nikon spinning disk confocal microscope at Cell Biology Department's Nikon imaging facility

at Harvard Medical School and analyzed them using Metamorph software (Universal Imaging).

Propidium iodide DNA content analysis. After the appropriate treatment, we washed Jurkat cells once, resuspended them in PBS and fixed the cells by adding 4 volumes of ice cold 100% ethanol. Cells remained on ice for 1 h, after which we discarded the fixing solution, washed the cells once in PBS, resuspended them in PBS supplemented with 50 μg/ml PI and 5 μg/ml RNAse A (Sigma) and incubated samples in the dark for 15 min at 37°C followed by analysis in FACSCalibur. We analyzed the data using ModFit software (Verity Software House).

Immunoblotting. We lyzed the cells in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate buffer supplemented with Complete Mini Protease Inhibitor tablet (Roche). We determined protein concentrations using Bio-Rad Protein Assay reagent and subjected equal amounts of protein to western blotting using antibodies described in the figure legends. In case of ischemic brain samples, we dissected out injured regions of the cortexes, lysed them in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, supplemented with Complete Mini protease inhibitors) and subjected equal amounts of proteins to western blotting. Results of the western blotting were quantified using Scion Image software (Scion Corporation).

Electroporation of Jurkat cells. pcDNA3-RIP-(1-580)-Fpk3-Myc, pcDNA3-RIP-(1-287)-Fpk3-Myc (pFR-KD) and pcDNA3-RIP-(1-580)-K45M-Fpk3-Myc, encoding RIP protein lacking DD domain, kinase domain of RIP and kinase dead mutant RIP,

respectively, fused to three copies of FKBP12 protein, and a control vector, pcDNA3-Fpk3-Myc (pFpk), were a generous gift of G. Nunez (University of Michigan). We amplified full length RIP using corresponding cDNA and cloned it into pcDNA3-RIP-(1-580)-Fpk3-Myc, replacing truncated RIP, to generate full length RIP-encoding dimerization construct (pFR). We used this construct to generate kinase dead mutant (pFR-K45M) with the help of QuikChange mutagenesis kit (Stratagene).

To generate pFF construct encoding FADD dimerization cassette, consisting of myristoylation signal followed by two copies of the 36v mutant FKBP12 (Fv2E) and coding region of FADD, we PCR amplified Fv2E and FADD using pC4M-Fv2E plasmid (Ariad Pharmaceuticals) and FADD cDNA, respectively, and cloned these fragments into pcDNA6 vector (Invitrogen).

For electroporation, we resuspended $20*10^6$ FADD-deficient Jurkat cells in 1 ml hypoosmolar buffer (Eppendorf), supplemented with 1.25% DMSO, mixed cells with 18 μg of the RIP vector/ and 2 μg of empty pEGFP vector and performed electroporation using Gene Pulser II (Bio-Rad). We allowed cells to rest for 5-10 min and then transferred them into 4 ml of RPMI1640 media, supplemented with 1.25% DMSO, 1% glutamine, 1% antibiotic-antimycotic mixture (Invitrogen) and 10% heat-inactivated fetal calf serum. Three hours later, we separated live cells using Ficoll-Paque (Pharmacia), washed them once and resuspended them in the media containing dimerizing agent AP1510 (Ariad Pharmaceuticals) and other chemicals as described. After treatment for indicated period of time, we added 2 μg/ml of PI to each sample, and determined percentages of viable PI-negative GFP-expressing cells. Alternatively, we selected a

stable clone of Jurkat cells expressing dimerizable FADD (JK-FF) in blasticidin (Invitrogen).

Generation of viral RNAi vectors. We directly ligated oligonucleotides containing RNAi sequences into pSRP vector (a gift of T. Jacks, Massachusetts Institute of Technology), which is based on pMSCV-puro (Invitrogen) viral vector and contains H1 promoter. We used published sequence of murine beclin-1 [5]. We generated the virus by co-transfection of the pSRP-based vector with the plasmids encoding retroviral Gag/Pol and VSV-G proteins into human HEK 293T cells. We infected Balbc 3T3 cells 3 times with each virus, followed by selection with puromycin. We used stable populations of cells to analyze target protein expression and cell viability.

Supplementary References

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