Supplementary Information

Supplementary Methods

DNA constructs, buffers and antibodies

The following DNA plasmids were used: HA-DAPk, HA-ΔCaM DAPk (Cohen et al., 1997), Flag-Beclin-1 or Flag-Beclin-1 ΔBcl-2 binding domain (a kind gift from B. Levine), Bcl-X_L (a kind gift from G.Kroemer), GST, GST-Beclin-1 (Erlich et al., 2007). The threonine substitutions in the GST-Beclin-1 T119A, Flag-Beclin-1 T119A and Flag-Beclin-1 T119E were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene).

The following antibodies were used: monoclonal antibodies to actin, DAPk, Flag (Sigma), to Bcl-2 (Santa Cruz Biotechnology), or to HA (BAbCO). Polyclonal antibodies to Beclin-1 or Bcl-X_L (Santa Cruz Biotechnology). The anti-T119 `phospho-Beclin-1 antibodies were developed in collaboration with Sigma-Aldrich. Rabbits were immunized with a synthetic phosphorylated peptide corresponding to amino acids 115-123 (pThr 119) of human Beclin-1, conjugated to KLH. The specific antibody was purified by first negatively absorbing the antiserum on the corresponding non-phosphorylated Beclin-1 peptide, to remove undesired antibodies to non-phosphorylated Beclin-1, and then affinity-purifying the non-absorbed fraction using the immunizing phosphorylated peptide immobilized on agarose.

HRP-conjugated goat anti-mouse, or anti-rabbit secondary antibodies (Jackson Immuno-Research), were used as required. Antibodies were visualized by enhanced chemiluminescence (Supersignal; Pierce) according to the manufacturer's instructions.

The following buffers were used: B buffer (0.4% NP-40, 0.5mM EDTA, 100mM KCl, 20mM Hepes pH-7.6 and 20% glycerol), NP-40 buffer (300 mM NaCl,

10 mM Tris pH-7.4, and 0.5% NP-40). PLB buffer (100 mM NaCl, 10 mM NaPO4, 5 mM EDTA pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS).

Pull-Down Assay

COS7 cells or HEK293T cells were washed once with ice-cold phosphate-buffered saline, and lysed in 600 ml CHAPS solubilization buffer (20 mM Tris pH-7.5, 5mM EDTA, 5mM EGTA, 100mM NaCl, 1% CHAPS) supplemented with 0.2mM Na3VO4 and protease inhibitors. Soluble extracts were prepared by centrifugation at 14,000 g for 10 min at 4˚C. Beclin-1 interacting proteins were precipitated from the cell lysates at 4˚C for 2 hours, using GST-Beclin-1 fusion protein conjugated to glutathione agarose beads. GST protein conjugated to glutathione agarose beads were used as a negative control. Beads were collected by centrifugation, washed with the lysis buffer and re-suspended in protein sample buffer. Proteins were separated by 10% SDS-PAGE, and blotted onto nitrocellulose membranes which were incubated with the indicated antibodies.

***In vitro* Kinase Assay**

HEK293T cells were transiently transfected by the standard calcium phosphate technique with full length Flag-DAPk or Flag-tagged Beclin-1. After 24 hours cell pellets were lysed in B buffer, supplemented with protease inhibitors, and with 1mM NaF and 50mM β−glycerolphosphate . Extracts were immunoprecipitated with anti-Flag M2 beads (Sigma), and proteins were eluted with excess Flag peptide. When high salt concentration was used, Beclin-1-bound beads were first washed stringently in 0.5 M LiCl followed by 0.5 M KCl to remove co-immunoprecipitating kinases.

For *in vitro* kinase assays, full length DAPk and Flag-tagged Beclin-1 or bacterially purified GST/GST-Beclin-1 were used as indicated in the figure legends, and were incubated at 30°C in kinase buffer (50mM Hepes pH 7.5, 20mM $MgCl_2$) supplemented with 0.116 μCi/μl [γ-^{33}P]ATP, 50 μM ATP, 1 μM bovine calmodulin, and 0.2 mM $CaCl_2$. Reactions were terminated by boiling in SDS-loading buffer, and were resolved on 10% polyacrylamide gels, blotted onto nitrocellulose membranes, and exposed to MR X-ray film (Kodak).

For peptide phosphorylation, DAPk's catalytic domain (DK1) was expressed in TOP10 bacteria (Invitrogen) upon induction with tetracycline, and affinity purified from bacterial lysates using the StrepTactin column (Genosys Biotechnologies) according to the manufacturer's instructions. A peptide corresponding to Beclin's BH3 domain (aa 108-127) was synthesized as well as the same peptide where T119 was substituted to alanine, and used in P-81 Whatman filter assays. DK1 (8 ng) was incubated for 15 min at 30°C with increasing concentrations of peptide (5-50 nmoles) in reaction buffer of 50 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 133 mM ATP and 4 mCi [γ-^{33}P]ATP. Reactions were applied to P-81 filters and washed extensively in 1% phosphoric acid. Total counts were measured by scintillation counting.

Transmission Electron Microscopy

HEK293 cells were transfected with Flag-Beclin, Bcl-X_L, and with DAPk ΔCaM or pcDNA3-luciferase plasmids After 24 hrs the cells were fixed for 1 hour in Karnovsky's fixative (3% paraformaldehyde, 2% glutaraldehyde, 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4, containing 3% sucrose). Cells were harvested and embedded with agar noble to a final concentration of 1.7% and post fixed with 1% OsO_4, 0.5% potassium dichromate and 0.5% potassium hexacyanoferrate in 0.1 M

cacodylate buffer. The pellet was stained and blocked with 2% aqueous uranyl acetate followed by ethanol dehydration, and embedded in graded Epon 812. Ultrathin sections (70-90nm thickness) were sectioned with Ultramicrotome Leica UCT, analyzed under 120kV at Tecnai 12 Transmission Electron Microscope (TEM) and imaged with Eagle 2k x 2k CCD camera FEI (Eindhoven Netherlands).

In gel proteolysis and mass spectrometry analysis

For *in vitro* kinase assays, 60ng Flag-tagged DAPk and 1050ng bacterially purified GST-Beclin-1 were incubated at 30°C in kinase buffer (50mM Hepes pH 7.5, 20mM $MgCl_2$) supplemented with 500 μM ATP, 1 μM bovine calmodulin, and 0.2 mM $CaCl_2$. After 2 hours 60ng DAPk were added to the reaction mix and the reactions were terminated 2 hours later by boiling in SDS-loading buffer, and were resolved on 10% acrylamide gels.

The proteins in the gel were reduced (10mM DTT), modified with 40 mM iodoacetamide and proteolyzed (modified trypsin or chymotrypsin (Promega)) at a 1:100 enzyme-to-substrate ratio. The resulting peptides were resolved by reverse-phase chromatography on 0.075 X 200-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 90 minutes gradients of 5 to 45% and 15 minutes at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 μl/min. Mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo) in a positive mode using repetitively full MS scan followed by collision induces dissociation (CID) of the 7 most dominant ion selected from the first MS scan.

Fe^{+2} columns (Sigma) were used to enrich phosphopeptides (binding in 250mM acetic acid, 30%acetonitrile and elution in 400mM ammonium hydroxide).

These peptides were analyzed similarly except the usage of multistage activation in the fragmentation method. The mass spectrometry data was analyzed using the Sequest 3.31 software (J. Eng and J.Yates, University of Washington and Finnigan, San Jose) and Pep-Miner (Beer et al., 2004) searching against the human part of the NCBI- NR database.

Supplementary References

Beer, I., Barnea, E., Ziv, T. and Admon, A. (2004) Improving large-scale proteomics by clustering of mass spectrometry data. Proteomics, 4, 950-960.

Cohen, O., Feinstein, E. and Kimchi, A. (1997) DAP-kinase is a Ca2+/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. Embo J, 16, 998-1008.

Erlich, S., Mizrachy, L., Segev, O., Lindenboim, L., Zmira, O., Adi-Harel, S., Hirsch, J.A., Stein, R. and Pinkas-Kramarski, R. (2007) Differential interactions between Beclin 1 and Bcl-2 family members. Autophagy, 3, 561-568.

Supplementary Figures Legends

Fig.S1 **DAPk phosphorylates bacterially purified Beclin-1.** Flag-tagged DAPk was incubated with GST or GST-Beclin-1 (the numbers indicated in the figure are the amount of protein used in ng) in the presence of Ca^{2+}, calmodulin and [γ-^{33}P] ATP for 15 min. Phosphorylated proteins were visualized by X-ray film exposure. The autophosphorylation of DAPk indicates that its catalytic activity was intact.

Fig.S2 **DAPk phosphorylates Beclin-1 on T119 located within its BH3 domain.** Bacterially-purified DAPk's catalytic domain was incubated for 15 min at 30°C with increasing concentrations (5-50 nmoles) of a peptide corresponding to Beclin's BH3 domain (aa 108-127) as well as with the same peptide where T119 was substituted to alanine. *In vitro* kinase assay was performed, and the reactions were applied to Whatman filters. Total levels of TCA insoluble counts were measured and plotted against substrate concentration.

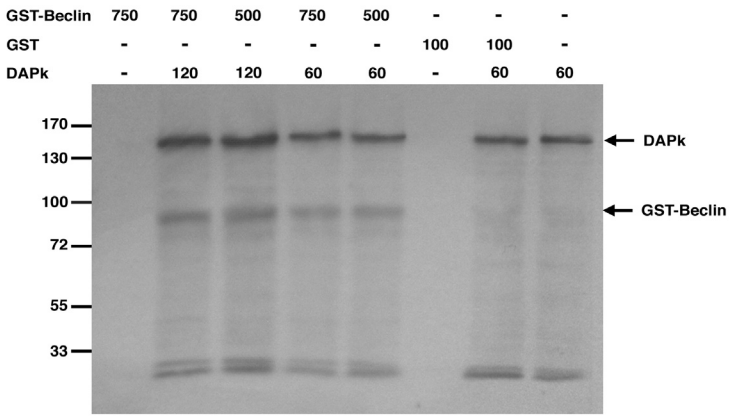
Fig.S3 **Phosphopeptide mapping of GST-Beclin-1 after its *in vitro* phosphorylation by DAPk** Fragmentation spectra of identified phosphopeptides 115-122 (A) and 103-118 (B).

Fig.S4 **DAPk phosphorylates Beclin-1 purified from mammalian cells *in vitro* on T119.** Flag-Tagged DAPk (60 ng) was incubated with Flag-tagged Beclin-1 (300 ng) purified from HEK293T cells by anti Flag antibodies and eluted from the beads by excess of Flag peptide. The kinase assay was performed for 30 min. Eluates obtained from similar amounts of untransfected cells which went through the same

purification procedure as the cell lysates prepared from the Beclin-1 transfected cells, were incubated with DAPk as a control. Phosphorylated proteins were visualized by X-ray film exposure (autoradiogram). After the radioactivity on the blots decayed, the phosphorylation on T119 was visualized by western blot analysis using anti-pT119 Beclin-1 antibodies (Western blot, upper panel). Total amount of eluted Beclin-1 was visualized by anti-Flag antibodies (Western blot, lower panel).

Fig S5 **ΔCaM DAPk promotes Beclin's dissociation from Bcl-X_L.** HEK293 cells were co-transfected with Flag-tagged Beclin-1 and Bcl-X_L with or without HA-tagged ΔCaM DAPk. Beclin-1 was immunoprecipitated using anti-Flag antibodies, and the co-immunoprecipitated proteins as well as the total cell extracts were blotted using anti Beclin-1, anti Bcl-X_L, or anti-HA antibodies. These experiments were repeated five times with similar results.

Fig.S1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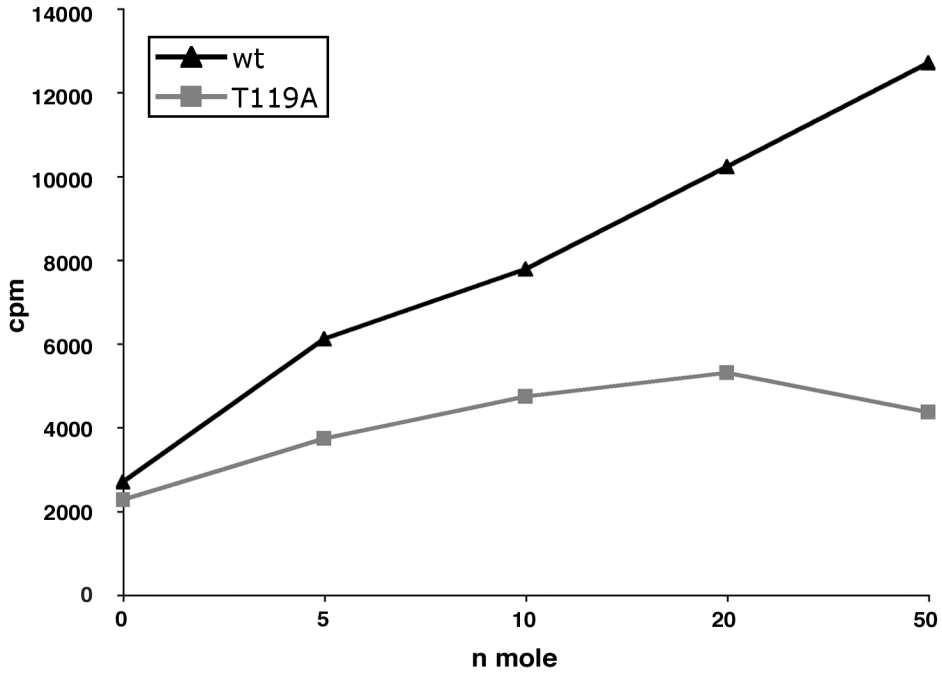
33
DAPk
GST-Beclin

Fig.S2


14000
12000
10000
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4000
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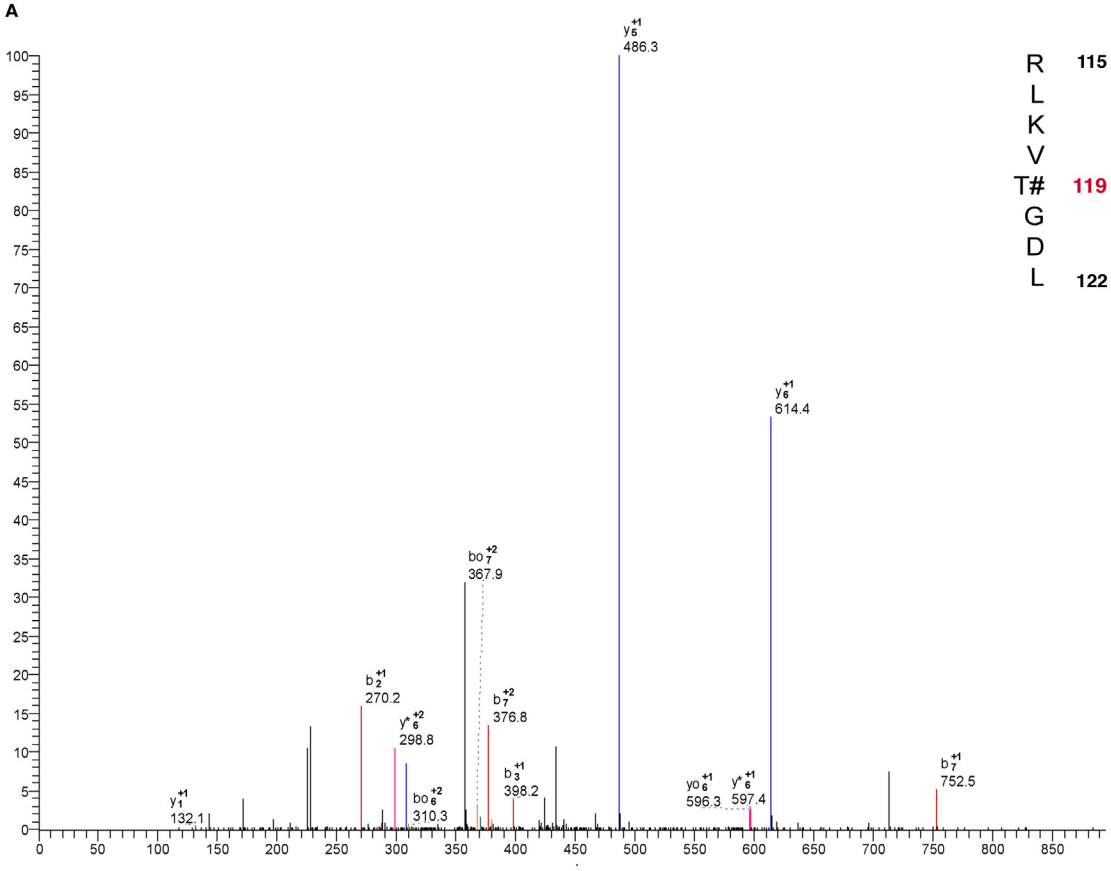
cpm
wt
T119A
0
5
10
20
50
n mole

Fig.S3

A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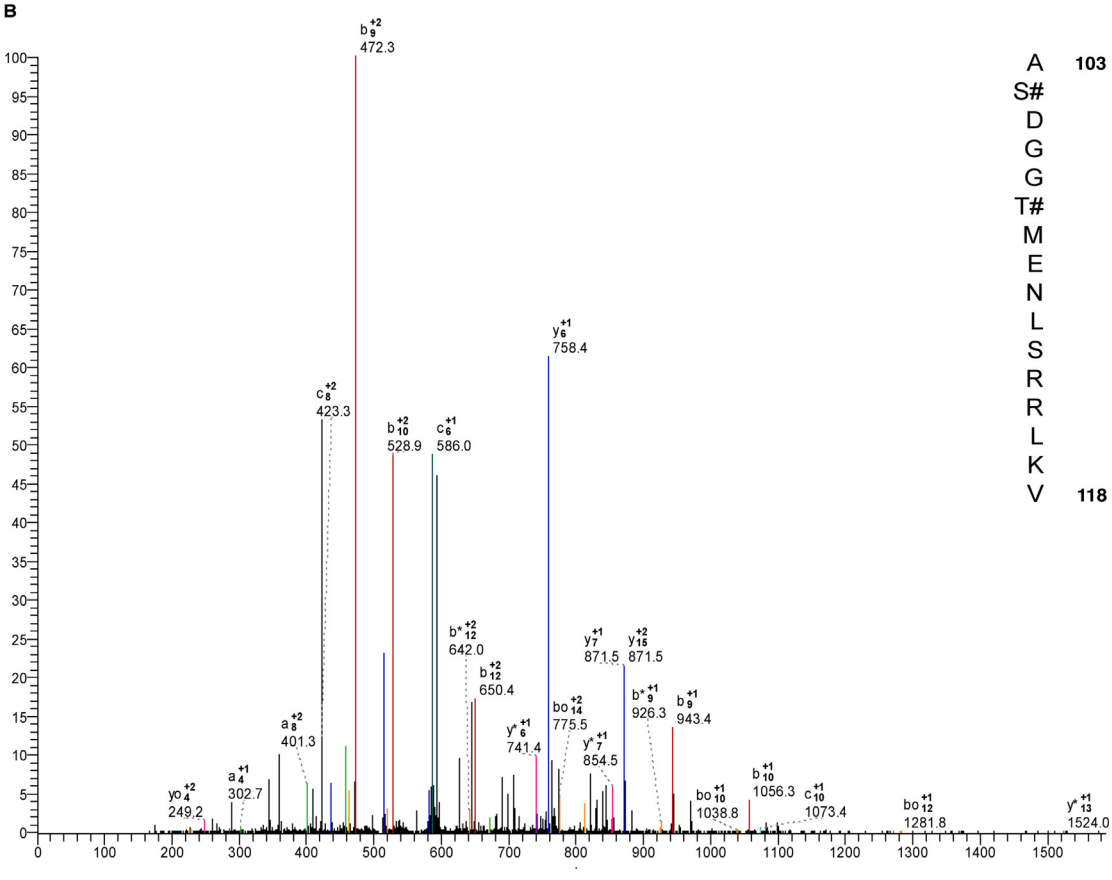
B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y7+1 871.5
y15+2 871.5
b*9+1 926.3
b9+1 943.4
bo14+2 775.5
y*6+1 741.4
y*7+1 854.5
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a4+1 302.7
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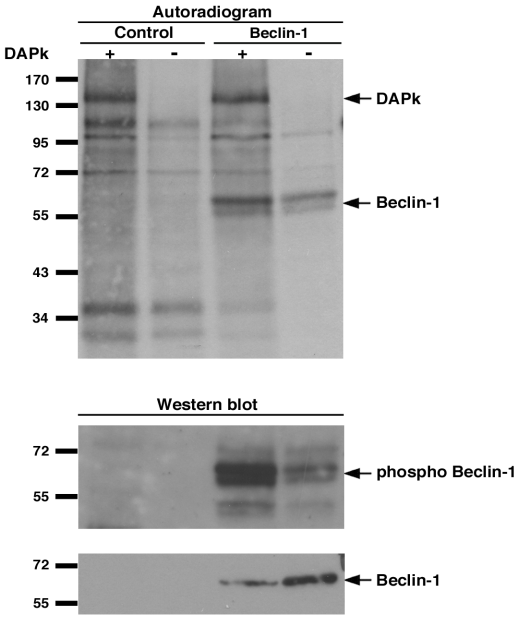
Fig.S4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-
+
-
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130
95
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Western blot
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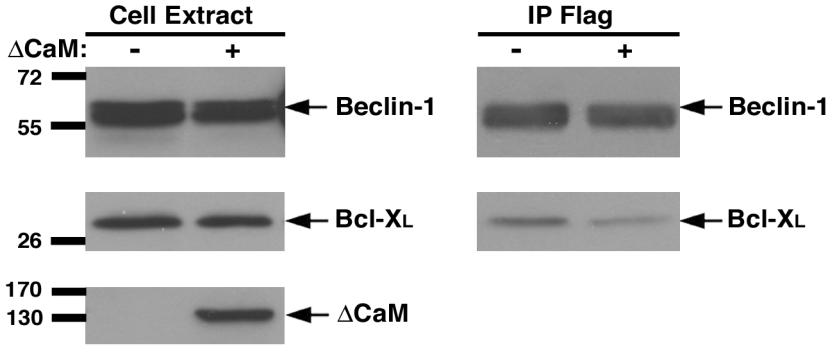
55
phospho Beclin-1
72
55
Beclin-1

Fig.S5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ΔCaM