Supplemental figures

Fig. S1 Purification of full-length recombinant Bim_L

A ArcticExpress *Escherichia coli* overexpressing 6His-SUMO-tagged Bim_L-Intein were lysed using a French press and centrifuged at 100,000 x g at 4°C for 1h. The supernatant was loaded on a Chitin-column (Input). The beads were then incubated for 40h at 4°C in buffer containing 100 mM β-mercapthoethanol to induce Intein cleavage. The elution fraction was collected and applied to a Ni^{2+}-NTA column (Elute). 6-HisSUMO-Bim_L was eluted using a buffer containing 300 mM Imidazole (Fractions 1-7). All the fractions were analyzed using gel electrophoresis followed by an o/n Coomassie Blue staining.

B Fractions containing purified 6His-SUMO-Bim_L prepared as detailed under (A) were joined (Input) and concentrated while diluting the imidazole to 20 mM (Concentrated). The SUMO-tag was then cleaved off o/n at 4°C with SUMO Protease 1 (Cleaved). After purification over a Ni^{2+}-NTA column, Bim_L was collected in the flow-through (Flow). Samples were analyzed as in (A).

Fig. S2 Bax activating activity of recombinant Bim_L.

Mitochondria prepared from *$Bax^{-/-}Bak^{-/-}$* MEFs were incubated for 30 min at 30 °C with 100 nM of recombinant Bax in combination with either various concentrations of recombinant 6His-SUMO-Bim_L or 20 nM recombinant tBid. 1µl of the elution buffer used during SUMO-Bim_L purification was added as an additional control (Buffer). Mitochondria were then pelleted by centrifugation and the presence of cytochrome c in the pellet (P) or in the supernatant (S) was determined by Western blot.

Fig. S3 Diffusion time of Bim_L-R and Bim_L-G increases in presence of DLC1 indicating formation of Bim-Bim complexes.

A The dot plot shows the quantification of diffusion time of Bim_L-R and Bim_L-G from three independent solution FCCS experiment (refers to Fig. 1 E and F). Note that the diffusion time of Bim increased in presence of DLC1 indicating complex formation. The higher diffusion

time of Bim_L-R compared to that of Bim_L-G is due to the larger detection volume of red channel (a function of the wave length). (*** p<0.001, two tailed paired t-test).

B The bar diagram represents the fold change (mean±SD) in diffusion time of Bim_L-R and Bim_L-G in presence of DLC1. Data were calculated from (S3A).

Fig. S4 Western blots showing results of subcellular fractionations of MEF cells. Mitochondria or cytosol from *Bim*$^{-/-}$ cells reconstituted with murine 3xHA-tagged Bim_{EL} under a 800 bp fragment of the Bim promotor (lanes 1-4) or from *Bax*$^{-/-}$ *Bak*$^{-/-}$ cells overexpressing murine V5-tagged Bim_{EL} under a retroviral promotor (lanes 5-8) were analyzed. Tubulin and Hsp60 serve as markers for cytosol and mitochondria. Data are representative of at least three independent experiments.

Fig. S5 Complex formation by Bim-splice variants

A Schematic overview of murine Bim splice variants and mutants used in this study. H1=helix 1, H2=helix 2 (H1, unstructured and H2 are structural domains predicted by *in silico* modeling), TM= transmembrane domain

B The splice variants Bim_{EL} and Bim_L but not Bim_S form Bim-Bim-complexes as detected by co-IP. Western blots showing results of IP-experiments with anti-HA antibodies in extracts from cells overexpressing either untagged or 3xHA-tagged murine Bim splice variants. Whole cell lysates were prepared with 1% Triton X-100. For the interaction studies with 3xHA-mBim_{EL}, *Bax*$^{-/-}$ *Bak*$^{-/-}$ MEFs carrying a tamoxifen-inducible murine 3xHA-Bim_{EL} construct and stably overexpressing the other indicated murine Bim-proteins were used (tamoxifen (100 nM) was added 24 h prior to extraction). For interaction studies with murine 3xHA-Bim_L or 3xHA-Bim_S HEK 293FT cells were transfected 24 h prior to lysis with the indicated constructs (10 µM QVD-OPH was added to prevent cell death). Data are representative of five (3xHA-Bim_{EL} with Bim_{EL}) or three (all other combinations) independent experiments.

*, endogenous Bim_{EL}-signal.

Fig. S6 Formation of Bim-complexes requires the region of amino acids 98-113 in Bim.

A 293FT cells were co-transfected with murine V5-tagged N-terminal truncation mutants of Bim and murine 3xHA-tagged Bim_{EL} 24 h prior to lysis and cultured in the presence of the caspase-inhibitor QVD-OPH (10 μM). Whole cell lysates were prepared with 1% triton X-100. HA-tagged Bim was immunoprecipitated using anti-HA-antibodies, and co-precipitated V5-Bim was detected by Western blotting. Data are representative of three independent experiments. *, anti-V5-signal from first blot.

B Western blots show results of immunoprecipitation experiments using anti-V5 antibodies in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs expressing different murine V5-tagged Bim versions under a retroviral promoter as indicated. Mitochondria enriched (mito.) or cytosolic fractions prepared in the presence of 1% triton X-100 were used. The membrane was re-probed for detection of V5-Bim versions (lower panel). *, antibody light chain. Data are representative of 3 independent experiments.

Fig. S7 Proximity ligation assay (PLA) showing interaction (close proximity) of 3xHA-tagged and V5-tagged murine Bim_{EL} wt but not Bim_{EL}AA.

Murine V5-tagged Bim_{EL} wt or Bim_{EL}AA was expressed constitutively in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs while murine 3xHA-tagged Bim_{EL} was induced by addition of 100 nM tamoxifen for 24 h as indicated. Images are representative of 3 independent experiments and were acquired under identical conditions and exposure time.

Fig. S8 Loss of DLC1 sensitizes HCC827 cells to gefitinib induced apoptosis.

HCC827 cells were treated with gefitinib (10 μM, 72 h) after 24 h of siDLC1. Induction of apoptosis was determined by measuring the activation of caspase-3 by flow cytometry. The bar diagram denotes the mean of two experiments while the circles and squares denote values for the individual experiments.

Fig. S9 Immunoprecipitation of endogenous Mcl-1 and DLC1 in HeLa and 1205Lu cells.

Mcl-1 (A) and DLC1 (B) immuno-precipitations were performed with the whole cell lysate of HeLa and 1205Lu cells and the interacting Bcl-2 family proteins were detected by Western

blotting. Data are representative of three independent experiments for Mcl-1 IP and two for DLC1 IP.

Fig. S10 Loss of DLC1 in combination with ABT-737 treatment induces activation of Bax and Bak.

HeLa (A and C) and 1205Lu (B and D) cells were treated with ABT-737 (2 μM) for 24h after siRNA mediated knockdown of DLC1 for 24h. Activation of Bax and Bak was detected by either cross linking (A and B) or by intracellular flow cytometry (C and D) using conformation specific antibodies. Cross linking was performed with the isolated mitochondria of the cells with BMH (0.5 mM BMH) and the samples were analyzed by SDS-PAGE, and different Bax/Bak species were detected by Western blotting. In the siDLC1 treated samples, oligomeric species of Bax and Bak were detected along with decrease in their monomeric forms. The asterisk indicates bands of unknown origin probably due to non-specific cross linking of Bak. HeLa (C) and 1205Lu (D) cells were treated as mentioned above and fixed using 4% formalin. The fixed cells were stained with primary antibodies specifically detecting the activated forms of Bax (6A7) and Bak (NT). Species specific Alexa fluor-647 or Cy5 conjugated secondary antibodies were used to detect activated Bax and Bak by flow cytometry. Data are representative of three independent experiments.

Fig. S11 Loss of DLC1 has no effect on cell cycle.

Cell cycle analysis was performed after siRNA mediated knockdown of DLC1 (24 h) in HeLa and 1205Lu cells. Cells were fixed overnight in 70 % ethanol before staining with PI. The stained cells were analysed by flow cytometry. (A) Histogram shown is representative of two independent flow cytometry experiments. (B) The bar diagram (mean of two independent experiments) shows the distribution of cells in different phases of cell cycle after DLC1 knockdown. The dots and square on the bar indicates the results of individual experiments.

Fig. S12 Effect of DLC1 loss on ERK signaling and Noxa levels.

Whole cell lysates were prepared from HeLa and 1205Lu cells after 48h of siRNA mediated knockdown of DLC1. Samples were separated by SDS-PAGE and levels of indicated

proteins were detected by Western blotting. Data are representative of three independent experiments.

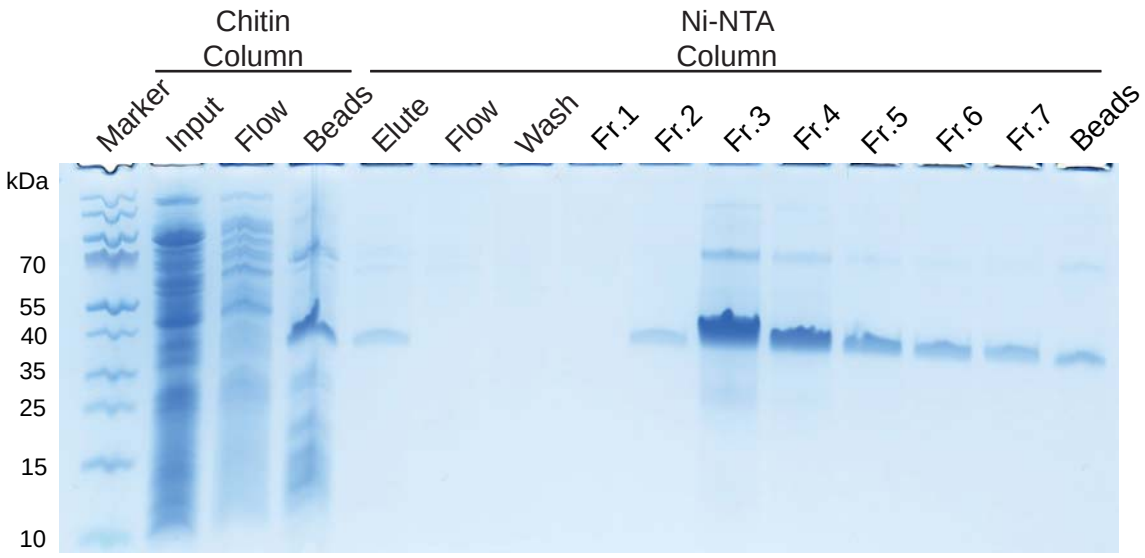
Fig. S13 Expression levels of tamoxifen-inducible wt Bim_{EL} or Bim_{EL}AA in MEFs by Flow cytometry.

Bim_{EL} expression was induced with 100 nM tamoxifen (+4HT) for 24 h where indicated and QVD-OPH (10 μM) was added together with tamoxifen in order to avoid cell death and protein degradation.

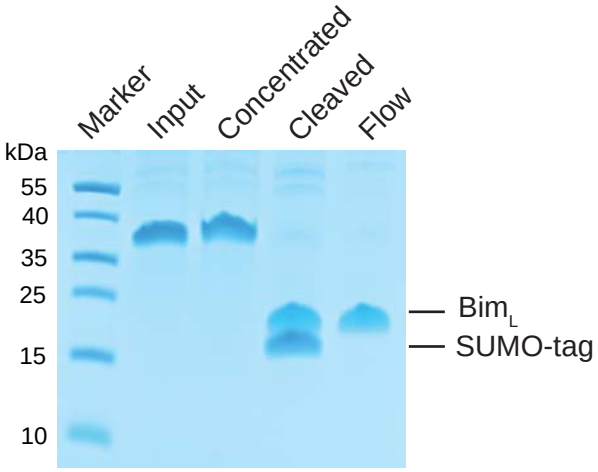
Supplementary Table 1: Functions used for the FCS analysis. *G* is the autocorrelation and G_x the cross-correlation function; τ the lag time for correlation; $F(t)$ the fluorescence intensity as a function of time (t); $\langle\ \rangle$ corresponds to time averaging ($\delta F(t) = F(t) - \langle F(t)\rangle$); F_R and F_G are the fluorescence intensity in the red and green channels; C_{RG} is the concentration molecules in the two color complex; $C_{RG,t}$ the concentration of all particles having both colours; $G_{0,x}$ is the cross-correlation amplitude; $G_{0,R}$ ($G_{0,G}$) is the amplitude or the red (or green) autocorrelation curve; D is the diffusion coefficient; N is the average number of detected molecules; t_D is the average time the fluorophore stays in the focal volume; G_{12} is the spatial cross-correlation curve comparing the two foci; and d is the distance between the two foci. The %CC is used to calculate unbound concentration of the red and the green-labelled molecules (C_r and C_g, respectively), as well as the bound concentration (C_{rg})

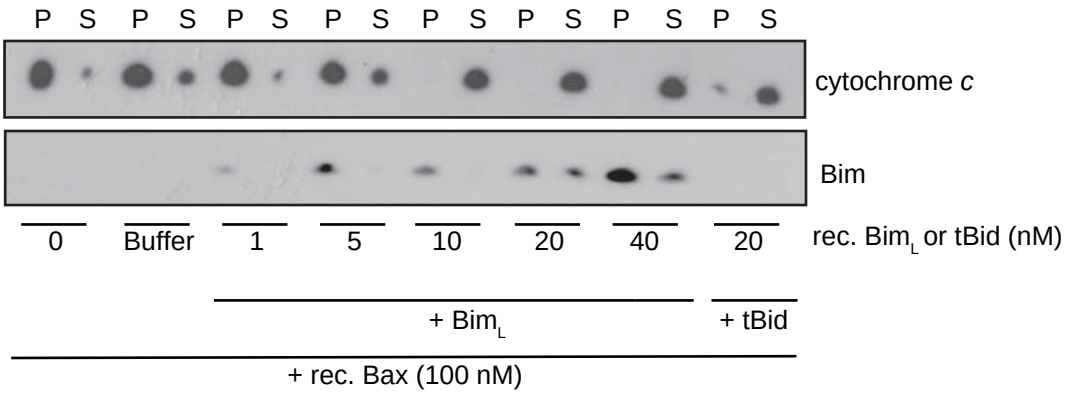
Name	Function
Auto-correlation function	$G(\tau) = \frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}$
Cross-correlation function	$G_x(\tau) = \frac{\langle \delta F_R(t)\ \delta F_G(t+\tau)\rangle}{\langle F_R(t)\rangle\langle F_G(t)\rangle}$
Fraction of molecules in two color complex	$C_{RG} = \frac{G_{0,x}}{G_{0,G}}$ or $C_{RG} = \frac{G_{0,x}}{G_{0,R}}$
Diffusion coefficient	$D = \frac{\omega_0^2 4t_D}{4t_D}$
3D diffusion for solution measurements	$G_{3D}(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\frac{1}{\sqrt{1+\frac{\tau}{S^2\tau_D}}}$
Dual-focus scanning FCS for measurements in membranes	$G_{12}(\tau) = \frac{1}{C\pi s\omega_0}\left(1+\frac{4D\tau}{\omega_0^2}\right)^{-1/2}\left(1+\frac{4D\tau}{\omega_0^2 s^2}\right)^{-1/2}\exp\left(-\frac{d^2}{\omega_0^2+4D\tau}\right)$
Cross correlation calculations: $\%CC_r = \frac{C_{rg}}{C_{rg}+C_g}$	$\%CC_g = \frac{C_{rg}}{C_{rg}+C_r}$

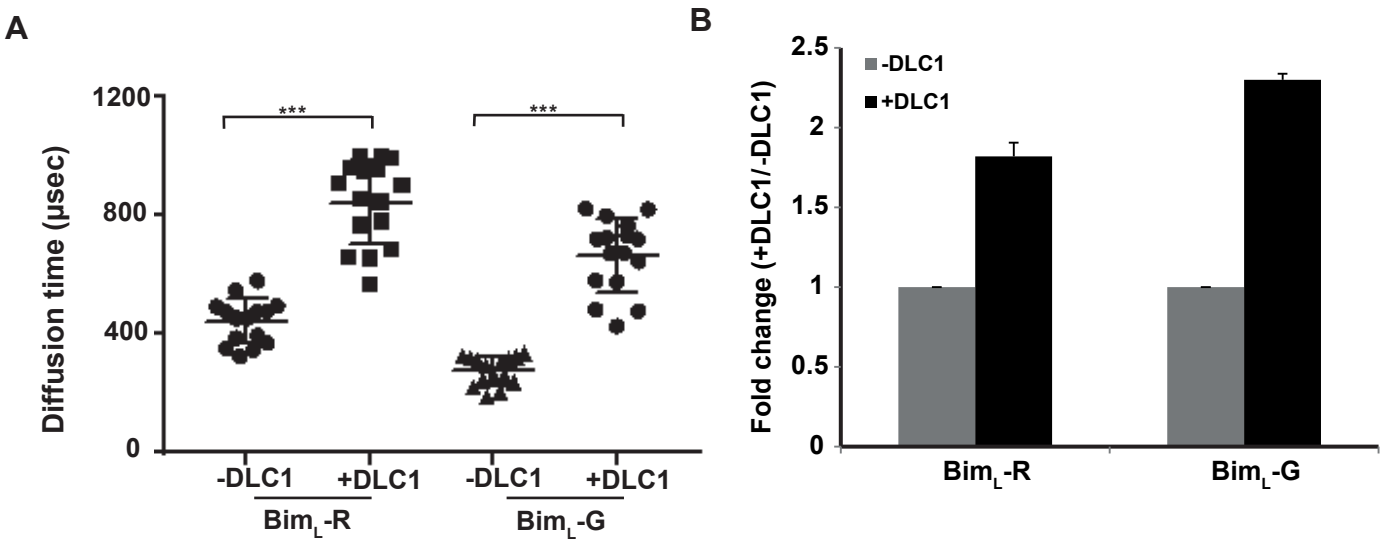
A

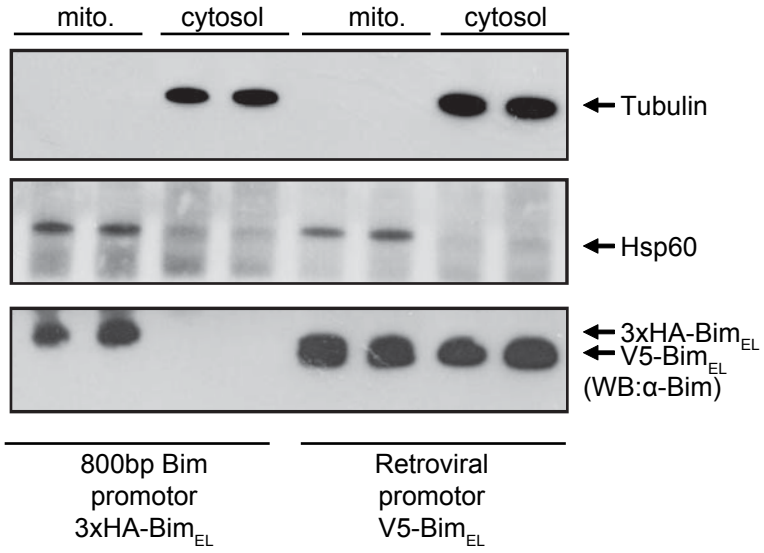

Chitin Column
Ni-NTA Column
Marker
Input
Flow
Beads
Elute
Flow
Wash
Fr.1
Fr.2
Fr.3
Fr.4
Fr.5
Fr.6
Fr.7
Beads
kDa
70
55
40
35
25
15
10

B

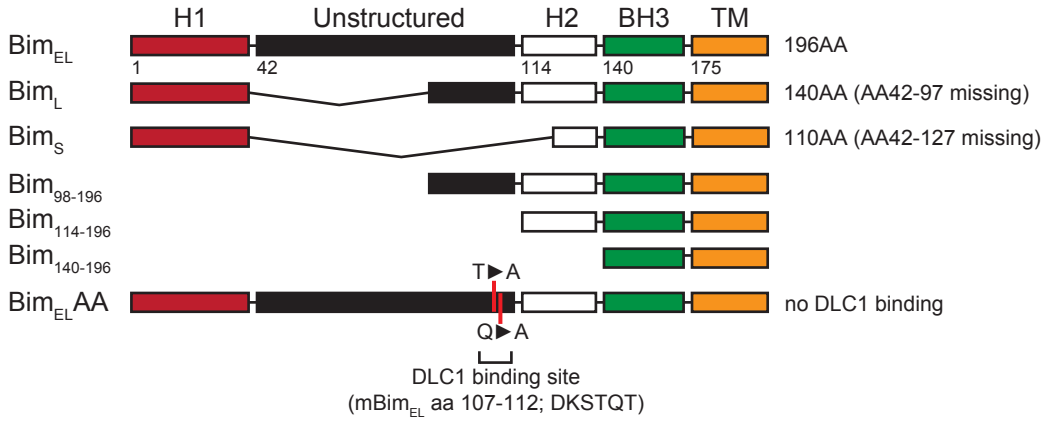

Marker
Input
Concentrated
Cleaved
Flow
kDa
55
40
35
25
15
10
Bim$_L$
SUMO-tag


P S P S P S P S P S P S P S P S
cytochrome c
Bim
0
Buffer
1
5
10
20
40
20
rec. Bim$_L$ or tBid (nM)
+ Bim$_L$
+ tBid
+ rec. Bax (100 nM)

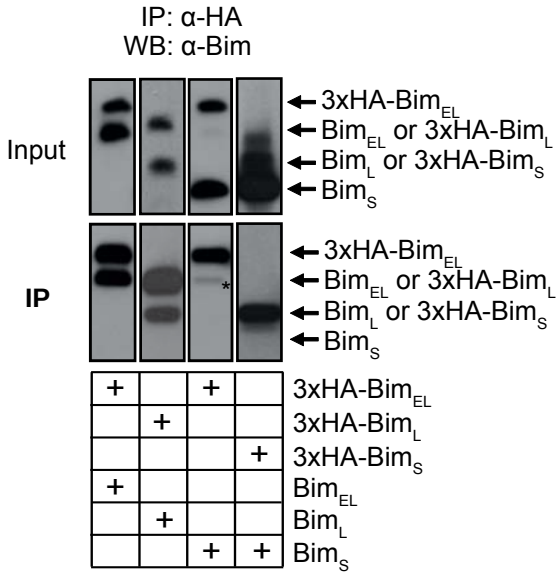

A
Diffusion time (µsec)
1200
800
400
0
***
***
-DLC1
+DLC1
-DLC1
+DLC1
Bim$_L$-R
Bim$_L$-G
B
Fold change (+DLC1/-DLC1)
2.5
2
1.5
1
0.5
0
-DLC1
+DLC1
Bim$_L$-R
Bim$_L$-G

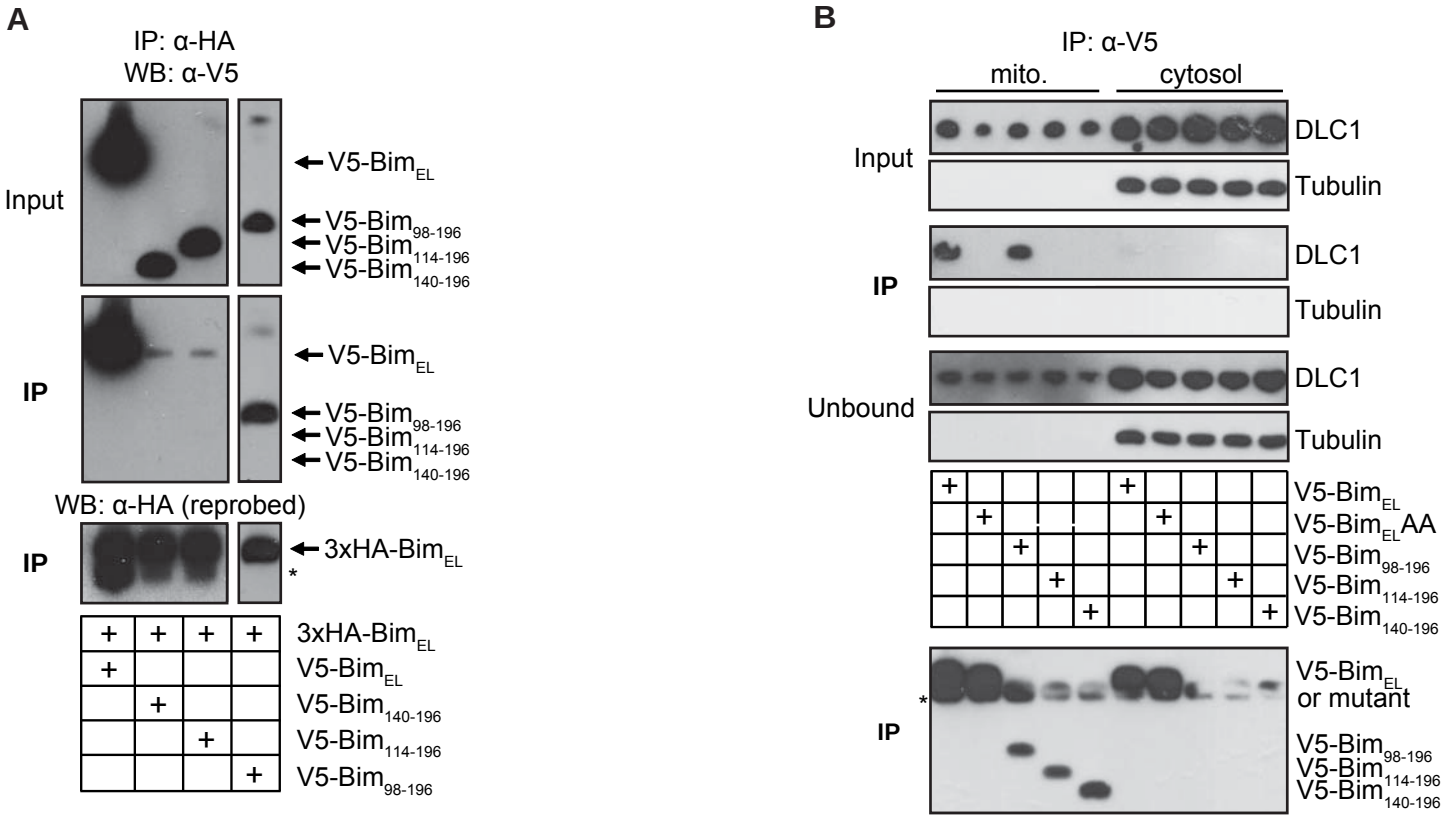

mito.
cytosol
mito.
cytosol
Tubulin
Hsp60
3xHA-Bim$_{EL}$
V5-Bim$_{EL}$
(WB:α-Bim)
800bp Bim promotor 3xHA-Bim$_{EL}$
Retroviral promotor V5-Bim$_{EL}$

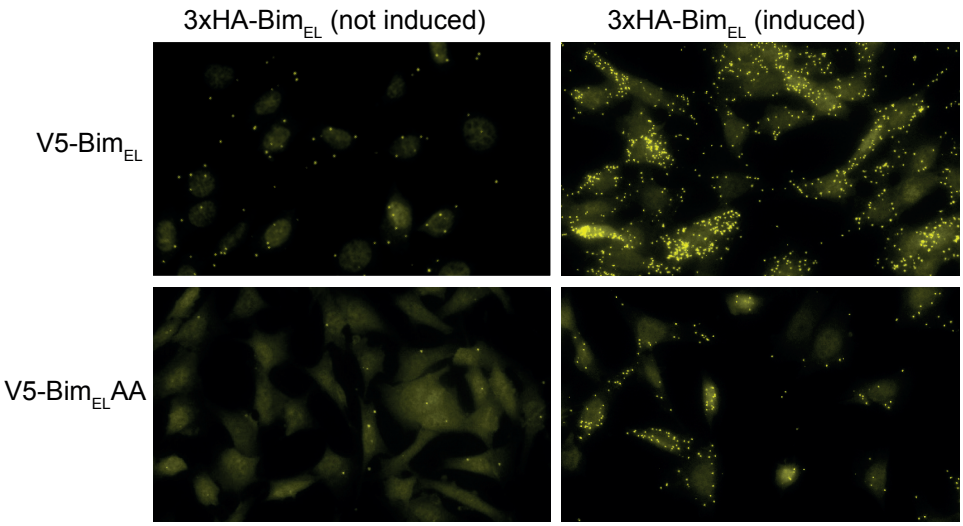
A

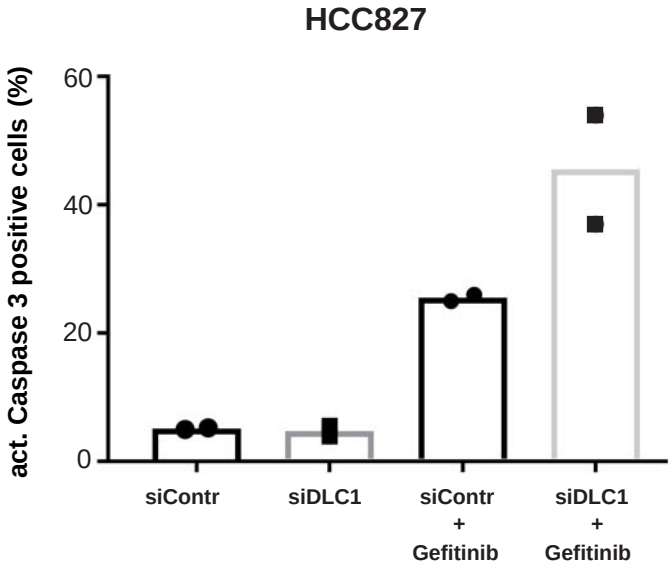

H1
Unstructured
H2
BH3
TM
Bim$_{EL}$
196AA
1
42
114
140
175
Bim$_{L}$
140AA (AA42-97 missing)
Bim$_{S}$
110AA (AA42-127 missing)
Bim$_{98-196}$
Bim$_{114-196}$
Bim$_{140-196}$
T▶A
Bim$_{EL}$AA
no DLC1 binding
Q▶A
DLC1 binding site
(mBim$_{EL}$ aa 107-112; DKSTQT)

B


IP: α-HA
WB: α-Bim
Input
IP
*
3xHA-Bim$_{EL}$
Bim$_{EL}$ or 3xHA-Bim$_{L}$
Bim$_{L}$ or 3xHA-Bim$_{S}$
Bim$_{S}$
3xHA-Bim$_{EL}$
3xHA-Bim$_{L}$
3xHA-Bim$_{S}$
Bim$_{EL}$
Bim$_{L}$
Bim$_{S}$

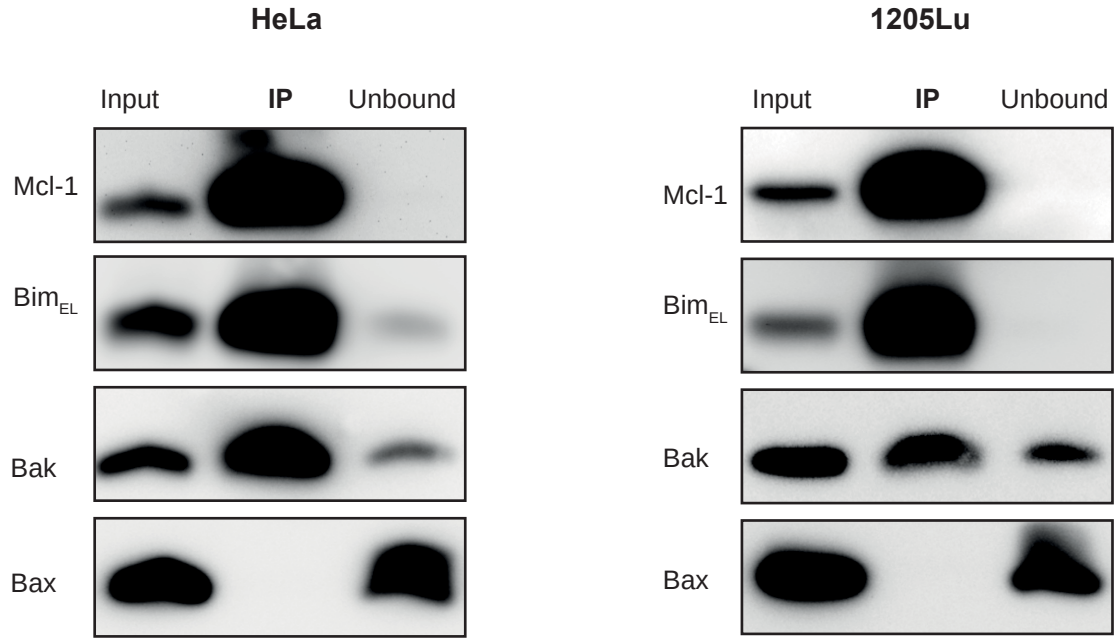

A
IP: α-HA
WB: α-V5
Input
IP
V5-Bim$_{EL}$
V5-Bim$_{98-196}$
V5-Bim$_{114-196}$
V5-Bim$_{140-196}$
WB: α-HA (reprobed)
3xHA-Bim$_{EL}$
*
3xHA-Bim$_{EL}$
V5-Bim$_{EL}$
V5-Bim$_{140-196}$
V5-Bim$_{114-196}$
V5-Bim$_{98-196}$
B
IP: α-V5
mito.
cytosol
Input
Unbound
IP
DLC1
Tubulin
V5-Bim$_{EL}$
V5-Bim$_{EL}$AA
V5-Bim$_{98-196}$
V5-Bim$_{114-196}$
V5-Bim$_{140-196}$
V5-Bim$_{EL}$ or mutant
*


3xHA-Bim$_{EL}$ (not induced)
3xHA-Bim$_{EL}$ (induced)
V5-Bim$_{EL}$
V5-Bim$_{EL}$AA
yellow: interaction spots


HCC827
act. Caspase 3 positive cells (%)
60
40
20
0
siContr
siDLC1
siContr + Gefitinib
siDLC1 + Gefitinib

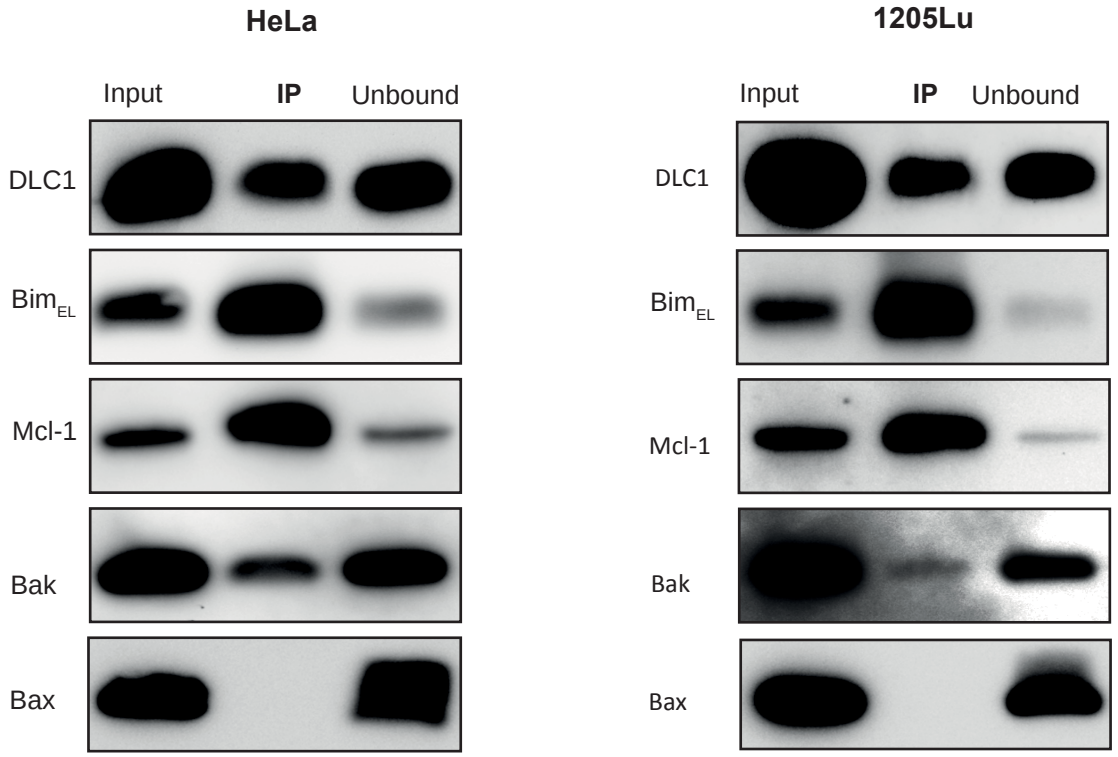
A

IP: α-Mcl-1



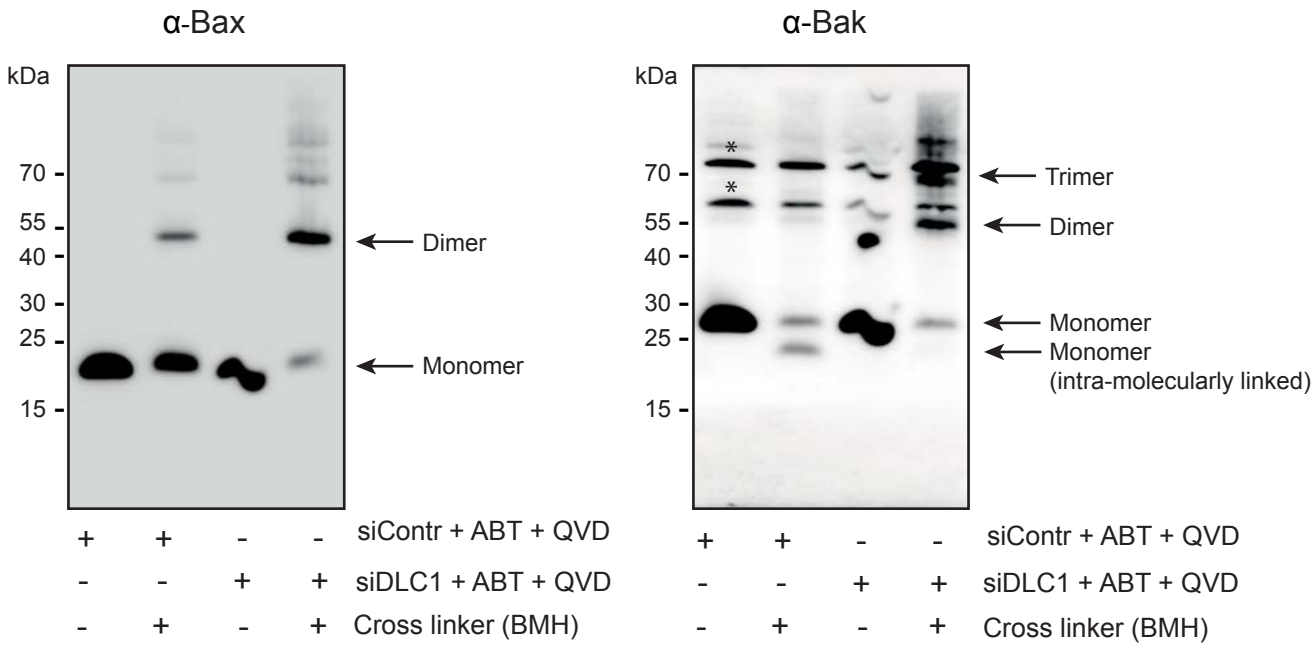
B

IP: α-DLC1



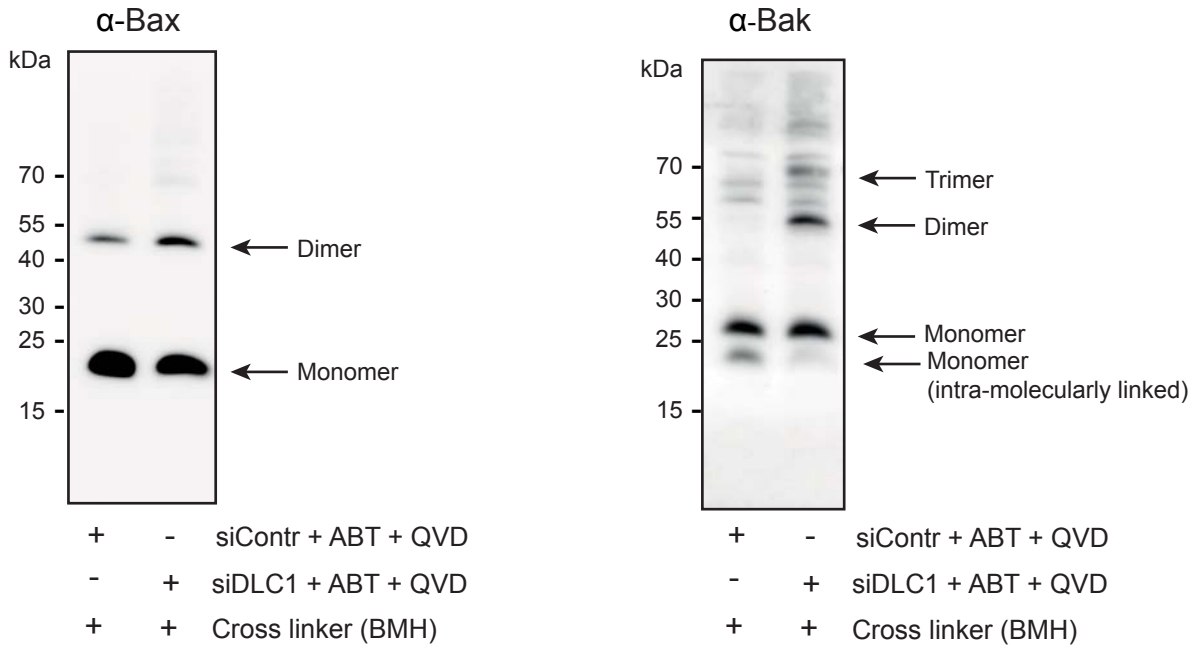
A

HeLa



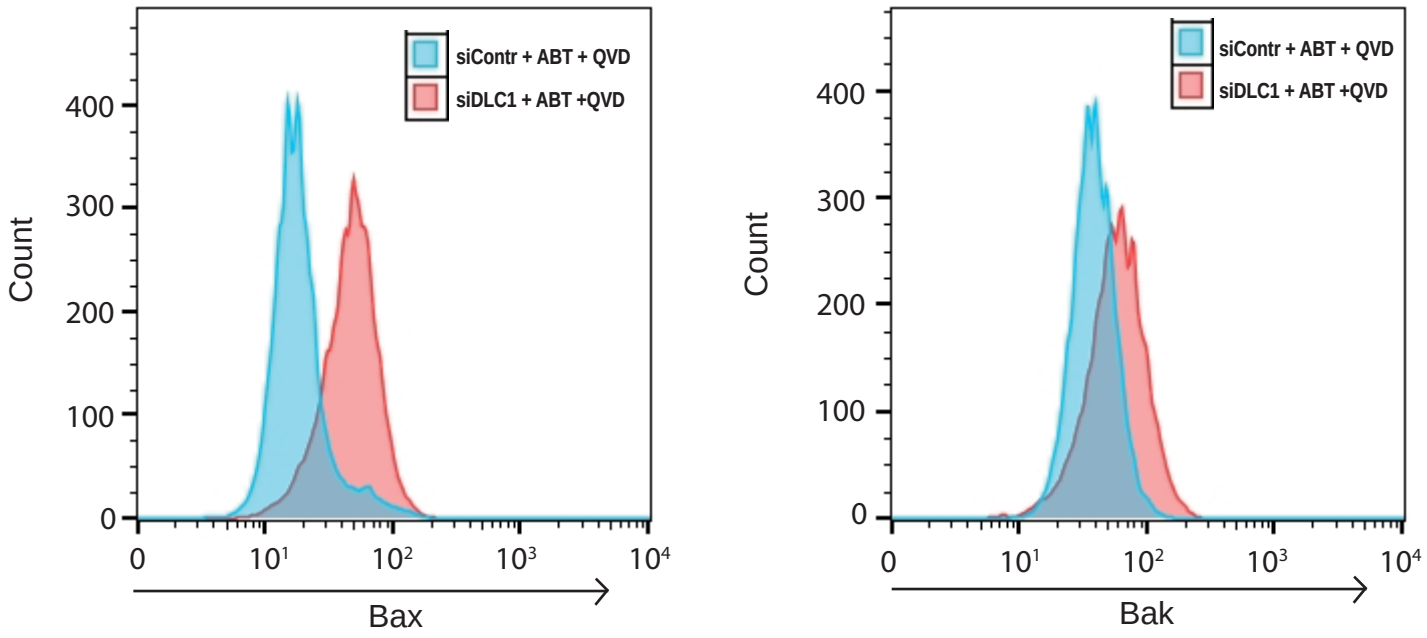
B

1205Lu



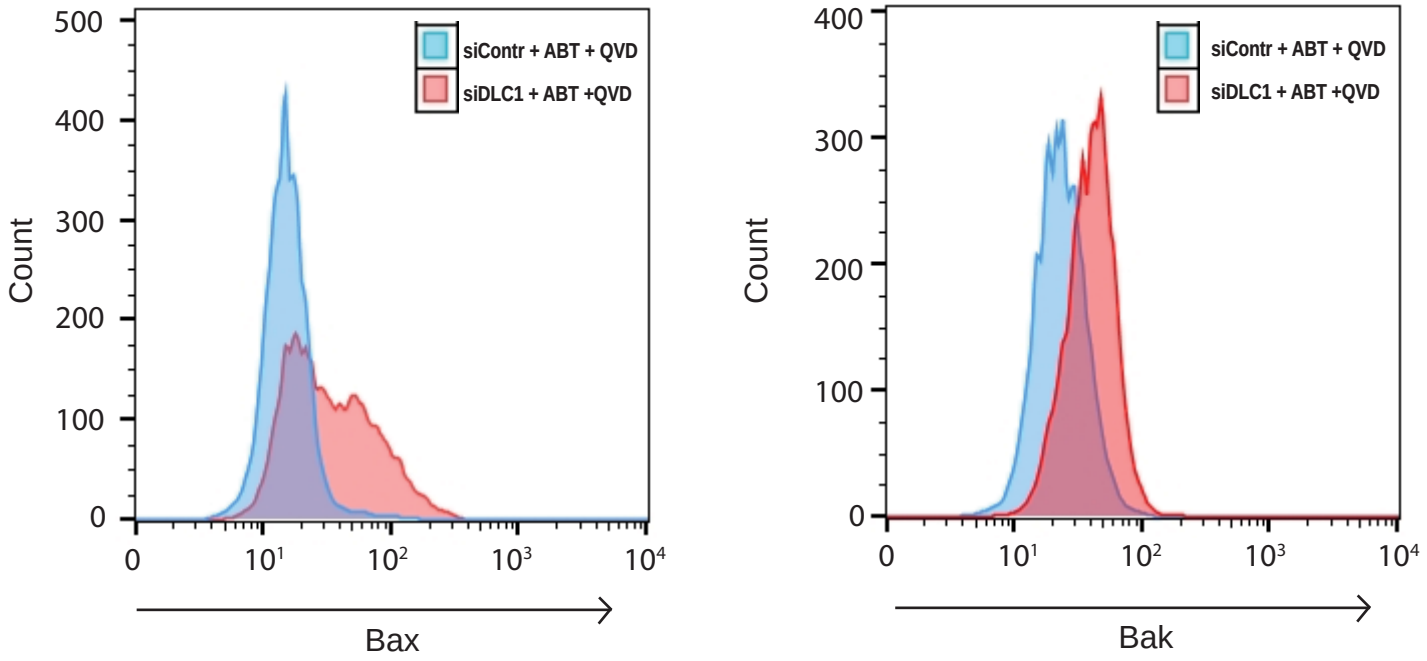
C

HeLa

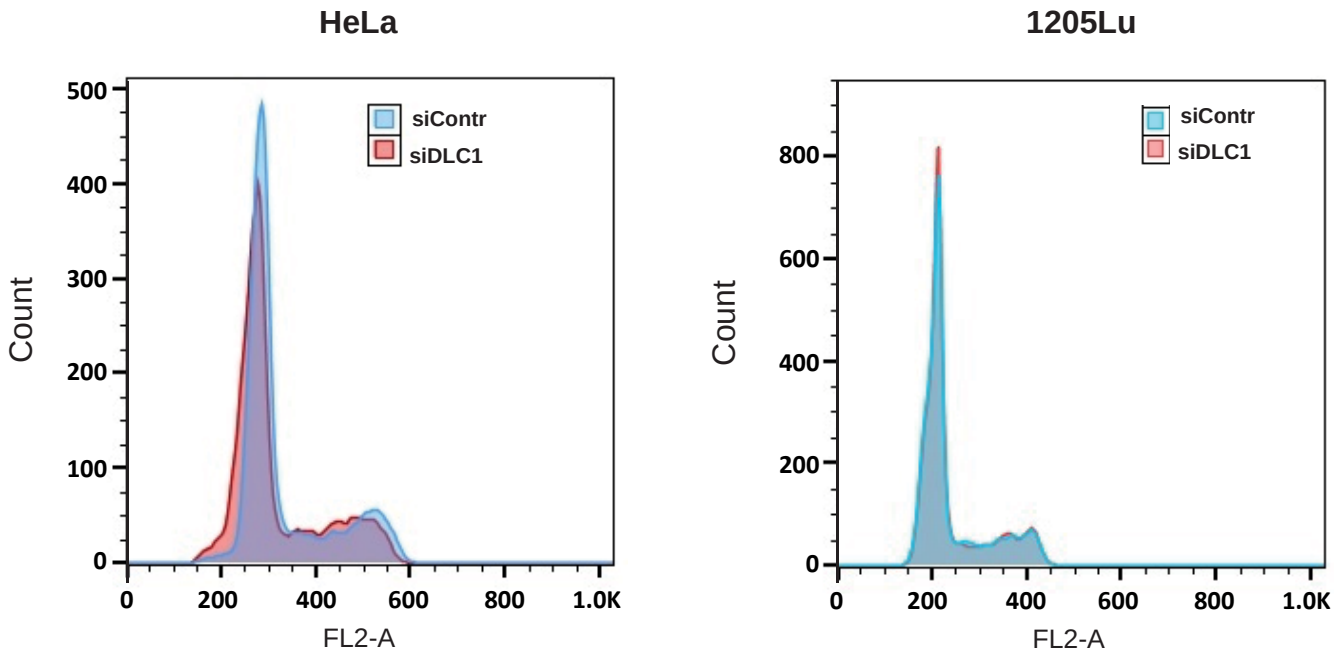


D

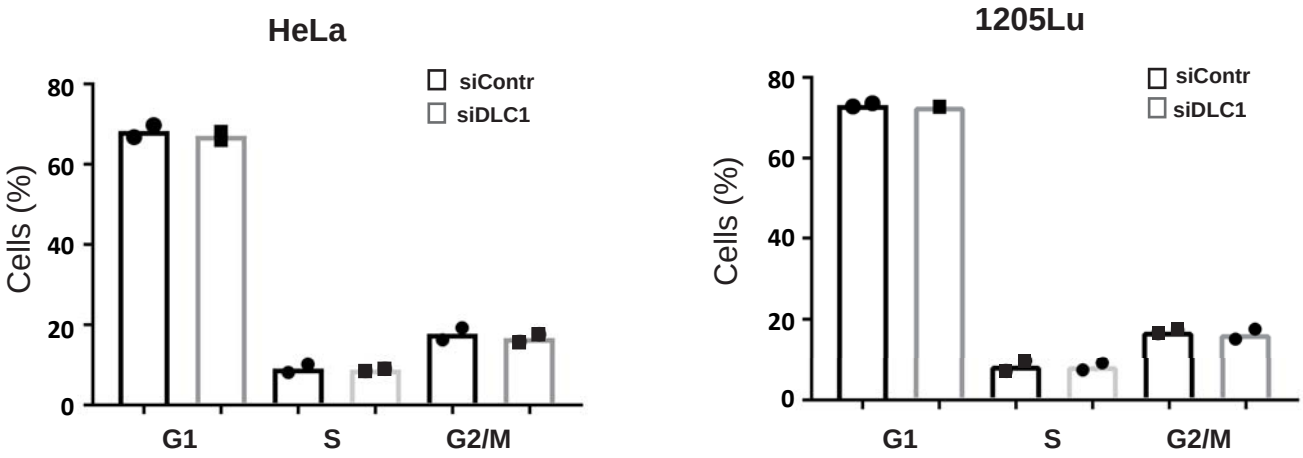
1205Lu

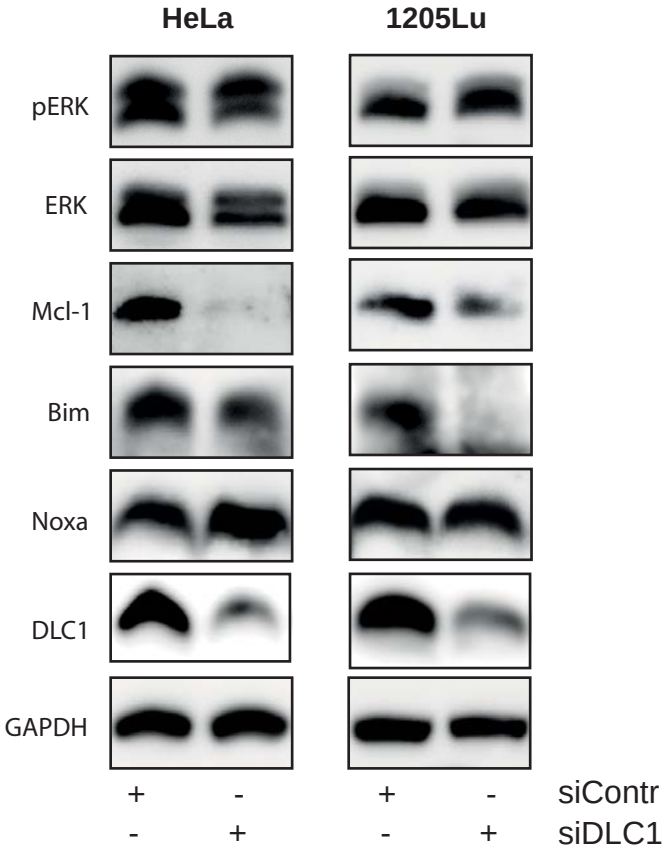


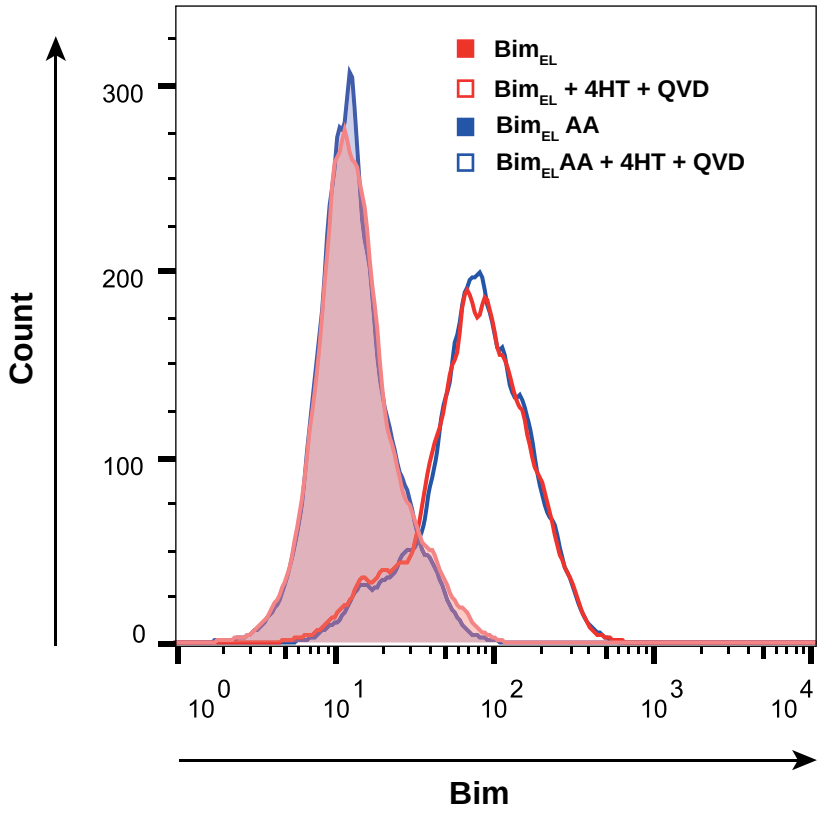
A


HeLa
1205Lu
siContr
siDLC1
Count
FL2-A
0
100
200
300
400
500
600
800
1.0K

B


HeLa
1205Lu
siContr
siDLC1
Cells (%)
0
20
40
60
80
G1
S
G2/M


HeLa
1205Lu
pERK
ERK
Mcl-1
Bim
Noxa
DLC1
GAPDH
+ - + - siContr
- + - + siDLC1


Bim_EL
Bim_EL + 4HT + QVD
Bim_EL AA
Bim_EL AA + 4HT + QVD
Count
300
200
100
0
10^0
10^1
10^2
10^3
10^4
Bim