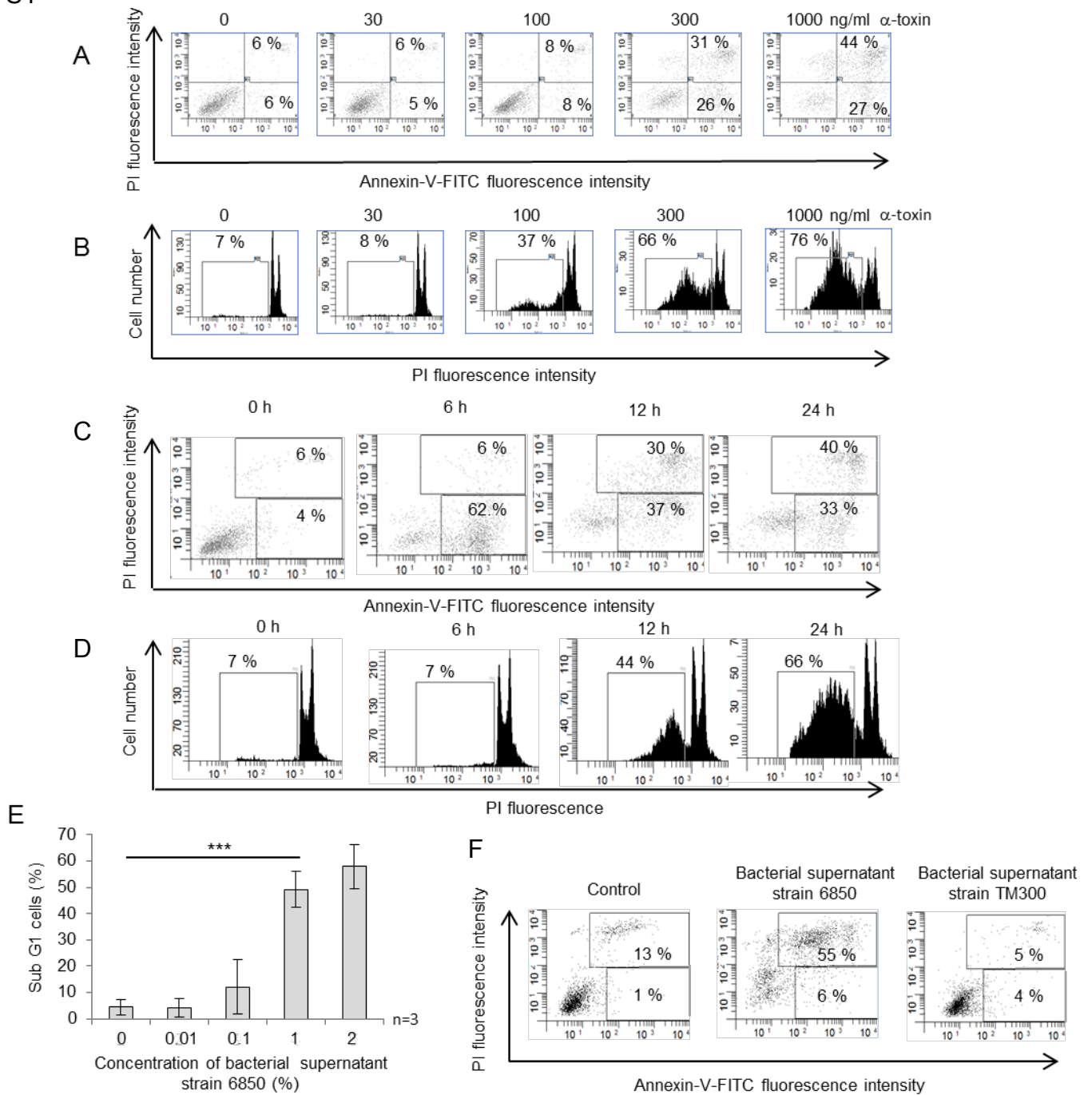
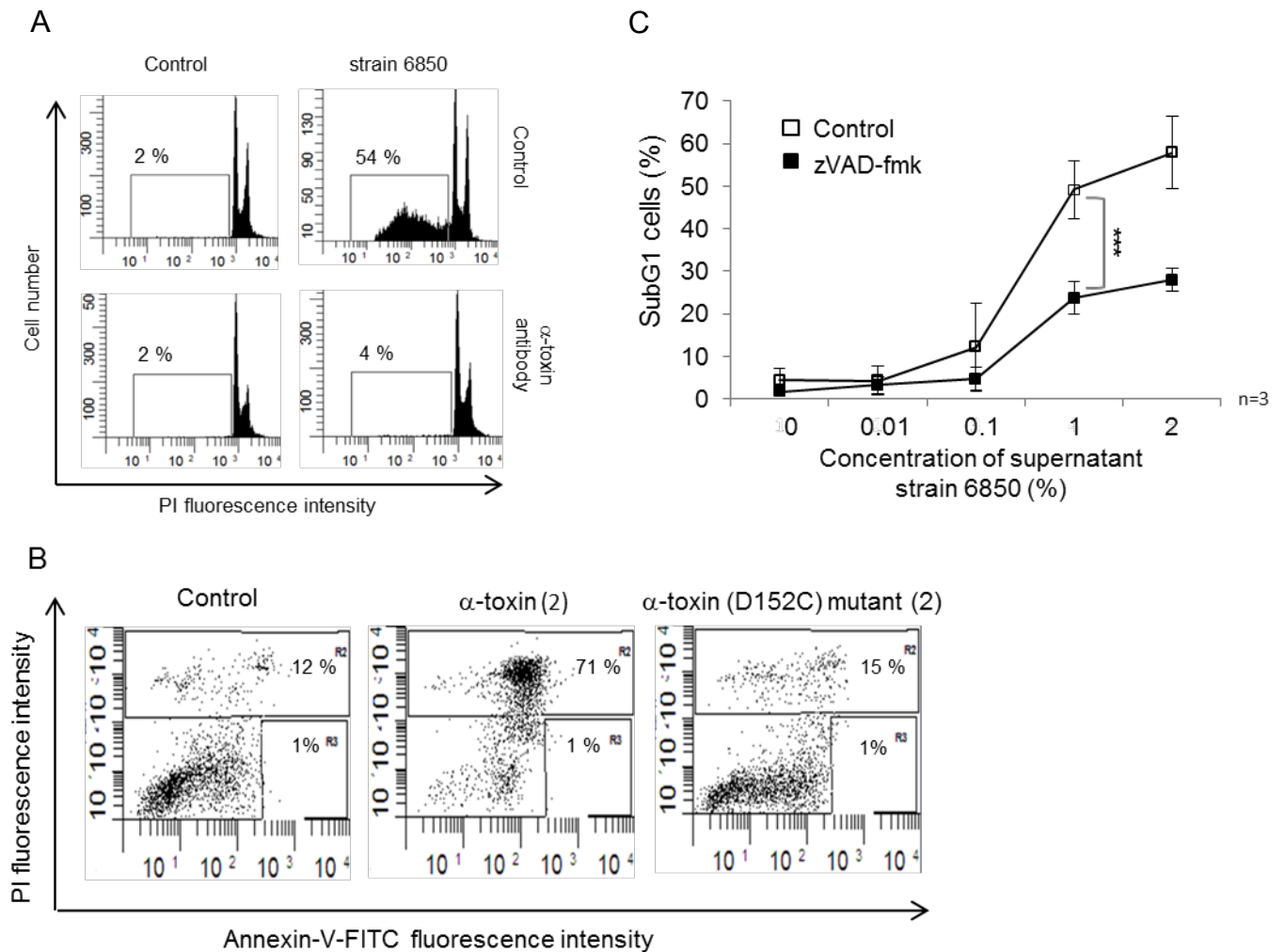


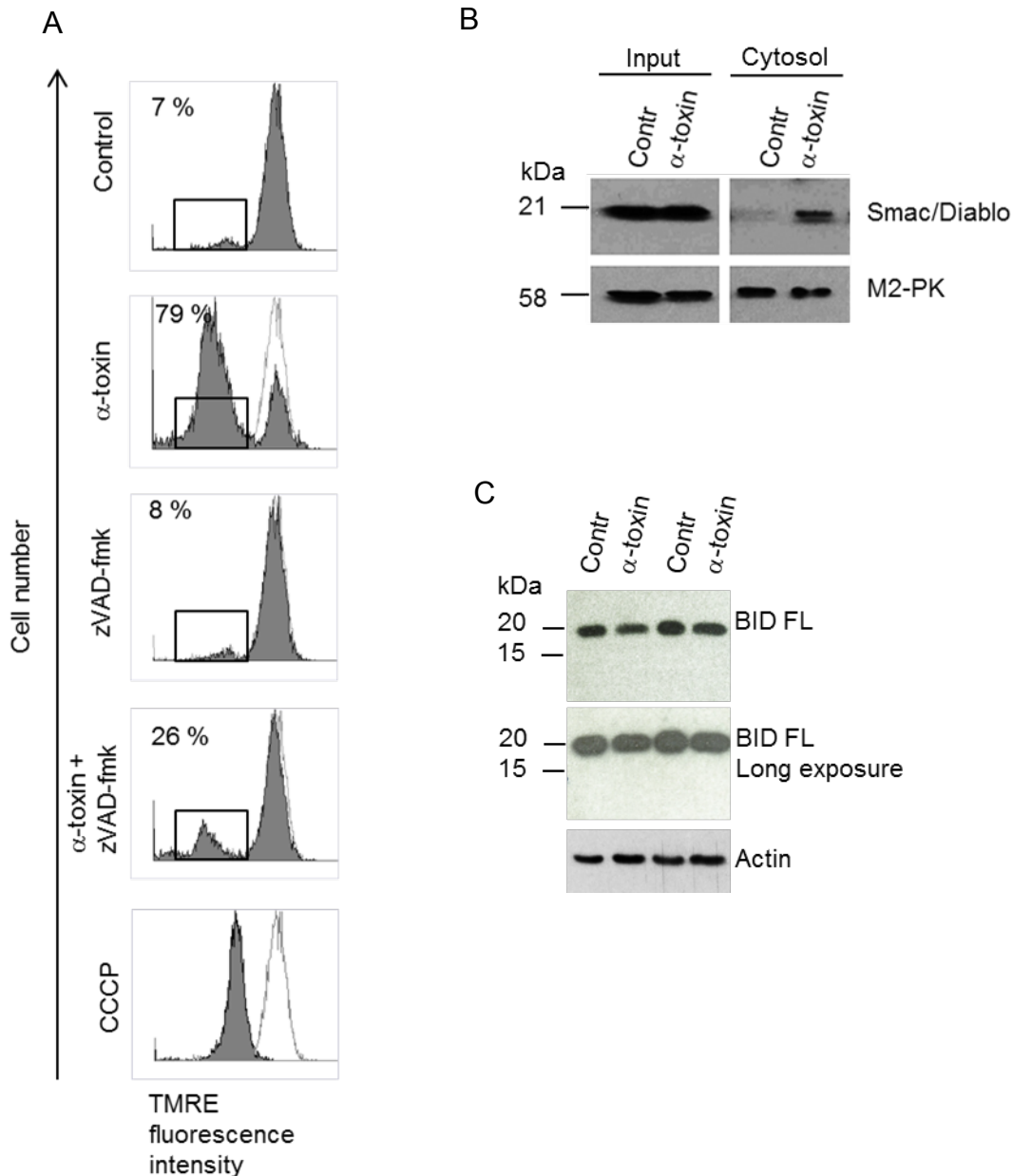
S1



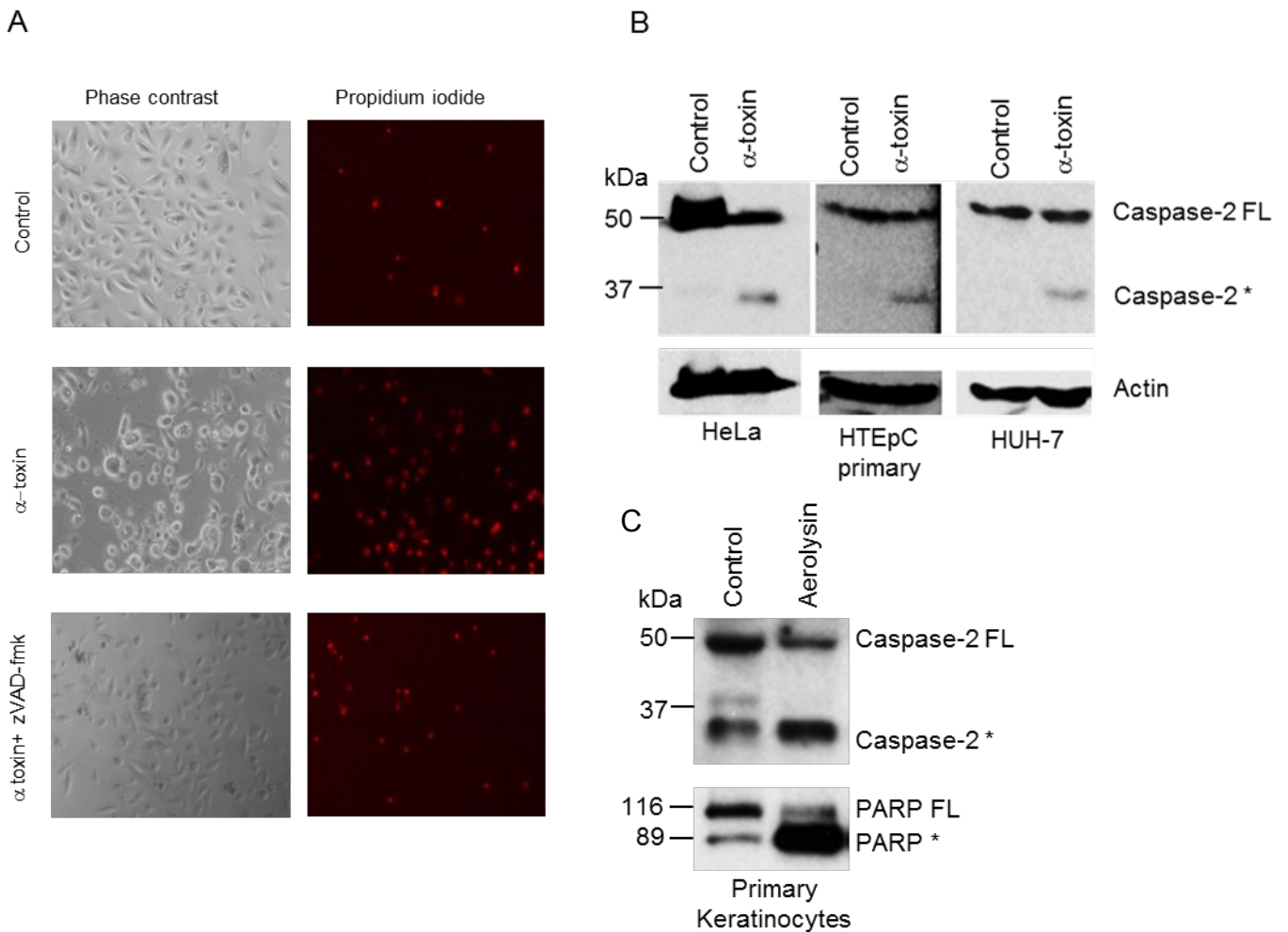
**Figure S1 (A)** Flow cytometric analysis of phosphatidylserine translocation (Annexin-V single positive cells) and membrane damage (PI and Annexin-V double positive cells). HeLa cells were treated with different concentration of  $\alpha$ -toxin (see concentrations above) for 24 hours. Values indicate the percentage of the gated population. **(B)** Flow cytometric detection of Sub G1 (apoptotic DNA fragmentation) population in control and  $\alpha$ -toxin treated cells. HeLa cells were treated with different concentrations of  $\alpha$ -toxin (24 h). The percentage of cells representing sub G1 is denoted. **(C)** The kinetics of cell death of  $\alpha$ -toxin (300 ng/ml) treated HeLa cells. The samples were analyzed as mentioned in (A). **(D)** The kinetics of  $\alpha$ -toxin (300 ng/ml)-mediated cell death was measured by the appearance of sub G1 population of cells. **(E)** HeLa cells were treated with different concentrations of bacterial culture supernatant from strain 6850 and cell death was measured by the appearance of Sub G1 cells. Shown are data from three independent experiments. **(F)** Representative dot plots of Annexin-V, PI analysis. The cells were treated with the bacterial culture supernatant (1%, 24 hours) of two different *S.aureus* strains: strain 6850 (virulent) and strain TM300 (non-virulent).



**Figure S2.** (A) Representative histograms of Sub G1 detection by flow cytometer. HeLa cells were treated with 1 % of bacterial supernatant (strain 6850) (24h) with or without  $\alpha$ -toxin antibody. Gated population exhibit the Sub G1 cells. (B) Representative scatter plots of Annexin-V, PI analysis with flow cytometry. HeLa cells were treated with  $\alpha$ -toxin (24h), (2) indicates a second source of  $\alpha$ -toxin. Population above represents PI and Annexin-V double positive cells and the population in the right corner below corresponds to the Annexin-V single positive cells. Values show the percentage of these populations. (C) HeLa cells were treated with various concentrations of bacterial culture supernatant (strain 6850). The induction of cell death in the presence or absence of caspase inhibitor (zVAD-fmk) was monitored by the appearance of Sub G1 population of cells 24 hours after toxin treatment. Shown are data from three independent experiments  $\pm$ SD.

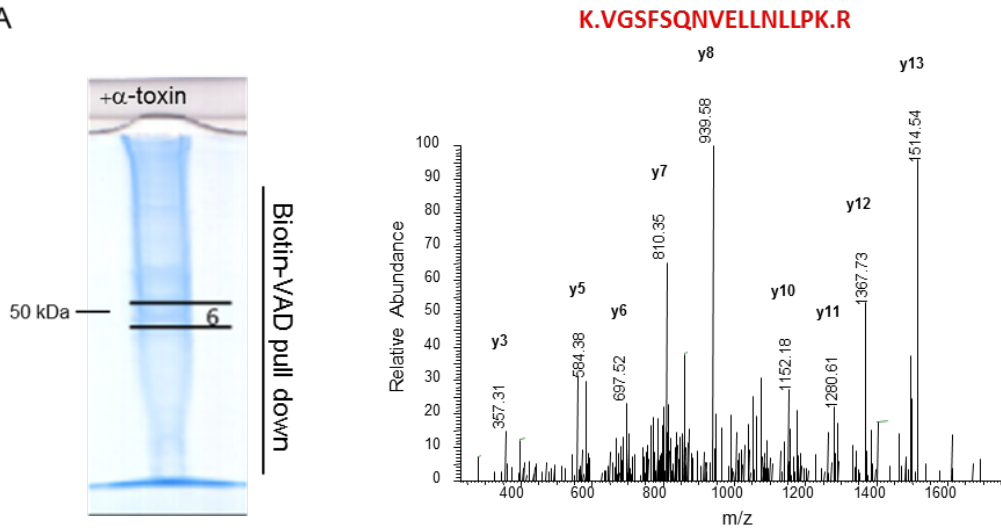


**Figure S3** (A) Flow cytometric analysis of mitochondrial depolarization (loss of TMRE fluorescence). HeLa cells were treated with  $\alpha$ -toxin (300 ng/ml) in the presence or absence of zVAD-fmk for 24 hours. Values indicate the percentage of the gated population. Filled peaks are the treated samples. Unfilled peaks (in histogram 2 to 5) show the distribution of the control sample (first histogram) as a reference. CCCP was employed as a positive control. (B) Detection of Smac/Diablo release in the cytosol by Western blot analysis. HeLa cells were treated with  $\alpha$ -toxin (24 h) and then subjected to subcellular fractionation and western blot analysis. M2-PK is provided as marker (cytosol) protein for loading. (C) Western blot analysis of BID protein cleavage from control and toxin treated cell lysates obtained from two independent experiments. HeLa cells were treated with  $\alpha$ -toxin (24 h) and harvested for western blot. BID FL- full length BID.

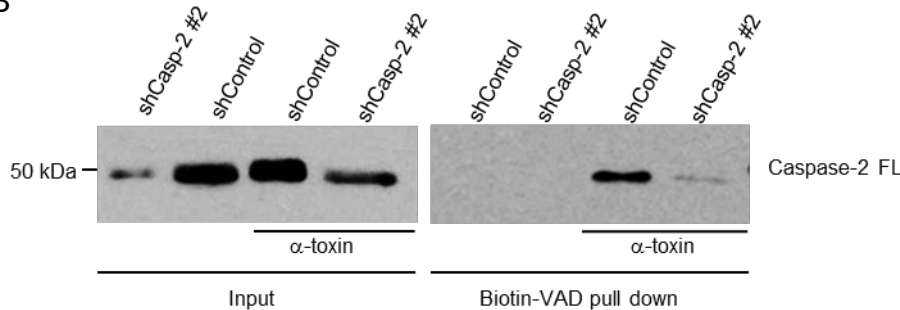


**Figure S4.** (A) Microscopy analysis (left panel: phase contrast, right panel: PI staining) of HTEpC cells treated with  $\alpha$ -toxin either alone or in combination with zVAD-fmk (50  $\mu$ M). Images were taken at 24 hours post treatment. (B) Caspase-2 cleavage in response to  $\alpha$ -toxin was monitored in various cell types. HeLa, HUH-7 and primary tracheal epithelial cells were treated with  $\alpha$ -toxin (300 ng/ml, 24h). (FL- full length, \*-processed from). (C) Cleavage of caspase-2 and PARP was monitored by immunoblots in human primary keratinocytes 24 hours after aerolysin treatment (5 ng/ml).

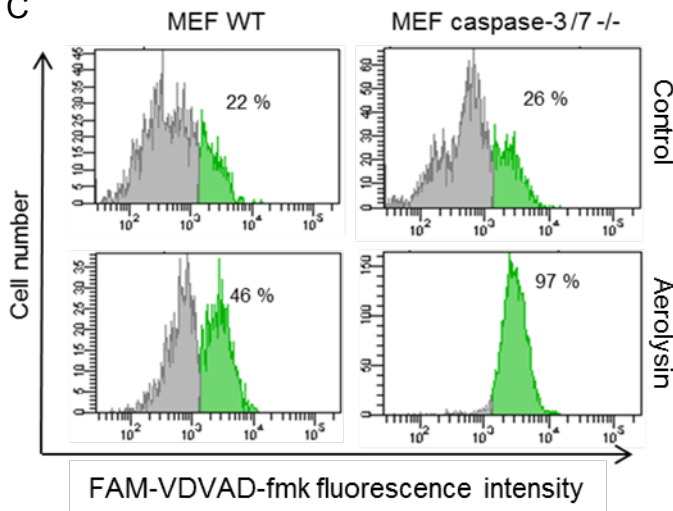
S5 A



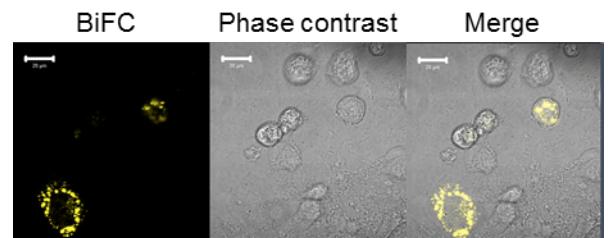
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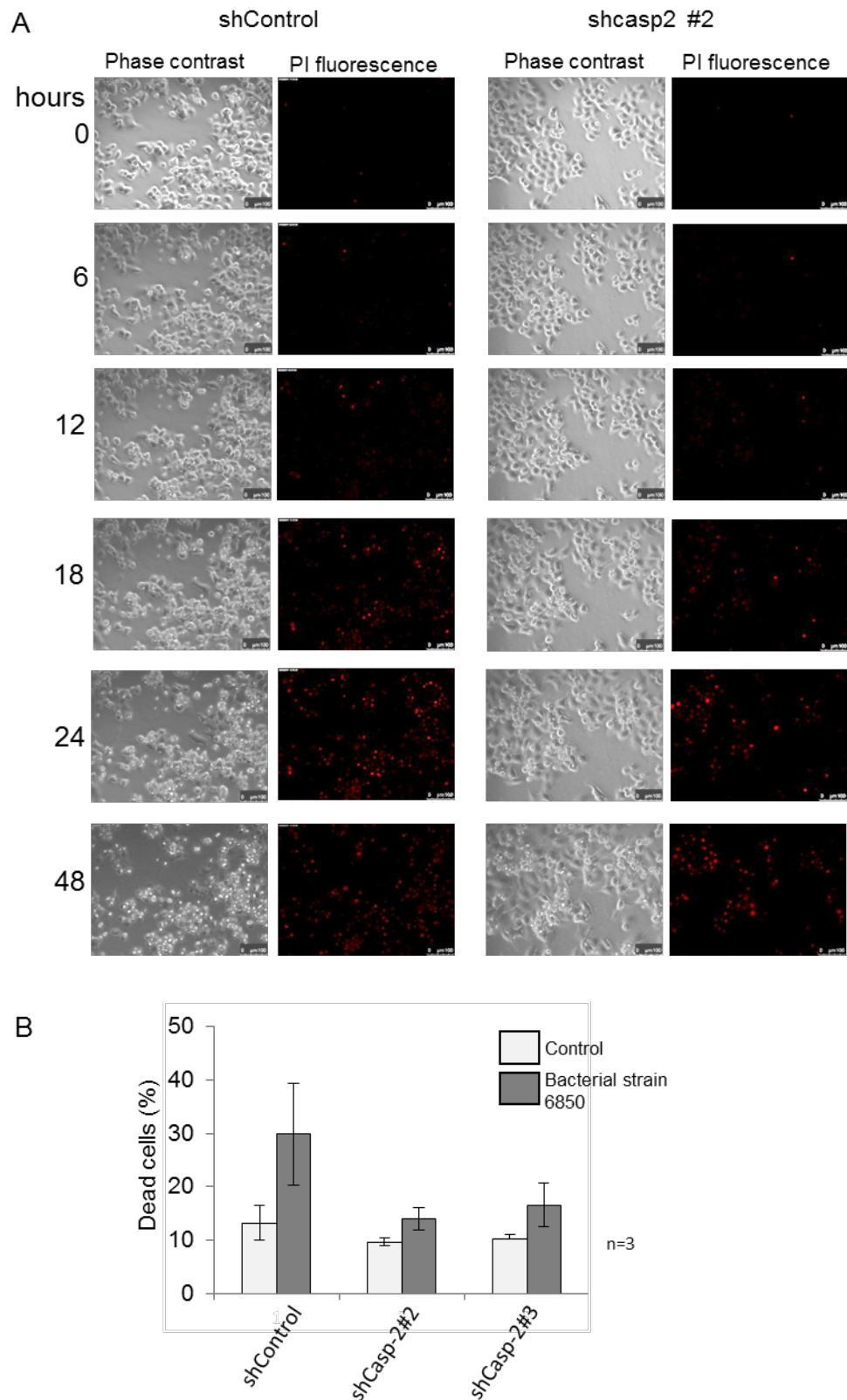
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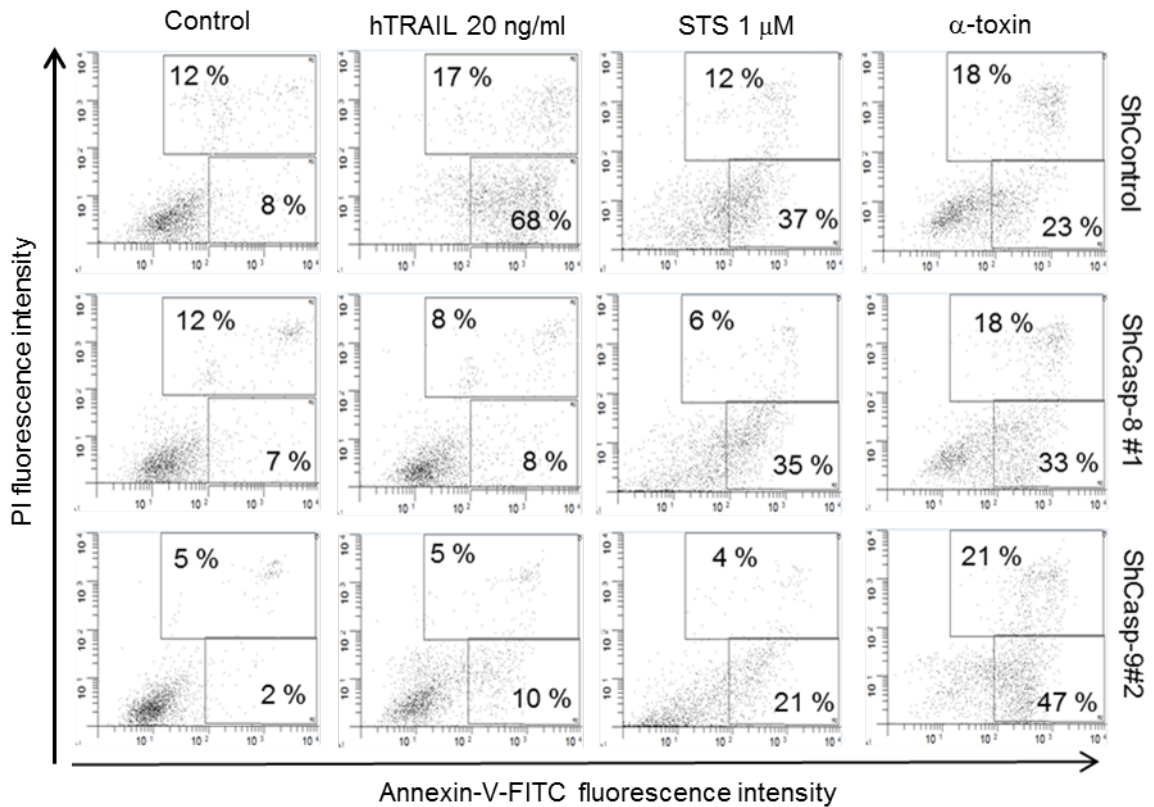


**Figure S5** (A) HeLa cells pretreated with biotin-VAD and treated with  $\alpha$ -toxin. The activated caspases are precipitated as mentioned in the methods section. The proteins were separated by SDS-PAGE and subjected to mass spectrometric analysis. Slice no.6 (denoted) encompasses peptides representing caspase-2. The MS/MS spectrum of the caspase-2 peptide obtained is presented. (B) HeLa cells stably expressing control and Caspase-2 shRNAs were loaded with biotin-VAD and treated with  $\alpha$ -toxin (300 ng/ml for 6h) as mentioned in methods section. The presence of caspase-2 was monitored by immunoblot analysis (FL-full length). (C) Representative experiment of flow cytometry analysis of caspase-2 activity upon aerolysin (5ng/ml, 24 h) in Caspase 3 and 7 deficient MEF cells. Numbers indicate the percentage of cells with increased caspase-2 substrate (FAM-VDVAD-fmk) fluorescence (green). (D) Representative images of CARD-BiFC in HeLa cells treated with  $\alpha$ -toxin (300 ng/ml, 6,5 h).

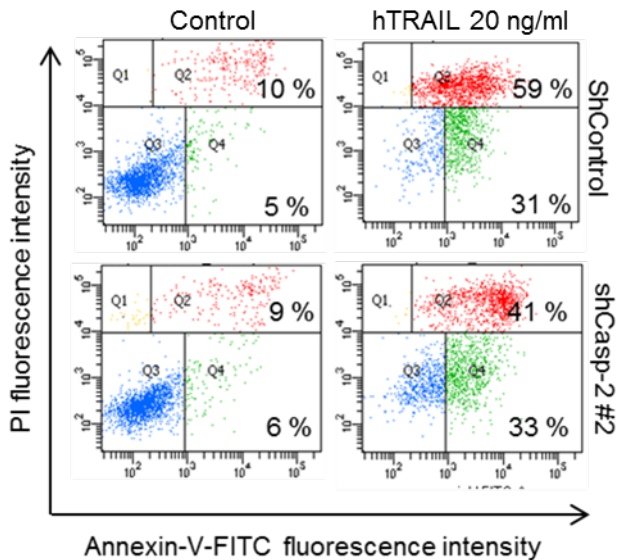


**Figure S6. (A)** Timelapse microscopy analysis (left panel: phase contrast, right panel: PI staining) of control and caspase-2 knock down HeLa cells upon  $\alpha$ -toxin treatment. shControl and shCaspase-2 #2 cells were incubated with alpha toxin (300 ng/ml). Shown are representative images from Supplementary movies ( movie 1,2). **(B)** shControl and shCaspase-2 #2 and #3 cells were incubated with or without bacterial culture supernatants from strain 6850 for 24 hours and subjected to Annexin-V, PI staining and flow cytometry analysis. Percentage of dead cells represents the percentage of cells exhibiting both annexin-V single positivity and annexin-V, PI double positivity compared to the total number of cells.

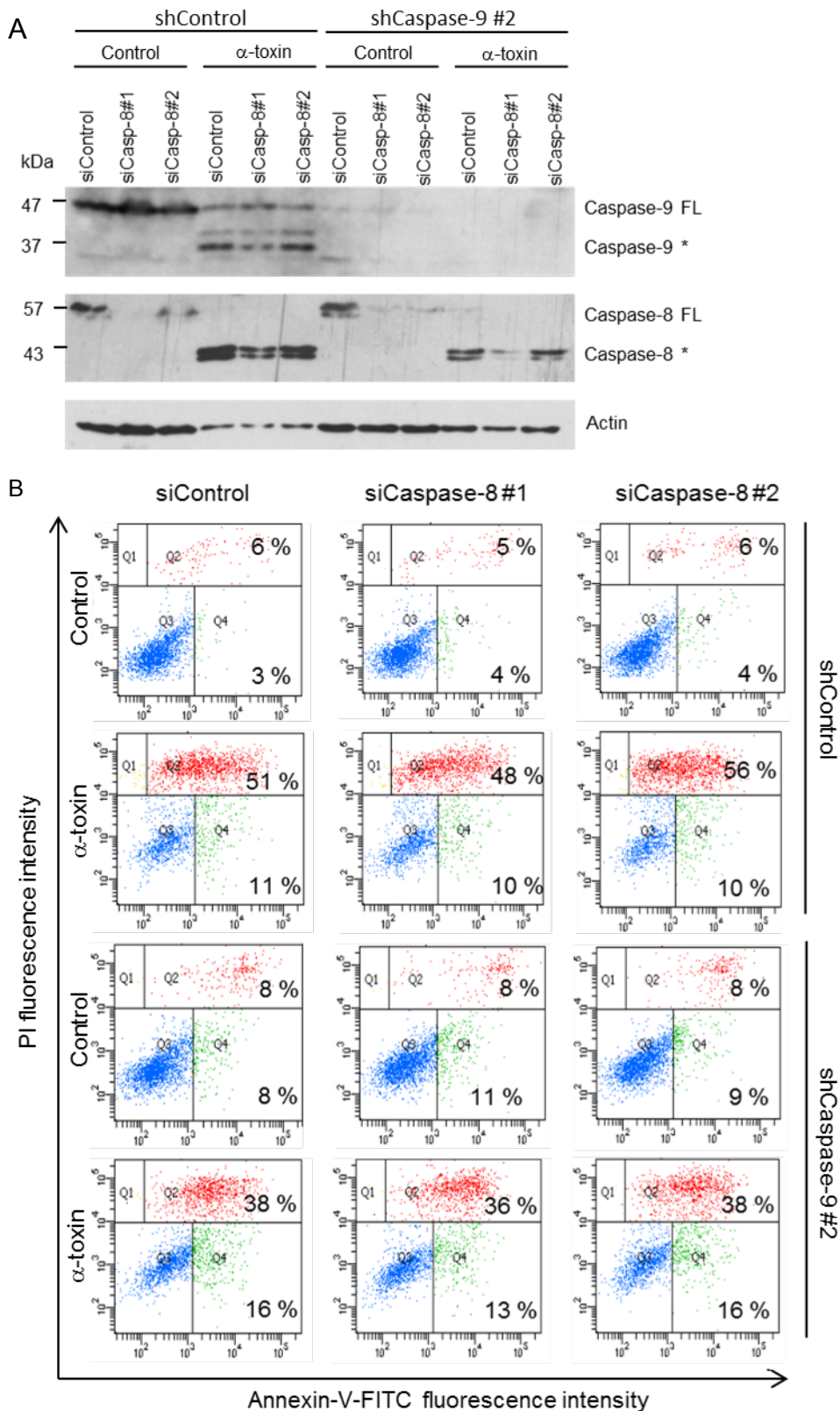
A



B



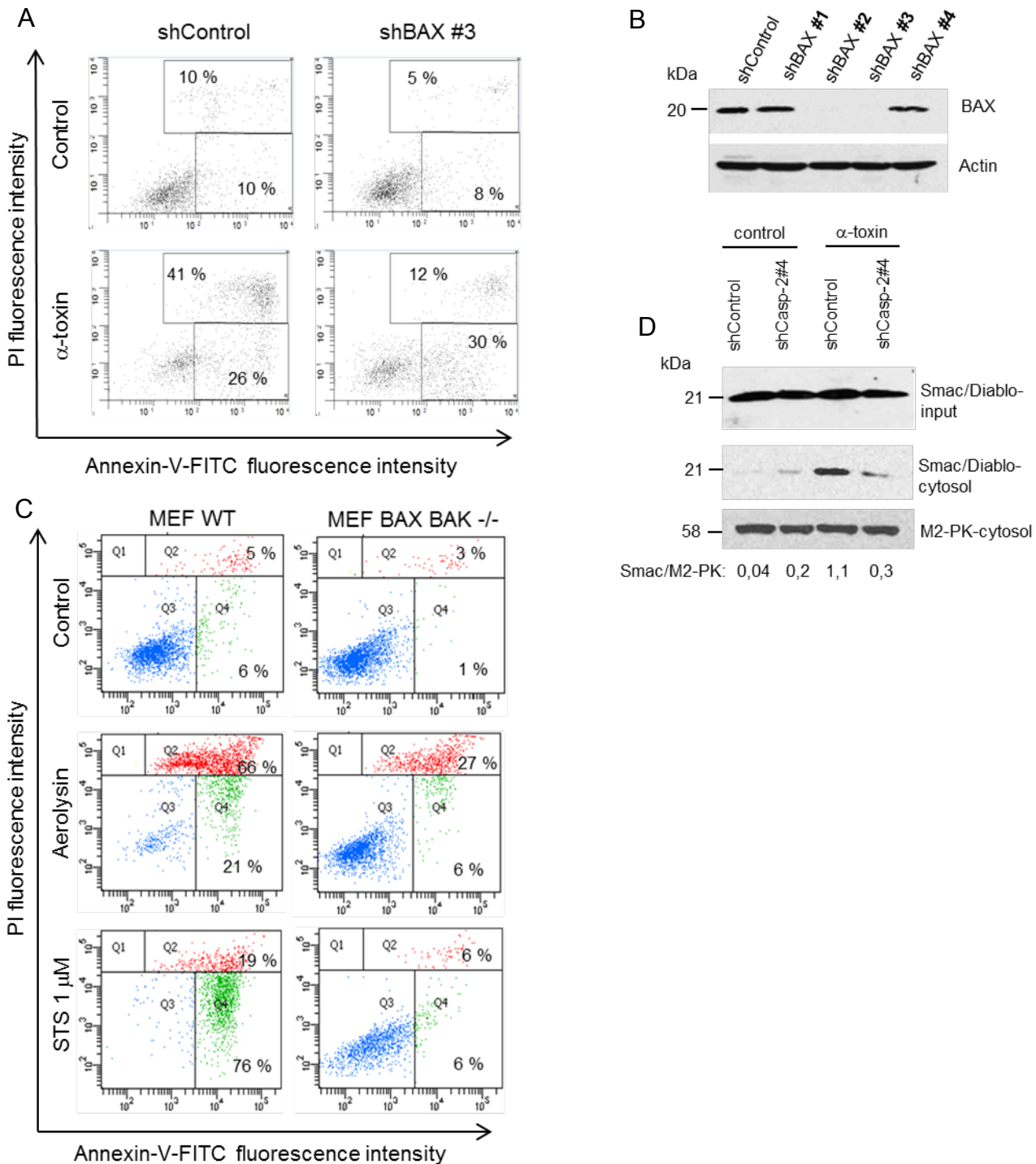
**Figure S7 (A, B)** Validation of Caspase-2, -8 and -9 knock down efficiency. HeLa cells were treated with Staurosporine (1  $\mu$ M) (STS), hTRAIL (20 ng/ml) and  $\alpha$ -toxin (300 ng/ml) and 24 hours later were subjected to Annexin-V, PI staining and subsequent flow cytometry measurement. Numbers indicate the percentage of the gated population.



**Figure S8 (A)** Western blot and **(B)** Flow cytometry detection of Caspase-8/9 double knock down cells upon  $\alpha$ -toxin (300 ng/ml) treatment. HeLa shCaspase-9 knock down cells were transfected with two sets of siRNA against Caspase-8. After 24 hours incubation the cells were treated and 24 hours later were subjected to western blot analysis (A), Annexin-V, PI staining and flow cytometry measurement (B). Numbers indicate the percentage of the gated population. (A) FL- full length, \* -processed form.

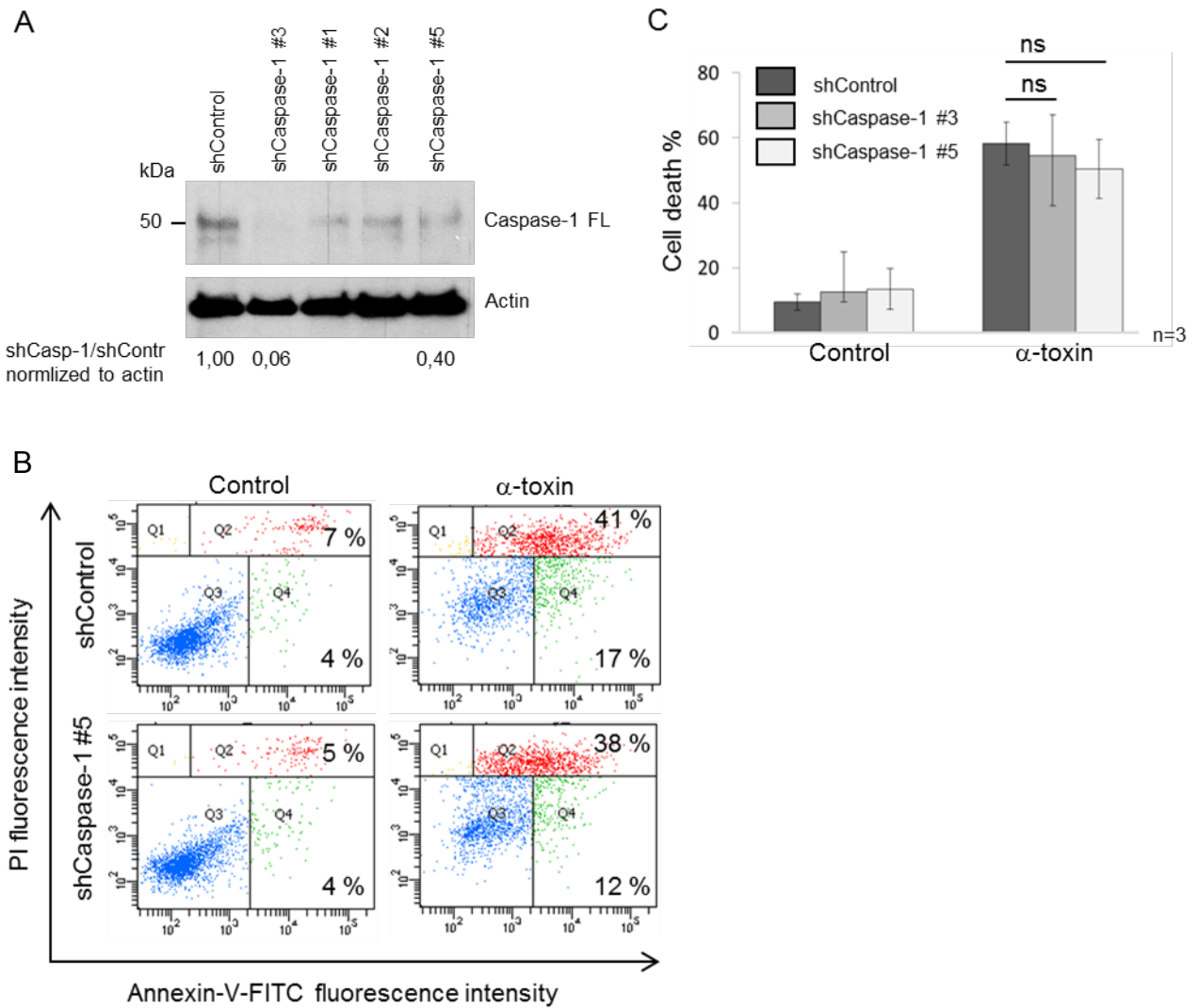


S9

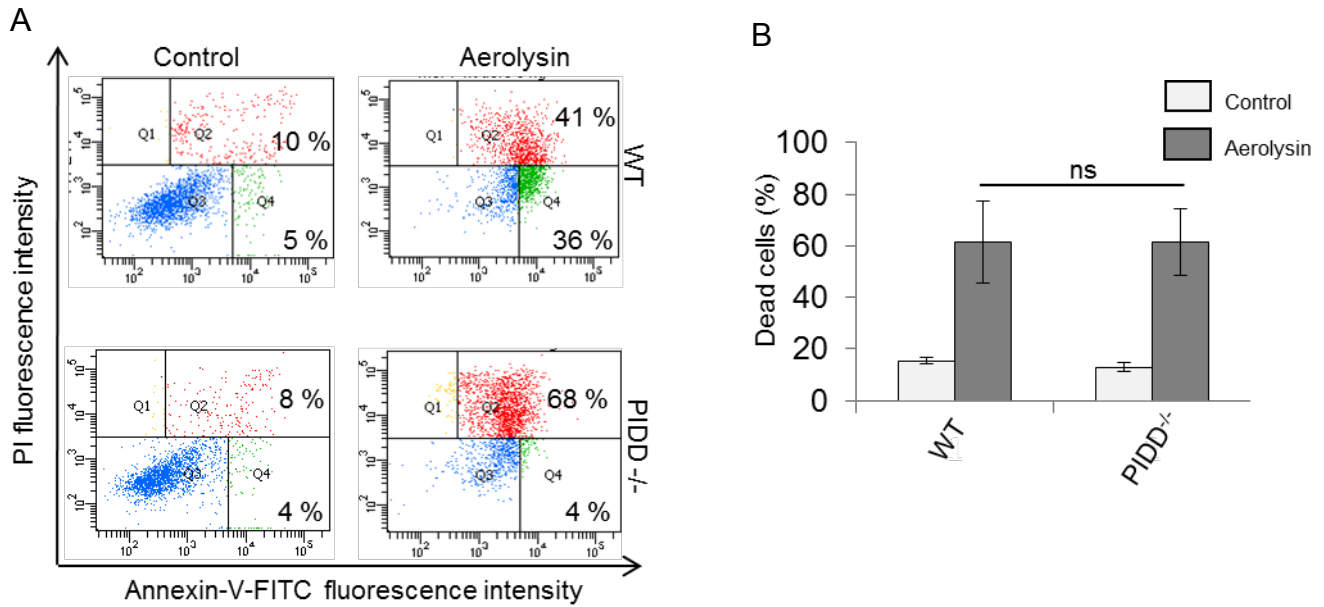


**Figure S9 (A)** Annexin-V, PI flow cytometry analysis of BAX knock down HeLa cells upon  $\alpha$ -toxin (300 ng/ml, 24 h). Numbers are the percentage of gated populations. **(B)** Western blot validation of BAX knock down efficiency in HeLa cells. **(C)** Representative plots of Flow cytometry detection of BAX/BAK double knock out MEF cells after aerolysin (5 ng/ml, 24 hours) and staurosporine treatment. Numbers show the percentage of the gated populations. **(D)** Western blot of subcellular fractionation. ShControl and shCaspase-2 knock down HeLa cells were treated with  $\alpha$ -toxin and harvested for subcellular fractionation and western blot analyses. The membranes were immunoblotted for Smac/Diablo and M2PK antibodies. Numbers below show quantification of Smac bands normalized to M2PK levels.

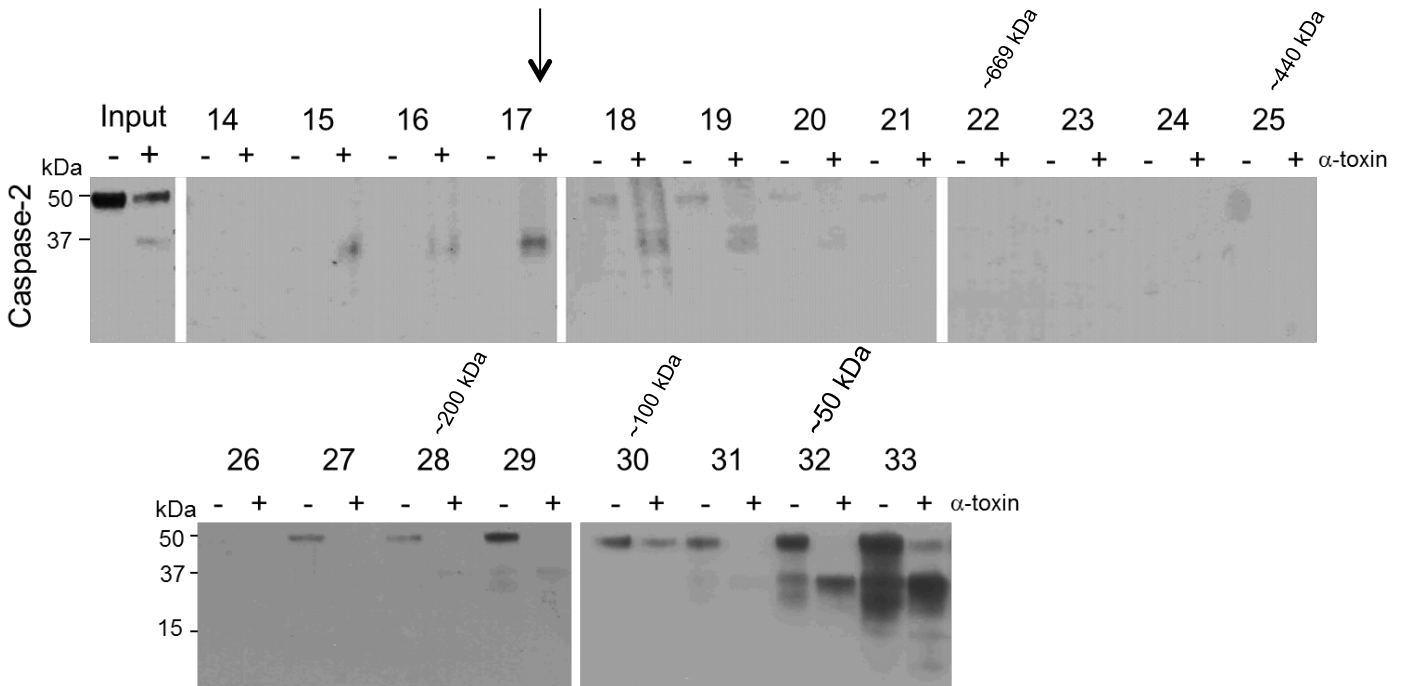
S10



**Figure S10** (A) Western blot of shCaspase-1 knock down HeLa cells. FL= full length. The numbers below show the quantification of Caspase-1 bands compared to the shControl cells. All values were normalized to actin level. (B) Representative histograms and (C) column diagrams of flow cytometry detection of Caspase-1 knock down cells after  $\alpha$ -toxin (300 ng/ml, 24 hours) treatment. Numbers show the percentage of the gated populations. Cell death % = the sum of the Annexin-V single and annexin-V, PI double positive cells. ns= not significant.



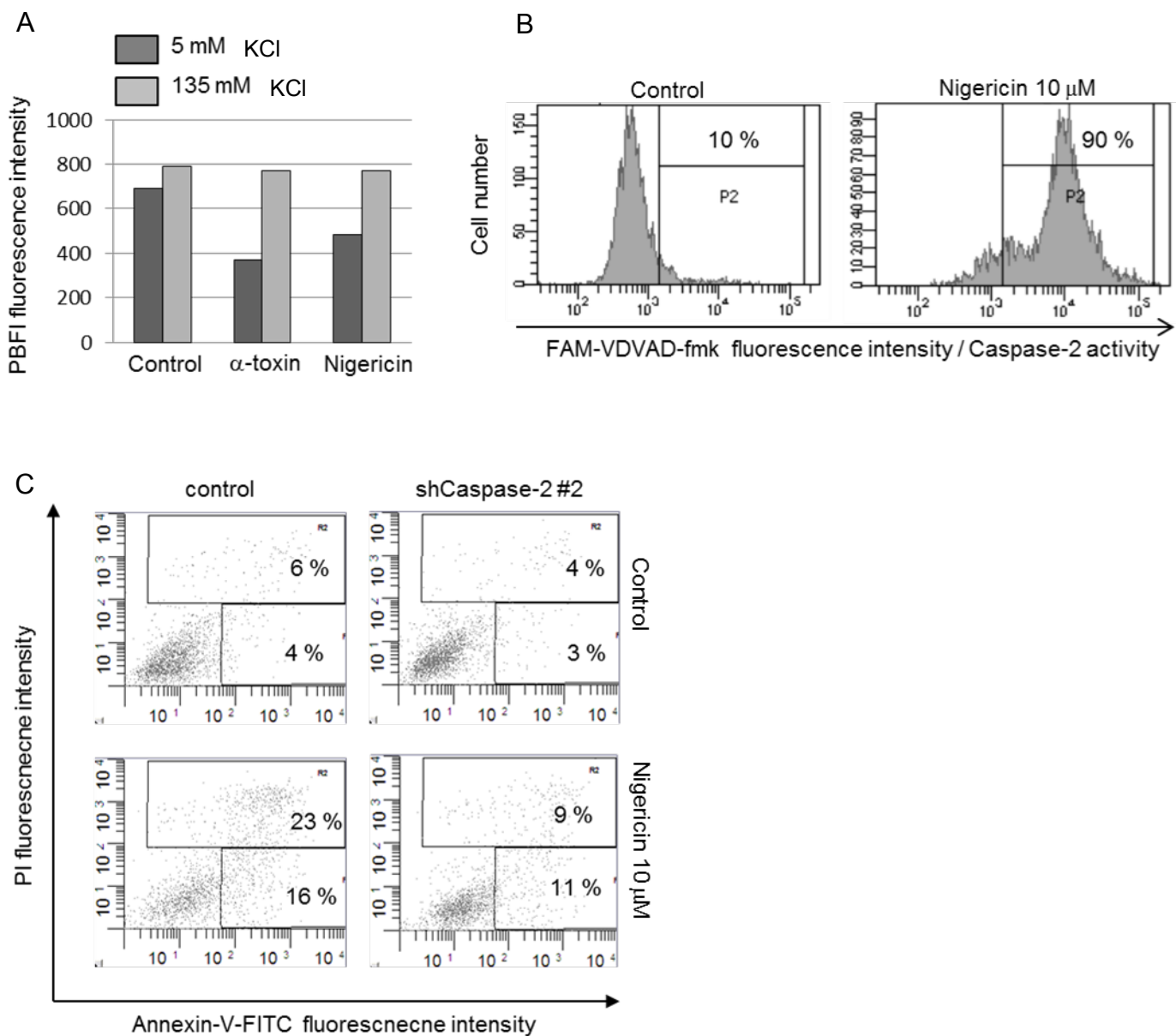
**Figure S11. (A)** Representative scatter plots from the flow cytometry analysis of PIDD<sup>-/-</sup> MEFs. Wild type and PIDD<sup>-/-</sup> MEFs cells were incubated with or without aerolysin (5 ng/ml) for 24 hours and harvested for analysis. The appearance of both annexin-V, PI double positive population (loss of membrane integrity) and annexin-V single positive population was monitored. The values in the scatter plots indicate the percentage of the gated populations. **(B)** Shown here are data from three independent experiments. Dead cells refer to both Annexin-V single positive and Annexin-V, PI double positive population.



**Figure S12.** Caspase-2 is recruited to high molecular weight complexes in  $\alpha$ -toxin treated HeLa cells. Control and  $\alpha$ -toxin treated cells (5 hours) were harvested for gel filtration analysis as mentioned in the methods section (gel filtration column: superose 6- 10/30 GL). The individual fractions (fraction numbers are indicated above) were analysed for the presence of caspase-2 by immuno blot analyses after precipitating all the proteins in the fractions. Since the peak maximum of the HMW complex overlaps with the void volume of the column only a lower limit of its molecular weight of 1 MDa can be estimated. The void volume could also contain aggregated protein. To distinguish a real HMW complex from aggregated proteins we performed gel filtration experiments with a Sephacryl S-500 HR column which confirmed the existence of the activated caspase-2 form within a HMW complex. The small bands of full length protease in fractions 18-21 of the non-treated cells probably are caused by aggregation. These bands are no longer visible in experiments carried out with the Sephacryl S-500 HR column.



S14



**Figure S14** (A) PBF1 fluorescence was measured with nigericin and  $\alpha$ -toxin treated HeLa cells. Normal cell culture media was replaced with low (5 mM) and high potassium (135 mM) containing media and 1 hour later cells were treated accordingly. The measurements were done after two hours. (B) Representative experiment of Flow cytometry analysis of caspase-2 activity upon Nigericin (10  $\mu$ M, 24 h) in HeLa cells. Numbers indicate the percentage of cells with increased caspase-2 substrate (FAM-VDVAD-fmk) fluorescence. (C) Flow cytometric analysis of phosphatidylserine translocation (Annexin-V single positive cells) and membrane damage (PI and Annexin-V double positive cells). HeLa cells were treated with nigericin for 48 hours. Values indicate the percentage of the gated population.

## Supplementary Information

### Methods

#### Cell culture and apoptosis induction

HeLa cells were cultured in RPMI-1640 medium and HUH-7 cell lines were cultured in DMEM (both Gibco BRL), both supplemented with 10 % FCS (Gibco BRL), and 0.2 % penicillin (100 U/ml) / streptomycin (100 µg/ml) (Gibco BRL) and 2 mM L-glutamine at 37°C in 5 % CO<sub>2</sub>. HTEpC cells were cultured in Airway Epithelial Growth Medium according to supplier's recommendations (Promocell). Human primary foreskin keratinocytes were kindly provided by Prof. H. Muehl (University of Frankfurt). WT, caspase-2- and PIDD- deficient MEFs (kindly provided by Prof. Andreas Villunger, Innsbruck, Austria), BAX/BAK DKO and Caspase-3/7 DKO MEFs were cultured in supplemented DMEM medium (as above). Most of the apoptosis induction experiments were performed with purified  $\alpha$ -toxin (alpha-haemolysin from *Staphylococcus aureus*) (Sigma) for various time points. A second batch of  $\alpha$ -toxin (indicated with (2) in the figures) was produced by the Husmann lab. Wild type and pore-dead single amino acid exchange mutant toxin D152C (Walker and Bayley, 1995) were also employed. HeLa cells were also induced to apoptosis with various dilutions of bacterial culture supernatants. *Staphylococcus aureus* strain 6850, a virulent clinical isolate producing  $\alpha$ -toxin (Balwit et al., 1994) expressed and strain Wood 46 (National Collection of Type Cultures 10344) a strong  $\alpha$ -toxin producer, used for  $\alpha$ -toxin purification were used for preparation of bacterial supernatants, purified as well as the food grade organism *Staphylococcus carnosus* strain TM300 as a negative control, as described (Bantel et al., 2001; Haslinger et al., 2003). Briefly five ml BHI (brain heart infusion, Merck) was inoculated with 2-3 colonies of staphylococci grown on blood agar plates (BD) in inclined loosely capped 25 ml glass tubes and

incubated at 37 °C on a rotatory shaker (200 rpm). After 14 h, bacteria were pelleted by centrifugation at 4000 rpm and the cleared supernatants were sterile-filtered (0.22 µm), aliquoted and stored at -20 °C until used. Sterility was checked by plating 200 µl onto blood agar plates (48 hrs at 37 °C).

Recombinant ProAerolysin was produced in *Aeromonas salmonicida* as previously described (Buckley, 1990). Nigericin (Sigma), a potassium ionophore was added to the cells at 10 µM concentration. For inhibition of caspase activity, zVAD(OMe)-fmk (zVAD-fmk) and q-VD-oph (BACHEM) pancaspase inhibitors were used (50 µM). Control cells were treated with DMSO alone. Polyclonal, rabbit anti- $\alpha$ -toxin antibody (1:100, Sigma) was used to specifically block the effect of  $\alpha$ -toxin. Rabbit IgG was used as control (1:100, Santa Cruz). Staurosporine (1 µM) (Sigma) and human recombinant TRAIL (20 ng/ml) (Natutec) were availed as reference controls of intrinsic and extrinsic apoptosis pathways.

For disrupting potassium efflux, cell culture media was replaced to either high potassium concentration-containing media (500 µM CaCl<sub>2</sub> / 500 µM MgSO<sub>4</sub> / 10 mM glucose / 10 mM Hepes, pH 7.4 and 135 mM KCl / 5 mM NaCl) or normal potassium concentration containing media (500 µM CaCl<sub>2</sub> / 500 µM MgSO<sub>4</sub> / 10 mM glucose / 10 mM Hepes, pH 7.4 and 5 mM KCl / 135 mM NaCl) one hour prior to  $\alpha$ -toxin treatment.

### **TCA precipitation of proteins**

500 µl of TCA (final concentration : 4%) was added and incubated with the fractions for 15 minutes (-20 °C). Samples were centrifuged (10.000g, 4 °C, 20 minutes). The precipitate was dissolved in 1 ml ice cold 80% acetone and centrifuged again. The supernatant was discarded and the precipitate was resuspended with 5x Laemmli buffer (with 5%  $\beta$ -mercapto-ethanol).



## **Time lapse imaging analysis**

Cells seeded on 6-well plates were treated with toxins in the presence of PI (5 µg/ml). The plates were placed into the Leica fluorescent imaging system (LEICA DMI 6000) fitted with an incubator (PECON) (37°C, 5 % CO<sub>2</sub>). Phase contrast and PI fluorescent images were taken with a digital camera connected with the microscope.

## **Protein identification by mass spectrometry**

The Coomassie Brilliant Blue G-250 stained gel lanes were excised into 8 slices for in-gel digestion with 0.1 µg trypsin (Promega, Madison, WI, USA) in 20 µl 25 mM ammonium bicarbonate, pH 7.8 at 37°C for 16 h. The tryptic peptides of each gel slice were purified with µ-C18 ZipTips (Millipore, Billerica, MA, USA), and dried using a Speed Vac concentrator (Savant, Holbrook, NY, USA). The dried peptides were dissolved in 10 µl 1% formic acid in water/5% acetonitrile and 5 µl were injected into an Ultimate 3000 nanoLC system (Dionex, Sunnyvale CA, USA) connected to a linear quadrupole ion trap-orbitrap (LTQ-Orbitrap XL) mass spectrometer (ThermoScientific, Bremen, Germany) equipped with a nano-electrospray ion source. An Acclaim PepMap 100 column (C18, 3 µm, 100 Å) (Dionex) with a capillary of 12 cm bed length was used for separation by liquid chromatography. A flow rate of 300 nl/min was employed with a solvent gradient of 7% B to 50% B in 40 minutes. Solvent A was 0.1% formic acid, whereas aqueous 90% acetonitrile in 0.1% formic acid was used as solvent B. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 2,000) were acquired in the Orbitrap with resolution  $R = 60,000$  at m/z 400 (after accumulation to a target of 1,000,000

charges in the LTQ). The method used allowed sequential isolation of the most intense ions, up to six, depending on signal intensity, for fragmentation on the linear ion trap using collisional induced dissociation (CID) at a target value of 100,000 charges. For accurate mass measurements the lock mass option was enabled in MS mode and the polydimethylcyclsiloxane (PCM) ions generated in the electrospray process from ambient air were used for internal recalibration during the analysis. Other instrument parameters were set as previously described (Koehler et al., 2009). Raw LTQ Orbitrap XL data were processed using DTA supercharge software to generate mgf files. Then, a database search was performed by tandem mass spectrometry ion search algorithms from the Mascot in-house version 2.2.1 by database comparisons with human entries (20,349 sequences) from Swiss-Prot (2010 v57.13). Trypsin was selected as enzyme with one missed cleavage site and tolerance of 10 ppm for the precursor ion and 0.6 Da for the MS/MS fragments was applied. In addition, methionine oxidation, acetylation at protein N-terminus, pyroglutamine formation of N-terminal glutamines and propionamide formation of cysteines were allowed as variable modifications. Proteins were considered to be identified by Mascot if a probability < 0.05 was achieved with a minimal peptide ion score cut-off of 20. The false discovery rate was < 1.5%.

### **Movie Legends:**

**Movie S1:** HeLa cells stably expressing control shRNAs were treated with  $\alpha$ -toxin (300 ng/ml) for 48h. Propidium iodide (5  $\mu$ g/ml) was added to the cells to check for loss of membrane integrity. The cells were observed under a Leica

time lapse imaging system and the images were processed using Leica software tools.

**Movie S2:** HeLa cells stably expressing Caspase-2 shRNA #2 were treated with  $\alpha$ -toxin (300 ng/ml) for 48h. Propidium iodide (5  $\mu$ g/ml) was added to the cells to check for loss of membrane integrity. The cells were observed under a Leica time lapse imaging system and the images were processed using Leica software tools.

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