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Title	Protease activity of procaspase-8 is essential for cell survival by inhibiting both apoptotic and nonapoptotic cell death dependent on receptor-interacting protein kinase 1 (RIP1) and RIP3.
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Protease activity of procaspase-8 is essential for cell survival by inhibiting both apoptotic and nonapoptotic cell death dependent on receptor interacting protein kinase 1 (RIP1) and RIP3*

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Running title: Caspase-8 inhibits multiple types of cell death including apoptosis

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Keywords: apoptosis, autophagy, caspase-8, necroptosis, ROS

Background: Caspase-8 inhibits necrosis by regulating RIP1 and RIP3.
Results: Caspase-8 knockdown T-cells simultaneously underwent apoptosis and nonapoptotic cell death.
Conclusion: Procaspase-8 prevents T-cells from multiple types of RIP1/3-dependent cell death.
Significance: RIP1/3 can regulate caspase-3-dependent apoptosis as well as non-apoptotic cell death.

SUMMARY

Caspase-8 has an important role as an initiator caspase during death receptor-mediated apoptosis. Moreover, it has been reported to contribute to the regulation of cell fate in various types of cells including T-cells. In this paper, we show that caspase-8 has an essential role in cell survival in mouse T-lymphoma-derived L5178Y cells. The knockdown of caspase-8 expression decreased the growth rate and increased cell death, both of which were induced by the absence of protease activity of procaspase-8. The cell death was associated with reactive oxygen species (ROS) accumulation, caspase activation and autophagosome formation. The cell death was inhibited completely by treatment with ROS scavengers, but only partly by treatment with caspase inhibitors, expression of Bcl-xL, and knockdown of caspase-3 or Atg-7 which completely inhibits apoptosis or autophagosome formation, respectively, indicating that apoptosis and autophagy-associated cell death are induced simultaneously by the knockdown of caspase-8 expression. Further analysis indicated that RIP1 and RIP3 regulate this multiple cell death, because the cell death as well as ROS production was completely inhibited by not only treatment with the RIP1 inhibitor necrostatin-1, but also by knockdown of RIP3. Thus, in the absence of protease activity of procaspase-8, RIP1 and RIP3 simultaneously induce not only nonapoptotic cell death conceivably including autophagic cell death and necroptosis but also apoptosis through ROS production in mouse T-lymphoma cells.

Apoptosis has important physiological roles in embryonic development, immune regulation and the maintenance of homeostasis in adult tissues. Caspases, members of the cysteine protease family, are essential to the induction of apoptosis (1,2). Two major pathways of apoptosis are known; a mitochondrial pathway (intrinsic pathway) and a death receptor pathway (extrinsic pathway) (3). Both require the activation of a variety of caspases. Moreover, caspases are known to be pro-enzymes which become activated by cleavage at aspartate residues upon death signaling (4).

Caspase-8 was originally identified as an initiator caspase and mainly functions in the death receptor pathway of apoptosis. Upon ligation of a death receptor such as Fas, caspase-8 is recruited to a complex known as a death-inducing signaling complex (DISC) together with other factors including the Fas-associated death domain (FADD) (5,6). Within the complex, caspase-8 becomes catalytically activated by undergoing proximity-induced auto-cleavage through homo-oligomerization. The activated caspase-8 conveys the death signal to downstream factors mainly to executor caspases including caspase-3 and 7, which then cleave various cellular proteins to complete the apoptosis-inducing process (7,8).

In addition to the main function of caspase-8

in apoptosis, some findings imply a non-apoptotic function. In mice, the knockout of caspase-8 caused embryonic death at 10.5 to 12 days with an associated deficiency in heart development, neural tube formation and angiogenesis (9,10). This has been also observed in knockout of FADD, a death-domain containing adaptor protein, and of cellular-FLICE-like inhibitory protein (c-FLIP), a catalytically inactive caspase-8 homolog (11,12). Therefore, the components of DISC are essential to embryonic development.

In the immune system, apoptosis is important for lymphocyte development and homeostasis such as the elimination of auto-reactive T- and B-cells and activated T-cells after their initial expansion (13-15). In mice, however, conditional caspase-8 knockout in T-cells caused decrease in numbers of mature T-cells in pancreas and lymph gland, and the caspase-8-deficient T-cells were highly defective in clonal expansion upon mitogenic stimulation (11,16-19). Furthermore, in T-cell-derived cell lines, a decrease and an increase in the number of proliferating and dying cells, respectively, were reported to be induced by treatment with z-VAD-fmk, a pan-caspase inhibitor (20-22). Since z-VAD-fmk inhibits the protease activity of pan-caspases, the protease activity of caspases may have another essential function in cell survival. Despite many reports indicating a crucial non-apoptotic function of caspase-8 in T-cell development, the precise mechanism by which caspase-8 regulates the clonal expansion or survival of T-cells is unclear.

A loss of caspase-8 was reported to result in the accumulation of reactive oxygen species (ROS), which induces non-apoptotic cell death or caspase-independent cell death such as autophagic cell death or necrotic cell death also known as necroptosis (23,24). Necroptosis is initiated by receptor-interacting serine/threonine-protein kinase (RIP) 1 and RIP3 in response to the activation of death receptors, and can be blocked by a RIP1 inhibitor, necrostatin-1 (Nec-1) (25). The most recent works showed that caspase-8 constantly inhibits necroptosis, an alternative programmed cell death pathway, by regulating RIP1 and RIP3 during embryonic development and T-cell expansion (26,27). Also, RIP1 deficiency was found to restore the viability of FADD-null T-cells (28). It is highly likely that caspase-8 and FADD regulate cell fate in embryogenesis by regulating RIP1- and RIP3-dependent necroptosis.

In this study, the significance of the non-apoptotic function of caspase-8 and its mechanism were explored. We confirmed that cell death was induced in a mouse lymphoma-derived T-cell line, L5178Y, by the knockdown of caspase-8 expression. The caspase-8 knockdown cells showed apoptosis and non-apoptotic cell death including autophagic cell death, both of which were simultaneously induced by the accumulation of ROS. Moreover, the cell death was completely reversed by treatment with Nec-1 or by knockdown of RIP3 expression. It is suggested that protease activity of procaspase-8 in T-lymphoma cells inhibits RIP1 and/or RIP3, which triggers cells multiple types of cell death including apoptosis, autophagic cell death, and necroptosis.

EXPERIMENTAL PROCEDURES

Cell lines – The mouse leukemic lymphoblast cell line L5178Y was maintained in a suspension culture in RPMI 1640 medium (Nacalai Tesque Inc.) supplemented with 5% fetal bovine serum (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Nacalai Tesque Inc.), and 50 μM β-mercaptoethanol (Sigma), at 37˚C in 5% CO_2. Human embryonic kidney-derived 293T cells, and Balb/C wild type and Caspase-$8^{-/-}$ mouse embryonic fibroblasts obtained in our laboratory were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc.) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque Inc.).

Plasmids - The expression vector for wild-type caspase-8 was sub-cloned from the vector as described before (29). A protease inactive form of caspase-8 (CS mutant) (See Fig. 2A) was constructed with the primer, 5'-ATTCAGGCTAGCCAAGGAAGT-3' (for C362S). A mutant without 7 putative cleavage sites (DE mutant) (See Fig. 2A) was constructed with 5'-GAGGAGATGAGCCT CAAAAT-3' (for D201E), 5'-CTGTGTGAGTCGCCAAGAGAACAAGA GAGTGAGTCA-3' (for D212E and D218E), 5'-GGAGT GCCTGAGGAGGCAGG-3' (for D373E), 5'-AAGTGGAGTCATCATCTCAC-3' (for D387E), and 5'-GAGGAGGCAGAGTTTCTGCT-3' (for D397E and D400E). Mouse caspase-8 with silent mutations in the target nucleotides of shRNA for caspase-8 (WT*) was constructed with the primer, 5'-CGGGAAGAAATGGTGAGGGAG-3' (encoding R83-E89), and the CS and DE mutants were constructed as substituents of WT* mouse caspase-8 with a flag-tag. Human Bcl-xL cDNA was amplified by RT-PCR from cDNA of Jurkat cells. All constructs generated by PCR were verified by DNA sequencing. For expression of LC3, the original plasmid, pEGFP-C1-LC3, was kindly provided by T. Yoshimori (Osaka University, Japan). EGFP-conjugated LC3 cDNA was sub-cloned and inserted into a lentiviral expression vector, pCSII-EF-EGFP-LC3-IRES- puro.

Lentiviral expression vectors - Lentiviral vectors, provided by H. Miyoshi (RIKEN, Japan), were prepared as described earlier (30). For the expression of the WT*, CS mutant, and DE mutant of caspase-8, cDNA fragments were inserted into both CSII-PGK-MCS-IRES-EGFP and CSII-PGK-3xFLAG-MCS-IRES-puro. For Bcl-xL expression, cDNA was inserted into both CSII-PGK-3xFLAG-MCS-IRES-puro and CSII-EF-MCS-IRES-puro.

shRNA expression system - To achieve the specific knockdown of caspase-8, the tetracycline-inducible short hairpin RNA (shRNA) expression system (Tet-On shRNA system) with lentivirus-based vectors was utilized as described previously (31). shRNA expression was induced by the treatment of cells with 10 ng/ml doxycyclin (Dox) (Nacalai Tesque Inc.). The oligonucleotides of shRNA for mouse caspase-8 (shCasp-8) were generated from nucleotides 248 to 266 of caspase-8 cDNA (GeneBank no. NM_009812.2). The oligonucleotides of shRNA for silencing *lacZ* (32), and for silencing ATG7 (23) were previously described. For the specific knockdown of RIP3, shRNA for mouse RIP3 was generated from nucleotides 413 to 431 of RIP3 cDNA (GeneBank no NM_019955.2). shRNA for mouse caspase-3 was generated from nucleotide 762 to 780 of caspase-3 cDNA (GeneBank no NM_009810).

Detection of cell death – The percentage of cells undergoing cell death was quantified by trypan blue exclusion method using a hemocytometer after the addition of an equal amount of 0.4% trypan blue solution in PBS to the suspended culture. Cell death was also quantified by lactate dehydrogenase (LDH) release method; the integrity of the cell membrane was measured by quantifying the amount of LDH released from dead cells using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) as described (31). Results obtained by trypan blue exclusion method were essentially same as those by LDL release method. The DNA ladder assay was performed as described (33). To quantify apoptotic cells, cells were stained with propidium iodide (PI) and percentage of sub-diploid (sub-G1) cells was measured by flow cytometric analysis as previously described (30). Apoptotic cells were also examined by analyzing nuclear morphology of cells stained with DAPI as described previously (31).

Reagents –Inhibitors used in this study were z-VAD-fmk (PEPTIDE INSTITUTE, INC.), z-IETD-fmk (R&D Systems), z-DEVD-fmk (R&D Systems), z-LEHD-fmk (R&D Systems), wortmanin (Santa Cruz), and Necrostatin-1 (Nec-1) (Santa Cruz). Butylated hydroxyanisole (BHA) (Sigma) was used as a ROS scavenger. *tert*-butylhydro-peroxide (Sigma) was used as a ROS inducer.

Western blot analysis and antibodies - Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH7.4 with 10% glycerol, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, and 1 mM EDTA) containing a protease inhibitor cocktail (Roche Molecular Biochemicals). Cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then analyzed by Western blotting as described previously (34). The antibodies used for detection were anti-caspase-8 (1:1000; 1G12; Alexis Biochemicals), anti-caspase-3 (1:1000; Cell Signaling), anti-caspase-9 (1:500; Cell Signaling), anti-PARP (1:1000; Cell Signaling), anti-Bcl-xL (1:1000; Cell Signaling), anti-RIP3 (1:1000; Enzo Life Sciences), anti-Flag (1:2000; M2; Sigma), anti-GFP (1:1000; 1E4; MBL), and anti-actin (1:5000; Chemicon).

qRT-PCR - Total RNA was isolated with a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The reverse transcription (RT) reaction was carried out with 8-20 ng of total RNA using a SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. RT products were analyzed with an ABI PRISM 7500 (Applied Biosystems). The level of each mRNA was normalized to that of GAPDH, and expressed as a ratio to the level in control shRNA (shRNA for LacZ: shLacZ)-expressing cells.

Detection of Reactive Oxygen Species (ROS) - Cells were washed with PBS twice and then exposed for 15 min at 37°C to 160 μM hydroethidine (DHE) dissolved in PBS. Analyses were performed on an EPICS XL flow cytometer (Beckman Coulter). The data were analyzed with FlowJo (Tree Star, Inc.).

RESULTS

Caspase-8 is essential for cell survival in L5178Y cells - To examine the role of caspase-8 in T-cell survival, we first treated mouse T-lymphoma-derived L5178Y cells with z-VAD-fmk, a pan-caspase inhibitor which blocks protease activities of various caspases including caspase-8, and quantified the cell death by trypan blue exclusion method. We found that L5178Y cells treated with z-VAD-fmk showed a decrease in cell growth and an increase in cell death (Fig. 1A and B). Also, we performed the same experiment with a caspase-8-specific inhibitor, z-IETD-fmk, and found that z-IETD-fmk-treated cells underwent

cell death (Supplemental Fig. 1A). These results suggested that the cell death induced by z-VAD-fmk is caused by the inhibition of caspase-8 activity.

To further investigate the function of caspase-8 in T-cells, gene-specific downregulation of caspase-8 expression was performed by utilizing the previously established Tet-On shRNA expression system with lentivirus-based expression vectors (31). In L5178Y cells expressing Tet-On shCasp-8, caspase-8 expression started to be down-regulated at the protein level after 24 h of Dox treatment (Fig. 1C). We observed a reduced cell proliferation rate and an increased rate of cell death specifically in the caspase-8 knockdown cells (Fig. 1D and E). It was confirmed that caspase-8 has an essential role in cell survival in L5178Y cells.

Protease activity but not auto-cleavage activity of procaspase-8 is required for cell survival - Caspase-8 knockdown by induced expression of shCasp-8 provoked cell death in mouse L5178Y cells. We further examined whether cell survival can be recovered by expression of exogenous mouse caspase-8 with silent mutations in the target nucleotides of shCasp-8 (WT*). In addition, we constructed two caspase-8 mutants; CS and DE, which hold a Cys to Ser mutation in the protease domain (C362S) and 7 Asp to Glu mutations in expected cleavage sites of caspase-8 itself and other caspases, respectively, as substituents of WT* mouse caspase-8 with a flag tag (Fig. 2A). These CS and DE mutants were expressed in caspase-8 knockout mouse embryonic fibroblasts by utilizing lentivirus expression vectors. By treatment of the cells with agonistic anti-Fas antibody, we confirmed that the CS and DE mutants did not undergo auto-cleavage since cleaved forms of caspase-8 were not detected upon the apoptosis-inducing stimulation (Fig. 2B).

To find out whether the protease activity and/or cleavage-associated activation of caspase-8 are necessary for cell survival in L5178Y cells, we exogenously expressed either WT*, CS or DE mutant in Tet-On shCasp-8-expressing cells and examined their levels (Fig. 2C). After the induction of shRNA expression by treatment with Dox, cell proliferation and cell death population were quantified (Fig. 2D and E). Expression of WT* caspase-8 enhanced cell proliferation and inhibited cell death in shCasp-8-expressing cells. DE mutant-expressing cells showed essentially the same rates of growth and cell death as WT*-expressing cells, indicating that activation of caspase-8 by auto-processing and cleavage by other caspases are not involved in cell survival. On the other hand, CS mutant-expressing cells showed essentially the same number of cells undergoing death as in the shCasp-8-expressing parental cells. Taken together, protease activity of procaspase-8 is indispensable for cell survival in L5178Y cells, but cleavage-associated activation of procaspase-8 is not necessary for cell survival.

Caspase-8-deficient L5178Y cells undergo apoptosis - We next examined the type of cell death induced by the knockdown of caspase-8 in L5178Y cells. Although cell death among caspase-8-deficient cells was previously shown to be non-apoptotic (35) or necroptosis (36-38), we first explored the characteristics of apoptosis in the knocked down cells. Cleaved forms of caspase-3 and PARP, a substrate of caspase-3, as well as decreased levels of procaspase-9 were observed after 36 and 48 h treatment with Dox in Tet-On shCasp-8-expressing L5178Y cells (Fig. 3A). Moreover, a DNA ladder, another characteristic of apoptosis, was also observed in the caspase-8-deficient cells (Fig. 3B). Thus, apoptosis was induced in L5178Y cells by the knockdown of caspase-8 expression.

To further determine whether apoptosis is the only type of cell death induced by knockdown of caspase-8, we at first blocked the protease activities of caspase-3 and -9 with z-DEVD-fmk and z-LETD-fmk, specific inhibitors for caspase-3/7 and caspase-9, respectively. These inhibitors could only partially inhibit the caspase-8 knockdown-induced cell death (Supplemental Fig. 1B and C). We then blocked the intrinsic pathway of apoptosis by over-expressing the anti-apoptotic protein Bcl-xL (Supplemental Fig. 2). The levels of the Bcl-xL expressed under the control of promoters for eEF1A1 or PGK, were much higher than the endogenous levels, and apoptosis induced by staurosporin was blocked in the established cells expressing Bcl-xL. Upon shCasp-8 expression, however, cell death in Bcl-xL-expressing cells was still induced; the number of dead cells was more than half of that in Bcl-xL-unexpressing cells. These results suggested that not only apoptosis but also other types of cell death might be induced by knockdown of caspase-8 expression in L5178Y cells.

To make certain apoptosis and other types of cell death to be simultaneously induced in each of caspase-8 knockdown cells, we generated caspase-3 knockdown cells and closely analyzed cell death in these cells after induced knockdown of caspase-8. Expression of caspase-3 was effectively down-regulated by expression of shRNA specific for caspase-3 (shCasp-3) in L5178Y cells with Tet-On shCasp-8 (Fig. 3C), and the caspase-3 knockdown cells were resistant to staurosporin-induced apoptosis (data not shown). The cell growth retardation and cell death, quantified by trypan blue exclusion method, were not affected by the knockdown of caspase-3 expression (Fig. 3D and E). Cell death

was also quantified by LDH release method (Fig. 3F), and the results obtained by the both methods were same. These results indicate that cell death population was not affected in caspase-3 knockdown cells. To exclude the possibility that apoptosis is still induced in part of the caspase-3 knockdown cells, we examined sub-G1 population and nuclear morphology on cell per cell basis. While knockdown of casepase-8 effectively increased the number of sub-G1 cells, knockdown of caspase-3 expression, which could not inhibit the caspase-8 knockdown-induced cell death, clearly inhibits the generation of sub-G1 population (Fig. 4A). Moreover, apoptosis-specific chromosomal fragmentation and chromosomal condensation near the nuclear periphery, both of which were induced by caspase-8 knockdown, were inhibited by caspase-3 knockdown (Fig. 4B). Interestingly, the size of nucleus of the caspase-3 knockdown cells was smaller than that of viable cells not treated with Dox, and the morphology of nucleus in caspase-3 knockdown cells was quite different from that of the viable cells. All the data suggest that caspase-8 knockdown simultaneously induces caspase-3-dependent apoptosis and other types of cell death in each of cells.

Autophagy was also observed in caspase-8 down-regulated L5178Y cells - We then examined the onset of autophagy in caspase-8 knockdown cells. During the formation of autophagosomes, light chain associated protein 3 (LC3) is transformed from LC3-I to LC3-II by lipidation, and this transformation is an indicator of autophagy (39). We detected this transformation in caspase-8 knockdown cells but not in control cells (Fig. 5A). Expression of ATG7, which is indispensable for the formation of autophagosomes, was down-regulated by expression of shRNA specific for ATG7 in caspase-8 knockdown L5178Y cells. Although the knockdown of ATG7 expression is partial (Fig. 5B), transformation of LC3 was attenuated by expression of shATG7 in caspase-8 knockdown cells (Fig. 5A). Importantly, cell death induced by caspase-8 knockdown was partly reduced by the knockdown of ATG7 expression (Fig. 5C). Taken together, autophagic cell death was also induced when the expression of procaspase-8 was down-regulated. To further confirm this, caspase-8 knockdown cells were treated with wortmanin, which was utilized as an inhibitor of autophagy. Upon treatment with wortmanin, both growth retardation and cell death were partially reduced in caspase-8 knockdown cells (Fig. 5D and E). Taken together, not only apoptotic cell death but also autophagic cell death is induced in cells lacking procaspase-8 activity.

Cell death in caspase-8 knockdown T-cells was mediated by ROS accumulation - We then analyzed whether ROS was accumulated in the caspase-8 knockdown cells. Firstly, we examined the effect of BHA, a ROS scavenger, on the cell death. Cell death in caspase-8 knockdown cells was almost completely rescued by treatment with BHA (Fig. 6A and B). We then measured intracellular levels of ROS by using hydroethidine (DHE). ROS accumulation was detected in Tet-On shCasp-8-expressing L5178Y cells after 36 and 48 h treatment with Dox, and BHA treatment inhibited its accumulation (Fig. 6C). These results indicated that oxidative stress is the cause of apoptosis and autophagic cell death in caspase-8 knockdown L5178Y cells.

RIP1 and RIP3 are key factors inducing cell death in caspase-8-deficient L5178Y cells - We next explored whether RIP1 and RIP3, which were reported to be involved in the induction of necroptosis in caspase-8-deficient cells, contribute to the cell death. We first examined the effect of Nec-1, a RIP1 inhibitor (25), on cell proliferation and viability in caspase-8 knockdown L5178Y cells. The cell viability was almost completely restored by treatment with Nec-1 (Fig. 7A). We then examined the involvement of RIP3 in the cell death. The cell death induced in caspase-8 knockdown cells was almost completely prevented by expression of shRNA specific for RIP3 (Fig. 7B and C). In RIP3 and caspase-8 double-knockdown cells as well as Nec-1-treated caspase-8 knockdown cells, the accumulated ROS level was significantly decreased compared to that in caspase-8 single knockdown cells not treated with Nec-1 (Fig. 7D). All the results in this study clearly indicate that knockdown of caspase-8 simultaneously induces apoptosis and non-apoptotic cell death including autophagic cell death through RIP1/RIP3-mediated ROS accumulation.

DISCUSSION

Treatment with z-VAD-fmk, a pan-caspase inhibitor, was reported to induce cell death in some types of cells (20,22). Recently, evidence has emerged that the cell death could be caused by the absence of the non-apoptotic function of caspase-8 in T-cells (23). Here, we observed the induction of cell death in mouse T lymphoma-derived L5178Y cells not only by treatment with z-VAD-fmk but also by down-regulation of caspase-8 expression. A similar phenomenon has been reported in mouse fibroblast-derived L929 cells as well as primary T-cells derived from the conditional knockout mice of caspase-8 (16,17,23). These results strongly indicate that caspase-8 has an essential

function in cell survival in several types of cells. Meanwhile, we also analyzed the effects of caspase-3- or caspase-9-specific inhibitors, and down-regulation of caspase-3 expression; however, neither cell death nor delayed cell proliferation was observed in L5178Y cells. Therefore, the cell death induced by caspase inhibition should be specific to caspase-8.

Even though we show here the importance of caspase-8 to prevent cell death in mouse T-lymphoma-derived L5178Y cells, caspase-8 was originally found to function in death receptor-mediated apoptosis. Activation of procaspase-8 is prompted by auto-cleavage at Asp210, Asp216, Asp374, and Asp384 (in human) in DISC. After oligomerization within a DISC, procaspase-8 undergoes auto-cleavage, and then translocates into the cytosol as an activated dimer harboring the active pocket with the active site Cys283 (40,41). Procaspase-8 was also reported to be cleaved by caspase-6 resulting in the formation of the active dimer, which process might function to form the positive feedback loop of caspase activation in induction of apoptosis (42,43). Our DE mutant does not retain the Asp residue in linker sequences where procaspase-8 is cleaved by caspase-8 itself and other caspases. Therefore, it would not be activated under any conditions, while previously reported DE mutants have the potential to be activated by other caspases. In fact, the exogenously expressed mouse DE mutant was confirmed never to be activated in human KB cells with endogenous caspase-8 where apoptosis was induced through both intrinsic and extrinsic pathways (data not shown). The results of this study showed that the DE-mutant compensated for the function of WT* caspase-8, whereas the CS-mutant did not. Taken together, our data clearly showed that the activation of caspase-8 is not necessary for cell survival in L5178Y cells, but protease activity of procaspase-8 to cleave its target substrates is indispensable for cell survival.

Whereas caspase-8 deficiency was reported to induce multiple types of cell death, such as autophagic cell death and necrosis (44,45), it is still controversial what type of cell death is actually induced as a result of the absence of procaspase-8 activity (20,24,45,46). In L929 cells, caspase-8 deficiency was reported to induce degradation of a ROS scavenger protein, catalase, resulting in ROS accumulation and autophagic cell death (20,23). Other reports showed that FADD and caspase-8 form a feedback loop to limit autophagy, a pathway important for rapid T-cell proliferation (46). In mouse T-lymphoma- derived L5178Y cells, we indeed observed autophagy-dependent cell death, which was inhibited by expression of shATG7 and by treatment with wortmanin, an inhibitor of autophagosome formation. In addition, caspase-8 and FADD were reported to form a salvage pathway from necroptosis dependent on RIP1 kinase (27,28). Recently, both RIP1 and RIP3 were reported to be involved in caspase-8-deficiency-induced embryonic lethality and cell death in caspase-8-deficient T-cells (26-28). According to those reports, it is likely that caspase-8 constantly suppresses necroptosis by inhibiting the activation of RIP1 and RIP3 not only in T-cells but also in other types of cells during embryonic development. Therefore, caspase-8-deficient cells must have the capacity to undergo necroptosis. In L5178Y cells, it was also observed that the cell death induced by caspase-8-deficiency was rescued by either treatment with a RIP1 inhibitor, Nec-1, or expression of shRIP3 (Fig. 7). These results indicate that RIP kinases are involved in the outcome of the events induced by caspase-8 dysfunction, and necroptosis would occur in L5178Y cells. Furthermore, we clearly indicated that apoptosis is also induced by caspase-8 deficiency in addition to previously reported autophagic cell death in mouse T-lymphoma-derived L5178Y cells. Taken together, protease activity of procaspase-8 would inhibit various types of cell death including apoptosis, autophagic cell death and necroptosis, all of which are simultaneously induced in the absence of caspase-8.

ROS accumulation was reported to be also observed in caspase-8 knockdown cells (23,46). Indeed, caspase-8 deficiency-induced cell death was completely rescued in caspase-8 knockdown L5178Y cells by treatment with BHA, a ROS scavenger. Moreover, an elevation of intracellular ROS levels was observed in caspase-8 knockdown cells downstream of the activation of RIP1 and RIP3. Taken together, knockdown of caspase-8 simultaneously induces apoptosis, and other types of cell death through RIP1- and RIP3-mediated ROS accumulation. One of the plausible interpretations of multiple types of cell death induced in caspase-8 knockdown L5178Y cells would be that apoptosis and autophagic cell death might be induced as secondary events of ROS accumulation along with necroptosis induced by activation of RIP1 and RIP3. Activated RIP1 and RIP3 were reported to increase the energy metabolism which results in accumulation of ROS (47). Taken together, procaspase-8 might constantly regulates activation of RIP1 and/or RIP3 in L5178Y cells to protect cells from unusual energy metabolism, therefore, cells survive by maintaining intracellular ROS levels. Even though there still remain many unknown reasons why casapase-8 deficiency leads to cell death, our study clearly showed that the protease activity of procaspase-8 is essential for cell survival in L5178Y cells through inhibiting ROS accumulation induced by activation of RIP1 and RIP3. Since it is known that mutation of caspase-8 in humans leads to immune deficiency

(18), further analysis of procaspase-8 function in T-cells may contribute better understanding of immune deficiency disease.

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FOOTNOTES

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Abbreviations: BHA, butylated hydroxyanisole; DISC, death-inducing signaling complex; Dox, doxycyclin; FADD, Fas-associated death domain; LC3, light chain-associated protein 3; LDH, lactate dehydrogenase; Nec-1, necrostatin-1; PI, propidium iodide; RIP1, receptor-interacting serine/threonine-protein kinase 1; RIP3, receptor-interacting serine/threonine-protein kinase 3; ROS, reactive oxygen species; shRNA, short hairpin RNA; shCasp-3, shRNA for mouse caspase-3; shCasp-8, shRNA for mouse caspase-8; tetracycline-inducible short hairpin RNA expression system, Tet-On shRNA system.

FIGURE LEGENDS

FIGURE 1. Caspase-8 has an essential role in cell survival in L5178Y cells. **A** and **B**. Cells were treated with z-VAD-fmk in DMSO or DMSO only for the periods indicated. z-VAD-fmk was added every 24 h. Numbers of viable and dead cells were quantified by trypan blue exclusion method using a hemocytometer. Total viable cell number (A) and % of dead cells (B) are indicated. Results obtained by trypan blue exclusion method were essentially same as those by LDL release assay. Results representative experiments carried out in triplicate are shown. (All error bars: ±S.D.) **C**. Tetracycline repressor (TetR)-expressing L5178Y cells were infected with lentiviral vectors with the Tet-On shRNA-expression system encoding shRNA specific for LacZ (shLacZ) or caspase-8 (shCasp-8). After induction of shRNA expression by treatment with 10 ng/ml Dox for the periods indicated, cell lysate was analyzed by Western blotting with anti-caspase-8 and anti-actin antibody. *: a non-specific band. **D** and **E**. After treatment with 10 ng/ml Dox for indicated periods, numbers of viable and dead cells were quantified as described in A and B. Total cell number (D) and % of dead cells (E) are indicated.

FIGURE 2. Protease activity of procaspase-8 is necessary for the survival of L5178Y cells. A. Schematic view of caspase-8. Mutated sites for caspase-8 without protease activity (CS mutant) and without sites of cleavage by caspases including caspase-8 itself (DE mutant) are shown. **B**. Flag-tagged wild type, CS mutant and DE mutant of caspase-8 (casp-8) were expressed in mouse embryonic fibroblasts (MEFs) derived from caspase-8 knockout mice. Cells were treated with 100 ng/ml anti-Fas antibody (Jo2) and 1μg/ml cycloheximide for 24 h, and then cleavage of caspase-8 was analyzed by Western blotting with anti-Flag antibody. *: nonspecific bands. **C**. Flag-tagged forms of caspase-8 (casp-8) were expressed in Tet-On shCasp-8-expressing L5178Y cells. Cells without Dox treatment were analyzed by Western blotting with anti-Flag, anti-caspase-8 (casp-8), and anti-actin antibodies. **D** and **E**. After treatment with 10 ng/ml Dox for the periods indicated, numbers of viable and dead cells were quantified as described in Fig. 1. Results representative of experiments carried out in triplicate were shown. (All error bars: ±S.D.)

FIGURE 3. Down-regulated procaspase-8 activity resulted in apoptotic cell death. A. After induction of shLacZ or shCasp-8 expression by treatment with 10 ng/ml Dox for the indicated periods, cell lysate was analyzed by Western blotting with antibodies to caspase-3, caspase-9, PARP, and actin. **B**. Cleaved genomic DNA was extracted from cells expressing Tet-On shLacZ or shCasp-8 after treatment with 10 ng/ml Dox for the periods indicated, and resolved by agarose gel electrophoresis. **C**-**F**. L5178Y cells with Tet-On shCasp-8 were infected with a lentiviral expression vector encoding shRNA specific for caspase-3 (shCasp3) or shLacZ. Cell lysate was analyzed by Western blotting with anti-caspase-3 and anti-actin antibodies (C). After treatment with 10 ng/ml Dox for the periods indicated, numbers of viable and dead cells were quantified as described in Fig. 1 (D and E). Percentage of dead cells was also quantified by LDL release assay (F). Cell death [%] = (amount of LDH released in culture medium) / (amount of LDH released in culture medium plus that retained in viable attached cells) × 100. Results representative of experiments carried out in triplicate were shown. (All error bars: ±S.D.).

FIGURE 4. Effects of casepase-3 knockdown on caspase-8 knockdown-induced cell death. A. Cells described in Figure 3C were cultured with or without 10 ng/ml Dox for 36 h. After staining with propidium iodide (PI), percentage of sub-diploid (sub-G1) cells was measured by flow cytometric analysis to quantify apoptotic cells. **B**. Cells, cultured with or without Dox for 48 h, were stained with DAPI and analyzed under a fluorescence microscope. Arrows and arrowheads indicate nuclei with condensed chromatin near the nuclear periphery and fragmented chromatin, respectively.

FIGURE 5. Down-regulated procaspase-8 activity induces autophagy. A. GFP-fused LC3 was over-expressed in L5178Y cells with either Tet-On shLacZ, Tet-On shCasp-8, or Tet-On shCasp-8 and shATG7 vectors. After treatment with 10 ng/ml Dox for the periods indicated, cell lysate was analyzed by Western blotting with antibodies to LC3 and actin. **B**. L5178Y cells with Tet-On shCasp-8 or shLacZ were infected with a lentiviral vector encoding shRNA for ATG7 (shATG7). Seven days after infection, the expression of ATG7 was quantified by QRT-PCR. **C**. L5178Y cells with the indicated Tet-On shRNA were treated with 10 ng/ml Dox for the periods indicated. Numbers of viable and dead

cells were quantified. **D** and **E**. L5178Y cells with Tet-On shCasp-8 or shLacZ were treated together with 10 ng/ml Dox and 500 nM wortmanin in DMSO or DMSO only for the periods indicated. Dox and wortmanin were added every 24 h. Numbers of viable and dead cells were quantified.

FIGURE 6. Caspase-8 knockdown cells undergo death through induction of ROS accumulation.
A and **B**. L5178Y cells with Tet-On shCasp-8 or shLacZ were treated with 10 ng/ml Dox and either 20 μM BHA in DMSO or DMSO only for the periods indicated. Numbers of viable and dead cells were quantified. Total viable cell number (A) and % of dead cells (B) are indicated. **C.** Expression of shLacZ or shCasp-8 was induced by treatment with Dox for the periods indicated in the presence or absence of 20 μM BHA. Amounts of accumulated ROS were measured by flow cytometric analysis using DHE.

FIGURE 7. RIP1 and RIP3 regulate both apoptotic and non-apoptotic cell death in L5178Y cells.
A. L5178Y cells with Tet-On shCasp-8 or shLacZ were treated with 10 ng/ml Dox and either 50 μM Nec-1 in DMSO or DMSO only for the periods indicated. Numbers of viable and dead cells were quantified. **B**. L5178Y cells with Tet-On shCasp-8 were infected with a lentiviral expression vector encoding shRNA specific for RIP3 (shRIP3) or shLacZ. After 48 h cultivation, cell lysate was analyzed by Western blotting with anti- RIP3 and anti-actin antibodies. **C**. L5178Y cells with Tet-On shCasp-8 and either shLacZ or shRIP3 were treated with 10 ng/ml Dox together with or without 50 μM Nec-1 for 72 h. Numbers of viable and dead cells were quantified. **D**. ROS levels in cells described in C were measured by flow cytometric analysis using DHE after 48 h treatment with Dox.

Figure 1

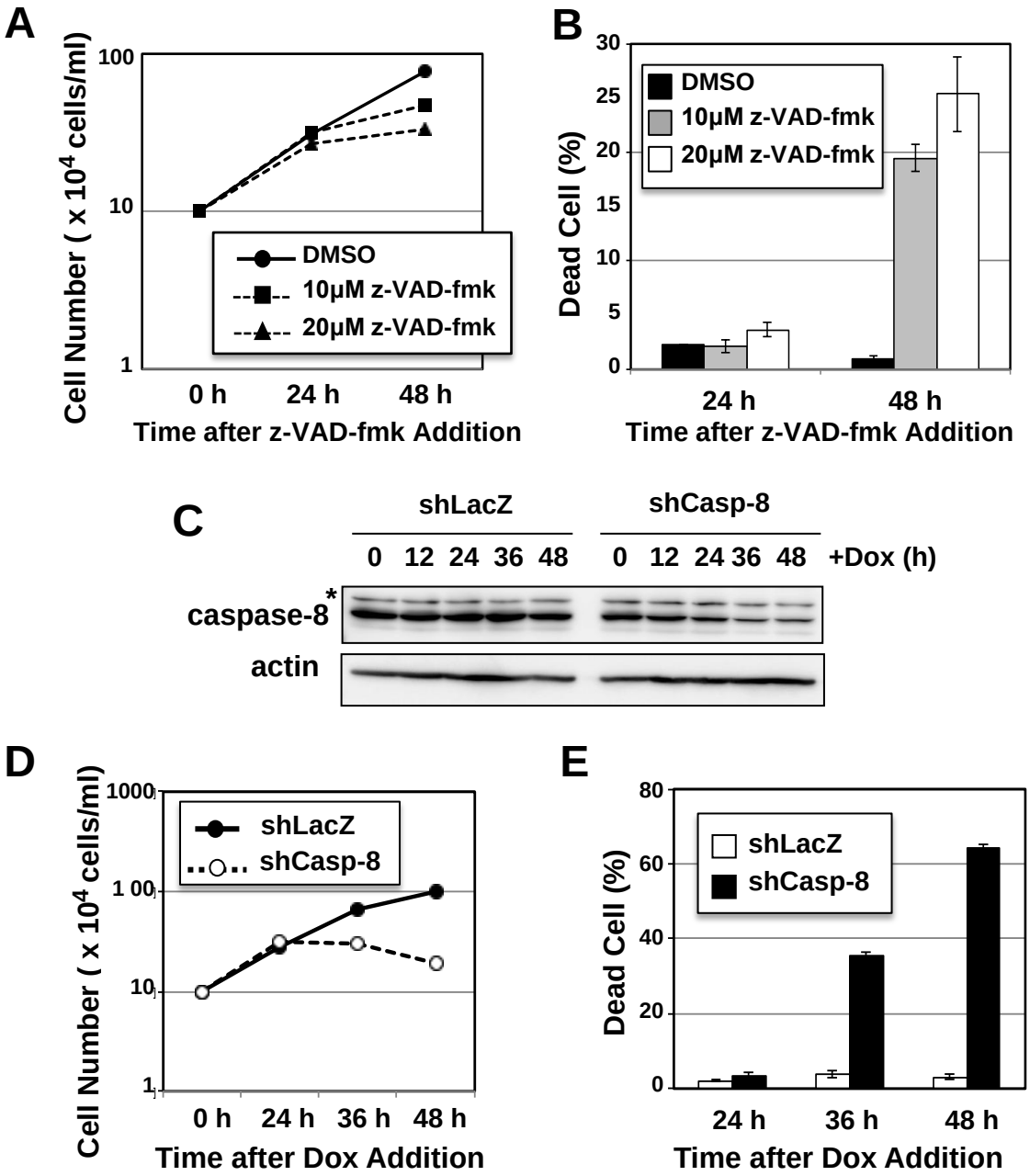

A
Cell Number (x 10^4 cells/ml)
100
10
1
DMSO
10μM z-VAD-fmk
20μM z-VAD-fmk
0 h
24 h
48 h
Time after z-VAD-fmk Addition
B
Dead Cell (%)
30
25
20
15
10
5
0
DMSO
10μM z-VAD-fmk
20μM z-VAD-fmk
24 h
48 h
Time after z-VAD-fmk Addition
C
shLacZ
shCasp-8
0 12 24 36 48 0 12 24 36 48 +Dox (h)
caspase-8*
actin
D
Cell Number (x 10^4 cells/ml)
1000
1 00
10
1
shLacZ
shCasp-8
0 h
24 h
36 h
48 h
Time after Dox Addition
E
Dead Cell (%)
80
60
40
20
0
shLacZ
shCasp-8
24 h
36 h
48 h
Time after Dox Addition

Figure 2

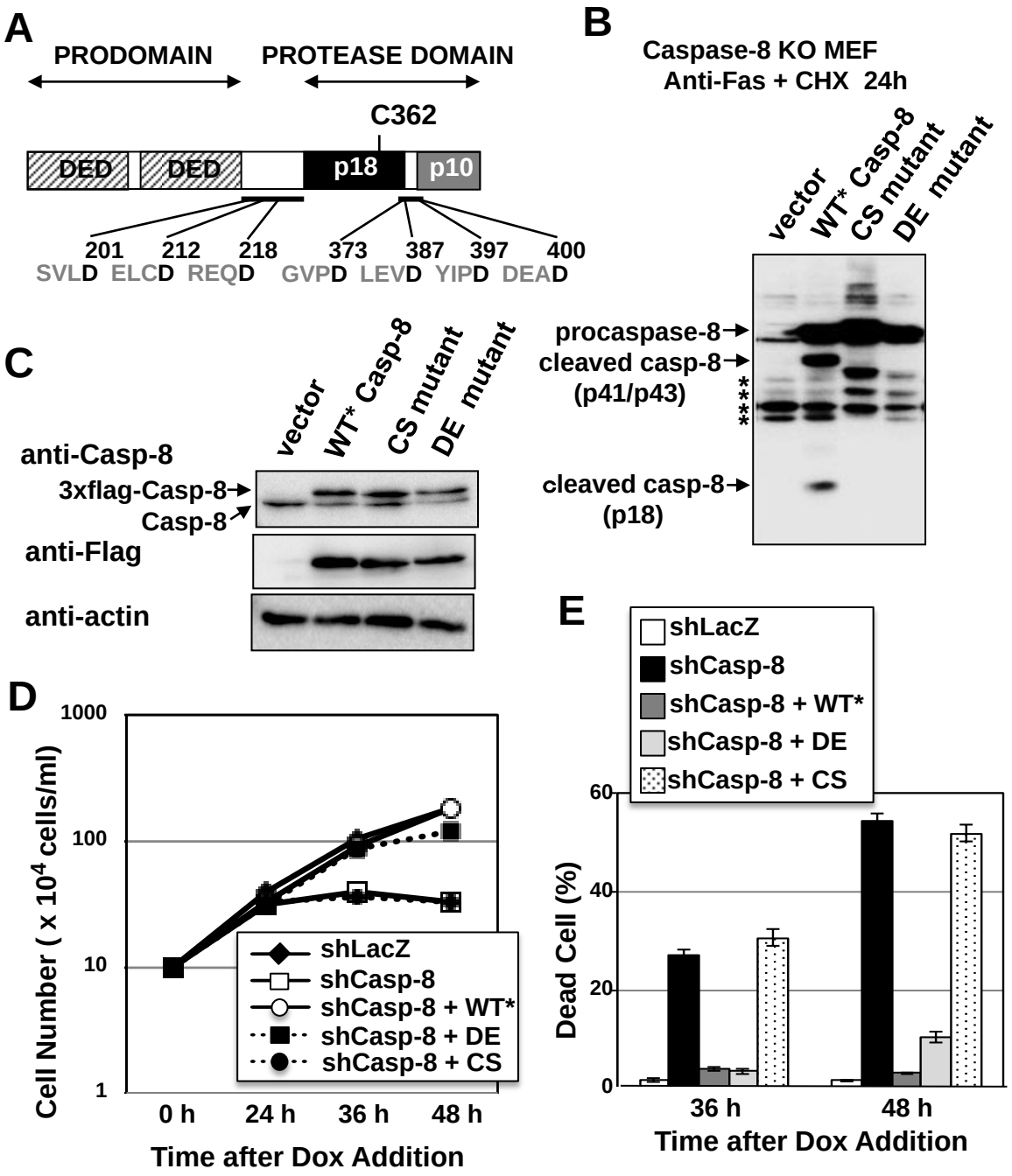

A
PRODOMAIN
PROTEASE DOMAIN
C362
DED
DED
p18
p10
201 212 218 373 387 397 400
SVLD ELCD REQD GVPD LEVD YIPD DEAD
B
Caspase-8 KO MEF
Anti-Fas + CHX 24h
vector
WT* Casp-8
CS mutant
DE mutant
procaspase-8
cleaved casp-8
(p41/p43)
****
cleaved casp-8
(p18)
C
vector
WT* Casp-8
CS mutant
DE mutant
anti-Casp-8
3xflag-Casp-8
Casp-8
anti-Flag
anti-actin
D
Cell Number (x 10^4 cells/ml)
1000
100
10
1
0 h
24 h
36 h
48 h
Time after Dox Addition
shLacZ
shCasp-8
shCasp-8 + WT*
shCasp-8 + DE
shCasp-8 + CS
E
shLacZ
shCasp-8
shCasp-8 + WT*
shCasp-8 + DE
shCasp-8 + CS
Dead Cell (%)
60
40
20
0
36 h
48 h
Time after Dox Addition

Figure 3

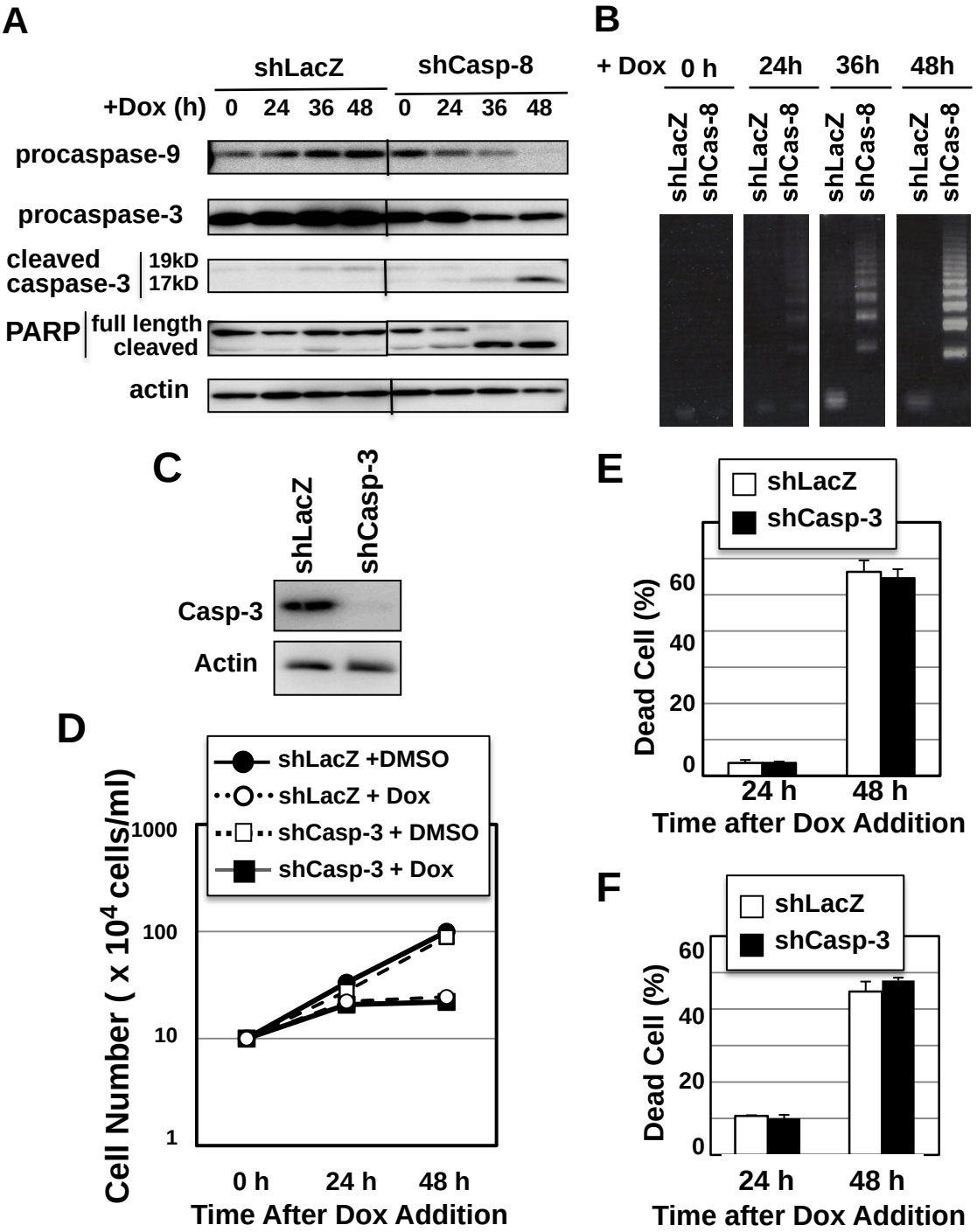

A
shLacZ
shCasp-8
+Dox (h)
0 24 36 48 0 24 36 48
procaspase-9
procaspase-3
cleaved caspase-3
19kD
17kD
PARP
full length
cleaved
actin
B
+ Dox
0 h
24h
36h
48h
shLacZ
shCas-8
C
shLacZ
shCasp-3
Casp-3
Actin
D
shLacZ +DMSO
shLacZ + Dox
shCasp-3 + DMSO
shCasp-3 + Dox
Cell Number (x 10^4 cells/ml)
1000
100
10
1
0 h
24 h
48 h
Time After Dox Addition
E
shLacZ
shCasp-3
Dead Cell (%)
60
40
20
0
24 h
48 h
Time after Dox Addition
F
shLacZ
shCasp-3
Dead Cell (%)
60
40
20
0
24 h
48 h
Time after Dox Addition

Figure 4

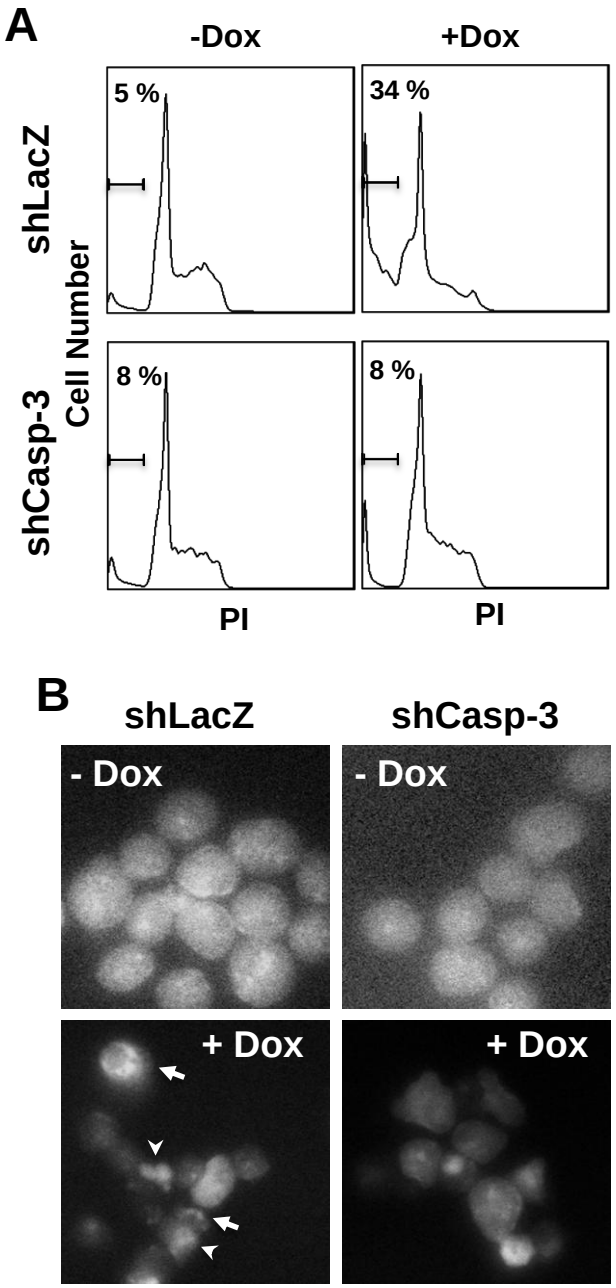

A
-Dox
+Dox
shLacZ
shCasp-3
Cell Number
5 %
34 %
8 %
8 %
PI
PI
B
shLacZ
shCasp-3
- Dox
- Dox
+ Dox
+ Dox

Figure 5

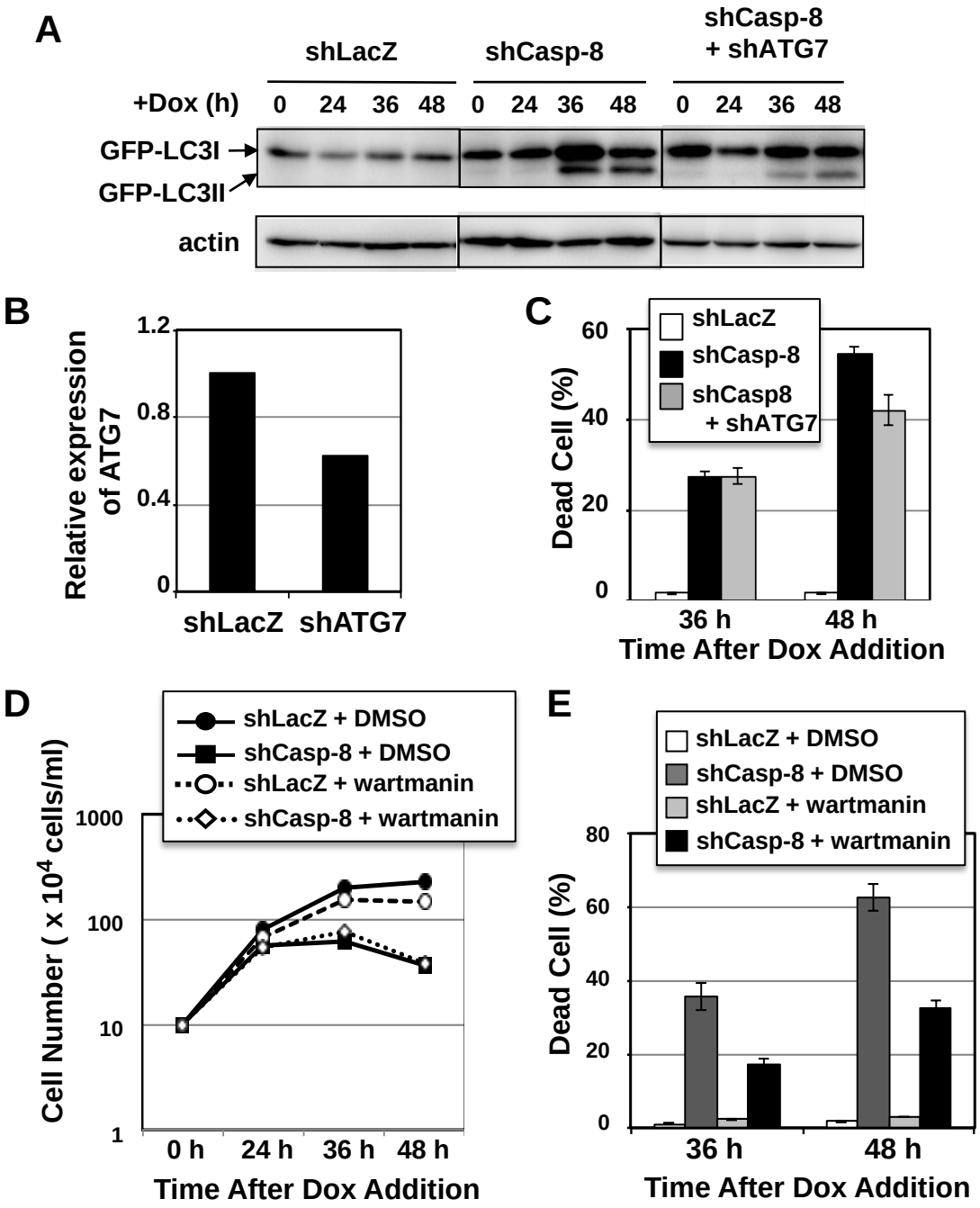
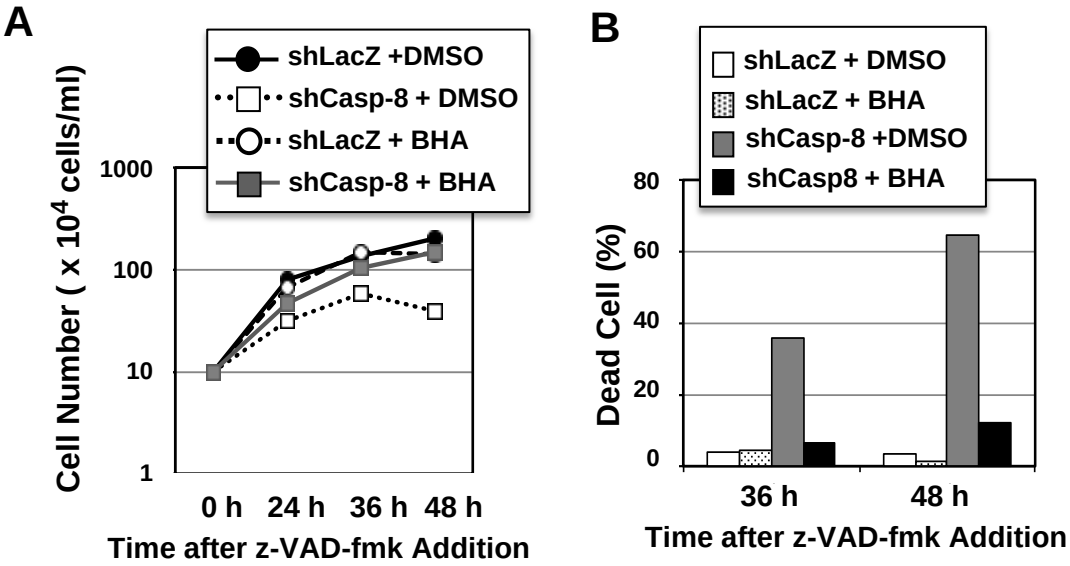

A
shLacZ
shCasp-8
shCasp-8 + shATG7
+Dox (h)
0 24 36 48 0 24 36 48 0 24 36 48
GFP-LC3I
GFP-LC3II
actin
B
Relative expression of ATG7
1.2
0.8
0.4
0
shLacZ shATG7
C
shLacZ
shCasp-8
shCasp8 + shATG7
Dead Cell (%)
60
40
20
0
36 h
48 h
Time After Dox Addition
D
shLacZ + DMSO
shCasp-8 + DMSO
shLacZ + wartmanin
shCasp-8 + wartmanin
Cell Number (x 10^4 cells/ml)
1000
100
10
1
0 h
24 h
36 h
48 h
Time After Dox Addition
E
shLacZ + DMSO
shCasp-8 + DMSO
shLacZ + wartmanin
shCasp-8 + wartmanin
Dead Cell (%)
80
60
40
20
0
36 h
48 h
Time After Dox Addition

Figure 6


A
shLacZ +DMSO
shCasp-8 + DMSO
shLacZ + BHA
shCasp-8 + BHA
Cell Number (x 10^4 cells/ml)
1000
100
10
1
0 h
24 h
36 h
48 h
Time after z-VAD-fmk Addition
B
shLacZ + DMSO
shLacZ + BHA
shCasp-8 +DMSO
shCasp8 + BHA
Dead Cell (%)
80
60
40
20
0
36 h
48 h
Time after z-VAD-fmk Addition

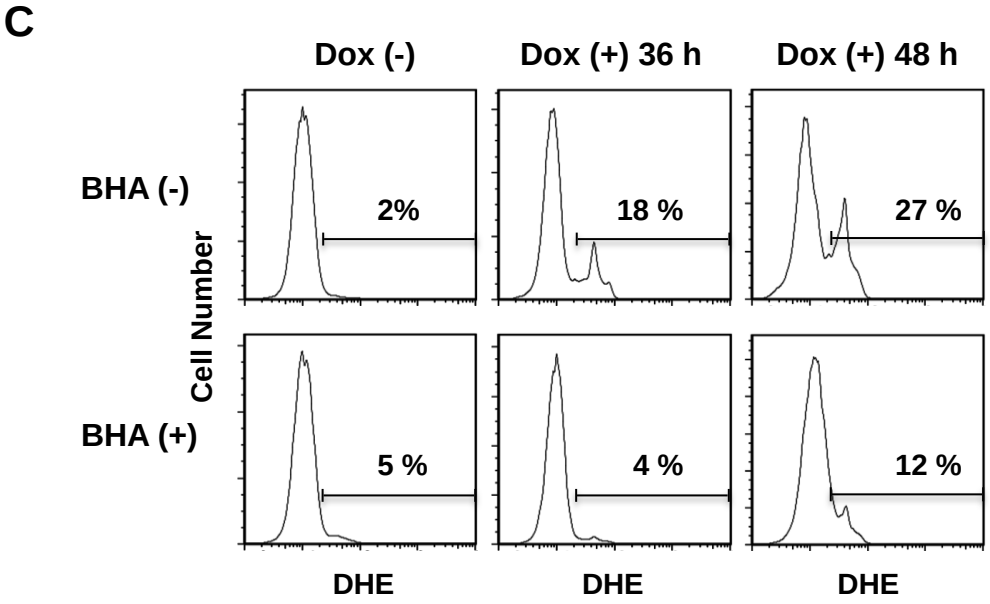
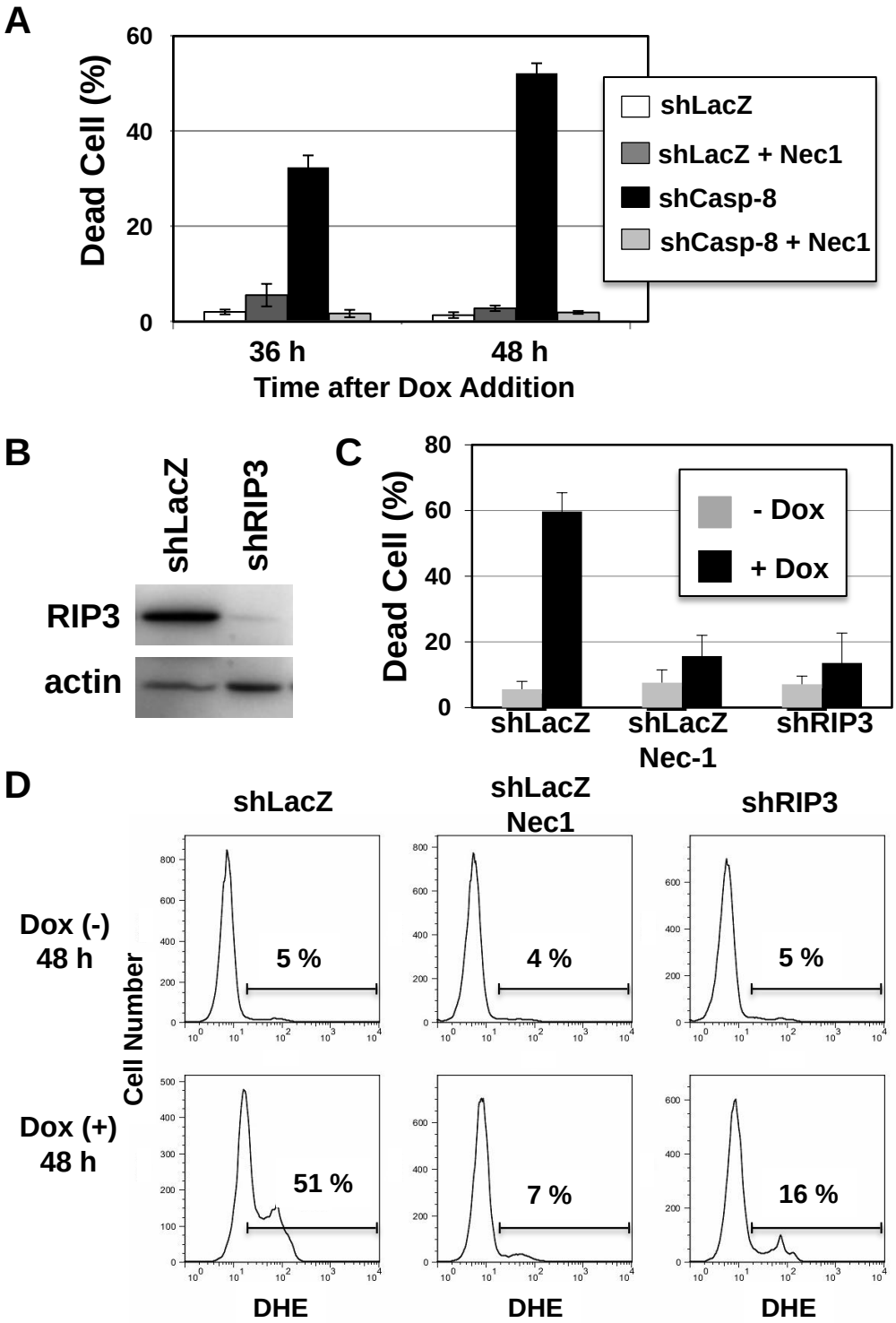

C
Dox (-)
Dox (+) 36 h
Dox (+) 48 h
BHA (-)
BHA (+)
Cell Number
2%
18 %
27 %
5 %
4 %
12 %
DHE
DHE
DHE

Figure 7

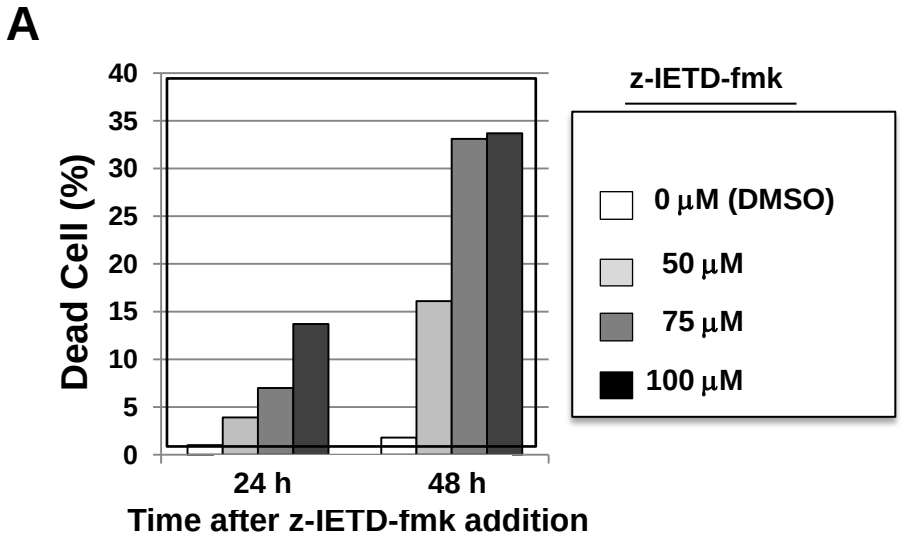

A
Dead Cell (%)
60
40
20
0
shLacZ
shLacZ + Nec1
shCasp-8
shCasp-8 + Nec1
36 h
48 h
Time after Dox Addition
B
shLacZ
shRIP3
RIP3
actin
C
Dead Cell (%)
80
60
40
20
0
- Dox
+ Dox
shLacZ
shLacZ Nec-1
shRIP3
D
shLacZ
shLacZ Nec1
shRIP3
Dox (-) 48 h
Dox (+) 48 h
Cell Number
5 %
4 %
5 %
51 %
7 %
16 %
DHE
DHE
DHE

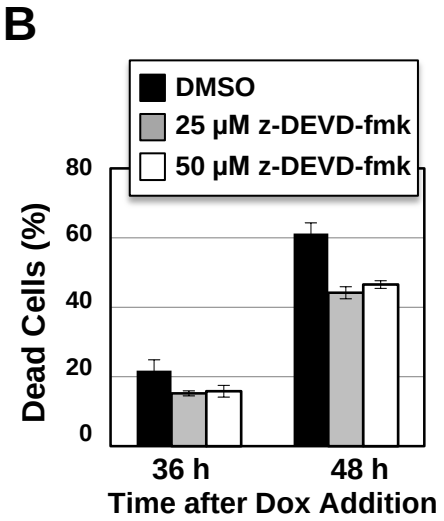
SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. Effects of caspase inhibitors on L5178Y cells with or without Tet-On shCasp-8.
A. L5178Y cells were treated with indicated amounts of z-IETD-fmk in DMSO or DMSO only for 24 or 48 h. Numbers of viable and dead cells were quantified as described in Fig. 1. **B** and **C**. Tet-On shCasp-8-expressing L5178Y cells were treated with 10 ng/ml Dox together with indicated amounts of z-DEVD-fmk (B) or z-LETD-fmk (C) in DMSO or DMSO only for the periods indicated. z-DEVD-fmk, z-LETD-fmk and Dox were added every 24 h. Numbers of viable and dead cells were quantified as described in Fig. 1.

FIGURE S2. Effects of exogenously expressing Bcl-xL on staurosporin-induced apoptosis and cell death induced by caspase-8 knockdown.
A. Flag-tagged and untagged Bcl-xL were over-expressed in L5178Y cells with Tet-On shCasp-8 by utilizing lentiviral expression vectors at various concentrations (concentration rates: x60 – x10) under the control of eEF1A1 (EF) and PGK promoters, respectively. Cell lysate was analyzed by Western blotting with antibodies to Bcl-xL, Flag and actin. **B** and **C**. L5178Y cells with Tet-On shCasp-8 and exogenous Bcl-xL as described in A were cultured with or without 2.5 μM staurosporin (STS) for 3h. Numbers of dead cells were quantified as described in Fig. 1 (B). Cell lysate was analyzed for the detection of cleaved-caspase-3 by Western blotting with anti-caspase-3 antibody (C). **D**. shLacZ and shCasap-8 expression were induced in exogenous Bcl-xL-expressing cells in A by treatment with 10 ng/ml Dox for the periods indicated. Numbers of viable and dead cells were quantified as described in Fig. 1.

Figure S1


A
z-IETD-fmk
0 μM (DMSO)
50 μM
75 μM
100 μM
Dead Cell (%)
40
35
30
25
20
15
10
5
0
24 h
48 h
Time after z-IETD-fmk addition


B
DMSO
25 μM z-DEVD-fmk
50 μM z-DEVD-fmk
Dead Cells (%)
80
60
40
20
0
36 h
48 h
Time after Dox Addition

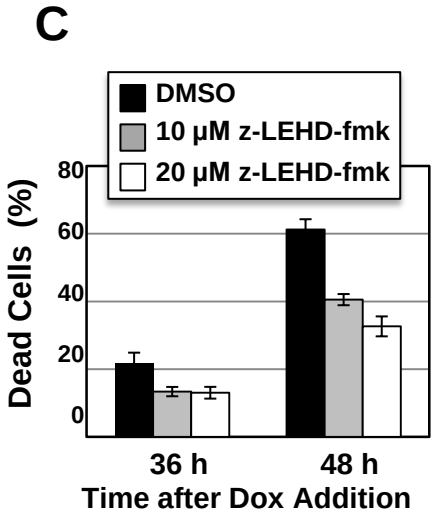
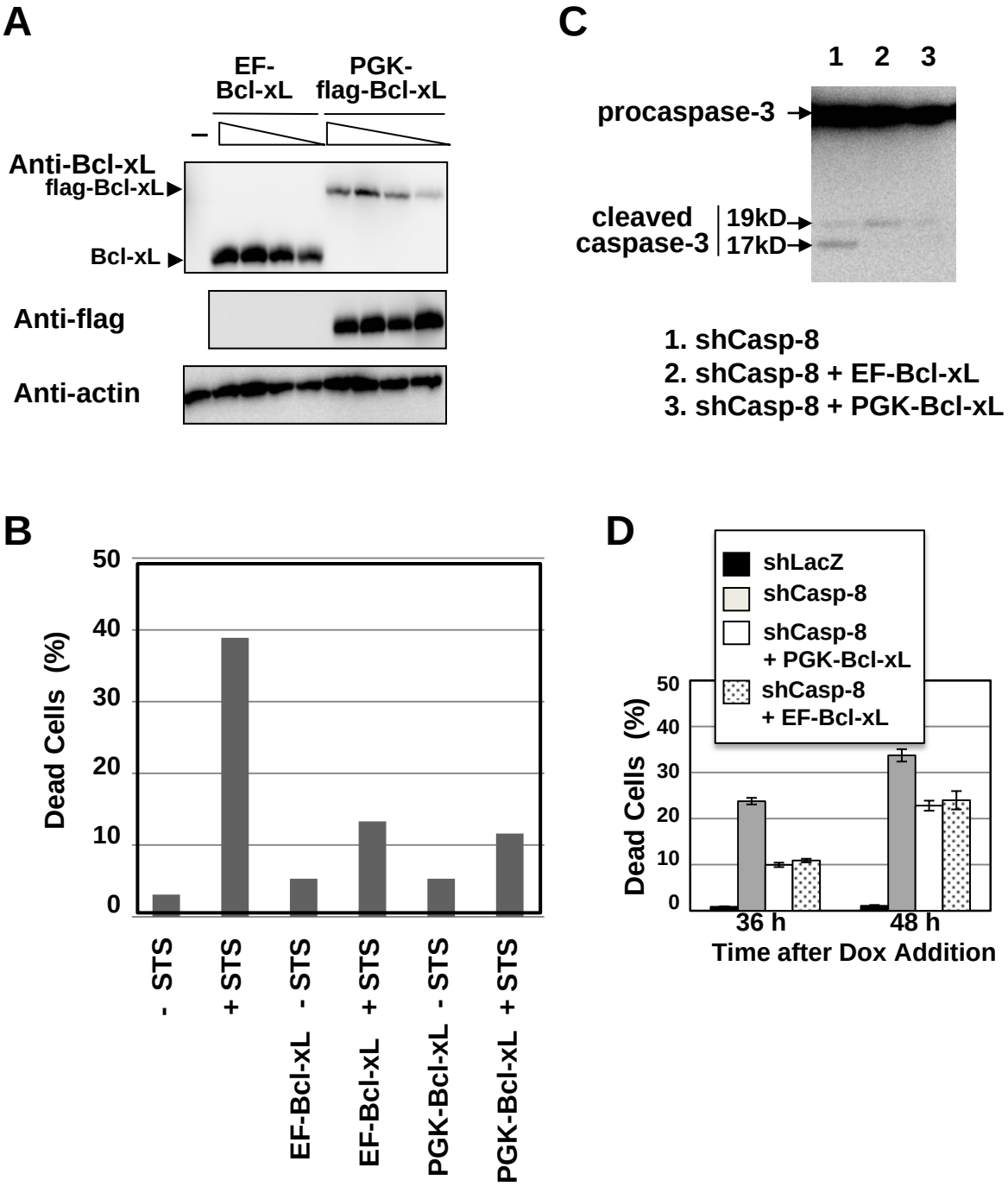

C
DMSO
10 μM z-LEHD-fmk
20 μM z-LEHD-fmk
Dead Cells (%)
80
60
40
20
0
36 h
48 h
Time after Dox Addition

Figure S2


A
EF-Bcl-xL
PGK-flag-Bcl-xL
–
Anti-Bcl-xL
flag-Bcl-xL
Bcl-xL
Anti-flag
Anti-actin
C
1 2 3
procaspase-3
cleaved caspase-3
19kD
17kD
1. shCasp-8
2. shCasp-8 + EF-Bcl-xL
3. shCasp-8 + PGK-Bcl-xL
B
Dead Cells (%)
50
40
30
20
10
0
- STS
+ STS
- STS
EF-Bcl-xL
+ STS
EF-Bcl-xL
- STS
PGK-Bcl-xL
+ STS
PGK-Bcl-xL
D
shLacZ
shCasp-8
shCasp-8 + PGK-Bcl-xL
shCasp-8 + EF-Bcl-xL
Dead Cells (%)
36 h
48 h
Time after Dox Addition