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Apaf-1- and Caspase-8-independent apoptosis

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Two major apoptosis pathways, the mitochondrial and death receptor pathways, are well recognized. Here we established cell lines from the fetal thymus of *Apaf-1-*, *Caspase-9-*, or *Bax/Bak*-deficient mice. These cell lines were resistant to apoptosis induced by DNA-damaging agents, RNA or protein synthesis inhibitors, or stress in the endoplasmic reticulum. However, they underwent efficient apoptosis when treated with kinase inhibitors such as staurosporine and H-89, indicating that these inhibitors induce a caspase-dependent apoptosis that is different from the mitochondrial pathway. CrmA, a Caspase-8 inhibitor, did not prevent staurosporine-induced apoptosis of fetal thymic cell lines, suggesting that the death receptor pathway was also not involved in this process. The staurosporine-induced cell death was inhibited by okadaic acid, a serine/threonine phosphatase inhibitor, suggesting that dephosphorylation of a proapoptotic molecule triggered the death process, or that phosphorylation of an antiapoptotic molecule could block the process. Cells of various types (fetal thymocytes, bone marrows, thymocytes, and splenocytes), but not embryonic fibroblasts, were sensitive to the noncanonical staurosporine-induced apoptosis, suggesting that the noncanonical apoptosis pathway is tissue specific.

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Apoptosis serves to eliminate cells that are useless (e.g., interdigital cells, nonreactive lymphocytes) or harmful (e.g., tumor cells and autoreactive lymphocytes). Deregulated apoptosis causes a variety of diseases. That is, a defect in apoptosis can lead to the development of tumors and autoimmune diseases, whereas an excess of apoptosis can cause organ failure.[1] Two apoptotic signaling pathways have been well characterized in mammals, the death receptor and mitochondrial pathways (also known as the extrinsic and intrinsic pathways, respectively).[2–4] In the death receptor pathway, the binding of a death factor such as Fas ligand (FasL), TNF, or TNF-related apoptosis-inducing ligand (TRAIL) to its receptor triggers a signaling cascade that leads to the activation, via an adaptor, Fas-Associated protein with Death Domain (FADD), of a caspase cascade consisting of Caspase-8 and Caspase-3. In the mitochondrial pathway, cytotoxic insults upregulate or activate BH3-only proteins that activate and oligomerize Bax and Bak, and the oligomerized Bcl-2 family members Bax/Bak cause the release of cytochrome *c* from mitochondria into the cytosol.[5] Cytochrome *c* then forms a complex with Apaf-1, and the cytochrome *c*/Apaf-1 complex, called the apoptosome, activates Caspase-9, which in turn activates Caspase-3.[6,7]

The role of FADD and Caspase-8 in the death receptor-induced apoptosis pathway was confirmed by the establishment of their knockout mice.[8,9] On the other hand, conflicting reports have been published on the roles of the signaling molecules in the mitochondrial pathway. Establishing knockout mice, Cecconi *et al.*,[10] Yoshida *et al.*,[11] and Kuida *et al.*[12]

originally reported that *Apaf-1* and *caspase-9* are indispensable for genotoxic agent-induced apoptosis. However, this idea was later challenged by Marsden *et al.*,[13] who showed that hematopoietic cells that lack *Apaf-1* or *Caspase-9* can undergo Bcl-2-regulated Caspase-dependent apoptosis. We recently reported that staurosporine, but not etoposide, activates Caspase-3 in embryonic day 14.5 (E14.5) fetal thymocytes in an Apaf-1-independent manner.[14] Moreover, activated Caspase-3 can be detected *in situ* in the E14.5 *Apaf-1*^{−/−} fetal thymus, suggesting that an *Apaf-1*-independent intrinsic apoptotic pathway exists in these cells.

Here, to characterize this *Apaf-1*-independent pathway, we established cell lines (immortalized fetal thymocytes (IFETs)) from the fetal thymus of wild-type and mutant mice. The *Apaf-1*^{−/−} IFETs were resistant to genotoxic agent-induced apoptosis, but staurosporine caused efficient caspase-dependent apoptosis. Neither a null mutation of *Caspase-9* nor double mutation of *Bax/Bak* abolished the staurosporine-induced apoptosis of IFETs. The expression of CrmA, a Caspase-8 inhibitor,[15] also did not inhibit staurosporine-induced apoptosis, indicating that the death receptor pathway was not used in this death process. This noncanonical Apaf-1- and Caspase-8-independent apoptosis signaling pathway triggered by staurosporine was observed in lymphoid and myeloid tissues such as fetal thymocytes and bone marrow cells, but was barely detectable in embryonic fibroblasts. Together, these results suggest the existence of a new tissue-specific apoptotic pathway that is distinct from the death receptor and mitochondrial pathways.

Results

Establishment of *Apaf-1*-null fetal thymocyte cell line.

We previously observed that Caspase-3 is activated in *Apaf-1*-null fetal thymus.[14] To characterize the Apaf-1-independent apoptosis biochemically, it was essential to establish *Apaf-1*-null cell lines. Mouse fetal thymocytes were immortalized by infecting them with mouse retrovirus carrying c-*myc* and H-*ras^{V12}*, using the RetroNectin-bound virus method. Several hundreds of transformants were obtained from the pair of thymic lobes of an *Apaf-1$^{+/+}$* or *Apaf-1$^{-/-}$* E14.5 embryo. The transformants were cloned by limiting dilution, and termed IFETs. The targeted disruption of the *Apaf-1* gene, or deletion of its exon 5, in *Apaf-1$^{-/-}$* IFETs was confirmed by PCR analysis of the genomic DNA (Supplementary Figure S1A).

Incubating cell lysates with cytochrome *c* and dATP activates Caspase-3 in an Apaf-1-dependent manner.[16] Accordingly, when extracts of *Apaf-1$^{+/+}$* but not *Apaf-1$^{-/-}$* IFETs were incubated with cytochrome *c* and dATP, the Caspase-3 activation was observed (Supplementary Figure S1B), confirming that Apaf-1 was functionally inactivated by the deletion of its exon 5. The *Apaf-1$^{+/+}$* and *Apaf-1$^{-/-}$* IFETs grew equally well, with no requirement for specific growth factors. The surface phenotype of both *Apaf-1$^{+/+}$* and *Apaf-1$^{-/-}$* IFETs was Thy1.2$^+$CD25$^-$CD44$^+$ (Supplementary Figure S2), although Thy1.2 was gradually downregulated after repeated passages. These results suggested that the IFETs established here were T-cell lineage cells at the early stage of development.

Apaf-1-independent Caspase-dependent cell death induced by staurosporine.

Next, the wild-type and *Apaf-1$^{-/-}$* IFETs were treated with staurosporine or etoposide. As shown in Figure 1a, the *Apaf-1*-null mutation blocked the etoposide-induced cell death. However, it had no effect on staurosporine-induced cell death, and >90% of staurosporine-treated *Apaf-1$^{-/-}$* IFETs died within 12 h. A low concentration (50 µM) of caspase inhibitor (Q-VD-OPh)[17] efficiently blocked the staurosporine-induced death of *Apaf-1$^{-/-}$* as well as of wild-type IFETs (Figure 1b), although Q-VD-OPh failed to rescue the staurosporine-induced cell-cycle arrest (Figure 1c). Not only procaspase-3 but also other caspases such as procaspase-2, -6, -7, -8, and -9 were efficiently processed into its active form upon staurosporine treatment without Apaf-1 (Figure 1d), in sharp contrast to the lack of processing of procaspases by etoposide without Apaf-1. The concentration of staurosporine required to activate Caspase-3 in *Apaf-1$^{-/-}$* IFETs seems to be higher than that required for the wild-type IFETs, but 0.1 µM staurosporine could still activate Caspase-3 in *Apaf-1$^{-/-}$* IFETs in a time-dependent manner (Supplementary Figure S3).

During apoptotic cell death, poly(ADP)ribose polymerase (PARP) is cleaved, and phosphatidylserine is exposed to the cell surface.[18,19] Staurosporine, but not etoposide, caused PARP processing (Figure 1d) and phosphatidylserine exposure without Apaf-1 (Figure 1e), confirming that executor caspases were activated by staurosporine through an Apaf-1-independent mechanism.

### No requirement for Caspase-9, Bax/Bak, or Caspase-8 in staurosporine-induced cell death.

Caspase-8 and -9 are essential for executing the extrinsic and intrinsic pathways of apoptosis, respectively.[8,12] To examine the requirement for Caspase-9 in staurosporine-induced activation of Caspase-3, IFETs were established from *Caspase-9$^{-/-}$* fetal thymocytes (Supplementary Figure S1C). As expected, etoposide could not activate Caspase-3 in the absence of Caspase-9, but staurosporine readily activated Caspase-3 without Caspase-9 (Figure 2a). Accordingly, overexpression of Caspase-9 in *Caspase-9$^{-/-}$* IFETs had little effect on staurosporine-induced Caspase-3 activation (data not shown). IFETs were then established from fetal thymocytes of *Bax$^{-/-}$ Bak$^{-/-}$* embryos[20] (Supplementary Figure S1D). Staurosporine but not etoposide activated Caspase-3 in the *Bax$^{-/-}$ Bak$^{-/-}$* IFETs (Figure 2a), and caused phosphatidylserine exposure. The caspase inhibitor, Q-VD-OPh, efficiently blocked staurosporine-induced phosphatidylserine exposure in *Bax$^{-/-}$ Bak$^{-/-}$* IFETs, but could not rescue staurosporine-induced cell-cycle arrest (Figure 2b). The release of cytochrome *c* is one of the major characteristics of the mitochondrial pathway of apoptosis.[21] When the wild-type and *Apaf-1$^{-/-}$* IFETs were treated with staurosporine, the release of cytochrome *c* was observed, but there was no release of cytochrome *c* in *Bax$^{-/-}$ Bak$^{-/-}$* IFETs (Figure 2c), indicating that cytochrome *c* was not involved in the new apoptosis pathway. Accordingly, transformation of *Apaf-1$^{-/-}$* IFETs with human BCL-2 had no effect on staurosporine-induced Caspase-3 activation in *Apaf-1$^{-/-}$* IFETs (Supplementary Figure S4). These results confirmed that staurosporine could activate Caspase-3 in IFETs without mitochondrial apoptotic pathway.

To examine the involvement of the extrinsic pathway of apoptosis in staurosporine-induced caspase activation, *Caspase-9$^{-/-}$* IFETs were stably transformed with CrmA, a cowpox virus protein that inhibits Caspase-1 and -8.[15] At their early developmental stage, T cells do not express Fas.[22,23] Thus, as expected, Fas was not expressed in *Caspase-9$^{-/-}$* IFETs, and the IFETs were resistant to FasL-induced apoptosis (data not shown). When the *Caspase-9$^{-/-}$* IFETs were transformed with Fas (Supplementary Figure S5), the FasL treatment efficiently activated Caspase-3, and this activation was inhibited by CrmA (Figure 2d). On the other hand, staurosporine activated Caspase-3 even in the presence of CrmA in *Caspase-9$^{-/-}$* IFETs (Figure 2d). These results indicated that staurosporine causes a caspase-dependent cell death that does not use the mitochondrial or the death receptor pathway.

Various reagents activate Caspase-3 to induce apoptotic cell death. As shown in Figure 3, treatment of wild-type IFETs with 2.5 µg/ml tunicamycin, 3 µg/ml actinomycin D, 10 µg/ml cycloheximide, or γ-ray (5 Gy) irradiation efficiently activated Caspase-3, and the cells were dead within 48 h in most cases (data not shown). In contrast, IFETs lacking *Apaf-1*, *Caspase-9*, or *Bax/Bak* were completely resistant to apoptosis induced by these reagents (Figures 3a–c, and e). Similarly, Caspase-3 was not activated when *Bax$^{-/-}$ Bak$^{-/-}$* IFETs were treated with γ-rays (Figure 3d). These results indicated that most apoptosis-inducing reagents, such as DNA-damaging agents and protein and RNA synthesis inhibitors, required the mitochondrial pathway to activate Caspase-3.
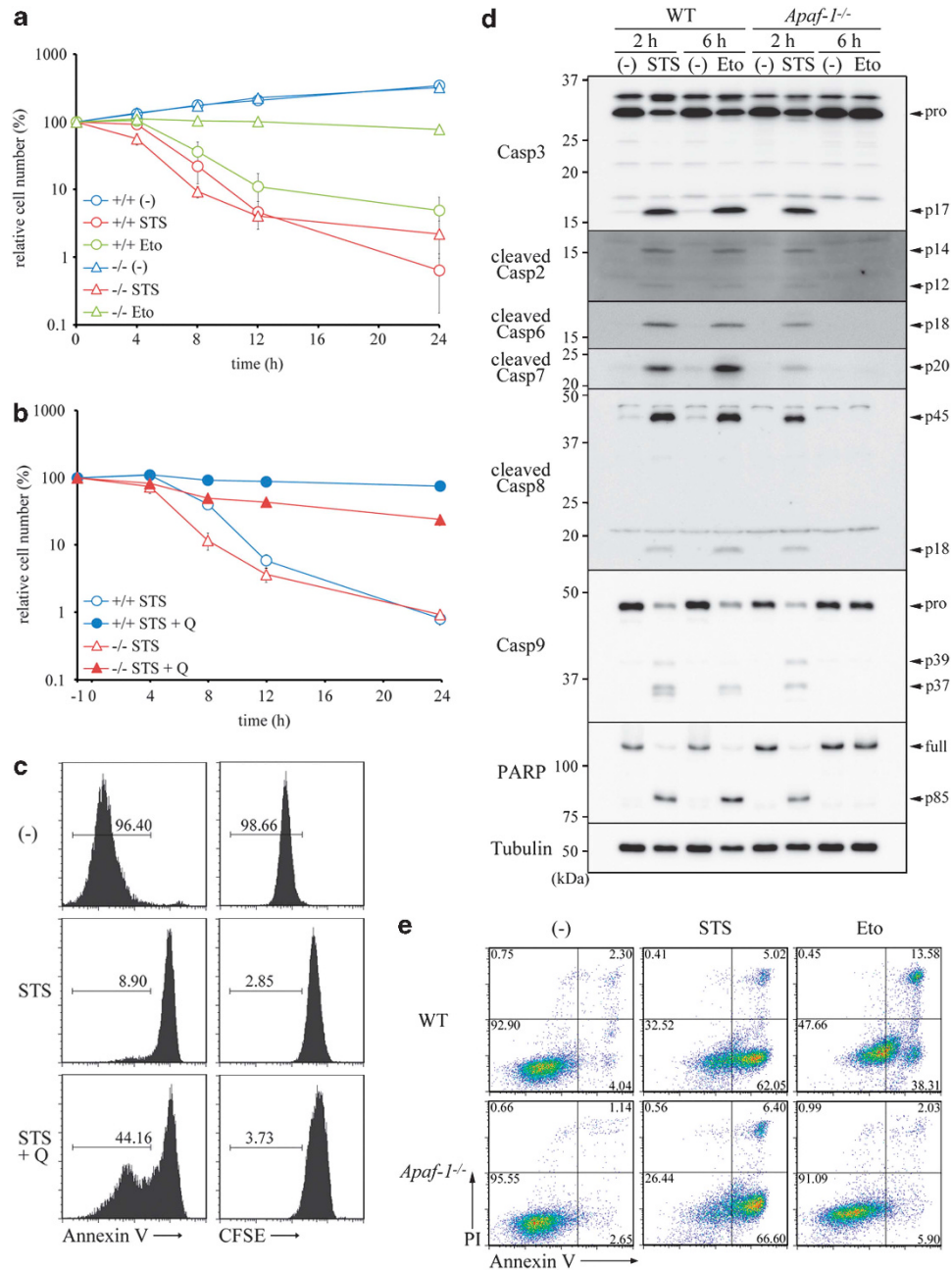
Figure 1 Staurosporine-induced *Apaf-1*-independent apoptosis. (**a**) Apaf-1-independent cell death induced by staurosporine but not etoposide. *Apaf-1$^{+/+}$* ($+/+$) and *Apaf-1$^{-/-}$* ($-/-$) IFETs were cultured with 10 μM staurosporine (STS) or 50 μM etoposide (Eto). At the indicated time, living cells were counted by Trypan blue exclusion assay, and expressed as a percentage of the initial cell number. The experiments were carried out independently for three times, and the average number was plotted with S.D. (bars). (**b**) Caspase-dependent Apaf-1-independent cell death induced by staurosporine. *Apaf-1$^{+/+}$* ($+/+$) and *Apaf-1$^{-/-}$* ($-/-$) IFETs were pretreated with 50 μM Q-VD-OPh (Q) for 1 h, and cultured with or without 10 μM STS and 50 μM Q-VD-OPh for the indicated times. Living cells were counted by Trypan blue exclusion assay, and expressed as a percentage of the initial cell number. Experiments were performed independently for three times, and the average values were plotted with S.D. (bars). (**c**) No effect of caspase inhibitor on the clonogenicity of IFETs treated with staurosporine. The CFSE-labeled *Apaf-1$^{-/-}$* IFETs were cultured in the absence or presence of Q-VD-OPh, and treated with 10 μM STS for 30 min. The cells were further cultured for 2 days in medium with or without Q-VD-OPh, stained with Annexin V/Cy5, and analyzed by FACSCalibur for CFSE and Annexin V. (**d**) *Apaf-1*-independent caspase activation by staurosporine but not etoposide. *Apaf-1$^{+/+}$* (WT) and *Apaf-1$^{-/-}$* IFETs were treated with 10 μM STS for 2 h or 50 μM Eto for 6 h. Cell lysates were analyzed by western blot with anti-Caspase-3, anti-Caspase-2, anti-cleaved Caspase-6, anti-cleaved Caspase-7, anti-cleaved Caspase-8, anti-Caspase-9, anti-PARP or anti-α-tubulin Abs. Except for the western blot for PARP and Caspase-9, where the cell lysates were separated by 7.5 or 10% SDS-PAGE, respectively, the cell lysates were separated by 10–20% SDS-PAGE. Arrows indicate the pro- and activated forms of Caspase, and noncleaved (113 kDa) and cleaved PARP (85 kDa). (**e**) *Apaf-1*-independent phosphatidylserine exposure. *Apaf-1$^{+/+}$* (WT) and *Apaf-1$^{-/-}$* IFETs were treated with 10 μM STS for 2 h or with 50 μM Eto for 8 h, stained with Cy5-conjugated Annexin V and PI, and analyzed by FACS. Cells with no treatment ($-$) were analyzed similarly, and FACS profiles are shown at left. Numbers indicate the percentage of the cells in each quadrant
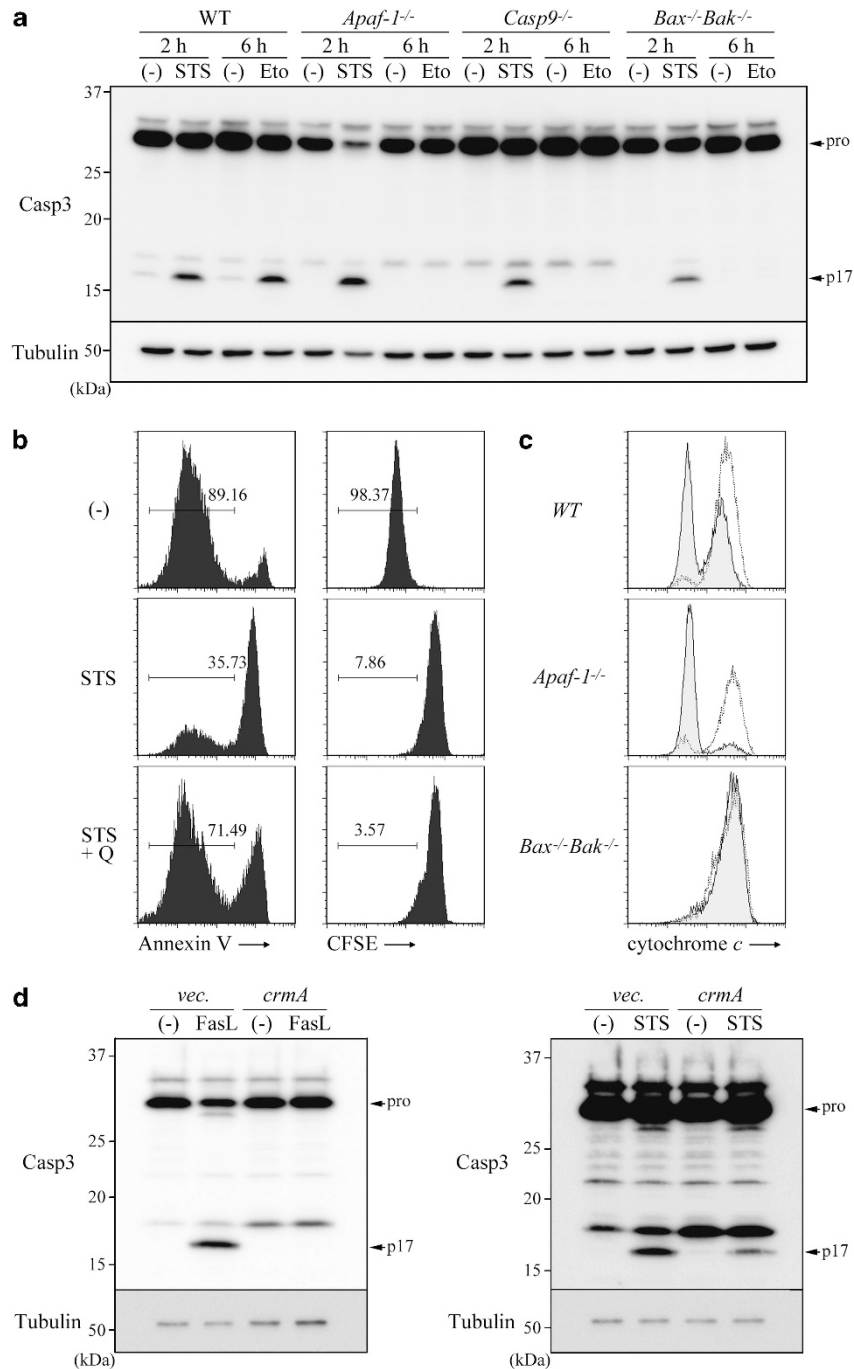
Figure 2 No requirement for the mitochondrial or death receptor apoptosis pathway for the staurosporine (STS)-induced Caspase activation. (**a**) Requirement of the mitochondrial pathway for etoposide-induced but not STS-induced Caspase-3 activation. Wild-type (WT), $Apaf-1^{-/-}$, $Caspase-9^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ IFETs were treated with $10\,\mu M$ STS for 2 h or with $50\,\mu M$ etoposide (Eto) for 6 h. The cell lysates were analyzed by western blotting with anti-Caspase-3 or anti-tubulin Abs. Arrows indicate pro- and cleaved Caspase-3. (**b**) No effect of caspase inhibitor on the clonogenicity of IFETs treated with STS. The CFSE-labeled $Bax^{-/-}Bak^{-/-}$ IFETs were cultured in the absence or presence of Q-VD-OPh, and treated with $10\,\mu M$ STS for 30 min. The cells were further cultured for 2 days in medium with or without Q-VD-OPh, stained with Annexin V/Cy5, and analyzed by FACSCalibur for CFSE and Annexin V. (**c**) No requirement of the cytochrome c release from mitochondria in STS-induced Caspase-3 activation. The WT, $Apaf-1^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ IFETs were treated with $10\,\mu M$ STS, permeabilized with digitonin, fixed with parafolmaldehyde, and stained with anti-cytochrome c (straight line), followed by FACS analysis. The cytochrome c staining profile before STS treatment is shown by dotted line. (**d**) No requirement for the death receptor pathway in STS-induced Caspase-3 activation in IFETs. Fas-$Casp9^{-/-}$ IFETs transformed with empty vector or CrmA were treated with 6.6 U/ml Fas ligand (FasL; left) or with $10\,\mu M$ STS (right) for 2 h. The cell lysates were analyzed by western blotting with anti-Caspase-3 and anti-α-tubulin Abs. Arrows indicate pro- and cleaved Caspase-3
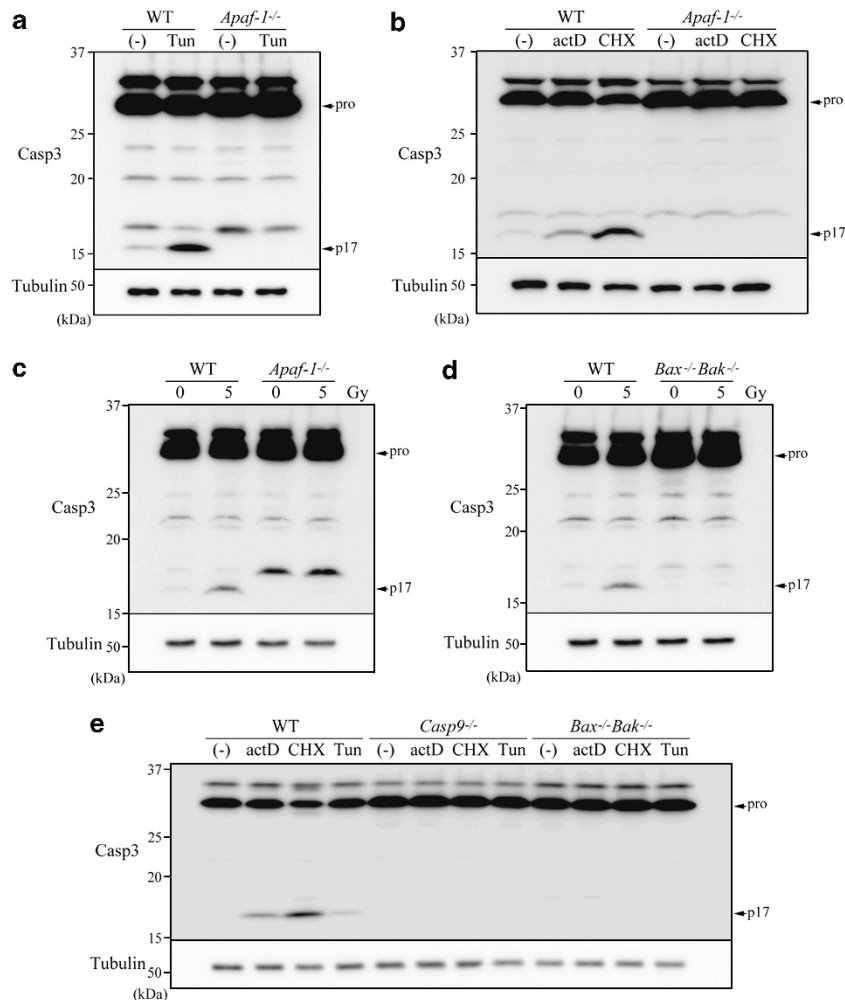
Figure 3 Requirement of the mitochondrial pathway for the tunicamycin-, actinomycin D-, cycloheximide-, and γ-ray-induced caspase activation in IFETs. Wild-type, $Apaf-1^{-/-}$ (a–c), $Caspase\ 9^{-/-}$ (e), or $Bax^{-/-}\ Bak^{-/-}$ (d and e) IFETs were treated with 2.5 μg/ml tunicamycin (Tun) for 6 h (a and e), 3 μg/ml actinomycin D (actD), 10 μg/ml cycloheximide (CHX) for 2 h (b and e), or 5 Gy γ-irradiation (c and d), and incubated at 37 °C for 4 h. The cell lysates were analyzed by western blotting with anti-Caspase-3 or anti-α-tubulin Abs. Arrows indicate pro- and cleaved Caspase-3

Effect of phosphatase inhibitors on staurosporine-induced cell death. Staurosporine is a multikinase inhibitor, but it also inhibits other enzymes such as ABC transporters and topoisomerase.[24,25] Thus, it is unclear whether the inhibition of a kinase or other targets led to the induction of Apaf-1-independent apoptosis. To address this question, we investigated whether phosphatase inhibitors suppressed staurosporine-induced apoptosis in $Apaf-1^{-/-}$ IFETs. As shown in Figure 4, okadaic acid, which is a serine/threonine phosphatase inhibitor, suppressed staurosporine-induced Caspase-3 activation (Figure 4a) and cell death in $Apaf-1^{-/-}$ IFETs (Figure 4b). On the other hand, sodium orthovanadate, a tyrosine phosphatase inhibitor, had only a small effect on staurosporine-induced Caspase-3 activation and cell death in $Apaf-1^{-/-}$ IFETs (Figure 4a and data not shown). These results suggest that staurosporine induces Apaf-1-independent apoptosis by inhibiting serine/threonine kinase(s); however, we cannot formally rule out the possibility that the phosphatase inhibitors simply enhanced an antiapoptotic pathway.

To confirm that inhibition of kinases induced apoptotic cell death, we examined the effect of various kinase inhibitors on apoptotic cell death. We used staurosporine analogs that had different specificities for target kinases,[24,25] H-7 and its derivatives,[26] and inhibitors of MAPK and PI3K signaling pathways. Among the 10 staurosporine analogs tested, 4 (UCN-01, GF109203X, Ro31-8220, and K252a) activated Caspase-3 in $Apaf-1^{-/-}$ IFETs at 2.5–50 μM, whereas the others had no effect, at least at the same concentration (Figure 4c). The treatment of $Apaf-1^{-/-}$ IFETs with 1.0 mM H-7, H-8, or H-9 did not kill them, but the derivative of H-8, H-89, efficiently killed $Apaf-1^{-/-}$ IFETs and activated Caspase-3 at 50 μM. On the other hand, none of the MAPK/PI3K inhibitors tested (U0126, SP600125, SB239063, and LY294002) activated Caspase-3 in $Apaf-1^{-/-}$ IFETs (Supplementary Figure S6).

Cell-specific mitochondrial death pathway-independent Caspase-3 activation by staurosporine. To examine whether the new apoptosis pathway that was independent
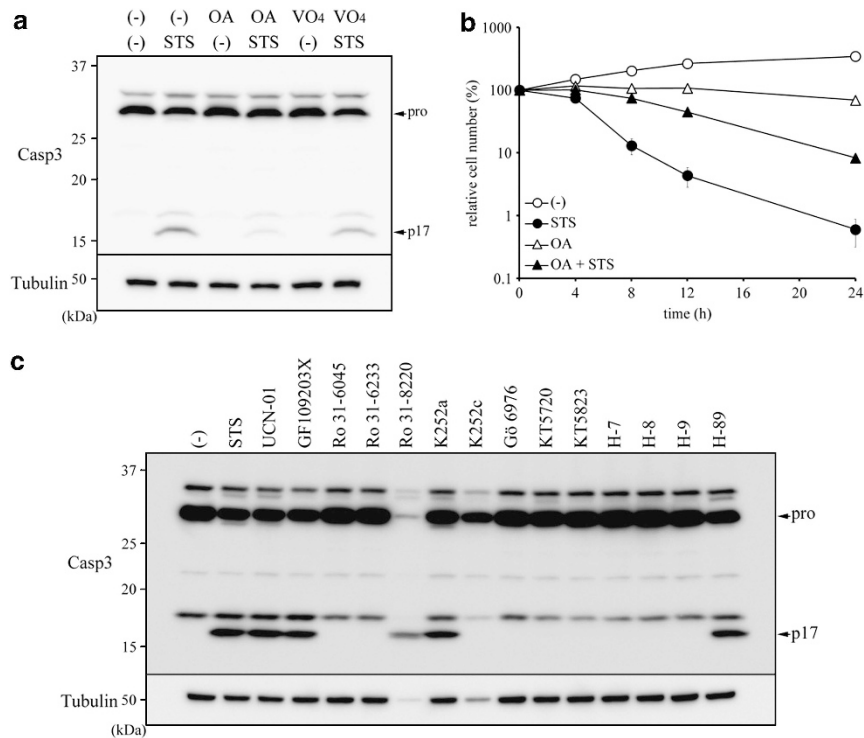
Figure 4 Involvement of serine/threonine kinase in the staurosporine (STS)-induced Apaf-1-independent apoptosis. (**a**) Effect of phosphatase inhibitors on the STS-induced Apaf-1-independent Caspase-3 activation. $Apaf-1^{-/-}$ IFETs were treated with 10 μM STS and 1 μM okadaic acid (OA) or 1 mM orthovanadate (VO$_4$) for 2 h. The cell lysates were analyzed by western blotting with anti-Caspase-3 or anti-α-tubulin Abs. Arrows indicate pro- and cleaved Caspase-3. (**b**) Effect of OA on STS-induced Apaf-1-independent cell death. $Apaf-1^{-/-}$ IFETs were cultured without or with 10 μM STS or 1 μM OA, or both. At the indicated times, living cells were counted by Trypan blue exclusion assay, and are expressed as a percentage of the initial cell number. Experiments were carried out independently for three times, and the average values are plotted with S.D. (bars). (**c**) Apaf-1-independent Caspase-3 activation by kinase inhibitors. $Apaf-1^{-/-}$ IFETs were treated at 37 °C for 2 h with 10 μM STS or its derivatives (25 μM UCN-01, 50 μM GF109203X, 50 μM Ro31-6045, 50 μM Ro31-6233, 25 μM Ro31-8220, 2.5 μM K252a, 25 μM K252c, 50 μM Gö 6976, 50 μM KT5720, or 50 μM KT5823) or H-series kinase inhibitors (1 mM H-7, H-8, or H-9, or 50 μM H-89). The cell lysates were analyzed by western blotting with anti-Caspase-3 or anti-α-tubulin Abs

from the death receptor and mitochondrial pathways was present in other cell types, $Apaf-1$-null embryonic fibroblasts were prepared. As shown in Figure 5a, treatment of the primary wild-type mouse embryonic fibroblasts (MEFs) with 10 μM staurosporine generated active Caspase-3, whereas no active Caspase-3 was observed with $Apaf-1^{-/-}$ MEFs, indicating that MEFs carry only the canonical apoptotic pathway.

To examine whether the noncanonical pathway was present in adult hematopoietic and lymphoid cells, $Tie2Cre^{+}$ $Bax^{fl/fl}Bak^{-/-}$ mice,[27–29] in which the floxed allele of the Bax gene was deleted in hematopoietic and endothelial cells by Cre-mediated recombination (Supplementary Figure S7A), were analyzed. Western blotting analysis showed that adult thymocytes, bone marrow cells, and splenocytes from $Bax^{fl/fl}Bak^{-/-}$ mice at the age of 7–9 weeks contained the 21-kDa Bax. In contrast, the thymocytes, bone marrow cells, and splenocytes from the $Tie2Cre^{+} Bax^{fl/fl}Bak^{-/-}$ mice did not show this band (Figures 5c–e), indicating that the Bax gene was fully deleted in these cells. As expected from the null mutation of Bak, the thymocytes, bone marrow cells, and splenocytes from the $Bax^{fl/fl}Bak^{-/-}$ mice did not carry the 28-kDa Bak (Supplementary Figure S7B). Thymocytes from $Bax^{fl/fl}Bak^{-/-}$ mice spontaneously die, and this death process was accelerated by treating with staurosporine

(Figure 5b). The lack of Bax/Bak in $Tie2Cre^{+} Bax^{fl/fl}Bak^{-/-}$ strongly inhibited the spontaneous death, yet staurosporine killed the $Tie2Cre^{+} Bax^{fl/fl}Bak^{-/-}$ thymocytes.

When the thymocytes, bone marrow cells, and splenocytes from the $Bax^{fl/fl}Bak^{-/-}$ mice were treated with staurosporine or etoposide, both reagents produced the active form of Caspase-3 (Figures 5c–e), as in wild-type fetal thymocytes. Deleting Bax and Bak in the $Tie2Cre^{+} Bax^{fl/fl}Bak^{-/-}$ mice completely abolished the ability of etoposide to activate Caspase-3 in these cells. On the other hand, staurosporine could still activate Caspase-3 in the absence of Bax and Bak. These results indicated that the noncanonical apoptosis pathway is present in lymphoid and myeloid cells such as thymocytes, splenocytes, and bone marrow cells, but not in fibroblasts.

Discussion

Caspase-dependent apoptotic cell death has been thought to be mediated by two pathways: the intrinsic mitochondrial pathway and the extrinsic death receptor pathway.[30] Here we showed that staurosporine, a nonselective inhibitor of diverse kinases,[24] could activate Caspase-3 to kill cells via two different pathways. One pathway required $Apaf-1$, whereas the other did not. The $Apaf-1$-independent caspase activation
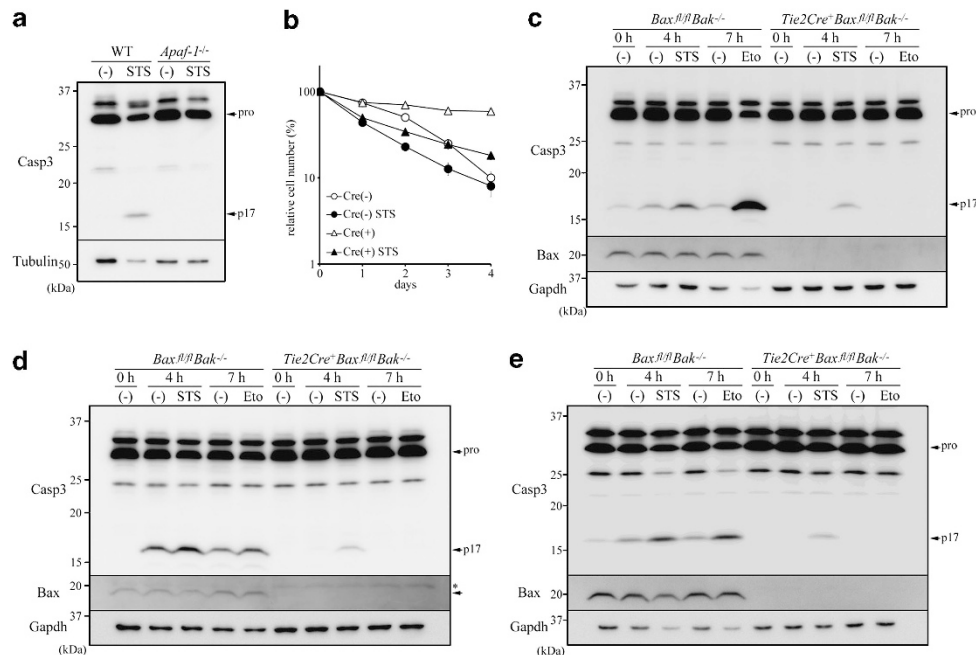
Figure 5 Tissue specificity of the noncanonical apoptotic pathway. Wild-type and $Apaf-1^{-/-}$ embryonic fibroblasts (MEFs) at passage 3 (**a**), and thymocytes (**b** and **c**), bone marrow cells (**d**), and splenocytes (**e**) from $Bax^{fl/fl}Bak^{-/-}$ or $Tie2Cre^+ Bax^{fl/fl}Bak^{-/-}$ mice were treated with 10 μM staurosporine (STS) for 4 h, or 50 μM etoposide (Eto) for 7 h. In (**a**), (**c**), (**d**), and (**e**), the cell lysates were analyzed by western blotting with anti-Caspase-3, anti-Bax, anti-tubulin, or anti-GAPDH Ab. Arrows indicate pro- and cleaved Caspase-3. In (**b**), at the indicated times, living cells were counted by Trypan blue exclusion assay, and are expressed as a percentage of the initial cell number. Experiments were carried out independently for three times, and the average values are plotted with S.D. (bars)

did not require *Bax/Bak* or *Caspase-9*, and was not inhibited by Bcl-2, and occurred without the release of cytochrome *c* from mitochondria, suggesting that the mitochondrial pathway was not involved in this death process. CrmA, an inhibitor of Caspase-8,[15] also had little effect on Apaf-1-independent caspase activation, suggesting that the death receptor pathway was also not involved in this process. Recently, the activation of inflammasomes by pathogens was shown to cause Caspase-1-dependent cell death of macrophages and dendritic cells, which is called pyroptosis.[31,32] CrmA inhibits not only Caspase-8 but also Caspase-1,[15] suggesting that the staurosporine-induced apoptotic pathway identified here was not pyroptosis.

The *Apaf-1-* and *Caspase-1/8-*independent noncanonical apoptotic pathway was activated by various kinase inhibitors, such as staurosporine, UCN-01, a staurosporine-derivative, and H-89. The activation of Caspase-3 by these kinase inhibitors was inhibited by okadaic acid, a phosphatase inhibitor. A simple idea is that growing cells carry a phosphatase(s) that leads to the activation of Caspase-3, and specific kinases prevent the phosphatase-induced Caspase-3 activation by keeping its target phosphorylated. Phosphatase inhibitors were previously shown to inhibit the mitochondrial and death receptor pathways,[33] in which phosphatases seem to increase the death signaling that requires dephosphorylation. Therefore, it is possible that cells carry proapoptotic molecule(s) that has the potential to induce caspase-dependent apoptosis if it is not phosphorylated. Thus, a kinase(s) in growing cells may prevent the dephosphorylation of this molecule, and kinase inhibitors block this

process to generate active proapoptotic molecule(s). Phosphatase inhibitors could prevent dephosphorylation of the proapoptotic molecule, or enhance antiapoptotic pathway such as the NF-κB pathway, by keeping the signaling molecules phosphorylated. Staurosporine, H-8, and their derivatives have different target specificities.[24,26,34] A more detailed analysis of the death-inducing ability of various kinase inhibitors may help elucidate the noncanonical death signaling pathway.

Staurosporine was originally found as a molecule that induces a Bcl-2-inhibitable apoptotic death, suggesting it causes cell death via the mitochondrial pathway.[35,36] In fact, Yoshida et al.,[11] by preparing *Apaf-1*-deficient mice, showed that staurosporine cannot activate caspase in the absence of *Apaf-1*. On the other hand, Stepczynska et al.[37] suggested that a mitochondrial-independent pathway was responsible for staurosporine-induced apoptosis because a dominant-negative Caspase-9 cannot inhibit it. Manns et al.[38] recently claimed that a noncanonical staurosporine-induced apoptosis pathway requires Caspase-9 but not Apaf-1. In this report, we showed that staurosporine could activate caspase-dependent apoptosis via two different intrinsic pathways. The first, probably present ubiquitously, is the canonical mitochondrial pathway that requires Bax/Bak, Apaf-1, and Caspase-9. The second pathway does not require the components for the mitochondrial pathway, and seems to work in a cell-type-specific manner. That is, staurosporine strongly activates the second pathway in the lymphocytes, but not embryonic fibroblasts, which apparently do not express the necessary components. This cell specificity of the noncanonical intrinsic

apoptotic pathway may explain the previous controversial results on staurosporine-induced cell death, which were obtained with different cell lines and tissues.[11,37,38]

Fetal thymocytes that lacked the canonical mitochondrial apoptotic pathway, and the cell lines derived from them, efficiently underwent caspase-dependent apoptosis by staurosporine treatment, which agrees with the observation that many apoptotic cells are present in *Apaf-1*-null E14.5 fetal thymus.[14] These apoptotic cells are observed in E14.5 but not E17.5 fetal thymus of *Apaf-1*-null embryos. In the E14.5 fetal thymus, most of the thymocytes are at the CD4$^-$CD8$^-$ stage, whereas in the E17.5 or neonate thymus, most of the thymocytes are at the CD4$^+$CD8$^+$, CD4$^+$CD8$^-$, or CD4$^-$CD8$^+$ stage. Three major selection processes occur during thymocyte development. In one of them, β-selection, immature thymocytes that productively rearrange the gene segments of the TCRβ locus undergo proliferative expansion and mature to the CD4$^+$CD8$^+$ stage, whereas those failing to do so die by apoptosis.[39] It is tempting to speculate that the noncanonical *Apaf-1*- and *Caspase-1/8*-independent apoptotic pathway is involved in the apoptosis accompanying β-selection. In any case, staurosporine and its derivatives (in particular, UCN-01) are under clinical trial as anticancer drugs.[40,41] Understanding how these kinase inhibitors cause apoptotic cell death will be essential for developing efficient treatment paradigms.

Materials and Methods

Mice. C57BL/6 mice were purchased from Nippon SLC (Hamamatsu, Japan). The *Apaf-1*$^{-/-}$[11] and *Caspase-9*$^{-/-}$[12] mice were maintained on a C57BL/6 background, and the *Bax*$^{-/-}$*Bak*$^{-/-}$[20,42] and *Tie2Cre*$^+$*Bax*$^{fl/fl}$*Bak*$^{-/-}$[28,29] mice were on a mixed genetic background between C57BL/6 and 129/Sv. The mice were housed in a specific pathogen-free facility at the Kyoto University, Graduate School of Medicine, and all animal experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee of the Kyoto University Graduate School of Medicine. To determine the genotype of the *Apaf-1*, *Caspase-9*, *Bax*, *Bak*, and *Tie2Cre* alleles, DNA from embryonic tissues or adult tail-snip tissue was analyzed using PCR. For *Apaf-1*, wild-type (5′-CTCAAACACCTCCTCCACAA-3′) and mutant-specific (5′-GGGCCAGCTC ATTCCTC-3′) sense primers were used with a common antisense primer (5′-G TCATCTGGAAGGGCAGCGA-3′). For *Caspase-9*, 5′-AGGCCAGCCACCTC CAGTTCC-3′ and 5′-CAGAGATGTGTAGAGAAGCCCACT-3′ for wild-type, and 5′-CGTGCTACTTCCATTTGTCACGTC-3′ and 5′-ACACCCTTGCAAGTCAGAGG TATG-3′ for the mutant were used. For *Bax*, wild-type (5′-GAGCTGATCAGAAC CATCATG-3′) and mutant-specific (5′-CCGCTTCCATTGCTCAGCGG-3′) sense primers and a common antisense primer (5′-GTTGACCAGAGTGGCGTAGG-3′) were used. For the floxed allele of the *Bax* gene, wild-type (5′-C TGGGGCGCGCGGATCCATTCCCACCG-3′) and mutant-specific (5′-TACGA AGTTATTAGGTCTGAAGAGGAG-3′) sense primers were used with a common antisense primer (5′-CCCTAGTAGTGACAAGTAGCATGGAAG-3′). For *Bak*, wild-type (5′-GGTGTCCACACTAGAGAACTACTC-3′) and mutant-specific (5′-CTATC AGGACATAGCGTTGG-3′) sense primers were used with a common antisense primer (5′-GAGCCATGCGAAGATGTTTTAGC-3′). For the *Tie2Cre* allele, a sense primer in the *Tie2* promoter region (5′-CCCTGTGCTCAGACAGAAATGAGA-3′) was used with an antisense primer in the *Cre* coding region (5′-CGCATAAC CAGTGAAACAGCATTGC-3′).

Cells, antibodies, and reagents. MEFs were prepared from E14.5 mouse embryos as previously described,[43] and cultured in DMEM containing 10% FCS (Gibco, Grand Island, NY, USA). Plat-E cells[44] were cultured in DMEM containing 10% FCS, 1 μg/ml puromycin (Clontech, Palo Alto, CA, USA), and 10 μg/ml blasticidin S (Invitrogen, Carlsbad, CA, USA).

Rat anti-mouse CD16/CD32 monoclonal antibody (mAb; 2.4G2; Mouse BD Fc Block), FITC-conjugated rat anti-mouse Thy1.2 mAb (53-2.1), PE-conjugated rat anti-mouse CD25 mAb (7D4), Cy-Chrome-conjugated rat anti-mouse CD44 mAb (IM7), PE-conjugated hamster anti-mouse Fas mAb (Jo2), and mouse anti-PARP mAb (C2-10) were purchased from BD PharMingen (San Diego, CA, USA). Rabbit mAbs against Caspase-3 (8G10), GAPDH (14C10), rabbit polyclonal antibodies against cleaved caspase-6, cleaved caspase-7, and mouse cleaved caspase-8 were from Cell Signaling (Danvers, MA, USA). Rabbit anti-caspase-2 was from Abcam (Cambridge, MA, USA), and mouse anti-caspase-9 mAb (5B4) was purchased from Medical Biological Laboratories (Nagoya, Japan). Mouse anti-α-tubulin mAb (Ab-1) was from Calbiochem (San Diego, CA, USA); rabbit anti-Bax (N-20) Ab and anti-Bcl-2 (C 21) Ab were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-Bak (NT) Ab was from Millipore (Temecula, CA, USA); and HRP-conjugated goat anti-rabbit and anti-mouse Ig Abs were from Dako (Copenhagen, Denmark). FITC-conjugated mouse anti-cytochrome *c* mAb (6H2.B4) was purchased from BioLegend (San Diego, CA, USA).

Leucine-zipper-tagged human FasL was prepared as previously described.[45] Cytochrome *c* from bovine heart, tunicamycin, cycloheximide, etoposide, sodium orthovanadate, K252a, UCN-01, SB239063, and LY294002 were purchased from Sigma-Aldrich (St Louis, MO, USA). Actinomycin D, 4-(2–aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF), and okadaic acid were from Wako Pure Chemical (Osaka, Japan). The Caspase inhibitor, Q-VD-OPh, was from R&D Systems (Minneapolis, MN, USA). GF109203X, Ro31-8220, Ro31-6045, Ro31-6233, K252c, KT5823, and H-7 were from Enzo Life Sciences (Farmingdale, NY, USA), H-9 was from Santa Cruz, U0126 was from Promega (Madison, WI, USA), Gö 6976 was from LC Laboratories (Woburn, MA, USA), KT5720 and SP600125 were from Calbiochem, and H-89 was obtained from Cayman Chemical (Ann Arbor, MI, USA).

Plasmids. The pCX4pur, pCX4bsr, pCX4hyg, pCX4pur/H-*ras*^{V12}, and pCX4bsr/c-*myc* retroviral vectors[46] were provided by Dr. Tsuyoshi Akagi (KAN Research Institute, Kobe, Japan). The pCX4 vector was constructed by removing the drug selection marker. The coding sequence of mouse Fas[47] was inserted into pCX4bsr (pCX4bsr/mFas), whereas the coding sequence of *CrmA*[48,49] was inserted into pCX4pur (pCX4pur/crmA). The human BCL-2-expression plasmid, pEF-BOS/BCL-2, was as previously described.[50]

Production of retroviruses. To produce retrovirus, Plat-E cultured in DMEM containing 10% FCS was transfected with expression vector using FuGENE 6 (Roche Applied Science, Basel, Switzerland). After culturing at 37 °C for 48 h, the supernatant was collected, passed through a 0.45-μm filter unit (Millipore, Billerica, MA, USA), and centrifuged at 6000 $\times$ *g* for 16 h at 4 °C.[51] After centrifugation, the pellet was resuspended in a 1/10 original volume of the culture medium and stored at -80 °C.

Establishment of IFETs. Cells from fetal thymus were immortalized by infection with retroviruses carrying H-*ras*^{V12} and c-*myc*. That is, fetal thymocytes were cultured in the presence of IL-7,[52] and then infected with retrovirus using RetroNectin kit (Takara Bio, Shiga, Japan) coupled with centrifugation, as recommended by the supplier. In brief, the wells of a 96-well plate (flat-bottom, BD Falcon) were coated with 6 μg of RetroNectin at 4 °C overnight, and then incubated at room temperature for 30 min in PBS containing 2% BSA. The retroviruses were added to the wells, and the plate was centrifuged at 2000 $\times$ *g* for 2 h at room temperature, and washed with PBS. Primary E14.5 mouse fetal thymocytes were suspended in culture medium (DMEM containing 10% FCS, 1 $\times$ NEAA (non-essential amino acids), 50 μM 2-mercaptoethanol, and 10 mM HEPES-NaOH (pH 7.4)) supplemented with 5 ng/ml mouse IL-7 (PeproTech, Rocky Hill, NJ, USA) and added to the wells. The cells were attached to the wells by centrifuging at 400 $\times$ *g* for 5 min, and the plate was incubated for 3 days at 37 °C. E14.5 *Apaf-1*$^{+/+}$ and *Apaf-1*$^{-/-}$ fetal thymocytes were infected with retroviruses from pCX4pur/H-*ras*^{V12} and pCX4bsr/c-*myc*. Other thymocytes were infected with retroviruses from pCX4/H-*ras*^{V12} and pCX4/c-*myc*. A single clone of each genotype was obtained by limiting dilution.

Transfection of IFETs. To express mouse Fas and CrmA, 1 $\times$ 10^5 IFETs in 12-well flat-bottom plates were suspended in culture medium containing retrovirus and 10 μg/ml polybrene (Sigma-Aldrich), centrifuged at 1000 $\times$ *g* for 1 h at room temperature,[53,54] and cultured at 37 °C for 24 h. Puromycin or blasticidin was added to the culture at a final concentration of 5 or 50 μg/ml, and the cells were further incubated for 7 or 10 days, respectively. The BCL-2 expression plasmid, pEF-BOS/BCL-2, was introduced into IFETs by electroporation together with

hygromysin-resistant gene pCX4hyg, and the stable transformants were selected by culturing in the presence of 4 mg/ml hygromycin B (Wako Pure Chemical).

FACS analysis. To examine the expression of surface antigens, 1×10^5 IFETs in 96-well round-bottom plates were centrifuged at $300 \times g$ for 3 min at 4 °C, washed with FACS staining buffer (2% FCS and 0.02% NaN_3 in PBS), pretreated with Fc block, stained with fluorescent dye-conjugated antibodies, and analyzed by FACSCalibur (BD Biosciences, San Jose, CA, USA).

To detect phosphatidylserine exposed on apoptotic cells, 6.25×10^4 cells were incubated at room temperature for 15 min with 500-fold diluted Cy5-conjugated Annexin V (BioVision, Mountain View, CA, USA) in 0.5 ml of Annexin V staining buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$). Propidium iodide (PI) was added to a final concentration of 2.5 μg/ml, and the samples were incubated at room temperature for 5 min, and analyzed by FACSCalibur.[55]

Cell-free assay for Caspase-3 activation. The cytochrome c-induced activation of Caspase-3 was assayed essentially as described.[56] In brief, IFETs were harvested by centrifugation at $400 \times g$ for 5 min at 4 °C, washed with extraction buffer (50 mM PIPES-KOH (pH 7.0), 20 mM KCl, 5 mM EGTA, 1 mM DTT, and 10 mM AEBSF), and suspended in 0.8 volumes of the extraction buffer. After incubation on ice for 20–30 min, the cells were disrupted in a Dounce homogenizer (Wheaton, Millville, NJ, USA) with 100 strokes of a tight pestle. The cell lysates were subjected to centrifugation at $10\,000 \times g$ for 10 min at 4 °C, and the supernatant was further centrifuged at $100\,000 \times g$ for 1 h at 4 °C to obtain the S-100 fraction. Protein (100 μg) of the S-100 fraction was incubated at 37 °C for 30 min with 200 μM dATP and 4 μM cytochrome c in 25 μl of the extraction buffer. The reaction was stopped by placing the reaction mixture on ice, and the sample was mixed with an equal volume of 2 × SDS sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.02% BPB, and 10% 2-mercaptoethanol) for western blotting.

Assay for apoptosis. To induce apoptosis, IFETs, primary thymocytes and splenocytes, bone marrow cells treated with erythrocyte lysis buffer, and MEFs at passage 3 were suspended at 1×10^6, 1×10^7, 5×10^6, and 5×10^4 cells per ml in culture medium, respectively, and incubated at 37 °C with 10 μM staurosporine and 50 μM etoposide. To induce apoptosis by γ-rays, the cells were irradiated with 5 Gy using a ^{137}Cs gamma-irradiator (dose rate of 0.744 Gy/min in a Gammacell 40 Exactor, Nordion International, Kanata, Ontario, Canada), and incubated at 37 °C. Viable cells were counted by Trypan blue exclusion assay.

Western blot analysis. Cells were harvested by centrifugation at $1100 \times g$ for 5 min at 4 °C, washed with PBS, suspended at 1.0–10×10^7 cells/ml in RIPA buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP40, 0.1% SDS, and 0.5% sodium deoxycholate) containing a protease inhibitor cocktail (cOmplete, Mini, Roche), and incubated on ice for 15 min. Samples were centrifuged at $13\,000 \times g$ for 15 min at 4 °C. The protein concentration of the supernatant was adjusted to 0.5 mg/ml with RIPA buffer, and the sample was mixed with an equal volume of 2 × SDS sample buffer. After incubating at 98 °C for 5 min, the proteins were separated by 10–20% gradient SDS-PAGE if not specified, and transferred to PVDF membranes (pore size: 0.22 or 0.45 μm, Millipore). After incubation at room temperature for 1 h with blocking buffer (TBST (Tris-buffered saline containing 0.05% Tween 20) supplemented with 5% non-fat dry milk), the membranes were stained with primary antibodies in blocking buffer or solution 1 (Can Get Signal Immunoreaction Enhancer Solution; Toyobo, Osaka, Japan) at room temperature for 1 h or at 4 °C overnight. After being washed with TBST, the membranes were incubated at room temperature for 1 h with HRP-conjugated secondary antibodies in blocking buffer or solution 2. The proteins recognized by the antibody were then visualized by a chemiluminescence reaction (Immobilon Western, Millipore) and detected by the LAS-4000 system (Fujifilm, Tokyo, Japan).

Clonogenic assay. After washing with PBS containing 0.1% BSA, IFETs were resuspended at a final concentration of 1×10^6 cells/ml in pre-warmed PBS containing 0.1% BSA and 10 μM CFSE (Molecular Probes, Eugene, OR, USA), and incubated at 37 °C for 10 min. The staining reaction was stopped by addition of 5 volumes of ice-cold IFET culture medium, and incubated on ice for 5 min. After washing twice with ice-cold culture medium, the CFSE-stained cells were pretreated with 50 μM Q-VD-OPh for 1 h in culture medium, and further treated at 37 °C for 30 min with 10 μM staurosporine in the presence of 50 μM Q-VD-OPh.

The cells were then washed with ice-cold culture medium, and cultured at 37 °C for 2 days in the culture medium containing 50 μM Q-VD-OPh. Then, the cells were stained at room temperature for 15 min with 1 : 1000 diluted Cy5/Annexin V in Annexin V staining buffer, and were analyzed by FACSCalibur.

Cytochrome c release assay. The apoptotic cytochrome c release was assayed as described previously.[57] In brief, 1×10^5 IFETs were treated at 37 °C for 2 h with 10 μM staurosporine, and treated for 5 min on ice with 50 μg/ml digitonin in 100 μl of PBS containing 100 mM KCl until >95% cells were permeabilized as assessed by Trypan blue exclusion. IFETs were washed with FACS staining buffer, fixed for 20 min at room temperature with 4% paraformaldehyde in PBS, washed three times, and incubated at room temperature for 1 h in blocking buffer (PBS containing 3% BSA and 0.05% saponin). The cells were then incubated for 1 h at room temperature in 1 : 200 diluted FITC-conjugated anti-cytochrome c mAb, washed, and analyzed by FACSCalibur.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)