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Synergistic Induction of Apoptosis by p53-inducible Bcl-2 Family Proteins Noxa and Puma

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Abstract

One critical tumor-suppressive function of p53 is the induction of apoptosis in oncogene-expressing cells. In this context, p53-inducible genes encoding the BH3-only proteins of the Bcl-2 family, Noxa and Puma, were identified. Gene knockout studies revealed that both Noxa and Puma are involved in apoptosis induction in oncogene-expressing cells. BH3-only proteins induce apoptosis, and activate the downstream apoptosis effectors Bax and Bak. In this study, we found that Noxa and Puma synergistically activate Bax and Bak, and induce apoptosis. Although Noxa activates Bak by inactivating Mcl-1 and Bcl-X_L, gene knockdown studies revealed that neither Mcl-1 nor Bcl-X_L is involved in this synergism. Moreover, Puma, but not Noxa, directly activated Bax in the absence of Bak, and Noxa enhanced Puma-mediated Bax activation in Bak-deficient cells. These results suggest the existence of a novel regulatory pathway for Noxa-mediated apoptosis. Although we detected synergistic induction of apoptosis by Noxa and Bim, a tumor suppressive transcriptional factor FoxO3-inducible protein, no such synergism was observed for other pairs of BH3-only proteins, Bim and Bid, or Bim and Puma. From these results, it can be considered that p53 carefully controls apoptosis by allowing two molecules to share full ability to induce apoptosis.

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Key words: p53, apoptosis, Noxa, Puma, mitochondria

Introduction

The tumor suppressor p53 functions as a transcriptional activator that induces the expression of genes regulating cell cycle, apoptosis, and DNA repair in response to various cellular stresses, such as DNA damage, hypoxia, and viral infection[1,2]. In many types of human cancer, p53 is inactivated by the mutation or deletion of *p53*[3], resulting in protection from p53-mediated apoptosis and cell cycle arrest. p53 selectively induces the apoptosis of oncogene-expressing fibroblasts such as adenovirus E1A and c-*myc*, and the replicative senescence of activated Ha-*ras*-expressing fibloblasts[1,2]. Recently, the activation of oncogenes has been shown to evoke a DNA-damage response, resulting in p53 activation[4,5]. More recently, the ectopic expression of oncogenes has been clearly shown to cause prematurely terminated DNA replication forks, DNA double-strand breaks, and DNA hyper-replication, resulting in the induction of DNA-damage responses

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to such aberrant DNA structures[6,7]. These findings suggest that the activation of oncogenes induces a DNA-damage response, i.e., "oncogenic stress", which activates p53 to eliminate such hazardous cells. Therefore, apoptosis induction may be an important function of p53 in tumor suppression. In this context, the molecular mechanism of p53-induced apoptosis was extensively analyzed, resulting in the identification of several p53 target genes encoding potential inducers of apoptosis, such as Noxa[8] and Puma (p53 upregulated modulator of apoptosis)[9,10].

Noxa and Puma are BH (Bcl-2 homology) 3-only proteins of the Bcl-2 family. Bcl-2 family proteins are critical regulators of apoptosis that reside immediately upstream of mitochondria. They possess conserved BH domains and are classified into "multidomain" and "BH3-only" proteins[11–13]. Among them, the proapoptotic multidomain members Bax and Bak function as mitochondrial executioners and directly open pores in the mitochondrial outer membrane, resulting in the release of apoptogenic factors, such as cytochrome c[11–13]. The released cytochrome c forms a complex with APAF-1 and caspase-9, termed the apoptosome. In this complex, caspase-9 is activated by protein cleavage and activates apoptosis effecter caspases, resulting in a widespread protein cleavage and apoptosis[14]. Analyses using mice lacking both *Bax* and *Bak* revealed that Bax and Bak are essential inducers of mitochondrion-mediated apoptosis in response to various stimulations[15–17]. In contrast, antiapoptotic multidomain proteins, such as Bcl-2, Bcl-X_L and Mcl-1, inhibit the pore formation of Bax and Bak. BH3-only proteins are critical for initiating apoptosis, functioning immediately upstream of multidomain proteins of Bcl-2 family[11–13]. BH3-only proteins are also divided into two classes; "activators" and "sensitizers"[18,19]. Bid and Bim are of the activator type, and induce the activation of Bax and Bak through direct binding. On the other hand, Noxa and Bad are of the sensitizer type and can not directly bind to Bax and Bak; however, they activate apoptosis by inhibiting antiapoptotic multidomain Bcl-2 members through direct binding. It is considered that activator-type BH3-only proteins directly activate Bax and Bak, resulting in cytochrome c release from mitochondria. In contrast, it is considered that the sensitizer-type of BH3-only proteins Noxa and Bad displace constitutively associated activators from antiapoptotic multidomain Bcl-2 members, and that displaced activators induce apoptosis[18,19]. Recently it has been also shown that Noxa activates Bak by inducing Mcl-1 degradation and Bcl-X_L inactivation[20]. Furthermore, we have found that Noxa-induced Bax activation is dependent on Bak, and that Noxa can not induce the apoptosis of Bak-deficient cells (Nakajima et al., manuscript in preparation). These findings suggest that Noxa induces Bak activation by inactivating Mcl-1, Bcl-X_L and other antiapoptotic multidomain Bcl-2 family proteins, and that activated Bak induces Bax activation. However, the precise mechanism of apoptosis induced by BH3-only proteins remains to be elucidated.

The roles of Noxa and Puma in p53-mediated apoptosis have been analyzed using gene knockout mice[21–23]. Mouse embryonic fibroblasts (MEFs) deficient in *Noxa* show notable resistance to oncogene-dependent apoptosis. Furthermore, *Noxa*-deficient mice show resistance to X-ray-irradiation-induced gastrointestinal death, accompanied by an impaired apoptosis of epithelial cells that is dependent on p53. MEFs from *Puma*-deficient mice also show resistance to oncogene-dependent apoptosis. In addition, thymocytes from *Puma*-deficient mice, but not *Noxa*-deficient mice, are resistant to DNA-damage-induced apoptosis. Therefore, Noxa and Puma show overlapping and, in some cases, different functions in p53-mediated apoptosis. In this study, we show that Noxa and Puma synergistically induce apoptosis. The synergistic induction of apoptosis is not a common effect of a pair for the BH3-only proteins, because no such synergism is observed in the BH3-only proteins Bim and Bid, which are critical inducers of apoptosis in response to various stimulations[11–13]. These findings may provide additional insights into the mechanism underlying p53-induced apoptosis.

Materials and Methods

Antibodies

The antibodies used for immunoblotting and immunoprecipitation were anti-Bcl-$X_{S/L}$ (S-18; Santa Cruz Biotechnology), anti-human Mcl-1 (S-19; Santa Cruz Biotechnology), anti-mouse Mcl-1 (Rockland) and anti-α-tubulin (DM 1A; SIGMA). The antibodies used for immunofluorescence analysis were anti-Bax (6A7; Exalpha Biologicals), anti-Bak (ab-1; Calbiochem) and anti-cytochrome c (6H2; Santa Cruz Biotechnology) antibodies. Immunoprecipitation and immunoblotting were performed as previously described[24] using CHAPS buffer (1% CHAPS, 300 mM NaCl, 25 mM 25 mM Hepes [pH 7.5], and a protease inhibitor cocktail) as a lysis buffer.

Immunofluorescence Microscopy

For immunofluorescence staining, 1×10^4 HeLa cells or MEFs were grown on 48-well glass-bottom plates (Iwaki). After 24 hrs, the cells were fixed with 4% paraformaldehyde for 30 min at 4℃ and permeabilized with methanol for 10 min at 4℃. The fixed cells were then incubated in 1% BSA-PBS for 30 min and then with anti-Bax (6A7; Exalpha Biologicals), anti-Bak (Ab-1; Calbiochem) and anti-cytochrome c (6H2; Santa Cruz Biotechnology) antibodies for 30 min at room temperature. After washing with PBS, the fixed cells were incubated for 30 min at room temperature with Alexa-488- or Alexa-568-conjugated anti-mouse IgG antibody. Immunofluorescence was recorded using an immunofluorescence microscope (Olympus). Images were taken using a Penguin 150CL camera (Pixera) and imaging software (Pixera).

Apoptosis Detection

HeLa cells and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For cell viability assay for apoptosis assessment, the trypan blue dye exclusion method was performed. Cell viability was measured using a Vi-Cell image analyzer (Beckman).

RNA Interference

To knockdown Bcl-X_L or Mcl-1 expression in MEFs, mouse Bcl-X_L shRNA-expressing vectors (siBcl-X_L) were generated using the pSUPER-retro-puro vector. 5'-GAAAGTGCAGTTCAGTAAT-3' was used for mouse Bcl-X_L and 5'-GAAGGACTTCTAGAATGAA-3' for mouse Mcl-1 (siMcl-1).

Adenoviral Infection

A recombinant adenovirus was generated with the pAxCAwt vector according to the manufacturer's protocol (Adenovirus Expression Vector kit; Takara). Noxa, Bim or Puma cDNA was tagged with the sequence encoding the influenza virus HA epitope at their N-terminus. Cells were infected with the adenoviruses as described previously[8].

Results

Noxa and Puma Synergistically Induce Apoptosis and Activation of Bax and Bak

To investigate the functional interaction of Noxa and Puma, we used an adenovirus-mediated gene expression system. As shown in **Figure 1a**, about 30% of HeLa cells had died 12 hrs after infection with adenovirus-expressing Noxa or Puma at amultiplicity of infection (moi) of 50. Interestingly, Noxa- or Puma-induced apoptosis was markedly enhanced by a combination of Noxa and Puma; more than 90% of cells died after infection with adenovirus-expressing Noxa (moi 25) and Puma (moi 25) **(Fig. 1a)**. The same effect was also observed with twice the amount of adenovirus (moi 100; **Fig. 1a**), indicating that this enhancement is caused by a synergistic effect, but not an additive effect. BH3-only proteins activate the proapoptotic multidomain Bal-2 family proteins Bax and Bak, and BH3-only-protein-induced apoptosis is dependent on Bax and Bak[15]. To detect Bax and Bak activations by Noxa and Puma, we performed immunofluorescence microscopy using an activated form of Bax- or Bak-specific antibody. As shown in **Figure 1b**, in Noxa- or Puma-expressing cells under the same conditions as those in **Figure 1a** (moi 50), the activated forms of

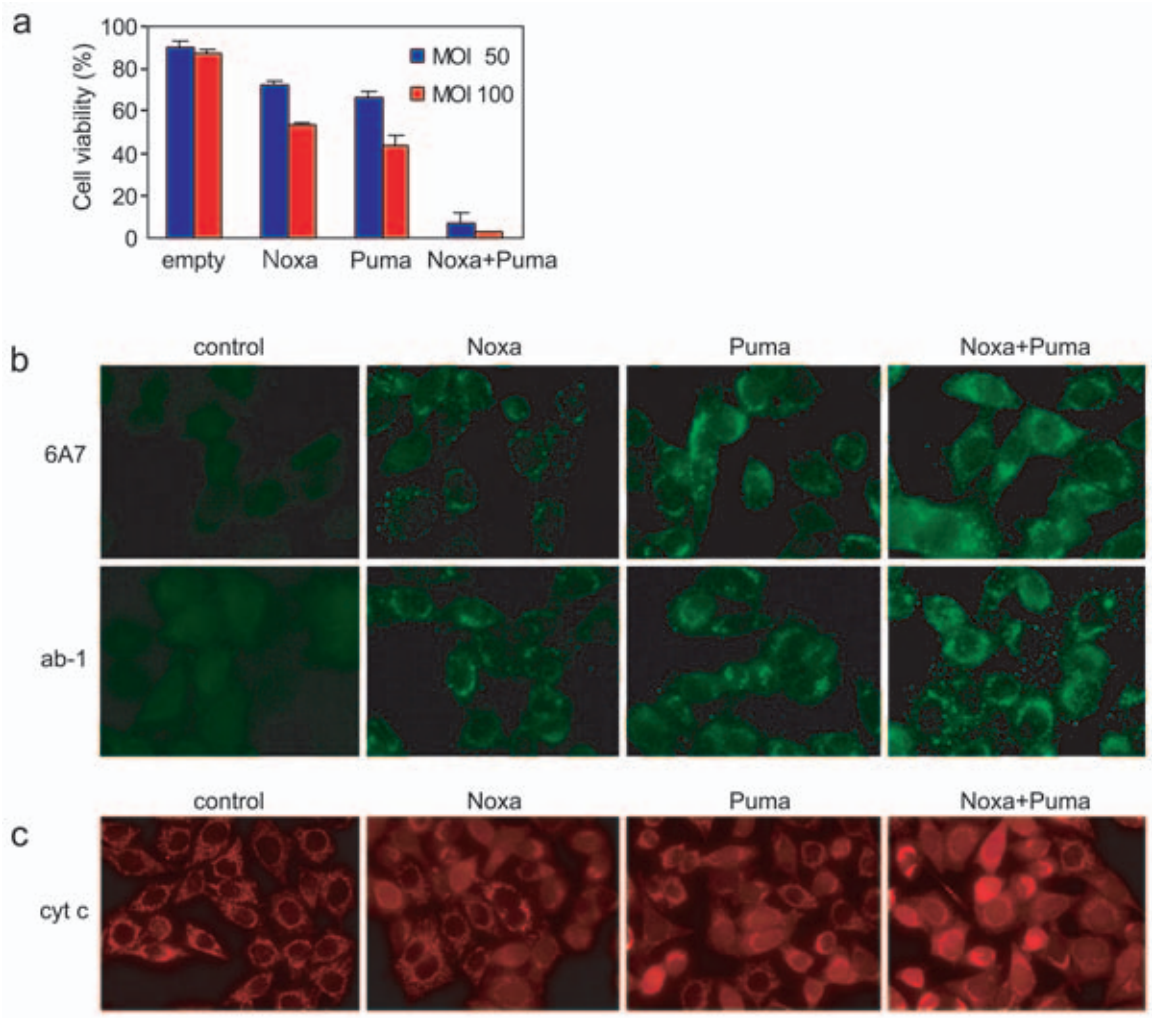

Fig. 1 **a**: HeLa cells were infected with an adenovirus expressing either of the BH3-only proteins, Noxa and Puma (moi 50 or 100), Noxa + Puma (moi; Noxa 25, Puma 25 or Noxa 50, Puma 50) or an empty adenovirus (moi 50 or 100). The cells were harvested 12 hrs after treatment and the percentage of viable cells was determined by the trypan blue dye exclusion method. The data are representative of at least three independent experiments. The error bars indicate standard deviations.
b, **c**: HeLa cells were infected with Ad-empty, Ad-Noxa, Ad-Puma, or Ad-Noxa + Ad-Puma for 12 hrs in the presence of 100 μM zVAD-fmk and visualized by immunofluorescence microscopy. The 6A7 epitope Bax N-terminus-recognizing anti-6A7 (green) and anti-cytochrome c (cyto c; red) were visualized by immunofluorescence microscopy.

Bax and Bak were weakly but definitively detected using the 6A7[25] and ab-1[26] antibodies, respectively. Generally apoptotic cells markedly change their morphology, resulting in the formation of apoptotic bodies. Therefore, to prevent the morphological change of cells during apoptosis caused by caspases, we performed this experiment in the presence of a caspase inhibitor (z-VAD fmk). It was previously shown that, in response to apoptotic stimulation, Bax and Bak change into their active forms, resulting in the formation of large clusters containing Bax and Bak on the mitochondrial outer membrane[27], and that staining images of Bax and Bak show such a cluster pattern **(Fig. 1b)**. In contrast, these antibodies did not stain uninfected cells. Next, we determined the effect of the combination of Noxa and Puma, and found that Noxa and Puma synergistically activate Bax and Bak **(Fig. 1b)**. Following cluster formation on the mitochondrial outer membrane, activated Bax and Bak induce cytochrome c release from mitochondria. As shown in **Figure 1c**, Noxa or Puma induces cytochrome c release from mitochondria. In normally growing cells, cytochrome c was located only in mitochondria,

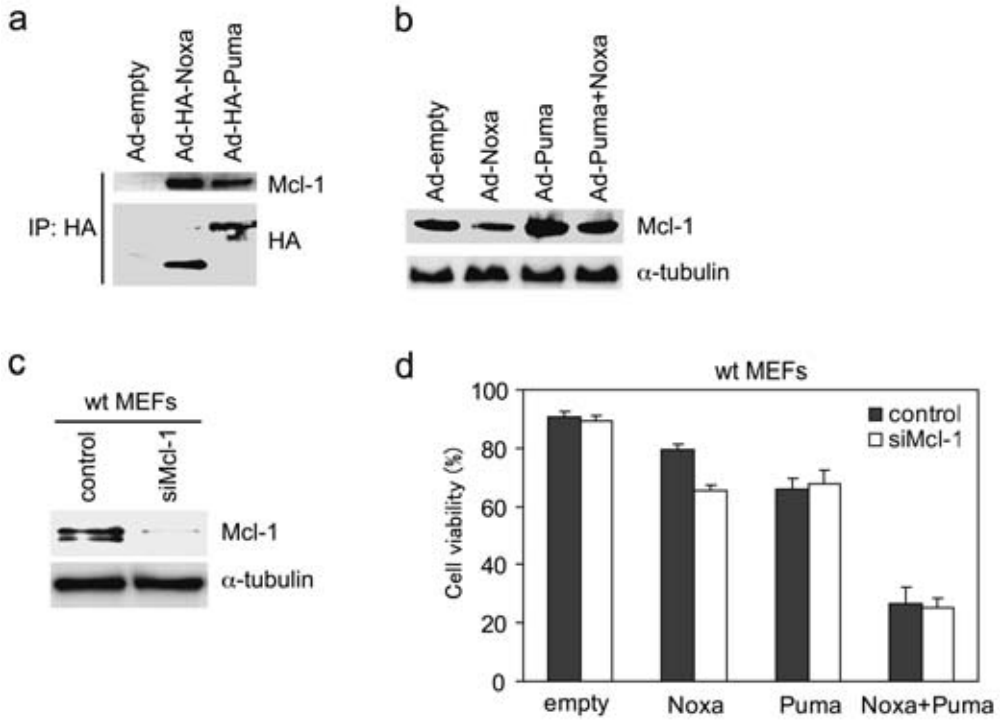

Fig. 2 **a**: HeLa cells were infected with an adenovirus expressing Noxa (moi 50) or Puma (moi 50) for 12 hrs in the presence of 100 μM zVAD-fmk and solubilized in CHAPS buffer. The cell lysate was subjected to immunoprecipitation with an anti-HA antibody and analyzed by immunoblotting using anti-Mcl-1 and anti-HA antibodies. **b**: HeLa cells were infected with Ad-empty (moi 50), Ad-Noxa (moi 50), Ad-Puma (moi 50), or Ad-Puma + Ad-Noxa (moi; Noxa 25, Puma 25). After 12 hrs, the lysate was analyzed by immunoblotting using antibodies to Mcl-1 and α-tubulin as a loading control. **c**: shRNA for Mcl-1 (siMcl-1) or the pSuper Vector (control) was expressed in SV40-transformed wild-type (wt) MEFs. The lysate was analyzed by immunoblotting using antibodies to Mcl-1 and α-tubulin as a loading control. **d**: Wt MEFs expressing siMcl-1 or control were infected with Ad-empty (moi 50), Ad-Noxa (moi 50), Ad-Puma (moi 50), or Ad-Puma + Ad-Noxa (moi; Noxa 25, Puma 25). The cells were harvested 12 hrs after treatment, and the percentage of viable cells was determined by the trypan blue dye exclusion method. The data are representative of at least three independent experiments. The error bars indicate standard deviations.

and, in response to apoptotic stimulations, cytochrome c release is determined diffuse cytochrome c staining patterns[24]. As shown in **Figure 1c**, cytochrome c release was clearly enhanced by a combination of Noxa and Puma.

The Suppression of Mcl-1 by Noxa is not Influenced by Puma-induced Apoptosis

Recently, Willis et al. have suggested that Noxa-induced apoptosis is mainly caused by Mcl-1 degradation by Noxa, resulting in Bak activation[20]. They also showed that Bak is sequestered by Mcl-1 and Bcl-X_L, but not by Bcl-2, until displaced by BH3-only proteins. In addition, we found that Noxa activates Bax in Bak-expressing cells, but not in Bak-deficient cells (Nakajima et al., manuscript in preparation). Furthermore, we also found that enforced Bax translocation to mitochondria in combination with Bcl-2 inhibition by siRNA and the inactivation of Bcl-X_L and Mcl-1 by Noxa are sufficient to induce cytochrome c release (Nakajima et al., manuscript in preparation). Therefore, we next analyzed the role of Mcl-1 in this synergistic induction of apoptosis by Noxa and Puma. In the study using peptides of the BH3 domain of known BH3-only proteins, the BH3 peptide of Noxa showed a high affinity to Mcl-1, but not to other antiapoptotic multidomain Bcl-2 family proteins, such

as Bcl-X_L, Bcl-2, Bcl-w, and A1/BFL-1[18,19]. In contrast, the BH3 peptide of Puma showed high affinities to Bcl-2, Bcl-w and A1/BFL-1, but relatively lower affinities to Bcl-X_L and Mcl-1. Therefore, we next analyzed the binding of Mcl-1 to Noxa and Puma. As shown in **Figure 2a**, immunoprecipitation analysis revealed that Noxa associates with Mcl-1 in under a relatively physiological condition using 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) buffer[25]. Puma also associates with Mcl-1, but at a lower efficiency than Noxa **(Fig. 2a)**. Therefore, we determined whether Noxa-induced Mcl-1 degradation is enhanced by Puma. As shown in **Figure 2b**, Noxa, but not Puma, induced a reduction in Mcl-1 protein expression level. In contrast, the Noxa-induced reduction in Mcl-1 protein expression level was relatively inhibited by Puma. However, no acceleration of Noxa-induced Mcl-1 degradation by Puma could be determined. Next, we analyzed the functional role of Mcl-1 in Puma-induced apoptosis using wild-type MEFs in which Mcl-1 expression is suppressed by siRNA using a retrovirus mediated shRNA expression system **(Fig. 2c)**. Noxa-induced apoptosis was partially accelerated by Mcl-1 suppression, but Puma-induced apoptosis had no effect on Mcl-1 suppression **(Fig. 2d)**. In this experiment, we used SV40-transformed MEFs, because siRNA efficiently suppressed Mcl-1 expression in the murine system under our experimental conditions. We also found that Noxa and Puma also synergistically induce apoptosis in MEFs, and that this synergism has no effect on the suppression of Mcl-1 expression **(Fig. 2d)**. These results clearly indicate that the

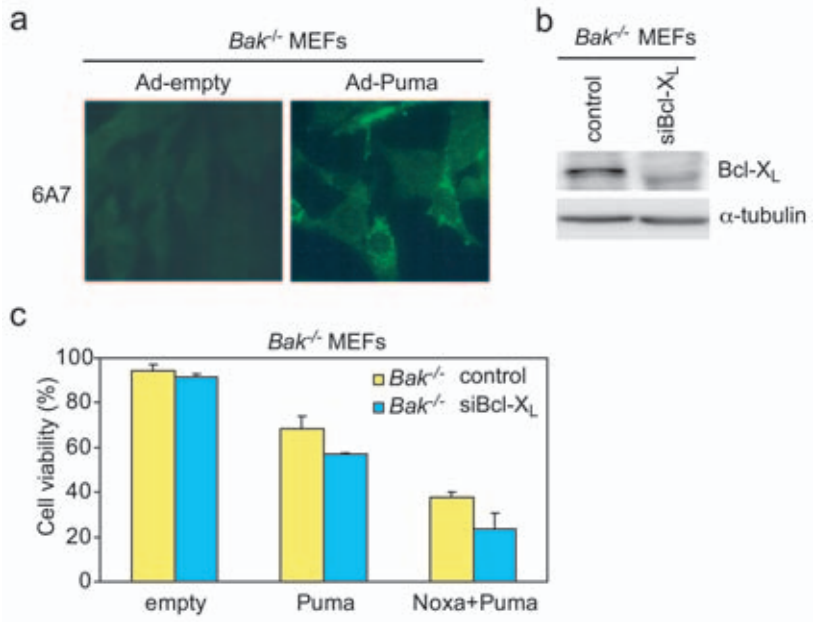

Fig. 3 **a**: *Bak* $^{-/-}$ MEFs were infected with Ad-empty (moi 50) or Ad-Puma (moi 50) for 12 hrs in the presence of 100 μM zVAD-fmk and visualized by imunofluorescence microscopy. The 6A7 epitope Bax N-terminus-recognizing anti-6A7 antibody (red) was visualized by imunofluorescence microscopy. **b**: siRNA for Bcl-X_L (siBcl-X_L) or the pSuper vector (control) was expressed in *Bak* $^{-/-}$ MEFs. The lysate was analyzed by immunoblotting using antibodies to Mcl-1 and α-tubulin. **c**: *Bak* $^{-/-}$ MEFs expressing siBcl-X_L or control vector were infected with Ad-empty (moi 50), Ad-Puma (moi 50), or Ad-Puma + Ad-Noxa (moi; Noxa 25, Puma 25). The cells were harvested 12 hrs after treatment, and the percentage of viable cells was determined by the trypan blue dye exclusion method. The data are representative of at least three independent experiments. The error bars indicate standard deviations.

suppression of Mcl-1 expression by Noxa is not influenced in Puma-induced apoptosis, suggesting that another Noxa-evoked mechanism enhances Puma-induced apoptosis.

Bak Activation by Noxa is not Influenced in Puma-induced Apoptosis

As described above, it was considered that Noxa-induced apoptosis is mainly mediated by Bak activation. Therefore, we determined whether Noxa-induced Bak activation enhances Puma-induced apoptosis by Noxa. As shown in **Figure 3a**, Puma induced apoptosis in *Bak*$^{-/-}$ MEFs. We also analyzed the role of Bcl-X_L using siRNA **(Fig. 3b)**, because Bcl-X_L is considered to be a major target of Noxa other than Mcl-1[8]. We previously found that Noxa-induced Bax activation is dependent on Bak using Bak-deficient cells (Nakajima et al., manuscript in preparation). As shown in **Figure 3b**, Noxa enhanced Puma-induced apoptosis in Bak-deficient SV 40-transformed MEFs. In addition, the suppression of Bcl-X_L expression did not markedly enhance Puma-induced apoptosis compared with the effect of Noxa **(Fig. 3c)**. These results suggest that the inhibition of antiapoptotic multidomain Bcl-2 family protein (s), other than Mcl-1 and Bcl-X_L, is important for the synergistic induction of apoptosis by Noxa and Puma.

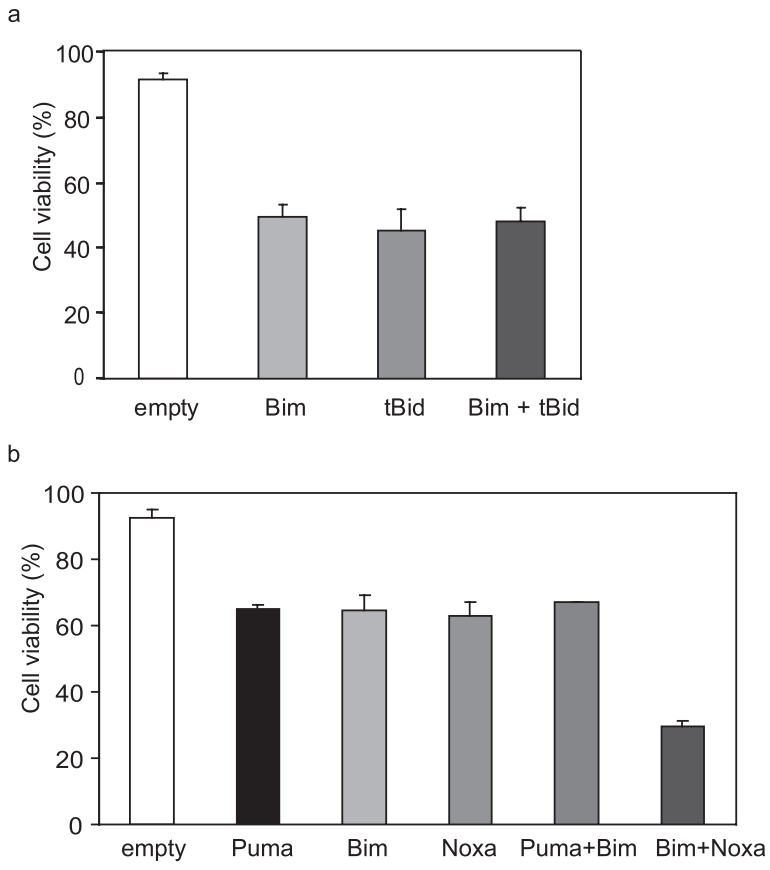

Fig. 4 **a**: HeLa cells were infected with an adenovirus expressing either of the BH3-only proteins, empty, Bim (moi 10), tBid (moi 10) or Bim + tBid (moi; Bim 5 + tBid 5). **b**: HeLa cells were infected with the adenovirus expressing BH3-only proteins, Puma (moi 50), Bim (moi 10), Noxa (moi 50), Puma + Bim (moi; Puma 25 + Bim 5), and Noxa + Bim (moi; Noxa 25 + Bim 5). The cells were harvested 12 hrs after treatment, and the percentage of viable cells was determined by the trypan blue dye exclusion method. The data are representative of at least three independent experiments. The error bars indicate standard deviations. (a, b)

The BH3-only Proteins Bim and Bid do not Show Synergistic Induction of Apoptosis

Is this synergistic induction of apoptosis specific for Noxa and Puma? To answer this, we analyzed the effect of the extensively analyzed BH3-only proteins Bim and Bid. As shown in **Figure 4a**, Bim and Bid showed only an additive effect of apoptosis induction, and no synergistic induction of apoptosis by Bim and Bid could be detected.

Furthermore, we analyzed the synergistic effect of Bim and Puma or Noxa. As shown in **Figure 4b**, no synergistic activation of Bim and Puma was observed. However, Bim-induced apoptosis was clearly enhanced by Noxa **(Fig. 4b)**.

Discussion

This study first demonstrated the synergistic induction of apoptosis by the p53-inducible Bcl-2 family proteins Noxa and Puma. No such synergism is observed for the BH3-only proteins Bim and Bid **(Fig. 4a)**, indicating that the synergistic induction of apoptosis is not a common effect of a pair of BH3-only proteins. Previously, it was considered that BH3-only proteins are divided into two classes, "activators" and "sensitizers"[18,19]. Bid and Bim are of the activator type and directly associate with Bax and Bak, resulting in the activation of Bax and Bak. In contrast, sensitizer type BH3-only proteins, e.g., Noxa, displace constitutively associated activators from antiapoptotic multidomain Bcl-2 members, resulting in the activator-mediated activation of Bax and Bak[18,19]. Initially, it was considered that Puma is of the sensitizer type[18]. However, recent findings suggested that Puma is also an activator, because the sensitizers Noxa and Bad induce apoptosis in wild-type MEFs, but not in Puma-deficient cells with Bim knockdown[28]. As shown in **Figure 4b**, Bim and Puma induced no synergism; however, Bim and Noxa synergistically induced apoptosis. It has been shown that Bim is a target gene for the tumor suppressive transcription factor FoxO3[29]. These findings raise the possibility that targets of tumor suppressive transcription factors synergistically activate apoptosis. However, this possibility remains to be elucidated. Nevertheless, it is possible that the synergistic induction of apoptosis is caused by a combination of an activator and Noxa. We also analyzed another sensitizer, Bad, using the same system. However, we found that Bad efficiently induces apoptosis in the absence of Bak (data not shown), suggesting that Noxa and Bad function in different pathways.

Although Noxa activates Bak through Mcl-1 degradation[20] and Bcl-X_L inactivation, we detected no involvement of Mcl-1 and Bcl-X_L in the enhancement of Puma-induced apoptosis by Noxa using a knockdown study utilizing siRNA **(Fig. 2 and 3)**. Moreover, Bak activated by Noxa is also not involved in such enhancement, because, in *Bak*-deficient cells, the synergistic induction of Noxa and Puma was observed **(Fig. 3)**. We previously demonstrated that Noxa can not activate Bax in the absence of Bak (Nakajima et al., manuscript in preparation). In contrast, Puma can activate Bax in the absence of Bak, and Noxa enhances Puma-induced apoptosis in *Bak*-deficient cells **(Fig. 3)**. Therefore, it can be considered that the inhibition of another antiapoptotic multidomain Bcl-2 family of proteins, e.g., Bcl-w, A1/BFL-1, BOO/DIVA and NR-13[11–13], by Noxa enhances Puma-induced apoptosis. In addition, it has been proposed that Puma displaces p53 from Bcl-X_L, allowing p53 to permeabilize mitochondria[30]. It can not be considered that p53 itself is involved in this synergism, because, in SV40-transformed MEFs, p53 is inactivated by the T antigen. However, from the occurrence of such a phenomenon, it can not be denied that other protein (s) may be involved in this synergism.

Our results clearly demonstrate the synergistic induction of apoptosis by Noxa and Puma. It has been shown that the expressions of Noxa and Puma are activated by other transcription factors; E2F1 activates Noxa and Puma expression[31] and FoxO3a activates Puma expression[32]. Moreover, it has been shown that the transcriptional repressor Slug antagonizes p53-mediated apoptosis by repressing Puma expresasion[33]. Therefore, the induction level of Noxa and Puma by p53 differ under cellular conditions, in which other transcription factors involved in the regulation of Noxa or Puma are differently activated. From this study and the

possibilities raised, it can be considered that p53 carefully controls apoptosis by allowing two molecules to share full ability to induce apoptosis.

Many biological processes are mediated by a specific molecular recognition of protein-protein interactions [34]. Recently, many small-molecule inhibitors or mimetics for targeting such protein-protein interactions have been discovered. During the course of these discoveries, several candidate mimetics have been reported[35,36]. Among them, the compound ABT-737, a BH3 domain mimetic, targets selective Bcl-2 proteins and efficiently induces apoptosis in cancer cells[37,38]. At present, critical targets for the synergistic induction of apoptosis by Noxa and Puma remain to be elucidated. However, such targets may be important as target molecules of a therapeutic drug for cancer. Therefore, we will continue to search for critical targets for the synergistic induction of apoptosis by Noxa and Puma.

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References

1. Lowe SW, Cepero E, Evan G: Intrinsic tumour suppression. Nature 2004; 432: 307–315.
2. Oren M: Decision making by p53: life, death and cancer. Cell Death Differ 2003; 10: 431–442.
3. Soussi T, Beroud C: Significance of TP53 mutations in human cancer: a critical analysis of mutations at CpG dinucleotides. Hum Mutat 2003; 21: 192–200.
4. Bartkova J, Horejsi Z, Koed K, et al.: DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005; 434: 864–870.
5. Gorgoulis VG, Vassiliou LV, Karakaidos P, et al.: Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005; 434: 907–913.
6. Bartkova J, Rezaei N, Liontos M, et al.: Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006; 444: 633–637.
7. Di Micco R, Fumagalli M, Cicalese A, et al.: Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 2006; 444: 638–642.
8. Oda E, Ohki R, Murasawa H, et al.: Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 2000; 288: 1053–1058.
9. Nakano K, Vousden KH: PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001; 7: 683–694.
10. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B: PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001; 7: 673–682.
11. Antignani A, Youle RJ: How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? Curr Opin Cell Biol 2006; 18: 685–689.
12. Gross A, McDonnell JM, Korsmeyer SJ: BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999; 13: 1899–1911.
13. Harris MH, Thompson CB: The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ 2000; 7: 1182–1191.
14. Wang X: The expanding role of mitochondria in apoptosis. Genes Dev 2001; 15: 2922–2933.
15. Cheng EH, Wei MC, Weiler S, et al.: BCL-2, BCL-X (L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001; 8: 705–711.
16. Lindsten T, Ross AJ, King A, et al.: The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 2000; 6: 1389–1399.
17. Wei MC, Zong WX, Cheng EH, et al.: Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001; 292: 727–730.
18. Certo M, Del Gaizo Moore V, Nishino M, et al.: Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell 2006; 9: 351–365.
19. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ: Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2002; 2: 183–192.
20. Willis SN, Chen L, Dewson G, et al.: Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005; 19: 1294–1305.
21. Jeffers JR, Parganas E, Lee Y, et al.: Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003; 4: 321–328.
22. Shibue T, Takeda K, Oda E, et al.: Integral role of Noxa in p53-mediated apoptotic response. Genes Dev 2003; 17: 2233–2238.
23. Villunger A, Michalak EM, Coultas L, et al.: p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science 2003; 302: 1036–1038.
24. Cheng EH, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ: VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science 2003; 301: 513–517.
25. Hsu YT, Youle RJ: Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J Biol Chem 1998; 273: 10777–10783.
26. Zhang L, Shimizu S, Sakamaki K, Yonehara S, Tsujimoto Y: A caspase-8-independent signaling

pathway activated by Fas ligation leads to exposure of the Bak N terminus. J Biol Chem 2004; 279: 33865–33874.
27. Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ: Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. J Cell Biol 2001; 153: 1265–1276.
28. Kim H, Rafiuddin-Shah M, Tu HC, et al.: Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol 2006; 8: 1348–1358.
29. Accili D, Arden KC: FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 2004; 117: 421–426.
30. Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR: PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science 2005; 309: 1732–1735.
31. Hershko T, Ginsberg D: Up-regulation of Bcl-2 homology 3 (BH3) -only proteins by E2F1 mediates apoptosis. J Biol Chem 2004; 279: 8627–8634.
32. You H, Pellegrini M, Tsuchihara K, et al.: FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J Exp Med 2006; 203: 1657–1663.
33. Wu WS, Heinrichs S, Xu D, et al.: Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. Cell 2005; 123: 641–653.
34. Cochran AG: Protein-protein interfaces: mimics and inhibitors. Curr Opin Chem Biol 2001; 5: 654–659.
35. Oltersdorf T, Elmore SW, Shoemaker AR, et al.: An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005; 435: 677–681.
36. Walensky LD, Kung AL, Escher I, et al.: Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. Science 2004; 305: 1466–1470.
37. Konopleva M, Contractor R, Tsao T, et al.: Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell 2006; 10: 375–388.
38. van Delft MF, Wei AH, Mason KD, et al.: The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 2006; 10: 389–399.

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