deficient mice, however, appears to be independent of the genetic background.[3,4] Genetic background differences have also been reported to drastically change the phenotype observed in mouse models lacking prominent cell death associated genes. Loss of p53 causes a very different tumour spectrum on different genetic backgrounds and embryonic lethality of female *p53*$^{-/-}$ mice ranges from 30 to almost 100%, due to neuronal tube-closure defects.[17] Caspase-3 or -9 deficiency cause phenotypes ranging from embryonic lethality due to massive fore-brain overgrowth to mild apoptosis defects.[18,19]

In conclusion, our study indicates that AKT1-mediated signals are not required to maintain the Bcl-2 rheostat in primary mouse thymocytes. Loss of *akt*1 does not change the cell death susceptibility of T lymphocytes and MEF to cell stresses as diverse as IL-2 or serum deprivation, TNF-RI or TCR ligation, glucocorticoids, kinase inhibition by Staurosporine and DNA-damage caused by UV- or γ-irradiation as well as topoisomerase II inhibition. This may be due to genetic background differences interfering with AKT1 regulated signaling. Finally, loss of *akt*1 does not cause compensatory upregulation of either AKT2 or AKT3, nor restoration of the overall AKT activity to wt levels underlining the strict separation of their true biological functions.

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DNA damage-inducing agent-elicited γ-secretase activity is dependent on Bax/Bcl-2 pathway but not on caspase cascades

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Dear Editor,

Accumulation of senile plaques composed of amyloid-α(Aβ) is a pathological hallmark of Alzheimer's disease (AD),[1] and Aβ is generated through the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases.[2] β-Secretase excises the ectodomain of APP (β-APPs)[3] to leave a 99-amino-acid long C-terminal fragment (APP-C99-CTF) in the membrane. γ-Secretase then cleaves this membrane-tethered APP-CTF within the transmembrane domain, releasing Aβ peptides and APP-intracellular domain (AICD). As such, β- and γ-secretase are regarded to perform the key steps in the pathogenesis of AD and have become important therapeutic targets in the prevention and treatment of AD. As a result of much effort on identifying the natures of these secretases, presenilin (PS) was found to be the first obligatory component of γ-secretase.[4] Further biochemical analyses revealed that γ-secretase is composed of multicomponent complexes, and

another membrane protein, nicastrin (NCT), was identified to be a component of the γ-secretase complex by co-immunoprecipitation studies.[5,6] Moreover, two other membrane proteins, anterior pharynx-defective phenotype 1 (APH-1) and PS enhancer 2 (PEN-2) were identified as components of γ-secretase by two independent studies using genetic screening in *Caenorhabditis elegans*.[7,8] Finally, it has been shown that γ-secretase activity can be reconstituted by coexpressing human PS, NCT, APH-1, and PEN-2 in yeast, a *Drosophila* cell line, or mammalian cells,[9–11] providing clear evidence that these four proteins compose the minimal constituents of the active γ-secretase complex.

Despite the importance of γ-secretase as a therapeutic target for AD and the enormous progress made in the biochemical characterization of γ-secretase over recent years, relatively few studies have elaborated the endogenous mechanism regulating γ-secretase activity. In this regard, some of the previous studies suggest a close relationship between apoptosis and the Aβ-mediated pathogenesis of AD. Galli *et al.*[12] reported increased amyloidogenic secretion when cerebellar granule cells were committed to apoptosis by KCl deprivation, and Tesco *et al.*[13] reported a marked increase in total Aβ and Aβ_{42} levels in Chinese Hamster Ovary (CHO) cells treated with staurosporine or etoposide. Ohyagi *et al.*[14] also reported increased level of cellular Aβ_{42} during apoptosis in fetal guinea-pig brain cells, suggesting that a death signal regulates the processing of APP by modulating secretase activity. Currently, however, no details are available concerning the mechanism underlying cell death-induced elevation of Aβ production. We undertook this study to elucidate the mechanism underlying γ-secretase-modulated enhancement of amyloidogenic processing of APP during cell death, based on luciferase reporter gene and *in vitro* peptide cleavage assay findings.[15]

Initially, we generated a stable cell line (CHO-C99) coexpressing UAS-controlled luciferase reporter gene and the cDNA for C99-containing GAL4/VP16-transactivating domain (C99-GV) using CHO cells, as described previously.[16] In these cells, γ-secretase-dependent cleavage of C99-GV released the APP intracellular domain containing GV transactivation domain (AICD-GV) from the membrane. The released APP domain then translocated to the nucleus and activated the expression of firefly luciferase reporter gene under the control of UAS *cis*-elements.

Using this cell line, we examined whether DNA-damage-inducing agents (DDIAs), that is, etoposide and camptothecin, can affect γ-secretase-mediated cleavage of APP. We found that etoposide-treated CHO-C99 cells displayed dramatically enhanced γ-secretase activity (Figure 1a). Moreover, this DDIA-induced increase in γ-secretase activity was markedly attenuated by NCT-specific siRNA. The treatment of a γ-secretase-specific inhibitor, inhibitor X (also known as L-685 458; 2 μM), also diminished γ-secretase activity to the control level, demonstrating the specificity of DDIA effect on γ-secretase activity (Figure 1a).

Etoposide-elicited regulation of γ-secretase activity was found to be a dose-dependent response (Supplementary Figure 1a). Treatment of camptothecin, another DNA-damaging agent, for 24 h after serum starvation for 1 day also activated γ-secretase in a dose-dependent manner (Supplementary Figure 1a), suggesting that γ-secretase activity enhancement is associated with apoptosis-inducing activity of DDIA.

To confirm whether the stimulatory effect of DDIA on γ-secretase activity is cell type- or assay method-specific, we stimulated ANPP cells, which overexpress all four γ-secretase components and Swedish mutant APP in HEK cell,[17] with various concentrations of etoposide or camptothecin. *In vitro* peptide cleavage assays showed the same stimulatory effect of DDIAs on γ-secretase activity in ANPP cells (Figure 1b). Moreover, Western blotting with APP-specific antibody 6E10 (epitope: 1–17 of Aβ sequence in APP C99, Signet) showed that APP C99 levels were significantly decreased by DDIA treatment (lower panel of Figure 1b). These results demonstrate similar DDIA effects on γ-secretase activity regardless of cell types or assay methods.

Under these conditions, we measured caspase-3 activity as a marker of apoptosis. Treatment of both CHO-C99 and ANPP cell lines with etoposide increased caspase-3 activity in a dose-dependent manner (Supplementary Figure 1b). Morphology and DNA fragmentation assay data obtained from etoposide- or camptothecin-treated cells showed the progress of cell death (Supplementary Figure 1c), indicating that DDIA triggered apoptosis in these cells. We also examined whether DDIA-triggered upregulation of γ-secretase activity affects S3 cleavage of Notch, which is another γ-secretase substrate. For this, we transiently expressed both Notch1 mutant construct with a deleted extracellular domain (ΔEN1-GV) and UAS-luciferase reporter gene in CHO cells. When we applied etoposide to these cells, γ-secretase activity was consistently enhanced, as observed in APP C99-GV (Supplementary Figure 1d), indicating that DDIA effect was not limited to APP as a γ-secretase substrate.

To examine whether stimulation of DDIA-induced γ-secretase activity affects Aβ generation, both secreted and intracellular forms of Aβ_{40} and Aβ_{42} levels were measured from conditioned media (CM) and cell lysates of HBA cells, which overexpress Swedish mutant APP and γ-secretase (BACE1) in HEK cell, after treating various concentrations of etoposide. Both Aβ_{40} and Aβ_{42} levels were significantly increased in both CM and cell lysates following etoposide treatment (Figure 1c and d, respectively). Etoposide-induced Aβ generation was blocked by inhibitor X treatment, indicating that DDIA-induced increase of Aβ generation is dependent on γ-secretase activity.

To elucidate the mechanism underlying DDIA-induced γ-secretase activity, the expression of each of the four γ-secretase components was examined by Western blotting using each specific antibody (Supplementary Figure 1e). No significant increase in the expression level of these components was observed. A slight reduction of immature NCT band was detected, but the reason for this is not clear.

We next determined whether caspase activation is involved in DDIA-triggered regulation of γ-secretase. It has been well documented that DDIA treatment can activate caspase, as shown in Supplementary Figure 1b.[18] We treated CHO-C99 cells with a potent cell-permeable caspase-3 inhibitor, z-DEVD-fmk (100 μM), or a pan-caspase inhibitor, z-VAD-fmk (100 μM), in the presence of etoposide. The treated caspase inhibitors effectively blocked the caspase-3 activities, as expected. However, DDIA-dependent stimulation of γ-secretase activity

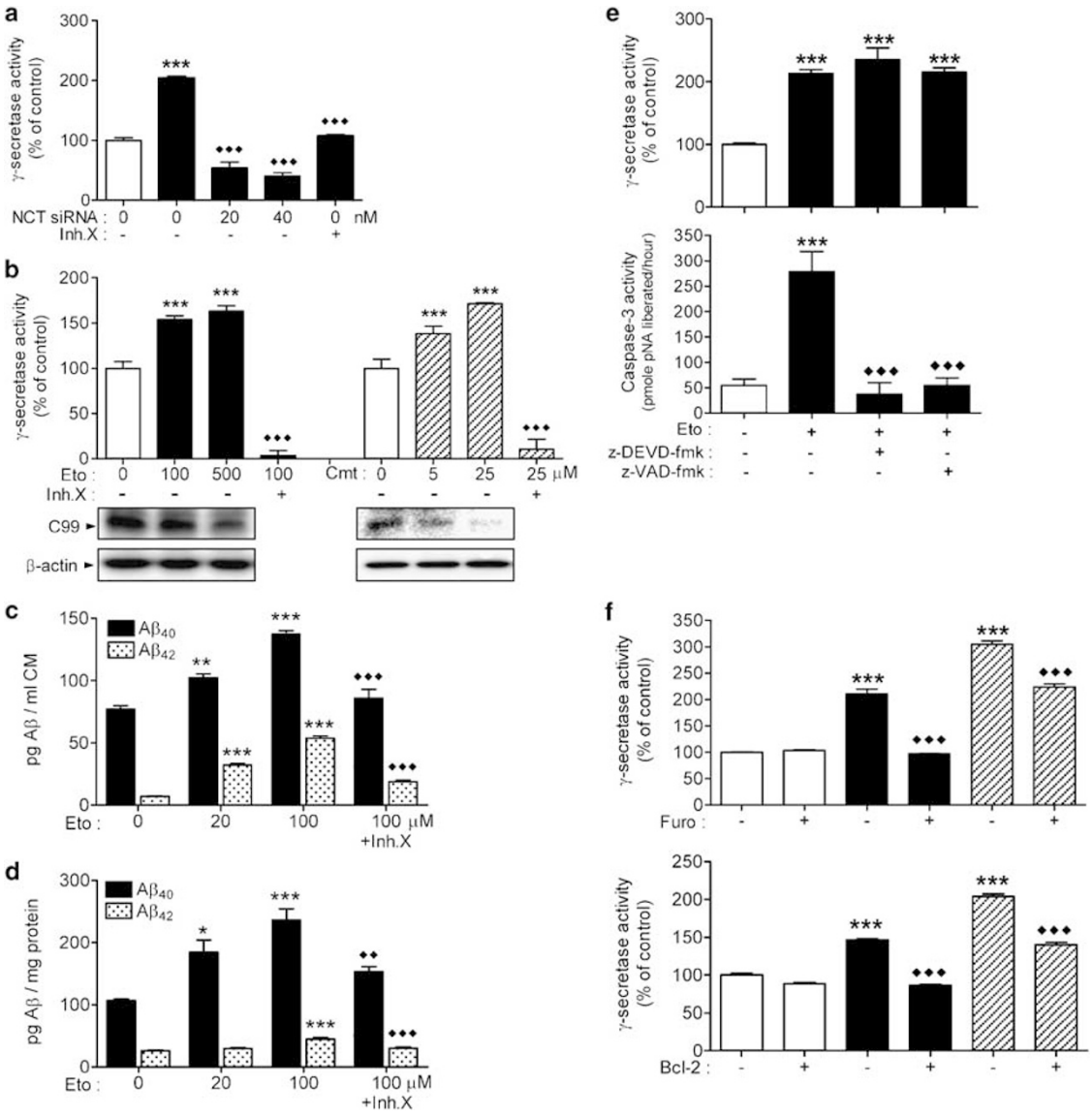

Figure 1 DDIAs increase γ-secretase activity and Aβ generation in a Bcl-2/Bax-dependent manner. (**a**) Etoposide treatment increased γ-secretase activity. Etoposide (100 μM; Sigma) was added to CHO-C99 cells for 48 h. Luciferase assay was performed with 10 μg of total protein using Luciferase Reporter Assay System (Promega), as instructed by the manufacturer. Expression levels of luciferase were measured using a Bio-Imaging Analyzer (LAS-3000; Fuji). To confirm the specificity of γ-secretase activity upregulation by etoposide, NCT-specific siRNA (Dharmacon) or the γ-secretase-specific inhibitor, inhibitor X (2 μM; Calbiochem), was administered. (**b**) *In vitro* peptide cleavage assay showing dose-dependent increases in γ-secretase activity by DDIAs. ANPP cells were treated with DDIAs at designated concentrations for 24 h. The preparations of crude membrane fraction and measurements of γ-secretase activity *in vitro* were performed as described previously.[15] Inhibitor X (10 μM) was added to confirm the specificity of the *in vitro* cleavage assay system. To verify *in vitro* peptide cleavage assay results, the protein levels of APP-CTF (C99) were examined by Western blotting. (**c**) Etoposide treatment increased Aβ accumulation in conditioned media (CM) of HBA cells. Etoposide was treated to HBA cells at the indicated concentrations with/without inhibitor X (5 μM). After 48 h, CM were collected and subjected to the sandwich ELISA using anti-Aβ N-terminal specific antibody (capturing antibody) and anti-Aβ_{40} or Aβ_{42} C-terminal specific antibody (detection antibody) according to the manufacturer's instruction (human β amyloid Immunoassay Kit; Biosource). (**d**) Etoposide treatment increased intracellular Aβ levels. Total protein extracts were prepared from HBA cells in (**c**) with RIPA buffer. Aβ levels in 300 μg of total protein were measured as above. (**e**) Increased γ-secretase activity was independent of caspase-3 activity. CHO-C99 cells were pretreated with a caspase-3-specific inhibitor, z-DEVD-fmk (100 μM; Calbiochem), or a pancaspase inhibitor, z-VAD-fmk (100 μM; Calbiochem), for 30 min and then with etoposide (100 μM) for 48 h for luciferase activity and 24 h for caspase-3 activity assay, respectively. Luciferase activity was measured as described in (**a**). Caspase-3 activity in 100 μg of cytosolic protein was measured using a CaspACE™ Assay System (Promega), as instructed by the manufacturer. (**f**) Bax inhibitors antagonize the effect of DDIAs on γ-secretase activity. Upper panel: γ-secretase activity enhancement was inhibited by furosemide, which blocks Bax translocation to the mitochondria. CHO-C99 cells were pretreated with furosemide (Furo; 2 mM; Sigma) for 30 min, and then etoposide or camptothecin was added for 48 or 24 h, respectively. Luciferase activity was measured as described in (**a**). Lower panel: Bcl-2 overexpression inhibited DDIAs-induced activation of γ-secretase. CHO-C99 cells were transiently transfected with Bcl-2 cDNA or pcDNA 3.1 and then treated with 100 μM etoposide or 25 μM camptothecin for 48 or 24 h, respectively. Luciferase activity was measured as described in (**a**). All results were presented as means $\pm$ S.E. and represent three independent experiments. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ *versus* vehicle-treated controls. $^{\blacklozenge\blacklozenge\blacklozenge}P<0.001$, $^{\blacklozenge\blacklozenge}P<0.01$ *versus* the DDIA-treated sample. Open bar: vehicle-treated control; black bar: etoposide-treated cells; hatched bar: camptothecin-treated cells; Eto: etoposide; Cmt: camptothecin; Vcl: vehicle; Inh: inhibitor

was not suppressed by these inhibitors (Figure 1e), indicating that the modulation of γ-secretase activity triggered by DDIA is not a downstream event of caspase cascades.

Because Bax can regulate apoptotic process in the upstream of caspase cascades,[19] we examined whether γ-secretase activity is regulated by Bax translocation. When etoposide or camptothecin was added to CHO-C99 cultures with/without furosemide (a Bax translocation inhibitor, 2 mM), the marked reductions in γ-secretase activity was observed in both cases (upper panel of Figure 1f).

Then, to determine whether Bcl-2/Bax system is essential for DDIA-elicited stimulation of γ-secretase, CHO-C99 cells were transiently transfected with Bcl-2 cDNA, which antagonizes Bax function.[20] The overexpression of Bcl-2 in CHO-C99 cells dramatically blocked γ-secretase activation triggered by DDIAs (lower panel of Figure 1f), indicating that Bcl-2/Bax-dependent death pathway mediates the DDIA-induced modulation of γ-secretase activity.

Although previous reports suggest a close correlation between cell death and the Aβ-mediated pathogenesis of AD, few studies have demonstrated how cell death can affect the proteolytic processing of APP. Here, we provide evidence that DDIA-elicited γ-secretase activity is dependent on Bax/Bcl-2 pathway, but not on caspase cascades. Based on these results, we propose that cell death pathways including Bax translocation, triggered by various apoptotic stimuli, critically facilitate the generation of Aβ by activating γ-secretase. Because increased level of Aβ acts as another death signal, a feedback loop between cell death and Aβ generation can result in the progress of cell death process in the sporadic AD brain. Our results suggest that blockade of apoptosis during the early pathologic stage presents as a good therapeutic target for the intervention in the pathogenesis of AD.

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Abrogation of signal-dependent activation of the cdk9/cyclin T2a complex in human RD rhabdomyosarcoma cells

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Dear Editor,

During skeletal myogenesis, the transcription factor MyoD promotes differentiation in myoblasts by its ability to arrest the cell cycle and activate muscle-specific transcription.[1,2] Recently, we showed that the cdk9/cyclin T2a complex plays an essential role in the MyoD-dependent activation of the myogenic program, describing a distinction with the cdk1 and cdk2 complexes, which are functionally downregulated during myotube formation and inhibit muscle-specific transcription when overexpressed.[3] The role of cdk9 in modulating promoter-restricted transcription appears to be dependent