

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 *akt1* 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 *akt1* 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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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)

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 (A β) is a pathological hallmark of Alzheimer's disease (AD),¹ and A β 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

S Kiessling¹, C Lutz-Nicoladoni², A Olsson¹, N Harald¹, G Baier² and A Villunger^{*1}

¹ Division of Experimental Pathophysiology & Immunology, Biocenter, Innsbruck Medical University, Innsbruck, Austria

² Department of Medical Genetics and Pharmacology, Molecular & Clinical Pharmacology, Innsbruck Medical University, Innsbruck, Austria

* Corresponding author: A Villunger, BIOCENTER, Division of Experimental Pathophysiology & Immunology, Innsbruck Medical University, Laboratory of Developmental Immunology, A-6020 Innsbruck, Austria.

Tel: +43-512-507-3105; Fax: +43-512-507-2867;

E-mail: andreas.villunger@i-med.ac.at

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A β 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 A β -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 A β and A β ₄₂ levels in Chinese Hamster Ovary (CHO) cells treated with staurosporine or etoposide. Ohyagi *et al.*¹⁴ also reported increased level of cellular A β 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 A β 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 μ M), 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), suggest-

ing 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 DDIA on γ -secretase activity in ANPP cells (Figure 1b). Moreover, Western blotting with APP-specific antibody 6E10 (epitope: 1–17 of A β 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 (Δ EN1-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 A β generation, both secreted and intracellular forms of A β ₄₀ and A β 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 A β ₄₀ and A β ₄₂ levels were significantly increased in both CM and cell lysates following etoposide treatment (Figure 1c and d, respectively). Etoposide-induced A β generation was blocked by inhibitor X treatment, indicating that DDIA-induced increase of A β 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 μ M), or a pan-caspase inhibitor, z-VAD-fmk (100 μ M), 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

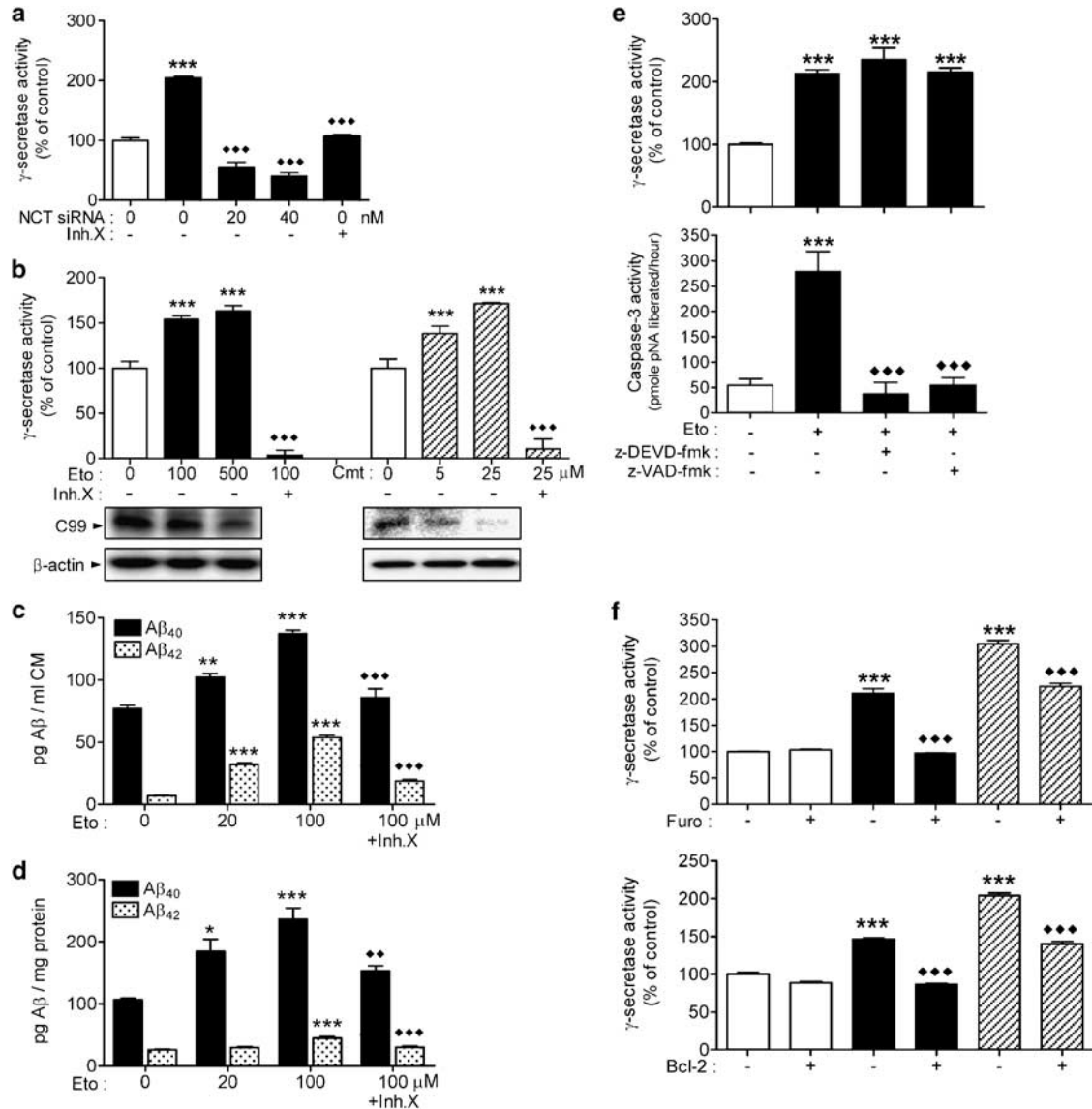


Figure 1 DDIAs increase γ -secretase activity and $A\beta$ generation in a Bcl-2/Bax-dependent manner. (a) Etoposide treatment increased γ -secretase activity. Etoposide (100 μ M; Sigma) was added to CHO-C99 cells for 48 h. Luciferase assay was performed with 10 μ g of total protein using Luciferase 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 μ M; 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 μ M) 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 $A\beta$ accumulation in conditioned media (CM) of HBA cells. Etoposide was treated to HBA cells at the indicated concentrations with/without inhibitor X (5 μ M). After 48 h, CM were collected and subjected to the sandwich ELISA using anti- $A\beta$ N-terminal specific antibody (capturing antibody) and anti- $A\beta_{40}$ or $A\beta_{42}$ C-terminal specific antibody (detection antibody) according to the manufacturer's instruction (human β amyloid Immunoassay Kit; Biosource). (d) Etoposide treatment increased intracellular $A\beta$ levels. Total protein extracts were prepared from HBA cells in (c) with RIPA buffer. $A\beta$ levels in 300 μ g 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 μ M; Calbiochem), or a pancaspase inhibitor, z-VAD-fmk (100 μ M; Calbiochem), for 30 min and then with etoposide (100 μ M) 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 μ g 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 μ M etoposide or 25 μ M 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. ♦♦♦ P < 0.001, ♦♦ 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 DDIA (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 A β -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 A β by activating γ -secretase. Because increased level of A β acts as another death signal, a feedback loop between cell death and A β 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.

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

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

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SM Jin^{1,2}, HJ Cho^{1,2}, MW Jung² and I Mook-Jung*¹

¹ Department of Biochemistry and Cancer Research Institute, College of Medicine, Seoul National University, 28 Yungun-Dong, Seoul 110-799, Korea

² Neuroscience and Technology Graduate program, Ajou University, Suwon, Korea

* Corresponding author: I Mook-Jung, Department of Biochemistry and Cancer Research Institute, College of Medicine, Seoul National University, 28 Yungun-Dong, Seoul 110-799, Korea. Tel: +82-2-740-8245; Fax: +82-2-3672-7352; E-mail: inhee@snu.ac.kr

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