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ZFAT is an antiapoptotic molecule and critical for cell survival in MOLT-4 cells

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ABSTRACT

***ZFAT* (also known as *ZNF406*), originally identified as a candidate gene for autoimmune thyroid disease, encodes a zinc-finger protein, however, its function has not been elucidated. Here, we report that human ZFAT protein is expressed in peripheral B and T lymphocytes and a human acute T lymphoblastic leukaemia cell line, MOLT-4 cells. Intriguing is that mouse ZFAT expression in $CD4^+$ lymphocytes is increased during blast formation. Furthermore, ZFAT-knockdown in MOLT-4 induces apoptosis via activation of caspases. These results suggested that ZFAT protein is a critical regulator involved in apoptosis and cell survival for immune-related cells.**

1. Introduction

Zinc-finger proteins constitute one of the largest classes of protein superfamily in the mammalian genome and are involved in a variety of cellular activities [1]. Cys2-His2 (C2H2) zinc-finger domains were originally identified as DNA-binding domains, however, there are even example of zinc fingers that support both DNA and protein interactions [2,3]. Several zinc-finger proteins such as BCL-6, BIRC5, PLZF and ZBP-89 are reported to be involved in apoptosis via these interactions [4–8], and some of them regulate fundamental aspects of the immune system [9,10].

ZFAT (zinc-finger gene in autoimmune thyroid disease susceptibility region; also known as *ZNF406*), originally identified as a candidate gene for autoimmune thyroid disease through linkage and association analyses on a cohort of Japanese autoimmune thyroid disease patients [11,12], encodes a 1243-amino-acids residue protein that contains one AT hook and 18 C2H2 zinc-finger domains [13]. Structurally, ZFAT is assumed to bind to DNA and function as a transcriptional regulator [13]. In mouse tissues, ZFAT is predominantly expressed in thymus, spleen and lymph nodes, but not in other tissues including bone marrow [13].

Here, we demonstrate that human ZFAT protein is expressed in human peripheral B and T lymphocytes and an acute T lymphoblastic leukaemia cell line, MOLT-4 cells, and that ZFAT-knockdown induces apoptosis via activation of caspases in MOLT-4. These results suggested that ZFAT protein is a critical regulator involved in apoptosis and cell survival.

2. Materials and methods

2.1. Protein extraction

Peripheral blood was collected from consented healthy volunteers, separated by Ficoll gradient, and the resulting mononuclear and leukocytes were further separated using the MACS system with CD19 and CD3 microbeads (Miltenyi Biotec.). $CD19^+$ lymphocytes, $CD3^+$ lymphocytes, and flow-through fraction, which is mainly monocytes, were subjected to protein extraction as described [14]. Mouse $CD4^+$ lymphocytes separated from C57BL/6J mouse spleens using CD4 microbeads (Miltenyi Biotec.) were stimulated with combinations of immobilized anti-CD3 (1 μg/ml; clone, 145-2C11; BD pharmingen) and anti-CD28 (5 μg/ml; clone, 37.51; BD pharmingen) antibodies in RPMI 1640 containing 10% fetal calf serum at 37 °C with 5% CO_2.

Abbreviations: ZFAT, zinc-finger gene in autoimmune thyroid disease susceptibility region; C2H2, Cys2-His2; siRNA, small inhibitory RNA; ZFAT-KD, ZFAT-knockdown

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2.2. Western blotting

Western blotting by anti-ZFAT or anti-actin (A2066, Sigma) antibodies were performed as described [13,14].

2.3. Quantitative reverse transcriptase-PCR

Real-time quantitative reverse transcriptase-PCR for the measurements of *ZFAT* mRNA levels in stimulated $CD4^+$ lymphocytes was performed using Absolute QPCR ROX Mix (ABgene) and TaqMan Gene Expression Assays (Applied Biosystems; Primer ID: Mm01195126_m1).

2.4. Cell culture and siRNA

Human acute T lymphoblastic leukaemia cell line MOLT-4 was cultured at 37 °C with 5% CO_2 in RPMI 1640 containing 10% fetal calf serum and transfected with a small inhibitory RNA (siRNA) using MicroPorator MP-100 (Digital Bio) according to the manufacturer's instruction. Two distinct siRNAs were designed to target the coding region of human *ZFAT* gene (nucleotides 200-224 and 319-343, GenBank Accession Number NM_020863). Scrambled RNAs containing the same number of each nucleotide as the siRNAs targeting the *ZFAT* gene were used as controls. The following siRNA duplexes (Invitrogen) were used in this study: ZFAT #1, 5′-A CGG CCA UCU UUA UGU GUA AAU GUU-3′ and 5′-A ACA UUU ACA CAU AAA GAU GGC CGU-3′; scramble #1, 5′-A CGA CCU UUA UGU GUA UAA UCG GUU-3′ and 5′-A ACC GAU UAU ACA CAU AAA GGU CGU-3′; ZFAT #2, 5′-C CUU AGG CCU CUG AGU ACA CCU GAA-3′ and 5′-U UCA GGU GUA CUC AGA GGC CUA AGG-3′; scramble #2, 5′-C CUC CGG GUC UAU GAC CAC UAU GAA-3′ and 5′-U UCA UAG UGG UCA UAG ACC CGG AGG-3′.

2.5. Cell proliferation assay

After transfection with ZFAT-siRNA or scramble RNA, 50 000 cells were cultured in 500 μl of medium with or without 100 μM z-VAD-fmk (R&D Systems), a broad spectrum caspase inhibitor. Cell proliferation was measured by using a Cell Counting Kit-8 (Dojindo) according to the manufacturer's instruction.

2.6. Flow cytometry

After transfection with ZFAT-siRNA or scramble RNA, MOLT-4 cells were fixed with cold 70% ethanol for 48 h at −20 °C, and stained with 10 μg/ml of propidium iodide after treated with 1 μg/ml RNase for 1 h. The cell suspensions were then analyzed with a fluorescence-activated cell sorter (FACS-Calibur, Becton Dickinson Co., Ltd.). Data was analyzed by CELL Quest software. Apoptosis quantification and detection of active CASP3 were carried out with TACS Annexin V Kit (TREVIGEN) and Active CASP3 PE MAb Apoptosis Kit (BD Pharmingen), respectively, according to the manufacturer's instructions.

2.7. Caspase activity

Activation of CASP8 and CASP9 were detected by using Caspase-Glo 8 Assay (Promega) and Caspase-Glo 9 Assay (Promega), respectively, according to the manufacturers' instructions. Luminescence was measured with a GloMax 96 Microplate Luminometer (Promega).

2.8. Statistical analysis

Data are presented as mean ± S.E.M., and statistical analysis was performed using an unpaired Student's *t*-test when comparing two groups means. Data for the quantitative-PCR and the cell proliferation assay were subjected to one-way ANOVA and two-way ANOVA, respectively, and followed by *post hoc* Fisher's PLSD multiple comparisons tests. Differences at $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. ZFAT protein expression in human peripheral B and T lymphocytes

We examined cell-type distribution of ZFAT protein in human peripheral blood by western blot analysis. ZFAT was detected as a 180 kDa protein in $CD19^+$ and $CD3^+$ lymphocytes, but not in the other cell fraction (Fig. 1A), suggesting that ZFAT is predominantly expressed in B and T lymphocytes but not in other cell types including monocytes in human peripheral blood. This result is consistent with the former analyses on ZFAT protein in mice [13].

To examine the expression level of ZFAT in activated peripheral T lymphocytes, purified naive $CD4^+$ T lymphocytes from mouse spleen were stimulated with combinations of anti-CD3 and anti-CD28 antibodies, showing that both ZFAT protein and mRNA levels were increased after the activation compared with those of the resting state (Fig. 1B and C). This result suggested that ZFAT will be a key molecule for blast formation in $CD4^+$ T lymphocytes.

3.2. Growth inhibition by ZFAT-knockdown in MOLT-4

To reveal the functions of ZFAT, ZFAT expression was inhibited by using siRNAs and the effect of ZFAT-knockdown (ZFAT-KD) on cellular activity in a human T lymphoblastic leukaemia, MOLT-4 cells, was examined. Treatment of MOLT-4 cells with two distinct

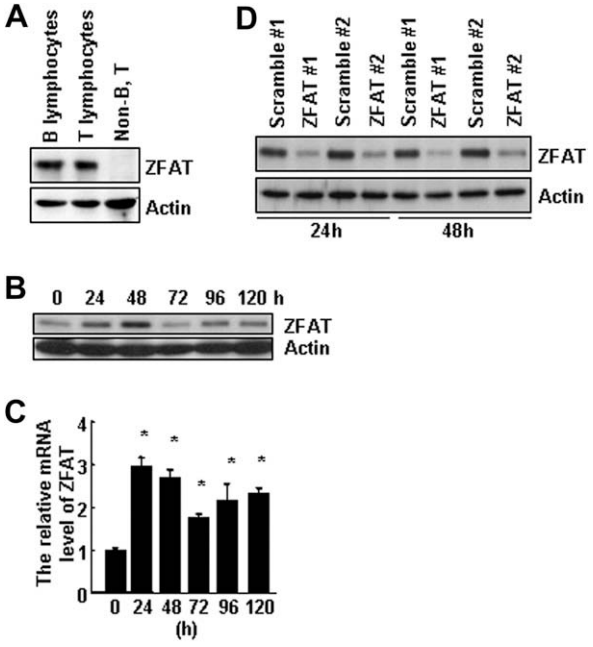

Fig. 1. Human ZFAT protein expression in human peripheral B and T lymphocytes and a human acute T lymphoblastic leukaemia cell line, MOLT-4. Western blot analyses for: (A) MACS-selected lymphocytes isolated from human peripheral blood and (B) purified mouse $CD4^+$ lymphocytes stimulated with combinations of anti-CD3 and anti-CD28 antibodies for the indicated period. (C) The relative mRNA levels of ZFAT in resting or activated $CD4^+$ lymphocytes were determined by real-time PCR. Representative data of two independent experiments in triplicates. $^*P < 0.001$ relative to resting control. (D) MOLT-4 cells treated with ZFAT-specific or scramble siRNAs. Affinity-purified anti-ZFAT antibody was used to detect endogenous ZFAT protein. Western blotting using anti-actin was performed as loading control (A, B and D).

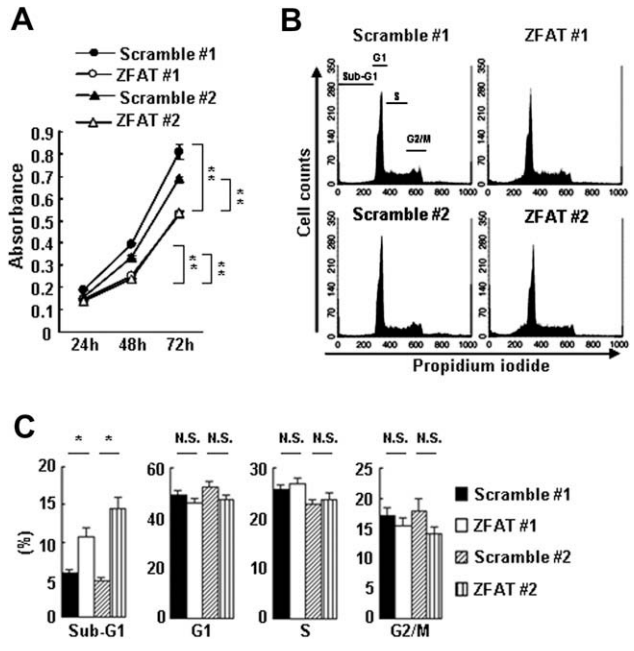

Fig. 2. Growth inhibition by ZFAT-knockdown in MOLT-4. (A) Cell growth of MOLT-4 cells 24, 48 and 72 h after transfection of siRNAs for ZFAT. (B) Cell cycle analysis by propidium iodide staining 48 h after the ZFAT-knockdown (ZFAT-KD). Representative data of eight independent experiments. (C) Quantifications of cell populations in sub-G1, G1, S, and G2/M phases shown in (B) determined by CELL Quest software. $n = 8$; $^{*}P < 0.05$; $^{**}P < 0.001$ relative to scramble siRNA-treated control; N.S., not significant.

siRNAs specific for *ZFAT* sequence led to significant decrease in ZFAT protein expression as compared to scramble RNA-treated controls (Fig. 1D). RT-PCR analysis of *ZFAT* mRNA expression in MOLT-4 cells treated with the siRNAs for *ZFAT* validated the specificities of the siRNAs used in this study (data not shown). We found that ZFAT-KD groups had decreased growth rates compared with the control groups (Fig. 2A).

To assess the exact molecular mechanisms of the growth inhibition of MOLT-4 induced by ZFAT-KD, flow cytofluorometric analysis of cell cycle by propidium iodide staining was done. Forty-eight hours after the knockdown treatment, significant increases in sub-G1 phase were detected in the ZFAT-KD groups relative to the control groups, whereas the other phases, including S-, G1- and G2/M-phases, were comparable between the groups (Fig. 2B and C). These results suggested that ZFAT-KD in MOLT-4 cells caused cell death and that the decreases in the cell numbers of ZFAT-KD groups observed in Fig. 2A would reflect the differences of survival population.

3.3. Apoptosis is induced by ZFAT-knockdown in MOLT-4

To examine whether apoptosis occurs in MOLT-4 cells treated by ZFAT-KD, flow cytofluorometric analysis by annexin V staining was performed, revealing that robust productions of annexin V-positive cells were observed in the ZFAT-KD groups (Fig. 3A and B). This result indicates that ZFAT-KD induced apoptosis in MOLT-4 cells. The apoptosis induced by ZFAT-KD was already detected at 24 h point and more evident at 48 h point after the transductions of siRNAs (Fig. 3B). When MOLT-4 cells were exposed to the conditioned medium in which ZFAT-KD cells were cultured for 48 h, apoptotic cells were not induced (Fig. 3C), suggesting that the ZFAT-induced apoptosis was not caused only by secretory factors from ZFAT-KD cells.

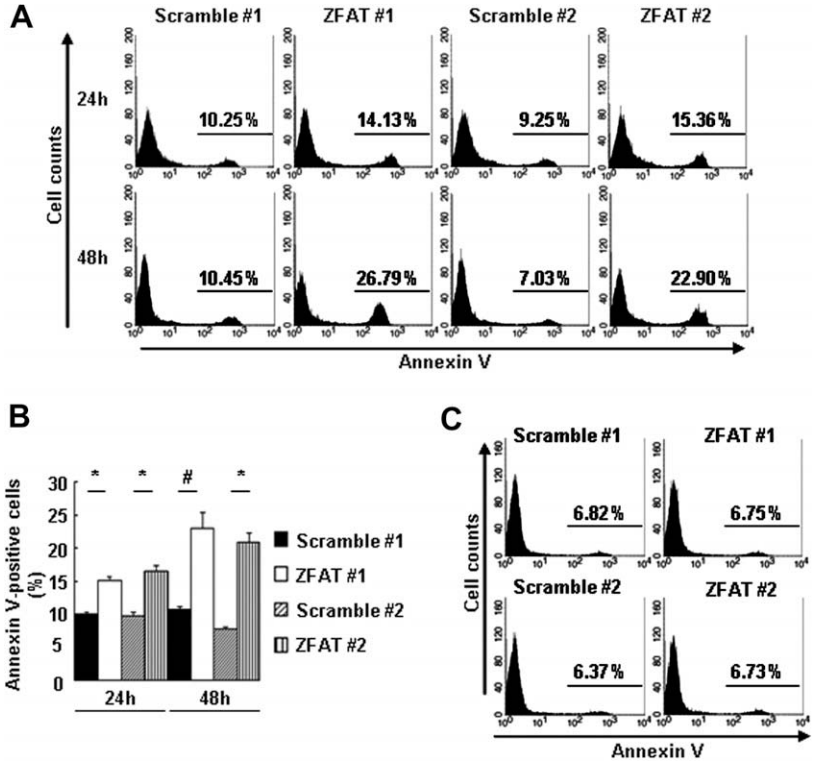

Fig. 3. Apoptosis is induced by ZFAT-knockdown in MOLT-4. (A) Annexin V staining 24 and 48 h after ZFAT-KD in MOLT-4 cells. Representative data of four independent experiments. (B) Quantifications of annexin V-positive cells shown in (A) determined by CELL Quest software. $n = 4$; $^{*}P < 0.01$; $^{\#}P < 0.05$ relative to scramble siRNA-treated control. (C) No induction of apoptotic cells by treatment of native cells with the conditioned medium from ZFAT-KD cells. MOLT-4 cells cultured in the conditioned medium for 48 h was subjected to annexin V staining. Representative data of two independent experiments.

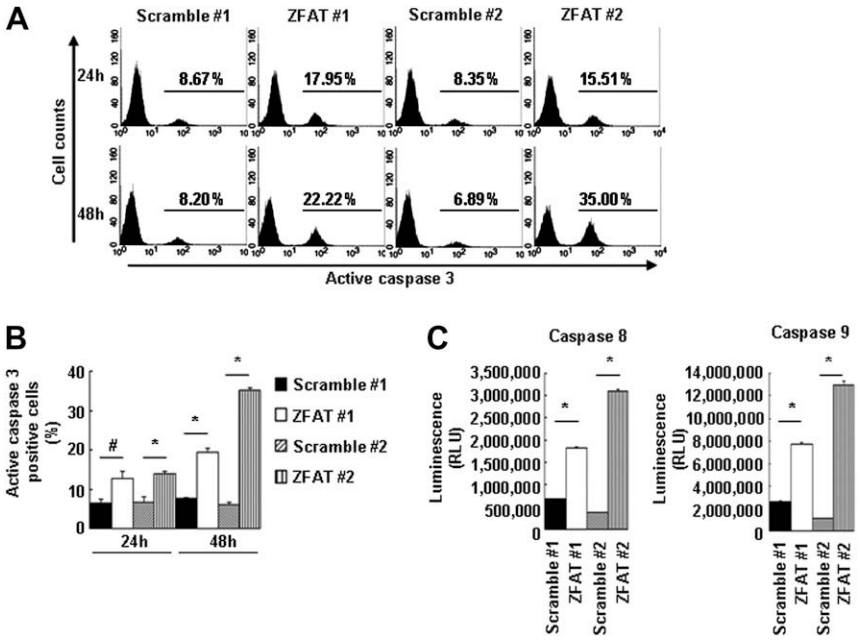

Fig. 4. CASP3, CASP8 and CASP9 are activated by ZFAT-knockdown in MOLT-4. (A) Active CASP3-staining 24 and 48 h after ZFAT-KD in MOLT-4 cells. Representative data of four independent experiments. (B) Quantifications of active CASP3-positive cells shown in (A) determined by CELL Quest software. $n = 4$; $^{*}P < 0.01$; $^{\#}P < 0.05$ relative to scramble siRNA-treated control. (C) CASP8 (left) and CASP9 (right) activities were determined 48 h after ZFAT-KD in MOLT-4 cells. $^{*}P < 0.01$ relative to scramble siRNA-treated control.

3.4. CASP3, CASP8 and CASP9 are activated by ZFAT-knockdown in MOLT-4

Next, to examine the contribution of caspases in the apoptotic event caused by ZFAT-KD in MOLT-4, flow cytofluorometric analysis by active CASP3-staining and luminescent assays measuring CASP8 or CASP9 activities were performed. Expectedly, CASP3 was significantly activated in the ZFAT-KD cells (Fig. 4A and B) and the degree of activation of CASP3 was more evident at 48 h point than 24 h point after the transductions of siRNAs (Fig. 4B), which is consistent with the result that annexin V-positive apoptotic cells gradually emerged in a time-dependent manner (Fig. 3A and B). Furthermore, both CASP8 and CASP9 were activated upon ZFAT-KD in MOLT-4 (Fig. 4C). These results demonstrated that ZFAT-KD in MOLT-4 cells caused apoptosis via CASP8, CASP9 and CASP3 activations.

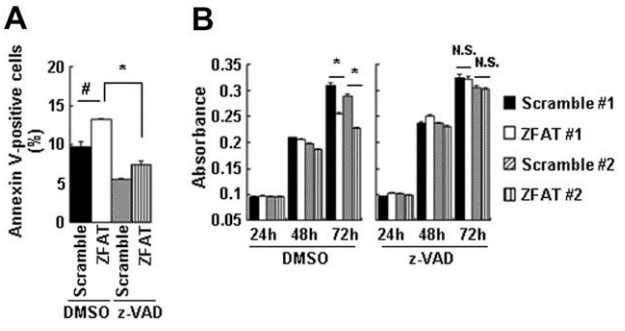

Fig. 5. Caspase inhibitor completely blocks growth inhibition induced by ZFAT-KD. (A) Annexin V staining of MOLT-4 cells 24 h after transfection with siRNAs in the presence of a caspase inhibitor, z-VAD. DMSO was used as the control. (B) Cell growth of MOLT-4 cells 24, 48 and 72 h after transfection of siRNAs in the presence of DMSO (left) or z-VAD (right). $^{*}P < 0.01$; $^{\#}P < 0.05$ relative to scramble siRNA-treated control.

3.5. Caspase inhibitor completely blocks growth inhibition induced by ZFAT-KD

Next, to confirm that apoptosis upon ZFAT-KD in MOLT-4 is mediated by caspases, ZFAT-KD cells were maintained in the presence of a caspase inhibitor, z-VAD, showing that the production of annexin V-positive cells was significantly attenuated (Fig. 5A) and the difference observed in growth rate caused by ZFAT-KD was completely diminished by the caspase inhibitor treatment (Fig. 5B). These results showed that ZFAT-KD cells fell into apoptosis via activations of caspases, but not into cell cycle arrest or reduction in cell cycle rate.

4. Discussion

We demonstrated that human ZFAT is expressed in human peripheral B and T lymphocytes, and that mouse ZFAT expression in T lymphocytes is increased during blast formation. Furthermore, ZFAT-KD induces apoptosis via activation of caspases including CASP8, CASP9 and CASP3 in MOLT-4. Taken together, ZFAT is a critical molecule in cell survival for immune-related cells and the blast formation of naive T lymphocytes.

What is the molecular mechanism of ZFAT in cell survival for MOLT-4? Considering the fact that both CASP8 and CASP9 are activated by ZFAT-KD in MOLT-4 (Fig. 4C), death receptor-mediated and mitochondrial death pathways would be involved in the apoptotic process [15–20]. Furthermore, since apoptosis by ZFAT-KD was not reproduced only by secretory factors (Fig. 3C), not only secretory proteins but also intracellular molecules such as death receptor-associated proteins and cellular inhibitor of apoptosis proteins might also play critical roles in the apoptosis. However, the precise molecular mechanisms underlying the apoptosis by ZFAT-KD should await further research on molecular functions of ZFAT, especially elucidation of the direct ZFAT target sites in the genome.

In the end, our result is the first report demonstrating that ZFAT is an antiapoptotic molecule and critical for cell survival in human

immune-related cells. The understanding of the molecular functions of ZFAT, especially in activated lymphocytes, will shed light on a novel gene-network in tumorigenesis and immune response, and lead to the intervention research aimed at preventing cell malignancies or immune-related diseases.

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References

[1] Ravasi, T., Huber, T., Zavolan, M., Forrest, A., Gaasterland, T., Grimmond, S., Hume, D.A., Group, RIKENGER and Members, GSL (2003) Systematic characterization of the zinc-finger-containing proteins in the mouse transcriptome. Genome Res. 13, 1430–1442.

[2] Brayer, K.J. and Segal, D.J. (2008) Keep your fingers off my DNA: protein–protein interactions mediated by C2H2 zinc-finger domains. Cell Biochem. Biophys. 50, 111–131.

[3] Brayer, K.J., Kulshreshtha, S. and Segal, D.J. (2008) The protein-binding potential of C2H2 zinc-finger domains. Cell Biochem. Biophys. 51, 9–19.

[4] Li, F., Ambrosini, G., Chu, E.Y., Plescia, J., Tognin, S., Marchisio, P.C. and Altieri, D.C. (1998) Control of apoptosis and mitotic spindle checkpoint by surviving. Nature 396, 580–584.

[5] Bai, L. and Merchant, J.L. (2001) ZBP-89 promotes growth arrest through stabilization of p53. Mol. Cell Biol. 21, 4670–4683.

[6] Tang, T.T., Dowbenko, D., Jackson, A., Toney, L., Lewin, D.A., Dent, A.L. and Lasky, L.A. (2002) The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J. Biol. Chem. 277, 14255–14265.

[7] Kang, S.I., Chang, W.J., Cho, S.G. and Kim, I.Y. (2003) Modification of promyelocytic leukemia zinc-finger protein (PLZF) by SUMO-1 conjugation regulates its transcriptional repressor activity. J. Biol. Chem. 278, 51479–51483.

[8] Asanuma, K., Tsuji, N., Endoh, T., Yagihashi, A. and Watanabe, N. (2004) Surviving enhances Fas ligand expression via up-regulation of specificity protein 1-mediated gene transcription in colon cancer cells. J. Immunol. 172, 3922–3929.

[9] Phan, R.T. and Dalla-Favera, R. (2004) The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature 432, 635–639.

[10] Parrado, A., Robledo, M., Moya-Quiles, M.R., Marín, L.A., Chomienne, C., Padua, R.A. and Alvarez-López, M.R. (2004) The promyelocytic leukemia zinc-finger protein down-regulates apoptosis and expression of the proapoptotic BID protein in lymphocytes. Proc. Natl. Acad. Sci. 101, 1898–1903.

[11] Sakai, K., Shirasawa, S., Ishikawa, N., Ito, K., Tamai, H., Kuma, K., Akamizu, T., Tanimura, M., Furugaki, K., Yamamoto, K. and Sasazuki, T. (2001) Identification of susceptibility loci for autoimmune thyroid disease to 5q31–q33 and Hashimoto's thyroiditis to 8q23–q24 by multipoint affected sib-pair linkage. Hum. Mol. Genet. 10, 1379–1386.

[12] Shirasawa, S., Harada, H., Furugaki, K., Akamizu, T., Ishikawa, N., Ito, K., Ito, K., Tamai, H., Kuma, K., Kubota, S., Hiratani, H., Tsuchiya, T., Baba, I., Ishikawa, M., Tanaka, M., Sakai, K., Aoki, M., Yamamoto, K. and Sasazuki, T. (2004) SNPs in the promoter of a B cell-specific antisense transcript, SAS-ZFAT, determine susceptibility to autoimmune thyroid disease. Hum. Mol. Genet. 13, 2221–2231.

[13] Koyanagi, M., Nakabayashi, K., Fujimoto, T., Gu, N., Baba, I., Takashima, Y., Doi, K., Harada, H., Kato, N., Sasazuki, T. and Shirasawa, S. (2008) ZFAT expression in B and T lymphocytes and identification of ZFAT-regulated genes. Genomics 91, 451–457.

[14] Fujimoto, T., Koyanagi, M., Baba, I., Nakabayashi, K., Kato, N., Sasazuki, T. and Shirasawa, S. (2007) Analysis of KRAP expression and localization, and genes regulated by KRAP in a human colon cancer cell line. J. Hum. Genet. 52, 978–984.

[15] Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. and Dixit, V.M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death–inducing signaling complex. Cell 85, 817–827.

[16] Chaudhary, P.M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J. and Hood, L. (1997) Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. Immunity 7, 821–830.

[17] Schneider, P., Thome, M., Burns, K., Bodmer, J.L., Hofmann, K., Kataoka, T., Holler, N. and Tschopp, J. (1997) TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity 7, 831–836.

[18] Wang, L., Du, F. and Wang, X. (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133, 693–703.

[19] Debatin, K.M., Poncet, D. and Kroemer, G. (2002) Chemotherapy: targeting the mitochondrial cell death pathway. Oncogene 21, 8786–8803.

[20] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates. Cell 91, 479–489.