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**Original Paper**

# Autoimmune Regulator Enhanced the Expression of Caspase-3 and Did Not Induce Massive Germ Cell Apoptosis in GC1-Spg Cells

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**Key Words**
Autoimmune regulator • Testis • Spermatogenesis • Caspase • Apoptosis

**Abstract**
***Background/Aims:*** AIRE is known for its involvement in autoreactive T-cell deletion in thymic epithelium. Though extrathymic expression of AIRE is well documented, the functional relevance of AIRE in non-thymus tissues is emerging. AIRE is expressed in neonatal and adult testis, and has been implicated in sporadic germ cell apoptosis in developing testis. In this study we examined whether AIRE has any role in inducing apoptosis in cultured spermatogonial cells. ***Methods:*** We over-expressed AIRE or CARD domain of AIRE in GC1-spg cells and evaluated its impact on cell cycle using fluorescence activated cell sorting following Hoechst 33342 staining. Apoptosis was assayed using Annexin-V staining. Caspase-3 cleavage was assessed on western blots and caspase-3 expression was quantitated using realtime PCR. ***Results:*** We report that C18-4 cells which are derived from Type A spermatogonia expressed AIRE, while GC1-spg which is closer to Type B spermatogonia was negative for AIRE expression. Overexpression of AIRE or CARD domain of AIRE induced Caspase-3 expression in GC1-spg cells. Silencing of AIRE in C18-4 cells inhibited Caspase-3 expression. When overexpressed, AIRE and CARD brought about a very negligible increase in germ cell death and resulted in altered cell cycle pattern with a reduction in G1 phase. This was not associated with any increase in activation of Caspase-3. ***Conclusion:*** We conclude that the CARD domain of AIRE enhances caspase-3 expression through possible direct DNA binding and triggers non-apoptotic downstream signaling in cultured spermatogonial cells.

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## Introduction

Autoimmune regulator (*AIRE*) was identified as a gene expressed in thymus which when mutated leads to a rare autoimmune disorder APECED [1, 2]. In thymus, AIRE is expressed in medullary epithelial cells [3] and it regulates the expression of tissue restricted antigens [4] to prevent autoimmunity. AIRE is a non-classical transcription factor [5, 6] with DNA binding properties [7, 8]. AIRE regulates gene expression by chromatin binding [9], using its histone binding domain [10, 11], and with the involvement of miRNAs [12, 13]. The presence of *Aire* transcript is not limited to thymus but is widely reported in different tissues of both human and murine systems [14]. Among the tissues outside immune system, presence of *Aire* in mouse testis is particularly interesting. Testis has abundant expression of AIRE protein [15] and *Aire* deficient mouse showed compromised fertility rates and reduced litter size [16]. A reduction in the number of apoptotic cells in testis at an early phase of germ cell apoptosis is a notable feature of *Aire*-/- mouse [17]. Apoptotic events operating at various stages of germ cell development are critical to a developing testis and caspase activation is known to play a crucial role in regulating the processes. AIRE has a Caspase Recruitment domain [18] and it has been shown to induce cell death in HEK 293 cells [19]. However, the role of AIRE in caspase mediated germ cell death has not been addressed. The expression of AIRE in the genital ridges of 12.5 day mouse embryos of both sexes and in embryonic stem cells of mouse and human origin along with other core pluripotency markers [20] have further strengthened the assumption that it might as well be involved in early development. In this work, we have evaluated the role of AIRE and its CARD domain in inducing germ cell death and whether AIRE/CARD dependent upregulation of caspase-3 in germ cells sensitizes these cells towards a drug induced apoptotic death.

## Materials and Methods

*Reagents*

Sourcing of antibodies used in this study were as follows: Caspase-3 and-9 (Cell signalling Technology Inc., Danvers, USA); AIRE-1 (M-300), OCT-3/4 (H-134), Actin (I-19), Goat anti-rabbit HRP, donkey anti-goat HRP (Santa Cruz Biotechnology Inc, CA, USA) and anti-GFP (Lifespan biosciences, Seattle, USA). Lipofectamine 2000 (Invitrogen, CA, USA); DMEM, DMEMF12, OptiMEM, FBS, Antibiotic-antimycotic cocktail, Glutamine and Non-essential amino acids (Invitrogen, Green Island, NY, USA); Pan caspase inhibitor Z-VAD-FMK (Bio vision, Mountain view, CA, California), Sodium pyruvate, Thapsigargin and Doxorubicin were from Sigma-Aldrich, MO, USA. Restriction enzymes EcoR1, Sal1, CIP, NEB buffer 3, EcoR1 Buffer, DNA Ligase and Ligase buffer from New England Biolabs, MA, USA. Nucleobond Xtra Midi Plus EF kit (Macherey Nagel, Duren, Germany), Gel extraction kit (GE Healthcare, NA, USA), Vybrant® Apoptosis Assay Kit #6 (Invitrogen, CA, USA) were also procured. A list of cell lines mentioned in this article and their source is included in Table 1.

*Cell culture and transfection*

GC1-spg (ATCC® CRL-2053™ from ATCC, VA, USA) and HeLa (Cell repository at Rajiv Gandhi Centre for Biotechnology, Trivandrum, India) cells were cultured in DMEM supplemented with 10% FBS and antibiotics. C18-4 cells were cultured in DMEM-F12 supplemented with 10% FBS, 1% non-essential amino acids and antibiotics. All transfections involving cell lines were carried out using Lipofectamine 2000 as per manufacturer's instructions. All transfections were carried out in Opti MEM, reduced serum medium. Four hours after transfection, the culture medium was replaced with fresh culture medium. In experiments involving drug treatment of cells in culture, thapsigargin or doxorubicin dissolved in DMSO (ATCC, VA, USA) was added to cells post transfection at a final concentration of 2

**Table 1.** Cell lines mentioned in this study and their sources

| Cell line | Source |
|---|---|
| C18-4 | Mouse type-A spermatogonia |
| GC1-spg | Mouse type-B spermatogonia |
| HEK 293 | Human embryonic kidney cells |
| HT-93 | Acute promyelocytic leukemia (APL) cells |
| SK-Hep-1 | Human endothelial cells |

μM /mL of medium. For inhibition of caspase activity, Z-VAD-FMK (2 μM/ mL) was added to cells two hours prior to transfection.

*Plasmid construction*

From pET-32a(+)-AIRE previously generated in our laboratory, full length AIRE (amino acids 1-545) and the N-terminal CARD domain (amino acids 1-197) were amplified with hAIRE 1F/hAIRE 545F and hAIRE 1F/hAIRE 197R primer pairs and were subcloned into pEGFPN1 vector. The constructs were authenticated by direct sequencing of the inserts and flanking vector regions. Details of primers used in RT-PCR and RT-qPCR are given under Table 2.

For over expression in GC-1spg cells, full length *AIRE*-GFP plasmid and CARD-GFP plasmids were isolated using Nucleobond Xtra Midi Plus EF kit (Macherey Nagel, Duren, Germany) as per the manufacturer's instructions and plasmids were quantitated using Nanodrop (Nano Drop 1000, Thermo Scientific, DE, USA)

*Protein extraction and Western blot analysis*

Protein extraction from mouse testis was carried out as described previously [15]. Cells were lysed by agitation at 4°C for 1 hour in RadioImmunoPrecipitationAssay(RIPA) buffer (150mM NaCl, 1.0 % NP 40, 0.5 % Sodium deoxycholate (HiMedia Laborataries, Mumbai, India), 50 mM Tris-HCl (pH 8.0) supplemented with protease inhibitor cocktail (Sigma, MO, USA) followed by centrifugation at high speed. Protein concentration was determined using DC protein assay kit (Bio Rad, Richmond, CA, USA). Cells obtained through FACS were washed with PBS and the cell pellet resuspended in residual PBS was mixed with an equal volume of Laemmli buffer and were heat- denatured before loading on SDS PAGE. Proteins were transferred to PVDF membrane (GE Healthcare, NA, USA) and were saturated with 5 % non fat dry milk. Detection was done using the respective primary antibodies. Secondary antibodies were conjugated to horse radish peroxidase. Detection was performed colorimetrically using 0.05 % DAB (Sigma-Aldrich, MO, USA), 0.1 % hydrogen peroxide (Qualigens, Glaxo Smithkline, Mumbai, India) and 0.04 % nickel chloride (Spectrum chemicals, Cochin, India). All western blot images were analysed using Phoretix Advanced 1D software (Non linear dynamics, California, USA) and results from 3 independent sets were averaged.

*RNA isolation and cDNA preparation*

RNA isolation from mouse tissues and transfected cell lines were carried out using TRI reagent. From each age group, 4 animals were used for RNA extraction. In the case of transfected cells 2 sets from two independent transfections were used. Total RNA (5 μg in 33μl RNase-free water) quantitated with Nanodrop and having 260/280 ratio of 2 or above were reverse transcribed using Ready-To-Go™ T-primed first strand cDNA synthesis kit (Amersham Biosciences, NJ, USA). RNA isolation from FACS sorted cells and primary culture of testicular cells after puromycin selection was carried out using RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1μg RNA was reverse transcribed using SUPERSCRIPT®VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA).

*Quantitative real time PCR*

The primers used for real time PCR are listed in Table 2. Quantitative Real time PCR was performed using SYBR green master mix (Applied Biosystems, WA, UK) using ABI PRISM® 96 well optical reaction plates. All reactions were performed in triplicates. Each 10 μL PCR reaction contained 1μL cDNA mixed with 2X Power SYBR-Green PCR master mix and primers diluted to a final concentration of 0.2 μM. The following cycling parameters were used: 50° C for 10 minutes, 90° C for 10 minutes and 95° C for 10 minutes. This was followed by 40 cycles at 95° C for 10 seconds and a combined annealing/extension temperature of 60° C for 2 minutes.

**Table 2.** List of primers used

| Primer Name | Sequence |
|---|---|
| hAire 1F | ATG GCG ACG GAC GCG GCG CTA |
| hAire 545R | GGA GGG GAA GGG GGC CGC CGG |
| hAire 197 R | CCC GGA GGA CAT GGC CAC AGC |
| mAire 4F | AAG ACT AAG CCC CCT AAG AAG C |
| mAire 8R | CAC ACT CAT CCT CGT TCT TCT G |
| beta actin F | TGT GAT GGT GGG AAT GGG TCA G |
| beta actin R | TTT GAT GTC ACG CAC GAT TTC C |
| For Real time PCR | |
| Aire F | GAA GCT GTA CCC ACC TCT GG |
| Aire R | GAC TCC AGG TCG TCC CTA TG |
| caspase 3 F | TCT GAC TGG AAA GCC GAA ACT C |
| caspase 3 R | TCC CAC TGT CTG TCT CAA TGC CAC |
| beta actin F | CAT TGC TGA CAG GATGCA GAA |
| beta actin R | GCT CAG GAG GAG CAA TGA TCT T |

*Analysing RT PCR data*

All PCR reactions were done in 96 well format and the average CT values were calculated. For relative quantification of gene expression mouse beta actin was used as reference gene. All groups were done in triplicate where each experiment was done in duplicate. Delta CT values of control and test were evaluated through Student's *t*-test to evaluate their statistical significance. Relative fold change is shown in log scale. In silencing experiments, the expression level of target gene in control samples were taken as 100 % and relative reduction in fold change is represented graphically.

*Annexin staining of transfected cells*

Cells grown in 6 well culture plates were transfected, harvested twelve hours post transfection and were stained following Vybrant apoptosis Assay kit (Molecular probes, Invitrogen, Oregon, USA) protocols. Briefly, the cell pellets were resuspended in annexin binding buffer and incubated with Biotin-X annexin-V antibody. After the incubation cells were resuspended in annexin binding buffer and incubated with AlexaFluor®350 streptavidin solution for 30 minutes. Propidium iodide was also added to the resuspended pellet and incubated for 10 minutes at room temperature. Analysis was performed using FACS. The data presented here are from three independent analyses from three independent transfections. Untransfected GC1-spg cells and cells transfected with pEGFPN1 were used as controls. For treatment with general caspase inhibitor, 2mM cell permeable Z-VAD-FMK was added to cells 2 hours prior transfection. Aire over expressing GC1-spg cells without Z-VAD-FMK treatment served as control.

*Cell cycle analysis of Hoechst 33342 stained GFP positive live cells*

DNA content of GC-1spg cells transfected with GFP-AIRE was analysed using flow cytometry. Transfected cells harvested by trypsinization were pelleted down at 1000 rpm. After PBS wash, the cell pellets were resuspended in 500 µL DMEM and incubated at 37° C for 45 min with 5 µL of 1 mg/mL Hoechst 33342 (Invitrogen, CA, USA). After gating the GFP positive population and double discrimination using area *vs.* width plot, DNA content of GFP positive populations were analysed. GFP positive population from GC1-spg cells transfected with pEGFP-N1 vector was used as control. Analysis was done with cells from four independent transfections.

## Results

*Expression of Aire in C18-4 and GC1-spg cells*

The C18-4 cells are mouse type A spermatogonia immortalized using the Simian virus 40 large T-antigen gene (LTAg) under the control of an ecdysone-inducible promoter and has neomycin resistance. The cells exhibit morphological features typical of spermatogonia at the light microscopic level and also express germ line stem cell markers like Oct-4, and GFRα-1 and Dazl [21]. The GC1-spg is a mouse testicular germ cell line, also immortalized using LTAg and has neomycin resistance. The cell line phenotype resembles to a stage between type B spermatogonia and primary spermatocyte, based on its characteristic features observed in phase contrast and electron microscopy. This cell line expresses two testis specific isoproteins, cytochrome c and lactate dehydrogenase C4 [22].

The basal level of expression of Aire in C18-4 and GC1-spg cells was evaluated using RT-PCR and western blot analysis. C18-4 cells expressed AIRE, while GC1-spg cells were negative for AIRE expression (Fig. 1).

*AIRE influenced the expression of CASP3 in C18-4 and GC1-spg cells*

Both full-length AIRE (Fig. 2B) and the CARD domain of AIRE (Fig. 2C) were expressed as EGFP fusion proteins in GC1-spg cells. Confocal microscopy indicated that the localization was predominantly on the cytoskeletal network, with clearly noticeable nuclear speckles visible in both the cases. An empty vector control showed a diffuse distribution of fluorescence (Fig. 2A). The expression of these constructs was further confirmed on western blots (Fig. 3). The efficiency of knocking down of AIRE in C18-4 was also confirmed (Fig. 4). The over-expression of AIRE-EGFP in GC1-spg cells enhanced the expression of Casp3 (Fig. 5A), while the silencing of AIRE lowered the levels of Casp3 (Fig. 5B).

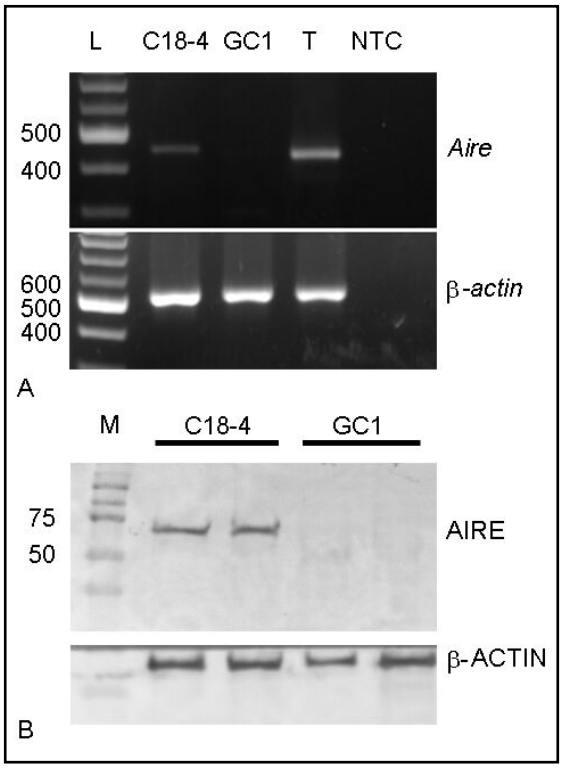


**Fig. 1.** (A) RT-PCR analysis of Aire expression in C18-4 and GC1-spg cells. L- 100 bp ladder, T – neonatal testis and NTC- no template control. (B) Western blot showing the expression of AIRE in C18-4 (lanes 2 and 3) and GC1-spg (lanes 4 and 5) cells. Lane 1 shows molecular weight markers. Beta-actin was used as loading control.

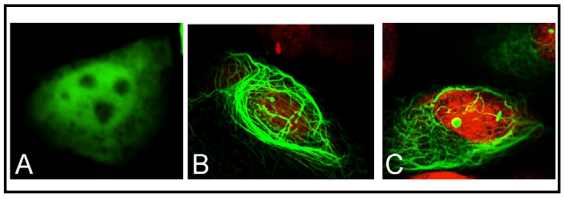


**Fig. 2.** EGFP was localized in a diffuse pattern both in the nucleus and the cytoplasm (A), while AIRE-EGFP (B) and CARD-EGFP (C) were localized heavily on the cytoskeletal network and nuclear speckles. Green channel shows EGFP localization, while the red channel shows propidium iodide staining of the nucleus.

*Over expression of AIRE/CARD in GC1-spg cells did not enhance apoptosis*

RT-PCR analyses on cDNA obtained from transfected cells confirmed the presence of GFP, CARD, and AIRE transcripts (Fig. S1, B – for all supplemental material see www.cellphysiolbiochem.com). Cell cycle analysis with Hoechst 33342 stained live GFP positive cells from control (Fig. 6A) and AIRE transfected GC1-spg cells (Fig. 6B) showed alterations in cell cycle pattern and accumulation of cells in G0 phase. Cell cycle analysis performed 24 hours after transfection showed that an average of 4.03 % cells were in G0 stage in controls when compared to the 12% cells in G0 state in AIRE expressing cells, indicating a four-fold increase in G0 population in the latter cells. The increase in G0 cells was followed by a corresponding reduction of the cells in G1 phase too, with 73 % G1 stage cells in AIRE expressing set when compared to the 79 % in the control set. A significant increase (with $p$ value ≤ 0.05, marked as **) in G0 phase along with a reduction in G1 phase was evident in AIRE over expressing germ cells (Fig. 6C). With alteration in cell cycle pattern, AIRE-positive cells also showed the externalization of canonical apoptotic marker phosphatidyl serine detected using BiotinX–annexin-Alexa Fluor®350 staining followed by FACS. The results are graphically represented in Fig. 6E. The basal level of cell death was only 0.8 % in GC1-spg cells transfected with empty vector. AIRE-expressing cells showed significant increase in annexin positivity ($p \leq 0.05$), when compared with untransfected cells or cells transfected with insertless vector. Annexin positivity in cells expressing CARD domain (1-197 amino acids) was also significantly higher

(Fig. 6D and 6E) when compared with the controls as mentioned above. However, the overall increase in cell death due to the expression of full length AIRE or the CARD domain was only 0.2 - 0.4 %. Treatment of AIRE-EGFP expressing GC-1spg cells with pan caspase inhibitor Z-VAD-FMK brought about 75 % reduction in annexin positive cells (Q4) when compared with untreated Aire expressing controls (Fig. 7A). When treated with Z-VAD-FMK, the percentage of apoptotic cells in GC1-spg cells expressing AIRE were comparable to empty vector controls (Fig. 7B). To evaluate the possibility of the activation of caspase mediated cell death pathway in AIRE expressing GC1-spg cells, protein from GC-1spg cells overexpressing Aire and three functional domains CARD, PHD, and SAND were evaluated for up/down regulation of caspases (Fig. 7C). Primary antibodies against caspase-2, -3, -6, -8, -12 and PARP were used to assess possible activation and cleavage of caspase. Cell lysate from GC1-spg cells treated with thapsigargin (1.5 μM) served as positive control for activated caspases. Untreated GC-

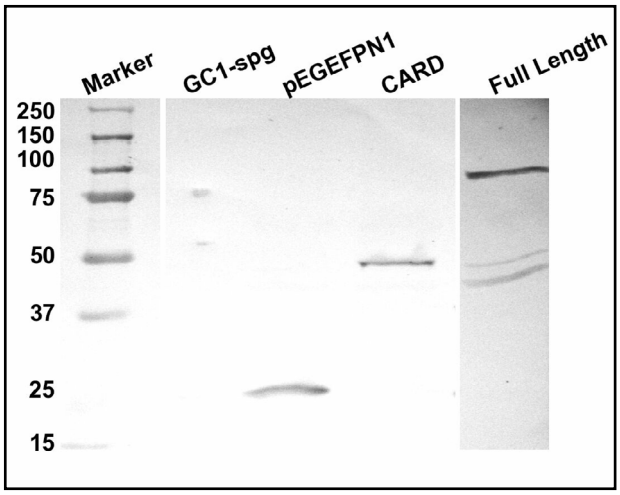


**Fig. 3.** Western blot of cell lysates from untransefected GC1-spg cells (lane 2) and those transfected with empty vector (lane 3), CARD-EGFP (lane 4) and AIRE-EGFP (lane 5), probed with anti-EGFP antibody. Lane 1 shows molecular weight markers.

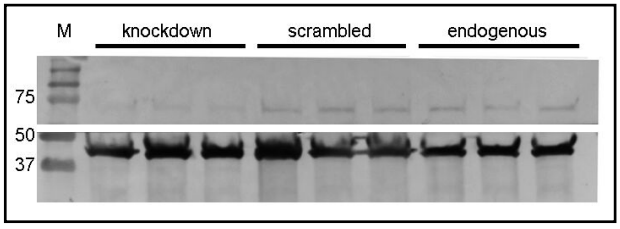


**Fig. 4.** Western blot showing AIRE expression in Aire shRNA transfected (knockdown), control shRNA transefected (scrambled) and untransfected (endogenous) C18-4 cells.

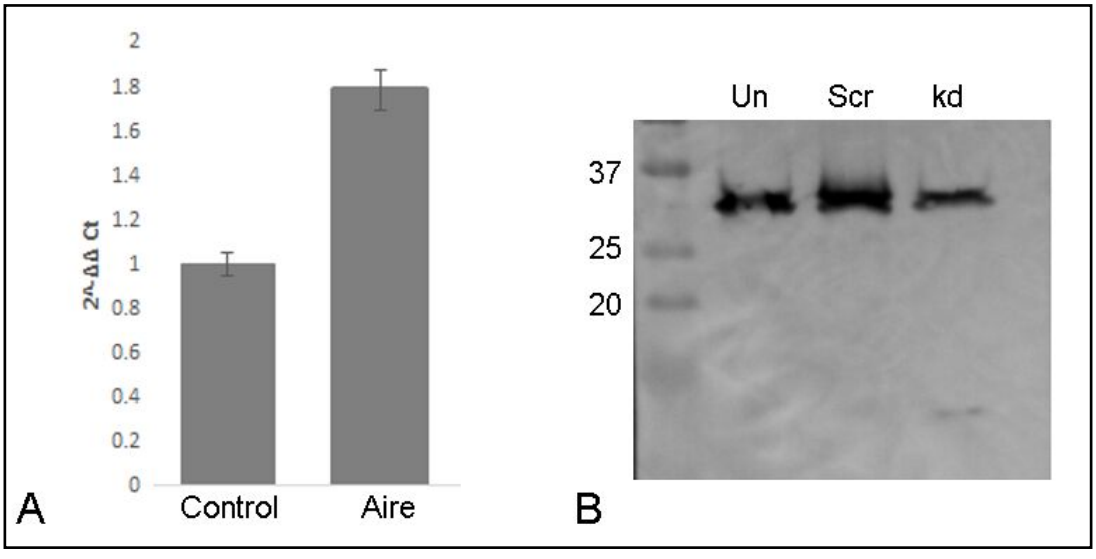


**Fig. 5.** (A) Real time PCR showing caspase 3 transcript level in GC1-spg cells transfected with empty vector and AIRE-EGFP (B) Western blot analysis of caspase 3 expression in untransfected (Un), control shRNA transfected (Scr) and Aire shRNA transfected (kd) C18-4 cells.

1spg cell lysate served as negative control for caspase activation and positive control for caspase expression. Endogenous caspase-2 and -8 were absent in GC1-spg lysate. Transient expression of AIRE in GC1-spg cells did not induce activation of any of the caspases tested, even though endogenous caspase-3 level was upregulated in Aire expressing cells.

*Aire expression did not enhance the sensitivity of GC-1spg cells to doxorubicin*

GC1-spg cells are sensitive to doxorubicin, an apoptotic inducer. Doxorubin induces caspase 3 mediated cell death in germ cells. We evaluated whether AIRE /CARD expression enhanced the sensitivity of GC1-spg cells to doxorubicin. Treatment with doxorubicin for 12 hours induced elevation in the levels of caspase-3 cleavage in GC1-spg expressing full length AIRE or the CARD domain when compared with that of the empty vector control (Fig. 8A). Moreover, significantly higher levels of caspase-3 were detected in GC1-spg cells overexpressing full-length AIRE or CARD when compared with the empty vector controls (Fig. 8B). As expected, activated caspase-3 (17kDa) was visible in all doxorubicin treated groups. Transfected cells maintained under similar conditions without drug treatment did not show any caspase-3 activation. However, the expression of AIRE or CARD did not enhance caspase-3 cleavage in response to doxorubicin treatment, as interpreted from the ratios of the normalized band intensities of uncleaved caspase 3 before and after treatment. While the reduction in band intensity was 1.8 fold in control, the same was 2.3 for AIRE transefected and 2.1 fold for CARD transfected cells. Real time qPCR analysis revealed a statistically significant increase in the expression caspase-3 at 16 hours post transfection in cells overexpressing full-length AIRE or CARD, when compared to vector controls (Fig. 8C).

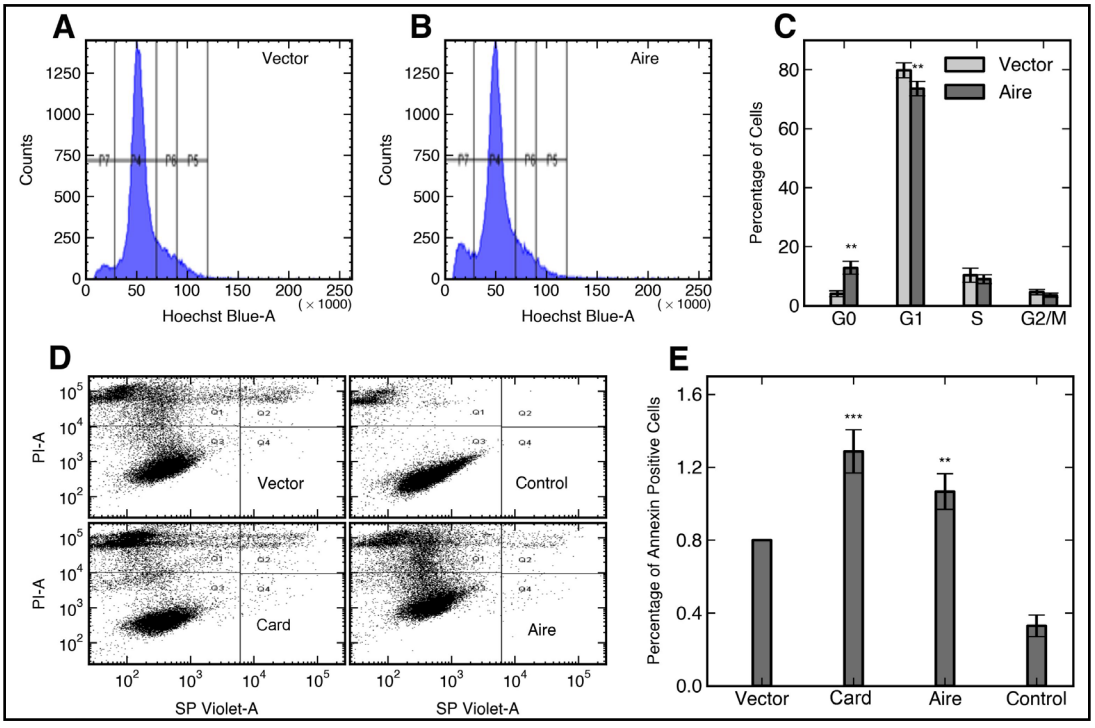


**Fig. 6.** (A) and (B) Histogram showing cell cycle analysis of GC1 cells over expressing Aire and control Vector (C). Percentage of cells in Sub $G_0$, $G_1$, S and $G_2$/M stages of cell cycle. Data represented here is from four independent transfection represented as Mean ± SEM. $p<0.05$ for $G_0$ and $p<0.05$ for $G_1$ phase. Asterisks indicate statistically significant difference versus control. (D) Flow cytometric analysis of Annexin positive cells in GC1 cells, transfected with Vector, CARD and Aire full length are shown. The Q4 population represents the apoptotic annexin positive cells. (E) Graphical representation of percentage of annexin positive cells normalised to vector controls from three independent experiments. Values are represented as Mean ± SEM and Students t-test showed $p< 0.001$ for card and $p< 0.05$ for full length.

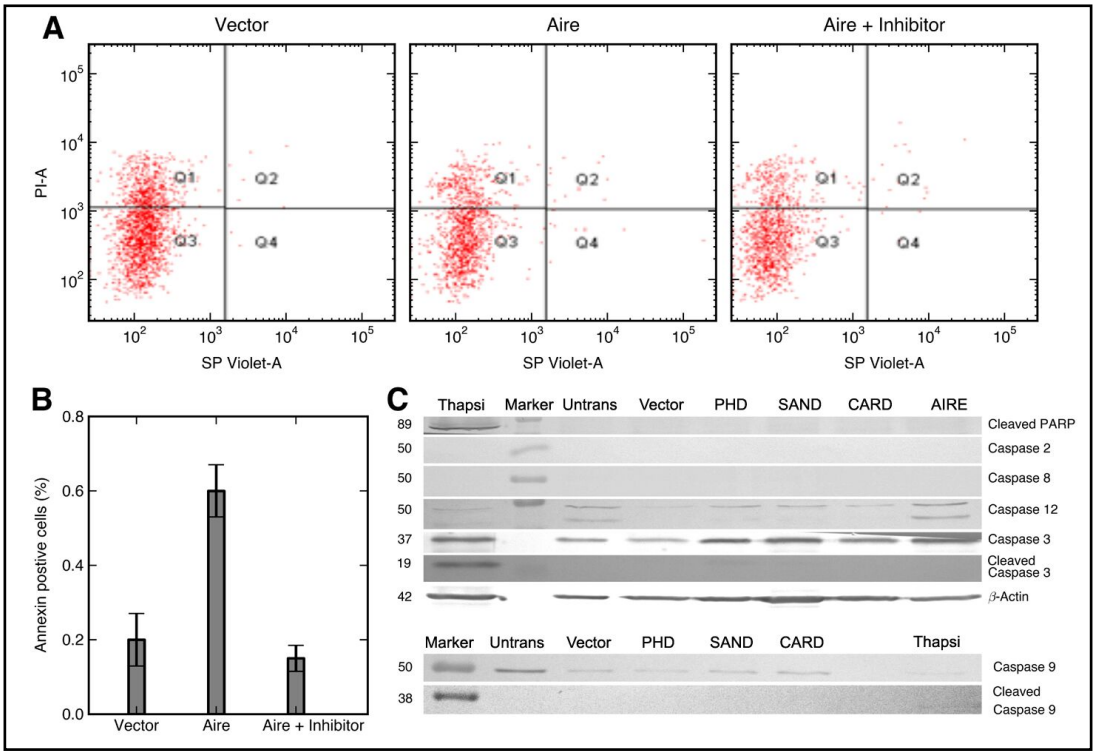


**Fig. 7.** (A) Dot plot showing reduction in annexin positivity of Aire expressing cells treated with general caspase inhibitor Z-VAD-FMK. GC1 cells over expressing GFP and untreated Aire expressing cells are taken as controls. (B) Percentage of annexin positive cells in cells transfected with Aire pre-treated with and without caspase inhibitor. Data from two independent transfection represented as Mean ± SEM. (C) Caspase-2,3,8,9,12 cleaved PARP and cleaved caspase 3 expression in total lysate from GC1 cells (untransfected and empty vector controls - negative controls), thapsigargin-treated cells (positive control) and cells transfected with full length AIRE, PHD, SAND and CARD constructs.

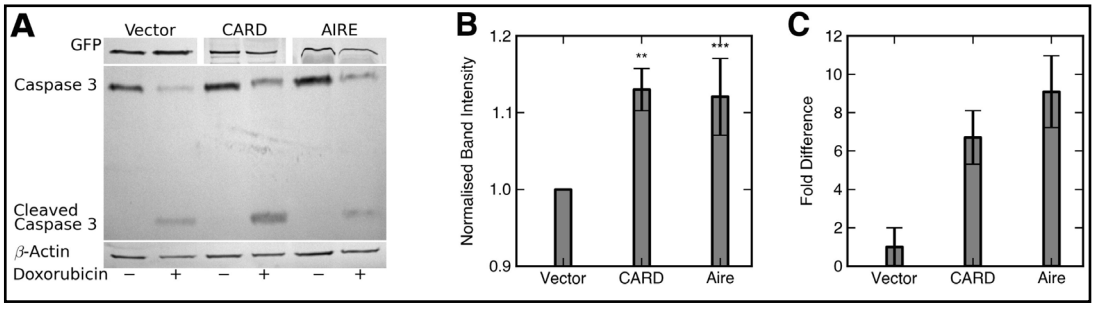


**Fig. 8.** (A) Representative image of western blot detection of caspase 3 and cleaved caspase 3 in cell lysates of GC1 cells over expressing GFP, CARD, and AIRE full length with and without doxorubicin tretment. (B) Normalised band intensities showing caspase 3 level. Values were calculated from three independent transfection with each set done in duplicates. Band intensities were calculated using Phoretix 1D advanced software and were normalised to beta actin. Stuents t Test showed $p<0.05$ (CARD) and $p< 0.001$ (full length AIRE). (C) Quantitative Real time PCR evaluations of caspase-3 levels in GC1 cells transfected with AIRE, CARD and insert less vector. The data shown here is from two independent transfection and each set RT-qPCR done in triplicates and fold change relative to beta actin is represented on log scale.

## Discussion

The presence of Aire mRNA in lung, kidney, adrenal gland ovary and testis of mouse [23, 24] suggested that it might also have function(s) outside the immune system [25, 26]. The detection of AIRE protein in mouse testis raised the possibility that it might regulate promiscuous gene expression in testis [17, 27]. Gonads share with the thymus the distinctive property of generating cells each of which is genetically unique. Promiscuous expression of proteins in these genetically unique germ cells, under the influence of AIRE and/or other similar proteins, could facilitate the detection of incompatibilities between proteins encoded by their unique genome and subsequent elimination of those cells which register incompatibilities [27]. Thus, AIRE-deficient mice showed reduction in scheduled apoptotic wave of germ cells which is necessary for normal mature spermatogenesis and increased incidence of sporadic apoptosis of germ cells in testis [17]. A reduction in early apoptotic event is reported in the germ cells of $Aire^{-/-}$ mouse, but AIRE was never shown to associate directly with any molecule of apoptotic cascade [17]. But, this was not the case in somatic cells. Overexpression of AIRE in two cultured epithelial cells (HT93 and SK-Hep-1) resulted in increased levels of several chaperones (HSC70, HSP27 and tubulin-specific chaperone A) and reduction in the levels of various cytoskeleton interacting proteins (transgelin, caldesmon, tropomyosin alpha-1 chain, myosin regulatory light polypeptide 9, and myosin-9) and differential expression of some apoptosis-related proteins. Moreover, the AIRE-positive cells suffered more spontaneous apoptosis and were less resistant to apoptosis induction [28].

AIRE expression was detected in keratinocytes and in tumors originating from stratified epithelia. An enrichment of translation-related pathways was observed in AIRE-expressing tumors [29]. A significant increase of cells in G1 phase and activation of caspase cascades was induced by AIRE transfection in breast cancer luminal cell lines [29]

We had previously amplified and cloned full length *Aire* from mouse testis [15]. We had also reported that GC-1 spg cells, a mouse spermatogonia-derived cell line, is negative for AIRE expression and that over-expression of AIRE-EGFP in them showed the nuclear dot like and filamentous pattern [30], similar to the previously established pattern of expression in other cell lines [31]. On the other hand, C18-4, another spermatogonia derived cell line which is closer to A-type spermatogonial cells expressed AIRE (Fig. 1). Ectopic expression of AIRE in GC1-spg cells followed by label-free LC-MS/MS detected increased levels of various nucleic-acid-binding proteins (proteins involved in transcription, translation and RNA processing) and a decreased level of various cytoskeletal and structural proteins in the AIRE overexpressing cells [30]. Thus, there are both similarities and differences in the ways in which AIRE influenced cellular proteomes of cultured epithelial cells [28] and spermatogonial cells [30].

This led us to examine whether overexpression of AIRE would enhance apoptosis in spermatogonial cells. The incidence of apoptosis in GC1-spg cultures was extremely low (0.4 %). Overexpression of AIRE elevated the percentage of apoptosis in GC1-spg cells to 1.3 % from the corresponding empty vector control values of 0.8 %. The expression of N-terminal CARD domain (amino acids 1-197) was sufficient to bring about this enhancement in apoptosis in GC1-spg cells (Fig. 6E). It should be noted that very minor fraction of only around 0.4 % of cells underwent apoptosis, though this enhancement in apoptosis was statistically significant. The observed inhibition of this effect by ZVAD-FMK (Fig. 7B) is suggestive of caspase-dependent processes in operation. This result also indicated that the CARD domain alone is sufficient to mediate the observed enhancement in cell death in GC1-spg cells. Our data also suggests an altered cell cycle pattern in germ cells that over express AIRE, favouring the accumulation of cells at the G0 state with a concomitant reduction in the population entering G1 phase. Germ cells undergo caspase mediated cell death and caspase-3 is an executioner caspase that operates at the cross roads of intrinsic, extrinsic, and endoplasmic reticulum mediated apoptotic pathways [32]. The absence of caspase-2 and caspase-8 in GC-1spg cells (Fig. 8C) is in agreement with an earlier report [33], and has strengthened the assumption that any caspase mediated cell death in AIRE expressing GC1-spg cells might

be through enhanced caspase-3 activity. We could observe a significant upregulation of endogenous caspase-3 in GC1-spg cells, both at the transcript (Fig. 5A) and the protein (Fig. 7C) level. This observation is relevant as studies have shown an up regulation of caspase-3 mRNA in events of cell death in nervous system and cellular stress [34]. The proteome profile data of GC1-spg cells over-expressing AIRE (PXD002511) also showed greater than 2-fold increase in the levels of caspase-3. On the other hand, silencing of *Aire* in C18-4 cells reduced the levels of caspase-3 (Fig. 5B), confirming that AIRE activates transcription and translation of caspase-3.

Aire is known to activate transcription of silent genes by releasing RNA polymerases from the promoter binding sites [35]. Our data shows that AIRE activates transcription of caspase-3 directly or indirectly and that the N-terminal CARD region (1-197) of AIRE is sufficient to bring about this transcriptional activation. In this context, it was reported that intact homogeneously staining region/caspase recruitment domain (HSR/CARD) and amino acids R113 and K114 were the key elements involved in AIRE binding to DNA [36]. AIRE-PHD1 is a highly specialized non-modified histone H3 tail reader [37, 38]. But, the transcriptional activation of caspase-3 by ectopic expression of CARD domain of AIRE is suggestive of the involvement of direct binding to DNA rather than chromatin-binding here.

Though AIRE/CARD expression increased caspase-3 expression in GC1-spg cells, there was notable absence of enhanced cell death response associated with it even after exposure to apoptosis inducers. This indicates that AIRE/CARD-dependent caspase-3 expression is a part of a non-apoptotic signalling pathway in GC1-spg cells. Several reports support this argument, and there are strong evidences that caspase-3 is involved in stem cell differentiation [37-39]. On the other hand, it could be argued that GC1-spg cells might have originated from a clonally distinct spermatogonium that was resistant to apoptosis. However, this doesn't seem to be the case, as GC1-spg cells responded to doxorubicin treatment by showing cleavage of caspase-3 (Fig. 7a), indicating that caspase-3 dependent cell death pathway is intact in these cells.

## Conclusion

We conclude that the CARD domain of AIRE enhances caspase-3 expression through possible direct DNA binding and triggers non-apoptotic downstream signalling in cultured spermatogonial cells. Further studies will be required to address whether AIRE is a cis- or trans-acting molecule in regulating caspase-3 expression in spermatogonial cells.

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*Author Contributions*

PGK conceived the idea, designed the work and revised the manuscript critically; KPB, RJA, KR and JS did the data acquisition and drafting of the manuscript; PGK, KPB, RJA, KR and JS analyzed and interpreted the data and all the authors approved the final version of this manuscript.

*Statement of Ethics*

The authors have no ethical conflicts to disclose.

## Disclosure Statement

The authors have no conflicts of interest to declare.

## References

1 Finnish-German APECED Consortium: An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet 1997;17:399-403.
2 Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, Krohn KJ, Lalioti MD, Mullis PE, Antonarakis SE, Kawasaki K, Asakawa S, Ito F, Shimizu N: Positional cloning of the APECED gene. Nat Genet 1997;17:393-398.
3 Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, von Boehmer H, Bronson R, Dierich A, Benoist C, Mathis D: Projection of an immunological self shadow within the thymus by the aire protein. Science 2002;298:1395-1401.
4 Anderson MS, Venanzi ES, Chen Z, Berzins SP, Benoist C, Mathis D: The cellular mechanism of Aire control of T cell tolerance. Immunity 2005;23:227-239.
5 Ilmarinen T, Kangas H, Kytomaa T, Eskelin P, Saharinen J, Seeler JS, Tanhuanpaa K, Chan FY, Slattery RM, Alakurtti K, Palvimo JJ, Ulmanen I: Functional interaction of AIRE with PIAS1 in transcriptional regulation. Mol Immunol 2008;45:1847-1862.
6 Pitkanen J, Rebane A, Rowell J, Murumagi A, Strobel P, Moll K, Saare M, Heikkila J, Doucas V, Marx A, Peterson P: Cooperative activation of transcription by autoimmune regulator AIRE and CBP. Biochem Biophys Res Commun 2005;333:944-953.
7 Kumar PG, Laloraya M, Wang CY, Ruan QG, Davoodi-Semiromi A, Kao KJ, She JX: The autoimmune regulator AIRE is a DNA-binding protein. J Biol Chem 2001;276:41357-41364.
8 Purohit S, Kumar PG, Laloraya M, She JX: Mapping DNA-binding domains of the autoimmune regulator protein. Biochem Biophys Res Commun 2005;327:939-944.
9 Org T, Rebane A, Kisand K, Laan M, Haljasorg U, Andreson R, Peterson P: AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. Hum Mol Genet 2009;18:4699-4710.
10 Koh AS, Kuo AJ, Park SY, Cheung P, Abramson J, Bua D, Carney D, Shoelson SE, Gozani O, Kingston RE, Benoist C, Mathis D: Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. Proc Natl Acad Sci U S A 2008;105:15878-15883.
11 Zumer K, Low AK, Jiang H, Saksela K, Peterlin BM: Unmodified histone H3K4 and DNA-dependent protein kinase recruit autoimmune regulator to target genes. Mol Cell Biol 2012;32:1354-1362.
12 Matsuo T, Noguchi Y, Shindo M, Morita Y, Oda Y, Yoshida E, Hamada H, Harada M, Shiokawa Y, Nishida T, Tominaga R, Kikushige Y, Akashi K, Kudoh J, Shimizu N, Tanaka Y, Umemura T, Taniguchi T, Yoshimura A, Kobayashi T, et al.: Regulation of human autoimmune regulator AIRE gene translation by miR-220b. Gene 2013;530:19-25.
13 Ucar O, Tykocinski LO, Dooley J, Liston A, Kyewski B: An evolutionarily conserved mutual interdependence between Aire and microRNAs in promiscuous gene expression. Eur J Immunol 2013.
14 Eldershaw SA, Sansom DM, Narendran P: Expression and function of the autoimmune regulator Aire gene in non-thymic tissue. Clin Exp Immunol 2011;163:296-308.
15 Brahmaraju M, Bhagya KP, Titus S, Sebastian A, Devi AN, Laloraya M, Kumar PG: AIRE1A might be involved in cyclin B2 degradation in testicular lysates. Biochem Cell Biol 2011;89:411-422.

16 Ramsey C, Winqvist O, Puhakka L, Halonen M, Moro A, Kampe O, Eskelin P, Pelto-Huikko M, Peltonen L: Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. Hum Mol Genet 2002;11:397-409.
17 Schaller CE, Wang CL, Beck-Engeser G, Goss L, Scott HS, Anderson MS, Wabl M: Expression of Aire and the early wave of apoptosis in spermatogenesis. J Immunol 2008;180:1338-1343.
18 Ferguson BJ, Alexander C, Rossi SW, Liiv I, Rebane A, Worth CL, Wong J, Laan M, Peterson P, Jenkinson EJ, Anderson G, Scott HS, Cooke A, Rich T: AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. J Biol Chem 2008;283:1723-1731.
19 Liiv I, Haljasorg U, Kisand K, Maslovskaja J, Laan M, Peterson P: AIRE-induced apoptosis is associated with nuclear translocation of stress sensor protein GAPDH. Biochem Biophys Res Commun 2012;423:32-37.
20 Nishikawa Y, Hirota F, Yano M, Kitajima H, Miyazaki J, Kawamoto H, Mouri Y, Matsumoto M: Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. J Exp Med 2010;207:963-971.
21 Hofmann MC, Braydich-Stolle L, Dettin L, Johnson E, Dym M: Immortalization of mouse germ line stem cells. Stem Cells 2005;23:200-210.
22 Hofmann MC, Millan JL: Establishment of mammalian testicular cell lines. Methods Cell Biol 1998;57:93-110.
23 Heino M, Peterson P, Sillanpaa N, Guerin S, Wu L, Anderson G, Scott HS, Antonarakis SE, Kudoh J, Shimizu N, Jenkinson EJ, Naquet P, Krohn KJ: RNA and protein expression of the murine autoimmune regulator gene Aire in normal, RelB-deficient and in NOD mouse. Eur J Immunol 2000;30:1884-1893.
24 Ruan QG, Wang CY, Shi JD, She JX: Expression and alternative splicing of the mouse autoimmune regulator gene Aire. J Autoimmun 1999;13:307-313.
25 Halonen M, Pelto-Huikko M, Eskelin P, Peltonen L, Ulmanen I, Kolmer M: Subcellular location and expression pattern of autoimmune regulator Aire, the mouse orthologue for human gene defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy APECED. J Histochem Cytochem 2001;49:197-208.
26 Kumar PG, Laloraya M, She JX: Population genetics and functions of the autoimmune regulator AIRE. Endocrinol Metab Clin North Am 2002;31:321-38, vi.
27 Forsdyke DR: When few survive to tell the tale: thymus and gonad as auditioning organs: historical overview. Theory Biosci 2019.
28 Colome N, Collado J, Bech-Serra JJ, Liiv I, Anton LC, Peterson P, Canals F, Jaraquemada D, Alvarez I: Increased apoptosis after autoimmune regulator expression in epithelial cells revealed by a combined quantitative proteomics approach. J Proteome Res 2010;9:2600-2609.
29 Bianchi F, Sommariva M, De CL, Triulzi T, Romero-Cordoba S, Tagliabue E, Sfondrini L, Balsari A: Expression and prognostic significance of the autoimmune regulator gene in breast cancer cells. Cell Cycle 2016;15:3220-3229.
30 Radhakrishnan K, Bhagya KP, Kumar AT, Devi AN, Sengottaiyan J, Kumar PG: Autoimmune Regulator AIRE Is Expressed in Spermatogenic Cells, and It Altered the Expression of Several Nucleic-Acid-Binding and Cytoskeletal Proteins in Germ Cell 1 Spermatogonial GC1-spg Cells. Mol Cell Proteomics 2016;15:2686-2698.
31 Rinderle C, Christensen HM, Schweiger S, Lehrach H, Yaspo ML: AIRE encodes a nuclear protein co-localizing with cytoskeletal filaments: altered sub-cellular distribution of mutants lacking the PHD zinc fingers. Hum Mol Genet 1999;8:277-290.
32 Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, Okazaki T, Yamamoto K, Sasada M: Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J Exp Med 1998;187:587-600.
33 Giampietri C, Petrungaro S, Coluccia P, D'Alessio A, Starace D, Riccioli A, Padula F, Srinivasula SM, Alnemri E, Palombi F, Filippini A, Ziparo E, De Cesaris P: FLIP is expressed in mouse testis and protects germ cells from apoptosis. Cell Death Differ 2003;10:175-184.
34 Citron BA, Arnold PM, Sebastian C, Qin F, Malladi S, Ameenuddin S, Landis ME, Festoff BW: Rapid upregulation of caspase-3 in rat spinal cord after injury: mRNA, protein, and cellular localization correlates with apoptotic cell death. Exp Neurol 2000;166:213-226.

35 Giraud M, Yoshida H, Abramson J, Rahl PB, Young RA, Mathis D, Benoist C: Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells. Proc Natl Acad Sci U S A 2012;109:535-540.
36 Maslovskaja J, Saare M, Liiv I, Rebane A, Peterson P: Extended HSR/CARD domain mediates AIRE binding to DNA. Biochem Biophys Res Commun 2015;468:913-920.
37 Chignola F, Gaetani M, Rebane A, Org T, Mollica L, Zucchelli C, Spitaleri A, Mannella V, Peterson P, Musco G: The solution structure of the first PHD finger of autoimmune regulator in complex with non-modified histone H3 tail reveals the antagonistic role of H3R2 methylation. Nucleic Acids Res 2009;37:2951-2961.
38 Gaetani M, Matafora V, Saare M, Spiliotopoulos D, Mollica L, Quilici G, Chignola F, Mannella V, Zucchelli C, Peterson P, Bachi A, Musco G: AIRE-PHD fingers are structural hubs to maintain the integrity of chromatin-associated interactome. Nucleic Acids Res 2012;40:11756-11768.
39 Aranha MM, Sola S, Low WC, Steer CJ, Rodrigues CM: Caspases and p53 modulate FOXO3A/Id1 signaling during mouse neural stem cell differentiation. J Cell Biochem 2009;107:748-758.