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Autoimmune Regulator protein interaction with DNA-dependent protein kinase and its role in apoptosis

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To my lovely daughter,
Maarja

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LIST OF ORIGINAL PAPERS

Current dissertation is based on the following original publications, which will be referred to in the text by their Roman numerals:

I **Liiv I**, Rebane A, Org T, Saare M, Maslovskaja J, Kisand K, Juronen E, Valmu L, Bottomley MJ, Kalkkinen N, Peterson P. DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. Biochim Biophys Acta 2008; 1783(1):74–83.

II 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(3): 1723–1731.

III Colomé N, Collado J, Bech-Serra JJ, **Liiv I**, Antón LC, Peterson P, Canals F, Jaraquemada D, Alvarez IJ. Increased apoptosis after autoimmune regulator expression in epithelial cells revealed by a combined quantitative proteomics approach. J Proteome Res 2010; 9(5):2600–2609.

IV **Liiv I,** Haljasorg U, Kisand K, Wang X, Maslovskaja J, Laan M, Peterson P. AIRE-induced apoptosis is associated with nuclear translocation of stress sensor GAPDH. Biochem Biophys Res Commun 2012; 423(1):32–37.

Contribution of Ingrid Liiv to original publications:

Study I: participation of study design; performing experiments, except for mass spectrometry, cell sorting and qPCR; manuscript preparation

Study II: performing the transactivation assays and immunofluorescence microscopy with AIRE's CARD domain mutants; manuscript preparation

Study III: performing apoptosis assays; manuscript preparation

Study IV: study design; performing all experiments; writing the manuscript

ABBREVIATIONS

aa	amino acid
AIRE/Aire	autoimmune regulator protein designation in human/mouse
AIRE/Aire	autoimmune regulator, gene designation in human/mouse
Apaf-1	apoptotic protease activating factor 1
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
ATF7ip	activating transcription factor 7 interacting protein
IFNB	interferon beta
bp	base pair
BrdU	bromodeoxyuridine
CARD	caspase recruitment domain
CASP	caspase
CBP	CREB-binding protein
CREB	cAMP response element-binding protein
CRM1	chromosome region maintenance or exportin 1
cTECs	cortical thymic epithelial cells
DAXX	death-domain associated protein
DC	dendritic cell
DOX	doxycycline
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dsDNA	double-strand DNA
EpCAM	epithelial cell adhesion molecule
FACT	chromatin-specific transcription elongation factor
FAS	apoptosis antigen 1 or CD95
FASL	FAS ligand
FOXP3	forkhead box protein 3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GST	glutathione S-transferase
H3	histone 3
γH2AX	histone H2A variant, phosphorylated form
HSP	heat shock protein
HSR	homogeneously staining region
IFNB	interferon beta
IL	interleukin
ILRA	interleukin 2 receptor alpha
INS	insulin
INV/*Inv*	involucrin
kb	kilobase
KO	knockout
KRT14	keratin 14

Ku70	Ku autoantigen 70 kDa
Ku80	Ku autoantigen 80 kDa
LOR/*Lor*	loricrin
MAR	matrix attachment region
MBD1	methyl CpG-binding protein 1
mRNA	messenger RNA
mTECs	medullary thymic epithelial cells
MHC	major histocompatibility complex
NHEJ	non-homologous end joining
NLS	nuclear localization signal
NO	nitric oxide
Oct-1	POU class 2 homeobox 1
p53	tumor suppressor protein p53
p300	histone acetyltransferase p300
PARP1	poly [ADP-ribose] polymerase 1
PCR	polymerase chain reaction
P-TEFb	positive transcription elongation factor b
PHD	plant homeodomain
PML	promyelocytic leukemia
qPCR	quantitative polymerase chain reaction or real-time PCR
RNA Pol II	RNA polymerase II
RNA	ribonucleic acid
S100A8	S100 calcium binding protein A8
SAND	Sp100, AIRE-1, NucP41/75, DEAF-1
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Siah-1	E3 ubiquitin-protein ligase Siah1
SOD	superoxide dismutase
Sp100	speckled 100 kDa
SPT1	salivary protein 1
STAT	signal transducer and activator of transcription
TOP2a	topoisomerase 2 alpha
TBS	Tris-buffered saline
Tet	tetracycline
Treg	T regulatory cell
TSA	tissue-specific antigen
TCR	T cell receptor
TBCA	tubulin-specific chaperone A
wt	wild type

I. INTRODUCTION

The function of the thymus was unknown for a long time. The ancient Greeks believed that the thymus is where the human soul or spirit is situated. The most famous physician of antiquity, Claudius Galen of Pergamum (c.130–200 AD), was the first to note that the thymus is at its largest during infancy, calling the thymus the "organ of mystery" (Jacobs et al., 1999). It was only in the 20th century that the role of the thymus in immunity was recognized.

The Autoimmune Regulator (*AIRE*) gene was discovered in 1997. Studies of the AIRE protein have expanded our understanding about the molecular mechanisms of immune tolerance and autoimmunity. These studies have helped answer the fundamental question of how the immune system discriminates between self and non-self within the thymic microenvironment. AIRE is one of indispensable proteins for the development and maintenance of immune tolerance and is needed to protect our bodies from self-reactive T and B lymphocytes. Mutations in the *AIRE* gene lead to a serious autoimmune syndrome known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). Various *AIRE* gene knockout (KO) mouse models have been used to study the monogenic mechanism of autoimmunity at the molecular level.

During the past several years, information on different aspects of AIRE function has accumulated and greatly advanced the understanding of tolerance mechanisms. Despite this expanding information, the molecular mechanisms by which AIRE mediates tolerance remain unclear. This thesis focuses on the molecular mechanisms of the AIRE protein, aiming to identify new interaction partners and explore the role of AIRE in apoptosis.

2. REVIEW OF LITERATURE

2.1. Monogenic autoimmune diseases

At least 70 autoimmune diseases have been described. Overall, autoimmune diseases affect 3–5% of the general population (Marrack et al., 2001, Danke et al., 2004). The etiology of autoimmune diseases is multifactorial, with a complex polygenetic background interacting with environmental factors. Only a few autoimmune diseases have a monogenic background. One of them is APECED (OMIM 240300), which is considered a model for endocrine autoimmunity. APECED is an autosomal recessive disease that is caused by mutations in the *AIRE* gene (Nagamine et al., 1997; Finnish-German APECED consortium 1997).

Other examples of rare monogenic autoimmune diseases are Autoimmune Lymphoproliferative Syndrome (ALPS) (OMIM 601859), Immunodysregulation Polyendocrinopathy X-linked (IPEX) (OMIM 304930) and interleukin receptor 2 alpha (ILR2A) or CD25 deficiency (OMIM 606367). ALPS is a disorder of apoptosis, resulting in the accumulation of autoreactive lymphocytes. The disease is caused by mutations in the *FAS*, *FASL*, *CASP8* and *CASP10* genes, which are all needed for FAS-mediated programmed cell death. Inheritance of ALPS is most often transmitted in an autosomal dominant manner, but autosomal recessive inheritance has also been described in patients with mutations in the *FAS* gene (Fisher et al., 1995). IPEX is an X-linked recessive immunologic disorder that is characterized by the onset in infancy of severe diarrhea due to enteropathy, type 1 diabetes mellitus, and dermatitis; other immunodysregulations have also been described in IPEX. IPEX is caused by mutations in the *FOXP3* gene, which is critical for regulatory T (Treg) cell development and function (Bennett et al., 2001; Ramsdell and Zieglery 2003). The mutations in *FOXP3* gene result in a defect in the differentiation of $CD4^+CD25^+$ Tregs, which are involved in immune homeostasis and protection against autoimmunity. Mutations in the *IL2RA* gene cause IPEX-like syndrome, resulting in the hyperproliferation and activation of $CD8^+$ T lymphocytes with impaired sensitivity to IL-2 (Goudy et al., 2013). Moreover, mutations in the *IL2RA* gene lead to defective IL-10 production in Tregs independent of their normal FOXP3 protein expression (Goudy et al., 2013). Disease caused by *IL2RA* defect leads to the development of progressive autoimmunity, such as enteropathy, erythrodermia and severe alopecia, due to autoimmune attacks by $CD8^+$ T cells and granzyme B production. The impairment of $CD8^+$ T cell effector functions causes decreased viral, bacterial and fungal antigen responses in those patients (Goudy et al., 2013).

2.1.1. APECED

APECED is a monogenic autosomal recessive disease. This syndrome is common in certain populations such as Finns, Sardinians and Iranian Jews, where the incidence is 1:25 000, 1:14 400 and 1:9000, respectively (Ahonen et al., 1990; Rosatelli et al., 1998; Zlotogora et al., 1992). APECED patients develop a progressive loss of tolerance against self-antigens and suffer from multiorgan failures due to autoimmune destruction. The clinical presentation of APECED is widely variable, and the list of possible disease components includes 30 different disorders (Perheentupa 2006). The first symptom of the syndrome is usually the chronic mucocutaneous infection of *Candida albicans*, which starts early in childhood, followed by hypoparathyroidism and adrenocortical insufficiency or Addison's disease. Other disorders, such as type 1 diabetes, hypogonadism, dystrophy of dental enamel and nails or ectodermal dysplasia, may occur during the patient's lifetime (Perheentupa 2006; Husebye et al., 2009; Peterson and Peltonen 2005). APECED patients have high titer autoantibodies that react with a large set of autoantigens that are expressed in many affected organs. The most prevalent autoantibodies are neutralizing autoantibodies against interferon α and ω (Meager et al., 2006, Kisand et al., 2008) and autoantibodies against the T-helper type 17 cell-associated cytokines IL-17A, IL-17F and IL-22 (Kisand et al., 2010; Kärner et al., 2013). Other common autoantigens among APECED patients are steroid 21-hydroxylase, 17-alpha-hydroxylase and side-chain cleavage enzyme (Peterson and Peltonen 2005).

2.2. Autoimmune Regulator (AIRE)

2.2.1. AIRE gene and APECED mutations

In 1997, two research groups simultaneously identified the human *AIRE* gene on chromosome 21q22.3 (The Finnish-German APECED consortium 1997; Nagamine et al., 2007). The *AIRE* gene (NC 000021.8; ID326) is approximately 13 kb in length, and the coding region is composed of 14 exons encoding a protein (accession nr O43918) with 545 amino acids and a theoretical molecular mass of 57 kDa (calculated pI is 7.53). The protein is proline rich (11.7%; Nagamine et al., 2007). *AIRE* is a single-copy gene whose structure has been conserved in vertebrates over more than 400 million years, with orthologues in mammals and, reportedly, in birds and fish (Saltis et al., 2008). Comparative genomic sequence analysis shows 70% homology between human *AIRE* and murine *Aire* genes (Mittaz et al., 1999; Wang et al., 1999; Blechschmidt et al., 1999).

AIRE is most highly expressed in the thymus, specifically in the medulla area in the thymic medullary epithelial cells (mTECs). *AIRE* transcripts have also been reported in many other human and mouse tissues, but at a much lower level than in thymus (Heino et al., 1999, 2000; Ruan et al., 1999; Blechschmidt et al., 1999; Halonen et al., 2001; Kont et al., 2008). Several alternatively spliced variants of *Aire* have been reported, but their biological function is not

known (Nagamine et al., 1997; Ruan et al., 1999). AIRE protein expression has also been described in peripheral immune organs (Gardner et al., 2008, Poliani et al., 2010; Cohen et al., 2010) and outside the immune system, such as in the spermatogonia and spermatocytes of the testes (Schaller et al., 2008).

More than 60 mutations have been reported in the *AIRE* gene associated with APECED (Peterson and Peltonen 2005; Husebye et al., 2009). The mutations are located throughout the coding region of the *AIRE* gene. *AIRE* has two mutation hotspots: 769C>T or R257X and a 13 bp deletion in exon 8 (967–979del, 13 bp). The first mutation is common among Finnish, Northern Italian and Eastern European APECED patients, whereas the second is a common mutation in Anglo-American patients but is also present in patients from many other populations (Peterson and Peltonen 2005). The genotype-phenotype correlation is still unclear. The missense mutations, especially R257X, seem to be more associated with mucocutaneous candidiasis (Kisand et al., 2011). The Y85C mutation, which is prevalent among Iranian Jewish patients, associates with hypoparathyroidism and less with mucocutaneous candidiasis (Peterson and Peltonen 2005). A rare dominant mutation of Italian origin, G228W, predisposes to autoimmune thyroiditis (Cetani et al., 2001). Another rare mutation C446G predisposes to type I diabetes (Wolff et al., 2007). Notably, identical AIRE mutations (c.967–979del 13/c.769C>T and c.879 1G>A/c.879 1G>A) of Norway APECED siblings in two family have been reported to have distinctive disease phenotypes (Wolff et al., 2007). This finding suggests that environmental factors or other stochastic mechanisms may influence the disease phenotype.

2.2.2. AIRE protein domains

The AIRE protein has domains that are common to transcription regulators and chromatin-binding proteins (Nagamine et al., 1997; Pitkänen et al., 2000). The AIRE protein consists of *homogenously staining region* (HSR), a nuclear localization signal (NLS), four leucine-rich or LXXLL motifs, a SAND (Sp100, Aire1, NucP41/P75, DEAF1) domain, two plant homeodomain zinc fingers (PHD1 and PHD2), and a proline-rich region (PRR) (Pitkänen et al., 2000; Ilmarinen et al., 2006) (Figure 1).

The HSR domain mediates protein dimerization, the subcellular localization of the protein in nuclear dots and intermediate filaments within the cytoplasm, transcription activation and interaction with the nuclear matrix (Pitkänen et al., 2000 and 2001; Ramsey et al., 2002; Halonen et al., 2004; Meloni et al., 2005). The HSR domain is similar to caspase recruitment domain (CARD), a domain structure that is often present in apoptotic proteins, as studied in this thesis.

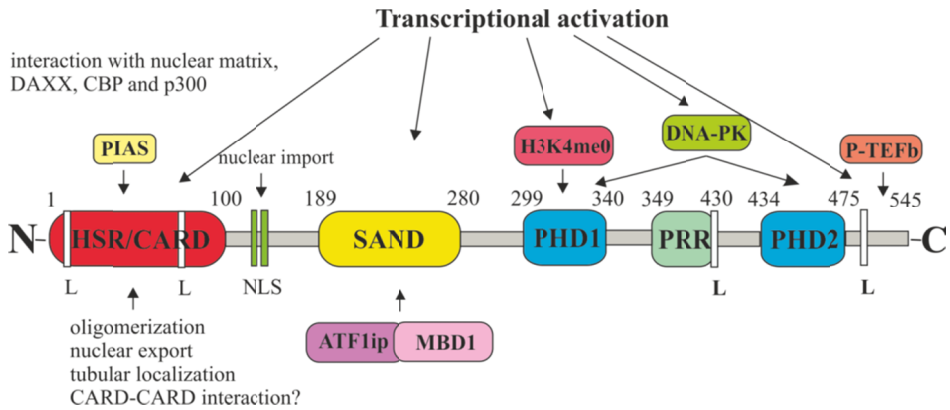

Figure 1. Schematic representation of the AIRE protein domains. AIRE contains several domains and motifs that are related to transcription factors and chromatin binding such as HSR/CARD, NLS, LXXLL (L), SAND, PHD1, PRR and PHD2. Functions of AIRE and its interacting partners such as PIAS, DAXX, P-TEFb, CBP, p300, DNA-PK, ATF1ip, MBD1, and H3K4me0 are indicated.

The SAND domain in AIRE has been shown to be important for proper subcellular localization, transactivation and dimerization and is a putative DNA binding domain (Kumar et al., 2001; Ramsey et al., 2002; Halonen et al., 2004). AIRE belongs to the Sp100/Sp140 family of proteins because they share a SAND domain and a conserved amino-terminal HSR region (Gibson et al., 1998). The structural similarity of AIRE to Sp100 proteins indicates a common ancestor (Gibson et al., 1998). However, the classical DNA-binding motif, KDWF (Lysine-Aspartate-Tryptophan-Phenylalanine), which is common among Sp100 family proteins, is lacking in AIRE (Kumar et al., 2001). Instead, AIRE has a KNKA (Lysine-Asparagine-Lysine-Alanine) motif (Bottomley et al., 2001).

The classical importin alpha/beta pathway mediates the nuclear import of AIRE by binding NLS to several importin-alpha family members (Ilmarinen et al., 2006). In addition to the consensus NLS, AIRE has another nuclear transport signal thus far undefined in the C-terminus (Pitkanen et al., 2001).

The LXXLL (L- Leucine; X- any amino acid) motif is a common signature for a nuclear receptor-binding site that could also function as a potential nuclear export signal (Heery et al., 1997). Moreover, LXXLL motifs are common in proteins with transcription regulation and chromatin modifying properties such as in TIF2, SRC1 and p300/CBP (Plevin et al., 2005). AIRE also has an exportin 1 (CRM1) dependent nuclear export signal at the N-terminus, and the protein was shown to shuffle between the nucleus and the cytoplasm (Pitkänen et al., 2001). PRR, which is located at the C-terminus of AIRE, may mediate protein-protein interactions (Kay et al., 2000). The PRR motif at the C-terminus of AIRE is important in the transactivation activity of AIRE (Uchida et al., 2004; Meloni et al., 2008).

AIRE has a two plant homeodomains, PHD1 and PHD2. A structure based on a sequence alignment of PHD domains of AIRE and other PHD-containing proteins, such as ING2 and BPTF, suggests that AIRE-PHD1 is a representative of a newly identified subclass of PHD fingers (Org et al., 2008). PHD1 of AIRE has an intrinsic E3 ubiquitin ligase activity (Uchida et al., 2004), although these findings have been debated (Bottomley et al., 2005). In AIRE, both PHD-fingers are important for transactivation activity (Org et al., 2008; Meloni et al., 2008; Gaetani et al., 2012; Yang et al., 2013). The PHD1 domain of AIRE is responsible for interaction with a nucleosome component, histone H3, interacting preferentially with unmodified lysine 4 (H3K4me0), which is an epigenetic mark associated with transcriptional repression of genes (Org et al., 2008; Koh et al., 2008; Chignola et al., 2009; Chakravarty et al., 2009). The *in vitro* binding experiments of AIRE-PHD1 have also confirmed interactions with other heterochromatin modifications of histone H3 such as H3K9me3 and H3S10Ph (Org et al., 2008). PHD2 in AIRE does not bind to any histones (Org et al., 2008; Koh et al., 2008; Gaetani et al., 2012).

2.2.3. AIRE subcellular localization

AIRE has a characteristic subcellular localization. The AIRE protein localizes to the cell nucleus and appears as a speckled pattern known as a nuclear body or nuclear dot. These nuclear bodies resemble but are distinct from promyelocytic leukemia (PML) nuclear bodies (Heino et al., 1999; Björses et al., 1999; Pitkänen et al., 2001; Seeler et al., 1998). Nuclear body formation by AIRE is dependent on the cell cycle (Akiyoshi et al., 2004). The nuclear dot pattern is characteristic of chromatin-associated proteins such as the Sp100 protein family and CBP/p300 proteins (Goodman and Smolik 2000). Studies of PML bodies have suggested that they function as nuclear protein depots or highly organized centers for gene expression or chromatin region organizers (Negorev and Maul 2001; Lallemand-Breitenbach and Thé 2010). Both PML and AIRE are nuclear matrix-associated proteins (Akiyoshi et al., 2004; Ching et al., 2005), although AIRE binding to the matrix attachment region (MAR), which is characteristic of PML, is still not described (Kumar et al., 2007). The MAR sequences contain an AT-rich DNA sequence, topoisomerase II (TOP2) consensus sequences that are characteristic of DNA replication origin regions, and enhancer regions for gene expression (Wang et al., 2010).

RNAi-mediated gene knock-down experiments demonstrated that the nuclear dot pattern of AIRE is dependent on several nuclear body-associated proteins, such as KPNB1, RanBP2, NUP93, and CRM1, which were also described as AIRE interacting proteins (Abramson et al., 2010).

In addition to nuclear staining, AIRE colocalizes with the fibrillar network of cytoplasmic filaments, which has also been described as colocalization with vimentin and γ-tubulin, as observed in cells that were transiently transfected with AIRE-expressing plasmids (Rinderle et al., 1999; Björses et al., 2000; Ramsey et al., 2002).

The majority of missense mutations in the HSR, SAND, and PHD2 domains alters the nucleus-cytoplasm distribution of AIRE and disturbs its association with nuclear dots and cytoplasmic filaments, as shown in Table 1. For example, the deletion of the first 84 amino acids of AIRE and missense mutations in the HSR, SAND, PHD1 and PHD2 domains, such as L93R, G228W, C311Y or C446G, respectively, impair nuclear dot and cytoplasmic fibrillar or tubular localization of AIRE (Björses et al., 2000; Pitkänen et al., 2001; Halonen et al., 2004; Uchida et al., 2004; Meloni et al., 2008; Gaetani et al., 2012). The deletion of PHD2 domain does not influence the protein folding and nuclear dot configuration of AIRE (Yang et al., 2013).

Table 1. The overview of AIRE missense mutations to study the transcriptional activity and subcellular localization

Mutation	Domain location	Acti-vation	Dots / Cyto	APECED	References
R15L	CARD	+	+/-	yes	Halonen et al., 2004; Liiv (unpubl.)
R15C	CARD	-	-/-	yes	Liiv (unpubl.)
T16M	CARD	+	-/-	yes	Halonen et al., 2004
T16M	CARD	-	-/-	yes	Liiv (unpubl.)
A21V	CARD	+	+/+	yes	Halonen et al., 2004; Liiv (unpubl.)
L28P	CARD	-	-/-	yes	Pitkänen et al., 2000, 2001; Halonen et al., 2004; Ilmarinen et al., 2005; Study II
L29P	CARD	-	-/-	yes	Halonen et al., 2004; Study II
T68A	CARD	-	+/+	no	Study I
W78R	CARD	-	-/-	yes	Halonen et al.,2004; Liiv (unpubl.)
V80L	CARD	+	+/+	yes	Halonen et al., 2004; Liiv (unpubl.)
K83E	CARD	+	+/+	yes	Pitkänen et al., 2001; Halonen et al., 2004; Study II
Y85C	CARD	+	+/+	yes	Björses et al., 2000; Liiv (unpubl.)
Y85C	CARD	+	-/-	yes	Ilmarinen et al., 2005
Y90C	CARD	-	+/+	yes	Halonen et al., 2004
Y90C	CARD	-	-/-	yes	Liiv (unpubl.)
L93R	CARD	-	-/-	yes	Halonen et al., 2004; Liiv (unpubl.)
R113A	NLS	nt	+/+	no	Ilmarinen et al., 2006
K114E	NLS	nt	+/+	no	Ilmarinen et al., 2006
K131E	NLS	nt	-/+	no	Ilmarinen et al., 2006
R132A	NLS	nt	-/+	no	Ilmarinen et al., 2006
K133A	NLS	nt	-/+	no	Ilmarinen et al., 2006
S156A	N-terminus	-	+/+	no	Study I

Mutation	Domain location	Acti-vation	Dots / Cyto	APECED	References
G228W	SAND	-	-/-	yes	Ramsey et al., 2002; Halonen et al., 2004; Ilmarinen et al., 2005
P252L	SAND	+	+/+	yes	Ilmarinen et al., 2005
K243R	SAND	+	+/+	no	Saare et al., 2012
K243Q	SAND	+	+/+	no	Saare et al., 2012
K245R	SAND	+	+/+	no	Saare et al., 2012
K245Q	SAND	+	+/+	no	Saare et al., 2012
K253R	SAND	+	+/+	no	Saare et al., 2012
K253Q	SAND	+	+/+	no	Saare et al., 2012
K259R	SAND	+	+/+	no	Saare et al., 2012
R257X	SAND	-	-/+	yes	Pitkänen et al., 2000; Björses et al., 2000; Halonen et al., 2001
D297A	PHD1	-	+/-	no	Org et al., 2008; Gaetani et al., 2012
C299A	PHD1	nt	+/+	no	Uchida et al., 2004
V301M	PHD1	-	+/-	yes	Gaetani et al., 2012
C302P	PHD1	-	+/+	no	Pitkänen et al., 2001
C311Y	PHD1	-	-/-	yes	Björses et al., 2000; Uchida et al., 2004; Meloni et al., 2008
D312A	PHD1	-	nt	no	Org et al., 2008, 2009
P326Q	PHD1	+	+/+	yes	Uchida et al., 2004; Halonen et al., 2004;
C434A	PHD2	-	+/+	no	Uchida et al., 2004
C437P	PHD2	-	+/+	no	Pitkänen et al., 2001
C446G	PHD2	-	-/-	yes	Gaetani et al., 2012; Meloni et al., 2008
L516A	LXXLL	+	nt	no	Meloni et al., 2008
L516X	LXXLL	-	nt	no	Meloni et al., 2008
W531X	C-terminus	-	nt	no	Meloni et al., 2008

"+" transcription activation, "–" no activation, nt – not tested, dots – nuclear dots, cyto – cytoplasmic filaments, unpubl. – unpublished results, LXXLL – leucine rich motif, X – stop codon

2.2.4. Transcriptional regulation by AIRE

The ability of AIRE to activate transcription of other genes has been shown in several studies (Pitkänen et al., 2000; Halonen et al., 2004; Ilmarinen et al., 2005, Meloni et al., 2005). Furthermore, AIRE is not only a transcriptional activator, but it also down-regulates the transcription of genes, as demonstrated in microarray studies with various cell lines as well as in mouse mTECs (Derbinski et al., 2001; Ruan et al., 2007; Org et al., 2008; Kont et al., 2008; Abramson et al., 2010).

AIRE transcriptional activation is mediated through multimerization and molecular interactions (Pitkänen et al., 2000; Halonen et al., 2004). In the nucleus, AIRE interacts with large multimolecular complexes (>670 kDa).

Many missense or deletional mutations, including APECED mutations, have been analyzed to study the transcriptional function of AIRE. As observed in the Table 1 listing of the missense mutations in AIRE, all domains of AIRE are important for transcriptional activation. Moreover, the functional studies of deletion constructs of AIRE protein domains, such as HSR (1–216 aa; Pitkänen et al., 2000), PRR (342–432 aa; Uchida et al., 2004), PHD1 (292–341 aa; Uchida et al., 2004) and PHD2 (434–475 aa; Yang et al., 2013), show that all of these deletions inactivate the transactivation ability of AIRE.

CREB-binding protein (CBP), a common transcriptional co-activator that has acetyltransferase activity, was the first reported AIRE interacting protein partner (Figure 1; Pitkänen et al., 2000). AIRE and CBP cooperatively activated transcription in assays with a minimal interferon beta (*IFNB*) promoter reporter (Pitkänen et al., 2005). However, acetyltransferase p300 did not similarly enhance the activation of AIRE-regulated genes, *INV* and *S100A8* but stabilized the AIRE protein (Saare et al., 2012). E3 Sumo-protein ligase, a protein inhibitor of activated STAT 8 (PIAS1), and AIRE both interact with the nuclear matrix and concurrently activate the human insulin (*INS*) gene promoter, although AIRE is not sumoylated by PIAS1 (Ilmarinen et al., 2008). Moreover, the transcription and apoptosis regulator protein, which is a death-domain associated protein (DAXX), was shown to interact and colocalize with AIRE in nuclear bodies (Meloni et al., 2010). DAXX also had a strong repressive function on the transcriptional activity of AIRE (Meloni et al., 2010).

AIRE interaction with nuclear matrix (Akiyoshi et al., 2004; Tao et al., 2006) and nucleosomal component, histone H3 (Org et al., 2008; Koh et al., 2008; Chignola et al., 2009; Yang et al., 2013) suggests that AIRE regulates genes epigenetically. In mTECs, AIRE target gene promoters have high H3K4me0 level, which is associated with inactive gene promoters and low H3K4me3 and acetylated histone 3 modification levels, which are common markers of active chromatin regions in another cell types (Mellor et al., 2006; Org et al., 2009). AIRE regulates the transcription of tissue-specific antigens (TSAs) in thymic medullary epithelial cells, a process known as promiscuous gene expression (Derbinski et al., 2001; Anderson, et al., 2002; Kyewski et al., 2002; Ruan et al., 2007) (Figure 2). Gene expression data derived from the microarray analysis of mTECs showed that the promiscuous gene expression comprises a highly diverse set of genes representing all tissues of the body, including sex-specific and development-dependent genes (Derbinski et al., 2001 and 2005; Anderson et al., 2002; Johnnidis et al., 2005; Org et al., 2009; Giraud et al., 2014). The epigenetic mechanisms that are involved in AIRE's function has been provided by the observation that the repertoire of genes induced by AIRE varies in individual mTECs (Derbinski et al., 2008; Villaseñor J et al., 2008; Guerau-de-Arellano et al., 2009).

The promiscuously expressed genes fall into two categories with respect to their transcriptional control, AIRE-dependent (induced/suppressed) and AIRE-independent genes (Derbinski et al., 2001 and 2005). For instance, AIRE-dependent genes in mTECs include structural proteins, such as involucrin, loricrin, and

keratins; hormones, such as insulin; and factors involved in transcription elongation, mRNA processing and polyadenylation, such as heterogeneous nuclear ribonucleoprotein L**,** and serine/arginine-rich splicing factors 1 and 3 (Derbinski et al., 2001 and 2005; Anderson et al., 2002; Giraud et al., 2014). Examples of AIRE-independent genes are glyceraldehyde-3-phosphate, C-reactive protein, and glutamate decarboxylase 2 (Derbinski et al., 2001 and 2005). The induction of the expression of endogenous tissue-specific antigens by AIRE has also been described in cell culture conditions, in HEK293 cells that stably expressed human AIRE (Org et al., 2009) and in 1C6 mTECs that over-expressed murine Aire (Kont et al., 2008). Approximately 2000 genes are estimated to be regulated by Aire in mouse mTECs (Derbinski et al., 2001; Kyewski et al., 2002; Derbinski et al., 2005). Those genes tend to colocalize in clusters, again supporting a role for AIRE in the epigenetic regulation of gene expression (Derbinski et al., 2005 and 2008; Johnnidis et al., 2005; Org et al., 2009).

The chromatin immunoprecipitation experiments demonstrated that the RNA Polymerase II (RNA Pol II) level is higher in AIRE-dependent gene promoters than it is in AIRE-independent promoters (Org et al., 2008). RNA Pol II catalytic subunit consists of repeats of serine residues at the C-terminus, which are phosphorylated by RNA Pol II carboxyl terminal domain kinases through different phases of transcription (Komarnitsky et al., 2000). The transcriptional elongation is regulated by the phosphorylation of serine 2 in the RNA Pol II C-terminal domain. Transcription initiation and elongation studies revealed that AIRE regulates the elongation rather than initiation of RNA Pol II (Oven et al., 2007). AIRE promotes the transcriptional elongation of RNA Pol II by increasing the phosphorylation of the Pol II subunit, Rbp1, at serine 2, which is a hallmark of transcription elongation (Žumer et al., 2011). This phosphorylation is catalyzed by the positive transcription elongation factor b (P-TEFb) complex, which is composed of the cyclin-dependent kinase 9 (CDK9) and the regulatory cyclins CycT1 and CycT2 (Peng et al., 1998). AIRE binds and recruits P-TEFb to target promoters of *INS* and *SPT1* genes and enables RNA Pol II to elongate (Oven et al., 2007). The APECED frameshift mutation (505fsx520) leads to the loss of C-terminal 40 residues of AIRE, which disrupts the interactions between AIRE and CDK9 (Žumer et al., 2011). Additionally, others recent studies described a role for AIRE in regulation of transcriptional elongation (Giraud et al., 2012; Giraud et al., 2014). It was shown in mTECs of Aire-KO mice that RNA elongation was impaired, as RNA Pol II was able to synthesize only the first 50–100 bp of mRNA of the target genes (Giraud et al., 2012). Recently, the same research group demonstrated that mRNA processing factor 7SK RNA, which plays a role in regulating transcription by controlling the P-TEFb activity, also cooperates with AIRE (Giraud et al., 2014).

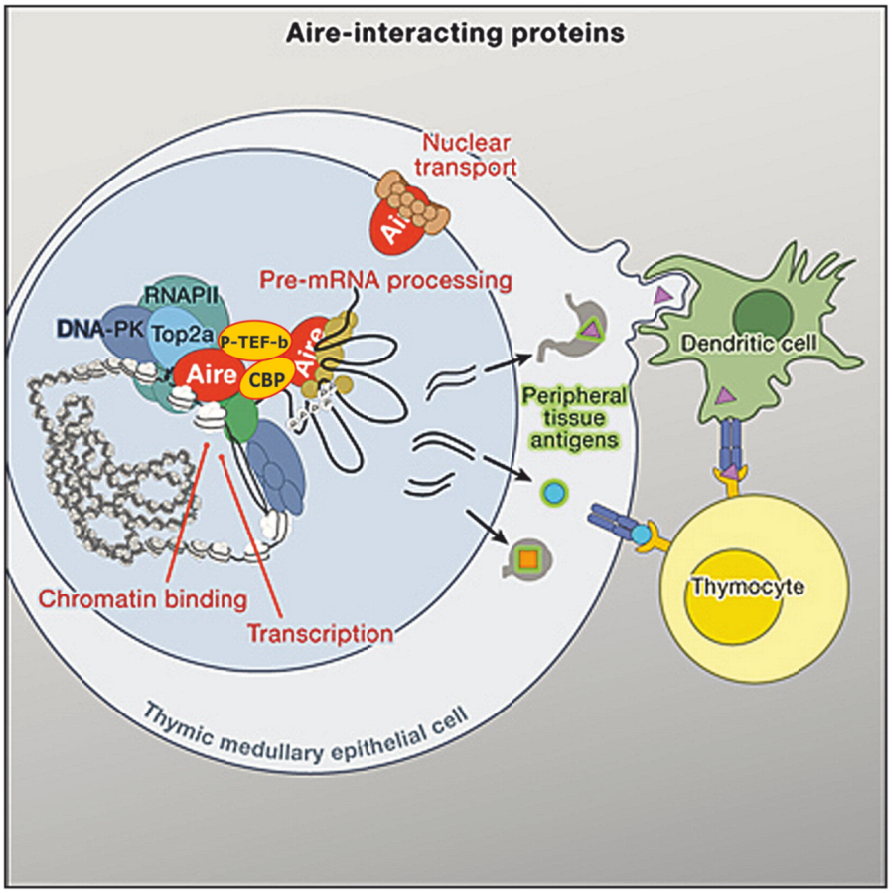

Figure 2. To promote immune tolerance, AIRE induces promiscuous expression of peripheral tissue antigens or tissue-specific antigens (TSAs) in the thymic medullary epithelial cells (mTECs), which are processed and then presented on cell surface-displayed MHC molecules. AIRE regulates transcription of TSAs with its partners, like RNA Pol II, CBP, TOP2a, DNA-PK and P-TEFb. AIRE with DNA-PK and TOP2a, and enhances formation of DNA double-strand breaks during transcription. AIRE also participates in the regulation of gene expression at level of pre-mRNA processing. After the induction of AIRE and TSAs, mTECs die by apoptosis. Immature thymocytes circulate through the medulla and can recognize MHC-TSA complexes directly on mTECs or indirectly on dendritic cells that have engulfed apoptotic mTECs or their fragments. If T cell receptors interact the MHC-TSA complex with too high affinity, they will be deleted in negative selection from the repertoire. In the absence of AIRE, the self-reactive thymocytes develop and escape to the periphery because of aberrant expression and presentation of TSAs to thymocytes by MHC complex. Adapted from (Abramson et al., 2010).

Moreover, recent *in vivo* and *in vitro* studies indicated importance of the interaction of AIRE with a common transcriptional repression complex MBD1-ATF7ip that target AIRE to repressed TSA-encoding loci leading to their expression (Waterfield et al., 2014). This study demonstrated that AIRE interacts via its SAND domain with ATF7ip and demonstrated that AIRE initiates the recruitment of RNA Pol II, MBD1-ATF7ip and P-TEFb to the silenced promoters in mTECs and enhances the transcription elongation.

The proposed mechanism to explain how AIRE promotes transcription is based on the idea that AIRE promotes the relaxation of condensed chromatin structure with the chromatin remodeling complex proteins. According to this hypothesis, AIRE interaction with unmethylated histone H3 recruits to the promoter a complex of proteins, including TOP2a, PARP1, FACT, and DNA-PK/Ku70/ Ku80 (Abramson et al., 2010), which are all involved in chromatin relaxation. The TOP2a unwinds DNA from a supercoiled structure by cleaving and ligating DNA during transcription (Champoux et al., 2001). This complex may also participate in histone eviction, removing and reassembling histones around elongating RNA Pol II, and thereby promoting transcriptional elongation (Abramson et al., 2010).

In addition to regulating transcription at the promoters, AIRE may influence the pre-RNA processing or splicing of TSA transcripts (Abramson et al., 2010; Giraud et al., 2014). Moreover, AIRE-induced pre-mRNA splicing was confirmed in the *Drosophila doublesex* minigene model system (Oven et al., 2007). Taken together, these studies confirm the importance of AIRE in transcriptional regulation by different mechanisms.

To date, many AIRE-interacting nuclear partners have been determined by immunoprecipitation and mass-spectrometry proteomic studies. Overall, AIRE was found to interact with nuclear proteins in the following different functional groups: transcriptional regulator proteins (e.g. CBP, p300, KAP1, RNA Pol II, P-TEFb, DNA-PK/Ku70/Ku80, PARP1, PCNA, TOP2a, FACT), chromatin binding proteins (e.g. histones H3, H2a, H2b, and γH2AX; MCM2, RAD21, LMNB1, RUVBL, MBD1, ATF7ip), nuclear transport proteins (e.g. XPO1, NUP93, RANBP2, RANB9, KPNB1), and preRNA-processing proteins (e.g. SFRS1, SFRS2, SFRS3, NOP56, GEMIN5, EFTUD2) (Abramson et al., 2010; Gaetani et al., 2012; Žumer et al., 2012; Yang et al., 2013; Waterfield et al., 2014). Recently, the PHD2 domain of AIRE was shown to interact with a very similar set of proteins as described by Abramson et al., 2010 (and DNA-PK; Figure 1) but not with preRNA-processing proteins (Yang et al., 2013).

2.2.5. The role of AIRE in immune tolerance

AIRE has a crucial role in shaping central immune tolerance. The small subset of mTECs in thymus where AIRE is primarily expressed has a critical role in establishing central immune tolerance through the negative selection of tissue-specific T cells (Derbinski et al., 2001; Anderson et al., 2002; Liston et al., 2003; Hubert et al., 2008). Approximately 20% of mTECs are AIRE-positive

(Heino et al., 1999), which are characterized by the high expression of major histocompatibility complex class II (MHC II; HLA-DR) molecules, co-stimulatory molecules CD40, CD80, and CD86, epithelial cell adhesion molecule (EpCAM), keratins 5 and 14, agglutinin UEA-1, and two tight junction proteins, claudins 3 and 4 (Heino et al., 1999; Hamazaki et al., 2007). AIRE is involved in mTEC differentiation (Gillard et al., 2007; Matsumoto 2011). AIRE positive cells are considered as terminally differentiated mTECs (Gäbler et al., 2007; Derbinski et al., 2008; Gray et al., 2007).

AIRE gene defects lead to a defective negative selection of autoreactive $CD4^+$ and $CD8^+$ T cells, and the production of a high titer of autoantibodies by B cells, and lymphocytic infiltrations in several organs in mice (Anderson et al., 2002; Liston et al., 2003). In mTECs of Aire-KO mice, the expression of several hundreds of peripheral tissue antigens is decreased (Anderson et al., 2002). In mice, the severity of the autoimmune phenotype depends on the specific mouse strain into which the gene defect has been introduced. Aire-KO on the BALB/c and NOD strain backgrounds tends to have a more severe phenotype, whereas the C57BL/6 strain is less affected by the *Aire* gene deficiency (Jiang et al., 2005; Gavanescu et al., 2007). In addition to knockout models, an *Aire* knock-in mouse model with a human dominant negative missense mutation G228W ($Aire^{GW/+}$) had a unique autoimmunity phenotype (Su et al., 2008). $Aire^{GW/+}$ mice do not have lymphocytic infiltration in the stomach, liver, retina, or gonads, as found in other Aire-KO mice models; instead, lymphocytic infiltration was detected only in the lacrimal and salivary glands of $Aire^{GW/+}$ mice (Su et al., 2008). However, in contrast to most APECED patients, Aire-KO mice are not susceptible to *Candida* infection (Kisand et al., 2011). Interestingly, Aire-KO mice can also develop autoimmune responses against certain autoantigens despite the normal expression of autoantigens in their mTECs. For example, autoimmune gastritis is a common autoimmune disease observed in the Aire-KO mice, though the gastritogenic autoantigens (ATP4a and ATP4b) are expressed at normal levels (Ruan et al., 2007). This observation suggests that there are other mechanisms beyond the transcriptional control of Aire-dependent autoantigen expression in mTECs.

AIRE is required for the proper migration of T lymphocytes. AIRE is also responsible for the mTEC expression of chemokines, such as CCL19, CCL21 (ligands for CCR7 receptor on $CD4^+CD8^+$ and $CD4^+$ T cells) and CCL22 (ligand for CCR4 receptor on $CD4^+CD8^+$ and $CD4^+$ T cells), which are all important for the controlled migration of double-positive $CD4^+CD8^+$ T cells during their negative selection (Laan et al., 2009). The overexpression of Aire results in the increased migration of $CD4^+CD8^+$ T cells and $CD4^+$ T cells, whereas the lack of Aire results in a delay in mature $CD4^+$ T cell emigration *ex vivo*, as demonstrated in a newborn mouse thymus organ culture assay (Laan et al., 2009).

AIRE is also important in the establishment and maintenance of $CD4^+CD25^+$ Tregs (Aschenbrennner et al., 2007; Hanabuchi et al., 2010; Malchow et al., 2013). In APECED patients, the *AIRE* gene defect impairs the loss of naive

Treg precursors and their suppressive function. APECED patients have highly activated Tregs, which express significantly less FOXP3 protein compared to the healthy controls, suggesting the impairment of peripheral immune tolerance (Laakso et al., 2010). However, neither the numbers nor the suppressive functions of Tregs were changed in Aire-KO mice (Kuroda et al., 2005). AIRE deficiency does not influence the proportion of dendritic cells (DCs) and metallophilic macrophages, neither their maturation state in thymus (Hubert et al., 2008; Milićević et al., 2009). Additionally, the development of natural killer T cells do not depend on Aire, as the frequency, distribution and cytokine production has been reported to be normal in Aire-KO mice (Pitt et al., 2008).

Ex vivo, isolated Aire-positive mTECs and DCs from Aire-hemagglutinin antigen transgenic mice showed a strong stimulation of $CD4^+$ T cells in an antigen presentation assay and no significant stimulation by cTECs (Aschenbrennner et al., 2007). It was also demonstrated that DCs take up, process and present mTEC-specific antigen or TSA to $CD4^+$ T cells (Figure 2). Furthermore, the transfer of intercellular MHC-peptide complex from mTECs to DCs was later demonstrated in *in vivo* experiments (Millet et al., 2008). Similar results were obtained by Hubert et al. (2011), who demonstrated direct antigen presentation by mTECs as well as the indirect presentation of mTEC-derived antigens by thymic DCs to $CD4^+$ and $CD8^+$ T cells (Hubert et al., 2011). These results indicated that cellular crosstalk occurred between mTECs and DCs during negative T cell selection.

Several studies have reported that AIRE might participate in the negative selection of T lymphocytes and in the maintenance of peripheral immune tolerance. In mice, *Aire* gene transcripts have been detected in several peripheral immune tissues such as the lymph nodes, tonsils and spleen (Heino et al., 2000; Kont et al., 2008; Suzuki et al., 2008). In human peripheral blood, *AIRE* mRNA expression was observed in B cells, granulocytes, and $CD14^+$ DCs/macrophages but was not observed in T cells (Suzuki et al., 2008). AIRE protein is also detectable in the peripheral immune organs, in DCs of the spleen and tonsils, and in the gut associated lymph nodes (Heino et al., 1999; Halonen et al., 2001; Gardner et al., 2008; Poliani et al., 2010). Previous studies have also reported AIRE protein expression in $CD14^+$ DCs/monocytes (Kogawa et al., 2002).

The extrathymic Aire-expressing cells (eTACs), which express a set of TSAs genes, have been described in mice lymph nodes and spleen (Gardner et al., 2008). Furthermore, these eTACs are different from canonical mTECs, as they express neither the co-stimulatory molecules CD80 and CD86 nor UEA-1 nor the DC marker CD11c; however, these eTACs do express the epithelial marker EpCAM as well as MHC II molecules on their membrane (Gardner et al., 2008). These eTACs were generally localized to the T-B cell boundary regions in spleen and lymph nodes. Surprisingly, as assessed by microarray analysis, there was only a very small overlap between the Aire-regulated genes in the eTACs and mTECs (of the 1835 TSAs expressed in mTECs, only seven TSAs overlapped with the 163 TSAs expressed in eTACs). These findings suggested that peripherally expressed Aire has a complementary role in the maintenance

of self-tolerance. Moreover, the authors demonstrated that eTACs directly interact with auto-reactive T cells and mediate deletional tolerance (Gardner et al., 2008). The lack of overlap in the expression of Aire-dependent genes in the eTACs and mTECs suggests that the Aire regulation of transcription is a complex process that may vary between cell types (Gardner et al., 2008).

Furthermore, studies by Poliani et al. (2010) revealed AIRE-expressing cells in human spleen that have myeloid dendritic cell morphology and express specific DC markers, such as CD11, S100, CD40, CD83, DC-LAMP/CD208, CCR7, and HLA-DR molecules on their membrane (Poliani et al., 2010). In cells, they also found the AIRE-dependent expression of genes, including insulin, steroid 17-alpha-hydroxylase (*CYP17A*), and steroid 21-hydroxylase (*CYP21A2*), as well as molecules associated with tolerogenic functions, such as indoleamine 2, 3-dioxygenase (*IDO1*) and interleukin-10 (*IL10*) (Poliani et al., 2010). It is probable that eTACs play an increasingly significant role with advancing age as the thymus involutes and the burden of maintaining self-tolerance shifts to the periphery (Gardner et al., 2008).

A recent study in mice demonstrated that lymph node stroma mediates $CD8^+$ T cell peripheral tolerance in an AIRE-independent manner (Cohen et al., 2010). This study suggests that AIRE has a partial and not absolute role in the establishment and maintenance of peripheral immune tolerance. Taken together, the findings discussed above on peripheral AIRE expression have provoked speculation about the role of AIRE in peripheral immune tolerance.

2.3. DNA-PK/Ku70/Ku80 protein complex

DNA-dependent protein kinase (DNA-PK) is a nuclear, DNA-dependent protein serine/threonine kinase (~470 kD). DNA-PK composed of a large catalytic subunit and two DNA-targeting proteins, Ku70 and Ku80, which are regulatory factors of enzyme (Smith and Jackson 1999). The human DNA-PK is coded by *PRKDC* gene from chromosome 8. DNA-PK is a member of phosphatidylinositol 3-kinase (PI3K) superfamily and is abundantly expressed in all mammalian cells (Smith and Jackson 1999). DNA-PK is crucial for B and T lymphocyte development because it participates in V(D)J gene recombination to form functional B and T cell receptors (Smith and Jackson 1999). DNA-PK is a molecular sensor of DNA damage and is a central component of DNA-damage repair pathways, including non-homologous end-joining (NHEJ) repair. DNA-PK regulates the proliferation of cells, telomere length of chromosomes, genomic stability (Ruis et al., 2008) and replication of DNA (Liu et al., 2012). DNA-PK is activated by double-strand DNA (dsDNA) ends or by dsDNA breaks (Anderson and Lees-Miller 1992). The cells defective in DNA-PK/Ku70/Ku80 proteins, which is characteristic to the SCID mice, are severely immunodeficient and hypersensitive to ionizing radiation owing to their inability to repair double-stranded DNA breaks effectively (Smith and Jackson 1999). The binding of Ku70/Ku80 to DNA ends recruits the catalytic subunit of DNA-PKs,

forming the active DNA-PK holoenzyme (Gottlieb and Jackson 1993). Although the enzymatic activity of DNA-PK depends on its interactions with DNA and the Ku70/Ku80 protein complex (Anderson and Lees-Miller 1992), it can be stimulated in *in vitro* assays in the presence of DNA sequences without targeting the Ku70/Ku80 protein complex (Hammarsten and Chu 1998). In addition to the dsDNA breaks, the DNA-PK and/or the Ku proteins are known to bind sequence-specific promoter elements (Xu et al., 2004), single-stranded DNA (Torrance et al., 1998), nucleosomes (Park et al., 2003), RNA (Zhang et al., 2004) and base-unpairing regions or BURs, which typically are found in matrix attachment regions in the genome (Galande et al., 1999).

DNA-PK phosphorylates many DNA-binding proteins and regulates their functions. The molecular targets of DNA-PK include Ku70/Ku80 proteins (Gottlieb and Jackson 1993), PARP1 (Ruscetti et al., 1998), γH2AX (Park et al., 2003), transcription factors such as p53, c-Fos, c-Jun, c-Myc, Sp1, Oct-1, NF-κB, and TFIID (Lees-Miller et al., 1992; Anderson 1993; Jackson 1996; Ju et al., 2010). For example, DNA-PK phosphorylates p53 on serine 15 and serine 37 leading to stabilization and inhibition of p53 degradation by MDM2 (Lees-Miller et al., 1992). The protein kinase activity of DNA-PK is autoregulatory; in the absence of a phosphorylation substrate, DNA-PK autophosphorylates and dissociates from the Ku70/Ku80/DNA complex (Smith and Jackson 1999).

Several reports have described the function of DNA-PK in transcriptional activation (Xu et al., 2004; Mayeur et al., 2005). The phosphorylation of TATA-binding protein (TBP) and transcription initiation factor IIB (TFIIB) by DNA-PK stimulates the basal transcription of RNA Pol II (Chibazakura et al., 1997). DNA-PK also phosphorylates RNA Pol II C-terminal serine residues, such as serines 2, 5 and 7, which are involved in the initiation and elongation phases of transcription (Tyagi et al., 2011). In addition to these examples, DNA-PK can modulate gene expression through the RNA-dependent phosphorylation of pre-mRNA binding or hnRNP proteins and the nuclear DNA helicase II/RNA helicase (Zhang et al., 2004). This RNA binding affinity has been ascribed to the Ku86 subunit.

Due to their high affinity for DNA ends, the Ku70 and Ku80 proteins can function independently of DNA-PK in mammalian cells (Anderson and Lees-Miller 1992). Ku70/Ku80 proteins also display afffinity to other DNA structures such as nicks, single strand gaps or any type of the single to dsDNA transitions (Tuteja and Tuteja 2000). Independent of DNA-PK, the Ku70/Ku80 heterodimer also possesses DNA helicase function (Tuteja et al., 1994). Furthermore, data have shown that Ku proteins bind to specific DNA sequences, transcription regulatory elements and origins of DNA replication (Toth et al., 1993; Dynan and Yoo 1998).

Interestingly, in autoimmune diseases such as systemic lupus erythematosus, scleroderma, polymyositis and Sjögren's syndrome, patients have autoantibodies to Ku70 and Ku80 (Mimori et al., 1981; Reeves et al., 1991).

2.4. Apoptosis

Apoptosis is genetically programmed cell death, which occurs during the development of multicellular organisms, aging, and maintenance of normal tissue homeostasis (Kerr et al., 1972; Raff 1998; Green 2011). The apoptotic cell has a characteristic morphology, which includes certain hallmarks such as shrinkage and blebbing of cells, chromosomal DNA fragmentation into nucleosomal size units (180 bp), and nucleus swelling and fragmentation (Kerr et al., 1972; Saraste and Pulkki 2000). Apoptosis can be induced by multiple external or environmental cues such as anti-cancer drugs, gamma radiation, ultraviolet light, heat, hypoxia, hormones, virus infections and deprivation of survival factors (Reuter et al., 2010). These stress factors generally induce the production of cellular "poisons", such as nitric oxide (NO) and other reactive oxygen species (e.g., hydrogen peroxide, hydroxyl radical, and superoxide anion), which act as signal molecules implicated in initiating apoptosis and/or inflammation (Brüne et al., 1999; Reuter et al., 2010). Defects in apoptotic processes can lead to pathological consequences, such as abnormal embryogenesis, autoimmune diseases and cancer development (Brüne et al., 1999; Reuter et al., 2010).

The key mediators of apoptosis are caspases, which are cysteine-dependent aspartate specific proteases (Pop and Salvesen 2009; Duprez et al., 2009). They exist as inactive pro-enzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits that dimerize to form an active enzyme. Several caspases have a long prodomain (~100 residues) termed the caspase recruitment domain (CARD) (present in caspases 1, 2, 4, 5, 9, 11, and 12) or a death effector domain (DED) (caspases 8, 10) (Hofmann et al., 1997; Pop and Salvesen 2009). The proteins with CARD, DED, the pyrin domain (PYD), and death domain (DD) belong to the death domain superfamily and primarily function in the regulation of apoptosis and inflammatory responses (Park et al., 2007). The CARD motif is common to several other apoptosis-related proteins, such as Apaf-1, RAIDD, and ICEBERG (Park et al., 2007). The secondary structure of CARD consists of six amphipathic helices and of highly conserved hydrophobic residues that form the core of the folded domain (Figure 3). CARD domains mediate the formation of larger protein complexes via direct interactions between individual CARDs and other death domain superfamily proteins (Park et al., 2007).

Apoptosis occurs in two major pathways, the extrinsic and intrinsic pathways, which both involve specific caspases (Duprez et al., 2009). The extrinsic pathway is mediated by a subgroup of the tumor necrosis factor receptor protein superfamily (Duprez et al., 2009). The activation of these so-called death receptors leads to the recruitment and activation of initiator caspases, i.e., the pro-caspases 8 and 10. The intrinsic pathway is regulated by mitochondria, whereas the signals for this stress can be genotoxic (DNA damage) or cytosolic. The cytosolic stress is induced by unfolded proteins or oxidized proteins and oxygen free radicals. In the intrinsic pathway, the activation of initiator

caspases, the pro-caspases 2 and 9, occurs with the release of cytochrome c from mitochondria. As a result, cytochrome c, caspase 9 and Apaf-1 proteins form the apoptosome complex through the interaction of their CARD domains (Acehan et al., 2002; Sanches-Pulido et al., 2007).

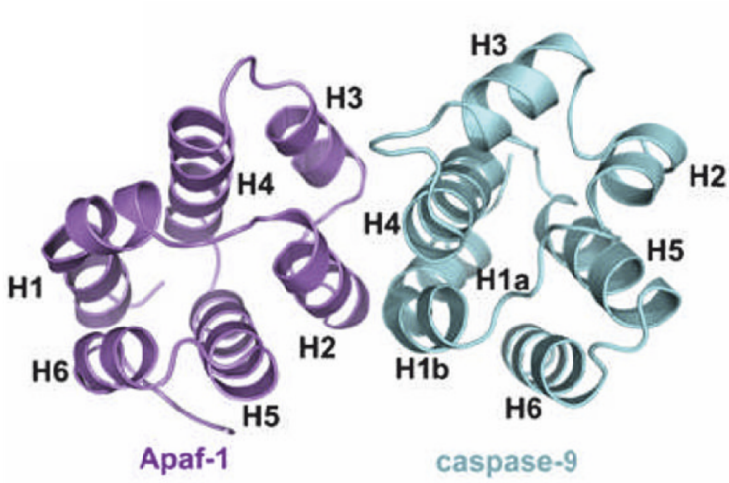

Figure 3. The six-helical bundle structural fold (H1-H6) as the death fold of CARD in Apaf-1 and caspase 9 proteins. Adapted from (Park et al., 2007).

Both the intrinsic and extrinsic apoptotic pathways lead to the activation of effector caspases such as pro-caspases 3, 6, and 7, which cleave multiple target proteins, also called death substrates. These biochemical changes lead to the irreversible impairment of DNA, RNA and proteins and, eventually, to cell death (Pop and Salvesen 2009; Duprez et al., 2009). Recent evidence has suggested that there might be a cross-activation of both apoptotic pathways because the activation of the mitochondrial pathway may occur after the activation of the extrinsic pathway (Duprez et al., 2009).

2.5. GAPDH as a sensor of cellular stress and apoptosis

GAPDH is a highly conserved protein that was initially known to have a role in glycolysis; it catalyzes reversible conversions between glyceraldehyde-3-phosphate and 1, 3-diphosphoglycerate, using nicotinamide adenine dinucleotide or NAD^+ as the cofactor and is involved in the basic energy production of ATP (Seidler 2013). GAPDH has been frequently used as a common housekeeping gene to normalize Northern blots and real-time PCR results. The GAPDH enzyme is highly expressed as a homo-tetramer (36 kDa), with an

expression level that does not vary under most normal biological conditions. The results from genomic library screenings suggest that there are 364 copies of GAPDH pseudogenes in the rat genome, 331 in the mouse genome, and 62 in the human genome, but none in the genomes of zebrafish, pufferfish, fruit flies, and worms (Liu et al., 2009). Only one functional GAPDH gene is known in the human, mouse, and rat genome (Liu et al., 2009).

GAPDH localizes to the cytosol, where the glycolysis occurs, in addition to multiple other cellular compartments such as the plasma membrane, mitochondria, endoplasmic reticulum, polysomes, Golgi complex, cytoskeletons and nuclei (Sirover 1997, 1999 and 2011). GAPDH has recently been implicated in several non-metabolic processes, such as endocytosis and membrane trafficking, and in the vesicular transport of secretory proteins (Tisdale et al., 2002).

In the nucleus, GAPDH has role in transcriptional regulation, as demonstrated by its activity as a transcriptional co-activator with POU class 2 homeobox 1 (Oct-1) on histone H2B promoter during the S-phase activation in HeLa cells (Zheng et al., 2003). Moreover, several other nuclear functions of GAPDH have been characterized, such as the regulation of nuclear tRNA export (Singh and Green, 1993), negative control of translation (Sampath et al., 2004; Arif et al., 2012), DNA replication (Mansur et al., 1993), DNA repair with the uracil glycosidase activity (Arenaz et al., 1983; Vollberg et al., 1989; Meyer-Siegler et al., 1991), and maintenance of telomeric DNA structure (Demarse et al., 2009). Additionally, GAPDH promotes the mRNA stability of colony-stimulating factor 1 (Zhou et al., 2008) and endothelin 1 (Rodríguez-Pascual et al., 2008). Intranuclear GAPDH exhibited decreased glycolytic activity compared to cytosolic GAPDH (Brown et al., 2003). In the nucleus, GAPDH interacts with nuclear matrix (Sawa et al., 1997, Hara et al., 2005) and RNA-dependent interaction between GAPDH and PML has been demonstrated (Carlile et al., 1998). GAPDH accumulation is observed in the nuclei during cell division arrest and the initiation of terminal differentiation, as demonstrated in rat neurons (Morgenegg et al., 1986).

GAPDH is the major target of oxidative stress. The enzyme of human GAPDH has previously been reported to be S-thiolated by hydrogen peroxide (Brodie and Reed 1987), S-nitrosylated by nitric oxide (Hara et al., 2006) and nitroalkylated by nitroalkene derivatives (Batthyany, et al., 2006). Different apoptotic stimuli, such as genotoxic stress, hypoxia, hyperglycemia, and cell starvation, induce GAPDH translocation into the nucleus (Saunders et al., 1999; Sawa et al., 1997; Brown et al., 2003; Dastoor et al., 2001; Kusner et al., 2004, Chuang et al., 2005; Hara et al., 2006). The connection of GAPDH to nuclear translocation and apoptosis was first recognized in primary cultures of cerebellar granule neurons and cortical neurons (Ishitani et al., 1996). In pathological conditions, GAPDH accumulates in the nuclei of apoptotic brain neurons, which has been implicated in neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's diseases (Chuang et al., 2005; Ishitani et al., 1996). Only apoptosis, but not cell necrosis, of cultured cerebellar granule neurons involves the overexpression and nuclear accumulation of GAPDH

(Ishitani et al., 1997). The GAPDH mRNA levels are highly upregulated in pathological conditions, as in certain malignant cells (Tokunaga et al., 1987; Bhatia et al., 1994), in neurodegenerative disorders (Berry 2004), in endothelial cells undergoing oxidative stress (Graven et al., 1994), and also in age-induced apoptosis of cerebellar neurons (Ishitani et al.,1996). The association of GAPDH with cellular stress and apoptosis has also been widely demonstrated in other non-neuronal cell lines such as macrophages, primary thymocytes, fibroblasts, epithelial and endothelial cells (Graven et al., 1994; Sawa et al., 1997; Sirover 1997 and 2011).

Cell stressors, such as neurotoxins or genotoxic agents (e.g. etoposide), activate nitric oxide synthase or NOS and increase the generation of NO in the cells. Hence, a high level of NO leads to the S-nitrosylation of GAPDH at the enzyme active site (Chuang et al., 2005). The S-nitrosylation of GAPDH abolishes its glycolytic activity and elicits the nuclear translocation of GAPDH (Hara et al., 2005). Moreover, the nuclear translocation depends on the interaction with E3 ubiquitin-protein ligase Siah-1, which has a nuclear localization signal and specifically binds and transports GAPDH to the nucleus (Hara et al., 2005). It has been shown that in the nucleus, GAPDH stabilizes the Siah-1 protein by enhancing its E3 ubiquitin ligase activity, thereby facilitating the degradation of nuclear proteins and initiating cell death (Hara et al., 2005). In the absence of stress, GAPDH is exported from the nucleus via its nuclear export signal, mediated by CRM1 (Brown et al., 2003). The ability of GAPDH to exert so many different functions has been explained by the variety of post-translational modifications observed in GAPDH (Sirover 2011).

3. AIMS OF THE STUDY

The aims of the current thesis were:

1. To identify AIRE interacting proteins.
2. To verify AIRE phosphorylation sites catalyzed by DNA-PK and to elucidate their functional importance.
3. To study the impact of APECED-associated mutations in AIRE HSR/CARD domain on activation of AIRE-dependent gene promoters.
4. To study the synergistic effect of CBP on transcriptional activity of AIRE and APECED-associated mutations.
5. To study AIRE role in apoptosis.

4. MATERIALS AND METHODS

4.1. Cloning of plasmid vectors

The plasmids generated in this thesis are given in Table 2 and 3. The GST-AIRE proteins were expressed in pGEX-1TλT vector, where glutatione S-transferase (GST) is located at N-terminus and is followed by a target protein. The pcAIRE was cloned into pcDNA 3.1B$^-$ Myc/His vector where Myc/His tag is located at the C-terminus of AIRE. The cDNA of AIRE K83E was cloned into pcDNA3.1A$^-$ Myc/His vector. All cloned vectors were verified by sequencing and by DNA restriction analysis in agarose gel electrophoresis. The expression of mutant AIRE constructs was verified by SDS-PAGE, Western blot and/or immunofluorescence microscopy.

Table 2. Overview of AIRE DNA plasmids used in this study

Plasmid	AIRE (aa)	Cloning sites (5', 3')	Study	Reference
GST-AIRE	1–545	EcoRI, XhoI	I	Pitkänen et.al., 2000
GST-PHD1	293–354	NcoI, KpnI	I	Bottomley et al., 2005
GST-SPP (SAND, PHD1, PHD2)	178–482	EcoRI, XhoI	I	Pitkänen et al., 2000
GST-AIRE R257X	1–256	EcoRI, XhoI	I	Pitkänen et al., 2000
GST-AIRE 293	1–293	EcoRI, XhoI	I	Pitkänen et al., 2000
GST-AIRE 348	1–348	EcoRI, XhoI	I	Pitkänen et al., 2000
GST-AIRE V80L	1–545	EcoRI, HindIII	I	Study I
GST-AIRE T68A	1–545	EcoRI, XhoI	I	Study I
GST-AIRE S156A	1–545	EcoRI, SacI	I	Study I
pSI-AIRE	1–545	EcoRI, SalI	I	Pitkänen et al., 2001
pSI-AIRE T68A	1–545	EcoRI, NotI	I	Study I
pSI-AIRE S156A	1–545	EcoRI, NotI	I	Study I
pcAIRE	1–545	EcoRI, HindIII	I, II, IV	Heino et.al., 2000
pcAIRE L28P	1–545	EcoRI, HindIII	II	Study II
pcAIRE L29P	1–545	EcoRI, HindIII	II	Study II
pcAIRE K83E	1–545	EcoRI, HindIII	II	Study II
pcAIRE 1–143	1–143	EcoRI, NotI	IV	Study IV
pcAIRE R15C	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE R15L	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE T16M	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE A21V	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE W78R	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE V80L	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE Y85C	1–545	EcoRI, HindIII	(II)	Ilmarinen et al., 2005
pcAIRE Y90C	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)
pcAIRE L93R	1–545	EcoRI, HindIII	(II)	Liiv (unpubl.)

aa – amino acids; unpubl. – unpublished results, (II) – corresponding plasmids used in Study II

Table 3. Other plasmids used in this thesis

Plasmid	Study	Reference
pGEX-2T-p53	I	A gift from Dr. T. Punga (University of Uppsala, Sweden)
pBL-LOR	I	Study I
pBL-INV	I, II	Studies I and II
pBL-IFNβ	II	Study II
pBL-KS	I, II	A gift from Dr. K. Saksela (University of Tampere, Finland).
pCDNA3.1B$^-$ myc/his	I, II, IV	Invitrogen (USA)
pSI	I	Promega (USA)
pdEYFP-N1	I	Clontech Laboratories (USA)
pGEX-1λT-SH3	I, IV	A gift from Dr. K. Saksela (University of Tampere, Finland)
pRc/RSV-mCBP-HA-RK	II	A gift from Dr. R. Goodman (Oregon Health and Science Institute, USA)
pTZhINV-nlbgal	I, II	A gift from Dr. A. Männik (FitBiotech, Estonia)
pTZhLOR1	I, II	A gift from Dr. A. Männik (FitBiotech, Estonia)
pTK-Hyg	IV	Clontech (USA)
pTRE vector	IV	Clontech (USA)
pmaxGFP	IV	Lonza (Germany)

4.2. Cell lines and transfections

HEK293 is a human embryonic kidney epithelial cell line (Graham et al., 1977). HT93 is a human epithelial cell line of thyroid origin (Belfiore et al., 1991). 1C6 cell line was derived from mouse primary mTECs (Mizuochi et al., 1992). MO59K and MO59J are human glioblastoma cell lines, whereas DNA-PK protein coding gene is defective in MO59J cells by frameshift mutation (in exon 31), terminating the reading frame early in exon 33 (Anderson et al., 2001). In addition to the mutation in *DNA-PK* gene in MO59J cell line, the line has mutations in *p53* and *ATM* genes (Ruis et al., 2008). MO59K, MO59J, HT93-AIRE, and doxycycline or DOX-inducible AIRE-HEK/Tet and mouse 1C6 mTECs were grown as monolayers in DMEM. DMEM media was supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics, 100 U/ml streptomycin/penicillin mix. AIRE stably expressing cell lines, HT93-AIRE and DOX-inducible AIRE-HEK/Tet were grown with the selective medium, containing 0.8 mg/ml G418 (Geniticin; Sigma-Aldrich). All media and supplements were obtained from PAA Laboratories. The cell cultures were grown in incubators at 37°C with 5% CO_2.

To perform Western blots, immunoprecipitations and immunofluorescence microscopy analysis, HEK293, MO59J, and MO59K cells were transfected with a corresponding plasmid vector DNA and Exgene *in vitro* 500 reagent (in studies I, II; Fermentas) or with Turbofect reagent (in study IV; Fermentas) according to kit protocols. All transfections were performed with appropriate

plasmid 18–20 h after splitting of cells in culture. DNA plasmids for transfections were purified by endotoxin free plasmid purification kit (MachereyNagel). Yellow and green fluorescence protein expressing plasmids, pdEYFP-N1 and pmaxGFP were used to control the efficiency of the transfections in HEK293 or 1C6 cells, respectively.

For apoptosis and immunofluorescence assays, $1x10^6$ cells of 1C6 mTECs were cotransfected by electroporation with plasmids of 1 μg pmaxGFP either with 2 μg pcDNA $3.1B^-$ (as negative control) or 2 μg pcAIRE, or 2 μg pcAIRE 1–143 using Basic Nucleofector Kit (Lonza). For electroporation, the Amaxa Nucleofector (T-023 program; Lonza) instrument was used. In apoptosis assays, 1C6 mTECs were seeded onto 100 mm Petri dishes after electroporation and cells were analyzed 5–20 h post-transfection. For immunofluorescence study, after electroporation of $1x10^6$ 1C6 cells, cells were divided onto 6-well plate and were grown on the glass coverslips for 20 h.

4.3. Human and mice thymi

C57BL/6J background wild type mice were maintained at the mouse facility of the Institute of Molecular and Cell Biology, University of Tartu. Thymi from 4- to 6-week-old mice were used for immunofluorescence studies and cell sorting. Thymic stromal cell isolation, mTEC, and cTEC cell sorting were carried out as described previously (Kont et al., 2008), using AutoMACS system (Milteny Biotec). Human thymus from 8-month-old child was obtained during heart surgery, from Children Hospital at University of Tartu. The microscope sections from thymi were prepared at the Service of Pathology (Tartu University Hospital). Study of human thymus was approved by the Ethics Review Committee on Human Research of the University of Tartu (permit number 170/T-7). Keeping, handling and experiments of the mice were authorized by the Ethical Committee of animal experiments of the University of Tartu (permit number 73).

4.4. Antibodies

Antibodies used in this thesis are shown in Table 4.

Table 4. Primary antibodies used in this thesis were for protein detections in Western blot (WB), immunoprecitation (IP), immunofluorescence (IF), flow cytometry (FC) or sorting of the cells (SC).

Antibody	Species	Method	Study	Reference
AIRE 6.1	Mouse mab	WB, IP, IF	I, II, IV	Pitkänen et al., 2001
DNA-PK (4F10C5)	Mouse mab	IP, WB	I	BD Pharmingen
CD45 MicroBeads	Mouse mab	SC		Miltenyi Biotech
H213-HBAb (anti-CDR1)	Rat mab	SC	I	Miltenyi Biotech
G8.8 (anti-EpCAM)	Rat mab	SC	I	Hybridoma Bank at University of Iowa (USA)
HA (sc-7392)	Mouse mab	IP	I	Santa Cruz Biotechnology
Myc (9E10)	Mouse mab	IP	I	Sigma-Aldrich
HuR (3A2)	Mouse mab	IP	I	A gift from Dr. J. Steitz, Yale University, USA
GAPDH (6C5)	Mouse mab	IP, WB	I, IV	Ambion, Abcam
GST (sc-459)	Rabbit polyclonal ab	IP	I	Santa Cruz Biotechnology
GAPDH (ab9485)	Rabbit polyclonal ab	IF	IV	Abcam
H3	Rabbit polyclonal ab	WB	IV	Abcam
β-actin (A2228)	Mouse mab	WB	IV	Sigma-Aldrich

mab – monoclonal antibody; ab – antibody

4.5. Western blot

The protein samples were separated on 8%, 10% or 12% Tris-glycine SDS-polyacrylamide gels. The semi-dry transfer system was used in Western blots (Bio-Rad Laboratories) and the proteins were transferred to PVDF filters (Immobilon-P, Milliopore). The dilutions of primary antibodies used in Western blots were anti-AIRE (6.1; 1:2000), anti-DNA-PK (1:1000), anti-H3 (1:5000) or anti-β-actin (1:5000), and anti-GAPDH (1:50 000). The secondary antibodies were conjugated goat anti-mouse HRP and goat anti-rabbit HRP antibodies and diluted to 1:10 000 (DAKO). The primary as well secondary antibodies were incubated for 1 h at room-temperature. The protein images on PVDF filters were visualized by Enhanced Chemiluminescence Advance Western Blotting Detection kit (Amersham, GE Healthcare) using ECL detection machine (ImageQuant RT ECL; GE Healthcare).

4.6. Immunofluorescence microscopy

In AIRE-HEK/Tet cell line, 2 μg/ml DOX alone (for AIRE expression) or 2 μM etoposide was added to the cells for 24 h before immunostaining. Immunostainings with antibodies were performed 18 or 24 h after the transfections with plasmids. The frozen thymus sections were used for immunoassaying. Cells or tissue sections were probed with a monoclonal anti-AIRE 6.1 (1:1000), and with polyclonal anti-GAPDH (1:250) antibodies for 1 h. The conjugated secondary antibodies were goat anti-mouse Alexa488 or goat anti-rabbit Alexa594 or goat anti-rabbit Alexa488 (all from Molecular Probes), were diluted to 1:1000. The primary and secondary antibodies were diluted in 0.1% Tween-20/ TBS/ 1% normal goat serum, and each incubation step was followed by washing with 0.1% Tween-20/ TBS. For microscopy, the cells were mounted and counterstained with fluorescense mounting medium containing DAPI (DAKO). All steps were performed at room temperature. The micrographs of cells were obtained using Olympus IX70 (63x water immersion objective; Figure 5 and Figure 12), Nikon Eclipse TE2000-U microscope (63x water immersion objective; used in Study II Figure 4A), Carl Zeiss (63x oil immersion objective; Figure 8) and a confocal microscope Carl Zeiss LSM5 DUO (63x water immersion objective; used in Study IV Figures 2A, 3A and 3B).

4.7. RNA purifications and real-time PCR

The total RNA was extracted with Trizol Reagent according to manufacturer's manual (Invitrogen). The cDNAs was synthesized with Superscript III Reverse Transcriptase (Invitrogen) and cDNA samples were analyzed in triplicate by quantitative PCR using the qPCR SYBR Green Core kit (Eurogentec) with compatible primers using ABI Prism 7900HT instrument (Applied Biosystems). The relative gene expression levels were calculated using comparative *Ct* (*ΔΔCt*) method (Applied Biosystems). Every sample was run in three parallel reactions. Data are presented as the mean ± SEM of three independent experiments. The primers used for quantitative RT-PCR are listed in Table 5. All primers were synthesized by TAG Copenhagen A/S, except GAPDH primer (PPH00150–200) that was obtained from Qiagen.

Table 5. Primers used in RT-PCR in this study

Primer	Gene	Sequence (5'-3')	Study	Sp.
2F	DNA-PK	CCAGCTGTTATAACTTGTGATGAG	I	mouse
2R	DNA-PK	TCTGAAAGCCCACTCTCTGGC	I	mouse
K2–8 F	keratin 8	AGGAGCTCATTCCGTAGCTG	I	mouse
K2–8 R	keratin 8	TCTGGGATGCAGAACATGAG	I	mouse
mLOR-F	loricrin	GTGCTTCAGGGTTCCCCTTCT	I	mouse
mLOR-R	loricrin	TCCTCCTCCACCAGAGGTCTT	I	mouse
mINV-F	involucrin	TCCCTCCTGTGAGTTTGGTTTGGT	I	mouse
mINV-R	involucrin	CACAGTCTTGAGAGGTCCCTGAA	I	mouse
Exon 6-F	HPRT	GACTTTGCTTTCCTTGGTCAGG	II	human
Exon 7-R	HPRT	AGTCTGGCTTATATCCAACACTTCG	II	human
hINV-F	involucrin	GCCTTACTGTGAGTCTGGTTGACA	II	human
hINV-R	involucrin	GGAGGAACAGTCTTGAGGAGCT	II	human
S1008A-F	S100A8	CTCAGTATATCAGGAAAAGGGTGC AGAC	II	human
S1008A-R	S100A8	CACGCCCATCTTTATCACCAGAATG AG	II	human
PPH00150–200 (Qiagen)	GAPDH	Sequence not published	IV	human
F-50	β-actin	CTGGAACGGTGAAAGGTGACA	IV	human
R-50	β-actin	CGGCCACATTGTGAACTTTG	IV	human

Sp. – species; F – forward; R – reverse

4.8. Expression and purification of GST-AIRE fusion proteins

GST-tagged protein expression and purification used has been described earlier (Pitkänen et al., 2000). The plasmid vectors GST-AIRE (1–545), GST-AIRE PHD1 (290–349), GST-R257X (1–256), GST-AIRE 293 (1–293), GST-AIRE 348 (1–348), AIRE-SAND (175–298), GST-AIRE T68A, GST-AIRE S156A and GST-AIRE V80L were expressed in *Escherichia coli* BL21(DE3) or M15 (Qiagen) strains. Full-length GST-AIRE and PHD-domain containing mutated AIRE proteins were expressed in the presence of 0.1 mM $ZnCl_2$. During lysis of bacteria, 1% N-laurylsarcosine and 3% Triton X-100 were added to increase solubility of the proteins and 50 μM $ZnCl_2$ was included when expressing PHD domain containing proteins. The proteins were affinity purified, using Glutathione Sepharose Fast Flow (Amersham Biosciences) or S-sepharose according to the manufacturer's instructions. The purified proteins were verified by SDS-PAGE and gels were stained with Coomassie Blue.

4.9. Co-immunoprecipitation assays

For co-immunoprecipitations (Co-IPs), approximately $2x10^6$ HEK293 cells were transfected with 10 μg of pcAIRE or either pcDNA3.1B$^-$ Myc/His (Invitrogen) or 10 μg pdEYFP-N1 (Clontech) vector. After 46 h, the total cell extract was prepared by lysis for 30 min with 0.25 ml of lysis buffer (0.3 M NaCl, 1% NP-40, 20 mM Tris pH 7.5, 0.1 mM $ZnCl_2$, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, 0.4 mM Na_3VO_4 and protease inhibitor mix; Sigma-Aldrich). The cell lysates were treated with the 25G syringe to shear genomic DNA and cleared by centrifugation at 4°C. The supernatants were diluted 5 times with the lysis buffer lacking NP-40. For Co-IP experiments, 1.5 μg of antibody was added to 700–900 μg of whole-cell extracts and incubated for 4 h at 4°C. Immunocomplexes were separated from protein total extract by 15 μl of packed Protein G-sepharose beads (AmershamPharmacia-Biotech) for 1 h at 4°C. Where indicated, with final concentration of 4 μg/ml RNaseA, 10 u/ml RNaseT (Fermentas) and 0.4 mg/ml ethidium bromide (EtBr) were added before Co-IPs by support protocol (Lai and Herr, 1992). The micrococcal nuclease (MNase; Fermentas) treatment was performed directly after Co-IP assay, where 1 units of MNase added to immunoprecipitates in 50 μl buffer (10 mM HEPES pH 7.0, 4 mM $CaCl_2$, 50 mM NaCl, 0.1 mM $ZnCl_2$) and were incubated for 10 min at 30°C accordingly to protocol (Nguyen and Goodrich 2006). The immunocomplexes were washed with the buffer (20 mM Tris pH 7.5, 0.2% NP-40, 300 mM NaCl, 0.1 mM $ZnCl_2$, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, protease inhibitors). The proteins were eluted from the beads with SDS sample buffer by boiling for 5 min. The eluted proteins were analyzed by Western blot.

4.10. Protein phosphorylation prediction

Two programs were used to predict phosphorylation sites in AIRE, Scansite 2.0 at http://scansite.mit.edu/cgi-bin/motifscanseq (Obenauer et al., 2003) and NetPhos 2.0 at http://www.cbs.dtu.dk/services/NetPhos (Blom et al., 1999).

4.11. Preparation of nuclear extracts

The nuclear extracts were prepared from HEK293 cells according to modified protocol (Dignam et. al., 1983). The cells were collected by centrifugation 4˚C, washed twice with ice-cold PBS and then once with ice-cold hypotonic buffer A (10 mM KCl, 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.4 mM Na_3VO_4, protease inhibitor mix). Cell membrane were lysed with 5x packed cell volume of ice-cold buffer A on ice for 10 min. Cell lysis was verified by Trypan Blue staining under the microscope. Nuclei were collected by centrifugation at 4°C and lyzed with 2–3x packed cell volume ice-cold buffer C (0.42 M NaCl, 20 mM HEPES, 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.4 mM Na_3VO_4, protease inhibitor mix) on ice for 30 min. Nuclear extract was cleared by centrifugation at 4°C. The soluble protein concentration (also the total cell protein extract) was measured with Bio-Rad Protein Assay reagent (Bio-Rad) in spectrophotometer (Ultrospec 2000, Pharmacia Biotech) at 595 nm.

4.12. Phosphorylation assays

The phosphorylation assays were performed with SignaTECT DNA-PK Kinase Assay System kit (Promega) according to the manufacturer's protocol. Preparations of total cell and nuclear extracts were made as described in sections of 4.9 and 4.11. Either 600 μg of total protein extract of immunoprecipitations, or 20 μg of HEK293, or 15 μg of MO59K, and MO59J nuclear extracts were used per phosphorylation reaction as source of kinases (DNA-PKcs) and dsDNA. Alternatively, 10 U of purified DNA-PKcs (Promega) in the presence of 250 ng of calf thymus DNA was used. 50 μM of LY294002 hydrochloride (Sigma-Aldrich) was added when indicated during kinase reactions. In assays using GST fusion proteins as the substrates, 10 μl of packed S-sepharose beads with GST-AIRE proteins were pre-washed with the kinase reaction buffer (SignaTECT kit buffer supplemented with 0.1 mM $ZnCl_2$ and 0.2 mM Na_3VO_4). The phosphorylation reactions were carried out in the presence of γATP-^{32}P. 25 μg of biotinylated-p53 peptide (EPPLSQEAFADLWKK; Promega) was as positive control substrate for DNA-PK (Lees-Miller et al., 1992). The immunoprecipitates were coupled to 10 μl of packed protein of Protein G or 10 μl of packed S-sepharose with purified GST-AIRE fusion proteins (4–5 μg) were used as substrates in phosphorylation assays. The phosphorylation reactions were done for 7 min at 30°C and were terminated with ice-cold 12.5 μl 7.5 M guanidine hydrochloride buffer. The sepharose beads were washed three times

with ice cold washing buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.1% BSA, 0.2% NP-40, 0.1 mM $ZnCl_2$ and 0.2 mM Na_3VO_4) after the phosphorylation reactions to measure the radioactivity of proteins. When was used biotinylated p53 peptide as control, it was incubated on streptavidin coated filters according to the kit protocol. After the washing of filters or sepharose-protein complexes, the efficiency of ^{32}P labeling of the substrates was measured in radioactivity counter (1414 Guardian, PerkinElmer, Wallac) in 1 ml scintillation liquid cocktail (OptiPhase HiSafe3; PerkinElmer, Wallac).

4.13. Transcription activation and luciferase reporter assays

The transcription activation assays were performed with Luciferase Assay System kit (Promega) according to the manufacturer's protocol. $4x10^4$ HEK293 cells were transfected 18–20 h after splitting with the plasmid vectors for 48 h. In study I, 0.1 μg of luciferase containing reporter plasmids with gene promoters of involucrin (INV-pBL) or with loricrin (LOR-pBL) were cotransfected with 0.3 μg pSI-AIRE, pSI-AIRE T68A, or pSI-AIRE S156A protein expression plasmids.

In study II, $4x10^4$ HEK293 cells were cotransfected with 0.1 μg of pBL-INV or pBL-IFNB (the full-length interferon beta gene promoter; nucleotides -604 to +94) reporter plasmid with 0.3 μg of pcAIRE or pcAIRE L28P, pcAIRE L29P or pcAIRE K83E protein expression plasmids. 0.5 μg of CBP protein expression vector (pRc/RSV-mCBP-HA-RK) was cotransfected with AIRE expression vectors or alone with negative control pc plasmid (pcDNA 3.1B$^-$ Myc/His). The luciferase activity was measured in luminescence counter (1450 LSC, MicroBetaTrilux, PerkinElmer, Wallac). For endogenous *INV* and *S1008A* promoter activation assays, 0.5 μg of pcAIRE or pcDNA 3.1B$^-$ Myc/His with 0.9 μg of CBP expression vectors were cotransfected to HEK293 cells.

4.14. Cell apoptosis and proliferation assays

To measure apoptosis level, $1x10^5$ cells were treated with Annexin V-PE (BD Pharmingen) and/or with 7-amino-actinomycin D or 7-AAD (Invitrogen) using a flow cytometer according to the manufacturer's protocol (BD Pharmingen). To estimate the effect of induced apoptosis in AIRE-HT93 cells, etoposide (100 nM as final concentration) or 5 μl of DMSO as negative control (same volume like etoposide solution in DMSO) was added to cell cultures at 43 h after seeding of the cells and incubated with etoposide for 5 h before cell harvest. In immunofluorescence studies of GAPDH nuclear translocation in AIRE-HEK/Tet cells, 2.0 μM etoposide was used as positive control, alone or with 2.0 μg/ml doxycycline for 24 h. 10.0 μM of R-(-) deprenyl (SigmaAldrich) was

added 1.5 h before induction of AIRE expression with doxycyclin (2 mg/ml) or immediately after electroporation of 1C6 mTECs.

Apoptosis percentage calculated from the GFP positive cell population in 1C6 cells or from Annexin V-PE positive and 7-AAD negative cell populations in AIRE-HEK/Tet, HEK293, HT93, and AIRE-HT93 cells, using cytometry software (FlowJo7). Data are the mean ± SEM of duplicate measurements of a representative sample of three independent experiments. *p* values were calculated by paired t-test using GraphPad Prism5 software.

In cell proliferation studies, BrdU incorporation into DNA was used for AIRE-HEK/Tet or HEK293 cells, accordingly to support protocol (BD Biosciences). 10 μM of BrdU was added into a fresh, serum-free medium for 1 h before cell trypsinization. $2x10^5$ cells were fixed, permeabilized, and stained with 1 μg anti-BrdU-FITC (BD Biosciences) and analyzed by flow cytometry. The flow cytometric studies were performed in FACS Calibur (BD Biosciences).

4.15. Fractionation of cytoplasmic and nuclear proteins

For subcellular fractionation, DOX-inducible AIRE-HEK/Tet or control, HEK293 cells were lyzed in the ice cold lysis buffer A (0.1% NP-40, 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 0.4 mM Na_3VO_4, proteinase inhibitor mix) for 8 min. The nuclear pellet was washed once with 1 ml lysis buffer A without NP-40 detergent. The half of the nuclear pellet was diluted in 5 volumes of SDS sample buffer for histone H3 detection by Western blot. The other half of the washed nuclei were lysed in 2 packed cell volumes of ice cold buffer B (300 mM NaCl, 0.3% NP-40, 20 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 0.4 mM Na_3VO_4, proteinase inhibitor mix), and incubated on ice for 45 min. The nuclear extracts were homogenized using a 23G syringe needle and cleared by centrifugation at 4°C. For GAPDH detection, either 20 μg cytoplasmic or 30 μg nuclear extracts were loaded to SDS-PAGE. To detect the total cell lysate, the unfractionated cells were lysed with SDS sample buffer. The protein extracts were analyzed by Western blot.

5. RESULTS

5.1. DNA-PK/Ku70/Ku80 proteins interact with AIRE's PHD1 domain

Only one AIRE interacting protein had been reported at the time that this study was initiated; a common transcriptional co-activator CBP (Pitkänen et al., 2000). Therefore, the aim of the present study was to find new AIRE protein partners. Because the PHD finger domains are potent transcription regulators and appear to mediate chromatin-related protein interactions (Bienz 2006), the AIRE PHD1 domain was used to identify novel AIRE interacting molecules. For this, bacterially expressed GST-AIRE PHD1 fusion protein was used to pull down interacting partners from THP-1 monocyte nuclear extracts (Figure 1A in Study I) that was performed by collegeas in the Universiy of Tampere (Autoimmunity Study Group at the Department of Pathology). Three protein partners were found with molecular masses of 70, 80 and over 250 kDa (Figure 1B in Study I). These bands were identified through MALDI-TOF peptide mass analysis and MALDI TOF/TOF amino acid fragmentation analysis as Ku70, Ku80 and DNA-PK catalytic subunit (Table 1 in Study I).

To further confirm the pull-down and MALDI-TOF results and to demonstrate that the full-length AIRE protein is able to interact with DNA-PK, we conducted co-immunoprecipitation (Co-IP) experiments. Because endogenous AIRE is not expressed in immortalized cell lines, we decided to use HEK293 cells for transfection to study the AIRE protein function and its interacting partners for two reasons. First, the HEK293 cell line can be easily transfected (approximately 80–100%), and second, the subcellular localization of AIRE in HEK293 cells is similar to that in mTECs. For Co-IPs in HEK293 cells, we transiently transfected AIRE expression plasmid (pcAIRE) with Myc/His tag or the negative control vectors (pcDNA3.1B$^-$ Myc/His). The AIRE protein was co-immunoprecipitated with the anti-DNA-PK monoclonal antibody, but not with the control antibodies (i.e., anti-GAPDH, anti-HUR, and anti-GST) (Figure 4A; Figure 2A in Study I). We also performed the converse of this experiment: the Co-IP was performed using anti-AIRE 6.1 monoclonal antibody, and anti-DNA-PK antibody was used for protein detection via Western blot (Figure 2B in Study I). Both Co-IPs confirmed the specific interaction between AIRE and DNA-PK.

As DNA-PK forms a complex on chromatin DNA together with Ku proteins, the next question was whether the interaction of AIRE and DNA-PK is stabilized by DNA, chromatin or RNA. Previously, it was shown that the formation of DNA-PK complex is DNA dependent (Suwa et al., 1994). The protein complex formed between DNA-PK and Ku70/Ku80 is disrupted by ethidium bromide (EtBr), whereas this protein complex is stabilized by DNA (Suwa et al., 1994). EtBr strongly and specifically interacts with DNA (and double helical RNA), unwinds the DNA helix and hence increases the distance between adjacent base pairs

(Olmsed and Kearns 1977). Therefore, the DNA- and RNA-protein associations are selectively inhibited by EtBr during the Co-IP reactions (Lai and Herr 1992).

To analyze the chromatin-independent protein interactions, we used a micrococcal nuclease (MNase) treatment. MNase is an endonuclease that cleaves single- and double-stranded DNA and RNA without sequence specificity and has been widely used to disrupt chromatin interactions (Alexander et al., 1961). MNase preferentially cuts the linker DNA that connects two nucleosomes, resulting in mononucleosomes and oligonucleosomes. MNase treatment strongly changes DNA or chromatin conformation and abolishes chromatin-dependent protein interactions (Nguyen et al., 2006). Using EtBr and RNase A/T in co-immunoprecipitation assays, these treatments did not influence the AIRE and DNA-PK interaction (Figure 4B; Figure 2C in Study I) and showed that AIRE and DNA-PK interaction is direct and independent of the presence of DNA or RNA. In contrast, the MNase treatment clearly diminished the interaction between DNA-PK and AIRE, as demonstrated by a weaker band of AIRE via Western blot (Figure 4B; Figure 2C in Study I).

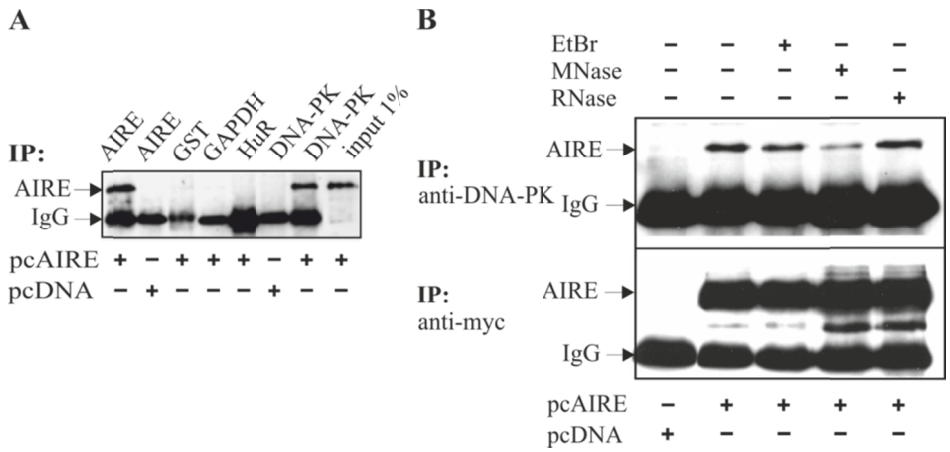

Figure 4. DNA-PK co-immunoprecipitates with AIRE. (A, B) The whole-cell extracts were prepared either from pcAIRE or control plasmid (pc) transfected HEK293 cells, and used in co-immunoprecipitations (Co-IPs). Western blots (A, B) were carried out with anti-AIRE antibody. (B) DNA-PK can interact with AIRE independently of DNA and RNA but interaction dependends on chromatin structure or chromatin binding proteins. Ethidium bromide (EtBr), micrococcal nuclease (MNase) and RNase A/T mix treatment were performed as described in section 4.9. Co-IPs were carried out with anti-DNA-PK and anti-Myc tag antibodies as indicated. Western blots (A, B) were performed with anti-AIRE 6.1 monoclonal antibody.

These observations showed that chromatin is required for the AIRE interaction with DNA-PK, which might be mediated by special chromatin conformation or by chromatin-interacting proteins that dissociate after the MNase treatment. To control the AIRE protein levels in these extracts, we used a total protein extract for Co-IPs

with anti-Myc tag antibody. The AIRE protein level was similar in all performed Co-IPs, thus validating our results (Figure 4B; Figure 2C in Study I). Collectively, these findings confirmed the AIRE protein interaction with DNA-PK.

To examine AIRE and DNA-PK colocalization in the cells by immunofluorescence assay, we transiently transfected HEK293 cells with pSI-AIRE expression vector for 24 h. As result, we could no detect any endogenous DNA-PK due to very low protein expression and therefore neither any colocalization between AIRE and DNA-PK (data not published). The next question was to investigate whether the subcellular location of AIRE is influenced by its interaction with DNA-PK. For this, we transiently transfected DNA-PK-negative MO59J and DNA-PK-positive MO59K cell lines with pcAIRE expression plasmid for 18 h and stained the cells with anti-AIRE monoclonal 6.1 antibody in an immunofluorescence assay. As observed by the immunofluorescence imaging of MO59K and MO59J cells, AIRE had a normal distribution of nuclear dots and fibrillar cytoplasmic structures in both cell lines (Figures 5**;** data not published). Thus, the lack of DNA-PK had no impact on the subcellular location of AIRE.

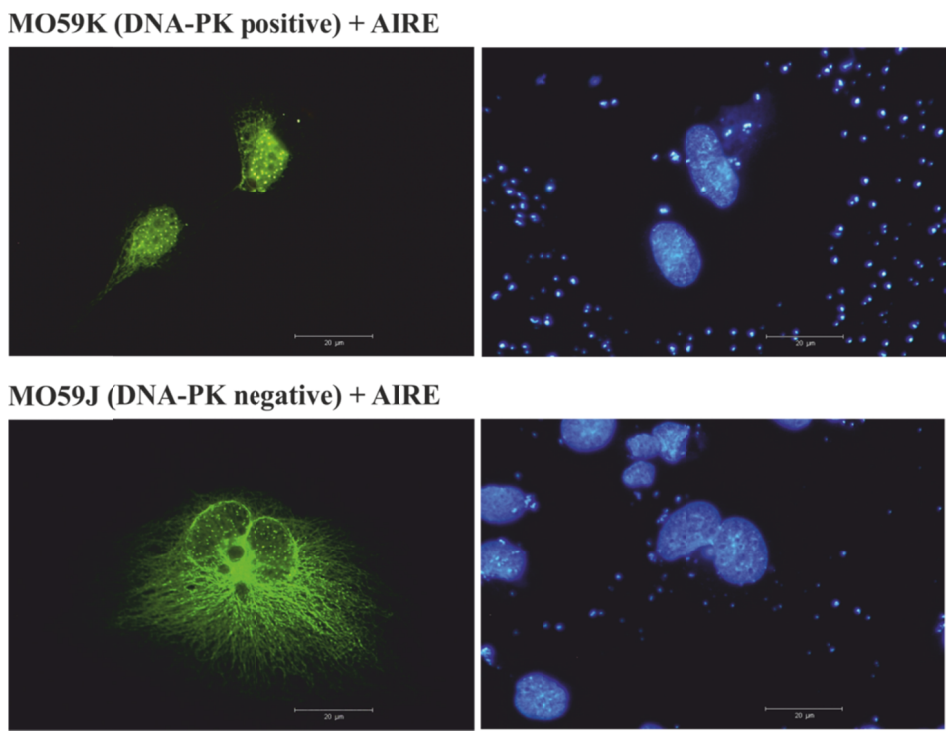

Figure 5. AIRE subcellular localization does not depend on DNA-PK. AIRE localizes in nuclear dots and cytoplasmic filaments in DNA-PK-positive MO59K and DNA-PK negative MO59J cell lines. The cells were cotransfected with pcAIRE expression vector and visualized by staining with anti-AIRE 6.1 monoclonal antibody (green). DAPI (blue) was used for nuclear counterstaining. 63x magnification.

5.2. DNA-PK phosphorylates AIRE protein

Previously, it was shown that bacterially expressed human AIRE is phosphorylated by cAMP-dependent PKA and PKC, but not by Abl protein kinase, casein kinases CK1 and CK2 (Kumar et al., 2001). Interestingly, AIRE phosphorylation was previously shown to trigger its dimerization (Kumar et al., 2001). To study whether DNA-PK phosphorylates AIRE, we used several different assays, which enabled us to measure the DNA-PK kinase activity. We first studied whether the AIRE protein co-immunoprecipitates the DNA-PK kinase activity. We transfected HEK293 cells with the plasmid expressing AIRE and performed Co-IPs with anti-DNA-PK, anti-Myc tag (for AIRE) and anti-GAPDH (negative control) antibodies. For positive and negative controls, we used immunoprecipitation with DNA-PK and GAPDH antibodies, respectively. The results showed that the anti-DNA-PK and anti-Myc tag (AIRE) antibody immunoprecipitated material contained DNA-PK kinase activity, as a similar amount of ^{32}P was incorporated into the p53 peptide from both immunoprecipitates (Figure 3A in Study I). As a negative control, we used GAPDH antibody-immunoprecipitated material, which had very low kinase activity. The positive control for the assay, the nuclear extract of HEK293, had a 2-fold higher kinase activity compared to the immunoprecipitates from AIRE and DNA-PK. With this experiment, we proved that both DNA-PK and AIRE immunoprecipitated material retained kinase activity and indicated that AIRE interacted with some p53 specific kinase, most likely with DNA-PK.

To study whether AIRE could be phosphorylated by DNA-PK, we conducted three different *in vitro* kinase assays in which purified GST fusion proteins (GST-AIRE and GST-p53) were used as the substrates of phosphokinase. The GST-p53 fusion protein was used as a positive control, and GST was used as a negative control. First, we performed the experiments using the nuclear extract from HEK293 cells as a source of kinases in the absence (Figure 6A) or presence of a specific inhibitor for DNA-PK, LY294002 (Figure 3B in Study I). LY294002 is a specific inhibitor of PI 3-family kinases and is a target of the ATP binding site in the enzyme active center (Vlahos et al., 1994). Second, we performed the kinase assays using the nuclear extracts from DNA-PK-positive (MO59K) and DNA-PK-negative (MO59J) cell lines (Figure 6A; Figure 3C in Study I). Third, we performed kinase reactions using a purified DNA-PK enzyme from HeLa cells (Promega) in the absence (Figure 6B) or presence of LY294002 (Figure 3D in Study I). We observed that the relative phosphorylation level of the AIRE protein reached up to 50–80%, compared to the positive control GST-p53 protein (Figures 3B, 3C and 3D in Study I). We also found that approximately 25–30% of the phosphorylation level of GST-AIRE protein was reduced when either DNA-PK inhibitor LY294002 or the DNA-PK-negative MO59J nuclear extract was used in the assay (Figures 3B and 3C in Study I). This result suggested that in addition to DNA-PK, other kinases may phosphorylate AIRE protein because the phosphorylation of AIRE was not totally inhibited by LY294002 and because the MO59J nuclear extract

yielded high (70%) kinase activity. Finally, to confirm that DNA-PK phosphorylates GST-AIRE, we used a purified DNA-PK enzyme in phosphorylation assays with or without the DNA-PK inhibitor LY294002 (Figure 6B; Figure 3D in Study I). As a result, DNA-PK was indeed able to phosphorylate AIRE as well as the positive control protein, GST-p53 (Figure 6B; Figure 3D in Study I).

To identify the AIRE protein region that is phosphorylated, we expressed different AIRE domains as GST fusion proteins and tested their phosphorylation using the HEK293 nuclear extracts as a source of kinases. The results showed that the HEK293 nuclear extract more efficiently phosphorylated the GST-fusions of the N-terminal AIRE containing the first 256 (1–256), 293 (1–293) or 348 (1–348) amino acids and that approximately two times less phosphorylation was observed with the GST-fusions of the AIRE C-terminal fragments containing SAND (175–298) or SAND-PHD1-PHD2 (178–482) domains (Figure 4B in Study I). A similar pattern of AIRE phosphorylation was observed when the phosphorylation assay was performed with DNA-PK-competent MO59K or DNA-PK-deficient MO59J cell extracts (Figure 4 C in Study I). These results showed that DNA-PK can phosphorylate AIRE *in vitro* and that the N-terminal region of AIRE is the predominant target of DNA-PK.

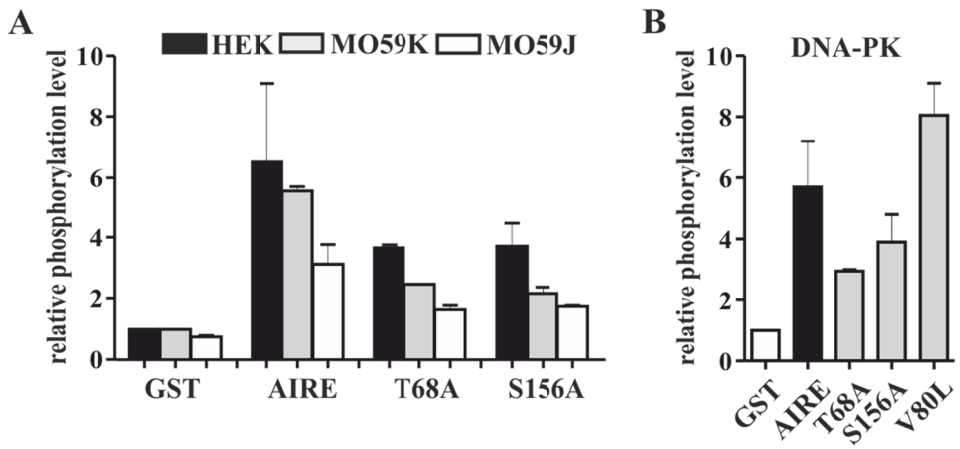

Figure 6. Amino acids T68 and S156 of AIRE are phosphorylated by DNA-PK. (A, B) The inhibitory effect of T68A and S156A mutations on AIRE phosphorylation efficiency. The kinase reactions were performed either by nuclear extracts prepared from HEK293, DNA-PK-positive MO59K or DNA-PK-negative MO59J cell lines or (B) by purified DNA-PK enzyme. (B) The GST-AIRE protein containing APECED mutation V80L in HSR/CARD domain was used as a control. (A, B) Phosphorylation levels are presented in relative values compared to phosphorylation level of GST (=1). The values (±SE) are means of two independent experiments.

5.3. DNA-PK phosphorylates AIRE at positions T68 and S156

Prior to the start of this work, the phosphorylation residues in AIRE had not been detected. We found two high scoring putative DNA-PK phosphorylation sites using the Scansite 2.0 software: threonine 68 (T68) and serine 156 (S156). To validate these sites, we used another program, NetPhos 2.0, which also predicted the same phosphorylation sites in AIRE. It should be mentioned that the computer programs also predicted lower scoring phosphorylation sites. The NetPhos 2.0 program predicted phosphorylation at 17 serines and 10 threonines. The Scansite 2.0 program predicted AIRE phosphorylation altogether at 19 sites.

To verify our high score predictions, we changed T68 and S156 sites in GST-AIRE to non-polar alanine (A) using PCR mutagenesis in wt GST-AIRE and truncated GST-AIRE (1–256) constructs and then tested the influence of these mutations on the phosphorylation efficiency. We used HEK293, MO59K and MO59J nuclear extracts as the sources of the kinases. The resulting phosphorylation levels of the T68A and S156A proteins were decreased by 50% compared to wt AIRE when the cell extracts from HEK293, MO59K or MO59J cells were used (Figure 6A; Figure 5B in Study I). The phosphorylation level of T68A and S156A mutants was reduced by up to 25% with nuclear extracts from DNA-PK-deficient MO59J cell line compared to the phosphorylation levels with DNA-PK-positive MO59K extract. Finally, we performed the phosphorylation assays using purified DNA-PK enzyme (Figure 6B; Figure 5C in Study I). In agreement with the previous experiments, a reduction of approximately 30–50% in the phosphorylation levels of the T68A and S156A mutants was detected with purified DNA-PK, whereas the APECED patient mutation V80L did not affect the AIRE phosphorylation level.

5.4. Inhibition of transcription by T68A and S156A AIRE mutants

Previously, it was demonstrated that several missense mutations in the HSR/CARD domain affected the cellular localization and transactivation ability of AIRE (Björses et al., 2000; Ramsey et al., 2002; Halonen et al., 2004; Ilmarinen et al., 2005). Thus, we tested whether T68A and S156A mutations in AIRE influence their transactivation activity. We first analyzed the wt AIRE transactivation ability of the *INV* and *LOR* gene promoters. For this, we cloned human *INV* and *LOR* promoter regions upstream of luciferase gene and performed luciferase assays to measure the transactivation activity of T68A and S156A AIRE mutants. We detected approximately 30–40 times higher luciferase activity with both promoters in AIRE-transfected cells compared to the AIRE-negative control cells (Figure 7; Figure 7A in Study I). Strikingly, the transactivation activity with T68A and S156A mutants was reduced by approximately 80–90% compared to the transactivation activity of wt AIRE. As a control for protein abundance, Western blot using the anti-AIRE antibody of lysates from the transfected cells showed that both mutant proteins are highly expressed (Figure 7B in Study I). To examine the effects of the T68A and S156A mutants on AIRE subcellular localization, we transfected HEK293 cells with pSI-AIRE, pSI-AIRE T68 and pSI-AIRE S156A plasmids for 24 h and immunostained with anti-AIRE 6.1 antibody. As result, we observed normal AIRE subcellular distribution with both mutants similar to wt AIRE (Figure 8; data not published).

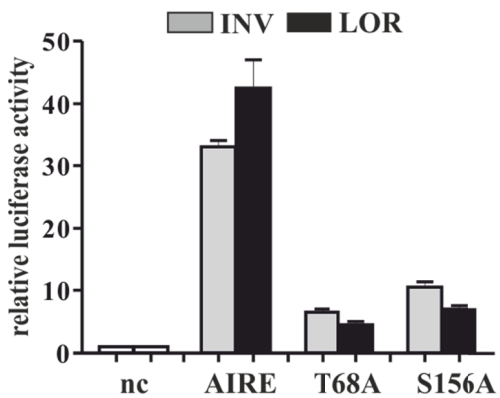

Figure 7. T68A and S156A mutations decrease transcriptional activity of AIRE. The HEK293 cells were transiently transfected with pSI (nc or negative control), pSI-AIRE (positive control), pSI-AIRE T68A, pSI-AIRE-S156A and either INV-pBL (involucrin) or LOR-pBL (loricrin) promoter reporter vectors with luciferase gene. Transcription activations are presented in relative values compared to luciferase activity of the control (=1). The values are means (±SE) of two independent experiments.

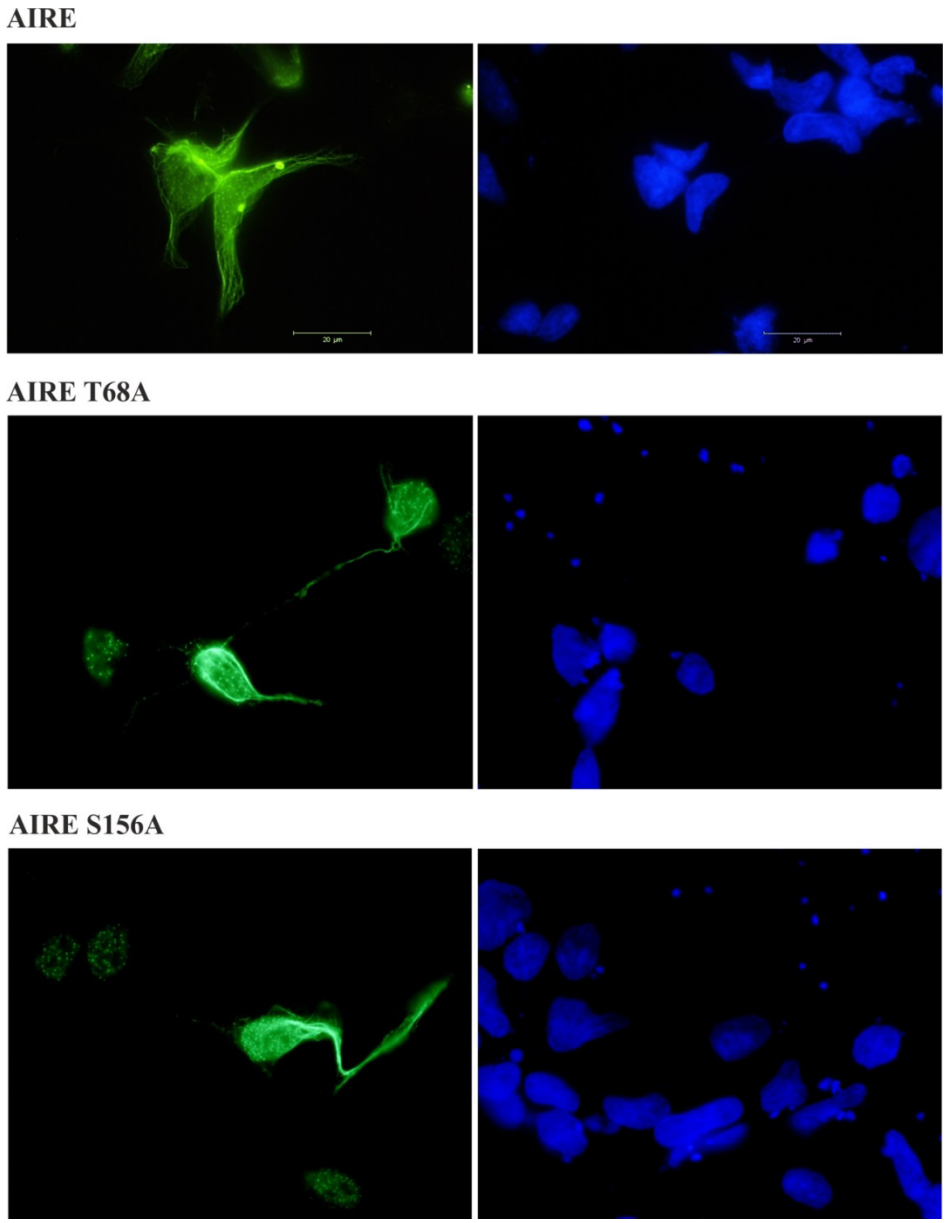

Figure 8. T68A and S156A AIRE mutations do not alter AIRE subcellular location. The HEK293 cells were cotransfected with pcAIRE, pcAIRE T68A or pcAIRE S156A expression vectors and visualized by staining with anti-AIRE 6.1 monoclonal antibody (green). DAPI (blue) was used for nuclear counterstaining. 63x magnification.

5.5. DNA-PK expression in mTECs and cTECs

DNA-PK is a ubiquitously expressed protein, of which the expression level is enhanced by the induction of double-strand breaks in DNA (Smith and Jackson 1999). To study whether the DNA-PK and AIRE interaction could occur *in vivo,* we analyzed the expression level of DNA-PK in Aire-positive mouse mTECs and Aire-negative cTECs. We separated mTEC and cTEC subpopulations by antibodies using accordingly the cell surface markers EpCAM and CDR1, as described previously (Kont et al., 2008). The expression of *Aire, DNA-PK, Inv,* and *Lor* genes was analyzed by real time RT-PCR using RNA from purified mTECs and cTECs. The *INV* and *LOR* genes are expressed during the terminal differentiation of skin epidermal keratinocytes (Henry et al., 2012), and also are AIRE target genes, which are downregulated in mTECs of Aire-KO mouse (Derbinski et al., 2005). For an endogenous control, the expression level of keratin 8 was used to normalize the relative mRNA levels of studied genes. The results showed that although DNA-PK mRNA was clearly expressed in both cell populations studied, there were no major differences in DNA-PK expression levels between mTECs and cTECs (Figure 6 in Study I). As characteristic to Aire-regulated genes in the thymus, the expression levels of *Inv* and *Lor* were relatively low, remarkable lower than those of *Aire* and *DNA-PK mRNA* expressions in mTEC subpopulation (Figure 6 in Study I).

5.6. Transactivation activity of APECED-associated AIRE mutants

The AIRE transcriptional activity was initially shown in assays using a reporter vector, which contained the *IFNB* minimal promoter upstream of the luciferase gene (Pitkänen et al., 2001).

Prior to the start of this study, APECED-associated AIRE missense mutations had not been studied using AIRE-dependent gene promoters, such as *INV* and *S1008A*. To analyze transcriptional activity of twelve APECED causing mutations in HSR/CARD domain (R15L, R15C, T16M, A21V, L28P, L29P, W78R, V80L, K83E, Y85C, Y90C, and Y93R), we used an AIRE-dependent *INV* promoter reporter construct with the luciferase gene. To analyze activity of L28P, L29P, and K83E mutants, we also used the full-length *INFB* promoter. For transactivation assays, we cotransfected promoter reporter vectors containing the *INV* or the full-length *INFB* promoter into HEK293 cells with wt AIRE or with corresponding HSR/CARD mutant protein expression vectors. We found that R15C, T16M, L28P, L29P, W78R, Y90C, and L93R mutants were completely inactive transcriptionally and that A21V, V80L, and Y85C were more active (10–78%) than wt AIRE (Figure 9; Table 1; Figure 4B in Study II; all data not published). Moreover, K83E or R15L mutants had moderate activity compared to wt AIRE on the *INV* promoter (Figure 9; Figure 4B in Study II), and K83E was fully active on the full-length *INFB* promoter (Figure 4B in Study II). Our results

were in agreement with other studies demonstrating transcriptionally inactive AIRE mutants L28P, L29P, W78R, Y90C, and L93R as well as studies with active AIRE mutants, such as A21V, V80L, K83E, and Y85C (Pitkänen et al., 2000, Halonen et al., 2004; Ilmarinen et al., 2005). Our result was different in transcription activation assays using T16M mutant from result by others, where the reporter construct with Adenoviral E1b minimal promoter and Cos-1 cells were used (Table 1; Halonen et al., 2004). It seems that transcription activation by AIRE also depends on the specific cell line and/or specific gene promoters.

To study the cooperative activity of CBP with AIRE HSR/CARD mutants, we analyzed L28P, L29P and K83E mutants. The acetyltransferase CBP collaboratively activates the AIRE-dependent transcription of a reporter construct containing GAL4 response elements and also elevates the transcription from the *INFB* minimal promoter up to a 3-fold in HEK293 cells (Pitkänen et al., 2005). In our experiments, co-expression of CBP increased AIRE activity on the *INV* promoter by up to a 2-fold and slightly increased the full-length *INFB* promoter activity in HEK293 cells (Figure 4B in Study II). CBP did not enhance the transcriptional activity of inactive L28P or L29P mutants on these promoters. Interestingly, K83E had full activity on the *INFB* promoter without CBP; however, with CBP, K83E transactivation activity did not enhance on both, the *INFB* and INV promoters (Figure 4B in Study II). These results showed that APECED-causing mutations can act differently, whereas CBP is not able to enhance the transcriptional activity of all AIRE mutants on *INV* and *INFB* promoters.

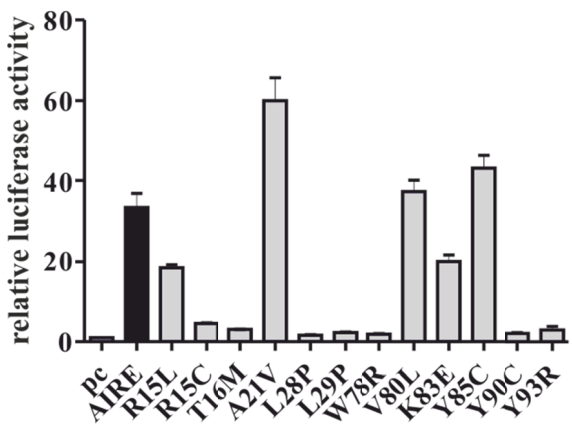

Figure 9. Transactivation activity of APECED-associated AIRE mutants located in the HSR/CARD domain. The HEK293 cells were transiently cotransfected with pc (negative control), pcAIRE (positive control), pcR15L, pcR15C, pcT16M, pcA21V, pcL28P, pcL29P, pcW78R, pcV80L, pcK83E, pcY85C, pcY90C, pcY93R plasmids and INV-pBL (involucrin) promoter reporter plasmid for 48 h. Transcription activations are presented in relative values compared to luciferase activity of the control (=1). The values (±SE) are means of parallel experiments of a representative sample of three independent experiments.

We also studied the transcriptional activation of endogenous promoters in HEK293 cells. Previous studies have demonstrated that wt AIRE activates the *INV* and *S100A8* genes in HEK293 cells (Org et al., 2009). We analyzed the activity of the transcriptionally inactive L28P, L29P and active (or moderately active) K83E AIRE proteins on the *INV* and *S1008A* promoters with and without CBP. We transfected HEK293 cells with L28, L29P, K83E, and wt AIRE expression plasmids and analyzed the relative mRNA levels of the *INV* and *S100A8* genes by real-time PCR. For an endogenous control, the expression level of HPRT was used to normalize the relative mRNA levels of studied genes. We found that AIRE activates transcription expression of the *INV* and *S1008A* promoters, 120- and 60-fold, respectively. Cotransfection with CBP further activated transcription by up to a 2-fold on both promoters (Figure 4B in Study I). The K83E AIRE mutant was able to activate transcription moderately, whereas L28P and L29P mutants did not exhibit activity on the endogenous promoters. CBP only weakly enhanced the activity of all studied mutants on both, *INV* and *S100A8* promoters.

In conclusion, our results showed that there is overall correlation between endogenous and exogenous promoters in transactivation studies. Our results confirmed that transcription from *INV* and *S100A8* genes is dependent on the AIRE protein, and we demonstrated that CBP has cooperative activity with AIRE at these two promoters.

5.7. AIRE induces apoptosis in epithelial cells

Several researchers have proposed the connection and induction of apoptosis by AIRE (Gray et al., 2007; Gillard et al., 2007; Dooley et al., 2008; Schaller et al., 2008; Meloni et al., 2010). To confirm this theory, we performed apoptosis assays using three different epithelial cell lines: AIRE-HT93, which stably expresses AIRE; a doxycycline AIRE-inducible AIRE-HEK/Tet cell line; and a 1C6 mouse thymic epithelial cell line that transiently expresses AIRE. In contrast to previous studies, we detected apoptosis with Annexin V-PE, which has a high affinity to cellular phospholipid phosphatidylserine. Phosphatidylserine translocates from the inner cell membrane to the outer plasma membrane during early apoptosis (Raynal et al., 1994). To separate late apoptotic cells, 7-AAD was used in assays with AIRE-HT93 and doxycycline-inducible AIRE-HEK/Tet cells. At late stages of apoptosis, 7-AAD intercalates dsDNA due to the disrupted nuclear membrane (Liu et al., 1991). For apoptosis assays with 1C6 cells, we used a transient cotransfection of AIRE-expressing plasmids. The transfection efficiency in 1C6 cells was 25–35%, according to the GFP signal obtained via flow cytometry (data not shown). As a note, we could not use 7-AAD to separate late apoptotic cells from early apoptotic cells in 1C6 cells because the GFP and 7-AAD emission spectra partially overlapped in the flow cytometer. Therefore, we analyzed the level of apoptosis only in GFP-positive cells.

As result, we detected a 2-fold increase of early apoptosis events in the AIRE-stable cell lines, AIRE-HT93 (Figure 5A in Study III) and AIRE-HEK/Tet, compared to AIRE-negative cells (Figure 10A; Figure 2B in Study IV). Moreover, higher levels of apoptosis were detected in 1C6 cells that were cotransfected with pcAIRE compared to the control cells (Figure 10B; Figure 1A in Study IV). Immortalized HT93 and HEK293 cell lines are more resistant to apoptosis, which might explain their lower apoptosis levels compared to transiently expressed AIRE protein in 1C6 cells. Moreover, we also analyzed the level of AIRE protein in 1C6 cells by Western blots after 5 h, 10 h, and 20 h transfections with pcAIRE plasmid. We detected a 40% decrease in AIRE protein level at 20 h post-transfection but not at 5 h and 10 h time points (Figure 1B in study IV). This result indicates the apoptosis of AIRE-positive cells.

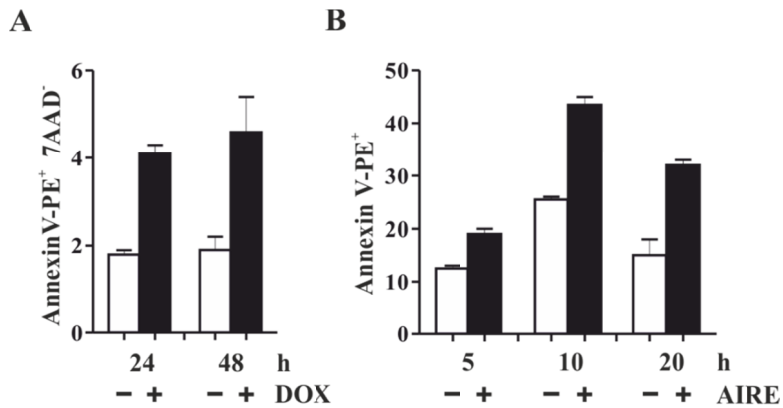

Figure 10. Expression of AIRE induces apoptosis in AIRE-HEK/Tet and in the thymic medullary epithelial 1C6 cell lines. (A, C) Overexpression of AIRE induces apoptosis in the AIRE-HEK/Tet cell line but not in AIRE-negative cell population. AIRE expression was induced with doxycycline (DOX), and apoptosis was measured 24 and 48 h later by flow cytometry of Annexin V-PE positive and 7-AAD negative cells. (B) 1C6 cells were cotransfected with pmaxGFP (negative control, NC) and pcAIRE expression vectors. Apoptosis was detected 5, 10 and 20 h later by flow cytometry of Annexin V-PE positive cells from the GFP-positive cell population. Data are the mean ± SEM of parallel experiments of a representative sample of four independent experiments.

We also studied AIRE-HT93 sensitivity to etoposide, the TOP2a inhibitor and inducer of apoptosis (Hande 1998). The experiments by Abramson and colleagues showed that AIRE and etoposide, but not hydrogen peroxide, had a similar effect on the transcription activation of AIRE-dependent genes such as *S100A8* and *KRT14*; however, these actions were not synergistic (Abramson et al., 2010). Our results demonstrated that etoposide strongly induced apoptosis in AIRE-HT93 cells by up to a 4-fold higher than the level of apoptosis in AIRE-HT93 cells without etoposide (Figure 5B in Study III). Moreover, the effect of

etoposide on apoptosis was a 3-fold lower in AIRE-negative HT93 or control cells compared to AIRE-positive cells. These results indicated a synergistic effect of AIRE and etoposide in the induction of apoptosis. Moreover, we confirmed the results of Gray et al., which indicated that AIRE induces apoptosis in 1C6 cells (Gray et al., 2007).

Because the N-terminal domain in AIRE is similar to several apoptotic proteins with a CARD domain, we cloned the first 1–143 amino acids of AIRE, which contains HSR/CARD, a two LXXLL nuclear receptor interacting motifs and the nuclear localization sequence. Transfection into 1C6 cells showed that the N-terminal 1–143 amino acid sequence of AIRE is sufficient to induce apoptosis at levels similar to wt AIRE protein (Figure 1C in Study IV).

Finally, we studied whether the expression of AIRE may alter the cell cycle or cell proliferation. Previously, it was shown that AIRE does not change cell proliferation in the 1C6 cells and in mouse mTECs ($Aire^{+}CD80^{high}$) (Gray et al., 2007). To analyze cell proliferation of the doxycycline-inducible AIRE-HEK/Tet cells, we also performed a 5-bromo-2-deoxyuridine (BrdU) incorporation assay. BrdU is is a synthetic nucleoside, a structural analogue of thymidine, which incorporates into DNA during the replication or S-phase of the cell cycle. BrdU is commonly used for cell proliferation assays and for the detection of apoptotic cells (Lehner et al., 2011). We measured the BrdU level by flow cytometry 48 h and 72 h after the induction of AIRE expression with doxycycline. As result, we did not observe any resultant changes in the proportion of the BrdU-labeled cell population between AIRE-positive and AIRE-negative HEK/Tet cells (Figure 2C in Study IV), suggesting that AIRE does not change the rate of cell proliferation.

5.8. AIRE induces GAPDH translocation to the nucleus

GAPDH translocation into the nuclei has been induced by various apoptotic stimuli such as hyperglycemia, hypoxia, and oxidative, nitric and genotoxic stress in the different cell types (Graven et al., 1994; Dastoor et al., 2001; Chuang et al., 2005; Brown et.al., 2003; Hara et al., 2005). Moreover, GAPDH increase is observed in differentiated rat cerebellar neurons at an early stage of apoptosis in the cell culture condition (Ishitani et al., 1997 and 1998).

To explore whether AIRE-induced apoptosis might cause GAPDH translocation into nuclei, we studied the doxycycline inducible AIRE-HEK/Tet cell line and 1C6 mTEC cells that transiently express AIRE. For that, we used two different methods: immunofluorescence staining of cells and Western blotting of nuclear and cytoplasmic extracts. Using immunofluorescence with GAPDH antibodies, we found that the majority of AIRE-positive HEK293 cells had nuclear staining of GAPDH, whereas the AIRE-negative control cells did not (Figure 3A in Study IV). GAPDH accumulation to nuclei has been described to be a result of the genotoxic stress agents such as etoposide (Hara et al., 2005). Therefore, we used an etoposide for positive control to induce GAPDH nuclear translocation. Via immunofluorescence staining, we again observed that

GAPDH localized to the nuclei of AIRE-positive cells after the transfection of AIRE into 1C6 mTECs (Figure 3B in Study IV).

To confirm the GAPDH nuclear location, we did subcellular fractionations from doxycycline-inducible AIRE-HEK/Tet cells. We detected the nuclear GAPDH in the doxycycline induced AIRE-positive cells and in etoposide treated cells, but not in control cells (Figure 11A; Figure 3C in Study IV).

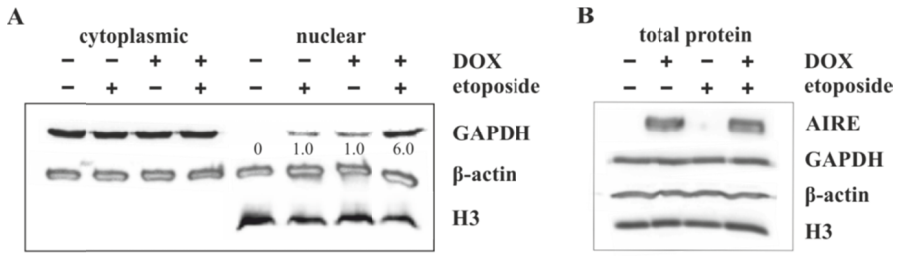

Figure 11. AIRE-induced apoptosis is associated with nuclear accumulation of GAPDH. (A) Doxycycline-inducible (DOX) AIRE-HEK/Tet cells were treated for 24 h with DOX and/or etoposide followed by Western blot of cytoplasmic and nuclear protein lysates for GAPDH, β-actin and histone H3. DOX (AIRE) alone induced nuclear accumulation of GAPDH, which was futher increased by etoposide, while the levels of cytoplasmic GAPDH remained unchanged. (B) An experiment was performed as in (A) except that the total cell extract of AIRE, GAPDH, β-actin and histone H3 proteins were analyzed by Western blot.

Moreover, we detected more nuclear GAPDH when we co-treated cells with etoposide and doxycycline to induce AIRE expression (Figure 11A; Figure 3C in Study IV). At the same time, the GAPDH levels in all cytoplasmic fractions remained similar (Figure 11A; Figure 3C in Study IV). Also, the GAPDH levels were similar in all unfractionated or in total protein extracts of cells from AIRE-negative and -positive, etoposide treated or co-treated with doxycycline (Figure 11B; Figure 3D in Study IV). For protein loading controls we used β-actin and histone H3 proteins to compare the amounts of cytoplasmic, nuclear and whole cell extract or total proteins in Western blots.

To see whether the nuclear accumulation of GAPDH correlates with its higher transcript levels, we used real-time PCR using β-actin as a house-keeping gene. The GAPDH mRNA levels were similar in each of the groups: the negative control cells, AIRE-expressing cells, etoposide treated cells and AIRE-expressing and etoposide-treated cells (Figure 3E in Study IV). These results were in agreement with studies on the AIRE-HEK293 cell line in which AIRE expression did not change the GAPDH mRNA expression level (Org et al., 2008).

Because of R-(-)deprenyl has an inhibitory effect on GAPDH-associated apoptosis and prevents its nitrosylation and translocation to nuclei (Kragten et

al., 1998; Hara et al., 2006), we analyzed the effect of R-(-) deprenyl on AIRE-induced apoptosis. R-(-) deprenyl inhibited approximately 25–30% of the AIRE-induced apoptosis in both AIRE-HEK/Tet cells (Figure 4A in Study IV) and the GFP-positive cell subset of AIRE-transfected 1C6 cells (Figure 4B in Study IV). Altogether, these studies showed that AIRE-induced apoptosis causes GAPDH translocation to the nucleus.

5.9. GAPDH nuclear localization in thymus

To study the possible GAPDH translocation to nuclei *in vivo*, we performed immunofluorescence microscopy with human thymus sections. Thymus medulla (Figure 12), as well as the cortical region (data not shown), had many cells with nuclear accumulation of GAPDH (data not published). GAPDH staining in thymus was similar to the immunofluorescence staining of the mouse brain neocortex, where many cells with GAPDH nuclear localization were observed (Chuang et al., 2005). Approximately 60% of AIRE-positive cells had an accumulation of GAPDH in their nuclei. In addition, a small number of AIRE-negative cells in thymus medulla also had nuclear GAPDH staining. Nuclear GAPDH staining in AIRE-positive cells might indicate a cell population in an early stage of apoptosis. It should be noted that GAPDH and AIRE do not interact with each other, as revealed by Co-IP studies (Figure 4A).

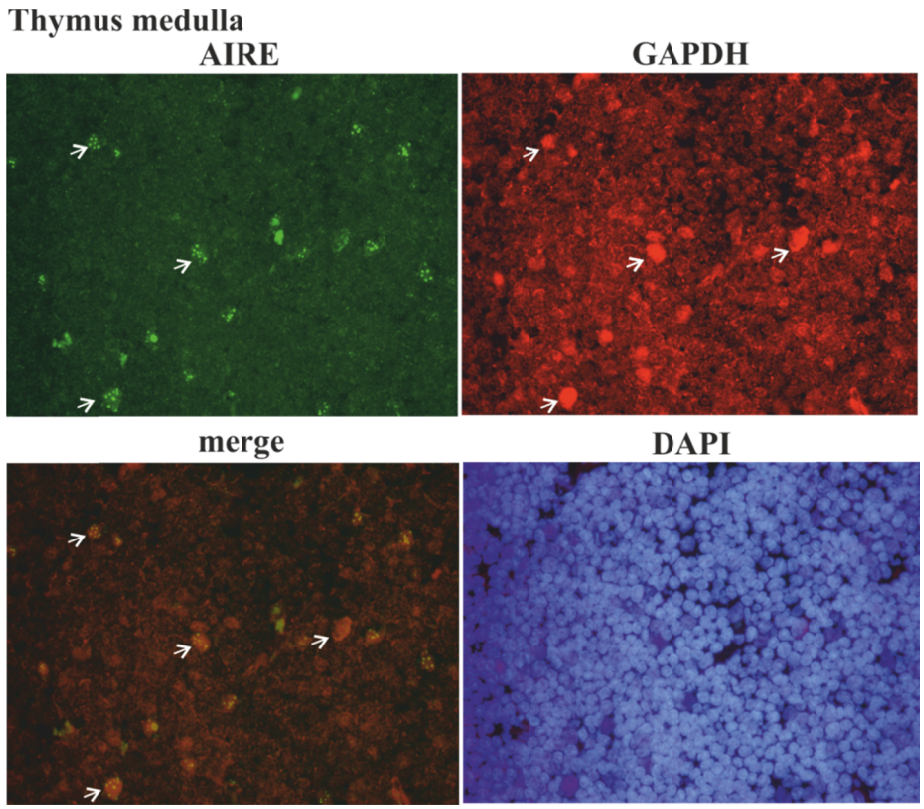

Figure 12. Nuclear localization of GAPDH in AIRE-positive mTECs. Thymus from 8-months-old child was stained with anti-AIRE 6.1 monoclonal (green) and anti-GAPDH polyclonal antibodies (red). Merged panel indicates colocalization between AIRE and GAPDH. The white arrows demonstrate some of AIRE-positive cells with nuclear GAPDH. 40x magnification.

6. DISCUSSION

Before the start of this thesis, only one AIRE interacting protein, a transcption activator CBP, was reported (Pitkänen et al., 2000). Therefore, the aim of this thesis was to identify novel AIRE partners using its PHD1 domain as bait. Using GST pull-down and subsequent co-immunoprecipitation experiments, we found that AIRE interacts with the DNA-PK/Ku70/Ku80 protein complex. Later, this finding was confirmed by other reports (Abramson et al., 2010; Gaetani et al., 2012). DNA-PK is a serine/threonine kinase with DNA-dependent activity (Smith and Jackson, 1999). Its main function is to recognize the dsDNA breaks and to catalyze a repair process known as non-homologous end joining. DNA-PK has been shown to phosphorylate proteins most effectively when it is bound to the same DNA molecule (Gottlieb and Jackson 1993), indicating that part of the activation caused by DNA is through the juxtaposition of DNA-PK and its target (Gottlieb and Jackson 1993). However, DNA also stimulates DNA-PK to phosphorylate non-DNA-binding peptide substrates, implying that binding to DNA must directly or indirectly induce an activating conformational change of DNA-PK (Smith and Jackson 1999). Whether there is a direct interaction of AIRE with DNA (Kumar et al., 2001) remains controversial; however, the interaction of AIRE with several DNA-binding proteins has now been shown (Abramson et al., 2010; Gaetani et al., 2012). Remarkably, these proteins are also DNA-PK target molecules, such as RNA Pol II, PARP1, γH2AX and TOP2a. Furthermore, all of these proteins have important roles in mRNA transcription.

We found that AIRE can bind DNA-PK without the presence of DNA and RNA, whereas this interaction is influenced by the chromatin structure or chromatin-bound proteins. At least two possibilities can be considered for how DNA-PK, AIRE and chromatin interact. First, chromatin or, for example, specifically modified chromatin may increase the affinity of AIRE for DNA-PK. Secondly, post-translationally modified AIRE protein may interact with DNA-PK in certain chromatin regions. It should be noted that because we could not exclude the influence of AIRE overexpression in our experimental system, further studies are needed to clarify the role of chromatin in AIRE and DNA-PK interactions *in vivo* and particularly in the thymic environment.

Post-transcriptional protein modifications, such as phosphorylation, glycosylation, acetylation, polyubiquitination, and sumoylation regulate the activity of transcription factors and their subcellular localization (Duprez et al., 1999; Ji et al., 2007; Zhang and Wang 2008; Abdel-Hafiz and Horwitz 2013). So far, we have a little information about AIRE post-translational modifications. The phosphorylation of AIRE by the cAMP-dependent protein kinase A and C has been reported previously (Kumar et al., 2001), though the phosphorylated amino acids of AIRE remained unidentified. AIRE dimerization was suggested to occur as a result of phosphorylation (Kumar et al., 2001).

Recently, the acetylation of the AIRE protein at NLS and SAND domain by p300 and CBP as acetyltransferases was reported (Saare et al., 2012). AIRE

acetylation at K243 and K253 was important for the activation of AIRE-dependent *INV* and *S1008A* gene promoters. Overexpression of p300 stabilized AIRE protein but did not enhance the transcriptional activation of AIRE-regulated genes (Saare et al., 2012). It remains to be investigated whether acetylation influences other functions of AIRE.

The results of this thesis show that the AIRE protein is phosphorylated by DNA-PK at two distinct residues, threonine 68 (T68) and serine 156 (S156), which are not APECED-related mutations in AIRE. Because both these phosphorylation sites are located outside the PHD1 domain, it indicates that multiple different contacts can occur between AIRE and DNA-PK. However, our *in vitro* phosphorylation studies also indicated that other kinases might phosphorylate AIRE protein.

The HSR/CARD domain of AIRE has been shown to be responsible for the dimerization and tetramerization of the protein (Pitkänen et al., 2000; Kumar et al., 2001). Many, though not all, APECED mutations within the HSR/CARD decrease AIRE transactivation activity and change its cellular localization (Halonen et al., 2004; Pitkänen et al., 2005). In this study, we demonstrated that AIRE activates involucrine (*INV*) and loricrine (*LOR*) gene promoters in transactivation assays. Moreover, we found that mutations of the amino acids T68 and S156 to alanines (T68A and S156A) strongly suppressed the AIRE transactivation of *INV* and *LOR* promoters but did not disturb the subcellular distribution. We proposed that AIRE phosphorylation at T68 and S156 residues by DNA-PK is important for AIRE transactivation activity. Recent studies have shown that AIRE transactivation activity is not dependent on AIRE phosphorylation by DNA-PK because the inhibition of DNA-PK catalytic activity by inhibitor Nu7441 did not abolish AIRE transactivity on the keratin 14 gene promoter (Žumer et al., 2012). According to the study by Žumer and colleagues, AIRE transactivation depends on the cooperative interaction between DNA-PK and H3K4me0, thus enabling AIRE recruitment to the keratin 14 gene promoter. These results suggest that transcriptional inactivation by T68A and S156A mutants is independent of phosphorylation by DNA-PK; instead, these mutations may abolish the interactions between AIRE and DNA-PK or between AIRE and H3K4me0. It is conceivable that AIRE T68 and S156 positions are important for interactions with other protein partners. The mutations of T68A and S156A in AIRE may change the protein structure, which may result in a low level of transcriptional activation. Moreover, we could not exclude that other protein kinases might phosphorylate T68 and S156 sites in AIRE that are important for transcription or for other AIRE functions.

Surprisingly, AIRE and its PHD1 D297A mutant activated the insulin promoter on an episomal from non-replicating plasmid target lacking histones (Žumer et al., 2012). The study indicated that AIRE does not need direct interaction with histone H3 to induce the gene expression of the plasmid reporter system, but it instead needs interaction with DNA-PK (Žumer et al., 2012). Furthermore, the D297A and V301M, which are the APECED-related mutations in PHD1, strongly attenuated the interactions of AIRE with

chromatin-associated proteins, such as DNA-PK, RuvB1/2, and GCN1 (Gaetani et al., 2012). Thus, these residues in AIRE PHD1 also might be important for interaction with DNA-PK.

The impact of DNA-PK on AIRE-dependent gene expressions was proven in different studies. For example, Abramson et al. demonstrated the importance of DNA-PK in mouse mTECs. They found a significant down-regulation of AIRE-dependent genes *S1008A, Spt1*, *Mup4*, and *Pcp4* in DNA-PK gene-deficient SCID mice (Abramson et al., 2010), showing that DNA-PK controls the expression of AIRE-dependent genes or tissue specific antigens *in vivo*. Moreover, Ku70/Ku80 (Mo et al., 2002) and DNA-PK associate with RNA Pol II elongation sites (Tyagi et al., 2011) as does AIRE (Žumer et al., 2011; Giraud et al., 2012). Recently, the role of DNA-PK in transcriptional regulation was demonstrated by its enzymatic activation and higher mRNA expression in response to ionizing radiation, which causes dsDNA breaks (Kress et al., 2008; Dhariwala et al., 2012). Although, we did not detect differences in mRNA expression levels of DNA-PK in Aire-positive mTECs and Aire-negative cTECs, DNA-PK might associate with increased numbers of dsDNA breaks in mTECs during the negative selection of T lymphocytes and in cTECs during the positive selection of T lymphocytes. This hypothesis is supported by the finding that wt mTEC ($CD80^{high}$) population has 3–4 times more dsDNA breaks than Aire-KO mice when assessed by γH2AX (Abramson et al., 2010), which is associated with dsDNA breaks and genomic instability (Mah et al., 2011). The expression of DNA-PK mRNA in cTECs might be needed for the expression of autoantigens in cTECs during the positive selection of T lymphocytes. Whether the gene expression in thymic epithelial cells is directly correlated with the activation of DNA-PK or other possible AIRE-specific protein kinases, requires further study.

Two-thirds of all APECED-associated missense mutations are clustered in the AIRE HSR/CARD domain (Halonen et al., 2004). We found (in collaboration with Dr. Rich, University of Cambridge, UK) that the HSR domain is similar to the caspase recruitment domain or CARD by the structure-based sequence alignment between apoptotic proteins, such as Apaf-1, caspase 9, ICEBERG, and RAIDD. As is characteristic of CARD domains in other proteins, AIRE HSR/CARD has a polarized distribution of surface charges, which is required for homotypic CARD-CARD interactions. Future work is needed to identify the protein partners of AIRE CARD domain.

We also noted a correlation between the AIRE HSR/CARD structure and the transcriptional activity of HSR/CARD domain mutants. The conserved hydrophobic amino acid residues that are located in the inner core of a helix (e.g., L28, L29, Y90 or L93) lead to the inactivation of transcription from the *INV*, *S1008A*, and *INFB* gene promoters. In contrast, mutations affecting surface amino acids, such as V80, K83 and Y85, did not influence the transcriptional activity. The protein structure of L28P and L29P mutants indicated the destabilization of the HSR/CARD domain, which is in accordance with their lower protein stability and transcriptional inactivity in experiments. In contrast,

transcriptionally active (or semi-active) K83E mutation was predicted to change the distribution of charge on the HSR/CARD domain and not to disrupt the overall fold. Our findings shed light on the direct connections between the mutated AIRE proteins changed structure and transcriptional activity as a probable cause of APECED. It remains unclear what AIRE functions are destroyed by the APECED mutations that were active in the transactivation assays. For example, a controversial result was obtained for the Y85C mutant, which, in experiments performed by us and others, showed 115% of the wt AIRE transcriptional activation activity (Björses et al., 2000), whereas the mutant protein appeared unstable with a short half-life (1.5 h for the mutant versus 15–20 h for wt AIRE; Ramsey et al., 2002). In addition, discordant results with Y85C mutant have been in the altered subcellular localization (Björses et al., 2000; Halonen et al., 2004; Ilmarinen et al., 2005). It is probable that all mutations in HSR/CARD domain (and other domains) in APECED patients change the charge distribution on the domain surface and disrupt protein-protein interactions between AIRE and its interaction partners important for normal protein functions. Unfortunately no clear genotype-phenotype associations have been detected for APECED patients.

The induction of apoptosis by AIRE has also been proposed by others (Gray et al., 2007; Schaller et al., 2008; Dooley et al., 2008). Our study confirmed that AIRE induces apoptosis in three different epithelial cell lines: 1C6 mTECs, AIRE-HT93 and doxycycline-inducible AIRE-HEK/Tet cells. In particular, we found that apoptosis is associated with the HSR/CARD domain in AIRE. In thymus, the apoptosis of AIRE-positive mTECs might enhance self-peptide presentation and cross-presentation of antigens by dendritic cells to developing thymocytes (Aschenbrennner et al., 2007; Millet et al., 2008; Hubert et al., 2011). AIRE expression induced cell death in the mouse 1C6 mTECs cell line (Gray et al., 2007) and in mouse mTECs (Gray et al., 2007; Dooley et al., 2008), whereas the Aire-positive medullary epithelial cell subset was almost postmitotic (Gray et al., 2007).

DNA fragmentation and the activation of caspases accompanying cell apoptosis were also increased in wt Aire mTECs ($K8^{+}K14^{+}$) compared to Aire-KO mouse mTECs ($K8^{+}K14^{-}$) (Dooley et al., 2008). AIRE interaction with cellular stress and apoptosis-related HSP70 family proteins and ubiquitin (Gaetani et al., 2012), and with DAXX has been reported (Meloni et al., 2010). DAXX has a death domain and can enhance FAS-mediated apoptosis (Meloni et al., 2010). It is also a transcriptional regulator, a SUMO-dependent transcription repressor (Lin et al., 2006). DAXX localizes to PML-containing nuclear bodies (Ishov et al., 1999), which are also considered sensors of DNA damage and cellular stress (Dellaire et al., 2004). Several PML-body locating proteins (p53, CBP, Sp100, GAPDH, and caspase 2) have been shown to be associated with apoptosis, transcriptional regulation and antiviral responses (Carlile et al., 1998; Regard and Chelbi-Alix 2001; Bernardi and Pandolfi 2003; Kurki et al., 2003; Dellaire et al., 2004; Sanchez-Pulido et al., 2007). In contrast, AIRE colocalizes not into PML bodies but into separate nuclear dots,

where it colocalizes with CBP (Pitkänen et al., 2005). Nevertheless, AIRE may mediate its apoptotic effect by interacting with the apoptotic protein DAXX, which also exerts a strong repressive transcriptional activity of AIRE (Meloni et al., 2010).

AIRE-induced apoptotic signals may originate from genotoxic or oxidative stress. Because AIRE interacts with proteins that are involved in dsDNA breaks, such as DNA-PK, TOP2a and γH2AX (Abramson et al., 2010), it might indicate the high level of DNA breaks during active transcription in mTECs. Alternatively, aberrant protein folding or protein oxidization, as a result of active tissue specific antigens expressions, may cause oxidative or cytoplasmic stress in the cell. In collaboration with Dr. Alvarez (Autonomous University of Barcelona), we studied the effect of AIRE on the cellular proteome in a stably transfected AIRE cell line (AIRE-HT93). The stably transfected AIRE-positive cells had increased levels of cytosolic stress proteins, such as HSP70, HSP27, TBCA, and SOD, indicating cellular stress. On the other hand, the anti-apoptotic protein Bcl-1 and the various cytoskeleton interacting proteins such as transgelin, caldesmon, tropomyosin alpha-1 chain and myosin 9 were decreased in AIRE-HT93 cells.

The housekeeping protein GAPDH is the sensor of nitric oxide (NO), cellular stress and apoptosis (Sawa et al., 1997; Dastoor et al., 2001; Hara et al., 2005; Hara et al., 2006; Sirover et al., 2012). Different apoptotic stimuli result in GAPDH translocation and accumulation to the nucleus due to its S-nitro-sylation by endogenous NO (Hara et al., 2005), and this event abolishes GAPDH glycolytic activity (Hara et al., 2005). In thymus, epithelial and dendritic cells in medulla and in corticomedullary junctions produce NO by inducible nitric oxide synthase in deletion of TCR-stimulated $CD4^{+}CD8^{+}$ double-positive thymocytes (Tai et al., 1997; Moulian et al., 2001). The nuclear accumulation of GAPDH has been reported in neurons (Morgenegg et al., 1986) and in non-neuronal cell lines such as HEK293, HeLa, and S49 (Sawa et al., 1997; Sirover 2012). In this study, we detected GAPDH accumulation in the AIRE-positive nuclei in AIRE-HEK/Tet and in AIRE-transfected 1C6 mTEC cell lines, and in the AIRE-expressing mTECs in human thymus medulla. In addition, we found that many cTECs in the thymus cortex, which are AIRE and EpCAM negative cells, were strongly nuclear GAPDH-positive. We found diminished AIRE-induced apoptosis by GAPDH specific inhibitor R-(-)-deprenyl, which inhibits the apoptosis through the blocking of GAPDH S-nitrosylation and its translocation to the nucleus (Hara et al., 2006). Altogether, our results underline the role of AIRE in apoptosis and its connection with NO-induced cellular stress.

Further studies are needed to confirm the cellular stress or apoptotic cell death processes in AIRE-positive mTECs.

7. CONCLUSIONS

The following conclusions can be drawn:

1. We showed that AIRE PHD1 domain interacts with the nuclear protein complex of DNA-PK/Ku70/Ku80. We found that DNA-PK phosphorylates AIRE at positions threonine 68 (T68) and serine 156 (S156) and that both residues in AIRE are important for its transcriptional activity.

2. We analyzed the transcriptional activity of APECED mutations in HSR/CARD domain using transactivation assay with *INV*, *IFNB* and *S100A8* gene promoters and HEK293 cells. We found that seven mutations (R15C, T16M, L28P, L29P, W78R Y90C and L93R) were transcriptionally inactive, two mutations (K83E and R15L) had moderate activity and three mutations (A21V, V80L, and Y85C) had even higher activity than the wt AIRE with *INV* promoter. The transcriptional activation of the *IFNB* and *S100A8* gene promoters detected only with K83E mutant but not with L28P and L29P mutants.

3. We found that the common activator CBP cooperatively enhanced the transcriptional activity of wt AIRE using transactivation assay with *IFNB*, *INV* and *S100A8* gene promoters. CBP did not enhanced transcription with L28P and L29P AIRE mutants on the exogenous *INV* and *IFNB* and neither on the endogenous *INV* and *S1008A* gene promoters in HEK293 cells. CBP enchanced transcriptional activity of K83E mutant only on the endogenous *INV* and *S100A8* gene promoters.

4. We confirmed that AIRE is able to induce apoptosis in epithelial cells. HSR/CARD domain in AIRE was found sufficient to induce apoptosis.

5. We found that the nuclear translocation of GAPDH is associated with AIRE-induced apoptosis in epithelial cell lines. In human thymus, GAPDH nuclear localization was also observed in the AIRE-expressing mTECs.

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SUMMARY IN ESTONIAN

Autoimmunregulaator valgu interaktsioon DNA-sõltuva proteiinkinaasiga ja seos apoptoosiga

Autoimmunregulaator valk (AIRE) on oluline tsentraalse ja perifeerse immuuntolerantsuse kujunemisel. Mutatsioonid *AIRE* geenis põhjustavad APECED sündroomi (*Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy*), mis on pärilik monogeenne autosoom-retsessiivne haigus. Seda sündroomi iseloomustab mitme erineva autoimmuunhaiguse koos esinemine (peamiselt endokriinorganites), millega kaasneb enamasti krooniline kandidoos.

AIRE valk ekspresseerub spetsiifiliselt tüümuse medulla ehk harkelundi säsi epiteelirakkudes (mTEC), kuid ka perifeersete immuunorganite põrna ja lümfisõlmede epiteeli- ja dentriitrakkudes. AIRE valk on transkriptsioonifaktor, mis reguleerib geene klastrite kaupa. Siiani on kirjeldatud ligikaudu kaks tuhat koespetsiifilist geeni, mille ekspressioone AIRE reguleerib. AIRE seondub geenide promootoraladel vaid inaktiivse kromatiini modifikatsiooni histoon H3-ga, milleks on metüleerimata neljas lüsiinijääk (H3K4me0). Sellist laiaulatuslikku geenide avaldumist on nimetatud "avatud" geeniekspressiooniks. Paraku AIRE ei reguleeri kõiki geene nii mTEC-s kui ka perifeersetes immuunorganites, mis viitab teiste, veel senikirjeldamata transkriptsioonivalkude rollile immuntolerantsuse kujunemisel. Autoantigeenide ekspressioon mTEC-s on vajalik küpsevate T lümfotsüütide "koolitamiseks", mis tagab tsentraalne immuuntolerantsuse organismi enda antigeenide suhtes. Selle protsessi ajal esitletakse autoantigeenide peptiidid epiteelraku membraanile suure koesobivuskompleksi (MHC) molekulide kaudu, et neid esitleda T lümfotsüütide retseptoritele. T rakkude selektsioon tüümuses toimub kahes etapis. Esimesena, tüümuse koore osas surevad apoptoosi kaudu positiivse selektsiooni käigus peptiididele liiga nõrga afiinsusega seondunud T rakud, kus peptiide esitlevad T rakkudele tüümuse koore epiteelrakud (cTEC). Seejärel läbivad ellujäänud T rakud negatiivse selektsiooni tüümuse medullas, kus surevad apoptoosi kaudu liiga tugeva afiinsusega peptiididele seondunud T rakud ehk autoreaktiivsed rakud. Mõlema selektsiooni käigus sureb tüümuses ligikaudu 95–98% T rakke. Kuna *AIRE* geeni mutatsioonide korral on AIRE valgu funktsioon häiritud, siis ei toimu AIRE-st sõltuvate autoantigeenide ekspressiooni, mis tingib autoreaktiivsete ehk "koolitamata" T rakkude teket ja autoimmuunhaiguste kujunemist. Samuti *AIRE*-defektsetes hiirtes tekivad ulatuslikud autoimmuunsed organite kahjustused.

Lisaks T rakkude selektsioonile vastutab AIRE mitmete kemokiinide ekspressiooni eest. Kemokiinid on vajalikud küpsevate T rakkude migratsiooniks läbi tüümuse nende selektsiooni ajal ja väljumisel tüümusest perifeeriasse. Samuti on AIRE valk oluline tüümuse arenguks ja mTEC-de diferentseerumiseks.

AIRE valgul on transkriptsioonifaktoritele iseloomulikud domäänid, milleks on HSR (oligomerisatsiooni vahendav ala), SAND (oletatav DNA seonduv ala), tsink-sõrmed (PHD1 ja PHD2) ja proliinirikas motiiv (PRR). AIRE-l on

signaaljärjestused nii impordiks tuuma kui tuumaekspordiks. AIRE lokaliseerub rakkude tuumas tuumakehadena sarnaselt paljudele transkriptsiooni regulaatorvalkudele. Tsütoplasmas AIRE lokaliseerub tsütoplasma filamentidel, kus ta on seondunud vimentiini ja γ-tubuliiniga. Kõik AIRE valgu struktuursed domäänid on olulised transkriptsiooniliseks aktiivsuseks. Tema õigeks rakusiseseks lokalisatsiooniks on samuti vajalikud kõik AIRE domäänid, kuid mitte PHD2 domään. Enamik APECED-i põhjustavaid mutatsioone *AIRE* geenis muudavad valgu lokalisatsiooni rakus ja inaktiveerivad AIRE transkriptsioonilist aktiivsust. Hiljutised avastused on kirjeldanud AIRE tähtsust transkriptsiooni elongatsioonil ja pre-mRNA splaissingul lisaks transkriptsiooni initsiatsioonile.

Antud doktoritöö eesmärgiks oli leida ja kirjeldada uusi AIRE valgu funktsioone. Doktoritöö koosneb kolmest projektist, mida ühendab AIRE valguga seotud temaatika.

Esimene projekt hõlmas AIRE uute valguliste partnerite leidmist ja vastavate interaktsioonide tähtsuse uurimist. Me leidsime, et AIRE seondub oma PHD1 domääni kaudu kolmest tuumavalgust koosneva kompleksiga: DNA-sõltuva proteiinkinaasiga (DNA-PK), Ku70 ja Ku80-ga. Meie avastust on hiljem kinnitanud mitmed teised töögrupid. DNA-PK on DNA-st sõltuv proteiinkinaas, mis osaleb DNA vigade parandamisel, T ja B rakkude antigeenidega seonduvate retseptorite geenirekombinatsioonil ja paljude geenide ekspressioonide regulatsioonil. Me leidsime, et AIRE ja DNA-PK interaktsioon on sõltuv kromatiini struktuurist või kromatiiniga seonduvatest valkudest, kuid ei sõltu otseselt interaktsioonist DNA- ja RNA-ga. Me tõestasime, et AIRE valk on üks paljudest märklaudadest, mida DNA-PK fosforüleerib. Eelnevalt oli teada, et vaid dimeerne AIRE valk omab transkriptsioonilist aktiivsust ja et dimeristasioon sõltub AIRE fosforüleerumisest. Siiani oli näidatud *in vitro* katsete tulemusena vaid kaks AIRE-t fosforüüleerivat proteiinkinaasi, cAMP-sõltuvad proteiinkinaasid A ja C, kuid siiani puudus kirjeldus, milliseid aminohappeid antud kinaasid AIRE-s fosforüleerivad. Selgitamaks, milliseid aminohappeid DNA-PK fosforüleerib AIRE valgus, selleks me kasutasime valkude fosforüleerimist ennustavaid arvutiprogramme *Scansite 2.0* ja *NetPhos 2.0*. *In vitro* AIRE fosforüleerimise katsete tulemusena kinnitati arvutiennusustusi. Nimelt leidsime, et AIRE-t fosforüleeritakse DNA-PK poolt treoniin 68 (T68) ja seriin 156 (S156) positsioonides, kuna antud aminohapete muteerimine alaniiniks (T68A ja S156A) põhjustasid märgatava fosforüülimise taseme languse AIRE valgus. Lisaks selgus, et AIRE valk on rohkem fosforüleeritud N-terminaalsel poolel, kasutades selleks erinevaid *AIRE* geenideletsiooni mutante. Rakkude immunofluorestsents uuringutel selgus, et AIRE tuuma ja tsütoplasma lokalisatsioon ei sõltu DNA-PK-st, kasutades selleks DNA-PK defektset rakuliini (MO59J) AIRE ekspressiooniks.

Eelnevalt oli teada, et AIRE aktiveerib *in vivo* hiire mTEC-s naha epiteeli spetsiifilisi, lorikriini (*Lor*) ja involukriini (*Inv*) geene. Me kinnitasime seda tulemust *in vitro* HEK293 rakuliinis transkriptsiooni aktivatsiooni katsetes, kasutades selleks inimese *INV* ja *LOR* geenide promootorjärjestusi ja lutsiferaasi geeni sisaldavat reporterplasmiide. Leidsime, et T68 kui ka S156 posit-

sioonid on olulised AIRE transkriptsiooniliseks aktivatsiooniks, kuna T68A ja S156A mutandid ei suutnud aktiveerida *LOR* ja *INV* promootoreid. Meie tulemused viitavad, et lisaks AIRE PHD1 domäänile on T68 ja S156 aminohapped olulised interaktsioonil DNA-PK-ga ja et AIRE transkriptsiooniline aktiivsus võiks sõltuda DNA-PK fosforüleerimisest. Katsete tulemused ei välista, et teised proteiinkinaasid lisaks DNA-PK-le võiksid fosforüleerida samuti T68 ja S156 aminohappe jääke AIRE valgus. Hilisem dr. Peterlini töögrupi avastus (California Ülikool, San Francisco, USA) näitas, et AIRE aktiivsus tema märklaudgeenide promootoritel sõltub interaktsioonist DNA-PK-ga, kuid mitte AIRE fosforülatsioonist DNA-PK poolt.

Kuna DNA-PK geeni ekspresseeritakse basaalselt kõikides rakkudes, siis meid huvitas, kas tema ekspressiooni tase on sarnane või erinev tüümuse säsi AIRE-positiivsetes mTEC-s ja tüümuse koore epiteeli cTEC-s. Me leidsime, et DNA-PK mRNA tase oli sarnane hiire AIRE-positiivsetes mTEC-s ja AIRE-negatiivsetes cTEC-s. Samas, DNA-PK mRNA ekspressioon oli aga kümneid kordi kõrgem AIRE-sõltuvate autoantigeenide, *Lor* ja *Inv* mRNA ekspressiooni tasemest mTEC-s. Hiljuti leiti AIRE-positiivsetes hiirte tüümuse epiteeli rakkudes (MHC^{high}) kolm kuni neli korda kõrgemal määral DNA ahelate katkeid võrreldes AIRE-defektsete (*AIRE-KO*) hiirte tüümuse epiteelirakkudega. Meie avastus võiks selgitada kõrget DNA-PK geeniekspressiooni mTEC-s toimuva aktiivse koespetsiifiliste antigeenide transkriptsiooniga, mis põhjustab ülegenoomselt DNA ahelate katkeid. On kirjeldatud, et DNA-PK/Ku70/Ku80 valkkompleks osaleb transkriptsiooni käigus kromatiini struktuuri ja DNA ahelate „avamisel“ koos mitmete transkriptsioonivalkudega, nagu Topo2a, PARP1, FACT-ga, millega on ka AIRE seondunud. Meie töö tulemusena leitud DNA-PK sarnane mRNA ekspressiooni tase cTEC-s ja mTEC-s võiks viidata, et DNA-PK osaleb samuti AIRE-sõltumatute autoantigeenide transkriptsioonil tüümuse koore epiteelis T rakkude positiivse selektsiooni ajal.

Teine doktoritöö projekt kirjeldas AIRE HSR domäänis paiknevate tähenduslike mutatsioonide mõju AIRE transkriptsioonilisele aktiivsusele, mis on seotud APECED-i sündroomiga. Me uurisime eksogeensete geenide, involukriini (*INV*) ja interferoon beeta (*INFB*) promootorite aktivatsioone HEK293 rakuliinis. Vastavates katsetes transfekteeriti rakke reporterplasmiididega, mis sisaldasid *INV* või *INFB* promootorjärjestusi koos lutsiferaasi geeniga ning AIRE HSR domääni mutante kodeerivate plasmiididega. Promootorite aktivatsioone hinnati seejärel lutsiferaasi suhtelise aktiivsuse järgi. Me leidsime, et kaheteistkümnest APECED-i punktmutasioonist *INV* promootoril olid seitse transkriptsiooniliselt inaktiivsed (R15C, L28P, L29P, T16M, W78R, Y90C, L93R), kolm aktiivsed (A21V, V80L ja Y85C) ning kaks (R15L ja K83E) olid osalise aktiivsusega. Lisaks uurisime L28P, L29P, K83E mutantide mõju *INFB* geeni promootorile. Selle katse tulemusena L28P ja L29P AIRE mutandid ei suutnud aktiveerida *INFB* promootorit erinevalt K83E mutandist, mil säilus transkriptsiooniline aktiivsus sarnaselt AIRE-le. Meie transkriptsiooni katsete tulemused AIRE mutantidega üldiselt kinnitasid eelnevalt avaldatud töid, kus olid kasutusel teised promotoor-reporter süsteemid. Kuna eelnevalt oli teada, et

üldine transkriptsiooni aktivaatorvalk CBP seondub AIRE-ga ja omab sünergistilist efekti AIRE-sõltuvale transkriptsiooni aktivatsioonile, siis otsustasime uurida, kas CBP mõjutab AIREL28P, L29P ja K83E mutantide transkriptsioonilist aktiivsust *INV* ja *INFB* promootoritel. Me leidsime, et CBP ei suutnud aktiveerida*INV* ja *INFB* geenide promootoreid ilma AIRE-ta ega koos L28P, L29P ja K83E AIRE mutantidega.

Lisaks me uurisime L28P, L29P ning K83E mutantideaktiivsust ka endogeensete geenide, *INV* ja S100 kaltsiumit seonduva A8 valgu (*S100A8*) promootoritel HEK293 rakuliinis. *S100A8* on samuti AIRE aktivatsioonist sõltuv koespetsiifiline geen, mis varem oli kirjeldatud hiire mTEC-s ja ka HEK293 rakuliinis. Katsete tulemusena selgus, et L28P, L29P mutandid ei aktiveerinud *S100A8* ja *INV* geenide ekspressiooni, mida hinnati nende mRNA taseme järgi kvantitatiivse PCR meetodiga. Samas, AIRE aktiveeris *INV* ja *S100A8* geenide promootoreid vastavalt 60- ja 120-kordselt HEK293 rakkudes. K83E mutant omas AIRE-ga sarnast transkriptsioonilist aktiivsust mõlemal promootoril. CBP lisamine katsesse ei tõstnud L28P, L29P mutantide transkriptsioonilist aktiivsust *S100A8* ja *INV* geenide promootoritel, kuid seevastu tõstis koos AIRE ja K83E mutandiga ligikaudu kahekordselt vastavate geenide ekspressiooni taset. Kokkuvõttes selgus, et eksogeensete ja endogeensete promootorite aktivatsioonikatsete tulemused olid üldiselt sarnased.

Koostöös dr. Richiga (Cambridge Ülikool, Inglismaa) leidsime uue avastusena, et AIRE HSR domään omab CARD (*caspase recruitment domain*) motiivi, mis on iseloomulik mitmetele apoptoosiga seotud valkudele, nagu Apaf-1, kaspaas 9, ICEBERG ja RAIDD. CARD-i sekundaarstruktuur koosneb kuuest α-heeliksist ja konserveerunud hüdrofoobsetest aminohapetest, mis paiknevad struktuuri sisemuses. Leidsime korrelatsiooni AIRE valgu struktuuri ja APECED-i põhjustavate mutatsioonide vahel, mida uuriti valgu struktuuri ennustava programmiga *Pymol* ning valkude stabiilsuse skoori mõõtva programmiga *Site-Directed Mutator*. Nimelt, CARD struktuuri sisemuses olevate evolutsiooniliselt konserveerunud leutsiinide, L28 ja L29 mutatsioonid proliiniks (L28P ja L29P) põhjustavad valgu struktuuri ebastabiilsust ja muutust, kuid mitte lüsiin K83 mutatsioon glutamaadiks (K83E), mis asub CARD-i pinnal. Antud avastus näitab otsest AIRE valgu struktuuri ja funktsiooni seost.

Kolmas doktoritöö projekt uuris AIRE seost apoptoosiga ehk rakkude programmeeritud surma. Meie tööd kinnitasid hiljutist avastust, et AIRE indutseerib apoptoosi, kuid ei mõjuta rakkude jagunemise kiirust. Me demonstreerisime, et AIRE indutseerib apoptoosi kolmes erinevas epiteeli rakuliinis (1C6 mTEC, AIRE-HT93 ja doksütsükliiniga indutseeritav AIRE-HEK/Tet). Leidsime esmakordselt, et AIRE HSR/CARD domään vastutab rakkude apoptoosi eest. Hiljuti tõestati, et AIRE-positiivsete mTEC-de apoptoos soodustab koespetsiifiliste antigeenide esitlemist apoptootiliste „kehade“ kaudu T rakkude negatiivsel selektsioonil, nii otseselt kui ka kaudselt läbi dendriitrakkude antigeen-MHC kompleksi. Lisaks eelnevale demonstreeriti AIRE interaktsiooni DAXX-ga, mis osaleb rakkude apotoosis.

Antud doktoritöös kirjeldasime esmakordselt, et GAPDH valk on akumuleerunud AIRE-positiivsete rakkude tuumadesse. Seda tulemust detekteerisime nii immunofluorestsents mikroskoopiaga, kuid ka AIRE-positiivsete tuumavalkude fraktsioonides immunoblot meetodil. Samas, AIRE ja GAPDH valgud ei seondu omavahel, mida me eelnevalt immunopretsipitatsiooni katsetega olime leidnud. Ajalooliselt on GAPDH eelkõige tuntud kui „koduhoidja" geen, mis kodeerib glükolüüsiraja ensüümi.

Paljud uuringud on näidanud GAPDH seost oküdatiivse stressi ja apoptoosiga. Rakkude oksüdatiivse stressi korral ning varases apoptoosi etapis GAPDH-d S-nitrosüleeritakse lämmastikoksiidi süntaasi poolt tsütoplasmas, mille kaudu toimub GAPDH seondumine E3 ubikvitiinligaas Siah-1-ga, mille kaudu GAPDH transporditakse tuuma. Tuumas GAPDH stabiliseerib Siah-1 valku, kus aktiveerub Siah-1 ensümaatiline aktiivsus. Siah-1 osaleb tuumavalkude degradatsioonil, mis põhjustab rakkudes apoptoosi. Seega, GAPDH tuumalokalisatsioon peegeldab rakus stressi ja apoptootilisi protsesse. On leitud, et GAPDH mRNA ekspressioon on tõusnud erinevate patoloogilistes protsessides, nagu kasvajates ja neurodegeneratiivsetes haiguste korral. Me uurisime, kas GAPDH akumuleerimine tuuma on seotud tema mRNA ekspressiooni tõusuga AIRE-HEK/Tet rakuliinis ja leidsime, et GAPDH mRNA tase ei tõusnud AIRE ekspressiooni korral.

Rakkude apoptoosi ja GAPDH translokatsiooni tuuma saab inhibeerida spetsiifiliselt GAPDH-ga seonduva R-(-) deprenüüliga, mis blokeerib GAPDH S-nitrosülatsiooni. Seetõttu otsustasime kontrollida, kas R-(-) deprenüül mõjutab ka AIRE poolt indutseeritavat rakkude apoptoosi AIRE-HEK/Tet rakuliinis. Töö tulemusena leidsime, et R-(-) deprenüül vähendas oluliselt AIRE poolt indutseeritavat apoptoosi, mis kinnatas veelgi AIRE seost GAPDH ja apoptoosiga.

Samuti tuvastasime inimese tüümuse koelõikudel, et 60%-l AIRE-positiivsetest mTEC-st olid GAPDH tuuma lokaliseerunud. Samuti täheldasime GAPDH tuumalokalistasiooni paljudes AIRE-negatiivsetes rakkudes tüümuse koores. Antud leid võiks viidata, et GAPDH tuumalokalisatsiooniga rakud tüümuse medullas kui koores on varases apoptoosi etapis.

AIRE rolli rakkude stressi ja apoptoosiga kinnatasid lisaks veel proteoomilised uuringud inimese kilpnäärmeepiteeli rakuliinis (AIRE-HT93) koostöös dr. Alvarezega (Barcelona Automoomne Ülikool, Hispaania). Töö tulemusena leidsime AIRE-positiivsetes rakkudes mitmete stressi ja apoptoosiga seotud valkude taseme tõusu, milleks olid mitmed *heat-shock* perekonna valgud ja superoksiid dismutaas (SOD). Lisaks täheldasime anti-apoptootilise valgu Bcl-1 vähenemist AIRE-HT93 rakkudes võrreldes kontrollrakkudega.

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PUBLICATIONS

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List of publications:

1. **Liiv I**, Rebane A, Org T, Saare M, Maslovskaja J, Kisand K, Juronen E, Valmu L, Bottomley MJ, Kalkkinen N, Peterson P. DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. Biochim Biophys Acta 2008; 1783(1):74─83.

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