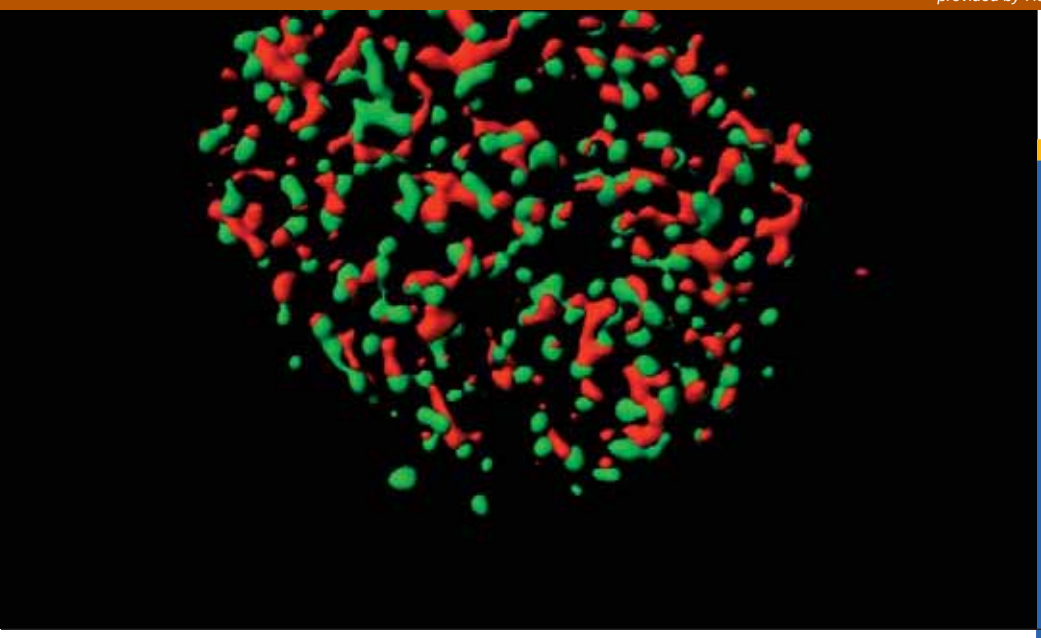

Tanja Ilmarinen

Functional and Cellular Analysis of Autoimmune Regulator (AIRE) Protein

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Department of Molecular Medicine,
National Public Health Institute
and
Division of Genetics, Department of Biological and Environmental Sciences,
Faculty of Biosciences, University of Helsinki
and
Helsinki Graduate School in Biotechnology and Molecular Biology

Helsinki, Finland 2007

Tanja Ilmarinen

FUNCTIONAL AND CELLULAR ANALYSIS OF AUTOIMMUNE REGULATOR (AIRE) PROTEIN

A c a d e m i c d i s s e r t a t i o n

To be presented with the permission of the Faculty of Biosciences, University of Helsinki, for public examination in the large lecture hall, Bulevardi 18, Helsinki, on November 16th, at 12 noon.

Department of Molecular Medicine, National Public Health Institute

and

Division of Genetics, Department of Biological and Environmental Sciences, Faculty of Biosciences, University of Helsinki

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AIRE (green) and PIAS1 (red) proteins in the nucleus of a Caco-2 cell presented as a 3D reconstruction of a cross-sectional confocal image. Figure by Tanja Ilmarinen.

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Remember that truth alone is the matter that you are in search after; and if you have been mistaken, let not vanity seduce you to persist in your mistake

-Henry Baker-

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ABSTRACT

Autoimmune diseases are a major health problem. Usually autoimmune disorders are multifactorial and their pathogenesis involves a combination of predisposing variations in the genome and other factors such as environmental triggers. APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) is one of the rare known autoimmune diseases caused by mutations in a single gene. APECED is inherited in a recessive manner. Patients with APECED suffer from several organ-specific autoimmune disorders, often affecting the endocrine glands. The defective gene, *AIRE,* has been identified and encodes a protein functioning as a transcriptional regulator. The AIRE (autoimmune regulator) protein controls the expression of hundreds of genes, representing a substantial subset of tissue-specific antigens which are presented to developing T cells in the thymus and has proven a key molecule in the establishment of immunological tolerance. However, the molecular mechanisms by which AIRE regulates the activity of its target genes, or other potential functions of AIRE, are still largely obscure.

The aim of this thesis has been to elucidate the pathogenesis of APECED by studying the molecular interactions involving AIRE, utilizing different cultured cell models. Exceptionally in one family, carrying a glycine 228 to tryptophan (G228W) mutation, APECED appears to be inherited as a dominant trait. It was shown in this thesis work that the AIRE polypeptide with G228W mutation is able to bind the wild type AIRE and inhibit its transactivation capacity *in vitro*. This provides a potential molecular mechanism for the dominant negative effect observed *in vivo* and emphasizes the importance of homomultimerization of AIRE. Furthermore, two novel protein families interacting with AIRE were identified: importin α and PIAS. The importin α molecules regulate the nuclear import of AIRE by binding to the nuclear localization signal of AIRE, delineated as a classical monopartite signal sequence. The interaction of AIRE with PIAS E3 SUMO ligases, indicates a link to the sumoylation pathway, which plays an important role in the regulation of nuclear architecture. It was shown that AIRE is not a target for SUMO modification but enhances the localization of SUMO1 and PIAS1 proteins to nuclear bodies, suggesting an interconnection between nuclear body formation of AIRE, SUMO and

PIAS proteins. Additional support for the suggestion that AIRE would preferably up-regulate genes with tissue-specific expression pattern and down-regulate housekeeping genes was obtained from transactivation studies performed with two models: human insulin and cystatin B promoters. Furthermore, AIRE and PIAS1 activate the insulin promoter concurrently in a model transactivation assay, indicating that their interaction is biologically relevant in the transcriptional regulation of the target gene of AIRE.

The identification of novel interaction partners for AIRE extend our knowledge of the molecular pathways involved in the establishment and maintenance of immunological tolerance. The observations presented in this thesis provide a foundation for further, *in vivo* studies.

Keywords: monogenic autoimmune disease, immunological tolerance, nuclear protein transport, regulation of gene expression, nuclear bodies

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TIIVISTELMÄ

Autoimmuunisairaudet ovat huomattava kansanterveydellinen ongelma. Yleensä autoimmuunisairaus syntyy usean perinnöllisen ja ympäristöllistä johtuvan tekijän summana. APECED (autoimmuuni polyendokrinopatia-kandidoosi-ektodermi dystrofia) on yksi harvoista tunnetuista autoimmuunisairauksista, joissa yksi virheellinen geeni riittää aikaansaamaan autoimmuunitaudin patogeneesin. APECED periytyy peittyvästi. APECED-potilailla esiintyy useita elinspesifisiä autoimmuunisairauksia, ja tyypillisesti immuunijärjestelmä hyökkää hormonirauhasia vastaan. APECED-taudissa virheellinen geeni, *AIRE*, on tunnistettu ja se koodaa transkription säätelijänä toimivaa proteiinia. AIRE (autoimmune regulator) -proteiini säätelee jopa satojen geenien ilmentymistä kateenkorvassa. Suuri osa näistä geeneistä ohjaa kehittyville T-soluille esiteltävien kudosspesifisten antigeenien synteesiä, ja AIRE on täten osoittautunut tärkeäksi molekyyliksi immunologisen toleranssin synnylle. AIREn kohdegeenien aktiivisuuden säätelymekanismeista, tai muista mahdollisista AIREn toiminnoista tiedetään kuitenkin vielä varsin vähän.

Tässä väitöskirjatutkimuksessa on pyritty valaisemaan APECED-taudin patogeneesia tutkimalla erilaisissa solumalleissa niitä molekyylitason vuorovaikutuksia, joissa AIRE-proteiini on osallisena. Poikkeuksellisesti yhdessä perheessä APECED näyttää periytyvän vallitsevasti. Tämän perheen jäsenillä toisesta *AIRE* alleelista tuotetussa proteiinissa glysiini 228 on muuttunut tryptofaaniksi (G228W). Tässä työssä osoitettiin, että kyseinen viallinen AIRE-proteiini kykenee sitomaan mutatoimatonta AIRE-proteiinia ja estämään sen kyvyn aktivoida transkriptiota *in vitro*, mahdollisesti selittäen vallitsevan periytymisen. Tämä työ tuo lisävahvistusta havainnoille, että AIRE-proteiinin multimerisaatio on tärkeä mekanismi sen toiminnalle *in vivo*. Tutkimuksessa kuvattiin myös kaksi uutta proteiiniperhettä, joiden kanssa AIRE on vuorovaikutuksessa: importiini α ja PIAS. Importiini α säätelee AIREn tumakuljetusta sitoutuen sen tumakuljetussignaaliin, joka identifioitiin tarkemmin tässä työssä yksiosaiseksi klassiseksi signaalisekvenssiksi. AIREn interaktio E3 SUMO ligaaseina toimivien PIAS-proteiinien kanssa viittaa yhteyteen sumoylaatio-reitin kanssa, mikä on yksi keskeisiä tuman toiminnallisen organisaation säätelijöitä. Tutkimuksessa osoitettiin, että AIREa ei sumoyloida,

mutta AIRE tehostaa SUMO1- ja PIAS1-proteiinien lokalisaatiota pistemäisiin tumarakenteisiin, mikä viittaa siihen, että AIREn, SUMOn ja PIAS-proteiinien sisältämien tumarakenteiden muodostuminen on kytköksissä toisiinsa. Kahdella mallilla, ihmisen insuliinin ja kystatiini B:n promoottorilla, saadut tutkimustulokset tukevat hypoteesia, jonka mukaan AIRE aktivoi monia kudosspesifisiä geenejä ja inhiboi useissa kudoksissa aktiivisten geenien ilmentymistä. Lisäksi AIRE- ja PIAS1-proteiinit säätelevät yhdessä insuliinipromoottorin aktiivisuutta transaktivaatiokokeessa osoittaen, että vuorovaikutus on biologisesti merkityksellinen AIREn kohdegeenin ilmentymisen säätelyssä.

Uusien AIREn kanssa vuorovaikutuksessa olevien proteiinien tunnistaminen lisää ymmärtämystämme siitä, mitkä molekyylireitit ovat osallisina immunologisen toleranssin synnyssä ja ylläpidossa. Tässä väitöskirjassa tehdyt havainnot luovat pohjan jatkotutkimuksille koko elimistön tasolla.

Avainsanat: monogeeninen autoimmuunisairaus, immunologinen toleranssi, proteiinien tumakuljetus, geenien ilmentämisen säätely, tumakappaleet

CONTENTS

ABBREVIATIONS

AADC	aromatic L-amino acid decarboxylase
AICD	activation induced cell death
AIRE	autoimmune regulator
ALPS	autoimmune lymphoproliferative syndrome
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APL	acute promyelocytic leukemia
APS	autoimmune polyendocrine/polyglandular syndrome
ARM	armadillo
CAS	cellular apoptosis susceptibility
cAMP	cyclic adenosine monophosphate
CB	cajal body
CBP	CREB-binding protein
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CPSF	cleavage and polyadenylation specificity factor
CREB	cAMP-responsive-element binding protein
Crm1	chromosome region maintenance 1
CSTB	cystatin B
CstF	cleavage stimulation factor
CTLA	cytotoxic T lymphocyte antigen
DBD	DNA binding domain
DC	dendritic cell
DEAF-1	deformed epidermal autoregulatory factor 1
DN	double negative
DP	double positive
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
GMEB-1	glucocorticoid-modulatory element-binding protein 1
GST	glutathione S-transferase
GTP	guanosine triphosphate
HDAC	histone deacetylase
HLA	human leukocyte antigen
HSR	homogeneously staining region
HSV AD	herpes simplex virus activation domain
IBB	importin β-binding
IL	interleukin
IFN	interferon
IPEX	immunodysregulation, polyendocrinopathy, and enteropathy, X-linked
LEF1	lymphoid enhancer factor 1
LRH-1	liver receptor homologue 1

MAR	matrix attachment region
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mTEC	medullary thymic epithelial cell
NB	nuclear body
NES	nuclear export signal
NKT cell	natural killer T cell
NLS	nuclear localization signal
NPC	nuclear pore complex
NUDR	nuclear DEAF-1 related
OMIM	online mendelian inheritance in man
PIAS	protein inhibitor of activated STAT
PHD	plant homeodomein
PML	promyelocytic leukemia
RARα	retinoic acid receptor α gene
rRNA	ribosomal RNA
SAND	Sp100, AIRE, NucP41/75 and DEAF-1
SAP	scaffold-attachment factor A and B, apoptotic chromatin-condensation inducer in the nucleus and PIAS
SATB1	special AT-rich sequence binding protein 1
Scc	side-chain cleavage
SIM	SUMO interacting motif
SMN	survival of motor neurons
Sn(o)RNP	small nucle(ol)ar ribonucleoprotein
SP	single positive
Sp100	speckled protein 100 kilodalton
SRP	signal recognition particle
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-like modifier
SV40 T Ag	simian virus 40 large T antigen
TCR	T cell receptor
TEC	thymic epithelial cell
TG	thyroglobulin
TGF	transforming growth factor
TH	tyrosine hydroxylase
Th cell	T helper cell
TNFRSF6	tumor necrosis factor receptor superfamily, member 6
TPH	tryptophan hydroxylase
TPO	thyroid peroxidase
Treg cell	regulatory T cell
Ubc9	ubiquitin-like protein SUMO-1 conjugating enzyme

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Ilmarinen Tanja**, Eskelin Petra, Halonen Maria, Rüppell Taina, Kilpikari Riika, Duran Torres Gilberto, Kangas Hannele and Ulmanen Ismo. Functional analysis of SAND mutations in AIRE supports dominant inheritance of the G228W mutation. Human Mutation, 26:322-31, 2005.

II **Ilmarinen Tanja**, Melén Krister, Kangas Hannele, Julkunen Ilkka, Ulmanen Ismo and Eskelin Petra. Monopartite nuclear localization signal of AIRE mediates its nuclear import and interaction with multiple importin α molecules. FEBS Journal, 273:315-24, 2006.

III **Ilmarinen Tanja***, Kangas Hannele*, Kytömaa Taina, Eskelin Petra, Saharinen Juha, Seeler Jacob, Tanhuanpää Kimmo, Chan Fiona Yih-Ling, Slattery Robyn Maree, Alakurtti Kirsi, Palvimo Jorma and Ulmanen Ismo. Functional interaction of AIRE and PIAS1 proteins in transcriptional regulation. Submitted.

*These authors have contributed equally to this work

INTRODUCTION

Autoimmune diseases are major causes of morbidity and mortality in the world. Autoimmunity has a tendency to run in families and the relatives of patients have an increased risk for developing an autoimmune disease. The genetic factors underlying most autoimmune disorders are complex and involve a combination of predisposing variations in the genome. In addition, other factors such as environmental triggers contribute to the aetiology of these diseases. Only a few monogenic autoimmune diseases exist. Although these diseases are rare, they can teach us important lessons about the pathways contributing to development of normal immunological tolerance and its breakdown in more common autoimmune diseases.

APECED is one of the few monogenic autoimmune diseases identified. Patients with APECED suffer from several concurrent organ-specific autoimmune disorders, due to loss of self-tolerance to certain autoantigens. The broad spectrum of autoimmune diseases in patients with APECED implies that AIRE may also play a role in the pathophysiology of other, more common autoimmune disorders. The gene defective in APECED was identified in 1997 and named autoimmune regulator (AIRE). The name autoimmune regulator has indeed proved felicitous, as the importance of AIRE in the establishment and maintenance of immunological self-tolerance is starting to emerge. Both *in vitro* and *in vivo* studies indicate a function for AIRE as a transcriptional regulator controlling the ectopic expression of self-antigens in the thymus. However, the underlying molecular mechanisms of this regulation have remained largely obscure.

The aim of this study was to further characterize the functional and cellular interactions involving AIRE in order to gain insight into the molecular mechanisms underlying the loss of tolerance to self molecules and development of APECED.

REVIEW OF THE LITERATURE

1 IMMUNOLOGICAL TOLERANCE

Our immune system faces the challenge of mounting immune responses to harmfull foreign antigens while remaining tolerant to autoantigens. Failure of the immune system to differentiate 'self' from 'nonself' can lead to an effective and specific immune response against host molecules and to the development of an autoimmune disease. In the control of self-tolerance T lymphocytes play a substantial role. Establishment and regulation of T cell tolerance takes place at two levels. First, in central tolerance, maturing T cells are selected against self-reactivity in the thymus (Kyewski and Klein, 2006). However, some potentially autoreactive T cells escape from the thymus and are normally present in the periphery of most healthy individuals. The pathogenicity of these lymphocytes is kept under control by the mechanisms of peripheral tolerance (Sommer et al., 1991; Lohmann et al., 1996; Walker and Abbas, 2002; Danke et al., 2004).

1.1 Thymic selection of T lymphocytes

Thymus remained a mysterious organ for centuries. It was thought to have no immunological function and the prevailing opinion was that the thymus is just a graveyard for dying lymphocytes (Maclean et al., 1957; Billingham et al., 1962). The importance of the thymus in the immune system was discovered in early 1960s by Jacques Miller who, based on his studies with mice thymectomized at birth, proposed that thymus was the source of immunocompetent lymphocytes (Miller, 1961).

Thymus consists of numerous lobules which are divided into outer cortical and inner medullary areas. The distinct microenvironments are characterized by the presence of specialized thymic stromal cells. The main structure is formed by various types of thymic epithelial cells (TECs), surrounded by other stromal cell types such as macrophages and dendritic cells (DCs). Signals from the stromal cells are required for the development of T lymphocytes (van Ewijk, 1988; Pearse, 2006; Takahama, 2006). For example chemokines produced by thymic stromal cells are crucial in directing the thymocytes (thymic T cells) within the thymus as they develop (Takahama, 2006). In addition, thymic stromal cells need communication with developing thymocytes in order to maintain their normal organization and maturation (Shores et al., 1991). As the T cell precursors migrate in the thymus, they go through several checkpoints which they must pass in order to continue maturing. This results in radical selection; only 1-3% of the developing thymocytes mature and eventually leave the thymus (Scollay et al., 1980; Egerton et al., 1990). The purpose of this stringent selection process is to ensure that the naïve T cells that are released into circulation are both functional and self-tolerant.

The course of T cell differentiation is characterized by the temporally and spatially coordinated expression of cell surface proteins on the thymocyte, including CD4, CD8, CD44, and CD25. The main stages of T cell development in the thymus are depicted in Figure 1. In postnatal thymus, early lymphocyte progenitors enter through the vasculatures in the cortico-medullary junction. Upon entry, the cells committed to T cell lineage lack expression of CD4 and CD8 and are called double negative (DN) (Scollay et al., 1984; Scollay and Shortman, 1985; Godfrey et al., 1993; Lind et al., 2001). The earliest events in thymocyte development, T cell receptor (TCR) gene rearrangement and positive selection, take place in the cortex. Those DN thymocytes that have successfully rearranged their TCRβ locus, can start to express CD4 and CD8 co-receptors and progress to the $CD4^{+}CD8^{+}$ double positive (DP) stage (Krimpenfort et al., 1989; Mombaerts et al., 1992; Lind et al., 2001). After the rearrangement of TCRα locus, thymocytes are selected based on interactions between the TCR they express and self-peptide-major histocompatibility complex (MHC) ligands on the surface of specific thymic stromal cells. Many of the randomly rearranged TCRs are not functional and cannot bind the MHC alleles present. Lack of binding leads to death of the thymocyte, in a process called death by neglect. Thymocytes with low to medium affinity for self-peptide-MHC complexes expressed on cortical epithelial cells are positively selected. Those cells that interact with MHC class II differentiate to CD4, and those interacting with MHC class I to CD8 single positive (SP) cells (Koller et al., 1990; Zijlstra et al., 1990; Cosgrove et al., 1991; Grusby et al., 1991; Starr et al., 2003). The SP cells then migrate to the medulla, where both common and tissue specific self-antigens are presented to them. Thymocyte recognizing the self-peptide-MHC complexes too strongly, thus being potentially dangerous, are destroyed (negative selection) and only those that react weakly are allowed to mature and leave the thymus (Starr et al., 2003).

Negative selection has been reported to occur both in cortex and medulla, but currently the prevailing opinion among immunologist appears to be that medulla is the main site for clonal deletion of autoreactive T cells (Hogquist et al., 2005; Kyewski and Klein, 2006). The presence of ectopic expression of peripheral proteins in the thymus has been known for nearly two decades (Linsk et al., 1989). Initially, this observation received little attention, but accumulating evidence suggests a functional role for the thymic expression of tissue specific genes, termed promiscuous gene expression, in the induction of T cell tolerance (Derbinski et al., 2001; Kyewski and Klein, 2006). Recently, medullary epithelial cells (mTECs) were identified as the specialized cell type which express of a broad range of tissue-specific genes (Derbinski et al., 2001). Self-antigens expressed by mTECs represent almost all parenchymal organs and they include developmentally and temporally regulated genes (Sospedra et al., 1998; Bruno et al., 2002; Gotter et al., 2004; Derbinski et al., 2005). The roles of different medullary antigen presenting cells

(APCs) in the deletion of autoreactive T cells are still under debate. In addition to presenting endogeneously expressed antigens, thymic DCs have been reported to obtain self-antigens from mTECs and cross-present them to the thymocytes. Also the mTECs themselves have been suggested to present tissue-restricted antigens to at least a subset of developing T cells (Zhang et al., 2003; Gallegos and Bevan, 2004; Kyewski and Klein, 2006). Additionally, it is possible that peripheral DCs which have taken up tissue-specific antigens may migrate to the thymus to supplement the range of self-antigens presented to the developing thymocytes (Bonasio et al., 2006).

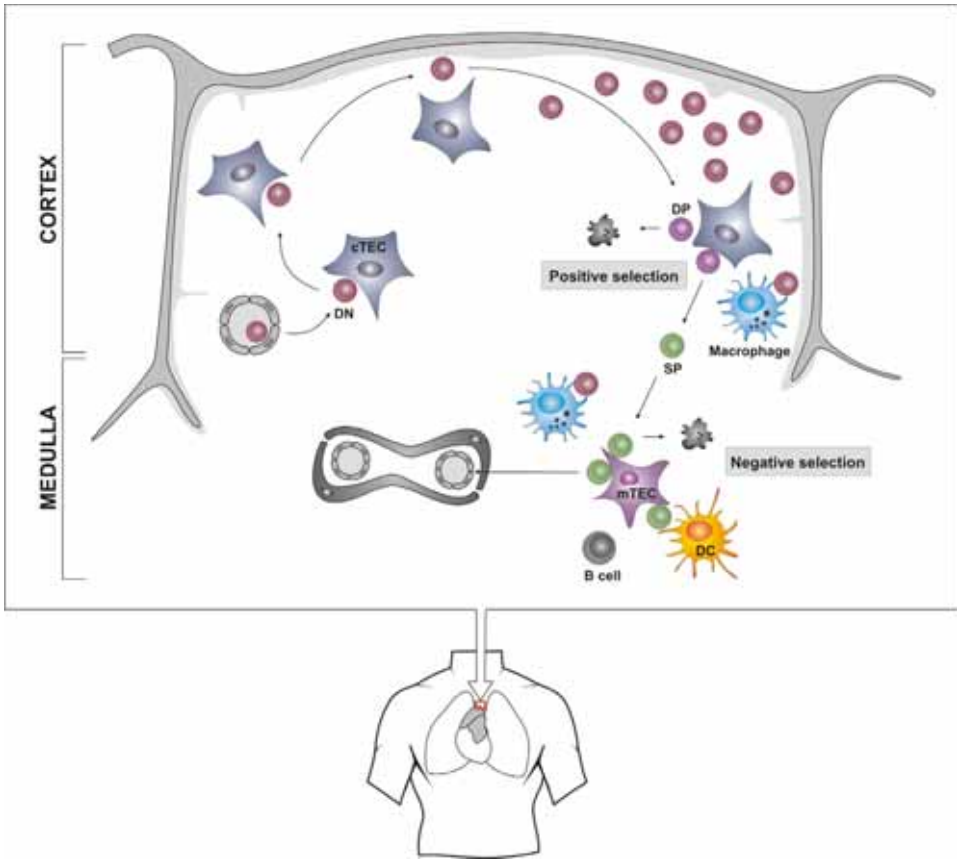

Figure 1. Schematic representation of the thymic development of T lymphocytes. The T cell progenitors enter the thymus through the vasculatures in the cortico-medullary junction. Double negative (DN) thymocytes move outwards to the capsule. Transition to double positive (DP) stage takes place in the outer cortex. Interactions with cortical stromal cells allows the positively selected thymocytes to differentiate to single positive (SP) cells and move to medulla, where negative selection leads to the death of autoreactive cells. Mature thymocytes return back to circulation. Modified from Takahama (2006) and from Kyewski and Klein (2006). cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; DC, dendritic cell.

1.2 Peripheral tolerance

Although most individuals have autoreactive T lymphocytes in their circulation, only 3–5% of the general population suffer from autoimmune diseases (Sommer et al., 1991; Lohmann et al., 1996; Jacobson et al., 1997; Danke et al., 2004). Indeed, there are several peripheral tolerance mechanisms by which the self-reactive T cells can be kept from causing harm to the host. Many of the signals involved in maintaining self-tolerance are in fact the same signals required for proper T cell responses to infections. The main mechanisms of peripheral tolerance can be divided into T cell intrinsic and T cell extrinsic (Walker and Abbas, 2002).

1.2.1 T cell intrinsic mechanisms

T cell intrinsic mechanisms comprise of those that act directly on the T cell and include ignorance, anergy, and deletion (Walker and Abbas, 2002). Ignorance encompasses the "innocuous" coexistence of naïve, autoreactive T cells and their target antigens. Ignorance can be the result of limited access of autoreactive T cells to certain self proteins due to spatial separation (Zinkernagel, 1996). It may also be that autoantigens are present in too low amounts to be detected by T cells, or that the avidity of the TCR for its cognate antigen is too low for effective recognition (Ohashi et al., 1991; Oldstone et al., 1991; Kurts et al., 1998). However, under certain conditions, such as immunization or viral infection, ignorance to these antigens may be broken and could lead to autoimmune responses (Ohashi et al., 1991; Oldstone et al., 1991).

In anergy, the T cell is functionally inactivated upon an antigen encounter, but remains alive in a hyporesponsive state. Anergic states of $CD4^+$/$CD8^+$ cells can be divided into two categories: clonal anergy and *in vivo* anergy or adaptive tolerance (Schwartz, 2003; Chiodetti et al., 2006). In addition to antigen recognition, T cells require other signals for full activation (Lafferty and Cunningham, 1975; Jenkins and Johnson, 1993). In clonal anergy, TCR occupancy in the absence of costimulation leads to unresponsiveness. The best characterized T cell costimulatory pathway is the B7-1/B7-2:CD28 pathway. Here, the costimulation is provided by the B7-1 (CD80) and B7-2 (CD86) molecules on APCs via their interaction with CD28 present on T cell surface (Greenwald et al., 2005). If the MHC-peptide complex is presented to the T cell by a resting APC not producing the appropriate costimulatory signals (e.g. in the absence of infection), the T cell does not get fully activated but becomes anergic (Jenkins and Schwartz, 1987). Clonal anergy leads to the down-regulation of certain cytokines (e.g. interleukin-2 and -3) and can be reversed by stimulation with interleukin-2 (IL-2) (Schwartz, 1996). Unresponsiveness in adaptive tolerance is most often initiated in naïve T cells *in vivo* by stimulation in the absence of costimulation or presence of coinhibition by e.g. cytotoxic T lymphocyte antigen-4 (CTLA-4) (Schwartz, 2003). In this type of anergy, all TCR-

induced cytokine production is down-regulated and continued encounter of antigen to maintain the anergic state of the T cells is required (Rocha et al., 1993; Schwartz, 2003). The reasons for maintaining such unresponsive and potentially self-reactive T cells are not known, but the reversibility of anergy has lead to the suggestion of anergy being a way to keep the T cell repertoire versatile by preserving this pool of potentially useful cells (Powell, 2006).

The ultimate way to control autoreactive T cells in the periphery is to delete these cells by activation induced cell death (AICD). The Fas pathway has a central role in AICD and defects in either the Fas death receptor or its ligand can lead to autoimmunity in both mice and humans (Watanabe-Fukunaga et al., 1992; Fisher et al., 1995). T cell growth factor IL-2, which is critical for proliferation of the T cells and their subsequent differentiation into effector cells, also has an important role in AICD (Lenardo, 1991; Gaffen and Liu, 2004). Mice deficient in IL-2, or IL-2 receptor, develop massive lymphoproliferation and an autoimmune syndrome as a result of failure in Fas-mediated AICD (Van Parijs et al., 1997; Abbas, 2003). It has been proposed, that in the absence of costimulation and presence of persistent antigen, the strength of interaction between the TCR and peptide-MHC complex determines whether the cell becomes anergic or is deleted, at least in the case of CD8 T cells: continuous exposure to high doses of antigen leading to anergy and low doses to clonal deletion (Rocha et al., 1995; Redmond et al., 2005).

1.2.2 DCs and regulatory T cells

T cell extrinsic tolerance mechanisms involve additional cells of the immune system, such as DCs and and regulatory T cells (Tregs) (Walker and Abbas, 2002). In addition to the key role in initiating adaptive immune reactions, DCs are also involved in the induction of tolerance. Migrating DCs have been shown to continuously sample self-proteins and carry them to lymph nodes, where the antigens are presented probably by both the migratory DCs and resident $CD8^+$ DCs to T cells, which leads to proliferation followed by deletion of the autoreactive T cells (Huang et al., 2000; Lee et al., 2007; Villadangos and Schnorrer, 2007). Increasing evidence also suggests that DCs are involved in the induction and maintenance of peripheral T cell tolerance by their interaction with special type of T cells, the suppressive Tregs; DCs e.g. appear to promote the differentiation of these cells (Hubert et al., 2007).

T cell mediated suppression was first described in the late 1960s, early 1970s, but remained controversial for years (Nishizuka and Sakakura, 1969; Penhale et al., 1973; Penhale et al., 1976; Sakaguchi et al., 1982). As more specific markers were identified to help to differentiate the potential regulatory cells from other T cells, it became clear that there is a population of T cells able to actively suppress the activation and proliferation of self-reactive lymphocytes (Sakaguchi, 2004).

Furthermore, it appears that there are in fact several different subset of Tregs involved in immunosuppression (Bach, 2003; Bluestone and Abbas, 2003). Some of these cells (natural Tregs), such as $CD4^+CD25^+$ T cells and natural killer T (NKT) cells, develop during T cell maturation in the thymus (La Cava et al., 2006). Others (adaptive Tregs), can be induced by antigenic stimulation in the periphery from conventional T helper (Th) or natural Treg cells (Bluestone and Abbas, 2003). Such cells include at least IL-10 producing Tr1 cells and transforming growth factor (TGF)-β producing Th3 cells (Wing et al., 2006). The mechanisms by which Tregs exert their suppressive function are still inconclusive. Due to the different developmental pathways and means of suppression, Bluestone and Abbas have suggested that the naturally occuring Tregs would function primarily to prevent immune responses against self-antigens and adaptive Tregs to keep adaptive immune responses in control (Bluestone and Abbas, 2003).

The $CD4^+CD25^+$ T cells in particular have received a lot of attention ever since Sakaguchi *et al.* (1995) showed that removal of this T cell subpopulation from normal animals leads to spontaneous development of various autoimmune diseases and excitingly, restoration of the $CD4^+CD25^+$ T cells is able to prevent the autoimmunity (Sakaguchi et al., 1995). One of the main regulators of the $CD4^+CD25^+$ T cell development and function is Foxp3, a member of the forkhead/winged-helix family of transcription factors (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Foxp3 appears to be expressed only in natural Tregs (Vieira et al., 2004). It is also the most specific marker for regulatory CD4+ cells since no cell-surface molecule unique for Tregs has been indentified to date. All, including CD25, are upregulated in naïve T cells during immune response and furthermore, some $CD25^-$ T cells (which also express Foxp3) can function as regulatory T cells, as well (Oida et al., 2003; Curotto de Lafaille and Lafaille, 2004; Ono et al., 2006; Wing et al., 2006).

$Foxp3^+$ Tregs are known to arise from the thymus, but the mechanisms behind their differentiation are still elusive. It has been suggested that they develop in the medulla from those thymocytes that have high affinity to self-antigens but escape negative selection due to Foxp3 expression (Jordan et al., 2001; Fontenot et al., 2005a; Fontenot and Rudensky, 2005; Liston and Rudensky, 2007). In addition, the presence of cofactors such as B7-CD28 costimulation, IL-2 and other cytokines, and thymic stromal lymphopoietin seem to be important (Salomon et al., 2000; Fontenot et al., 2005b; Tai et al., 2005; Watanabe et al., 2005; Liston and Rudensky, 2007). Liston and Rudensky (2007) have proposed a two-step model of Treg development. The first step involves the recognition of self-peptide with appropriate affinity and the second step exposure to cofactors leading to upregulation of Foxp3 expression. After this, the expression of Foxp3 is reinforced which provides additional protection from negative selection (Liston and Rudensky, 2007).

2 MONOGENIC AUTOIMMUNE DISEASES

In addition to APECED, which will be discussed in detail further on, ALPS (autoimmune lymphoproliferative syndrome) and IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked) are caused by defects in single genes (Ulmanen et al., 2005). ALPS (OMIM 601859) is a variable disease characterized by accumulation of double negative T cells, autoimmune cytopenias and susceptibility to malignancy. The inheritance is usually autosomal dominant (Straus et al., 1999). The first causative gene for ALPS was identified with the help of a spontaneous animal model, the *lpr* mouse, which lacks expression of Fas (CD95, Apo-1) (Watanabe-Fukunaga et al., 1992). Subsequently, the human homologue for Fas, the *TNFRSF6* (*FAS*) gene, was cloned, and its mutations were found to cause defective apoptosis in patients with ALPS (Behrmann et al., 1994; Fisher et al., 1995; Rieux-Laucat et al., 1995). Most, but not all, of the ALPS patients have mutations in the *FAS* gene. In some of the patients, the mutations lie in FAS ligand or in *caspase* 8 or 10 genes, all of which are also involved in the Fas-mediated signalling pathway needed for programmed cell death (Wu et al., 1996; Wang et al., 1999b; Chun et al., 2002; Walsh et al., 2003). Based on the molecular pathology, ALPS has been classified into types 1a and 1b, 2 and 3 (Rieux-Laucat et al., 2003; Sneller et al., 2003). Apoptosis is an important element in the regulation of lymphocyte homeostasis, self-tolerance and termination of immune responses. Studying the molecular defects in ALPS has increased our understanding of how the death receptor signalling functions in these immunological processes (Worth et al., 2006).

IPEX (OMIM 304790) has an X-linked recessive pattern of inheritance (Powell et al., 1982). It is a highly variable and very severe disease, which in most cases results in early death. The disease phenotype includes among others type 1 diabetes, severe enteropathy, hypothyroidism, and autoimmune skin disease that often appear sequentally (Wildin et al., 2002). Similar to ALPS, the first clues to the identity of the defective gene came from a mouse model, scurfy. The mouse disease had similar clinical, genetic and immunological features to the human disease, and the critical genomic regions were syntenic. The cloning of the mouse gene, *Foxp3*, led to the identification of the homologuous human gene *FOXP3* as the causative gene in IPEX (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). The phenotype of both the mouse and human diseases suggested that the defect could be in the development and/or function of $CD4^+CD25^+$ regulatory T cells and indeed, Foxp3 was showed to be needed for the thymic development of these cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Recent studies also suggest that peripheral naïve T cells can be induced to express Foxp3 and gain Treg function (Chen et al., 2003; Fantini et al., 2004; Fu et al., 2004; Bruder et al., 2005; Kretschmer et al., 2005).

The mechanisms by which Foxp3 exerts its functions are still largely elusive but some clues are beginning to emerge. Foxp3 can directly bind to the regulatory sequences of its target genes, e.g. *Il2* and *Ifng* which it downregulates and *Il2ra* and *Ctla4* which it upregulates. It is probably capable of recruiting chromatin remodeling complexes to its binding sites. Foxp3 interaction with NFAT (nuclear factor of activated T cells) and the binding of this complex to the corresponding regulatory regions appears essential for Treg differentiation and/or suppressor function. Foxp3 may also control the expression of other gene expression regulators and thus modulate the transcriptional activity of its target genes indirectly (Zheng and Rudensky, 2007).

In addition to IPEX, Treg deficiency has been associated with several other autoimmune diseases (Ehrenstein et al., 2004; Viglietta et al., 2004; Lindley et al., 2005). Further, increased levels of Tregs have been associated with cancer and persistent microbial infections (Curiel et al., 2004; Mendez et al., 2004). Indeed, due to the immunosuppressive properties of Tregs there are great expectations for their use in immunotherapy (Becker et al., 2006; Roncarolo and Battaglia, 2007).

3 APECED

APECED (OMIM 240300) belongs to a group of diseases called autoimmune polyendocrine/polyglandular syndromes (APS). In addition to APECED, these syndromes include APS-2 (also known as Schmidt's syndrome) which appears to be polygenic, and IPEX, which has been added to the group recently. Although a very heterogeneous group of disorders, a common feature to these diseases is that the patiens suffer from several organ-specific autoimmune diseases often attacking endocrine organs (Schmidt, 1926; Thorpe and Handley, 1929; Esselborn et al., 1956; Neufeld et al., 1980; Neufeld et al., 1981; Powell et al., 1982; Eisenbarth and Gottlieb, 2004). APECED has been called with several different names, but to date two of them have been established: APECED (used hereafter) for autoimmune polyendocrinopathy candidiasis ectodermal dystrophy reflecting the clinical features of the disease and APS-1 for autoimmune polyglandular or polyendocrine syndrome type 1 (Neufeld et al., 1980; Perheentupa, 1980; Neufeld et al., 1981).

Global prevalence of APECED is low. However, it is enriched in certain genetically isolated populations, including Finns (lifetime prevalence 1:25,000) belonging to the Finnish disease heritage (Perheentupa, 1972; Norio et al., 1973), Iranian Jews (1:9,000) (Zlotogora and Shapiro, 1992), and Sardinians (1:14,500) (Rosatelli et al., 1998). APECED is inherited as an autosomal recessive trait (Ahonen, 1985). Altogether, there are 91 identified Finnish patients with APECED. This is the largest cohort of APECED patients worldwide and the most extensive follow-up of the course of their disease has recently been reported by Perheentupa (2006). The clinical phenotype described here is largely based on that report. Most

autoimmune diseases are more common among women than men (Lockshin, 2006), but APECED affects both genders equally (Perheentupa, 2006). The clinical picture and course of the disease are highly variable, even among siblings. The age of onset varies from infancy to adolescence (0.2-18 years in Finnish patients). The patients can have a combination of even over ten different disease components caused by destructive autoimmune reactions toward endocrine and nonendocrine organs. The diagnosis is based on the occurrence of at least two of the three most common clinical manifestations, that is, chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenocortical failure (Addison's disease) (Ahonen, 1985).

All Finnish patients have candidiasis at least in some phase of their life. It is among the first symptoms in ~60% of the Finnish patients. In most other populations the prevalence of APECED-associated chronic mucocutaneous candidiasis is also high (Betterle et al., 1998; Collins et al., 2006; Stolarski et al., 2006; Wolff et al., 2007), except among the Iranian Jewish patients who suffer from it only rarely (Zlotogora and Shapiro, 1992). Candida infection can appear e.g. in the mouth, nails, esophagus, and intestine, and may predispose to squamous cell carcinoma of the mouth or esophagus. Hypoparathyroidism shows a gender difference, appearing earlier and more often in women (prevalence 98%) than men (prevalence 71%) (Gylling et al., 2003). The prevalence of most disease manifestations increases with age. For example the prevalence of adrenocortical failure is 8% among the age group of 5-year-olds and 84% among 50-year-olds (Perheentupa, 2006).

Other relatively common endocrine symptoms include type 1 diabetes, hypothyroidism, and testicular failure. Type 1 diabetes, hypothyroidism, and testicular failure become more common in older patients than other disease components. Females suffer from infertility and ovarian failure occurs in over half of the female patients over 20 years of age (Table 1). Characteristic ectodermal manifestations in APECED include alopecia, vitiligo, and rash with fever. Prevalence of enamel hypoplasia is also rather high (77%) (Ahonen et al., 1990). Hepatitis is a serious disease component that can lead to acute liver failure and death. Eye problems are also quite frequent and have led to blindness in 11% of the Finnish patients.

The patients have increased levels of various circulating, tissue-specific autoantibodies. Many of the antibodies are against intracellular enzymes functioning in hormone synthesis in affected organs such as the parathyroid and adrenal glands (Peterson and Peltonen, 2005). These antibodies may be responsible for the destruction of the target tissues and sometimes correlate with a clinical manifestation (Ahonen et al., 1987; Betterle et al., 1998; Betterle et al., 2002) (Table 2). Recently, neutralizing antibodies against type 1 interferons (IFN), especially IFN-α and IFN-ω, have been reported to be present in the sera of practically all tested APECED patients homozygous for *AIRE* gene mutations. These antibodies

were not present in heterozygotes, healthy controls, or patients with other endocrine diseases. Furthermore, in some cases the antibodies preceded the development of clinical symptoms (Meager et al., 2006; Wolff et al., 2007). In the future, testing for these antibodies may prove to be an important diagnostic tool which helps especially in the identification of patients with only some features of the disorder. Contact information for laboratories performing autoantibody assays is available at the web site of the European Union APS1 project EURAPS (www.apeced.net).

Table 1. Prevalence[1] (%) of the diagnostic dyad and the most common disease components by age (yr) and the age ranges (yr) at their appearance. Modified from Perheentupa (2006)

	Age									
	1	**2**	**5**	**10**	**15**	**20**	**30**	**40**	**50**	**Age range at appearance**
Diagnostic dyad[2]	0	0	21	70	85	94	97	99	99	2.2-35
Classic triad										
Candidiasis	17	30	48	83	93	96	98	100	100	0.2-31
Hypoparathyroidism	0	6	34	65	77	83	85	87	88	1.6-43
Adrenal failure	0	0	8	40	63	72	78	81	84	3.5-41
All three	0	0	3	25	50	56	64	71	76	3.5-43
Other endocrine disorders										
Ovarian failure					35	53	60	69		-36
Testicular failure						8	12	28		-37
Type 1 diabetes	0	0	2	3	7	10	13	23	33	4.1-58
Hypothyroidism	0	0	1	1	1	4	14	21	31	4.7-45
Skin disorders										
Alopecia	0	0	5	16	29	33	39	39	39	2.5-30
Vitiligo	1	1	2	9	17	20	27	31		0.7-45
Rash with fever	3	7	10	12	13	14	14	15		0.7-31
Gastrointestinal disorders										
Pernicious anemia	0	0	0	3	10	16	20	28	31	6.1-48
Severe obstipation	1	1	8	10	14	18	21	26		1.0-31
Chronic diarrhea[3]	0	0	8	13	16	17	22	22	22	2.5-27
Hepatitis	1	2	5	12	16	18	18	18	18	0.7-16
Eye disorders										
Keratoconjunctivitis	0	5	11	18	20	21	22	22	22	1.3-16

[1] in the series of 91 patients, estimated from the observed incidence rates over the age intervals assuming that all patients live until the age of 50 years

[2] at least two of candidiasis, hypoparathyroidism, and Addison's disease

[3] does not include the diarrhea associated with hypocalcemia

Table 2. Prevalence of autoantigens in APECED patients.
Modified from Peterson and Peltonen (2005) and from Meriluoto *et al*. (2001)

Antibody against	prevalence	Clinical manifestation
Type 1 interferons	~100%	
		Endocrine
P450c21 (Steroid 21 hydroxylase)	66%	Addison's disease
P450scc (Side-chain cleavage enzyme)	52%	Addison's disease, gonadal failure
P450c17 (Steroid 17-alpha hydroxylase)	44%	Addison's disease
GAD65 (Glutamic acid decarboxylase)	37%	Type 1 diabetes
TPO (Thyroid peroxidase)	36%	Hypothyroidism
TG (Thyroglobulin)	36%	Hypothyroidism
		Nonendocrine
AADC (Aromatic L-amino acid decarboxylase)	51%	Autoimmune hepatitis
TPH (Tryptophan hydroxylase)	45%	Malabsorption
TH (Tyrosine hydroxylase)	40%	Alopecia

4 AUTOIMMUNE REGULATOR (AIRE)

The defective gene in APECED was identified in 1997 by positional cloning in Finnish families indepently by two international collaborations, the other including our research group (Consortium, 1997; Nagamine et al., 1997). The APECED locus was assigned to chromosome 21q22.3 and the gene named autoimmune regulator (*AIRE*). The *AIRE* gene consists of 14 exons spanning 11.9 kilobases of genomic DNA and encodes a 545-amino-acid protein with a predicted molecular mass of 57.7 kDa.

4.1 APECED-causing mutations

To date, more than 60 patient mutations have been identified in the *AIRE* gene (Buzi et al., 2003; Harris et al., 2003; Peterson et al., 2004; Meloni et al., 2005; Podkrajsek et al., 2005; Dominguez et al., 2006; Stolarski et al., 2006; Ulinski et al., 2006; Wolff et al., 2007). Mutation registry for APECED can be found at bioinf.uta.fi/AIREbase. Mutations occur throughout the protein-coding region and include single nucleotide substitutions, small insertions and deletions. In addition, mutations affecting splicing consensus sequences have been described. In the isolated populations in which APECED is enriched, a clear founder effect is observed and there is one common mutation detected in the majority of the disease alleles. The common Sardinian APECED mutation, R139X, accounts for 90% of the mutant alleles in the Sardinian patients with APECED (Rosatelli et al., 1998). All the patients in the Iranian Jewish population are homozygous for a Y85C mutation and this mutation has not been detected in patients with any other ethnic background (Zlotogora and Shapiro, 1992). The most common mutation in the Finnish population is R257X. A total of 77% of the Finnish patients analyzed are homozygotes for the R257X mutation and 17% are compound heterozygotes (Perheentupa, 2006). Additionally, the R257X mutation is found in ~30% of non-Finnish patients (Björses et al., 2000), being the most common mutation in many

populations (Scott et al., 1998). The R257X accounts for e.g. 75% of Central and Eastern European, and 71% of Polish APECED alleles examined (Cihakova et al., 2001; Stolarski et al., 2006). Another frequently occurring mutation is a deletion of 13 base pairs (c.967-979del13bp). This mutation accounts for e.g. 82% of Irish, 70% of British, 53% of North American, and 48% of Norwegian chromosomes studied, and is also reported in several APECED patients from other populations (Pearce et al., 1998; Scott et al., 1998; Wang et al., 1998a; Heino et al., 1999b; Björses et al., 2000; Dominguez et al., 2006; Wolff et al., 2007). Identification of the patient mutations in APECED has greatly facilitated the diagnosis of APECED patients. Diagnostic DNA testing is currently available at HUSLAB Laboratory of Molecular Genetics in Finland, where the presence of three [c.769C>T (p.R257X); c.967-979del (p.L323SfsX51); c.1163-1164insA (p.M388IfsX36)] APECED mutations is tested (www.huslab.fi), and at the Haukeland University Hospital in Norway where all exons of the *AIRE* gene are sequenced (www.apeced.net).

Most of the mutations in the coding region of *AIRE* are nonsense mutations leading to a premature STOP codon. It is not known whether these messengers are translated into a truncated protein or, as the majority of nonsense transcripts, are degraded via nonsense-mediated mRNA decay. However, there are also several missense mutations in *AIRE*, which we and others have utilized to unravel the functionally critical regions of the AIRE protein (Björses et al., 2000; Pitkänen et al., 2000; Pitkänen et al., 2001; Halonen et al., 2004; Uchida et al., 2004; Pitkänen et al., 2005).

No obvious correlations between the genotypes and phenotypes of the patients have been observed. In a series of 104 Finnish, Swedish, Norwegian, and Italian patients, the only statistically significant association was detected between the absence of the R257X mutation and decreased frequency of mucocutaneous candidiasis (Halonen et al., 2002). In addition, among the Iranian Jewish patients, who all carry the Y85C mutation, mucocutaneous candidiasis is rare (Zlotogora and Shapiro, 1992). The variability in the severity of the disease and the range of affected organs, suggests that in addition to the *AIRE* gene also other genetic or environmental factors contribute to the phenotype of the disease. In the common autoimmune disorders genetic polymorphisms in the HLA (human leukocyte antigen) gene locus encoding the MHC molecules often associate to disease risk. In APECED, individual HLA class II alleles have been shown to modify the APECED phenotype. The most definite associations were for alopecia, Addison's disease and type 1 diabetes, the diabetes association being for a protective allele (Halonen et al., 2002). The same alleles are also found associated with these diseases in non-APECED patients (Maclaren and Riley, 1986; Weetman et al., 1991; Ettinger et al., 1998; Colombe et al., 1999; Yu et al., 1999; Halonen et al., 2004).

4.2 Functional domains

The AIRE protein consists of multiple structural domains which are often present in transcriptional regulators (Consortium, 1997; Nagamine et al., 1997) (Figure 2). All the predicted functional domains are conserved in the mouse homologue of AIRE (Aire) (Blechschmidt et al., 1999; Mittaz et al., 1999; Wang et al., 1999a). Both endogenous and transiently expressed AIRE is detected in the nucleus, where it is mainly distributed as nuclear bodies (NBs) which associate with the nuclear matrix fraction of the cells. In cultured cells expressing tagged or nontagged AIRE either transiently or stably, also even nuclear staining can be detected with or without NBs (Akiyoshi et al., 2004; Ilmarinen et al., 2005; Tao et al., 2006; our unpublished observations). When AIRE is expressed transiently in cultured cells, it is also detected in the cytoplasm associated with intermediate filaments and/or microtubules and aggregates of various sizes (Björses et al., 1999; Rinderle et al., 1999). Cytoplasmic AIRE has been detected in human thymus tissue sections, as well (Cavadini et al., 2005). The N-terminus of AIRE (amino acids 101–141) is able to direct GFP into the nucleus supporting the existence of a NLS within this region (Pitkänen et al., 2001). In addition to a functional NLS, AIRE has been suggested to contain a potential nuclear export signal (NES) in its HSR domain. Supporting evidence for the existence of NES comes from experiments, in which treatment of cells with leptomycin B, a specific inhibitor of Crm1 (chromosome region maintenance 1) -mediated nuclear export, leads to increased nuclear accumulation of AIRE under transient expression conditions (Pitkänen et al., 2001). The nuclear localization signal of AIRE is discussed in detail further on.

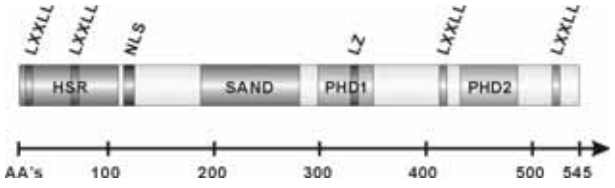

Figure 2. Schematic representation of the functional domains of the AIRE protein.

AIRE shares several domains with members of the Sp100 (Speckled protein 100 kDa) protein family. The Sp100 proteins interact with many transcriptional regulators and are involved in both transcriptional activation and repression (Bloch et al., 2000; Wasylyk et al., 2002; Moller et al., 2003; Ling et al., 2005; Yordy et al., 2005). The homogeneously staining region (HSR) in the N-terminus of AIRE is present also in all the Sp100 splice variants, in which it mediates homodimerization

(Sternsdorf et al., 1999). AIRE is also able to form homomers (Pitkänen et al., 2000; Kumar et al., 2001) and phosphorylation may be involved in this since AIRE oligomerizes spontaneously upon phosphorylation by cAMP dependent protein kinase A or C *in vitro*. Furthermore, thymic AIRE protein has been reported to be phosphorylated at the tyrosine and serine/threonine residues (Kumar et al., 2001). Several of the missense patient mutations are situated in the HSR domain of AIRE and many of them disrupt the homomultimerization, indicating that it is important for the proper functions of AIRE (Halonen et al., 2004). APECED-causing missense mutations predicted to alter the structure of HSR also disturb the nuclear body targeting of AIRE and its localization to high-molecular-weight complexes (Halonen et al., 2004). Although the composition of these AIRE-containing complexes still remains to be solved, it appears that in addition to homomultimerization, the HSR domain of AIRE may also mediate interactions with other proteins, possibly both in the nucleus and in the cytoplasm, since the attachment of AIRE with cytoplasmic filaments has also been proposed to be mediated by the HSR domain (Pitkänen et al., 2001; Ramsey et al., 2002a).

The SAND (Sp100, AIRE, NucP41/75 and DEAF-1) domain is another domain shared by AIRE and several members of the Sp100 family. This domain is characteristic of proteins involved in chromatin-dependent transcriptional regulation, and contains a conserved KDWK sequence motif (Gibson et al., 1998). The three-dimensional (3D) structure of the SAND domain has been solved from Sp100b and GMEB-1 (glucocorticoid-modulatory element-binding protein 1), and the KDWK motif mediates DNA-binding in Sp100b, NUDR (Nuclear DEAF-1 related), and in GMEB-1 (Bottomley et al., 2001; Surdo et al., 2003). The AIRE protein binds TTATTA and ATTGGTTA DNA motifs and its homomultimerization is needed for DNA-binding (Kumar et al., 2001). The SAND domain of AIRE does not contain the KDWK motif (Gibson et al., 1998). Instead, the DNA binding capacity to TTATTA motif appears to reside in the SAND domain involving residues 189-196 (QRAVAMSS) (Purohit et al., 2005).

The two PHD (plant homeodomein) zinc fingers in AIRE further indicated a function for AIRE in transcriptional regulation since these domains are often found in chromatin regulatory factors. Of the Sp100 family members, Sp100C, Sp110, and LYSp100B/Sp140 contain a PHD finger in their C-terminus (Bloch et al., 1996; Dent et al., 1996; Bloch et al., 2000; Seeler et al., 2001). When AIRE is fused to a yeast DNA-binding domain it acts as a powerful transcriptional transactivator, and the PHD fingers, especially the second PHD domain, are important for the transactivation capacity of AIRE (Björses et al., 2000; Pitkänen et al., 2001; Uchida et al., 2004; Meloni et al., 2007). The PHD fingers of AIRE have also been reported to have DNA binding capacity to ATTGGTTA motif (Purohit et al., 2005). In addition, AIRE has been suggested to have E3 ubiquitin ligase activity mediated by

the first PHD finger (Uchida et al., 2004), but this finding is still controversial (Bottomley et al., 2005). Based on the recently determined 3D structure of the first PHD zinc finger of AIRE this domain has been suggested to function in protein-protein interactions (Bottomley et al., 2005).

In addition, the AIRE protein contains four nuclear receptor-binding LXXLL motifs, a leucine zipper (LZ) motif within the first PHD finger, and a proline rich region between the two PHD domains (Consortium, 1997; Nagamine et al., 1997; Mittaz et al., 1999). LZ functions as a dimerization domain in several enhancer-type transcription factors (Landschulz et al., 1988; Deppmann et al., 2006), and it has been speculated that also AIRE could dimerize using the LZ motif (Kumar et al., 2001). Proline-rich motifs have been identified in several transcription factors and often mediate protein-protein interactions in situations requiring e.g. rapid recruitment of several proteins, such as during initiation of transcription, and deletion of this domain can demolish the transactivation capacity of the protein (Kay et al., 2000). The roles of these structural domains in AIRE remain to be experimentally examined. The LXXLL motifs are present in many coactivator proteins, including Sp110 (Bloch et al., 2000), and mediate their binding to nuclear receptors which consequently activates transcription (Heery et al., 1997; Savkur and Burris, 2004). The last LXXLL motif of AIRE was recently shown to be important for the transactivation capacity of AIRE (Meloni et al., 2007). Furthermore, the last 15 amino acids of the AIRE protein also have transactivation potential (Meloni et al., 2007). The importance of this region for the activity of AIRE is further emphasized by the presence of an APECED-causing missense mutation within this region (Meloni et al., 2002) and the ability of this mutation to severely disturb the transactivation capacity of AIRE *in vitro* (Meloni et al., 2007). In addition, several APECED-causing missense mutations in the HSR domain also disrupt the transactivation capacity of AIRE (Halonen et al., 2004).

4.3 Functions of AIRE

4.3.1 Aire and promiscuous gene expression in the thymus

The generation of several Aire-deficient mouse models has enabled further studies in the context of a whole organism. The disease in Aire-knockout mice on a C57BL/6x129/Sv genetic background is mild, although the mice show some autoimmune manifestations, such as multi-organ lymphocytic infiltrations and autoantibodies against several organs (Anderson et al., 2002; Ramsey et al., 2002b). The outcome is more severe on the nonobese diabetic background (Jiang et al., 2005). However, the similarity between the clinical outcome of AIRE deficiency in humans and mice has been questioned (Kekäläinen et al., 2007a).

Expression of both human and mouse AIRE/Aire protein is most abundant in immunologically relevant tissues, such as thymus, lymph nodes and spleen (Björses

et al., 1999; Heino et al., 1999a). The *AIRE* gene expression has also been detected in cells of the monocytic-dendritic cell lineage both in the thymus and in the periphery (Heino et al., 1999a; Kogawa et al., 2002; Sillanpää et al., 2004). In mouse, expression of the Aire protein has also been detected in several non-immunological tissues (Halonen et al., 2001). In the thymus, where AIRE/Aire is mainly expressed, expression localizes to medulla and the corticomedullary junction and is highest in mTECs (Björses et al., 1999; Heino et al., 1999a; Heino et al., 2000; Zuklys et al., 2000; Halonen et al., 2001). Since mTECs had been shown to express a vast range of tissue-specific antigens (Derbinski et al., 2001), and *in vitro* studies had shown that AIRE has transcriptional transactivation potential (Björses et al., 2000; Pitkänen et al., 2001), Klein *et al.* (2000) and Anderson *et al.* (2002) hypothesized that AIRE may regulate the ectopic transcription of genes encoding peripheral tissue-restricted antigens in mTECs (Klein and Kyewski, 2000; Anderson et al., 2002). Indeed, subsequent microarray and expression studies done with $Aire^{-/-}$ mice have shown that the expression of a substantial subset (hundreds or thousands of genes) of tissue-specific antigens in the mTECs is lost in the absence of Aire (Anderson et al., 2002; Derbinski et al., 2005). Some of these genes encode proteins known to be targets of autoantibodies in APECED patients, such as insulin (Gylling et al., 2000). Furthermore, Aire deficiency in mice causes almost complete failure to delete self-specific lymphocytes in the thymus (Liston et al., 2003). Additional support for the hypothesis that the widespread autoimmunity of APECED patients is caused by the lack of thymic ectopic expression and subsequently loss of tolerance to these antigens comes from recent studies indentifying two Aire-dependent antigens, a stomach and an eye antigen. In these studies, the authors presented a link between the lack of expression of the antigens in the thymus and the generation of pernicious autoantibodies against them in the peripheral tissues of Aire-deficient mice (DeVoss et al., 2006; Gavanescu et al., 2007). Recently, Aire was shown to directly bind the promoters of some of its target genes *in vivo*, and regulate their expression in the thymus (Ruan et al., 2007).

4.3.2 Other potential functions of Aire

Regulating the expression of peripheral tissue-specific antigens in mTECs may not be the only function of the Aire protein. Recently, Aire-expressing mTECs were reported to have a high turnover and the authors suggested that by inducing apoptosis of the mTECs, Aire would facilitate cross-presentation of the self-antigens produced (Gray et al., 2007). Aire-deficient mice also develop autoimmunity against antigens, such as α-fodrin, that are still expressed in the thymi of $Aire^{-/-}$ mice (Kuroda et al., 2005; Niki et al., 2006). Possible explanation for this could be the proposed role of Aire in antigen processing and presentation; perhaps the potential function of AIRE as a ubiquitin E3 ligase plays a role in this (Uchida et al., 2004).

Expression arrays on Aire$^{+/+}$ and Aire$^{-/-}$ mTECs show differences also in genes such as H2-M, which is a facilitator of MHC class II peptide loading, and its negative modulator H2-O. Aire$^{-/-}$ mTECs also appear to be less effective antigen presenters than Aire$^{+/+}$ mTECs (Anderson et al., 2005). It may also well be that the functions of Aire extend to the peripheral control of self-reactive T cells escaping the thymic negative selection, as AIRE/Aire has been reported to be involved in DC (Sillanpää et al., 2004) and Treg differentiation, and in the regulation of T cell activation by peripheral DCs. Although in mice Aire deficiency does not lead to obvious abnormalities in the Foxp3 Treg population (Anderson et al., 2005; Kuroda et al., 2005; Niki et al., 2006), in the APECED patients the Foxp3 Tregs are defective (Kekäläinen et al., 2007b). Furthermore, a study by Aschenbrenner *et al.* (Aschenbrenner et al., 2007) suggests that Aire-expressing mTECs would be involved in the development of Tregs in the thymus. Aire may also control the T cell activation by DCs in the periphery, since Aire$^{-/-}$ DCs activate naïve T cells more efficiently than do their Aire-expressing counterparts (Ramsey et al., 2006). Aire expression in lymph nodes was recently localized to stromal cells. Interestingly, these cells also express several tissue-specific antigens and directly present them to CD8^{+} T cells leading to clonal expansion and subsequent elimination of the T cells, a mechanism possibly reinforcing the peripheral tolerance induced by cross-presentation by DCs (Lee et al., 2007; Zehn and Bevan, 2007).

5 NUCLEAR PROTEIN TRANSPORT

The spatial separation of genetic material and transcription from the rest of the cell by nuclear envelope affords a mechanism to control different nuclear functions including gene expression. Transcription factors and other nuclear proteins are synthesized in the cytoplasm and have to be transported into the nucleus to execute their functions. Nucleocytoplasmic shuttling also operates as a mechanisms for regulating the activity of transcription factors (Rothwarf and Karin, 1999; Reich and Liu, 2006). Exchange of proteins and RNA between the nucleus and the cytoplasm occurs through the nuclear pore complexes (NPC) which are composed of ~30 different proteins called nucleoporins embedded in the nuclear envelope (Cronshaw et al., 2002). To reach the nucleus, macromolecules larger than ~40 kDa need an active, signal-mediated transport mechanism facilitated by soluble carrier proteins that cycle between the nucleus and cytoplasm. Several pathways for nucleocytoplasmic transport have been described, each transporting a specific range of molecules. In all transport pathways, a signal sequence in the cargo proteins targeting them to either nuclear import (nuclear localization signal, NLS) or export (nuclear export signal, NES) is required (Paine et al., 1975; Gorlich and Kutay, 1999; Pemberton and Paschal, 2005). Regulating the activity of the NLS by protein modifications such as phophorylation modulating its affinity to import receptors or by masking the signal via binding of an inhibitory

protein is one of the key control points in nuclear import (Jans et al., 2000; Poon and Jans, 2005). The nuclear transport of most proteins is mediated by an evolutionary conserved karyopherin-β family of carrier molecules, containing both import carriers (importins) and export carriers (exportins). The β-karyopherins can bind the localization signals in their cargoes directly or via an adapter protein (Weis, 1998; Pemberton and Paschal, 2005).

The best characterized transport system is the classical nuclear import pathway (Stewart, 2007). It has been estimated that ~100-1,000 cargoes would be transported via this pathway per minute per NPC (Ribbeck and Gorlich, 2001). The classical NLS consists of one (monopartite) or two (bipartite) clusters of basic amino acids separated by a 10–12 amino-acid linker. The simian virus 40 large T antigen (SV40 T Ag) NLS (PKKKRKV) is considered as the prototype of a monopartite NLS and the nucleoplasmin NLS (KRPAATKKAGQAKKKK) as a typical bipartite NLS (Kalderon et al., 1984; Dingwall and Laskey, 1991; Robbins et al., 1991). In the classical import pathway, cargo proteins containing the classical NLSs are recognized by an adapter protein importin α. The importin α molecules have a large NLS-binding domain consisting of 10 tandem, highly structured armadillo (ARM) repeats (Peifer et al., 1994). The NLS-binding domain can accommodate either a single bipartite or two monopartite classical NLS peptide(s) at two sites consisting of ARM repeats 2–4 (the major site) and 7–9 (the minor site). The C-terminal cluster of a bipartite NLS usually occupies the major site, while the N-terminal cluster is recognized by the minor site. Monopartite NLSs can bind to both of these sites but predominantly use the binding site formed by ARM repeats 2–4 of importin α (Conti et al., 1998; Conti and Kuriyan, 2000; Fontes et al., 2000; Melen et al., 2003). Altogether six importin α molecules (α1, α3-α7) have been identified in humans (Cortes et al., 1994; Cuomo et al., 1994; Kohler et al., 1997; Seki et al., 1997; Kohler et al., 1999). The importin α proteins are structurally and functionally conserved and, on the basis of their sequence similarity, can be divided into three subfamilies (importin α1-containing; importins α3- and α4-containing; importins α5-, α6-, and α7-containing). Within one subfamily, the identity of the proteins is over 80% and they differ mainly in the sequence outside the NLS-binding regions (Kohler et al., 1999; Melen et al., 2003).

Once importin α has bound the cargo, the importin α-cargo complex is bound to importin β by the N-terminal importin β-binding (IBB) domain of importin α (Gorlich et al., 1996). Importin β mediates interactions with the NPC and translocates the import complex into the nucleus in a RanGTPase-dependent manner (Gorlich et al., 1995; Izaurralde et al., 1997). In the nucleus, importin β is bound by RanGTP which results in a conformational change and the release of importin β from the importin α-cargo complex (Lee et al., 2005). The affinity of importin α to the cargo is decreased by the now free IBB domain which competes for binding to

importin α with the cargo-NLS, and the binding of nucleoporin Nup50 to importin α (Kobe, 1999; Matsuura and Stewart, 2005). Finally, importins are recycled back to the cytoplasm, importin β by RanGTP and importin α by CAS (cellular apoptosis susceptibility) (Kutay et al., 1997; Stewart, 2007) (Figure 3).

Of the nuclear export signals, the hydrophobic NES is the best characterized. It typically contains three to four hydrophobic residues, often leucines, and is recognized by the karyopherin Crm1 co-operatively with RanGTP, forming a trimeric export complex. In the cytoplasm, release of the cargo is mediated by hydrolysis of the Ran-GTP. Crm1-mediated nuclear export resembles the export of importin α by CAS (Figure 3). After cargo release, the exportin presumably returns empty to the nucleus (Fornerod et al., 1997; Stade et al., 1997; Sebastian et al., 2004; Pemberton and Paschal, 2005).

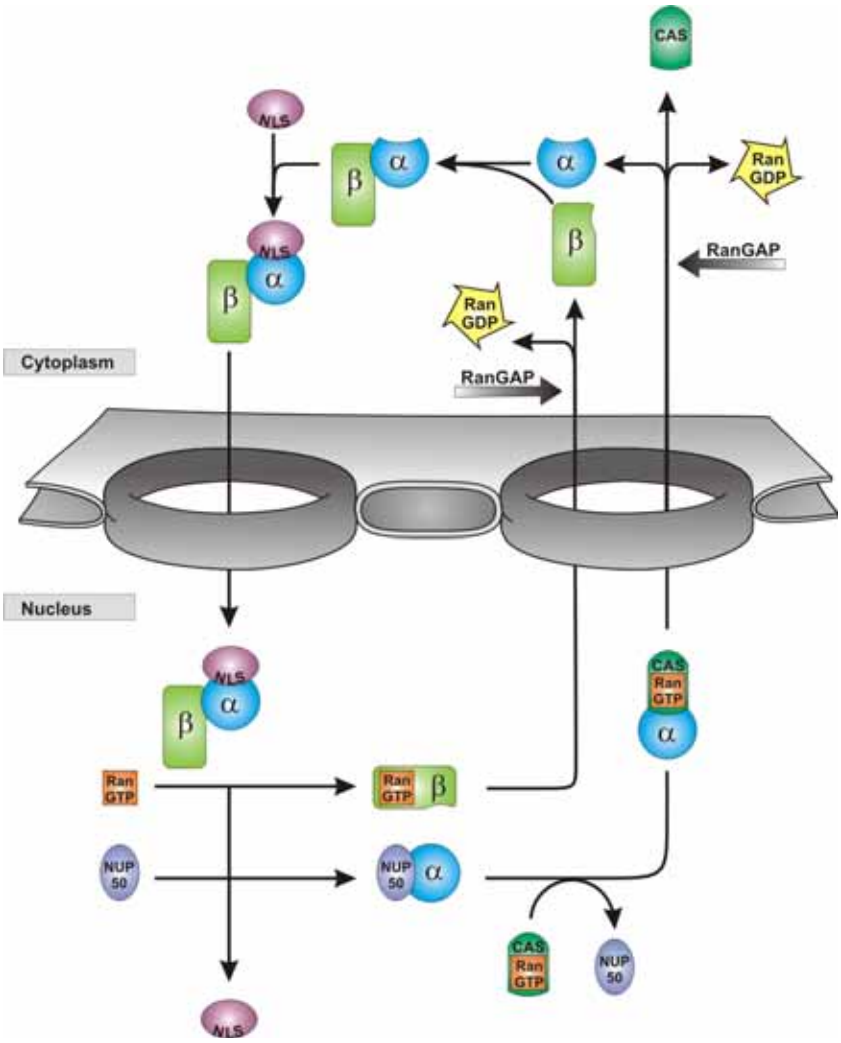

Figure 3. Schematic representation of the classical nuclear protein import pathway. The assembly status of the transport receptor–substrate complexes and the directionality of transport are regulated by the small GTPase Ran. Ran locates predominantly in the nucleus bound to GTP, whereas cytoplasmic Ran is in the GDP form. Cargoes with classical nuclear localization signals bind to the heterodimeric importin-α/-β receptor. After entering the nucleus through the nuclear pore complex, RanGTP binds to importin-β and dissociates it from importin-α. Nucleoporins like Nup50 catalyse dissociation of the cargo. Importin β is recycled back to the cytoplasm by RanGTP and importin α by its export receptor CAS in a complex with RanGTP. In the cytoplasm, the complexes are disassembled by GTP hydrolysis of Ran stimulated by RanGAP. Modified from Stewart *et al*. (2007).

6 FUNCTIONAL ORGANIZATION OF GENE-REGULATORY MACHINERY IN NUCLEUS

In order to ensure correct spatial and temporal expression of the 20,000–25,000 protein-coding genes and an ever growing number of different non-coding RNA genes of the human genome, transcription must be strictly regulated (Gustincich et al., 2006). There are still many open questions on how expression of genes is organized. However, it is clear that the mammalian nucleus is highly compartmentalized. For example transcription by RNA polymerase II (Pol II) takes place in several thousand distinct sites scattered across the nucleus (Wansink et al., 1993). These sites are highly enriched in Pol II and have been suggested to represent "transcription factories" where active polymerases remain immobilized while the templates are moved through them (Cook, 1999). Because there are fewer transcription factories than active genes, it appears that several genes are transcribed in each factory (Wansink et al., 1993; Cook, 1999). These genes can be situated far apart as it has been shown that genes separated by up to 40 Mb of chromosomal sequence or even genes in different chromosomes frequently colocalize in the same transcription factory (Osborne et al., 2004). In addition to gene expression, also DNA replication and repair are found in distinct subnuclear structures (Stein et al., 2003; Misteli, 2007).

Regulating the chromatin structure is an important mechanism affecting the transcriptional status of a cell. Histones are targets for several modifications affecting higher-order chromatin structure and the accessibility of different molecules, such as transcription factors and RNA polymerases, to the regulatory DNA sequences. These modifications include among others methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and remodeling by ATP-dependent enzymes. The localization of the modification is essential for its effect on transcription and tightly regulated (Li et al., 2007). Furthermore, the dynamic spatial organization of regulatory proteins and DNA within the nucleus has turned out to be essential. Two models have been suggested to explain the organization and function of genomes: a deterministic model and a self-organizing model. In the deterministic model functional sites are preformed and chromosomes interact with a nuclear scaffolds such as the lamin network (Misteli, 2007). According to self-organization model transcription factors are diffused around the nucleus scanning for their binding sites by a hit-and-run mechanism and gather around the genes upon their activation for a very short period of time. The stable structures in the nucleus may serve as assemble platforms but are not absolutely required as chromatin might be sufficient to serve this function (McNally et al., 2000; Misteli, 2001; Becker et al., 2002; Cook, 2002; Misteli, 2007) (Figure 4).

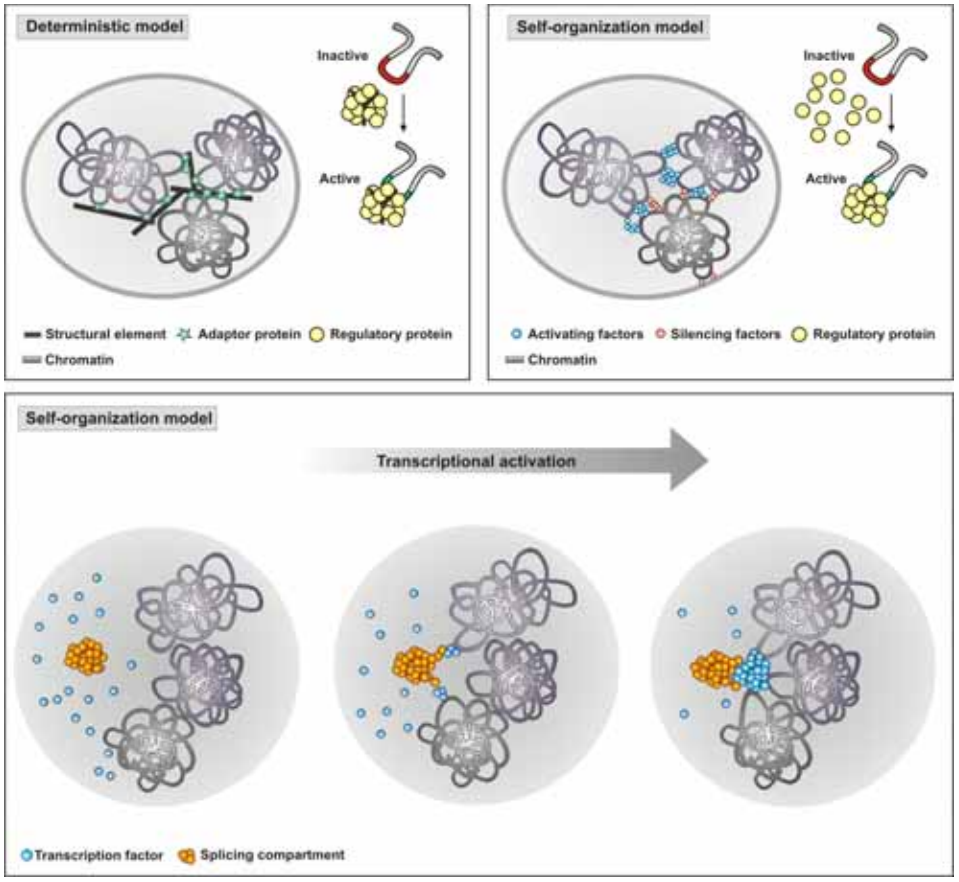

Figure 4. Models of nuclear organization. (Top) In a deterministic model functional sites that provide the environment for nuclear processes are preformed and contain structural elements. Chromosome position is maintained by interactions with a scaffold. In a self-organization model the functional site forms around the gene upon its activation. Chromosome position is established by chromatin itself by interactions with functional sites. (Bottom) Transcription factors diffuse freely in the nucleoplasm as they search for their binding sites. Upon activation chromatin is remodeled and transription factors gradually recruited to the gene(s) for formation of a transcription hub onto which splicing factors are recruited from their storage compartments. Modified from Misteli (2007).

6.1 Small ubiquitin-like modifier (SUMO) pathway

An increasing amount of evidence suggests that SUMO may be one of the key molecules in regulating the organization of chromatin and subnuclear compartments (Heun, 2007). SUMO belongs to a class of proteins having a common 3D structure with ubiquitin (Muller et al., 2001). The SUMO conjugation (sumoylation) pathway involves an E1 activating enzyme, an E2 conjugating enzyme Ubc9, and E3 ligases

and SUMO proteases. For conjugation, E1 activates the C-terminus of mature SUMO, activated SUMO is then transferred to Ubc9 which in conjunction with the substrate recognizing E3 ligase conjugates SUMO to the target protein (Dohmen, 2004). The ubiquitin and sumoylation systems can also have mutual interactions as same substrates can be both ubiquitinated and sumoylated, sometimes even in the same residue (Ulrich, 2005). So far four different SUMO genes have been identified in humans (Muller et al., 2001; Bohren et al., 2004). SUMO-2 and -3 can form polychains but SUMO-1 cannot and SUMO-4 may not be able to form covalent intractions with substrates at all (Tatham et al., 2001; Owerbach et al., 2005). Several proteins can also interact with SUMO noncovalently via a SUMO interacting motif (SIM) (Minty et al., 2000). At least SUMO1-3 are able to bind SIM (Song et al., 2004; Hecker et al., 2006).

In all, the SUMO pathway has been linked with several consequences: it can e.g. facilitate or inhibit protein-protein and protein-DNA interations, affect enzymatic activity or half-life of proteins, or change their subcellular localization (Herrmann et al., 2007). Studies done with Ubc9-deficient mice show that Ubc9 is essential for nuclear integrity and thus viability of the mice (Nacerddine et al., 2005). Similarly, mutation in a yeast E3 ligase Su(var)2-10 leads to defects in chromosome structure and lethality (Hari et al., 2001). Flaws in the SUMO protease pathway can also lead to severe nuclear defects (Di Bacco et al., 2006).

6.2 Nuclear bodies

Several nuclear proteins, including general and gene-specific transcription factors, are often detected as dotted structures called nuclear bodies (NBs). Additionally, many transcription-related proteins also localize to other nuclear domains, such as the nucleoli, Cajal bodies or gems, speckles, or promyelocytic leukemia bodies. Although many NBs can be localized in the vicinity of certain genes, they rarely appear to be active sites of transcription and the role played by these structures in transcriptional regulation is still not clear (Zimber et al., 2004; Corry and Underhill, 2005). There are several examples of a dynamic relationship between different nuclear subdomains and they are often found associated with each other (Grande et al., 1996; Wang et al., 2002). Changes in the composition and organization of these subnuclear structures are also found in many human diseases (Zimber et al., 2004).

6.2.1 Nucleolus

The nucleolus is the most noticeable and extensively studied subnuclear organelle. It is formed around the ribosomal RNA (rRNA) genes, which cluster at chromosomal loci called nucleolar organizing regions. A main function of the nucleolus is the production of 28S, 18S and 5.8S ribosomal RNAs and assembly of ribosomal subunits (Miller, 1981). In addition to being a ribosome factory, the nucleolus has

been suggested to be involved in various other cellular events, including regulation of cell-cycle progression and proliferation, many forms of stress response and the processing and/or maturation of different ribonucleoproteins (RNPs), including the signal recognition particle (SRP) (Boisvert et al., 2007). In some cases the nucleolus appears to regulate protein activity by retaining them until needed elsewhere (Maser and DePinho, 2002; Wsierska-Gadek and Horky, 2003). The nucleolus has also been linked to several human diseases. Many proteins defective in genetic disorders associate with nucleoli (Marciniak et al., 1998; Heiss et al., 1999; Isaac et al., 2000; Woo et al., 2006), whereas many forms of cancer and viral infections affect nucleolar structure or the biogenesis of ribosomes (Ochs et al., 1994; Dove et al., 2006; Boisvert et al., 2007).

6.2.2 Cajal bodies and gems

Cajal bodies (CBs, also known as coiled bodies) are highly dynamic structures present in most eukaryotic cells associated with the nucleolus or located in the nucleoplasm (Hardin et al., 1969; Raska et al., 1990; Gall et al., 1995; Boudonck et al., 1999). There are typically one to six CBs per nucleus (Andrade et al., 1993). CBs contain many proteins that are typically found in the nucleolus, including the P80-coilin characteristic for these structures (Andrade et al., 1991; Raska et al., 1991). One of the proposed functions for CBs is participation in the biogenesis of nuclear snRNPs and snoRNPs. After initial assembly in the cytoplasm, snRNPs return to the nucleus, where they accumulate in CBs probably for further modification prior to their release to splicing factor compartments (Sleeman and Lamond, 1999). In addition to the role in the maturation of nuclear RNPs, several other functions have been postulated for CBs, such as integration of cell cycle progression, involvement in the cellular stress responses, and telomerase RNA biogenesis (Cioce and Lamond, 2005). Many RNA pol II transcription factors have also been found enriched in CBs (Jordan et al., 1997). Although CBs do not contain DNA or nascent RNA, active genes have been found localized in the immediate vicinity of CBs in the nucleoplasm (Raska, 1995; Jordan et al., 1997; Cmarko et al., 1999). For example, histone gene clusters are frequently found adjacent to CBs, which are enriched in U7 snRNA required for 3' processing of the histone transcripts, suggesting a role for CBs in the production and maturation of transcripts from nearby genes (Melin et al., 1992; Frey and Matera, 1995). Additionally, the 100 kD subunit of the cleavage and polyadenylation specificity factor (CPSF) and the 64 kD subunit of the cleavage stimulation factor (CstF) localize in small spherical domains called cleavage bodies. The cleavage bodies often localize adjacent to coiled bodies, but can also be found partially or completely overlapping with them. The cleavage bodies adjacent to coiled bodies contain newly synthesized RNA, while inhibition of RNA polymerase II transcription results in complete

colocalization of coiled bodies and cleavage bodies (Schul et al., 1996). Thus, Schul *et al.* (1996) have proposed a model according to which the cleavage factors CstF 64 kD and CPSF 100 kD are concentrated in CBs and can be distributed to one or more active genes adjacent to the CB, but relocate to the CB when the genes are inactive. Gems (gemini satellites of Cajal body) are structures which pair frequently with Cajal bodies and have been suggested to be manifestations of the same structure. Gems contain the survival of motor neurons (SMN) protein, defective in a severe inherited form of human muscular wasting disease, spinal muscular atrophy (Hebert et al., 2001). SMN interacts with coilin, and the methylation status of coilin affects this interaction. Inhibition of the methylation of coilin decreases its interaction with SMN, resulting in the formation of gems that are separate from Cajal bodies. When coilin is methylated, Cajal bodies and gems merge again (Hebert et al., 2002).

6.2.3 Splicing speckles

In the eukaryotic nucleus, the pre-mRNA splicing factors localize mostly to distinct sites called speckles or SC35 domains, smaller proportion being diffusely distributed throughout the nucleus. About 10-50 nuclear speckles per nucleus can usually be visualized with an antibody against the spliceosome assembly factor SC35 (Fu and Maniatis, 1990). In addition to SC35, they contain poly(A) RNA and many proteins involved in the transcription, processing, and export of mRNAs (Carter et al., 1991; Carter et al., 1993; Xing et al., 1993; Hall et al., 2006). Although speckles themselves are not very mobile, the molecules in these structures are, moving in and out of speckles (Kruhlak et al., 2000; Phair and Misteli, 2000). Several active genes, many of which are highly expressed, are often found adjacent to nuclear speckles associating with the outer periphery of the domain but not the interior. Furthermore, corresponding mRNA has been detected within the SC35 speckles (Smith et al., 1999; Shopland et al., 2002; Moen et al., 2004). However, for the pre-mRNAs studied in detail, splicing is essentially completed before the mRNA enters the domain interior (Johnson et al., 2000). There are also evidence linking SC35 speckles to the export of mRNA. For example, various factors involved in mRNA export localize to these domains and a significant amount of the poly(A) containing RNA in the nucleus transits through them prior to export out of the nucleus (Zhou et al., 2000; Le Hir et al., 2001; Molenaar et al., 2004). Furthermore, it has been suggested that speckles may function as checkpoints in which mRNAs are screened (Molenaar et al., 2004) and if inappropriately processed detained, possibly for nonsense mediated decay (Kim et al., 2001). Based on these and other observations, Hall *et al.* (2006) have hypothesized that splicing speckles are subcompartmentalized, with transcription and splicing taking place at their periphery. Subsequently, more mature mRNA accumulate within the domains, a step perhaps required for screening of the mRNA or binding of export factors (Hall et al., 2006).

6.2.4 Promyelocytic leukemia (PML) bodies

The PML (promyelocytic leukemia) bodies are one of the most intensively studied nuclear domains. PML was originally identified in patients with acute promyelocytic leukemia (APL) and to date, seven isoforms have been identified (Jensen et al., 2001). PML associates to nuclear bodies also known as PML oncogenic domains, Kremer bodies or nuclear domain 10 (Ascoli and Maul, 1991; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). A vast majority of the APL patients have a chromosomal break t(15;17) that leads to the fusion of the *PML* gene with the *retinoic acid receptor* α gene (*RAR*α) and to the generation of oncogenic chimeric PML-RARα protein and disruption of the PML NBs (Rowley et al., 1977; de The et al., 1990; Goddard et al., 1991; Kakizuka et al., 1991). Administration of retinoic acid to the patients results in the reappearance of the PML bodies and remission of the patients (Weis et al., 1994). PML-deficient mice do not get spontaneous cancer, but are more susceptible to tumor formation when exposed to carcinogens and also to certain spontaneous infections (Wang et al., 1998b).

There are usually 5-30 PML bodies per nucleus and they can be found in most cell types, but apparently only in higher eukaryotes (Ascoli and Maul, 1991; Koken et al., 1995; Borden, 2002). In addition to NB association, diffuse nuclear distribution and cytoplasmic isoforms of PML have also been observed (Salomoni and Bellodi, 2007). PML NBs are dynamic, motile structures and their size, number and composition varies depending on the cell type, during the cell cycle and in response to different cellular stresses such as heat shock, IFN treatment and viral infection (Everett et al., 1999; Regad and Chelbi-Alix, 2001; Muratani et al., 2002; Nefkens et al., 2003). More than 40 different proteins have been reported to colocalize with PML NBs. In addition to PML, proteins constitutively present in these NBs include Sp100 and SUMO. PML is the main component of these NBs and in $PML^{-/-}$ cells no PML bodies are present and many of the other component proteins show more diffuse localization and no longer colocalize with each other. Furthermore, sumoylation of PML is essential for the NB formation (Zhong et al., 2000). PML also binds SUMO non-covalently and the SIM is also required for PML NB formation (Shen et al., 2006). Also other components of the PML NBs are modified by SUMO, including Sp100 (Sternsdorf et al., 1997b).

The highly variable morphology of the PML NBs have led to a vast number of potential functions for these structures, including roles in apoptosis, antiviral defence, DNA repair and replication, transcription, RNA stability and RNA transport (Strudwick and Borden, 2002). Especially the transcriptional role of the PML NBs has been vigorously debated and there are contradictory reports about whether or not the PML NB is a site of transcriptional activity. Some nascent RNA polymerase II transcripts have been found within PML NBs (LaMorte et al., 1998). In many cases sites of active transcription are situated in the periphery of the NBs,

but PML NBs are not themselves sites of transcription for these genes (Boisvert et al., 2000; Kiesslich et al., 2002; Wang et al., 2004). However, the localization of PML bodies to transcriptionally active sites may be cell cycle dependent, since in G1 phase of the cell cycle a majority of PML bodies were found to contain active transcription foci. These findings have led to the suggestion that PML NBs may be recruited to up-regulated transcription where they may serve as a scaffold for transcriptional regulators (Kiesslich et al., 2002). The PML NBs have also been suggested to function as protein reservoirs adjusting the levels of active molecules in the nucleoplasm, thus possibly controlling the availability of e.g. transcription factors to active chromatin domains (Chalkiadaki and Talianidis, 2005; Kumar et al., 2007). For example the sumoylated form of orphan nuclear receptor LRH-1 (liver receptor homologue 1) localizes exclusively in PML NBs. Desumoylation of LRH-1 leads to its release from the NBs and association with actively transcribed target genes (Chalkiadaki and Talianidis, 2005). PML has also been associated to chromatin modifications, it can e.g. interact with histone deacetylases (HDACs) *in vivo* and modulate histone deacetylation thus silencing transcription (Wu et al., 2001). Furthermore, a recent finding links PML to higher-order chromatin organization via its physical interaction with SATB1 (special AT-rich sequence binding protein 1) (Kumar et al., 2007). SATB1 organizes chromatin into distinct loops by anchoring matrix attachment regions (MARs) to the nuclear matrix (Cai et al., 2003). In the study by Kumar *et al*. (2007), PML and SATB1 were shown to work together to organize the MHC-I locus into this chromatin-loop structure by tethering MARs to the nuclear matrix. IFNγ treatment and silencing of SATB1 or PML altered the chromatin architecture, and affected the expression profile of a subset of MHC class I genes in a PML isoform-dependent manner (Kumar et al., 2007).

7 PIAS PROTEIN FAMILY

The PIAS (protein inhibitor of activated STAT) proteins are transcriptional coregulators originally identified as inhibitors of cytokine signaling mediated by STAT (signal transducer and activator of transcription) and named accordingly (Chung et al., 1997; Liu et al., 1998). To date, the activity of over 60 proteins is known to be regulated either positively or negatively by members of the PIAS family. A substantial number of the target proteins are transcription factors, and many function in immunological pathways. The sites mediating interaction with the target proteins localize throughout the length of the PIAS proteins, with no obvious hotspots for interaction (Schmidt and Muller, 2003; Shuai and Liu, 2005).

The mammalian PIAS family consists of four members: PIAS1, PIAS3, PIASx (also known as PIAS2) and PIASy (also known as PIAS4) which share several conserved domains (Chung et al., 1997; Valdez et al., 1997; Wu et al., 1997; Liu et al., 1998; Moilanen et al., 1999; Sturm et al., 2000). The most conserved

regions are the N-terminus containing a SAP domain and the central RING finger-like domain (SP-RING) (Shuai and Liu, 2005). The SAP domain (scaffold-attachment factor A and B, apoptotic chromatin-condensation inducer in the nucleus and PIAS domain) situated in the N-terminus of the PIAS proteins is present in many chromatin-binding proteins and binds (A+T)-rich DNA sequences found in MARs (Aravind and Koonin, 2000; Kipp et al., 2000). The SAP domain of PIAS1 and PIASy can also bind matrix-attachment DNA and PIASy has been shown to associate with nuclear matrix fraction of the cell *in vivo* (Sachdev et al., 2001; Okubo et al., 2004).

The SP-RING of the PIAS proteins resembles the RING domain present in many ubiquitin E3 ligases and all mammalian PIAS proteins have been found to function as E3 SUMO ligases. The SP-RING domain of PIAS proteins facilitates the transfer of SUMO from Ubc9 to substrates and is in most cases needed for their SUMO ligase activity (Kahyo et al., 2001; Sachdev et al., 2001; Kotaja et al., 2002; Deng et al., 2007). In addition to the SP-RING domain, the PINIT motif present in all the other PIAS proteins but the PIASyE6– splice variant has also been reported to be required for the SUMO ligase activity of Siz1, a yeast PIAS-like protein (Takahashi and Kikuchi, 2005). Most of the interaction partners for PIAS proteins undergo SUMO modification and the PIAS proteins themselves can also be sumoylated. However, the SUMO-modified residues in the PIAS proteins still remain to be mapped (Kotaja et al., 2002). The PIAS proteins also interact with other SUMO-modified proteins and free SUMO in a noncovalent fashion, most likely via the SIM situated within the acidic domain of all other PIAS proteins except PIASy and its isoform (Kotaja et al., 2002). The SIM motif is not needed for E3 SUMO ligase activity and the functional role of this motif remains to be further defined (Sachdev et al., 2001; Kotaja et al., 2002; Wong et al., 2004).

There are also several examples in which the regulatory effect of PIAS proteins on their substrate protein is separate from the sumoylation of the target and the PIAS proteins appear to have functions beyond their E3 ligase activity (Gross et al., 2004; Nishida et al., 2006; Sharrocks, 2006). Proposed mechanisms include blocking the DNA binding of a transcription factor, for example PIAS1 can inhibit the DNA binding of STAT1 (Liu et al., 1998). The PIAS proteins may also recruit other coregulators. A PIASx isoform PIASxβ and PIASy can interact with histone deacetylases (HDACs) and HDAC inhibitor abolishes the ability of PIASx to repress STAT4- and PIASy to repress SMAD3- or androgen receptor-mediated gene activation (Arora et al., 2003; Long et al., 2003; Gross et al., 2004). On the other hand, e.g. PIAS3 can recruit p300 or CBP (cyclic-AMP-responsive-element binding protein (CREB)-binding protein) to activate transcription (Long et al., 2004). A third mechanism by which PIAS proteins can mediate their functions is changing the subcellular localization of their targets. Msx1 homeodomain protein is a repressor of

muscle-specific genes. Msx1 is modified by SUMO but sumoylation appears to have little effect on the transcriptional repressive properties of Msx1. Interaction of Msx1 with PIAS1 is also required for the localization of Msx1 to the nuclear periphery where it interacts with its target genes keeping them inactive. Thus, it appears that it is the targeting of Msx1 to correct subnuclear compartment rather than sumoylation by PIAS1 that regulates its activity. Furthermore, after the removal of PIAS1, binding of Msx1 to its target promoters was not inhibited, but binding was shifted to more proximal promoter regions suggesting that PIAS1 affects the DNA binding specificity of Msx1 (Lee et al., 2006). In the case of LEF1 (lymphoid enhancer factor 1), PIASy represses its activity and targets it to nuclear bodies. Although LEF1 can be modified by SUMO and the interaction between PIASy and LEF1, repression of LEF1 activity, and targeting of LEF1 to nuclear bodies require an intact SP-RING domain of PIASy, they do not require the sumoylation sites in LEF1. Furthermore, LEF1 and PIASy colocalized with Sp100, suggesting that PIASy targets LEF1 to a subset of PML nuclear bodies (Sachdev et al., 2001).

AIMS OF THE STUDY

Due to its monogenic nature, APECED provides an intriguing model for autoimmunity. Since the identification of *AIRE* as the gene defective in APECED, both *in vitro* and *in vivo* studies indicate a function for AIRE as a transcriptional regulator. During the course of this study, it became evident that Aire regulates the expression of hundreds, if not thousands of genes. Furthermore, it appears that Aire can both up- and downregulate its target genes. However, the underlying molecular mechanisms of this regulation have remained largely obscure.

The aims addressed in this thesis study were:

- To experimentally evaluate the molecular mechanisms behind an exceptional dominant inheritance of APECED in patients heterozygous for a G228W mutation in the SAND domain of AIRE
- To analyze the potential consequences of other patient missense mutations in the SAND and HSR domain to the wt AIRE protein in a heterozygous situation *in vitro*
- To deliniate the nuclear localization signal of AIRE and identify the nuclear import receptors for AIRE
- To gain more knowledge about the molecular mechanisms of how AIRE controls the expression of its target genes by identifying novel protein interaction partners

MATERIALS AND METHODS

Materials and methods used in this thesis are presented in the original publications.

Table 3. Published materials and methods used in this study

Material or method	Original publication
analysis of molecular complexes	I
antibody production	I, III
plasmid construction and DNA sequencing	I, II, III
in vitro mutagenesis	I, II, III
cell culture	I, II, III
transfections	I, II, III
transactivation assay	I, III
yeast two-hybrid screening	II, III
mammalian two-hybrid assay	I, III
immunoprecipitation	I, III
protein production by *in vitro* transcription-translation	I, II, III
protein production in *E. coli*	II, III
protein detection by immunofluorescence	I, II, III
protein detection by autoradiography	I, II, III
protein detection by Western analysis	III
confocal microscopy	I, II, III
GST pull-down assay	II, III
image analysis	III
nuclear matrix extraction	III
sumoylation assays	III

RESULTS

1 FUNCTIONAL CONSEQUENCES OF AIRE MUTATIONS IN A HETEROZYGOUS SITUATION (I)

Preceeding this work, Cetani *et al.* (2001) identified an Italian family in which APECED appeared to be inherited dominantly. All of the seven affected family members had hypothyroid autoimmune thyroiditis (hAT). Three of them also expressed other components of the APECED phenotype. Genetic analysis of the *AIRE* gene revealed a novel missense mutation G228W in a heterozygous form in all the affected individuals (Cetani et al., 2001). Due to the homomeric nature of AIRE, it is possible that certain mutations can have a dominant negative effect. Thus, we proceeded to analyze the functional consequences of the G228W mutation on the wild-type (wt) AIRE in a simulated heterozygous situation *in vitro*. Since the G228W mutation locates in the SAND domain, we also studied the other missense patient mutation identified in this domain, P252L, and an artificial double mutation K243A+R247A affecting positively charged amino acids in the potential DNA binding region of the SAND domain. For comparison, we examined two HSR domain patient missense mutations, L28P and Y85C, with different predicted structural consequences.

1.1 The G228W mutant prevents wt AIRE from localizing to NBs

The effects of the mutant AIRE proteins on the subcellular localization of the wt AIRE were examined by cotransfecting human colon epithelial Caco-2 cells with expression constructs producing the wt AIRE as GFP fusion and mutant proteins with FLAG tags. We first analyzed the possible effects of the GFP- and FLAG-tagging on the subcellular distribution of AIRE. Dotted nuclear staining similar to the one detected with nontagged AIRE was observed with both GFP-AIRE and FLAG-AIRE. When both wt fusion proteins were coexpressed in the same cell, the distribution of the GFP-AIRE remained unchanged (Figure 5, Table 4). In addition, the overall staining pattern of the wt AIRE in Caco-2 cells was similar to that detected in COS-1 (African green monkey kidney) and rat thymus epithelial TuD-1 cells.

Consistent with our previous observations in COS-1 cells (Halonen et al., 2004), the G228W mutation severely disturbed the subcellular distribution of AIRE. In the nucleus, the mutant protein was detected as even staining. In addition to the nuclear staining, the mutant protein was observed as large cytoplasmic aggregates in a large proportion of the transfected cells. These aggregates may represent aggresomes, which have been proposed to form in response to exceeded capacity of the proteasome pathway by aggregation-prone misfolded proteins (Johnston et al., 1998). Coexpression of the G228W mutant with wt AIRE altered the subcellular

distribution of the wt to resemble the distribution of the mutant protein (Figure 5, Table 4). The G228W mutant and wt AIRE also colocalized almost completely (Figure 5).

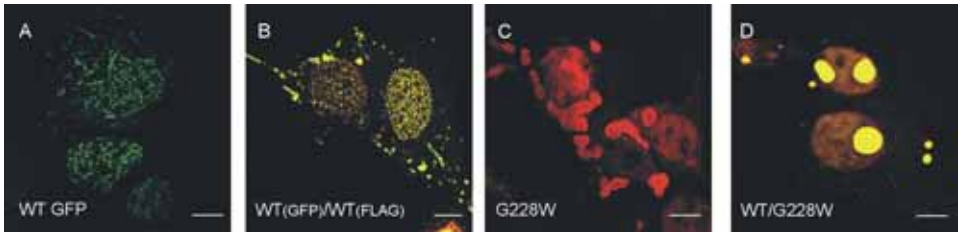

Figure 5. Effect of G228W mutant AIRE protein on the subcellular localization of wt AIRE in transiently transfected Caco-2 cells. FLAG M2 antibody was used to visualize the FLAG fusion proteins. A) Wt GFP-AIRE alone. B) Coexpression of AIRE as GFP and FLAG fusion proteins. C) G228W mutant protein alone and D) coexpressed with the wt AIRE. Scale bar 10 μm.

1.2 Effects of other mutants studied on subcellular distribution of wt AIRE

The other missense patient mutation in the SAND domain, P252L, did not disrupt the NB localization of AIRE. However, it somewhat reduced the nuclear localization of AIRE as 40% of the P252L mutant expressing cells displayed only cytoplasmic staining. In P252L- and wt-coexpressing cells, also this mutant protein altered the subcellular distribution of wt AIRE reducing its nuclear localization (Table 4). The third SAND domain mutation studied, K243A;R247A, had very similar effects as the P252L mutation. It also disturbed the nuclear localization of AIRE, and in coexpression situation the K243A;R247A mutant protein reduced the nuclear localization of wt AIRE protein (Table 4).

Based on the 3D homology model of the HSR domain (Pitkänen et al., 2000), the L28P mutation is located inside of the predicted bundle structure of HSR possibly causing severe structural changes and the Y85C is located on the surface, and may affect protein-protein interactions (Halonen et al., 2004). As previously detected in COS-1 cells (Halonen et al., 2004), the L28P mutant was observed mainly as even staining in the nucleus of Caco-2 cells. In coexpressing cells, the L28P mutant did not change the subcellular localization of the wt AIRE, which is consistent with the inability of this mutant to form multimers. The Y85C mutation disrupted the nuclear dot association and most of the mutant protein was observed in the nucleus as even staining. This is consistent with the observations by Ramsey et al. (2002) but differs from our previous observations in COS-1 cells, in which the Y85C mutant protein forms nuclear dots (Björses et al., 2000; Halonen et al., 2004). When the Y85C mutant was coexpressed with the wt, the localization of the wt protein in the nuclear dots was significantly reduced but not totally blocked (Table

4). In conclusion, all other mutants studied but the L28P mutant, affected the subcellular localization of the wt AIRE. However, the G228W mutant was the only one that totally prevented the wt from forming nuclear bodies.

Table 4A. Subcellular distribution of wt/mutant AIRE in transiently transfected Caco-2 cells[1]

	nuclear bodies +/- cytoplasm	even nucleus +/- cytoplasm	cytoplasm only
WT AIRE (FLAG)	97	0	3
WT AIRE (GFP)	78	11	11
G228W	2	94	4
K243A + R247A	50	1	49
P252L	60	0	40
L28P	0	95	5
Y85C	0	97	3

[1] % of cells expressing AIRE (n=100)

Table 4B. Subcellular distribution of wt AIRE in transiently transfected Caco-2 cells when coexpressed with mutant AIRE proteins[2]

	nuclear bodies +/- cytoplasm	even nucleus +/- cytoplasm	cytoplasm only
WT AIRE (GFP) + WT AIRE (FLAG)	89	1	10
WT/G228W	2	93	5
WT/K243A + R247A	50	0	50
WT/P252L	41	0	59
WT/L28P	83	0	17
WT/Y85C	13	58	29

[2] % of cells expressing AIRE (n=100)

1.3 The G228W mutant inhibits the transactivation capacity of wt AIRE

We had earlier shown that the G228W mutation totally abolishes the transactivation capacity of the protein (Halonen et al., 2004). To study the effects of the mutant AIRE on the transactivation capacity of the wt AIRE in an experimental heterozygous situation, we transiently expressed the wt AIRE fused with Gal4 DNA binding domain (DBD) together with the mutant proteins in COS-1 cells. In this assay, the G228W mutant dramatically reduced the transactivation potential of the wt lowering it to 18% compared to the wt/wt situation (Figure 6).

The other SAND domain mutations P252L and K243A;R247A did not affect the transactivation capacity of AIRE, nor did these mutant forms affect the activity of the wt AIRE in a cotransfection experiment. Of the HSR mutants, the transactivation deficient L28P mutant slightly affected the transactivation capacity of the wt which exhibited 80% of its activity when cotransfected with the mutant. This reduction may be caused by the presence of cells expressing only the mutant protein and is indeed similar to the result obtained with wt AIRE and vector control. The transactivation potential of the Y85C mutant varies between assays from 80% (present study) to over 100% compared with the wt (Björses et al., 2000). In the presence of the Y85C mutant, the wt showed ~60% of its activity (Figure 6). When

tested with Dunnett's test, the reduction in transactivating capacity of the wt AIRE caused by the Y85C mutant was statistically significant ($P<0.01$).

To examine whether the dramatic interference caused by the G228W mutant on the transactivation capacity of the wt is AIRE-specific and not due to the disrupting effect of the G228W mutant, e.g., on the transactivating assay system, we performed the cotransfection experiments with Herpes simplex virus (HSV) transactivator VP16 fused to the Gal4-DBD. In this assay, only the Y85C mutant somewhat reduced the transcriptional activation capacity of the VP16, although the change was not statistically significant in Dunnett's test ($P>0.05$). Taken together, G228W mutant form of the AIRE protein is able to disrupt the transactivation capacity of the wt AIRE in a heterozygous situation. The Y85C mutant also disturbed the transactivation potential of the wt AIRE to some extent.

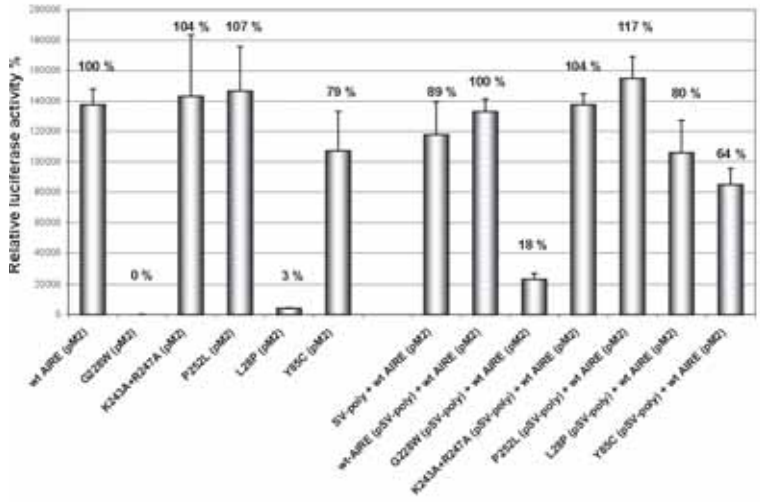

Figure 6. Effects of mutant AIRE proteins on the transactivation potential of wt AIRE in transiently transfected COS-1 cells. The cells were transfected with wt/mutant AIRE-Gal4 DBD construct alone or with wt/mutant AIRE-pSV-poly construct. In all transfections pG5LUC was used as the reporter. The luciferase activities from the cell lysates were normalized according to total protein concentration. The values represent means from three expreriments performed in triplicates and normalized according to an inner control.

1.4 The G228W mutant is able to multimerize with wt AIRE

To study whether the observed changes caused by the mutants to the subcellular distribution and transcactivation capacity of the wt AIRE could be due direct interaction between these two forms of AIRE, we performed mammalian two-hybrid assays and coimmunoprecipitations. Because AIRE in itself is a powerful

transactivator when fused with the Gal4-DBD the transactivation assays were performed with the wt AIRE fused in frame with the VP16-AD and the mutant forms fused with the Gal4-DBD. In this setup, the Y85C, K243A;R247A, and P252L mutant fusion proteins produced almost the same level of luciferase activity as the wt (89%, 82%, and 98%, respectively), which was expected because these mutants are able to activate transcription in the one-hybrid setup. The G228W mutant as well as the L28P mutant considerably reduced the luciferase activity (~9% and ~7% compared with the wt, respectively). Because the G228W mutant appeared to prevent the ability of the wt to activate transcription which might cause a false negative result in the mammalian two-hybrid assay, we further examined the interaction of mutant AIRE proteins with wt AIRE by coexpressing ^{35}S-labeled wt FLAG-AIRE and nontagged mutant AIRE proteins in same *in vitro* translation lysates and immunoprecipitated FLAG-AIRE with anti-FLAG antibody. In this assay, all but the L28P mutant protein bound to the wt protein. This data collectively indicates that *in vitro* the G228W mutant does have a dominant negative effect by binding to the wt AIRE and inhibiting the ability of the wt to form the complexes needed for transactivation.

2 NUCLEAR IMPORT OF AIRE (II)

It has been suggested that AIRE is shuttled between the nucleus and cytoplasm, which may be an important factor in the regulation of AIRE activity (Pitkänen et al., 2001; Halonen et al., 2004). However, the mechanism and amino acids needed for the nuclear localization of AIRE had not been previously identified. In this study, we aimed to further analyze the nuclear import of AIRE.

2.1 AIRE contains a monopartite nuclear localization signal

The N-terminus of AIRE contains two stretches of basic amino acids (110-RKGRK-114 and 131-KRK-133) separated by a linker sequence of 16 amino acids in length previously predicted to function as a classical bipartite NLS (Consortium, 1997). To define the functional NLS of AIRE, we mutated the positively charged lysines and arginines (R113A, K114E, R113A+K114A, K131E, R132A, or K133A) in the two predicted parts of the AIRE NLS and determined the subcellular distribution of these mutant proteins by immunofluorescence microscopy in TuD-1 and COS-1 cells. The AIRE proteins with mutations (R113A, K114E, or R113A+K114A) in the N-terminal part of the potential NLS showed a punctate nuclear expression pattern similar to that of wt AIRE with only a minor reduction in the nuclear fraction. However, mutations in the C-terminal part of the predicted bipartite NLS (K131E, R132A, or K133A) led to a significant reduction in the localization of the mutant proteins in the nucleus and to a complete absence of AIRE nuclear bodies. Similar results were obtained with both FLAG-tagged and nontagged proteins. The results

strongly indicate that the basic residues K131, R132, and K133 regulate nuclear import of AIRE and that the NLS of AIRE is monopartite (Table 5).

Table 5. Subcellular distribution of wt/mutant AIRE in transiently transfected COS-1 cells[1]

	nucleus only	nucleus + cytoplasm	cytoplasm only
WT AIRE	56	44	0
R113A	35	64	4
K114E	43	56	2
R113A + K114E	41	51	9
K131E	1	14	86
R132A	2	3	96
K133E	1	24	76

[1] % of cells expressing AIRE (n=200)

2.2 AIRE interacts with importin-α nuclear carrier molecules

From a yeast two-hybrid screen performed with human fetal liver cDNA library using the first 139 amino acids of the AIRE protein as bait, we identified 10 clones containing importin α1 protein sequences, suggesting that the NLS of AIRE is a true classical NLS and that similar to other proteins containing classical NLSs, also AIRE is transported into the nucleus via the importin α/β-mediated nuclear import pathway. To identify which of the six known human importin α receptors interact with AIRE, we performed glutathione S-transferase (GST) pull-down assays with the ubiquitously expressed importins α1, α3, α5, and α7, representing receptors from each importin α subfamily. *In vitro*-expressed full-length AIRE protein bound to all GST-importin α isoforms studied, binding being strongest to importins α3 and α5, and weakest to importin α7.

2.2.1 Importin-α proteins bind to the nuclear localization signal of AIRE

To determine whether the interaction between AIRE and importin α is mediated by the amino acids required for the nuclear localization of AIRE, the pull-down assays were performed with the NLS mutant AIRE proteins and importins α3 and α5. The R113A+K114A double-mutation had no effect on the interaction between AIRE and importin α3 or α5, whereas the K131E and K133E mutant AIRE proteins showed clearly reduced binding to both importins α3 and α5. The data suggest that nuclear import of AIRE is mediated by classical nuclear import receptors, importin α molecules, through a specific interaction with the AIRE NLS containing amino acids 131–133.

2.2.2 Importin-α3 and α5 interact with AIRE via their "minor" binding site

Comparison of the ARM repeats of the human importin α proteins shows that all the ARM repeats have a conserved asparagine residue and all except ARM 9 have a

conserved tryptophan residue (Melen et al., 2003). To map the ARM site(s) interacting with AIRE, we performed the GST pull-down assays with importins α3 and α5 that had both of the conserved residues in the ARM 3 (in the major binding site) or ARM 8 (in the minor binding site) repeats mutated. Mutations in ARM 3 of either importin α3 or α5 had no effect on binding to AIRE, whereas mutations in ARM 8 clearly reduced the binding of both importin α3 and α5 to AIRE. As a control, the binding experiment was carried out with SV40 T Ag known to bind to ARM 3 of importin α3 (Melen et al., 2003). These results show that, unlike most proteins with classical monopartite NLSs, AIRE binds to the 'minor' binding site of both importin α3 and α5.

3 INTERACTION OF AIRE WITH PIAS1 (III)

In order to identify novel interaction partners for AIRE, we utilized yeast two-hybrid screening as the first step. Because the full-length AIRE and especially the PHD fingers are potent activators of transcription, we used the N-terminus of AIRE lacking the PHD fingers and containing the HSR domain as bait in the screen. Since many of the patient mutations in the HSR domain disturb the complex formation of AIRE, this domain may be involved in interactions with other proteins (Björses et al., 2000; Pitkänen et al., 2001; Halonen et al., 2004).

3.1 AIRE interacts with PIAS proteins

The screening of a human thymus cDNA library yielded 34 positive clones, four of which contained sequences of PIAS1, PIASxα, and PIAS3 genes. We chose PIAS1 for further studies and verified the interaction with pull-down assay using GST-AIRE produced in bacteria and *in vitro*-translated PIAS1. Alignment of the PIAS clones obtained from the yeast two-hybrid screen showed that they all contained a common region of about 30 conserved amino acids including the SUMO interaction motif (SIM) (Minty et al., 2000). Furthermore, the SP-RING domain of the PIAS proteins can mediate protein-protein interactions (Shuai and Liu, 2005). Thus, we mutated these regions from PIAS1 (Figure 7) and performed the pull-down experiment also with the mutants (PIAS1-SIMΔ and PIAS1-RINGΔ). However, neither of these domains appear to be essential for the interaction between AIRE and PIAS1 since both of the mutants still bound to GST-AIRE. We also performed coimmunoprecipitation with nuclear extracts prepared of COS-1 cells transiently expressing AIRE and FLAG-PIAS1. Although consistently positive, the coimmunoprecipitation was very weak, despite our attemps to precipitate the complex with different antibodies and cell lysis systems.

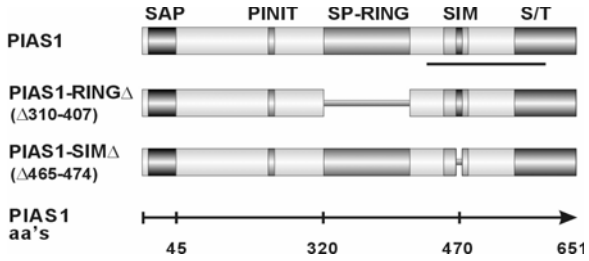

Figure 7. Schematic representation of wt and mutated human PIAS1 proteins. The line indicates the PIAS1 clone obtained from yeast two-hybrid screens.

We also analyzed whether wt or mutant PIAS1 proteins would have an effect on the nuclear matrix association of AIRE, or vice versa. A significant proportion of AIRE, wt PIAS1, PIAS1-RINGΔ, or PIAS1-SIMΔ was found in the nuclear matrix fraction. Coexpression of the AIRE and wt/mutant PIAS1 proteins did not notably affect the amount of any of the proteins in the nuclear matrix fraction.

3.2 AIRE is not modified by SUMO

Because the PIAS proteins function as E3 SUMO ligases, we studied whether AIRE is modified by SUMO. The sumoylation of PIAS1 was used as a control. No protein species potentially representing sumoylated forms of AIRE were detected from AIRE-transfected COS-1 cell lysates analyzed in SDS-PAGE in the presence of NEM, an inhibitor of SUMO proteases. The presence of neither SUMO1 or PIAS1 did not bring about any additional AIRE forms, indicating that AIRE is not sumoylated under these conditions. Sumoylation of AIRE was also tested in a cell-free system. SUMO E3 ligase is not required for *in vitro* sumoylation of several targets, thus the assay was performed with ^{35}S-labeled *in vitro*-translated AIRE incubated with human recombinant E1 and Ubc9 SUMO E2 conjugase in the absence of SUMO or with SUMO1 or SUMO2. Again, no SUMO-modification of AIRE was detected.

3.3 AIRE and PIAS1 localize to adjacent or partially overlapping NBs

Similar to AIRE, the PIAS proteins have been reported to be localized mainly in the nucleus where they associate to nuclear bodies when overexpressed (Sachdev et al., 2001; Tan et al., 2002; Duval et al., 2003). Since no cell line endogenously expressing AIRE was available, we transiently overexpressed AIRE and FLAG-tagged PIAS1 in Caco-2 cells and COS-1 cells to determine the mutual subcellular distribution of these proteins. The cells were immunostained with anti-AIRE and anti-FLAG antibodies and analyzed by confocal microscopy. In the majority of coexpressing cells, AIRE- and PIAS1-containing NBs did not colocalize. However,

AIRE-containing NBs and PIAS1-containing NBs situated frequently adjacently or were partially overlapping in all of the cells displaying AIRE and PIAS1 NBs (Figure 8).

3.4 Expression of AIRE enhances the formation of PIAS1 NBs

Endogenous PIAS1 and PIASy have been detected expressed uniformly in the nucleus (Liu et al., 2001; Liu et al., 2005). In addition to the NB staining, we also detected diffuse nuclear staining with both AIRE and PIAS1 in both cell types studied. We quantified the occurrence of the different subnuclear patterns with confocal microscopy. All quantifications were performed in COS-1 cells due to their higher transfection efficiency. The observed phenotypes were also present in Caco-2 cells. A total of 60-85% of AIRE-transfected cells displayed NBs with or without cytoplasmic staining. Even nuclear staining was detected in 10-30% of AIRE-expressing cells. Of the PIAS1-expressing cells, 43% displayed the NB staining pattern, and roughly the same proportion of the cells showed even nuclear staining. When AIRE and PIAS1 were coexpressed, PIAS1 had no evident effect on the NB localization of AIRE. However, AIRE increased the number of cells displaying PIAS1 NBs from 43% to 66% (Table 6).

3.4.1 Deletion of the SIM of PIAS1 leads to full colocalization of AIRE and PIAS1

We also examined the effects of the deletions of the SP-RING domain and the SIM to the subcellular localization of PIAS1 in the presence and absence of AIRE. Deleting the SP-RING domain did not prevent the localization of PIAS1 to NBs, which in the presence of AIRE were found adjacent to AIRE NBs (Figure 8). Furthermore, coexpression of AIRE with PIAS1-RINGΔ increased the number of cells containing PIAS NBs from 46% to 64%, similar to wt PIAS1 (Table 6).

The PIAS1-SIMΔ mutant was able to localize in NBs, as well. Further, when coexpressed with AIRE, a full colocalization was detected in almost all cells displaying NBs (Figure 8) and the number of cells containing PIAS1-SIMΔ NBs increased from 44% to 79% (Table 6).

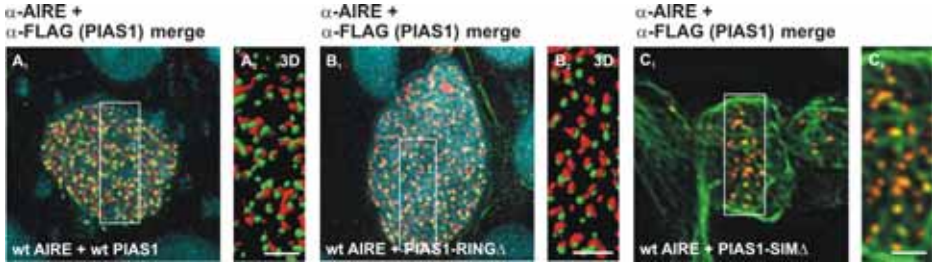

Figure 8. Subnuclear localization of AIRE and wt/mutant PIAS1 NBs in cotransfected Caco-2 cells analyzed with confocal immunofluorescence microscopy using sequental scanning. The overlays are shown as projections of deconvoluted stack images. AIRE was detected with an anti-HSR antibody and the FLAG-tagged wt/mutant PIAS1 proteins with a FLAG M2 antibody. Coexpression of A) AIRE and wt PIAS1, B) AIRE and PIAS1-RINGΔ, C) AIRE and PIAS1-SIMΔ. A2 and B2 show part of the nucleus enlarged as 3D reconstruction and C2 as projection. Scale bar 2 μm.

Table 6. Subnuclear localization of wt/mutant PIAS1 in the presence and absence of AIRE[1]

	subnuclear localization of PIAS1		
cells expressing	**nuclear bodies**	**diffuse staining**	**miscellaneous**
wt PIAS1	43	38	19
wt PIAS1 + AIRE	66	15	19
PIAS1-RINGΔ	46	31	23
PIAS1-RINGΔ + AIRE	64	34	2
PIAS1-SIMΔ	44	42	14
PIAS1-SIMΔ + AIRE	79	18	3

[1] % of (co)expressing cells (n=100-150)
Miscellaneous = cells containing only a few PIAS1-containing nuclear dots/aggregates of varied size.

3.5 Expression of AIRE enhances the recruitment of SUMO1 to NBs

The PIAS proteins can reqruite SUMO1 into same NBs with them (Kotaja et al., 2002). To study the effects of AIRE to the subcellular localization of SUMO1 in the presence and absence of PIAS1, we overexpressed the proteins in COS-1 cells. Similar to endogeneous SUMO1 (Muller and Dejean, 1999), in our overexpression conditions GFP-SUMO1 showed predominantly a nuclear diffuse staining (65% of coexpressing COS-1 cells). Coexpression of wt PIAS1 with SUMO1 effectively recruited SUMO1 into NBs since the percentage of cells displaying SUMO1 NBs increased from 19% to 91%. Deleting the SIM from PIAS1 did not disturb its ability to reqruite SUMO1 into NBs, but the PIAS1-RINGΔ mutant failed to target SUMO1 into NBs as efficiently as wt PIAS1 (from 19% to 35%). When AIRE and SUMO1 were coexpressed, they did not colocalize and the AIRE NBs and SUMO1 NBs did not localize to the vicinity of each other. However, the percentage of cells displaying SUMO1 NBs increased from 19% to 42% (Table 7).

Table 7. Subnuclear localization of SUMO1 in the presence and absence of AIRE or wt/mutant PIAS1[1]

	subnuclear localization of SUMO1		
cells expressing	**nuclear bodies**	**diffuse staining**	**miscellaneous**
SUMO1	19	65	16
SUMO1 +AIRE	42	54	4
SUMO1 + wt PIAS1	91	5	4
SUMO1 + PIAS1-RINGΔ	35	61	4
SUMO1 + PIAS1-SIMΔ	98	1	1

[1] % of (co)expressing cells (n=100-150)
Miscellaneous = cells containing SUMO1 localized in addition to the even nuclear staining in variable sizes of blurred speckles, or in large bright aggregates.

3.6 PIAS1 is able to attract AIRE into SUMO1-containing complexes

When AIRE, SUMO1 and wt PIAS1 were coexpressed, a complete colocalization of all three proteins in NBs was detected in 28% of the coexpressing cells. In the cells not showing colocalization of all three, SUMO1 and PIAS1 still fully colocalized, and AIRE and PIAS1 NBs were located juxtaposed to each other in most of the cells

(57%). In 12% of the cells a part of the AIRE NBs and in 3% all AIRE NBs were separated from the PIAS1/SUMO1 NBs. Deletion of the SP-RING domain from PIAS1 did not affect the targeting of AIRE into same structures with PIAS1 and SUMO1. However, in the remaining cells only 28% of AIRE and PIAS1 NBs were located adjacent to each other and in 26% of the triple-transfected cells AIRE NBs and PIAS1 NBs were totally separated (Table 8).

3.6.1 SIM of PIAS1 is needed for colocalization of AIRE and SUMO1

Deleting the SIM from PIAS1 disturbed the recruitment of AIRE into same subnuclear structures with PIAS1 and SUMO1 and full colocalization of AIRE, PIAS1-SIMΔ and SUMO1 was detected in only 12% of triple-transfected cells. In only 17% of the triple-transfected cells AIRE and PIAS1 NBs were found adjacent to each other and in the majority (54%) of triple-transfected cells, all the AIRE-containing NBs were distinctly separated from the PIAS1-SIMΔ/SUMO1-containing NBs (Table 8).

Table 8. Subnuclear association of AIRE and PIAS1 NBs in the presence and absence of SUMO1

	% of cells displaying AIRE and wt/mutant PIAS1-containing NBs[1]					
subnuclear association	**AIRE + wt PIAS1**		**AIRE + PIAS1-RINGΔ**		**AIRE + PIAS1-SIMΔ**	
of AIRE and PIAS1 NBs	**-SUMO1**	**+SUMO1**	**-SUMO1**	**+SUMO1**	**-SUMO1**	**+SUMO1**
adjacent	100	57	100	28	4	17
both adjacent and separate	-	12	-	20	-	17
separate	-	3	-	26	-	54
full colocalization	-	28	-	26	96	12

[1]n=100-150

3.7 PIAS1 and AIRE concurrently activate the human insulin promoter

We and others have previously shown that the AIRE protein acts as a powerful transcriptional transactivator, when AIRE is fused to the GAL4 DBD domain directing it to a reporter promoter containing GAL4 response elements (Björses et al., 2000; Pitkänen et al., 2000). Since PIAS proteins are known to modulate the activities of many proteins (Schmidt and Muller, 2003; Shuai and Liu, 2005), we analyzed whether PIAS1 would affect the transcriptional regulatory function of AIRE. Consistent with previous results, GAL4-AIRE enhanced the activation of the GAL4-responsive reporter ~70-fold in COS-1 cells. However, coexpression of PIAS1 with GAL4-AIRE had no effect on the transactivation capacity of AIRE (data not shown).

AIRE is known to control the expression of several peripheral tissue-specific antigens, including preproinsulin (Anderson et al., 2002; Chin et al., 2003; Sabater et al., 2005; Taubert et al., 2007). To study the functional consequences of the interaction between AIRE and PIAS1 in an AIRE target gene context, we performed transcription assays in COS-1 cells coexpressing the human insulin promoter-driven

luciferase, AIRE, and PIAS1. Expression of AIRE or PIAS1 alone stimulated the activity of the insulin promoter by 2-fold and scaling up the amount of either AIRE or PIAS1 expression plasmids did not significantly increase the induction. Coexpression of AIRE and PIAS1 with the reporter construct enhanced the transcription of the reporter by 3.5-fold. Although the combined effect of AIRE and PIAS1 was additive, coexpression of increasing amounts of PIAS1 with a constant amount of AIRE further increased induction of the insulin promoter in a dose-dependent manner, in contrast to when PIAS1 was expressed alone. This suggests that AIRE and PIAS1 interact also functionally. When the coexpression experiment was performed with increasing amounts of AIRE and a constant amount of PIAS1, no further induction was detected, indicating that in coexpression situations AIRE reaches its saturation limit at a lower concentration than PIAS1 (Figure 9A).

Deletion of the SP-RING domain abolished the ability of PIAS1 to activate the insulin promoter indicating that the E3 ligase activity of PIAS1 is required for the induction. Unlike with wt PIAS1, coexpression of AIRE with increasing amounts of the PIAS1-RINGΔ mutant construct did not show a dose-dependent increase in activation of the insulin promoter (Figure 9B).

Deletion of the SIM from PIAS1 did not affect its ability to activate the insulin promoter. Coexpression of AIRE and PIAS1-SIMΔ mutant led to a 6-fold activation of the insulin promoter compared with the 3.5-fold induction by AIRE together with wt PIAS1 (Figure 9C).

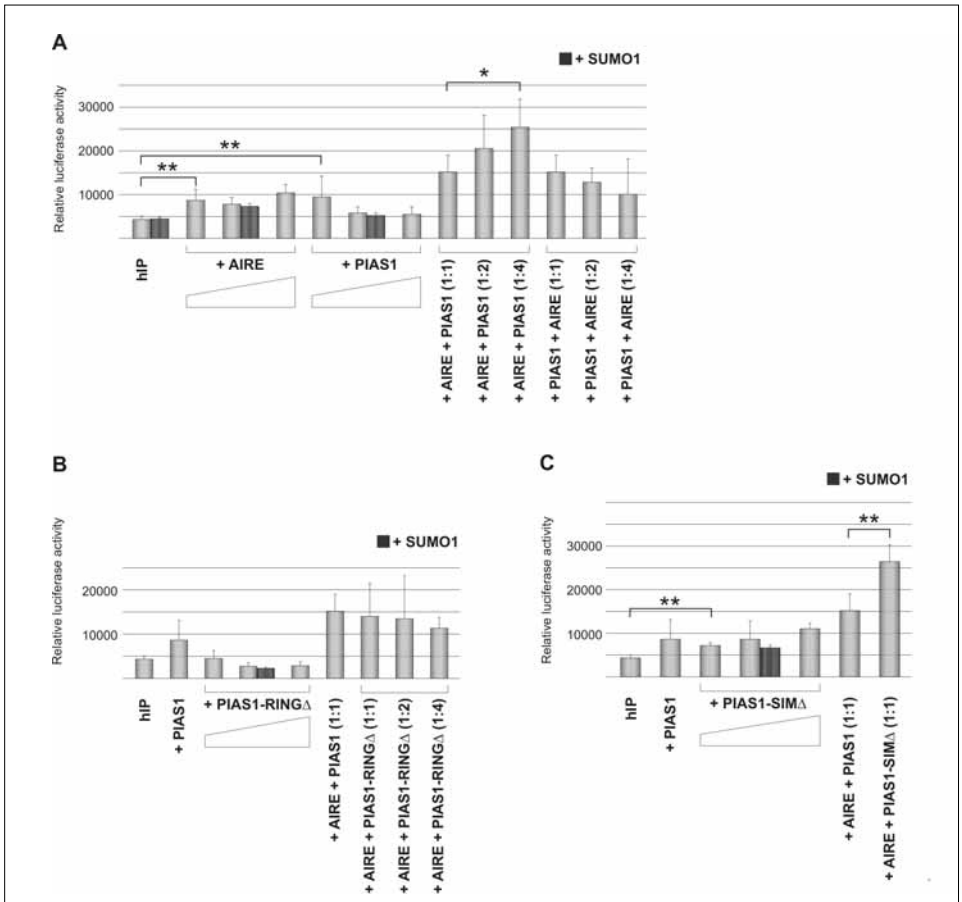

Figure 9. Transactivation of human insulin promoter by AIRE and wt/mutant PIAS1 in COS-1 cells. The values represent means from at least two independent experiments. (A) Effects of AIRE/wt PIAS1/SUMO1 alone or coexpressed, (B) PIAS1-RINGΔ alone or coexpressed with AIRE/SUMO1, (C) PIAS1-SIMΔ alone or coexpressed with AIRE/SUMO1, on the hIP promoter.

3.8 AIRE is able to reduce the activation capacity of PIAS1 on the human CSTB promoter

The expression pattern of insulin is highly tissue-specific. Since AIRE is also known to regulate the expression of genes expressed more ubiquitously, we studied the effects of AIRE and PIAS1 on a housekeeping gene. For this purpose, we studied the human cystatin B promoter (CSTB)-driven luciferase (Alakurtti et al., 2000) by coexpressing it with AIRE and/or PIAS1 in COS-1 cells. AIRE strongly repressed the CSTB promoter, whereas PIAS1 up-regulated it enhancing the induction 4-fold

in a dose-dependent manner. When AIRE and PIAS1 were coexpressed with the reporter construct, they showed a combined effect. AIRE was able to inhibit the activation effect of PIAS1 on the promoter. The activity of the cystatin B promoter was reduced more than if the effect were merely additive. Coexpression of increasing amounts of PIAS1 with a constant amount of AIRE enhanced the induction and coexpression of increasing amounts of AIRE attenuated the induction of the CSTB promoter in a dose-dependent manner (Figure 10A).

Deletion of the SP-RING domain totally abolished the ability of PIAS1 to activate the CSTB promoter and coexpression of AIRE with PIAS1-RINGΔ led to even further reduction in expression of the reporter gene (Figure 10B). Deletion of the SIM domain also decreased the capacity of PIAS1 to activate the CSTB promoter, although it did not disrupt it totally. When coexpressed with PIAS1-SIMΔ AIRE was also able to reduce the activity produced by this mutant (Figure 10C).

Since SUMO1 modified the subnuclear association of AIRE- and PIAS1-containing NBs, we tested whether addition of SUMO1 to the transactivation assay would change the transactivation potential of AIRE and/or wt/mutant PIAS1. However, coexpression of SUMO1 in any of the reporter gene experiments did not significantly alter the insulin or CSTB promoter activity. The only statistically significant ($P<0.05$) change was detected when SUMO1 was coexpressed with PIAS1-SIMΔ, which led to slightly increased activation of the CSTB promoter, which disappeared in the presence of AIRE (Figure 10C).

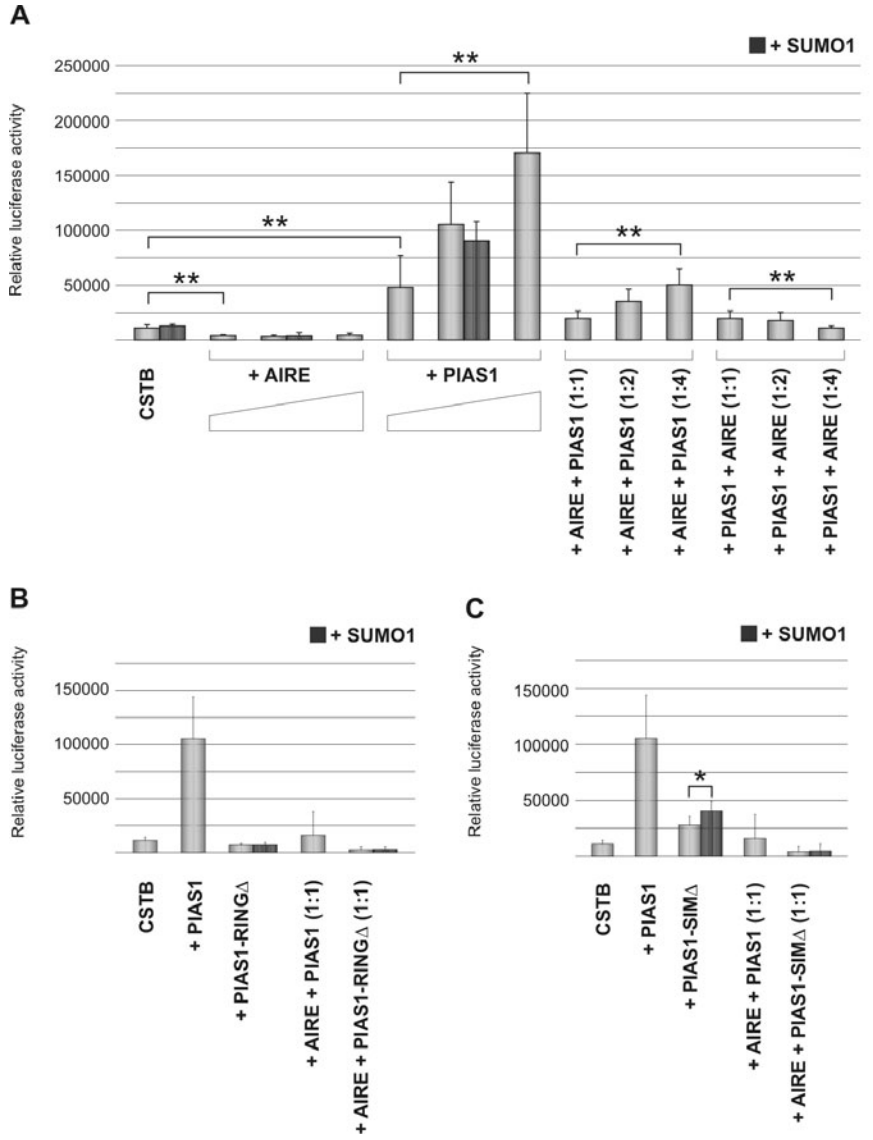

Figure 10. Transactivation of human cystatin promoter (CSTB) by AIRE and wt/mutant PIAS1 in COS-1 cells. The values represent means from at least two independent experiments. (A) Effects of AIRE/wt PIAS1/SUMO1 alone or coexpressed, (B) PIAS1-RINGΔ (300 ng) alone or coexpressed with AIRE/SUMO1, (C) PIAS1-SIMΔ (300 ng) alone or coexpressed with AIRE/SUMO1, on the human CSTB promoter.

DISCUSSION

1 CAN APECED BE INHERITED IN A DOMINANT MANNER? (I)

In the first study of this thesis, we aimed to experimentally evaluate the functional consequences of the G228W mutation proposed to cause dominant inheritance of hypothyroidism/APECED. Additionally, two other SAND domain mutations, and two HSR domain mutations were studied. For this, we transiently overexpressed the wt and mutant forms of AIRE with two different tags, wt fused with GFP and mutants with FLAG. Tagging of proteins is always potentially hazardous, especially with GFP which is a quite large molecule of 27 kDa. Indeed, in COS-1 cells, which tend to have high levels of expression of plasmids bearing an SV40 origin for replication, GFP-tagged AIRE can often be detected in large aggregates. To avoid this, and to study the subcellular distribution of wt and mutant AIRE proteins in an epithelial cell model, these experiments were carried out in Caco-2 cells, in which both GFP-AIRE and FLAG-AIRE displayed a distribution similar to that of the nontagged AIRE, except for the reduced cytoplasmic filament staining of GFP-AIRE. When coexpressed, both the GFP-AIRE and FLAG-AIRE colocalized in NBs, yet again indicating that AIRE forms homomers. The effects of the G228W mutant to the subcellular localization of the wt AIRE protein were dramatic. The subcellular localization of the wt was changed totally in the presence of the G228W mutant and it appeared that the mutant was able to prevent the wt from forming NBs as both wt and mutant AIRE were mostly distributed as diffuse nuclear staining. The ability of the G228W mutant to bind the wt was first tested with mammalian two-hybrid assay, in which a negative result for interaction was detected. The two-hybrid assay has the requirement for the fusion proteins to be able to reach the nucleus and to interact with the transcription initiation complex. The nuclear localization of the fusion proteins was verified by staining the cells with AIRE antibody, by which a diffuse nuclear distribution of AIRE similar to the G228W mutant was observed. Thus, it may be that the negative result was either because the wt and G228W mutant proteins do not interact or the GAL4-mutant protein is able to prevent the wt-AD protein from interacting with the transactivation initiation complex. The latter may be the case, since the G228W mutant and wt AIRE were able to interact as detected by coimmunoprecipitation. In the one-hybrid experiment, the G228W mutant was able to inhibit the transactivation capacity of the wt, further supporting the hypothesis that the G228W mutant is able to multimerize with the wt and prevent the wt from reaching the complexes required for transactivation. In all, our *in vitro* data supports the suggested dominant inheritance of APECED in the family carrying the G228W mutation.

During the preparation of the manuscript I, Purohit *et al.* (2005) mapped the region most likely mediating DNA binding within the SAND domain of AIRE to

amino acids 189-196. Although the G228W does not hit that region, it is still possible that changing a small amino acid residue, glycine, into a bulky and aromatic residue, tryptophan, may affect the physicochemical properties or conformation of AIRE interfering the DNA binding of AIRE. It is also possible, that the mutation disturbs protein-protein interactions, since based on the homology model it is located on the surface of the SAND domain (Halonen et al., 2004). Because in our *in vitro* experiments the mutant was able to reduce the transactivation capacity of the wt in conditions where the wt was forced to DNA, the disturbance of protein-protein interactions seems more likely. If the mutant protein multimerizes with the wt also *in vivo*, the inability of the wt/mutant complexes to bind the molecular interaction partners of AIRE, DNA or protein, could explain the dominant negative nature of this mutation.

In the previous studies of our group, in about 8% of a series of 104 clinically confirmed patients with APECED, no mutations or mutation in only one chromosome was detected (Björses et al., 2000; Halonen et al., 2002). The mutations found in heterozygous form include R257X and c.967-979del13bp, which are the two most common APECED mutations. Since no evidence for APECED in the heterozygous first-degree relatives of patiens has been reported, it is unlikely that these mutations would be inherited dominantly. The recent findings that the presence of anti-type 1 IFN antibodies highly correlates with APECED and that the asymptomatic heterozygous carriers of the R257X and c.967-979del13bp mutations tested so far do not have these autoantibodies (Meager et al., 2006) further supports the existence of other APECED-causing mutations or predisposing polymorphisms outside the coding region of *AIRE*, that could e.g. affect splicing. In addition to the coding region and exon-intron boundaries, we have sequenced a 5 kb region upstream from the translation initiation site from these patients. Although none of the identified variants were unique to the patients, we have not evaluated the effects of the found sequence variations to the expression levels of AIRE. Even if some of these polymorphisms would lead to only a modest decrease in the level of AIRE expression in the thymus not sufficient to cause a disease in itself, combined to a null allele this could increase the risk for autoimmunity enough, especially in an individual with a susceptible genetic background. For example in two patients with Omenn syndrome, a severe primary immunodeficiency due to mutations in the recombinase-activating genes, the levels of AIRE mRNA in the thymus were found to be reduced to 10–25% compared to healthy controls. The reduction in the AIRE expression is probably due to the defects in the compartmentalization of the thymus in these patients, resulting from the faulty thymocyte development. The authors also detected a significant reduction in the expression levels of insulin, cytochrome P450 1A2, and FABP mRNAs, and hypothesized that the few T cells that develop in Omenn syndrome escape negative selection causing the autoimmune manifestations in this disease (Cavadini et al.,

2005). Mice seem even more susceptible to changes in the Aire expression levels, since loss of a single copy of Aire was sufficient to diminish thymic expression of the insulin gene and dramatically increase the development of diabetes (Liston et al., 2004). Although heterozygotes do not appear to develop APECED, they may be more prone to autoimmunity in general. Especially the carriers of the Iranian Jewish Y85C mutations would be interesting in this respect, since the Y85C mutant AIRE also disturbed the subcellular localization and the transactivation potential of the wt to some extent in our experiments. However, studying this aspect in a systematic way is challenging, since the carrier frequencies of the APECED mutations are low, so very large sample sizes would be required.

It would be interesting to test the presence of autoantibodies against type 1 INFs from the individuals carrying the G228W mutation, since these autoantibodies have been speculated to predispose to candidiasis (Meager et al., 2006) and five (age range 28-82 yrs) of the seven affected individuals had not developed candida infection by the time the article reporting this mutation was published (Cetani et al., 2001). Candidiasis is also less common among the Iranian Jewish patients, so they would be of interest, as well.

2 NUCLEAR IMPORT OF AIRE (II)

Both endogenous AIRE and AIRE overexpressed in several different cell cultures localize mainly in the nucleus, indicating that the NLS of AIRE is constitutive. The presence of AIRE also in cytoplasm suggests that it continuously shuttles between nuclear and cytoplasmic compartments, with predominance in nuclear import. Furthermore, most of the analyzed missense patient mutations alter the nucleus-cytoplasm distribution of AIRE, leading to increased nuclear localization of AIRE (Halonen et al., 2004). In our study we showed that AIRE interacts via its monopartite NLS with several different importin αs, which all belonged to different importin α subfamilies. All human importin α proteins apart from α6 are ubiquitously expressed (Cuomo et al., 1994; Kohler et al., 1997; Nachury et al., 1998; Kohler et al., 1999), but previous studies have shown that although some nuclear-targeted proteins can be imported into the nucleus by several different human importin α isoforms, several substrates are transported specifically by particular isoform (Kohler et al., 1999; Quensel et al., 2004; Fagerlund et al., 2005; Reich and Liu, 2006). Thus, it is possible that particular importin α proteins are responsible for nuclear import of AIRE in different tissues *in vivo*.

The NLSs are necessary and sufficient for nuclear import via the importin α/β pathway (Lange et al., 2007), which also holds true for AIRE. However, for high selectivity for specific importin αs, contribution from other domains in the cargo protein are required, perhaps via additional, weaker interactions between these protein

regions and importin αs (Friedrich et al., 2006). In majority of DNA-binding proteins, NLSs are found adjacent or colocalizing with the DNA binding domains and mutations in one region can affect the other (LaCasse and Lefebvre, 1995; Cokol et al., 2000). There are no identified patient mutations in the NLS of AIRE, and majority of APECED missense mutations led to an increase rather than a decrease in the nuclear localization of AIRE. However, when we analyzed the SAND domain missense mutations, we noticed that two of the mutations significantly lowered the occurance of AIRE in nucleus. The SAND domain has been implicated to be involved in the nuclear localization of AIRE also previously, when deleting the SAND domain was shown to retain AIRE in the cytoplasm (Ramsey et al., 2002a). However, it is possible that deleting the whole domain leads to e.g. folding problems which causes the cytoplasmic retention. Furthermore, the NLS delineated in the first study of this thesis, is situated ~60 amino acids apart from the SAND domain, so at least in the amino acid level these domains do not appear to be connected. Of course, in the tertiary structure these domains could be in such relation to each other, that mutations in the SAND domain might disturb the nuclear import of AIRE, possibly by lowering the affinity of AIRE to importin α molecules.

3 INTERACTION OF AIRE WITH PIAS PROTEINS (III)

AIRE regulates the expression of hundreds of genes, which are clustered in chromosomes, indicating the involvement of epigenetic mechanisms (Gotter et al., 2004; Derbinski et al., 2005; Tao et al., 2006). On the other hand, as demonstrated by chromatin immunoprecipitation, AIRE binds *in vivo* to the regulatory regions of several genes, including APECED autoantigens, and genes involved in cytokine production, lymphocyte homeostasis, and posttranslational modification of surface proteins (Ruan et al., 2007). Furthermore, thymic expression of adjacent genes in the gene clusters can be regulated by Aire in an alternating manner, the expression of some of the genes being Aire-dependent and some Aire-independent, supporting the role of AIRE as a gene-specific regulator of gene expression (Derbinski et al., 2005; Johnnidis et al., 2005). To further understand the molecular mechanisms behind this interesting gene expression pattern influenced by Aire, it is important to identify other proteins AIRE interacts with. This has turned out to be challenging as so far only one transcriptional interaction partner for AIRE, the common transcriptional coactivator CBP (CREB-binding protein), has been identified (Pitkänen et al., 2000). In the third study of this thesis, we identified an interaction between AIRE and PIAS proteins, a family of transcriptional coregulators with E3 SUMO ligase activity. A large proportion of AIRE, as several other transcriptional regulators has been previously shown to associate with the nuclear matrix, and the SAP domain of PIAS1 was shown to bind AT-rich DNA sequences typical for matrix attachment

regions *in vitro* (Akiyoshi et al., 2004; Okubo et al., 2004; Corry and Underhill, 2005; Tao et al., 2006). The role of nuclear scaffold, such as the nuclear matrix, in the regulation of functional architecture of the nucleus is still controversial. However, whether or not absolutely required, nuclear scaffolds may serve as platforms onto which functional sites are assembled and thus enhance the efficiency of nuclear processes (Cook, 1999; Misteli, 2007). In our study, we further demonstrated that also PIAS1 is present in the lamin-containing nuclear matrix fraction in cultured cells and that neither the SP-RING or the SIM of PIAS1 were required for its localization in the nuclear matrix fraction, or association to NBs. The association of probably a large proportion of nuclear AIRE and PIAS1 with the nonsoluble nuclear matrix could also partly explain their weak co-immunoprecipitation in mammalian cells. Another possibility is that the interaction is very dynamic. The interaction was also demonstrated with pull-down experiments performed with bacterially expressed GST-AIRE and *in vitro*-translated PIAS1. However, without using purified proteins we cannot exlude the possibility that the interaction between AIRE and PIAS1 is mediated by another/other protein(s).

The SUMO-modified residues in the PIAS proteins have not yet been mapped. *In vitro*-translated PIAS1 showed also higher molecular weight bands on SDS-PAGE gel reminiscent of sumoylated forms of PIAS1. Deletion of both the SP-RING and SIM from PIAS1 lead to reduction in these forms potentially suggesting the presence of SUMO-modified amino acids within these regions. When we analyzed the COS-1 cell lysates in the sumoylation assays, we noticed a consistent decrease in the potentially sumoylated forms of PIAS1 in the presence of AIRE. There are several possible explanations for this, the most tempting ones being that either the interaction between AIRE and PIAS1 blocks some sumoylation sites in PIAS1 or that AIRE exerts its speculated ubiquitin ligase activity on PIAS1 and there is competition between sumoylation and ubiquitination of the same residue(s).

AIRE significantly enhanced the formation of both SUMO1- and PIAS1-containing NBs, indicating interdependence of the pathways in the assembly of AIRE, SUMO1, and PIAS1 NBs. One explanation for this could be that AIRE up-regulates the expression of genes coding for NB-associated proteins which are modified by or interact with SUMO/PIAS. AIRE could also enhance the expression of PIAS1 itself, but at least based on Western analyses, we did not detect an increase in the production of PIAS1. Another possibility is that AIRE, perhaps by ubiquitination, modifies either PIAS1 or other molecules involved in the reqruitement of PIAS1/SUMO into NBs. Whether or not AIRE has noncovalent interactions with SUMO remains to be verified, since although AIRE does not colocalize with SUMO1 in the cell models examined so far, it may be that AIRE has affinity to the other SUMO isoforms. Furthermore, a substantial subset of AIRE NBs colocalized with PIAS1 and SUMO1, and deleting the SIM of PIAS1 reduced

the colocalization, suggesting that under certain cellular conditions AIRE can be targeted to same nuclear complex with PIAS1 and SUMO1, and this is mediated by noncovalent interactions between PIAS1 and SUMO/sumoylated proteins.

It has been estimated that Aire-activated genes have a tendency towards tissue-specificity and Aire-repressed genes towards more ubiquitous expression (Hughes and Friedman, 2006). Thus, the functional consequences of AIRE and PIAS1 interaction were analysed using transactivation assays with two different promoters. We selected human preproinsulin as a representative of a tissue-specific promoter, as it is a well-established AIRE-upregulated gene both in humans and mice (Anderson et al., 2002; Chin et al., 2003; Sabater et al., 2005; Taubert et al., 2007). Human CSTB promoter was chosen as a representative of a household gene (Alakurtti et al., 2000). Our results support the abovementioned hypothesis, since AIRE repressed the CSTB promoter and as expected, up-regulated the insulin promoter. Cotransfection of the insulin or CSTB reporter with PIAS1 showed that also it was able to moderately stimulate the insulin promoter. On the CSTB promoter PIAS1 had a strong stimulatory effect. The effects of SP-RING deletion observed with both promoters suggest that E3 SUMO ligation activity is important for the PIAS1-mediated transcriptional effects, perhaps more generally than the interactions mediated by the SIM since the responses to SIM deletion varied between the two promoters examined.

When expressed alone, neither AIRE or PIAS1 showed a dose response. However, when expressed together with AIRE, PIAS1 showed dose-responsive stimulation of the insulin promoter-driven reporter, which was abolished by deleting the SP-RING, suggesting that both AIRE and the sumoylation activity of PIAS1 are required for the enhanced induction. Deleting the SIM from PIAS1 lead to an enhanced stimulation of the insulin promoter when coexpressed with AIRE. One could speculate that when PIAS1 is no longer able to interact with SUMO/SUMO-binding proteins, more of it is “released” to activate transcription with AIRE.

It has been recently reported that several target genes of Aire appear to have Aire-binding consensus motifs in their promoters (Ruan et al., 2007). Whether or not AIRE directly binds to the promoters tested here remains to be solved. *In vivo*, both the level of AIRE expression and promoter variations affect the expression of AIRE’s target genes in thymus (Liston et al., 2004; Sabater et al., 2005). For example in the case of insulin, variations in the intrathymic expression levels of insulin correlate with the susceptibility to diabetes in both mice and humans (Pugliese et al., 1997; Vafiadis et al., 1997; Liston et al., 2004).

The sensitivity of the PIAS1-directed activation of the insulin promoter to deletion of SP-RING and of the CSTB promoter to deletions of both SIM and SP-RING domains strongly suggests that sumoylation and/or binding to SUMO by PIAS1 is involved. Many of the transcription factors known to regulate the

preproinsulin are modified by SUMO, leading to transcriptional activation (Qiu et al., 2002; Kishi et al., 2003; Berberich-Siebelt et al., 2006). On the other hand, several factors acting on the CSTB promoter are negatively regulated by sumoylation (Alakurtti et al., 2000; Poukka et al., 2000; Schmidt and Muller, 2002; Spengler and Brattain, 2006). Clearly, the responses of the insulin and CSTB promoters to the overexpression of E3 ligase PIAS1 observed here, cannot be simply explained by the known separate effects of sumoylation on the transcription factors involved.

Because of the strong similarities between the domain structures of AIRE and Sp100, and the fact that Sp100 is one of the main components of the PML NBs (Sternsdorf et al., 1997a), it has been speculated whether also AIRE localizes to these nuclear structures. However, no colocalization between AIRE and PML or Sp100 has been detected (Björses et al., 1999; Akiyoshi et al., 2004; Pitkänen et al., 2005). On the other hand CBP, which is known to interact with AIRE, can localize into both PML and AIRE bodies, but with a preference to PML bodies (Akiyoshi et al., 2004). In human thymus, a partial colocalization was detected with CBP and AIRE NBs (Pitkänen et al., 2005). The AIRE NBs do not seem to be active sites of transcription, as they do not colocalize with acetylated histone H3. Although, the localization of AIRE NBs to transcriptionally active sites could be cell cycle dependent as is the case with PML NBs. A similar function that has been proposed for the PML NBs could be envisioned for AIRE, as well. AIRE could function as a scaffold, concentrating transcription factors (including itself), cofactors (e.g. CBP), and chromatin modifiers to the vicinity of transcription sites to facilitate transcription initiation. As proposed by Akiyoshi *et al.* (2004), one way to control the activity of AIRE is by regulating the localization of CBP in either AIRE NBs or PML NBs (Figure 11). The potential role of diffuse AIRE staining, which could of course be an experimental artefact, remains to be solved. However, it may be functionally relevant, since many other proteins involved in regulation of gene expression are also found as diffuse nuclear staining and it has been proposed that when not bound to DNA targets, for instance transcription factors freely diffuse through the nucleoplasm scanning the genome (Misteli, 2001; Hager et al., 2002).

Although overall Aire has hundreds of target genes, each tissue restricted antigen (Aire-dependent or -independent) appears to be expressed by only a small subset of mTECs (1-3%) in clusters of few cells (Derbinski et al., 2001; Klein et al., 2001). The factors leading to such expression pattern are still unknown. Thus far, Aire is the only molecule shown to be involved in this regulation *in vivo*. Since not all tissue-specific antigens are regulated by Aire there must be other unidentified players, as well. Furthermore, it is still unclear whether in addition to directly binding to the promoter regions of these genes, Aire also mediates epigenetic regulation. Most likely the effects of AIRE are regulated by interactions with other

transcriptional modulators (such as different members of the PIAS family), affecting the variable expression patterns of AIRE-responsive genes in each thymic cell clone.

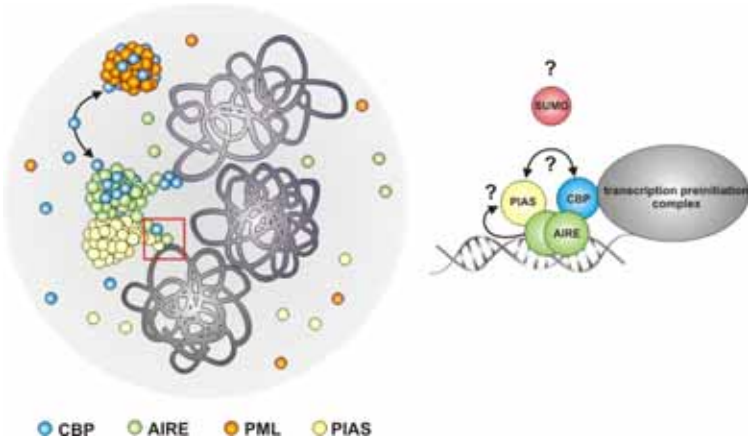

Figure 11. Hypothetical model for cellular functions of AIRE. AIRE could mediate its function by consentrating transcription factors, cofactors (e.g. PIAS, CBP), and chromatin modifiers to the vicinity of transcription sites to facilitate transcription initiation. Regulating the localization of CBP in either AIRE NBs or PML NBs could be a way to control the activity of AIRE.

CONCLUSIONS AND FUTURE PROSPECTS

Identification of molecular interactions can provide essential clues in the elucidation of the biological function and cellular mechanisms of proteins. Cellular processes can be regulated for example by the abundance of proteins, the subcellular targeting of proteins, and the dynamic association and dissociation of protein complexes. Transcriptional regulators need to be transported to the nucleus, and furthermore directed to correct subnuclear compartments via dynamic interactions with other molecules for the generation of appropriate cellular responses. In this thesis, these aspects of AIRE were addressed. The nuclear import of AIRE was identified to be regulated by the classical importin α/β pathway, and is potentially modulated, in addition to the monopartite NLS of AIRE, by other domains such as the SAND domain. The importance of homomultimerization to correct functions of AIRE were further emphasized by the data indicating that hetero-oligomerization between the G228W mutant and wt AIRE can result into incorrect subcellular localization of the wt protein and disrupt its transactivation capacity, potentially explaining the dominant negative effect of this mutation observed *in vivo*. Furthermore, a novel interaction between AIRE and the PIAS proteins was identified, and the results obtained indicate that although AIRE is not covalently modified by SUMO, sumoylation processes most likely play an important role also in the regulatory pathways involving AIRE. The localization of AIRE in the nuclear matrix may be physiologically critical, and future analysis of the effects of APECED patient mutations to the matrix association could be informatic. No cell lines expressing endogenous Aire are available, and it has been recently demonstrated that taking Aire-expressing stromal cells out of their natural three-dimensional environment to cell cultures leads to down-regulation of Aire expression (Kont et al., 2007). Thus, the nuclear relationship between AIRE, PIAS1 and SUMO1 was examined in transiently transfected cell cultures, and obviously the staining patterns may not entirely correspond to the situation in thymic mTECs. An important continuum to this study would be to examine the nuclear localization of these molecules by staining human thymus sections. Furthermore, the relevance of the AIRE-PIAS interaction *in vivo* remains to be examined. One approach could be the generation of an Aire/PIAS1 double knock-out mouse. Further characterization of the AIRE-PIAS interaction is important, as it may reveal novel mechanisms for regulation of gene expression in general, and deepen our understanding of the role played by AIRE not only in APECED but possibly also in several other autoimmune diseases.

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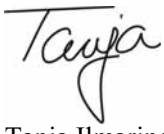

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