



The autoimmune regulator (AIRE) is a target of the E3-Ubiquitin ligase Seven-in-absentia homolog 1 (SIAH1).

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Received: 1 April 2026 / Accepted: 4 July 2026 / Published online: 14 July 2026
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Abstract

The autoimmune regulator (AIRE) is expressed in medullary thymic epithelial cells (mTECs) and is crucial for generating an immunocompetent T cell repertoire during central tolerance. Loss of AIRE function causes autoimmune polyglandular syndrome type 1 (APS-1), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Intracellular proteins are regulated by ubiquitination pathways. E3 ubiquitin ligases are specific proteins that confer selectivity to polyubiquitination, tagging proteins for proteasomal degradation while also performing other functions. In mTECs, peptides generated via the class I antigen-processing pathway are presented by HLA-I molecules to CD8 thymocytes to eliminate self-reactive T-cell precursors. AIRE induces promiscuous gene expression in mTECs, but little is known about the regulatory mechanisms of AIRE. Previously, we and others demonstrated that AIRE is an apoptosis inducer. Here, we demonstrate that AIRE increases the intracellular levels of SIAH-interacting protein (SIP), an adaptor protein of the SIAH E3 ubiquitin ligase family. We also show that AIRE interacts with SIAH1 in the human thymus. While AIRE contains two putative SIAH-interacting motifs, it interacts with SIAH proteins only through the sequence spanning residues 119–125. AlphaFold modeling indicated that the interaction between AIRE and SIAH1 is highly analogous to that observed between SIP and SIAH1, suggesting that both proteins compete for SIAH1 binding. The CARD domain and the SIAH-interacting motif is required to AIRE-mediated apoptosis. AIRE co-localized with SIAH proteins in the cytoplasm of HEK-293 cells. Finally, SIAH1 ubiquitinated AIRE, targeting it for proteasomal degradation. Collectively, these findings reveal a novel pathway for the degradation and regulation of AIRE.

Keywords Thymus · Autoimmune regulator (AIRE) · E3-ubiquitin ligase SIAH · Antigen processing · Apoptosis · Central tolerance

Abbreviations

AIRE	Autoimmune regulator
APS-1	Autoimmune polyglandular syndrome type 1
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
FBS	Fetal bovine serum
mTECs	Medullary thymic epithelial cells
mDCs	Medullary dendritic cells
pMHC	Peptide-MHC complex
SIP	Siah-1-interacting protein
SIAH1	Seven-in-absentia homolog 1
SIAH2	Seven-in-absentia homolog 2
TRAs	Tissue-restricted antigen

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Introduction

T cell progenitors exit the bone marrow and migrate to the thymus, where they undergo maturation, developing into naïve T cells. During this maturation process, potentially self-reactive thymocytes are eliminated in the thymic medulla through a mechanism known as negative selection. This involves the interaction of single positive thymocytes with self-peptide-MHC complexes (pMHC) presented by medullary thymic epithelial cells (mTECs) and medullary dendritic cells (mDCs) [1]. Thus, the final goal of thymic selection is the generation of an immunocompetent T cell repertoire that does not react against self-molecules and presents variability enough to react against many pathogens or aberrant cells. Ideally, all the self-proteome that T cells might encounter in the periphery should be expressed in the thymic medulla to efficiently eliminate self-reactive thymocytes.

The autoimmune regulator (AIRE) is predominantly expressed in mTECs, although it is also found in other extrathymic lymphoid tissues, including secondary lymphoid organs [2–4], the testis [5], and stem cells [6]. Loss of function of AIRE causes a disease called autoimmune polyglandular syndrome type 1 (APS-1), also denominated as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (OMIM entry # 240300) [7, 8], a rare disease affecting children and teenagers described in 1963 [9]. APECED patients can develop most of the known autoimmune disorders. Diagnosis is established by the presence of at least two of the following symptoms: (i) candida infection, (ii) autoimmune hypoparathyroidism, and (iii) autoimmune Addison's disease. APECED presents a higher prevalence among some populations such as Iranian Jews (1/9000) [10], Sardinians (1/14400) [11], and Finns (1/25000) [12].

AIRE controls the expression of many tissue-restricted antigens (TRAs) by mTECs, although the mechanism is not fully understood [7, 12, 13]. It has been reported that AIRE unleashes stalled RNA II polymerase [14], facilitates transcription and pre-mRNA processing [15], and interacts with specific unmethylated histones associated with inactive chromatin [16]. In addition, AIRE has been described as playing a role during differentiation and maturation of mTECs [17, 18].

AIRE-expressing cells exhibit increased apoptosis [19], suggesting the possibility that mDCs also present peptides to thymocytes derived from TRAs through cross-presentation [20]. In a previous analysis by our group, an increase of apoptosis was detected in AIRE-transfected HT93 cells (epithelial cells derived from human thyroid) [21]. One of the proteins detected as more abundant in AIRE⁺ cells was calyculin-binding protein, or SIAH-interacting protein

(SIP), an adaptor protein of the RING domain E3-ubiquitin ligase seven-in-absentia homolog (SIAH) family [22]. There are two functional proteins of this family, SIAH1 and SIAH2, which share some ligands but have also specific target proteins. E3 ubiquitin ligases are the members that confer specificity to the process of protein polyubiquitination, marking proteins for degradation in the proteasome. Furthermore, SIAH proteins have been involved in many cellular processes, such as DNA damage [23], hypoxia [24], RAS signaling [25], transcription [26], and p38 and JNK/NF- κ B pathways [27, 28]. SIAH1 and SIAH2 are expressed in several thymic cell types, including AIRE⁺ mTECs [29].

Proteins that interact with SIAH members contain the consensus motif PxAxVxP, where x represents any amino acid. SIP, for instance, harbors the motif PAAVVAP at residues 60–66. The structural interaction between SIP and SIAH1 has been characterized [30, 31].

Here, using the HEK-293 cell model, we observed that AIRE induced an elevation of SIP levels, validating the findings in HT93 cells. Additionally, we investigated the direct interaction of AIRE with SIP and SIAH proteins. AIRE did not exhibit interaction with SIP, but it did with SIAH1 and SIAH2. The SIAH-interacting motif present in AIRE was defined. AlphaFold interaction modeling indicated that the interaction between AIRE and SIAH1 is highly analogous to that observed between SIAH1 and SIP. Moreover, SIAH1 ubiquitinated AIRE, tagging it to proteasomal degradation. This interaction was also detected in the human thymus, indicating that it occurs *in vivo*. Our data demonstrate that AIRE is a novel SIAH1-interacting protein, identifying a novel putative pathway of AIRE regulation.

Materials and methods

Antibodies

The following antibodies were used:

Specificity	Isotype	Company	Cat. number	RRID
Anti-SIP (Clone D-8)	Mouse monoclonal IgG2a	Santa Cruz Biotechnology	sc-166,195	AB_2009821
Anti-Flag (Clone M2)	Mouse monoclonal IgG1	Sigma-Aldrich	F1804	AB_262044
Anti-6x His Tag (Clone HIS.H8)	Mouse monoclonal anti-body	Invitrogen	MA1-21315	AB_557403

Specificity	Isotype	Company	Cat. number	RRID
Anti-Vinculin (Clone 7F9)	Mouse monoclonal IgG antibody	Invitrogen	MA5-11690	AB_10976821
Anti- β -catenin (Clone 5H10)	Mouse monoclonal anti-body	Invitrogen	13-8400	AB_2533039
Anti- β actin (Clone C4)	Mouse monoclonal anti-body	Santa Cruz Biotechnology	sc-47,778	AB_626632
Anti-AIRE-1	Rabbit polyclonal IgG antibody	Invitrogen	PA5-78747	AB_2745863
Anti-Ubiquitin	Rabbit polyclonal antiserum	Sigma-Aldrich	U5379	AB_477667
Anti-SIAH1	rabbit polyclonal anti-body	Antibodies.com	A89218	AB_2892180
ECL anti-Mouse IgG	Horse-radish Peroxidase-Linked whole anti-body from sheep	GE Healthcare - Cytiva	NA931	AB_772210
ECL anti-Rabbit IgG	Horse-radish Peroxidase-Linked whole anti-body from donkey	GE Healthcare - Cytiva	NA934	AB_772206
Alexa Fluor 488 anti-mouse IgG	Goat polyclonal anti-body IgG (H+L)	Invitrogen (Thermo Fisher)	A-11,001	AB_2534069

Specificity	Isotype	Company	Cat. number	RRID
Alexa Fluor 488 anti-Rabbit IgG	Goat polyclonal anti-body IgG (H+L)	Invitrogen (Thermo Fisher)	A-11,008	AB_143165
Alexa Fluor 568 anti-mouse IgG	Goat polyclonal anti-body IgG (H+L)	Invitrogen (Thermo Fisher)	A-11,004	AB_2534072

DNA constructs

The pcDNA3.1-Flag-SIAH1 and pcDNA3.1-Flag-SIAH2 constructs were kindly gifted by Dr. Thilo Hagen (National University of Singapore (NUS) - Singapore).

The human *AIRE* gene and the constructs containing different regions of human *AIRE* were cloned in the pcDNA3.1-MycHis expression vector. The following constructs were used: AIRE-MycHis, AIRE-(1-106)-MycHis, AIRE-(107-545)-MycHis, AIRE-(1-180)-MycHis, AIRE-(181-545)-MycHis, AIRE-(1-343)-MycHis, and AIRE-(344-545)-MycHis. The human *CACYBP* gene was also cloned in pcDNA3.1 with a Flag tag. The human *AIRE* gene without any tag was also cloned in pcDNA3.1 (RRID: Addgene_138209).

Thymus samples, cell culture, and transfection

Thymus samples were obtained from children who underwent corrective heart surgery in the Department of Cardiac Surgery, Hospital Universitari Vall d'Hebron. Samples were cut into small pieces of about 1 cm³ and frozen at -80 °C. Samples were obtained after informed consent from the participants' families, and the experimental procedures were approved by the local ethical review boards of participating institution (ref PR AG-145/2011) in line with the principles of the Declaration of Helsinki.

Human Embryonic Kidney 293 (HEK-293; RRID: CVCL_0045) were used [32]. Cells were cultured in D-MEM (Gibco - ThermoFisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and cultured at 37 °C with 5% CO₂. Cells were authenticated by HLA typing, and regular absence of mycoplasma contamination was performed.

For transfection, approximately 2×10^5 cells were plated in each well of 6-well plates (Corning™) with 2 ml of DMEM 10% FBS. After 24 h at 37 °C and 5% CO₂ and 30 min prior to transfection, cells were serum-deprived

by replacing the medium with 2 ml of non-supplemented DMEM. Transfection was prepared by diluting 1 µg DNA in non-supplemented DMEM culture medium and, separately, the required volume of polyethyleneimine (PEI, Polysciences™) in non-supplemented DMEM, with a PEI: DNA ratio of 3:1. After brief shaking, the volume of PEI transfection solution was applied dropwise to the volume of the transfection solution with the DNA constructs. The transfection solutions were kept at room temperature, mixed periodically every 5 min for 30 min, and then applied dropwise to each well. Cells were incubated for 4 h at 37 °C, and then the culture medium was removed and replaced by fresh DMEM supplemented with 10% FBS. After 48 h, cells were recovered either mechanically or by trypsinization.

To generate stable transfectants, after 48 h of transfection, medium was replaced by DMEM 10% FBS and 1 mg/ml G418 and incubated for 2–3 weeks. The expression level of genes of interest was periodically checked by flow cytometry. In cases in which more than a single positive population was detected, limiting dilution was performed. The presence of a unique positive population was confirmed by flow cytometry.

Flow cytometry

About 5×10^5 cells were trypsinized, plated in 96-well plates, and fixed with PFA 3.7% for 20 min at room temperature (RT). Cells were rinsed twice with PBS and then permeabilized with staining buffer (SB: PBS, 0.5% Triton X-100, and 2% FBS) and incubated for 1 h with primary antibodies diluted 1/100 in SB. Cells were washed three times with SB and incubated with secondary antibody at a 1/200 dilution for 1 h. After three washing steps, cells were then resuspended in PBS, and data were collected with a FACSCalibur or a FACSCanto cytometer (BD FACSCalibur Flow Cytometry System (RRID: SCR_000401)) and analyzed by FlowJo_V10 software.

Western blot

Western blot was performed as previously described [33]. Twenty µg of cell lysates were loaded in 12% SDS-PAGE gels, and proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked for 1 h with 5% skimmed milk in 0.1% Tween-20 in PBS (T-PBS). Membranes were incubated for 1 h with the corresponding primary antibodies, washed three times with T-PBS, incubated for 1 h with peroxidase-labeled secondary antibodies, and washed 3 times with T-PBS. Vinculin (VNC) was selected to compare the abundance of different proteins in cell lysates, as it was usually used as a housekeeping protein for loading control. Detection was

performed by luminescence using Clarity Western ECL Blotting Substrate (Bio-Rad).

Immunofluorescence

A total of 2×10^5 cells were seeded in 24-well plates and cultured for 24 h. The cells were washed three times with PBS and fixed in 3.7% formaldehyde for 20 min at room temperature (RT). After fixation, cells were washed and permeabilized with SB for 5 min. They were then incubated for 1 h with the primary antibody (1:200 in SB), followed by three washes with SB and a 1 h incubation with the secondary antibody (1:400 in SB). Finally, the cells were washed three times with SB, twice with PBS, and mounted using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were acquired using an Olympus Fluoview 1000 confocal microscope.

Co-immunoprecipitation assay

Transiently transfected cells were treated with 20 µM MG132 for 4 h, collected, and washed twice with cold PBS. Cells were fixed in 500 µl of 0.37% formaldehyde in PBS for 10 min at RT. The fixation was quenched by adding 500 µl of cold PBS containing 1.25 M glycine (Sigma-Aldrich™), followed by centrifugation at $6,000 \times g$ for 10 min at 4 °C. Pellets were washed with 1 ml of cold PBS and resuspended in 200 µl of cold immunoprecipitation lysis buffer (IP lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM EDTA, supplemented with cOmplete™ protease inhibitor cocktail (Roche)). After a 1-h incubation on ice, samples were sonicated (three 10-sec cycles at 60 Hz). Simultaneously, anti-c-Myc (Pierce) or anti-Flag (Sigma-Aldrich Cat #M8823, RRID: AB_2637089) magnetic beads were equilibrated and kept on ice. Lysates were centrifuged at $13,000 \times g$ for 10 min at 4 °C, and the supernatants were collected. A 20-µl aliquot of each sample was reserved as the total lysate (input), mixed with 20 µl of RBS, and stored at −20 °C. The remaining volume was incubated with the magnetic beads for 4 h at 4 °C under rotation. The beads were then washed once with 300 µl of IP lysis buffer and three times with 300 µl of cold IP wash buffer (IPW buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP40, 1 mM EDTA, with protease inhibitors) using a magnetic separator. Finally, the beads were resuspended in 40 µl of RBS, boiled for 15 min, and analyzed by Western blot.

For ex vivo experiments, thymic tissues were mechanically disrupted using a Potter-Elvehjem homogenizer in ice-cold isotonic lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, with protease inhibitors). The homogenate was sonicated at 25 Hz for 10 min (1 s on / 5 s off) on ice and centrifuged at $13,000 \times g$ for 10 min at 4 °C. Protein concentration

in the supernatant was determined using a BCA kit (Thermo Fisher). While 10 µg of total protein was reserved as input, approximately 1 mg was used for the co-immunoprecipitation assay. Lysates were pre-cleared with 25 µL of protein G-Sepharose for 1 h under rotation, centrifuged at 6,000 x g for 2 min at 4 °C, and transferred to new tubes. Samples were incubated with 10 µg of primary antibodies for 2 h under rotation, followed by an overnight (O/N) incubation with 25 µL of protein G-Sepharose. The beads were washed five times with 300 µL of ice-cold isotonic lysis buffer (6,000 x g, 2 min, 4 °C). Finally, the protein complexes were resuspended in 40 µL of RBS, boiled for 5 min, and analyzed by Western blot.

Modeling of SIAH1 interactions with CACYBP and AIRE motifs

To model the complexes between SIAH1 (residues 125–282) and peptides from SIP (residues 58–70) or AIRE (residues 117–129 and 414–426, modeled as independent complexes), AlphaFold v2.2.4 [34] was employed. The software was installed and executed within a Docker container, with the source code and required databases (full versions) downloaded on October 18, 2022, from the official repository (<https://github.com/deepmind/alphafold>). AlphaFold was run using the “multimer” model with default parameters; multiple sequence alignments were constructed using all templates available at the time of download. For each complex, 24 models were generated, and the one with the highest ranking score was selected for further analysis.

Apoptosis assays

HEK-293 cells (RRID: CVCL_0045) or AIRE-transfected variants were seeded at a density of 1×10^5 cells per well. After 48 h of incubation at 37 °C, the cells were treated with either DMSO (as a control for basal apoptosis) or 50 µM etoposide (Sigma-Aldrich™) to induce apoptosis, followed by an additional 24 h incubation. Apoptosis was assessed using an Annexin V-Alexa Fluor 488 and Propidium Iodide (PI) Detection Kit (Thermo Fisher Scientific™) according to the manufacturer’s instructions. Samples were subsequently analyzed by flow cytometry.

Ubiquitination assays

Stable clones expressing AIRE-Myc/His, AIRE-(1–343)-Myc/His, or AIRE-(181–545)-Myc/His were transiently transfected with Flag-SIAH1 and Ub-Flag constructs and treated with 20 µM MG132 (Sigma-Aldrich) for 4 h at 37 °C. Cells were then harvested and lysed in lysis buffer (LB: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5%

NP40, 1% Triton X-100, supplemented with cOmplete™ protease inhibitor cocktail) for 1 h at 4 °C. Lysates were homogenized by passing the samples several times through a 30-gauge needle. AIRE-Myc/His proteins were immunoprecipitated using anti-Myc magnetic beads (Thermo Fisher Scientific Cat# 88843, RRID: AB_2861398) as described above. Proteins were resolved by 12% SDS-PAGE and transferred onto PVDF membranes. Ubiquitinated proteins were detected using a rabbit polyclonal anti-ubiquitin antibody (Sigma-Aldrich Cat# U5379, RRID: AB_477667).

AIRE degradation assays

Approximately 2×10^5 HEK293 cells (RRID: CVCL_0045) or stable AIRE-expressing cells were seeded in 6-well plates (Corning™) in 2 ml of DMEM supplemented with 10% FBS. After 24 h at 37 °C, cells were transiently transfected as described above with AIRE-Myc/His, AIRE-V3E-Myc/His, AIRE-P4E-Myc/His, Flag-SIAH1, or Flag-ΔSIAH1 (a catalytic domain-deficient mutant). Cells were harvested by scraping, washed twice with ice-cold PBS, and lysed in lysis buffer (LB) for 1 h on ice. Lysates were homogenized by passing them several times through a 30-gauge needle. Supernatants were then collected by centrifugation at 13,000 x g for 10 min at 4 °C. Protein concentrations were determined, and samples were analyzed by SDS-PAGE and Western blot. Densitometric analysis was performed using Fiji/ImageJ software (RRID: SCR_003070).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. The Mann-Whitney *U* test ($p < 0.05$, $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$) was used to compare the corrected total cell fluorescence (CTCF) between control and AIRE-transfected cells. The Wilcoxon signed-rank test ($p < 0.05$, $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$) was employed to compare densitometric values of protein levels between the control and transfected groups.

Results

CACYBP/SIP levels are upregulated in AIRE-expressing cells

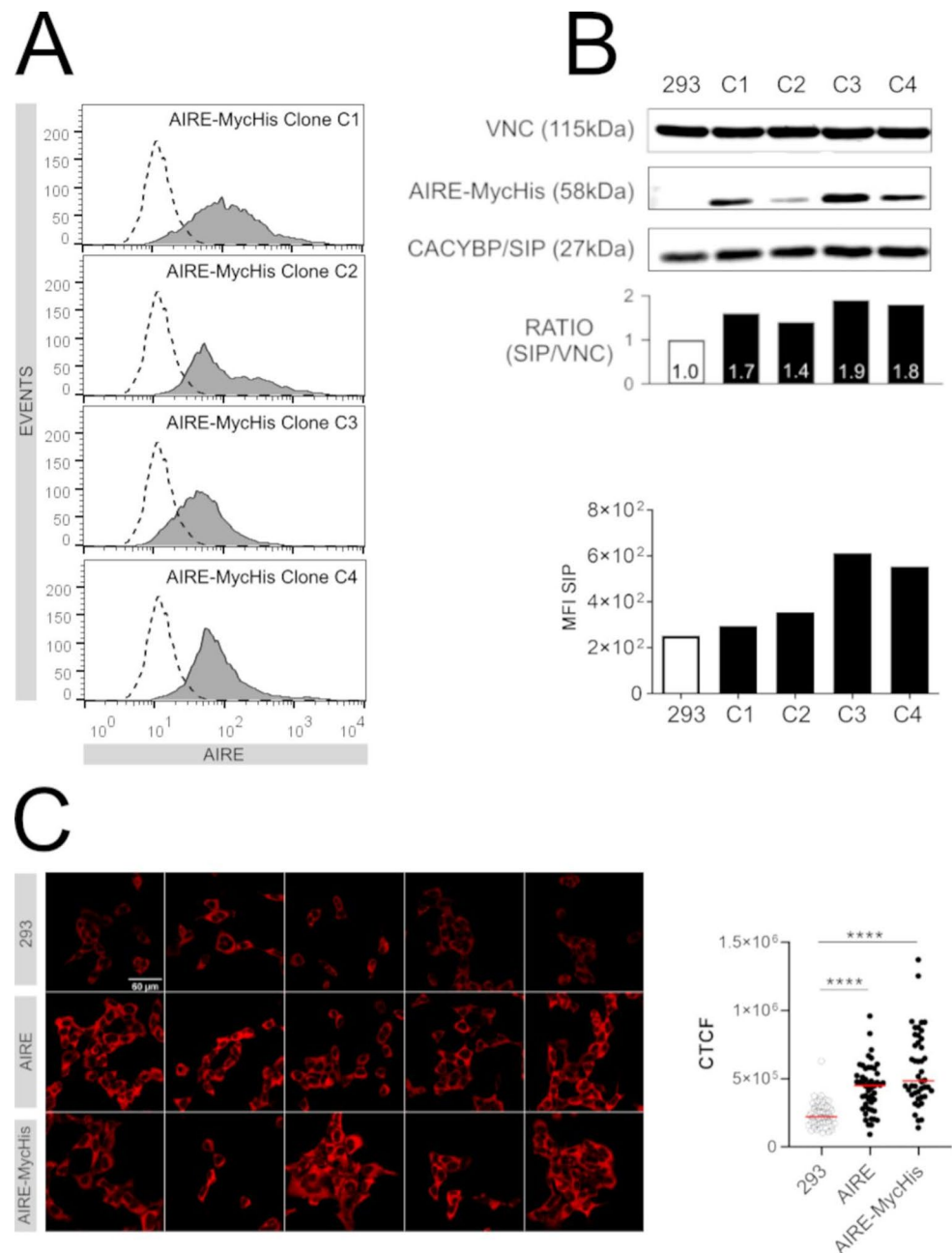
We previously characterized the proteomic profiles of HT93 epithelial cells (thyroid origin) and AIRE-transfected HT93 cells, demonstrating that AIRE⁺ cells exhibit increased apoptosis. Additionally, higher levels of SIP were detected in AIRE⁺ cells compared to control groups. To verify that this effect is mediated by AIRE expression and is not

cell-line specific, we generated a stable HEK-293 transfectant overexpressing AIRE with a C-terminal Myc/His tag (AIRE-MycHis). Four AIRE⁺ clones were isolated via limiting dilution (Fig. 1A). Increased SIP protein expression was confirmed across all four clones using Western blot, flow cytometry (Fig. 1B), and immunofluorescence (IF, Fig. 1C). To exclude potential tag-induced artifacts, a tag-free AIRE transfectant was also generated. IF analysis revealed SIP elevation levels comparable to the AIRE-MycHis clones (Fig. 1C). These findings, consistent with our prior observations in HT93 cells, demonstrate that AIRE expression increases SIP protein abundance.

To determine whether AIRE modulates SIP through transcriptional induction, total RNA from HEK-293 and AIRE⁺ clones was purified and reverse-transcribed into cDNA. PCR analysis of *CACYBP* mRNA transcripts revealed higher transcript abundance in all AIRE⁺ clones (Figure S1).

Furthermore, we examined the reciprocal effect of SIP on AIRE expression. Human *CACYBP* (encoding SIP) was cloned with a Flag tag into pcDNA3.1 (SIP-Flag) and transiently transfected into AIRE-expressing HEK-293 clones. Western blot analysis showed an increase in AIRE levels in three SIP-Flag transfectants compared to non-transfected controls (Figure S2). This mutual upregulation of

Fig. 1 CACYBP/SIP levels are upregulated in AIRE-expressing cells. **A** Flow cytometry analysis of HEK-293 clones stably transfected with AIRE-MycHis. Protein expression was detected using an anti-Myc antibody. Dashed line: parental HEK-293; filled histogram: AIRE-transfected clones. **B** Analysis of SIP protein expression in AIRE⁺ clones. Upper panel: Representative Western blot of SIP levels. The bar graph represents the SIP/vinculin ratio determined by densitometric analysis, normalized to parental HEK-293 cells. Lower panel: Mean fluorescence intensity (MFI) quantified by flow cytometry of SIP expression in HEK-293 cells and AIRE-expressing clones (C1–C4, as described in panel A). In the figure a representative experiment is shown, but 3 different experiments were made, and SIP was increased in all clones. **C** Confocal immunofluorescence microscopy of SIP expression in parental HEK-293, HEK-293-AIRE (untagged), and HEK-293-AIRE-MycHis. Cells were fixed and stained with an anti-SIP antibody (red). Micrographs were acquired at 60× magnification. Right panel: Quantification of Corrected Total Cell Fluorescence (CTCF) from 50 randomly selected cells per group. CTCF was calculated as: Integrated Density – (Area of selected cell × Mean fluorescence of background readings). Statistical significance was determined using the Mann-Whitney U test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)



intracellular SIP and AIRE levels suggests a bidirectional regulatory mechanism between these two proteins.

AIRE and SIP do not interact in transfected HEK-293 cells

To investigate a potential direct interaction between AIRE and SIP *in vivo*, co-immunoprecipitation (Co-IP) assays were performed. HEK-293 cells were co-transfected with AIRE-MycHis and SIP-Flag constructs and subsequently treated with the proteasome inhibitor MG-132 for 4 h. Following incubation, AIRE was immunoprecipitated using an anti-Myc monoclonal antibody (mAb), and the presence of both proteins was assessed via immunoblotting with specific anti-Myc and anti-Flag antibodies. Reciprocal Co-IP experiments were also conducted by immunoprecipitating SIP with an anti-Flag mAb, followed by Western blot analysis for Flag and Myc. As shown in Fig. 2A, no physical interaction was detected between AIRE and SIP under these conditions. Furthermore, confocal microscopy was employed to evaluate intracellular localization in HEK-293 cells stably expressing AIRE; however, no significant co-localization was observed (Fig. 2B).

AIRE interacts with SIAH1 and SIAH2 in transfected HEK-293 cells and in the thymus

Since SIP serves as an adaptor protein for the E3 ubiquitin ligases SIAH1 and SIAH2, we employed confocal microscopy to investigate the potential co-localization of AIRE with these ligases. HEK-293 cells stably expressing AIRE were transiently transfected with SIAH1 or SIAH2

constructs. As shown in Fig. 3A, AIRE co-localized with both SIAH1 and SIAH2 within the cytoplasm. The expression of AIRE outside the nucleus was in fibrillar structures, what has been previously reported in our laboratory [21].

SIAH proteins recognize a consensus binding motif, PxAxVxP (where x represents any amino acid). *In silico* analysis of the AIRE primary sequence identified two putative SIAH-interacting motifs (SIMs): a canonical PKALVPP sequence (residues 119–125, SIM1) and a non-canonical PLLCVGP sequence (residues 416–422, SIM2), the latter featuring a leucine substitution for alanine at the second position (Figure S3).

To evaluate the physical association between AIRE and SIAH1, Co-IP assays were performed using AIRE-MycHis and Flag-SIAH1 constructs. Following immunoprecipitation of AIRE with an anti-Myc mAb, Flag-SIAH1 was detected via Western blot (Fig. 3B), confirming an interaction between AIRE and SIAH1 in transfected HEK-293 cells. Analogous experiments using Flag-SIAH2 yielded comparable results (Fig. 3B), indicating that AIRE also associates with SIAH2.

Finally, to validate this interaction in a physiological context, Co-IP was performed using human thymus tissue lysates. Following AIRE immunoprecipitation, SIAH1 was recovered and detected by Western blot (Fig. 3C), demonstrating that the AIRE-SIAH1 interaction occurs endogenously within the human thymus.

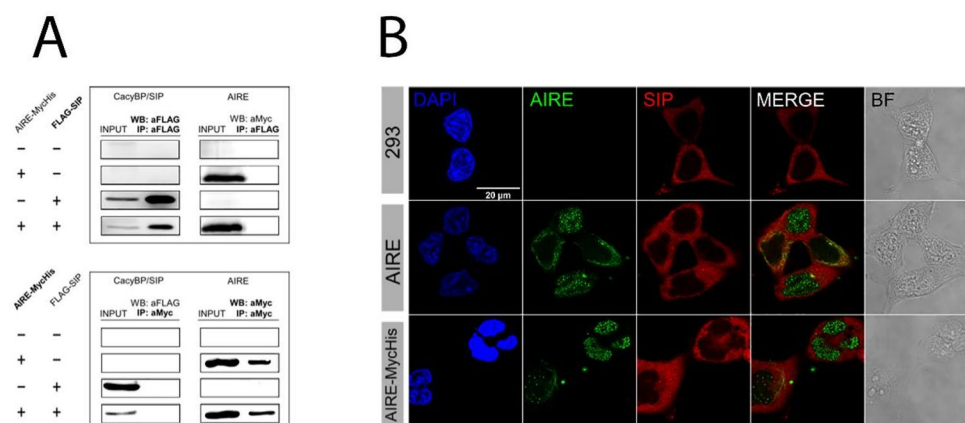
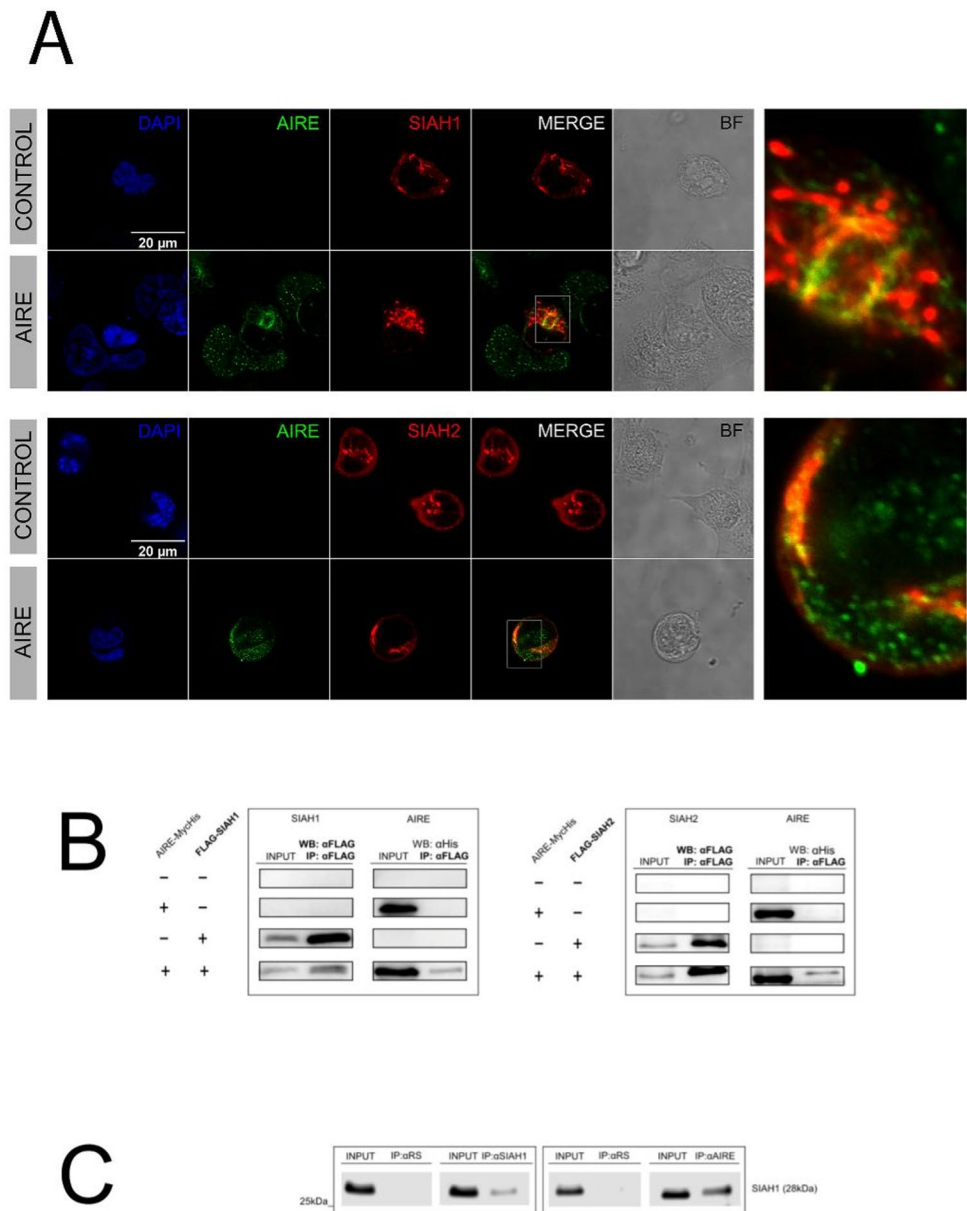


Fig. 2 AIRE does not physically associate with SIP in transfected HEK-293 cells. **A** Co-immunoprecipitation (Co-IP) assay of HEK-293 cells transiently co-transfected with AIRE-MycHis and SIP-Flag. Lysates were subjected to immunoprecipitation (IP) using anti-Flag or anti-Myc antibodies, followed by Western blot (WB) analysis for the indicated tags. INPUT: Total cell lysate (1/100 of the starting material). No reciprocal interaction was detected between AIRE and SIP.

B Confocal immunofluorescence microscopy of HEK-293 cells stably expressing either untagged AIRE or AIRE-MycHis. Fixed cells were stained with anti-AIRE (green) and anti-SIP (red) antibodies; DAPI (blue) was used for nuclear counterstaining. Merged images (green and red channels) and brightfield (BF) micrographs are shown. No intracellular co-localization between AIRE and SIP was observed. Images were acquired at 60× magnification with a 4× digital zoom

Fig. 3 AIRE co-localizes and interacts with SIAH1 and SIAH2.

A Confocal immunofluorescence analysis of parental HEK-293 cells or HEK-293 cells stably expressing AIRE, transiently transfected with Flag-SIAH1 or Flag-SIAH2. Fixed cells were stained with anti-AIRE (green) and anti-Flag (red) antibodies; DAPI (blue) was used for nuclear counterstaining. Merged images (green and red channels) and brightfield (BF) micrographs are shown. Intracellular co-localization between AIRE and SIAH1/2 was observed in the cytoplasm. Images were acquired at 60× magnification with a 4× digital zoom. **B** Co-immunoprecipitation (Co-IP) assay of HEK-293 cells transiently co-transfected with AIRE-MycHis and Flag-SIAH1 or Flag-SIAH2. SIAH proteins were immunoprecipitated using an anti-Flag antibody, followed by Western blot (WB) analysis for Flag or His tags. INPUT: Total cell lysate (1/100). **C** Endogenous interaction in human thymus. Thymus tissue samples were homogenized (potterized), and protein extracts were subjected to IP using normal rabbit serum (α RS) as a negative control, a rabbit anti-SIAH1 polyclonal antibody α SIAH1), or a rabbit anti-AIRE polyclonal antibody α AIRE). Following immunoprecipitation, SIAH1 was detected by WB. INPUT: Total thymus lysate (1/100)



AIRE interacts with SIAH proteins via its N-terminal SIAH-binding motif

To map the specific AIRE regions mediating the interaction with SIAH proteins, several C-terminally Myc/His-tagged AIRE truncation mutants were cloned into the pcDNA3.1 expression vector. These constructs were designed to include or exclude the two putative SIAH-interacting motifs: (1) Fragment 1–106 (CARD domain, lacking both SIMs); (2) Fragment 107–545 (containing both SIMs and the SAND, PHD1, PRR, and PHD2 domains); (3) Fragment 1–180 (CARD and SIM1); (4) Fragment 181–545 (SAND, PHD1, PRR, PHD2, and SIM2); (5) Fragment 1–343

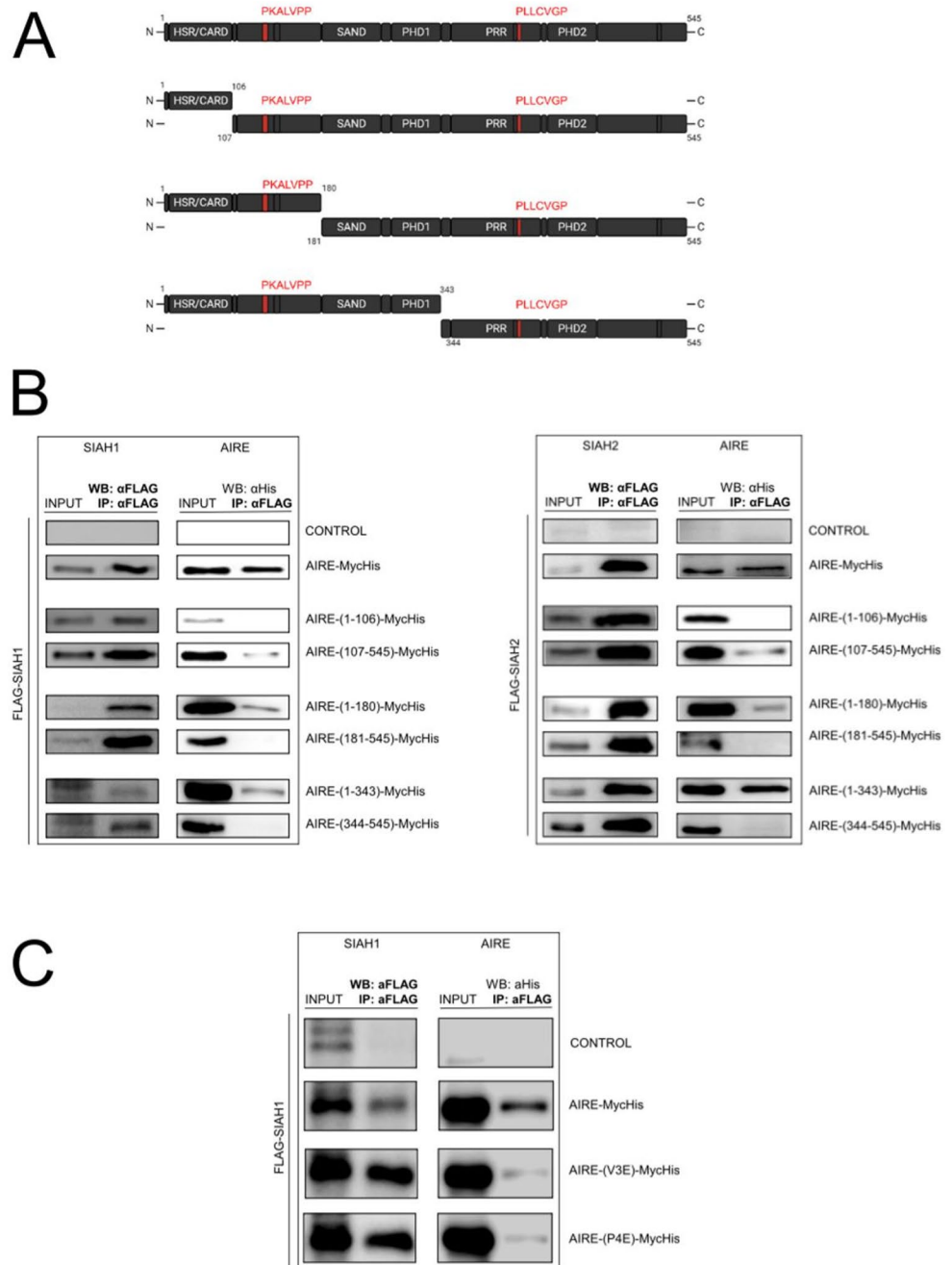
(CARD, SAND, PHD1, and SIM1); and (6) Fragment 344–545 (PRR, PHD2, and SIM2) (Fig. 4A).

Co-transfection assays with Flag-SIAH1 revealed that only the full-length AIRE and constructs containing SIM1 (107–545, 1–180, and 1–343) were recovered after anti-Flag immunoprecipitation. In contrast, constructs lacking this motif (1–106, 181–545, and 344–545) showed no interaction with SIAH1 (Fig. 4B). Equivalent results were obtained in experiments with SIAH2 (Fig. 4B).

To further validate the requirement of SIM1, site-directed mutagenesis was performed to replace Val123 and Pro125 with Glutamic acid (V123E and P125E). Co-IP analysis showed a marked reduction in AIRE recovery following SIAH1 immunoprecipitation for both mutants (Fig. 4C),

Fig. 4 AIRE interacts with SIAH1 and SIAH2 via the first SIAH-interacting motif (SIM1).

A Schematic representation of full-length AIRE and various truncation constructs. The two putative SIAH-interacting motifs (SIM1 and SIM2) are highlighted in red. **B** Co-immunoprecipitation (Co-IP) assay in HEK-293 cells transiently co-transfected with Flag-SIAH1 or Flag-SIAH2 and either full-length or truncated AIRE-MycHis constructs. SIAH proteins were immunoprecipitated using an anti-Flag antibody, followed by Western blot (WB) analysis for Flag and His tags. **C** Site-directed mutagenesis analysis. HEK-293 cells were co-transfected with Flag-SIAH1 and either wild-type AIRE-MycHis or the point mutants V123E (AIRE-V3E) and P125E (AIRE-P5E) within the first motif. Following anti-Flag immunoprecipitation, AIRE recovery was assessed by anti-His WB. **INPUT**: Total cell lysate (1/100).



confirming that the motif spanning residues 119–125 is the primary SIAH-binding site.

Finally, we performed a competitive binding assay to determine if AIRE and SIP compete for SIAH1 association. Recombinant StII-ΔSIAH1 (a catalytically inactive protein, lacking N-terminal residues 1–89) was incubated with HEK-293-AIRE cell lysates in the presence or absence of purified SIP-His protein. The addition of SIP significantly reduced the amount of AIRE recovered, suggesting that AIRE and SIP compete directly for the same binding site on SIAH1 (Figure S4).

Structural modeling predicts that SIAH1 selectively interacts with the AIRE₁₁₉₋₁₂₅ motif

To assess the structural basis of the AIRE-SIAH1 interaction, we performed in silico modeling using AlphaFold. We first validated the model by superimposing the predicted SIAH1-SIP peptide complex with the existing crystallographic structure (PDB 2A25). Subsequent modeling was conducted for the two putative SIMs of AIRE. As shown in Fig. 5A, the modeled SIAH1-SIP₅₈₋₇₀ complex aligns precisely with the experimental crystal structure. Similarly, the

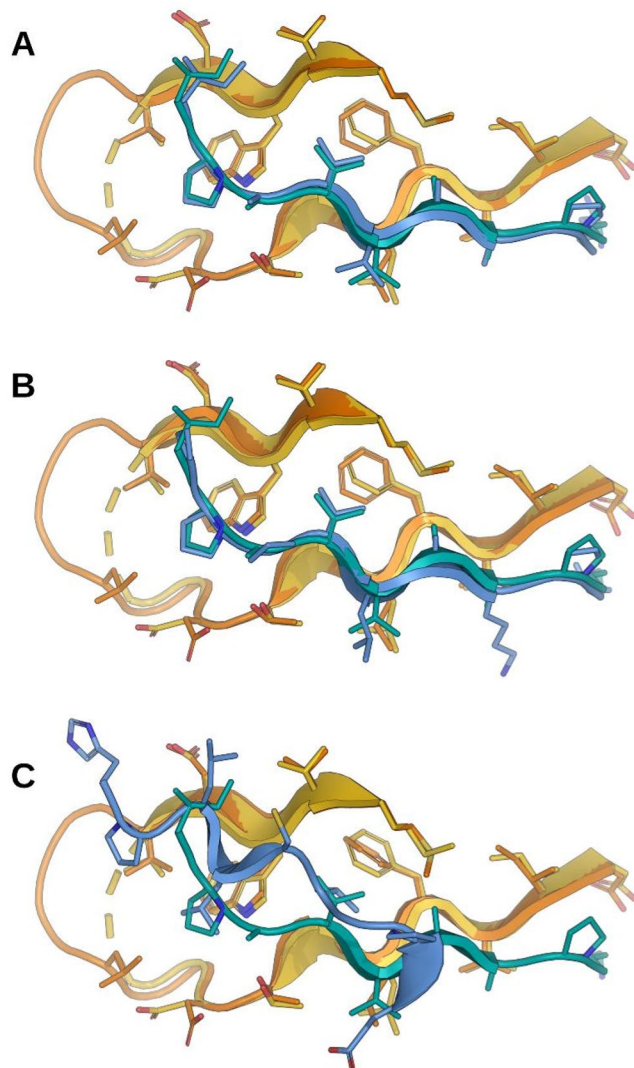


Fig. 5 Structural characterization of SIAH1-peptide complexes at the binding interface. The panels illustrate the structural superposition of the crystallographic SIAH1-SIP₅₉₋₆₇ complex (PDB 2A25) with the complexes modeled by AlphaFold: **A** SIAH1-SIP₅₈₋₇₀, **B** SIAH1-AIRE₁₁₇₋₁₂₉, and **C** SIAH1-AIRE₄₁₄₋₄₂₆. Color code: Gold: crystallographic SIAH1 structure (dashed lines indicate unresolved loops); Green: crystallographic SIP₅₉₋₆₇ peptide; Orange: AlphaFold-predicted SIAH1 structure; Blue: AlphaFold-predicted peptide structure. Structural representation: Both ribbon diagrams (highlighting β -strand secondary structures) and stick representations (side chains) are shown. The models display SIAH1 residues 162–180, which constitute the primary binding surface, and nine residues from each peptide corresponding to the crystallographically resolved region. Sequences are as follows (core binding residues in bold): KPAAVVAPI (SIP₅₉₋₆₇), VPKALVPPP (AIRE₁₁₈₋₁₂₆), and HPLLVCVGPE (AIRE₄₁₅₋₄₂₃). Note that the AIRE₄₁₅₋₄₂₃ sequence adopts an inverted orientation within the binding pocket relative to the canonical SIP₅₉₋₆₇ and AIRE₁₁₈₋₁₂₆ binding modes

SIAH1-AIRE₁₁₇₋₁₂₉ complex exhibited an identical binding mode, overlapping perfectly with the SIAH1-SIP₅₈₋₇₀ interface (Fig. 5B). In contrast, the SIAH1-AIRE₄₁₄₋₄₂₆ peptide appeared significantly less structured and adopted an

inverted orientation (Fig. 5C), suggesting a lack of stable interaction with SIAH1.

Model reliability was further evaluated using AlphaFold confidence metrics (Figure S5A (SIAH1-SIP₅₈₋₇₀), S5B (SIAH1-AIRE₁₁₇₋₁₂₉) and S5C (SIAH1-AIRE₄₁₄₋₄₂₆)). The pLDDT (per-residue confidence score) for AIRE₄₁₄₋₄₂₆ was below 50, a threshold indicative of disordered regions [35]. Conversely, pLDDT values exceeded 90 for the core residues of both SIP₅₈₋₇₀ and AIRE₁₁₇₋₁₂₉, predicting high structural accuracy. Furthermore, Predicted Aligned Error (PAE) matrices confirmed high confidence for the interchain interactions in the SIP and AIRE complexes, while confidence for the AIRE₄₁₄₋₄₂₆ interaction remained low.

Both SIP₅₈₋₇₀ and AIRE₁₁₇₋₁₂₉ formed a parallel β -sheet with SIAH1 residues Val164–Thr168, mediated by residues Pro60–Val64 in SIP, seen in the crystal (Figure S6A) and with AlphaFold (Figure S6B) and Pro119–Val123 in AIRE (Figure S6C). Although AIRE₄₁₄₋₄₂₆ contains a potential SIM (PxxxVxP), we hypothesized that the substitution of the canonical Ala62 (found in SIP) with a bulky Leu418 in AIRE results in steric hindrance with SIAH1 residues Thr156 and Met180. To test this, we re-evaluated the AIRE₄₁₄₋₄₂₆ model after introducing a L418A mutation. The AlphaFold prediction confirmed that this single substitution restores the canonical binding pose observed in SIP₅₈₋₇₀ and AIRE₁₁₇₋₁₂₉ (Figure S7A), with a significant improvement in confidence scores (Figure S7B). These results demonstrate that an alanine at the second position of the motif is a critical determinant for SIAH1 binding.

The CARD domain and the SIAH1-interacting motif are essential for AIRE-mediated apoptosis

Previous studies by our group and others have established that AIRE expression induces apoptosis. To determine whether the SIAH1-interacting motif (degron) is required for this proapoptotic activity, we analyzed stable transfectants expressing full-length AIRE (with or without a Myc/His tag) and four AIRE truncation mutants: AIRE₁₋₁₈₀ (containing the CARD domain and the degron), AIRE₁₋₁₀₆ (CARD domain only), AIRE₁₀₇₋₅₄₅ (degron sequence without CARD), and AIRE₁₈₁₋₅₄₅ (lacking both domains).

Cells were treated with DMSO to assess spontaneous apoptosis or with etoposide to evaluate sensitized apoptotic responses. Apoptosis levels were quantified by Annexin V staining and flow cytometry (Figure S8 shows a representative workflow of the flow cytometry analysis). A significant increase in spontaneous apoptosis (Fig. 6A) or etoposide-induced apoptosis (Fig. 6B) was observed only in cells expressing full-length AIRE or the AIRE fragment, which retains both the CARD domain and the SIM1. In contrast, constructs lacking either the CARD domain or the

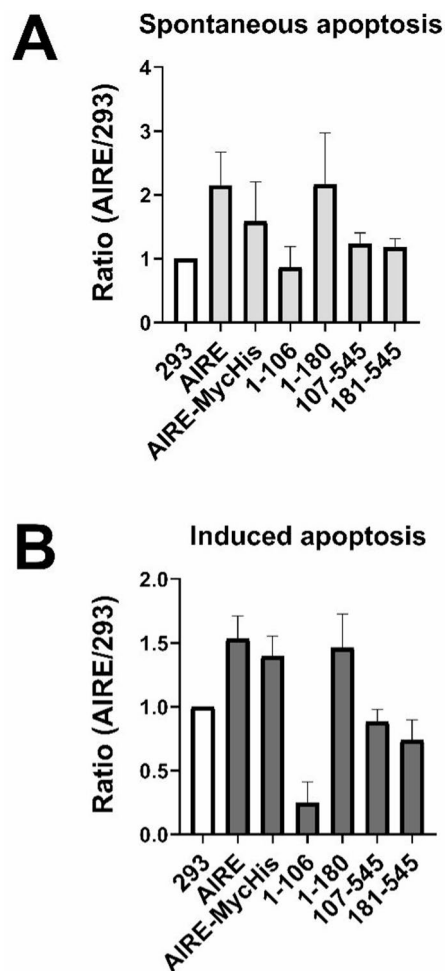


Fig. 6 The CARD domain and the SIAH1-interacting motif (SIM1) of AIRE are essential for apoptosis induction in HEK-293 cells. Parental HEK-293 cells and various stable AIRE transfectants were treated with DMSO (vehicle control) to measure spontaneous apoptosis or with etoposide (50 μ M) to evaluate chemically induced apoptosis. Apoptotic levels were assessed by flow cytometry using Annexin V-FITC and Propidium Iodide (PI) staining. Upper panel: Fold increase in spontaneous apoptosis of different stable AIRE transfectants relative to parental HEK-293 cells after 24 h. Lower panel: Fold increase in etoposide-induced apoptosis across the indicated cell lines after 24 h. Data represent the mean of $n=4$ independent experiments (measures range, from 6 to 20). Statistical significance was determined using the Kruskal-Wallis test followed by post-hoc comparisons (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). Values in (A) and (B) represent total apoptosis, calculated as the sum of early apoptotic (Annexin V⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) populations, normalized to parental HEK-293 controls

degron sequence failed to promote apoptosis, indicating that both regions are strictly required for AIRE's proapoptotic function.

SIAH1-mediated ubiquitination and proteasomal degradation of AIRE

To determine if the physical association between AIRE and SIAH1 leads to post-translational modification, we performed ubiquitination assays in HEK-293 cells. AIRE-MycHis was co-transfected with Flag-SIAH1 and Flag-Ubiquitin (Flag-Ub). Additionally, cells stably expressing AIRE₁₋₃₄₃ (containing SIM1) or AIRE₁₈₁₋₅₄₅ (lacking the motif) were analyzed. Following immunoprecipitation of AIRE with an anti-Myc mAb, polyubiquitination was detected using a specific anti-Ub antibody. As shown in Fig. 7, robust polyubiquitination was observed for both full-length AIRE and the AIRE₁₋₃₄₃ fragment, whereas no ubiquitination was detected in control HEK-293 cells or the AIRE₁₈₁₋₅₄₅, confirming that the SIAH1-interacting motif is required for AIRE ubiquitination.

E3 ubiquitin ligases provide substrate specificity for polyubiquitination, often targeting proteins for proteasomal degradation. To investigate whether SIAH1 modulates AIRE stability, AIRE-expressing HEK-293 cells were transiently transfected with SIAH1. Western blot analysis, normalized to Vinculin, revealed a statistically significant reduction in AIRE levels upon SIAH1 overexpression (Fig. 8A). This decrease was highly reproducible across independent experiments. Notably, this effect was abrogated when cells were transfected with a catalytically inactive SIAH1 mutant (FLAG- Δ SIAH1, lacking residues 1–89), demonstrating that the E3 ligase activity of SIAH1 is essential for AIRE degradation (Fig. 8A). A similar reduction was observed for β -catenin, a well-characterized SIAH1 substrate, further validating the assay.

To confirm the functional relevance of the identified degron (residues 119–125), degradation assays were repeated using the V123E and P125E mutants. As shown in Fig. 8B, these single-point mutations within the interaction motif impaired SIAH1-mediated degradation. Taken together, these data demonstrate that SIAH1 functions as an E3 ubiquitin ligase that targets AIRE for proteasomal degradation through a specific interaction with the motif spanning residues 119–125.

Discussion

AIRE plays a pivotal role in the thymic medulla by ensuring an immunocompetent T-cell repertoire through the induction of promiscuous gene expression (PGE) in medullary thymic

Fig. 7 SIAH1 mediates the polyubiquitination of AIRE. Parental HEK-293 cells and stable transfectants expressing AIRE-MycHis, AIRE₁₋₃₄₃-MycHis (containing the SIAH-interacting motif), or AIRE₁₈₁₋₅₄₅-MycHis (lacking the motif) were transiently co-transfected with Flag-SIAH1/2 and Flag-Ubiquitin (Flag-Ub). After 48 h, proteasomal degradation was inhibited with MG-132 for 4 h. AIRE was immunoprecipitated (IP) using an anti-Myc antibody. AIRE proteins were detected by Western blot (WB) using an anti-His antibody. SIAH proteins and Flag-Ub were detected with an anti-Flag antibody, and polyubiquitinated AIRE species were visualized using an anti-Ub antibody. (*) Denotes the IgG heavy chain from the immunoprecipitating antibodies. VNC: Vinculin (loading control); β -CAT: β -catenin; β -ACT: β -actin

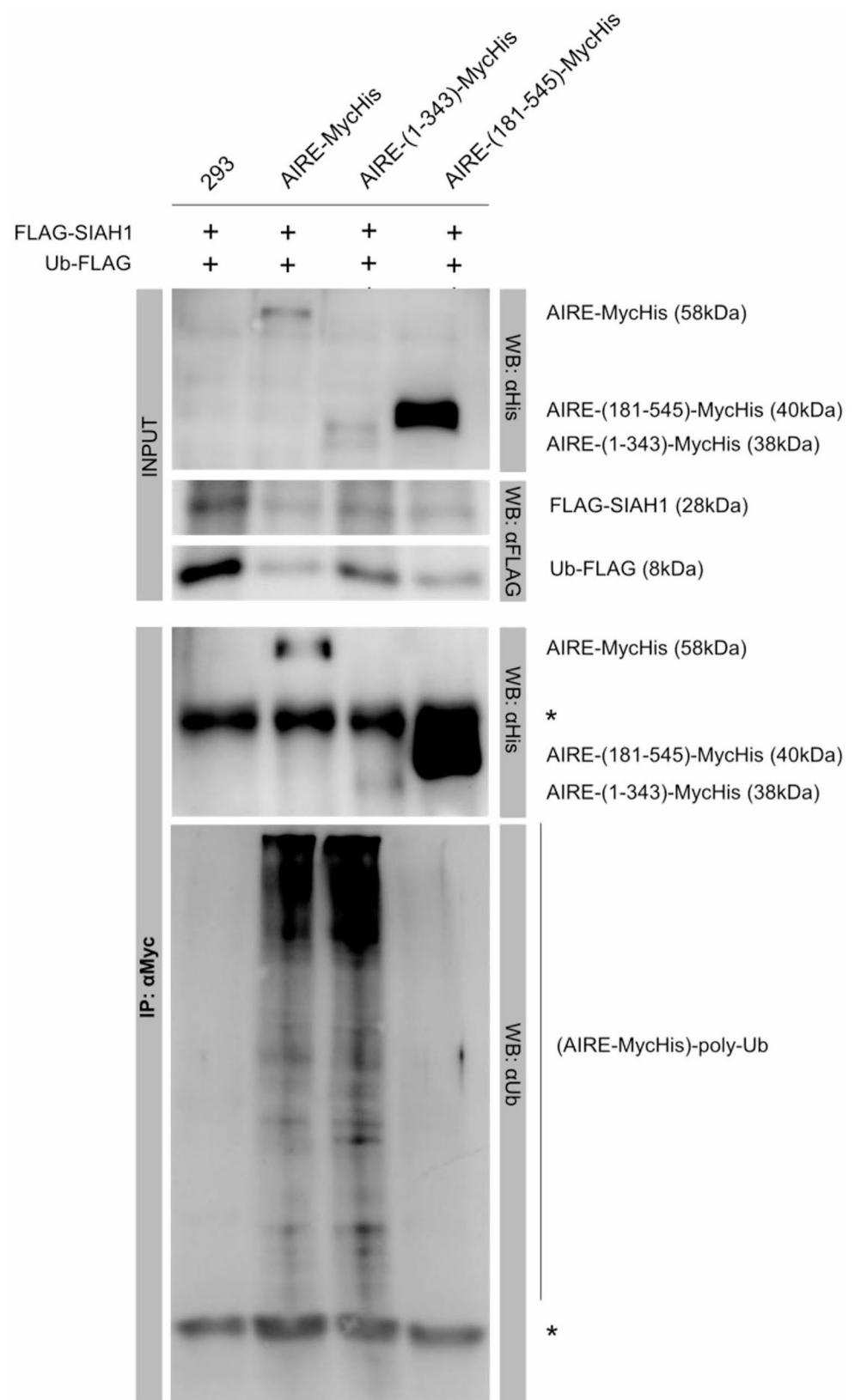
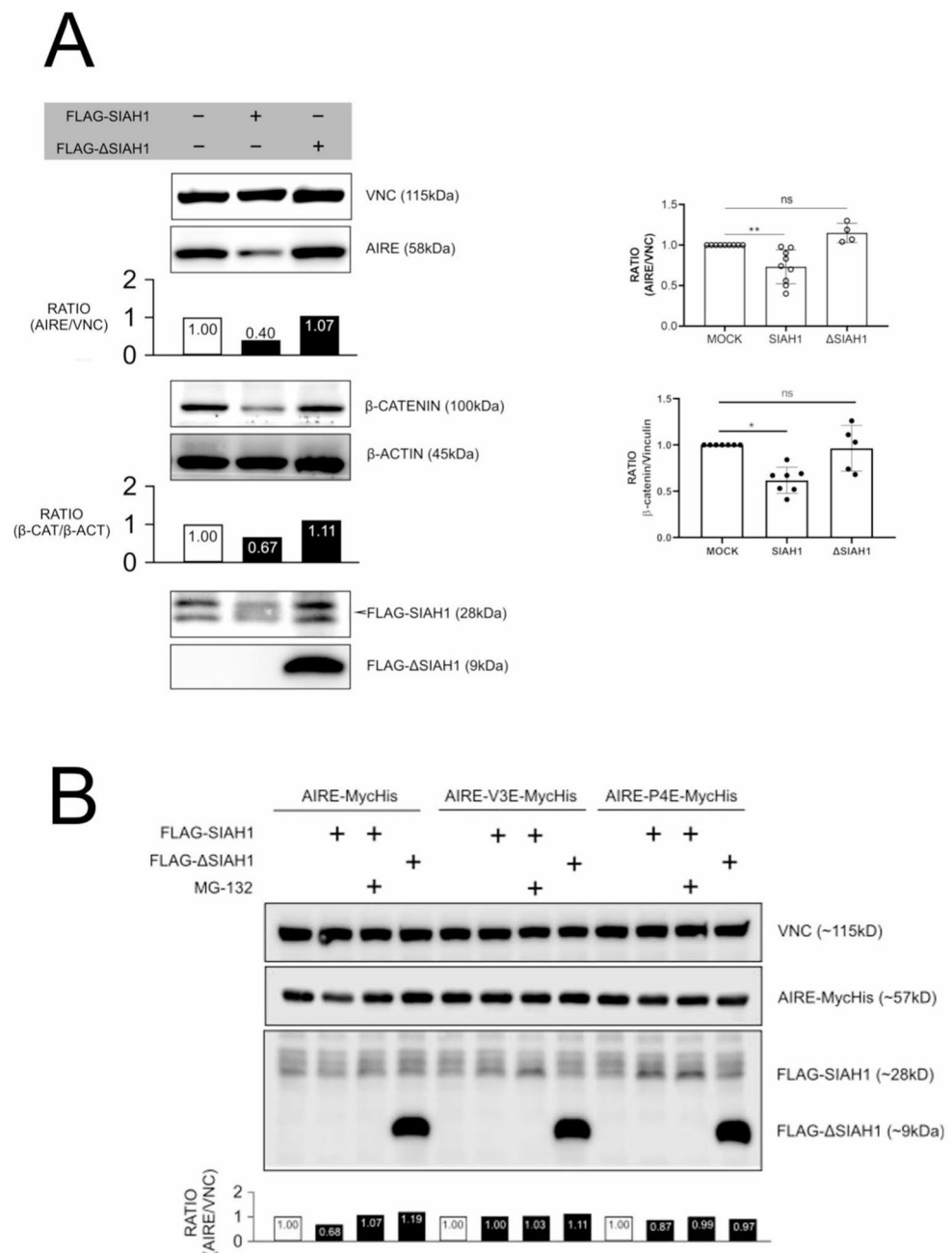


Fig. 8 SIAH1 mediates the proteasomal degradation of AIRE. **A** HEK-293 cells stably expressing AIRE were transiently transfected with SIAH1 or a catalytically inactive protein, Δ SIAH1 (lacking residues 1–89). Protein levels were assessed by Western blot (WB). Bar graphs represent the AIRE/Vinculin or β -catenin/Vinculin densitometric ratios. Right panel: Normalized values for AIRE/Vinculin ratios in non-transfected ($n=9$), SIAH1-transfected ($n=9$), and Δ SIAH1 transfected cells ($n=4$), and β -catenin/Vinculin ratios in non-transfected ($n=7$), SIAH1-transfected ($n=7$), and Δ SIAH1 transfected cells ($n=5$). Statistical significance was determined using the Wilcoxon signed-rank test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). **B** Mutational analysis of the AIRE degon. HEK-293 cells were transiently co-transfected with AIRE-MycHis (wild-type or the V123E and P125E mutants) and Flag-SIAH1 or Flag- Δ SIAH1. Cells were treated with or without the proteasome inhibitor MG-132 to evaluate the involvement of the ubiquitin-proteasome pathway. Relative AIRE/Vinculin densitometric ratios are shown for each condition



epithelial cells (mTECs) [7, 12–15, 17–19]. Mutations in the AIRE gene lead to the development of APS-1/APECED, a severe autoimmune disorder characterized by a breakdown in central tolerance [36]. Understanding the post-translational regulation of the AIRE protein is, therefore, essential for unraveling the mechanisms of immune tolerance and the development of autoimmunity. In this study, we identified and characterized the interaction between AIRE and members of the SIAH E3-ubiquitin ligase family, demonstrating that AIRE undergoes ubiquitination and subsequent proteasomal degradation mediated by SIAH1.

Our findings reveal a reciprocal regulation between AIRE and SIP (CACYBP). Stable AIRE-expressing HEK-293 clones showed consistent SIP upregulation, confirming that this process is AIRE-dependent. Conversely, SIP overexpression led to increased AIRE levels, suggesting a compensatory co-regulation mechanism potentially driven by their competition for SIAH binding.

The canonical SIAH-interacting motif (PxAxVxP) is present in SIP (residues 60–66; PAAVAP) [37]. In human AIRE, we identified two putative motifs: AIRE_{119–125} (PKALVPP) and AIRE_{416–422} (PLLCVGP). Although the second motif contains a basic residue that has been suggested

to enhance SIAH1 interaction [38], it features a leucine substitution for the canonical alanine at the second position. Our biochemical data and structural modeling demonstrate that AIRE interacts with SIAH exclusively through the first motif (residues 119–125). Truncated proteins lacking this region failed to co-immunoprecipitate with SIAH1/2, and AlphaFold models confirmed that AIRE_{414–426} cannot adopt the correct binding orientation. These results indicate that a canonical sequence is more critical for SIAH1 recruitment than the presence of flanking basic residues. Furthermore, competitive binding assays with purified proteins confirmed that AIRE and SIP directly compete for the same SIAH1 binding site.

The AIRE PKALVPP motif is in a structurally flexible region between the CARD and SAND domains, adjacent to the bipartite nuclear localization signal (NLS; residues 110–114 and 131–133). This motif is highly conserved across mammals (humans, mice, rats, and simians), with only minor variations in species such as horses, cows, and pigs. In contrast, the SIP motif remains entirely unchanged across mammalian evolution. While many proteins, such as ACK1 [39], OBF1 [40], DCC [41] and KLF10 [37], utilize this canonical motif for SIAH interaction, others like Axin-1 [42] or KIF22 [43] rely on non-canonical sequences. Our study highlights that AIRE belongs to the former group, utilizing a conserved, canonical degron to regulate its intracellular stability.

Our results demonstrate that AIRE undergoes SIAH1-mediated degradation. Although the reduction in protein levels was modest, it was remarkably consistent across both transient and stable transfection models. In contrast, SIAH2 did not show a consistent effect on AIRE stability; while some experiments suggested a decrease in AIRE abundance, others showed an increase, indicating that SIAH2 may not effectively degrade AIRE under the tested conditions. However, a potential degradative role for SIAH2 in different cellular contexts cannot be entirely excluded. To validate the specificity of these findings, we analyzed β -catenin, a well-known substrate of the SIP/SIAH1 pathway. The degradation kinetics observed for β -catenin mirrored those of AIRE, further supporting the role of SIAH1 as a regulator of AIRE stability. Notably, AIRE expression also correlated with reduced β -catenin levels, likely due to the AIRE-induced upregulation of SIP.

The interaction with SIAH1 appears to be functionally distinct from other AIRE-associated E3 ligases. For instance, FBXO3 co-localizes with AIRE in nuclear bodies to promote its transcriptional activity through ubiquitination [44]. In contrast, the AIRE–SIAH association is predominantly cytoplasmic, and we found no evidence of nuclear co-localization. Therefore, SIAH1 is unlikely to modulate

AIRE's transcriptional functions directly, acting instead as a regulator of its protein turnover and steady-state levels.

Furthermore, we cannot exclude the possibility that the interaction with SIAH proteins is required for AIRE to execute alternative functions. This could involve AIRE acting as a scaffolding component within a degradation complex, analogous to the role described for SIP. Alternatively, it was previously proposed that AIRE itself might possess E3-ubiquitin ligase activity [45], however, this hypothesis was largely dismissed following the NMR structural analysis of the PHD1 domain [46]. In addition, the AIRE–SIAH1 interaction may be linked to apoptosis induction. It is well established that AIRE⁺ mTECs frequently undergo apoptosis. Interestingly, SIAH1 is known to interact with S-nitrosylated GAPDH, facilitating its nuclear import to trigger cell death [47]. AIRE-induced apoptosis requires both the CARD domain and the nuclear translocation of GAPDH [48]. Our data show that the SIAH1-interacting motif (degron), in conjunction with the CARD domain, is essential for AIRE-mediated apoptosis. This suggests a model where SIAH1 regulates AIRE abundance in cells committed to the apoptotic pathway, potentially abrogating its transcriptional role once the cell has reached its final developmental stage.

SIAH1 and SIP are integral components of the β -catenin degradation pathway [49], and SIP has been shown to be essential for thymocyte development [50]. Recent studies have further demonstrated that SIAH1 mediates the degradation of Axin—the primary scaffolding protein of the β -catenin destruction complex—through a direct interaction [42, 51]. Notably, while the ubiquitination of β -catenin by SIAH requires the adaptor function of SIP, Axin ubiquitination is SIP-independent. Consequently, AIRE-induced upregulation of SIP may act as a rheostat for β -catenin levels. Given that AIRE directly interacts with SIAH1, the increase in intracellular SIP could represent a compensatory response to compete for SIAH1 binding. Thus, AIRE expression potentially modulates Wnt/ β -catenin signaling by influencing both SIP abundance and its availability for SIAH1-mediated complexes.

This AIRE–SIAH1 axis may extend beyond the thymus, as both proteins are co-expressed during spermatogenesis and may play a role in meiotic regulation [5, 52]. Despite their high sequence homology, SIAH1 and SIAH2 exhibit distinct substrate specificities, which likely explains the inconsistent degradation of AIRE by SIAH2 observed in our assays. Furthermore, their biological roles are divergent: SIAH1 functions as a tumor suppressor [53], whereas SIAH2 acts as a proto-oncogene, driving progression in various malignancies, including melanoma, lung, breast, pancreatic, prostate, and liver cancers [54–60]. Therefore, the differential regulation of these ligases may significantly

impact AIRE protein stability and its downstream physiological effects.

Conclusion

In summary, our findings identify a novel regulatory pathway for AIRE mediated by SIAH1. We have demonstrated that AIRE undergoes SIAH1-dependent ubiquitination and proteasomal degradation through a specific, conserved SIAH-interacting motif (degron). This regulatory module not only advances our understanding of AIRE's protein turnover and its role in apoptosis but also identifies the AIRE–SIAH1 interaction as a potential therapeutic target for APECED patients, particularly those carrying mutations that disrupt AIRE's functional stability.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10495-026-02398-9>.

Acknowledgements The authors thank Dr. Michael Edel for a critical revision of the manuscript. The authors thank Dr. Thilo Hagen for providing the SIAH1 and SIAH2 constructs.

Author contributions AT-H: conceptualization, methodology, and data acquisition; AP-M, MA-N, JN-V, and OC: methodology and data acquisition; JRP: data curation, manuscript editing, and review; XD: methodology, data curation, and original draft preparation writing; IA: conceptualization, original draft preparation writing, supervision, and validation.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This work was supported by Grant RTI2018-097414-B-I00 from the Spanish Ministry of Economy and Competitiveness and Grant PPC2024_38 from the Universitat Autònoma de Barcelona to IA.

Data availability Data will be made available on request.

Declarations

Conflict of interest The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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References

1. Irla M (2022) Instructive Cues of Thymic T Cell Selection. *Annu Rev Immunol* 40:95–119
2. Gardner JM, Devoss JJ, Friedman RS et al (2008) Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science* 321:843–847
3. Halonen M, Peltto-Huikko M, Eskelin P, Peltonen L, Ulmanen I, Kolmer M (2001) Subcellular location and expression pattern of autoimmune regulator (Aire), the mouse orthologue for human gene defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). *J Histochem Cytochem* 49:197–208
4. Kogawa K, Nagafuchi S, Katsuta H et al (2002) Expression of Aire gene in peripheral monocyte/dendritic cell lineage. *Immunol Lett* 80:195–198
5. Schaller CE, Wang CL, Beck-Engeser G et al (2008) Expression of Aire and the early wave of apoptosis in spermatogenesis. *J Immunol* 180:1338–1343
6. Nishikawa Y, Hirota F, Yano M et al (2010) Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. *J Exp Med* 207:963–971
7. Finnish-German AC (1997) An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet* 17:399–403
8. Nagamine K, Peterson P, Scott HS et al (1997) Positional cloning of the APECED gene. *Nat Genet* 17:393–398
9. Blizzard RM, Kyle M (1963) Studies of the Adrenal Antigens and Antibodies in Addison's Disease. *J Clin Invest* 42:1653–1660
10. Zlotogora J, Shapiro MS (1992) Polyglandular autoimmune syndrome type I among Iranian Jews. *J Med Genet* 29:824–826
11. Rosatelli MC, Meloni A, Meloni A et al (1998) A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients. *Hum Genet* 103:428–434
12. Anderson MS, Venzani ES, Klein L et al (2002) Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395–1401
13. Derbinski J, Gabler J, Brors B et al (2005) Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med* 202:33–45
14. Giraud M, Yoshida H, Abramson J et al (2012) Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells. *Proc Natl Acad Sci U S A* 109:535–540
15. Abramson J, Giraud M, Benoist C, Mathis D (2010) Aire's partners in the molecular control of immunological tolerance. *Cell* 140:123–135
16. Org T, Rebane A, Kisand K et al (2009) AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. *Hum Mol Genet* 18:4699–4710
17. Akiyama T, Shimo Y, Yanai H et al (2008) The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity* 29:423–437
18. Hikosaka Y, Nitta T, Ohigashi I et al (2008) The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. *Immunity* 29:438–450
19. Gray D, Abramson J, Benoist C, Mathis D (2007) Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J Exp Med* 204:2521–2528
20. Mathis D, Benoist C (2009) Aire. *Annu Rev Immunol* 27:287–312
21. Colomé N, Collado J, Bech-Serra JJ et al (2010) Increased apoptosis after autoimmune regulator expression in epithelial cells revealed by a combined quantitative proteomics approach. *J Proteome Res* 9:2600–2609

22. Matsuzawa SI, Reed JC (2001) Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol Cell* 7:915–926
23. Winter M, Sombroek D, Dauth I et al (2008) Control of HIPK2 stability by ubiquitin ligase Siah-1 and checkpoint kinases ATM and ATR. *Nat Cell Biol* 10:812–824
24. Nakayama K, Frew IJ, Hagensen M et al (2004) Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. *Cell* 117:941–952
25. Nadeau RJ, Toher JL, Yang X, Kovalenko D, Friesel R (2007) Regulation of Sprouty2 stability by mammalian Seven-in-Absentia homolog 2. *J Cell Biochem* 100:151–160
26. Zhang J, Guenther MG, Carthew RW, Lazar MA (1998) Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes Dev* 12:1775–1780
27. Habelhah H, Frew IJ, Laine A et al (2002) Stress-induced decrease in TRAF2 stability is mediated by Siah2. *EMBO J* 21:5756–5765
28. Xu Z, Sproul A, Wang W, Kukekov N, Greene LA (2006) Siah1 interacts with the scaffold protein POSH to promote JNK activation and apoptosis. *J Biol Chem* 281:303–312
29. Sansom SN, Shikama-Dorn N, Zhanybekova S et al (2014) Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia. *Genome Res* 24:1918–1931
30. Matsuzawa S, Li C, Ni CZ, Takayama S, Reed JC, Ely KR (2003) Structural analysis of Siah1 and its interactions with Siah-interacting protein (SIP). *J Biol Chem* 278:1837–1840
31. Santelli E, Leone M, Li C et al (2005) Structural analysis of Siah1-Siah-interacting protein interactions and insights into the assembly of an E3 ligase multiprotein complex. *J Biol Chem* 280:34278–34287
32. Graham FL, Smiley J, Russell WC, Nairn R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36:59–74
33. Marcilla M, de Castro JA, Castano JG, Alvarez I (2007) Infection with *Salmonella typhimurium* has no effect on the composition and cleavage specificity of the 20S proteasome in human lymphoid cells. *Immunology* 122:131–139
34. Jumper J, Evans R, Pritzel A et al (2021) Highly accurate protein structure prediction with AlphaFold. *Nature* 596:583–589
35. Varadi M, Anyango S, Deshpande M et al (2022) AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res* 50:D439–D444
36. Ramsey C, Winqvist O, Puhakka L et al (2002) Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet* 11:397–409
37. House CM, Frew IJ, Huang HL et al (2003) A binding motif for Siah ubiquitin ligase. *Proc Natl Acad Sci U S A* 100:3101–3106
38. Zhang Q, Wang Z, Hou F et al (2017) The substrate binding domains of human SIAH E3 ubiquitin ligases are now crystal clear. *Biochim Biophys Acta Gen Subj* 1861:3095–3105
39. Buchwald M, Pietschmann K, Brand P et al (2013) SIAH ubiquitin ligases target the nonreceptor tyrosine kinase ACK1 for ubiquitinylation and proteasomal degradation. *Oncogene* 32:4913–4920
40. Tiedt R, Bartholdy BA, Matthias G, Newell JW, Matthias P (2001) The RING finger protein Siah-1 regulates the level of the transcriptional coactivator OBF-1. *EMBO J* 20:4143–4152
41. Hu G, Zhang S, Vidal M, Baer JL, Xu T, Fearon ER (1997) Mammalian homologs of seven in absentia regulate DCC via the ubiquitin-proteasome pathway. *Genes Dev* 11:2701–2714
42. Ji L, Jiang B, Jiang X et al (2017) The SIAH E3 ubiquitin ligases promote Wnt/beta-catenin signaling through mediating Wnt-induced Axin degradation. *Genes Dev* 31:904–915
43. Germani A, Bruzzoni-Giovanelli H, Fellous A, Gisselbrecht S, Varin-Blank N, Calvo F (2000) SIAH-1 interacts with alpha-tubulin and degrades the kinesin Kid by the proteasome pathway during mitosis. *Oncogene* 19:5997–6006
44. Shao W, Zumer K, Fujinaga K, Peterlin BM (2016) FBXO3 Protein Promotes Ubiquitylation and Transcriptional Activity of AIRE (Autoimmune Regulator). *J Biol Chem* 291:17953–17963
45. Uchida D, Hatakeyama S, Matsushima A et al (2004) AIRE functions as an E3 ubiquitin ligase. *J Exp Med* 199:167–172
46. Bottomley MJ, Stier G, Pennacchini D et al (2005) NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1). Insights into autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. *J Biol Chem* 280:11505–11512
47. Hara MR, Agrawal N, Kim SF et al (2005) S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat Cell Biol* 7:665–674
48. Liiv I, Haljasorg U, Kisand K, Maslovskaja J, Laan M, Peterson P (2012) AIRE-induced apoptosis is associated with nuclear translocation of stress sensor protein GAPDH. *Biochem Biophys Res Commun* 423:32–37
49. Liu J, Stevens J, Rote CA et al (2001) Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol Cell* 7:927–936
50. Fukushima T, Zapata JM, Singha NC et al (2006) Critical function for SIP, a ubiquitin E3 ligase component of the beta-catenin degradation pathway, for thymocyte development and G1 checkpoint. *Immunity* 24:29–39
51. Chen L, Liu YP, Tian LF et al (2023) Structural Basis of the Interaction between Human Axin2 and SIAH1 in the Wnt/beta-Catenin Signaling Pathway. *Biomolecules* 13(4):647
52. Dickins RA, Frew IJ, House CM et al (2002) The ubiquitin ligase component Siah1a is required for completion of meiosis I in male mice. *Mol Cell Biol* 22:2294–2303
53. Wong CS, Moller A (2013) Siah: a promising anticancer target. *Cancer Res* 73:2400–2406
54. Ahmed AU, Schmidt RL, Park CH et al (2008) Effect of disrupting seven-in-absentia homolog 2 function on lung cancer cell growth. *J Natl Cancer Inst* 100:1606–1629
55. Malz M, Aulmann A, Samarin J et al (2012) Nuclear accumulation of seven in absentia homologue-2 supports motility and proliferation of liver cancer cells. *Int J Cancer* 131:2016–2026
56. Qi J, Nakayama K, Cardiff RD et al (2010) Siah2-dependent concerted activity of HIF and FoxA2 regulates formation of neuroendocrine phenotype and neuroendocrine prostate tumors. *Cancer Cell* 18:23–38
57. Qi J, Nakayama K, Gaitonde S et al (2008) The ubiquitin ligase Siah2 regulates tumorigenesis and metastasis by HIF-dependent and -independent pathways. *Proc Natl Acad Sci U S A* 105:16713–16718
58. Sarkar TR, Sharan S, Wang J et al (2012) Identification of a Src tyrosine kinase/SIAH2 E3 ubiquitin ligase pathway that regulates C/EBPdelta expression and contributes to transformation of breast tumor cells. *Mol Cell Biol* 32:320–332
59. Schmidt RL, Park CH, Ahmed AU et al (2007) Inhibition of RAS-mediated transformation and tumorigenesis by targeting the downstream E3 ubiquitin ligase seven in absentia homologue. *Cancer Res* 67:11798–11810
60. Wong CS, Sceneay J, House CM et al (2012) Vascular normalization by loss of Siah2 results in increased chemotherapeutic efficacy. *Cancer Res* 72:1694–1704

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