MCB
Journals.ASM.org

Unmodified Histone H3K4 and DNA-Dependent Protein Kinase Recruit Autoimmune Regulator to Target Genes

Kristina Žumer,[a,b] Audrey K. Low,[b] Huimin Jiang,[b,c] Kalle Saksela,[a] and B. Matija Peterlin[a,b]

Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland[a]; Department of Medicine, Microbiology and Immunology, Rosalind Russell Medical Research Center, University of California San Francisco, San Francisco, California, USA[b]; and Complete Genomics Inc., Mountain View, California, USA[c]

Autoimmune regulator (AIRE) directs the expression of otherwise tissue-restricted antigens (TRAs) in medullary thymic epithelial cells, allowing their presentation to developing T cells, which leads to central tolerance. We addressed the conundrum of how AIRE is recruited to these otherwise silent genes in cells. Our studies confirmed that interactions between AIRE and the unmodified histone H3K4 (H3K4me0) are important for targeting AIRE to the mouse insulin promoter in chromatin. By replacing its H3K4me0-binding module with one that binds to the methylated H3K4me3, we redirected the mutant AIRE.ING protein to an actively transcribed gene. Nevertheless, the mutant AIRE D297A protein, which could not bind to H3K4me0, still activated the human insulin promoter on an episomal plasmid target. This targeting was due to DNA-dependent protein kinase (DNA-PK). Thus, in cells that lacked the catalytic subunit of DNA-PK (DNA-PKcs), the assembly and activity of AIRE on DNA, whether in chromatin or on episomal plasmids, was abrogated. However, by the heterologous tethering of AIRE to DNA, we could restore its activity on a plasmid target in DNA-PKcs-negative cells. Importantly, mutations in the putative DNA-binding residues in its SAND domain had no effect on the transcriptional effects of AIRE. Thus, AIRE is recruited to TRA genes in chromatin via cooperative interactions with H3K4me0 and DNA-PK.

The negative selection of developing T cells in the thymus is an essential element of the mammalian immune system. In medullary thymic epithelial cells (mTECs), the protein that governs central tolerance is the autoimmune regulator (AIRE) (3, 26, 29, 39). Mutations in AIRE lead to a rare genetic autoimmunity called the autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which affects mostly endocrine and exocrine organs, such as salivary, adrenal, thyroid, and parathyroid glands, as well as the β cells of the pancreas (1, 13, 33). In mTECs, AIRE activates the expression of otherwise silent tissue-restricted antigen (TRA) genes, whose peptides are presented on major histocompatibility complex class II (MHC-II) determinants to thymocytes so that autoreactive T cells are eliminated (3, 26, 29, 39).

AIRE is a transcription factor (TF) of 57 kDa and contains 545 residues (13, 33). It forms oligomers that migrate as dimers, tetramers, and higher order aggregates, which can exceed 670 kDa (17). Thus, 12 or more AIRE proteins could be present in these large complexes. However, the stoichiometry of the transcriptionally active complex is unknown. From its N terminus, AIRE contains a homogenous staining region (HSR); an Sp100, Aire-1, NucP41/75, and DEAF-1 (SAND) domain, which has been suggested to bind DNA; two plant homeodomains (PHD1 and PHD2), of which PHD1 binds unmodified histone H3K4 (H3K4me0) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs); and finally a transcriptional activation domain (TAD), which binds the positive transcription elongation factor b (P-TEFb) at its C terminus (24, 34, 40, 47). HSR and SAND domains also form homo-oligomers (17, 18).

Whereas the trimethylated histone H3K4 (HeK4me3) marks sites of active transcription, H3K4me0 is found at and near silent genes (6, 43). Since DNA-PKcs can phosphorylate AIRE at threonine and serine residues at positions 68 (T68) and 156 (S156), it also interacts with the HSR and sequences N-terminal to the SAND domain (28). The AIRE TAD recruits P-TEFb to RNA polymerase II (RNAPII) (36, 47). Indeed, we observed previously that unmodified RNAPII is already engaged at TRA promoters in the absence of AIRE (36, 47). After its recruitment, P-TEFb phosphorylates negative transcription elongation factors and the C-terminal domain of RNAPII, thus modifying transcription complexes for the productive elongation and cotranscriptional processing of nascent transcripts (38). On most TRA genes, processing events, i.e., pre-mRNA splicing, rather than transcription elongation *per se* predominate (2, 47).

However, the targeting of AIRE to TRA promoters remains poorly understood, especially since only a small number of genes, some of which are found in clusters, are activated in clones of mTECs (9, 14, 20). Moreover, several studies suggested that interactions between AIRE and H3K4me0 are not sufficient for this recruitment. In knock in mice expressing the mutant AIRE protein defective in H3K4me0 binding, only low levels of TRA gene expression were found, levels which were insufficient to induce central tolerance in mice (23). A similar situation was observed with DNA-PK, which also binds to AIRE. Again, low levels of expression of TRA genes were observed in mTECs from bone marrow-reconstituted SCID mice (2). Further proteomic analyses revealed 45 candidate proteins for mediating effects of AIRE in cells. They were divided into four groups, those involved with nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing (2). In the group of proteins involved in transcription were topoisomerase II, poly(ADP-ribose) poly-

Received 28 September 2011 Returned for modification 1 November 2011
Accepted 26 January 2012
Published ahead of print 6 February 2012
Address correspondence to B. Matija Peterlin, matija.peterlin@ucsf.edu.
K.Ž., A.K.L. and H.J. contributed equally to this work.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/MCB.06359-11

merase 1 (PARP-1), and DNA-PK. The silencing of any of these three proteins abrogated effects of AIRE on genes in chromatin, but only DNA-PK was critical for this phenotype on episomal plasmid targets (2).

We were intrigued by additional reports indicating multiple roles for DNA-PK in transcription (16). Although its primary function is in the repair of DNA double-strand breaks, in particular nonhomologous end joining (NHEJ), DNA-PK also binds specific DNA sequences, TATA boxes, and single-stranded regions of DNA, as well as those traversed by an elongating RNAPII (16, 31). In these situations, topoisomerase II and PARP-1 unwind and cleave the DNA, which then binds DNA-PK and is marked by the histone H2AX phosphorylated at serine 139 (γH2AX). Whereas its smaller subunits, Ku70 and Ku80, bind to DNA, DNA-PKcs phosphorylates γH2AX as well as many TFs and RNAPII itself (10). γH2AX marks DNA breaks (8). Near promoters, they increase transcriptional activation by the steroid hormone receptors AP-1 and USF-1 (21, 46). We reasoned that DNA-PK, which is found ubiquitously on DNA and chromatin, might also recruit AIRE to transcription units. Thus, we investigated the ability of H3K4me0 and DNA-PK to recruit AIRE to TRA genes in chromatin and on episomal plasmids.

MATERIALS AND METHODS

Cell lines. 293T and 293 human embryonic kidney cells were obtained from the ATCC and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Mouse 1C6 mTECs were a kind gift from M. Kasai (National Institute of Infectious Diseases, Tokyo, Japan) (30) and were cultured in S-MEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 5 mM Glutamax (Invitrogen), 0.2 μM β-mercaptoethanol (Invitrogen), 0.5 μg/ml hydrocortisone hemisuccinate (Sigma-Aldrich), and 1 mM sodium pyruvate (Invitrogen). Murine embryonic fibroblasts (MEFs) were a kind gift from B. Chen (UT Southwestern Medical Center, Dallas, TX) and were cultured in DMEM supplemented with 10% FBS and antibiotics.

Plasmids. Myc.AIRE and GAL.AIRE were a kind gift from P. Peterson (University of Tartu, Tartu, Estonia). hInsLuc was a kind gift from M. German (UCSF, San Francisco, CA). G5Luc was generated by the restriction ligation of the luciferase reporter gene in place of chloramphenicol acetyltransferase (CAT) in G5CAT, which was described previously (22). GST.PHD1 was constructed by inserting a PCR fragment encoding human AIRE PHD1 into the pGEX-6p-3 vector. AIRE.ING was generated by swapping out the AIRE PHD1 for the PHD of ING2 (QuikChange II XL site-directed mutagenesis kit; Stratagene) into the Myc.AIRE backbone using PCR primers 5′-TTC CAT CCC TCC CCA GTG AGC CCC AGC CTG CCG ACC TCC CCA TC-3′ and 5′-TAG CTC CGG GGG CCT GGA CAC CCT GTT GTA AGC CCT CTC-3′. The E296A, D297A, K243A, K245A, and KKR-A mutations were generated in Myc.AIRE or GST.PHD1 by PCR mutagenesis with a QuikChange II site-directed mutagenesis kit (Stratagene).

RNA isolation by RT-qPCR. 1C6 cells or MEFs were harvested in Trizol reagent (Invitrogen), and RNA was isolated according to the manufacturer's specifications. RNA was treated with DNase I (Invitrogen) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) (Invitrogen) and random primers. Levels of *Ins2* mRNA were determined by quantitative PCR (qPCR) and normalized to control β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. qPCRs were performed using SYBR green JumpStart *Taq* ready mix for quantitative PCR (Sigma-Aldrich) on an Mx3005p (Stratagene). Fold activation represents the ratio between the effector-activated mRNA levels and the levels with empty vector, which were calculated using the threshold cycle ($\Delta\Delta C_T$) method. Primer sequences are available upon request.

Immunoprecipitation. 293T cells were transfected using calcium phosphate and harvested after 24 h. They were resuspended in hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM EDTA, 0.05% NP-40) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and incubated on ice for 15 min. Cell lysates then were centrifuged for 10 min at 1,000 × *g* (4°C), and supernatant was removed as completely as possible and discarded. Nuclei were resuspended in nuclear lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM KCl, 5 mM $CaCl_2$) supplemented with protease inhibitor cocktail and incubated for 1 h on ice. EDTA was added to a final concentration of 10 mM. Nuclear lysates were centrifuged at maximum speed for 20 min to remove insoluble material, and supernatants were used for immunoprecipitation. Two μg of antibodies was added, the samples were incubated for 4 h at 4°C, protein A and/or G Dynabeads (Invitrogen) were added, and samples were incubated for an additional hour. Precipitates were washed at room temperature 4 times with cold phosphate-buffered saline (PBS) supplemented with 0.05% NP-40 and protease inhibitor cocktail. Precipitated proteins were eluted by the addition of 1× SDS-PAGE loading buffer and heated to 95°C for 5 min. Precipitates then were separated by SDS-PAGE, and proteins were detected by Western blotting with specific antibodies.

ChIP. Chromatin immunoprecipitations (ChIPs) were performed as described previously (36). Briefly, 1C6 or MEF cells were transfected using Fugene 6 (Roche Diagnostics) according to the manufacturer's instructions. A portion of the cells was used for Western blot analysis to determine comparable expression levels of AIRE proteins. Cross-linking was performed with 1% formaldehyde at room temperature for 10 min and stopped with 0.125 M glycine. Nuclei were isolated and sonicated to generate DNA fragments of 200 to 500 bp. The chromatin was precleared with protein G agarose (GE Healthcare). Chromatin immunoprecipitations were performed using 2 μg specific antibody or normal IgG. Precipitated DNA was reverse cross-linked and treated with RNase A and proteinase K. DNA then was purified by phenol-chloroform extraction. Levels of DNA in the immunoprecipitates were detected by qPCR and normalized to inputs.

GST pulldown. Hybrid glutathione *S*-transferase (GST)-PHD proteins were expressed in *Escherichia coli* strain BL21(DE3) (Novagen). Proteins were extracted with bacterial protein extraction reagents (B-PER) (Pierce) supplemented with 150 mM NaCl, 1× protease inhibitor (Roche, Indianapolis, IN), and 100 U of DNase I (Pierce) according to the manufacturer's instructions. Glutathione-Sepharose beads (GE Healthcare, Piscataway, NJ) were added to the bacterial lysate, diluted 2-fold with an equal volume of chilled 1× PBS, incubated at 4°C with rotation for 4 h, and washed 3 times with cold 1× PBS, and then GST proteins were eluted with 30 mM glutathione and subjected to desalting using a Zeba desalting column (Pierce). The purity of the eluted proteins was examined by the Coomassie blue staining of SDS-PAGE. GST pulldown assays were performed by incubating purified GST fusion proteins with purified calf thymus histones (Sigma-Aldrich) in histone binding buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, 1% NP-40) supplemented with 1× protease inhibitor (Sigma-Aldrich) at 4°C overnight, adding glutathione-Sepharose beads (GE Healthcare) to the reaction for an additional hour, washing the beads three times in histone binding buffer, and eluting bound proteins in 1× SDS sample buffer. Eluted proteins were resolved by SDS-PAGE on a 4 to 20% gradient gel and analyzed by Western blotting.

Luciferase assay. 1C6 mTECs or MEFs were transfected using Fugene 6 (Roche) to coexpress AIRE proteins, hInsLuc, and pRL-CMV (Promega). Cells were harvested after 24 h, and luciferase assays were performed using the dual luciferase kit (Promega) according to the manufacturer's instructions. Firefly luciferase readings of each sample were normalized to *Renilla* luciferase readings to determine relative activity. Averages were calculated from at least three separate transfections. Fold

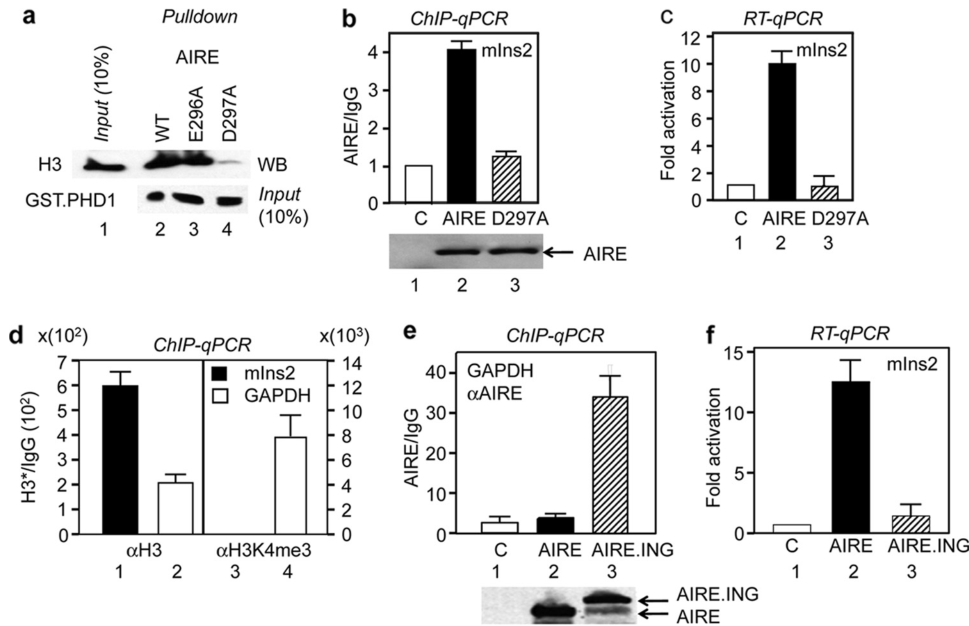

FIG 1 AIRE must bind histone H3 for its recruitment to a TRA gene in chromatin. (a) AIRE PHD1 binds to histone H3. WT and mutant GST.PHD1 fusion proteins were incubated with purified histones. Immunoprecipitated proteins were analyzed by Western blotting with the indicated antibodies. Input GST fusion proteins (10%) are presented in the lower panel. Western blotting of histone H3 is presented in the upper panel. (b) AIRE must bind H3 for its recruitment to an endogenous TRA gene. ChIPs were performed with anti-AIRE antibodies from 1C6 mTECs expressing the empty plasmid vector (C), WT AIRE, or mutant AIRE D297A (D297A) proteins. Binding to the mIns2 promoter is presented as fold enrichment above the level of the IgG control (AIRE/IgG). Expression levels for AIRE proteins are presented below the bar graph. (c) AIRE must bind histone H3 to induce the expression of an endogenous TRA gene. mIns2 RNA levels from WT AIRE and mutant AIRE D297A protein-expressing 1C6 mTECs are presented as fold activation above the level of the empty plasmid vector (C). Levels of Ins2 RNA were normalized to those for GAPDH. (d) H3K4me3 is enriched at the GAPDH but not the mIns2 promoter. ChIPs were performed with anti-H3 (αH3) and anti-H3K4me3 (αH3K4me3) antibodies from 1C6 mTECs. Binding to promoter regions is presented as fold enrichment for specific antibodies above the level of the IgG control (H3*/IgG). (e) Only the mutant AIRE.ING protein with PHD1 replaced with the PHD from ING2 is recruited to the GAPDH promoter. ChIPs were performed with anti-AIRE antibodies (αAIRE) from 1C6 mTECs expressing the empty plasmid vector (C), WT AIRE, or mutant AIRE.ING proteins. Binding to the GAPDH promoter is presented as fold enrichment above the level of the IgG control (AIRE/IgG). Expression levels for AIRE proteins are presented below the bar graph. (f) The mutant AIRE.ING protein cannot induce the expression of an endogenous TRA gene. mIns2 RNA levels from WT AIRE and mutant AIRE.ING protein-expressing 1C6 mTECs are presented as fold activation above the level of the empty plasmid vector (C). Levels of mIns2 RNA were normalized to those of GAPDH.

activation represents the ratio between the effector relative activity and the activity of the reporter with the empty plasmid vector.

Antibodies. Anti-AIRE (ab13573 [Abcam] and sc-33188 [Santa Cruz Biotechnology, Inc.]), anti-H3 (ab1791; Abcam), anti-H3K4Me3 (ab8580; Abcam), anti-HDAC1 (Santa Cruz Biotechnology, Inc.), anti-DNA-PKcs (Ab-4; Thermo Scientific), anti-GST (Santa Cruz Biotechnology, Inc.), anti-Ku80 (ab80592; Abcam), and anti-γH2AX (JBW301; Millipore) antibodies were used.

Inhibition of DNA-PKcs. HEK293T cells were transfected transiently using Fugene 6 (Roche) to express AIRE or the empty plasmid vector. Four h following transfection, the medium was changed to include 1 μM DNA-PK inhibitor Nu7441 (Tocris Bioscience) or its solvent, dimethyl sulfoxide (DMSO). After a total of 24 h of incubation, cells were harvested and lysed in Trizol (Invitrogen), and aliquots were subjected to Western blotting. RNA was isolated according to the manufacturer's instructions (Invitrogen). Residual contaminating DNA was removed with DNAfree (Ambion) for 1 h. Two μg RNA was reverse transcribed using random primers with M-MLV reverse transcriptase (Invitrogen). cDNA was used for subsequent RT-qPCR amplification using Power SYBR green PCR master mix (Applied Biosystems) and an Mx3005P qPCR system (Stratagene). All reactions were analyzed in triplicate. Fluorescent signals were normalized to the ROX passive dye, which was included in the master mix. Levels of *KRT14* mRNA were determined with forward (5′-CAGTCCCT ACTTCAAGACCATTGA-3′) and reverse (5′-ACTGTGGCTGTGAGAA TCTTGTTC-3′) primers and normalized to those of *GAPDH* analyzed with forward (5′-TCAAGTGGGGCGATGCTGGC-3′) and reverse (5′-T GGGGGCATCAGCAGAGGGG-3′) primers in the same sample. The same cells were analyzed for the autophosphorylation of DNA-PKcs after the induction of double-strand DNA breaks. Cells were treated with 1 μM etoposide (Sigma) or DMSO for an additional 1 h to induce DNA double-strand breaks. They then were analyzed for changes in the autophosphorylation of DNA-PKcs on the serine at position 2056 (S2056P) with a specific antibody (ab18192; Abcam) by Western blotting. Protein and phosphorylation levels were visualized and quantified with an Odyssey infrared imaging system (LI-COR).

RESULTS

Binding to histone H3 is required for AIRE to activate a TRA gene in chromatin. To extend previous studies on interactions between AIRE and H3K4me0, we first confirmed that a specific mutation in PHD1 no longer associated with histone H3 (24, 34). Wild-type (WT) and mutant GST.PHD1 chimeras were expressed from *E. coli* and incubated with purified calf thymus histones. In GST pulldowns, WT GST.AIRE and mutant GST.AIRE E296A, but not GST.AIRE D297A, fusion proteins bound to histone H3 (Fig. 1a, lanes 1 to 4). This finding confirmed previous reports on the failure of this aspartate-to-alanine mutation (positions 297 and 299 in human AIRE and mouse AIRE proteins, respectively) to bind to H3K4me0. Using various peptides corresponding to different modifications in the tail of histone H3, we also noted that

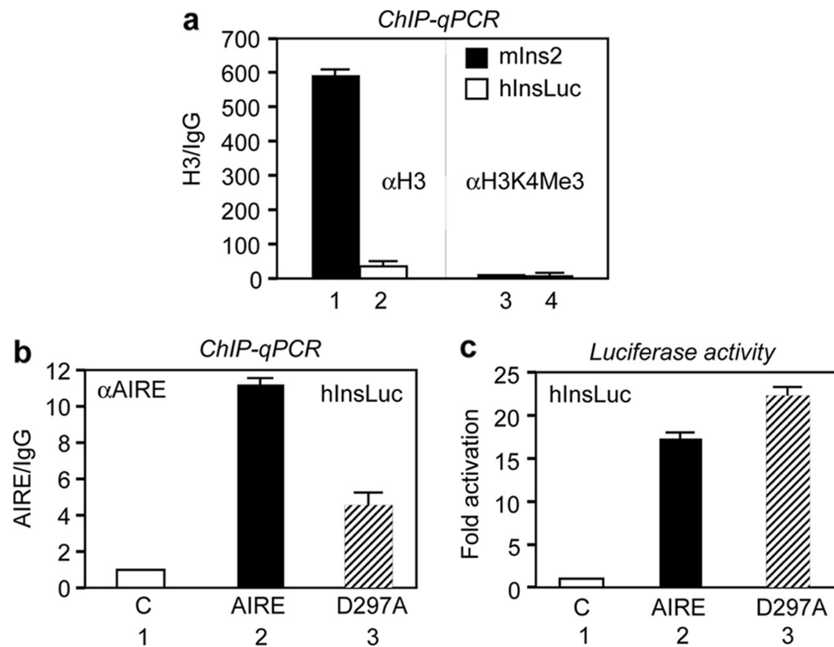

FIG 2 Mutant AIRE protein, which does not bind histone H3, still activates a TRA promoter on a plasmid target. (a) The episomal, nonreplicating human insulin plasmid target does not recruit histone H3. ChIPs were performed with anti-H3 (αH3) and anti-H3K4me3 (αH3K4me3) antibodies on the expressed hInsLuc plasmid from 1C6 mTECs. The binding of histones to endogenous (mIns2) and plasmid (hInsLuc) promoter regions is presented as fold enrichment above the level of the IgG control (specific antibodies/IgG). (b) The mutant AIRE protein, which does not bind histone H3, still binds a TRA promoter on a plasmid target. ChIPs were performed with anti-AIRE antibodies (αAIRE) on the expressed hInsLuc plasmid from 1C6 mTECs that coexpressed the empty plasmid vector (C), WT AIRE, or mutant AIRE D297A (D297A) protein. Binding to the hInsLuc promoter is presented as fold enrichment above the level of the IgG control (AIRE/IgG). (c) AIRE does not need to bind to histone H3 to induce expression from a TRA promoter on a plasmid target. The relative luciferase activity of hINSLuc for WT AIRE and mutant AIRE D297A proteins is presented as fold activation above the level of the empty plasmid vector (C).

AIRE binds to H3K4me0 almost exclusively (data not presented). Armed with this knowledge, we also confirmed that the WT AIRE, but not the mutant AIRE D297A protein binds to the mouse insulin 2 (mIns2) promoter and activates its expression in chromatin (Fig. 1b and c, lanes 1 to 3). Moreover, chromatin immunoprecipitations (ChIPs) revealed that the WT AIRE binds to this promoter 4-fold better and activates its expression 10-fold better than its mutant AIRE D297A counterpart (Fig. 1b and c, compare lanes 2 and 3). In these experiments, the mutant AIRE D297A protein behaved no better than the empty plasmid vector control (Fig. 1b and c, compare lanes 1 and 3).

We wanted to determine if we could redirect the targeting of AIRE to a constitutively expressed gene by exchanging its PHD1 with that from another protein that is known to bind to H3K4me3, which marks sites of active transcription (7, 37). GAPDH is expressed constitutively at high levels in all cells, and as presented in Fig. 1d (lanes 1 and 2), its promoter contains up to 3-fold less total histone H3 than the mIns2 promoter. In contrast, whereas abundant H3K4me3 was found on the GAPDH promoter, almost none was detected on the mIns2 promoter in 1C6 mTECs (Fig. 1c, compare lanes 3 and 4). Finally, ChIPs revealed that the WT AIRE could not bind to the GAPDH promoter (Fig. 1e, lane 2). However, when we replaced PHD1 from AIRE with the PHD from ING2 that binds H3K4me3, the mutant AIRE.ING protein bound efficiently to the GAPDH promoter (Fig. 1e, lane 3). In addition, the WT AIRE, but not the mutant AIRE.ING, protein could activate the mIn2 promoter in chromatin (Fig. 1f, compare lanes 2 and 3). Since GAPDH is already expressed at high levels, its expression did not change upon the addition of the mutant AIRE.ING protein (data not presented). We conclude that the binding between AIRE and H3K4me0 is essential for the targeting of AIRE to relatively silent TRAs in mTECs, which activates their expression. These findings complement those of others and indicate that one can redirect AIRE to different genes by modifying its recognition of specific histone modifications.

Mutant AIRE D297A protein, which does not bind to histone H3, still activates a TRA promoter on an episomal plasmid target. Despite its targeting to H3K4me0, we and others found that additional proteins and/or DNA-binding events must be involved in the recruitment of AIRE to TRA genes. For example, the mutant AIRE D299A protein still was able to activate TRA genes in knock-in mice, albeit to levels that were insufficient for central tolerance in the mouse (23). Others found that AIRE can bind to DNA directly, rather promiscuously and primarily to purine-rich sequences, via its SAND domain (25, 41).

To extend these findings, we asked if the mutant, PHD1-defective AIRE D297A protein could still activate a TRA promoter on an episomal, nonreplicating plasmid target (hInsLuc). To this end, we first determined that these plasmids contained relatively little to no histone H3. As presented in Fig. 2a (compare lanes 1 and 2), the human insulin (hIns) promoter on a plasmid target contained less than 10% of total histone H3 level of the mIns2 gene in chromatin. Moreover, H3K4me3 was not abundant on either of these two targets (Fig. 2a, compare lanes 3 and 4). We examined the occupancy of the WT AIRE and mutant AIRE D297A proteins on hInsLuc. To our surprise, both proteins bound to the hIns promoter (Fig. 2b, lanes 3 and 4) and were able to activate hInsLuc to a similar extent (Fig. 2c, compare lanes 2 and 3). These findings

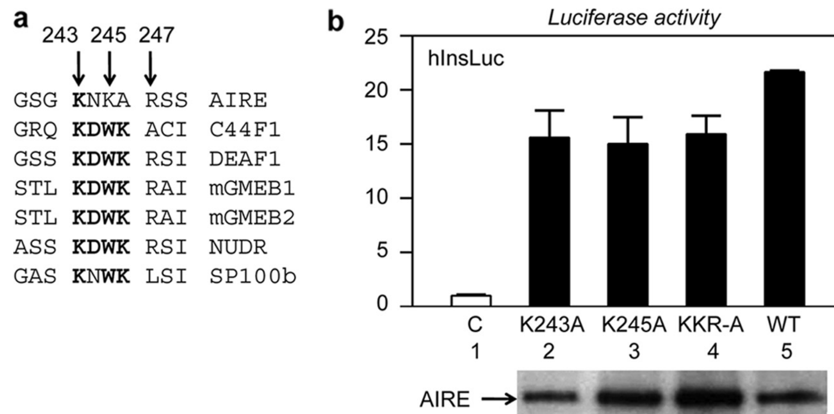

FIG 3 Putative DNA-binding residues in the SAND domain are not required for the recruitment of AIRE to a TRA on a plasmid target. (a) Alignment of SAND domain residues. Numbers above the sequences point to the KDWK DNA-binding motif (positions 243 to 247). (b) Residues required for the DNA binding of other SAND domains are not required for AIRE to activate a TRA promoter on a plasmid target. Relative luciferase activity from hInsLuc for WT AIRE (WT) and mutant AIRE (K243A, K245A, and KKR-A) proteins is presented as fold activation above the level of the empty plasmid vector (C). Expression levels for AIRE proteins are presented below the bar graph.

extend those of others and indicate clearly that AIRE can be recruited to DNA in the absence of histone H3 and its binding to H3K4me0. Thus, additional targeting mechanisms must operate for AIRE to activate the expression of TRA genes in mTECs.

Mutations in the putative DNA-binding motif in the SAND domain do not affect the activity of AIRE. The SAND domain in AIRE has been implicated in DNA binding. However, the KDWK motif that is required for this binding in other SAND domain-containing proteins contains KNKA residues in AIRE (Fig. 3a) (4). Nevertheless, the arginine after these four residues is conserved among all of these proteins (Fig. 3a). To determine if these basic residues are required for the recruitment of AIRE to plasmid targets, we replaced these residues singly and in combination with alanine. We next investigated the activity of mutant AIRE K243A, AIRE K245A, and AIRE KKR-A proteins on hInsLuc. As presented in Fig. 3b (lanes 2 to 5), all KNKAR mutant AIRE proteins functioned like their WT counterparts. These data indicate clearly that the KNKAR residues in the SAND domain are not involved in the targeting of AIRE to the hIns promoter. Thus, the putative SAND-mediated and promiscuous binding to DNA can be excluded as the mechanism for the recruitment of AIRE to TRA genes.

DNA-PKcs is required for the recruitment and activity of AIRE in chromatin and on a plasmid target. To investigate the possibility that additional proteins beside H3K4me0 are required for the recruitment of AIRE to TRA genes, we noted that DNA-PK was found to be essential for effects of AIRE in cells and in the mouse (2, 28). DNA-PKcs was identified in a proteomic screen with the GST.PHD1 chimera, and this binding was confirmed with the entire protein in cells (28). Moreover, in mTECs from bone marrow-reconstituted SCID mice, TRAs were expressed at very low levels (2). Thus, we thought that DNA-PK, which has been implicated in transcriptional effects of AIRE, was a good candidate for targeting AIRE to DNA.

To examine the importance of DNA-PK in the function of AIRE, we compared DNA-PKcs$^{-/-}$ and matched WT MEFs (45). As presented in Fig. 4a, when expressed transiently, AIRE activated the mIns2 promoter in WT but not DNA-PKcs$^{-/-}$ MEFs (Fig. 4a, compare lanes 2 and 4). In addition, AIRE could not activate the hIns promoter on hInsLuc when this plasmid target was cotransfected into DNA-PKcs$^{-/-}$ MEFs (Fig. 4b, compare lanes 2 and 4). Finally, AIRE was not recruited to this hIns promoter in DNA-PKcs$^{-/-}$ MEFs (Fig. 4c, compare lanes 2 and 4). Importantly, we confirmed that AIRE was expressed to the same extent in WT and DNA-PKcs$^{-/-}$ MEFs (Fig. 4b and c, top) and that these cells did not express DNA-PKcs (Fig. 4b and c, middle), whereas HDAC1 was expressed equivalently and was used as the loading control (Fig. 4b, lower). Thus, DNA-PK is required for the recruitment to and activity of AIRE on TRA genes, be it in chromatin or on episomal plasmids. We conclude that DNA-PK cooperates with H3K4me0 to recruit AIRE to TRA genes in chromatin.

The kinase activity of DNA-PKcs is not required for effects of AIRE on an endogenous TRA gene. AIRE binds to DNA-PKcs, which can phosphorylate it on T68 and S156 *in vitro* (28). Mutating each of these residues to alanine also reduced the activity of AIRE on episomal plasmid targets in cells (28). Thus, we wanted to know whether the kinase activity was required for the DNA-tethering function of DNA-PK. To this end, we expressed AIRE in the presence and absence of Nu7441, which is a potent inhibitor of DNA-PKcs, in 293T cells (27). In these cells, we measured the ability of AIRE to activate the expression from another endogenous TRA gene, *KRT14* (Fig. 5a) (2, 47). Indeed, AIRE increased levels of *KRT14* transcripts by 29- and 23-fold in WT and Nu7441-treated cells, respectively (Fig. 5a, compare lanes 3 and 4). To demonstrate that Nu7441 inhibited DNA-PKcs at these concentrations, we also examined the autophosphorylation of DNA-PKcs on serine at position 2056 (S2056P), which can be detected with phosphospecific antibodies (5, 11). To activate the kinase activity of DNA-PKcs, we also treated 293T cells at the end of our incubations for 1 h with etoposide, which induces DNA double-strand breaks (32). As presented in Fig. 5b, etoposide increased levels of S2056P only in cells that were not treated with Nu7441. We conclude that its kinase activity is not required for effects of DNA-PKcs on the function of AIRE.

Heterologous DNA tethering of AIRE restores its activity in DNA-PKcs$^{-/-}$ MEFs. If DNA-PK is required only for the recruitment of AIRE to TRA genes, then heterologous DNA tethering should also restore AIRE activity in DNA-PKcs$^{-/-}$ MEFs. To this end, we linked the Gal4 DNA-binding domain (DBD) to AIRE

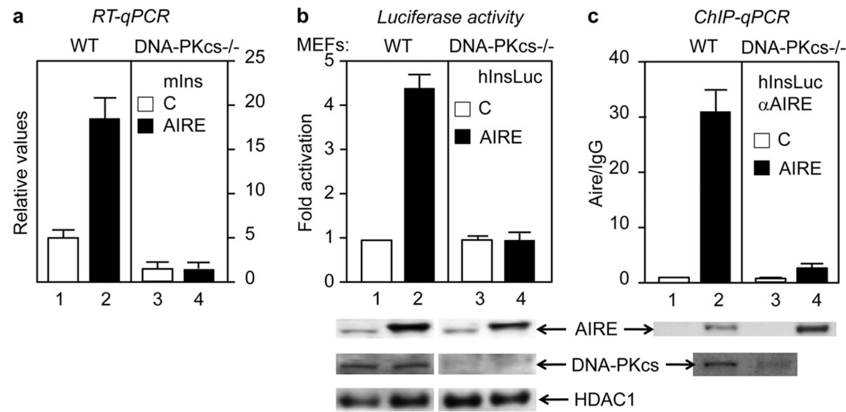

FIG 4 DNA-PKcs is required to recruit AIRE to a TRA gene in chromatin and on a plasmid target. (a) AIRE does not activate mIns in cells lacking DNA-PKcs. mIns2 RNA levels from WT AIRE protein-expressing WT or DNA-PKcs$^{-/-}$ MEFs are presented as fold activation above the level of the empty plasmid vector (C). Levels of *Ins2* RNA were normalized to those for GAPDH. (b) AIRE does not activate a TRA promoter on a plasmid target in cells lacking DNA-PKcs. The relative luciferase activity of hInsLuc for WT AIRE protein-expressing WT or DNA-PKcs$^{-/-}$ MEFs is presented as fold activation above the level of the empty plasmid vector (C). (c) AIRE requires DNA-PKcs to interact with a TRA promoter on a plasmid target. ChIPs were performed with anti-AIRE antibodies on hInsLuc from WT or DNA-PKcs$^{-/-}$ MEFs coexpressing the WT AIRE protein or empty plasmid vector (C). Binding to hInsLuc is presented as fold enrichment above the level of IgG. The expression of AIRE and DNA-PKcs proteins was confirmed by Western blotting and is presented below the bar graph.

and expressed the Gal.AIRE chimera with a plasmid target that contained 5 Gal4 DNA-binding sites (upstream activating sequences [UASs]) upstream of a minimal promoter, the luciferase reporter gene, and the simian virus 40 (SV40) polyadenylation signal (Fig. 5a). When these plasmid effectors and target were cotransfected into cells, only the Gal.AIRE chimera was able to activate G5Luc (Fig. 6b, lanes 3 and 6). WT AIRE protein had no effect (Fig. 6b, lanes 2 and 5). Importantly, the Gal.AIRE fusion protein activated G5Luc 18- and 10-fold in WT and DNA-PKcs$^{-/-}$ MEFs, respectively (Fig. 6b, compare lanes 3 and 6). All of these proteins were expressed in cells (Fig. 6b, lower). Thus, heterologous DNA tethering rescued the recruitment to and activity of AIRE on a plasmid target. Combined with results shown in Fig. 4, we conclude that DNA-PK is required for the recruitment of AIRE to TRA genes.

The role of DNA-PK and DNA breaks in the recruitment of AIRE to TRA genes. Since H3K4me0 and DNA-PK recruit AIRE to TRAs in chromatin and DNA-PK is required for this targeting on episomal plasmids, we wanted to determine if they also interacted with their appropriate targets on DNA. To this end, we performed additional binding studies between AIRE and DNA-PKcs. As presented in Fig. 7, lane 2, AIRE and DNA-PKcs always immunoprecipitated each other. In addition, our AIRE immunoprecipitations revealed the presence of histone H3 and γH2AX by Western blotting (Fig. 7, lane 2). This finding indicates that both proteins also interact with their targets on DNA, AIRE with histone H3 and DNA-PK with DNA breaks, which are marked by γH2AX. Note that inputs of all proteins were equivalent (Fig. 7, right, input). These binding studies reveal the targeting of AIRE to TRA genes in chromatin, to H3K4me0 on inactive genes, and DNA-PK at sites of DNA breaks.

DISCUSSION

In this study, we confirmed that the binding between PHD1 and H3K4me0 is required to target AIRE to TRA genes in cells. Indeed, replacing this PHD finger with one that interacts with H3K4me3 redirected the mutant AIRE.ING protein to an actively expressed gene in cells. However, the mutant AIRE D297A protein, which could not bind to a TRA gene in chromatin, still activated the hIns promoter on an episomal plasmid, which contained only small amounts of histone H3. This targeting depended on DNA-PK. In DNA-PKcs$^{-/-}$ MEFs, AIRE did not bind to a TRA promoter in

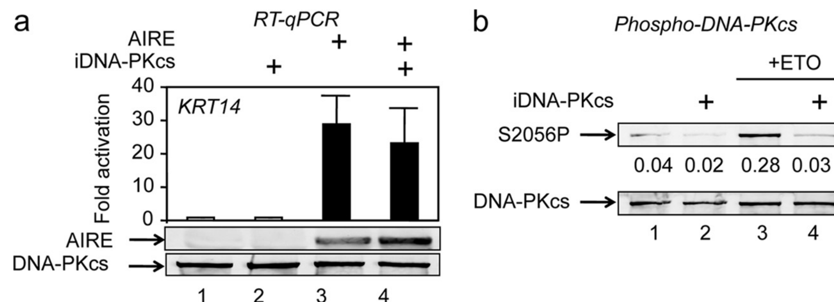

FIG 5 Kinase activity of DNA-PKcs is not required for effects of AIRE in cells. (a) Catalytic activity of DNA-PKcs is dispensable for the AIRE-induced expression of the *KRT14* gene. *KRT14* mRNA levels were analyzed in 293T cells, which transiently expressed AIRE or the empty plasmid vector in the presence or absence of 1 μM Nu7441 (iDNA-PKcs). (b) Inhibition of DNA-PKcs prevents its autophosphorylation after the induction of double-strand DNA breaks. After the standard incubation period, etoposide (ETO) was added for 1 h to 293T cells in the presence or absence of 1 μM Nu7441 (iDNA-PKcs). Levels of DNA-PKcs autophosphorylated on serine at position 2056 (S2056P) were quantified relative to those of total DNA-PKcs and are presented in numbers below the panels.

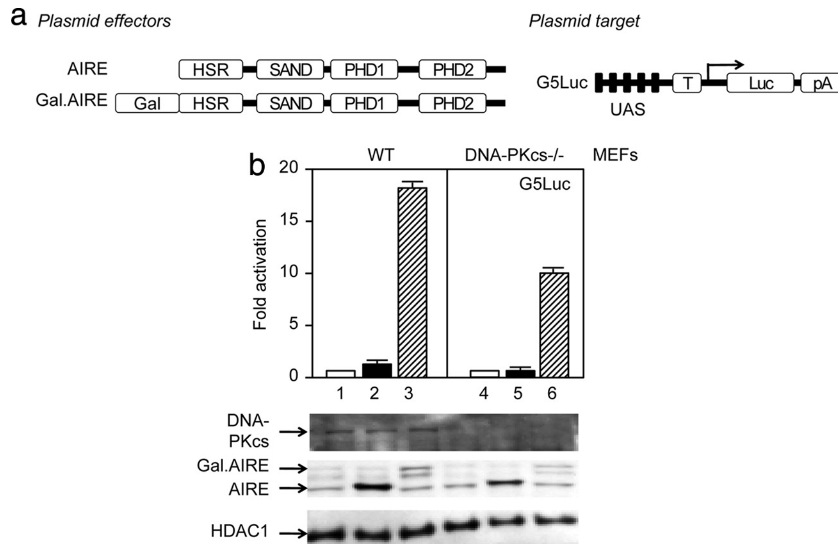

FIG 6 When AIRE is tethered heterologously to DNA, DNA-PKcs is not required for its activation of transcription. (a) Schematic representation of plasmid effectors and targets. Plasmid effectors are given on the left. From the N terminus, domains of AIRE are the homogenously staining region (HSR); Sp100, Aire-1, NucP41/75 and DEAF-1 (SAND) domain; and plant homeodomains 1 and 2 (PHD). The hybrid Gal.AIRE protein additionally contains the heterologous Gal4 DNA-binding domain (Gal). The plasmid target is presented on the right. G5Luc contains five GAL binding sites (UAS) and the TATA box (T) upstream of the luciferase gene (Luc) and polyadenylation signal (pA). (b) Via heterologous DNA tethering, the Gal.AIRE chimera activates a plasmid reporter. The bar graph presents relative luciferase activities of G5Luc in WT or DNA-PKcs$^{-/-}$ MEFs expressing WT AIRE or hybrid GAL.AIRE proteins as fold activation above the level of the empty plasmid vector (C). The expression of AIRE and DNA-PKcs proteins was confirmed by Western blotting and is presented below the bar graph.

chromatin or on an episomal plasmid. It also did not activate transcription from this DNA target. Nevertheless, via heterologous DNA tethering, the Gal.AIRE chimera was able to activate a plasmid target with appropriate DNA-binding sites in these DNA-PKcs$^{-/-}$ MEFs. Importantly, the kinase activity of DNA-PKcs was not required for effects of AIRE on another endogenous TRA gene, *KRT14*. We also confirmed that AIRE binds to DNA-PKcs in cells. Moreover, this complex contained histone H3 as well as γH2AX, which marks DNA breaks. We conclude that AIRE is recruited to chromatin via H3K4me0 and DNA-PK, and that these interactions account for the targeting of AIRE to TRA genes in individual mTECs.

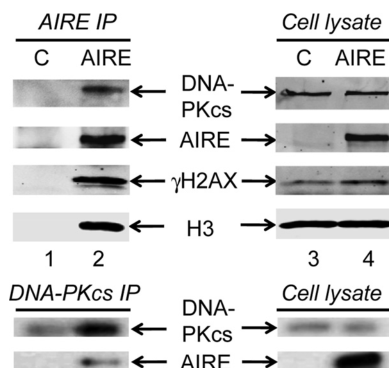

FIG 7 AIRE interacts with DNA-PKcs, histone H3, and γH2AX in cells. WT AIRE protein interacts with DNA-PKcs, histone H3, and γH2AX. AIRE was immunoprecipitated with anti-AIRE antibodies from cells expressing the empty plasmid vector (C) or the WT AIRE protein (top 4 panels). DNA-PKcs interacts with the WT AIRE protein in cells. DNA-PKcs was immunoprecipitated with anti-DNA-PKcs antibodies. Western blotting was performed with specific antibodies (bottom 2 panels). Analyzed proteins are indicated by arrows. The Western blottings of inputs (2% cell lysate) are presented in panels on the right, and immunoprecipitations (IP) are presented in the panels on the left.

We used a variety of assays to confirm previous findings on interactions between AIRE and H3K4me0 as well as DNA-PKcs. Interestingly, these binding partners came out from two independent proteomic screens and were confirmed by functional studies (2, 28). H3K4me0 binds to PHD1 in AIRE (24, 34). DNA-PKcs, which measures 450 kDa, binds to PHD1, HSR, and other sequences near the N terminus of AIRE (28). We found that AIRE binds to H3K4me0 and DNA-PKcs. Thus, nonoverlapping surfaces are involved in these interactions, or individual AIRE proteins in these higher order aggregates (up to 12 subunits) bind to them individually to tether the entire complex (17). Importantly, γH2AX was also present in this complex, which indicates that DNA-PK binds to DNA breaks in TRA genes. In previous work, it was also demonstrated that in the absence of H3K4me0 binding or with the genetic inactivation of DNA-PKcs, transcriptional effects of AIRE were diminished in cells and in the mouse (2, 23, 24). However, some expression of TRA genes could still be demonstrated in mTECs, which suggested that both of these binding partners are required for optimal effects of AIRE. Our work confirms and extends these findings and reveals that these interactions are indeed required for the recruitment of AIRE to TRA genes in chromatin. We focused our analysis on the insulin and *KRT14* genes, which are bona fide targets of AIRE in mouse and human TECs and are induced by AIRE in somatic cells. Nevertheless, we and others have found that what pertains to these TRA genes is true for other AIRE-induced genes in cells and in the animal (2).

Thus, we and others addressed the targeting of AIRE to TRA genes in mTECs. These cells contain low levels of DNA methylation, which means that promoters are more accessible to TFs (44). Indeed, unmodified RNAPII is already present on all examined TRA genes (36, 47). H3K4me0 also predominates on or near

these genes (35). With the help of topoisomerase II, DNA breaks form, chromatin becomes more relaxed, and RNAPII elongates, albeit poorly. These DNA breaks (12) attract PARP-1 and DNA-PK (21, 46). DNA breaks are marked by γH2AX. Indeed, mature mTECs from WT mice have 3- to 4-fold larger populations of cells expressing high levels of γH2AX than their $AIRE^{-/-}$ littermates (2). H3K4me0 and DNA-PK recruit AIRE, which brings P-TEFb to RNAPII (36, 47). P-TEFb modifies RNAPII for the productive elongation and cotranscriptional processing of TRA transcripts (38). Multiple P-TEFb molecules are required to modify RNAPII, and thus the higher-order complexes of AIRE would be ideally suited for such recruitment (17, 42). P-TEFb also activates genes at a distance, thus conveying effects of transcriptional enhancers (19, 42), which might well explain the observation that AIRE-responsive genes occur in clusters (15, 20).

The scenario proposed above is in keeping with recent findings that the complex consisting of topoisomerase II, PARP-1, and DNA-PK potentiates the transcriptional activation of a number of genes in chromatin, e.g., those that respond to steroid hormone receptors, AP-1 and USF-1 (21, 46). On episomal plasmids, which contain little chromatin and abundant DNA breaks, DNA-PK is already present at multiple sites and topoisomerase II and PARP-1 are not required for the transcriptional activation by AIRE. In the organism, optimal effects of AIRE will then depend on levels of H3K4me0, DNA breaks, DNA-PK, P-TEFb, and RNAPII at TRA genes in individual mTECs. Stress, such as that which occurs with DNA damage, will also release active P-TEFb from its inactive complex (38). These processes are expected to be stochastic and to target different TRA genes in individual mTECs. However, the sum total of these events will ensure that sufficient TRA genes are transcribed and expressed for the presentation of these self peptides in the context of MHCII determinants to developing T cells. Central tolerance will ensue, and the organism will be protected against autoimmunity.

ACKNOWLEDGMENTS

We thank B. Chen, M. German, M. Kasai, P. Peterson, and J. Kamine for reagents, Peter Cimermancic for discussions on the domains and structure of AIRE, and members of our laboratories for frequent input on the work and comments on the manuscript. H. Jiang, A. K. Low, K. Žumer, B. M. Peterlin, and K. Saksela designed the experiments; H. Jiang, A. K. Low, and K. Žumer performed the studies; and H. Jiang, A. K. Low, K. Žumer, B. M. Peterlin, and K. Saksela analyzed the data and wrote the manuscript.

We declare no competing financial interests.

This work was supported by the Nora Eccles Treadwell Foundation and the FIDIPRO funds to the University of Helsinki, Finland. Huimin Jiang was funded by the Larry L. Hillblom Foundation.

REFERENCES

1. **Aaltonen J, Björses P, Sandkuijl L, Perheentupa J, Peltonen L.** 1994. An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. Nat. Genet. **8**:83–87.
2. **Abramson J, Giraud M, Benoist C, Mathis D.** 2010. Aire's partners in the molecular control of immunological tolerance. Cell **140**:123–135.
3. **Anderson MS, Su MA.** 2011. Aire and T cell development. Curr. Opin. Immunol. **23**:198–206.
4. **Bottomley MJ, et al.** 2001. The SAND domain structure defines a novel DNA-binding fold in transcriptional regulation. Nat. Struct. Biol. **8**:626–633.
5. **Chen BPC, et al.** 2005. Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks. J. Biol. Chem. **280**:14709–14715.
6. **Chi P, Allis CD, Wang GG.** 2010. Covalent histone modifications–miswritten, misinterpreted and mis-erased in human cancers. Nat. Rev. Cancer **10**:457–469.
7. **Chignola F, et al.** 2009. The solution structure of the first PHD finger of autoimmune regulator in complex with non-modified histone H3 tail reveals the antagonistic role of H3R2 methylation. Nucleic Acids Res. **37**:2951–2961.
8. **Ciccia A, Elledge SJ.** 2010. The DNA damage response: making it safe to play with knives. Mol. Cell **40**:179–204.
9. **Derbinski J, et al.** 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. J. Exp. Med. **202**:33–45.
10. **Dobbs TA, Tainer JA, Lees-Miller SP.** 2010. A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation. DNA Repair **9**:1307–1314.
11. **Douglas P, et al.** 2007. The DNA-dependent protein kinase catalytic subunit is phosphorylated in vivo on threonine 3950, a highly conserved amino acid in the protein kinase domain. Mol. Cell. Biol. **27**:1581–1591.
12. **Dynan WS, Yoo S.** 1998. Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids. Nucleic Acids Res. **26**:1551–1559.
13. **Finnish-German Consortium APECED.** 1997. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat. Genet. **17**:399–403.
14. **Gillard GO, Farr AG.** 2006. Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. J. Immunol. **176**:5815–5824.
15. **Gotter J, Kyewski B.** 2004. Regulating self-tolerance by deregulating gene expression. Curr. Opin. Immunol. **16**:741–745.
16. **Haffner MC, De Marzo AM, Meeker AK, Nelson WG, Yegnasubramanian S.** 2011. Transcription-induced DNA double strand breaks: both oncogenic force and potential therapeutic target? Clin. Cancer Res. **17**:3858–3864.
17. **Halonen M, et al.** 2004. APECED-causing mutations in AIRE reveal the functional domains of the protein. Hum. Mutat. **23**:245–257.
18. **Ilmarinen T, et al.** 2005. Functional analysis of SAND mutations in AIRE supports dominant inheritance of the G228W mutation. Hum. Mutat. **26**:322–331.
19. **Jiang H, Peterlin BM.** 2008. Differential chromatin looping regulates CD4 expression in immature thymocytes. Mol. Cell. Biol. **28**:907–912.
20. **Johnnidis JB, et al.** 2005. Chromosomal clustering of genes controlled by the aire transcription factor. Proc. Natl. Acad. Sci. U. S. A. **102**:7233–7238.
21. **Ju B-G, et al.** 2006. A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription. Science **312**:1798–1802.
22. **Kamine J, Subramanian T, Chinnadurai G.** 1991. Sp1-dependent activation of a synthetic promoter by human immunodeficiency virus type 1 Tat protein. Proc. Natl. Acad. Sci. U. S. A. **88**:8510–8514.
23. **Koh AS, Kingston RE, Benoist C, Mathis D.** 2010. Global relevance of Aire binding to hypomethylated lysine-4 of histone-3. Proc. Natl. Acad. Sci. U. S. A. **107**:13016–13021.
24. **Koh AS, et al.** 2008. Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. Proc. Natl. Acad. Sci. U. S. A. **105**:15878–15883.
25. **Kumar PG, et al.** 2001. The autoimmune regulator (AIRE) is a DNA-binding protein. J. Biol. Chem. **276**:41357–41364.
26. **Kyewski B, Klein L.** 2006. A central role for central tolerance. Annu. Rev. Immunol. **24**:571–606.
27. **Leahy JJJ, et al.** 2004. Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. Bioorg. Med. Chem. Lett. **14**:6083–6087.
28. **Liiv I, et al.** 2008. DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. Biochim. Biophys. Acta **1783**:74–83.
29. **Mathis D, Benoist C.** 2009. AIRE. Annu. Rev. Immunol. **27**:287–312.
30. **Mizuochi T, Kasai M, Kokuho T, Kakiuchi T, Hirokawa K.** 1992. Medullary but not cortical thymic epithelial cells present soluble antigens to helper T cells. J. Exp. Med. **175**:1601–1605.
31. **Mo X, Dynan WS.** 2002. Subnuclear localization of Ku protein: functional association with RNA polymerase II elongation sites. Mol. Cell. Biol. **22**:8088–8099.
32. **Muslimović A, Nyström S, Gao Y, Hammarsten O.** 2009. Numerical analysis of etoposide induced DNA breaks. PLoS One **4**:e5859.
33. **Nagamine K, et al.** 1997. Positional cloning of the APECED gene. Nat. Genet. **17**:393–398.

34. **Org T, et al.** 2008. The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression. EMBO Rep. **9**:370–376.
35. **Org T, et al.** 2009. AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. Hum. Mol. Genet. **18**: 4699–4710.
36. **Oven I, et al.** 2007. AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. Mol. Cell. Biol. **27**: 8815–8823.
37. **Peña PV, et al.** 2006. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature **442**:100–103.
38. **Peterlin BM, Price DH.** 2006. Controlling the elongation phase of transcription with P-TEFb. Mol. Cell **23**:297–305.
39. **Peterson P, Org T, Rebane A.** 2008. Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. Nat. Rev. Immunol. **8**:948–957.
40. **Peterson SR, et al.** 1997. Characterization of two DNA double-stranded break repair-deficient cell lines that express inactive DNA-dependent protein kinase catalytic subunits. J. Biol. Chem. **272**:10227–10231.
41. **Purohit S, Kumar PG, Laloraya M, She J-X.** 2005. Mapping DNA-binding domains of the autoimmune regulator protein. Biochem. Biophys. Res. Commun. **327**:939–944.
42. **Taube R, Lin X, Irwin D, Fujinaga K, Peterlin BM.** 2002. Interaction between P-TEFb and the C-terminal domain of RNA polymerase II activates transcriptional elongation from sites upstream or downstream of target genes. Mol. Cell. Biol. **22**:321–331.
43. **Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ.** 2007. How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. Nat. Struct. Mol. Biol. **14**:1025–1040.
44. **Tykocinski L-O, et al.** 2010. Epigenetic regulation of promiscuous gene expression in thymic medullary epithelial cells. Proc. Natl. Acad. Sci. U. S. A. **107**:19426–19431.
45. **Weterings E, Chen DJ.** 2007. DNA-dependent protein kinase in non-homologous end joining: a lock with multiple keys? J. Cell Biol. **179**:183–186.
46. **Wong RHF, et al.** 2009. A role of DNA-PK for the metabolic gene regulation in response to insulin. Cell **136**:1056–1072.
47. **Zumer K, Plemenitas A, Saksela K, Peterlin BM.** 2011. Patient mutation in AIRE disrupts P-TEFb binding and target gene transcription. Nucleic Acids Res. **39**:7908–7919.