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# Aire expands: new roles in immune tolerance and beyond

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## Introduction

The maintenance of immune tolerance to self is an essential mechanism to prevent untoward immune responses and autoimmunity. The breakdown of tolerance is often a complex process that is challenging to dissect. Despite this challenge, there have been major advances of our understanding of autoimmunity through the study of monogenic forms of the disease [1]. This approach has identified a number of key regulators of immune tolerance with direct relevance to human disease including *FoxP3* [2–4], *CTLA4* [5, 6], and *AIRE* [7, 8]. Aire is best known as a critical transcriptional regulator that functions by promoting the display of a wide array of tissue specific antigens (TSA's) in the thymus. Encounters with TSAs by developing T cells leads to negative selection of autoreactive T cells that recognize TSAs with high affinity [9, 10]. Despite the fact that AIRE was identified more than 15 years ago, we continue to learn more about how this key tolerance regulator operates and how it impacts the immune system. Here we highlight some of the recent advances that have expanded our understanding of the biology of Aire.

## Autosomal Dominant AIRE

*AIRE* was originally identified as the culprit mutated gene in patients with an autosomal recessive form of autoimmunity called Autoimmune Polyglandular Syndrome Type 1 (APS1) [7, 8]. Although patients classically present with rare AIRE mutations on both alleles, it has now become increasingly appreciated that there are subjects that harbor single point mutations on one allele that have increased susceptibility to autoimmunity [11–13]. These patients often present later in life and only with a portion of the features of APS1. To date, these autosomal dominant mutations appear to cluster in the SAND domain and the PHD1 domain of AIRE (Figure 1). Aire has a number of subdomains that include HSR/CARD, SAND, PHD1, and PHD2. Of note, the HSR/CARD domain appears to be involved in promoting Aire to multimerize to itself [14] and thus one can envision a model where point mutations in other domains of Aire can result in interference of the activity of the multimerized Aire complex. The PHD1 domain of Aire functions as a histone code reader

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(see below) while the SAND domain appears to be involved in promoting a protein-protein interaction with a transcriptional repressive complex (see below). These recent findings raise several interesting questions. First, how widespread could autosomal dominant AIRE mutations be in the general population? The recent study by Husebye and colleagues provides evidence that this may be more frequent then previously thought with a frequency of nearly 1/1000 individuals harboring a potential point mutation in the PHD1 domain. More study will be needed to determine if rare variants in AIRE such as this are major contributors with subjects that have autoimmunity, especially in kindreds with a strong family history. Second, how do these mutations operate at a molecular level to interfere with AIRE activity? Work by our group demonstrated that a particular point mutation in the SAND domain interferes globally with Aire function by severely reducing Aire-dependent thymic TSA expression [12]. Similar data for the effect of PHD1 domains *in vivo* is lacking and it will be interesting to see if such mutations result in a global interference of Aire's activity or if there is some predilection for a subset of Aire-target genes.

## New molecular insights into Aire's functional activity

### Properties of Aire-dependent TSA expression

Aire is highly expressed in thymic medullary epithelial cells (mTEC's) where as outlined above it promotes the expression of thousands of TSAs [15]. How Aire impacts transcription of such a large number of genes, most of which are subject to strict spatio-temporal regulation in peripheral tissues, has been of great interest. Despite the fact that Aire was identified more than 15 years ago, progress on the exact mechanisms by which it does this has been technically challenging. There currently is not a robust transfection model or a cell line to assay its activity that is identical to that which occurs in mTEC's. Furthermore, the number of mTEC's available from a thymus is relatively small (<50,000 in a single murine thymus). Recently, we have learned a large amount about the properties of TSA expression in the thymus and the target genes of Aire through sophisticated transcriptional analyses of cell sorted mTEC's that now includes single cell RNA-sequencing analyses [15–17]. The overall features of TSA-expression within mTEC's appear to have the pattern of being both ordered and stochastic (Figure 2). On an individual cell level, each mTEC will randomly express a given TSA gene; however, within that given mTEC one frequently observes that a particular set of other genes will often be co-expressed in that cell. At one level, these co-expressed genes within a given cell are often clustered at a positional level on the same chromosome [16]. At another level, these co-expressed genes are also clustered between chromosomes (interchromosomal clustering) [17]. Many of these co-expressed genes frequently do not show other commonalities such as being part of a similar transcriptional program or being expressed in the same peripheral tissue. Despite this, within individual cells the TSA transcript areas do show evidence of increased chromatin accessibility. These recent findings complement a series of similar observations in the past that show that individual mTEC's have a somewhat random pattern of expressing TSA's that are clustered in the genome [18–20] such that on a population level a broad array of self is being constantly displayed to the thymic T cell repertoire by a diverse pool of mTEC's that harbor different TSA's on an individual cell level. In line with these observations, it also has become clear that TSA-expression within mTEC's does not follow the same rules as that which occurs in

peripheral organ tissues. In support of this notion, is the recent observation that transcription of insulin occurs in the thymus of mice deficient for the key pancreatic islet regulator Pdx1 which do not produce pancreatic insulin [21].

Given these unique properties of Aire-dependent TSA expression, the molecular basis of Aire's function in mTECs has been the subject of intense interest. A picture has emerged in which Aire partners with multiple other proteins to exert its effect on a broad number of target genes [22–25]. Using various approaches including co-IP, yeast two hybrid, and RNAi knockdown approaches, intensive efforts have now identified dozens of Aire's interacting partners. These partners initially could be broadly classified into 4 functional groups: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing [22]. More recent reports have suggested additional partners and/or functions as well as provided confirmation of earlier findings (Figure 3A). For instance, interacting proteins have been described that help Aire to recognize and target TSA genes[25–30], release stalled RNA polymerase to allow RNA elongation[24, 31–33], and regulate Aire itself [34–38]. Additionally, inroads have been made into mapping the regions of Aire that bind to interacting partners [25, 38, 39] (Figure 3B). These recent developments in understanding how Aire functions at a molecular level are described in detail below.

## Targeting Aire to TSA genes

A key to understanding how Aire regulates TSA expression is the molecular mechanism(s) used by Aire to target loci encoding TSAs. Aire appears to be atypical among transcription factors in that it does not have a clear DNA binding motif, but instead recognizes genes that possess silenced chromatin states (Figure 3A) [15, 25–27]. Unmethylated histone H3 lysine 4 (H3K4) is a repressive epigenetic mark that is selectively recognized by the first plant homeodomain (PHD1) of Aire (Figure 3B) [26–29]. Aire's binding to H3K4 is not sufficient for Aire to drive TSA expression, however, since global demethylation of H3K4 by overexpression of a H3K4 demethylase does not alter TSA expression [29]. Thus, additional mechanisms independent of unmethylated H3K4 are likely important in Aire driven TSA expression.

Concordant with this, Aire associates with additional marks of a repressive chromatin state. First, Aire targets activating ATF7ip-MBD1 (activating transcription factor 7-interacting protein-methyl CpG-binding protein 1) complexes, which are normally associated with gene repression (Figure 3A) [25]. ATF7ip-MBD1 associates with the histone methyltransferase ESET-SETDB1 to target it to methylated CpG dinucleotides, which are enriched in the promoters of inactive genes. Additionally, ATF7ip is an essential cofactor in the generation of the repressive trimethylated histone 3 lysine 9 (H3K9me3) epigenetic mark [40]. Aire-mediated expression of TSAs require the ATF7ip and MBD1 proteins, since shRNA knockdown of these two proteins *in vitro* prevented Aire-dependent TSA expression. Furthermore, genetic deletion MBD1 in mice also prevented Aire-dependent TSA expression in mTECs and predisposed mice to autoimmunity. The development of autoimmunity mapped to MBD1 deficiency in the thymus, since transplantation of MBD1 deficient thymus into athymic nude mice was sufficient to cause autoimmune disease development. Second, Aire-regulated TSAs in mTECs are highly associated with the

repressive H3K27me3 epigenetic mark [15]. Aire does not directly bind H3K27me3 [27], but may interact indirectly through chromatin binding proteins, such as CHD6 (Chromdomain Helicase-DNA 6) [22].

DNA-PK, a nuclear kinase with multiple roles including the repair of double- strand DNA breaks and DNA replication, may also play a role in targeting Aire to TSAs (Figure 3A) [30]. Double strand DNA breaks are marked by the variant histone H2AX phosphorylated at Ser 139 (γH2AX), and DNA PK appears to target Aire to these (γH2AX marks. [30] Since transient double stranded DNA breaks have been associated with transcription initiation [41], DNA-PK may direct Aire to TSAs poised for transcription [42]. mTECs derived from reconstituted SCID mice that carry a mutation in DNA-PK have reduced expression of a number of Aire-dependent TSAs, suggesting that efficient mTEC expression of Aire-dependent TSAs requires DNA-PK. Of note, however, additional roles for DNA-PK in Aire's function, including relaxation of surrounding chromatin[22] and phosphorylation of Aire [37] (see below), have also been proposed. Which of these DNA-PK functions are most relevant to Aire's function remains to be determined. In any case, the prevailing evidence points to Aire as an unusual transcriptional regulator that recognizes a combination of chromatin signals to target TSA genes in mTECs.

## Aire releases stalled RNA polymerase

Another atypical aspect of Aire is that Aire does not act primarily by initiating TSA gene transcription. Rather, Aire promotes TSA expression through the release of stalled RNA polymerase to elongate RNA transcripts. Microarray analysis using mRNA spanning probes showed no differences in transcription initiation within mTECs of wildtype and Aire-deficient mice [33]. Instead, RNA lengths were reduced (50–100 bp) in Aire-deficient mTECs, suggesting that Aire has a role in promoting RNA elongation. Furthermore, Aire's interacting partners also suggest that Aire promotes TSA expression through RNA elongation. Aire interacts with positive transcription elongation factor b (P-TEFb), a protein that controls release of stalled RNA polymerase (Figure 3A) [31, 32]. This interaction occurs through Aire's extreme C-terminus, in which patient mutations that disrupts this interaction can result in multi-organ autoimmunity (Figure 3B) [32]. Aire's interaction with P-TEFb was independently confirmed using an RNAi screening approach for Aire's functional allies [24]. Interestingly, this RNAi screen identified 51 candidates involved in Aire's transactivating function. While many of these candidates had known roles in RNA elongation, none of them had known roles in transcription initiation [24]. Thus, Aire primary effect appears to be in RNA elongation rather than initiation of transcription.

The identification of HnRNPL (heterogenous nuclear ribonucleoprotein L) as an Aire binding partner lends support to Aire's role in releasing stalled RNA polymerase (Figure 3A) [24]. HnRNPL knockdown in mTEC cells decreased transcripts that are controlled by the P-TEFb components CCNT2 and CDK9, suggesting that HnRNPL has a role in RNA elongation[24]. Furthermore, HnRNPL co-immunoprecipitates with Aire and the P-TEFb components CDK9 and HEXIM1. Finally, the 7SK snRNA, a noncoding RNA that regulates P-TEFb activity, co-precipitated with Aire and HnRNPL knockdown decreased this interaction, suggesting that HnRNPL may facilitate Aire's interaction with the P-TEFb

regulator, 7SK snRNA. Thus, Aire appears to interact with multiple P-TEFb complex members that are important in productive RNA elongation.

In addition to promoting RNA elongation, Aire may also regulate pre-mRNA splicing through P-TEFb and other factors [22, 24, 32]. Aire increases the pre-mRNA splicing of a heterologous minigene and the Aire-regulated KRT14 gene, and this increase is P- TEF-b dependent, since inhibition of the kinase subunit of P-TEFb blocked Aire-induced pre-mRNA splicing[32]. Consistent with a role in pre-mRNA splicing, Aire associates with multiple interacting partners that have known roles in pre-mRNA splicing (e.g. EFTUD2, SNRPB, SRSF1) [12, 22]. Rather than initiating transcription, then, Aire primarily functions to promote RNA elongation and splicing of target TSAs.

## Aire interacts with its functional regulators

Interestingly, Aire interacts with a number of proteins that exert control over Aire's function through post-translational modifications [34–38]. First, Aire associates with deacetylase and acetyltransferase proteins that act directly upon Aire [34–36]. For instance, Aire physically binds to Sirtuin 1 (Sirt1), a protein deacetylase that is highly expressed within mTECs (Figure 3A) [36]. Sirt1 deacetylates lysine residues in Aire, a process that activates Aire transcription. Moreover, eptihelial cell-specific ablation of Sirt1 disrupted Aire-dependent TSA expression without affecting Aire expression, which suggests a role for Sirt1 in Aire-regulated TSA expression. At the same time, Aire also interacts with CREB-binding protein (CBP) and p300, both of which have acetyltransferase activity. CBP and p300 add acetyl groups to the Aire protein, a process that downregulates Aire transcriptional activity and alters the profile of TSAs regulated by Aire[35]. Thus associations with proteins that acetylate and deacetylate Aire appear to regulate Aire's function.

In addition to factors that modulate acetylation, proteins that mediate phosphorylation are also associated with Aire [37, 38]. Aire interacts with DNA dependent protein kinase (DNA PK), which in expressed in mTECs and phosphorylates Aire *in vitro* [37]. Mutation of Aire Thr68 and Ser156, which are targets of DNA-PK phosphorylation, reduces Aire's ability to promote transcription in *in vitro* reporter systems. However, the requirement for DNA-PK's nuclear kinase activity has been called into question, since addition of Nu7441, an inhibitor of DNA-PK catalytic activity, did not abrogate Aire's effect on TSA expression in 293T cells[30]. A possible explanation for these discrepant results is that DNA-PK's catalytic activity is required for a subset of TSA expression, since a limited number of promoters were tested in these studies. At the same time, Aire's phosphorylation may also be regulated by homeodomain-interacting protein kinase 2 (HIPK2), a protein that partially-colocalizes with Aire in co-transfected cell lines and human thymus [38]. In an *in vitro* reporter system, HIPK2 decreased Aire transactivation activity in a kinase dependent manner; unexpectedly, however, eptihelial cell-specific deletion of HIPK2 affected largely Aire-independent TSAs. Thus, Aire's association with this kinase may not affect Aire's function in promoting mTEC TSA expression. Instead, it is possible the HIPK2 may affect a non-Aire, yet-to-be-identified, regulator of mTEC TSA expression.

## Aire promotes positive selection of thymic Tregs

Aire's major influence on the negative selection of autoreactive T cells is well-established [43–46]; however, its influence on thymic positive selection of FoxP3+ Treg's has been more controversial. Ectopic antigen expression in Aire-positive mTECs promotes the development of antigen-specific CD4+ Tregs in the thymus [47] , which, early on, suggested the possibility that Aire-expressing mTECs can promote thymic Treg development. Subsequent studies have provided evidence that Aire may influence thymic Treg development [48–51]. A decrease in thymic CD4+ Tregs is particularly evident during an early perinatal time period and decreased absolute numbers of CD4+ Tregs up to Day 10 [49]. In addition, decreased thymic CD4+ Tregs have also been reported in adult mice [48, 50], however, data are conflicting on this issue [49].

Regardless of whether Aire transiently or permanently influences thymic Treg numbers, Aire appears to influence, at least in part, specific clones of the thymic Treg repertoire. Certain CD4+ Treg clones seem to be entirely dependent on thymic Aire expression for their development. One particular Treg clone, for instance, that was originally isolated from tumor-infiltrating lymphocytes, is normally generated in the Aire-replete thymus. In the Aire deficient thymus, however, this CD4+ Treg clone is entirely absent [50]. Thus, thymic development of this particular Treg clone appears to be completely Aire-dependent. Conversely, some Treg clones, appear to develop independently of Aire within the thymus. Perry et al. used limited TCR repertoire approach to demonstrate that the most frequent thymic Treg clones are Aire independent [51]. Morisita Horn similarity index analysis of Treg TCR repertoires showed a high degree of similarity between Aire deficient and Aire wildtype mice. On the surface, this finding suggested that Aire does not have a major effect on Treg TCR selection. Remarkably, removal of the three most frequent Treg TCRs from this analysis significantly lowered the similarity, which pointed to the most frequent clones erasing the effects of Aire. These findings suggest that the most frequent Treg TCRs are Aire independent, whereas rare Treg TCRs include those that are Aire dependent.

The factors governing whether a given CD4+ Treg clone is Aire-dependent or independent are not currently known. It is tempting to speculate that the Aire-dependent clones are those that are specific for Aire-dependent TSAs. Further investigation is required to test this hypothesis, however, as the studies of Perry et al. and Malchow et al. did not directly identify the antigen specificity of the Aire-dependent Treg clones. Interestingly, Aire may also indirectly regulate Treg development through its effects on thymic APC migration [48]. Aire regulates mTEC expression of XCL1, a chemokine that directs dendritic cells into the thymus. Similar to Aire deficient mice, XCL1 deficient mice also have reduced frequency and absolute numbers of thymic CD4+ CD8- FOXP3+ CD25+ Tregs. Thus, Aire may affect thymic Treg selection by means other than TSA upregulation.

Given that Aire appears to influence the thymic Treg repertoire, an interesting question is how much does a defect in this process contribute to the autoimmunity present in Aire-deficient individuals? Recent work by Yang et al. [49] suggests that a defect in the neonatal output of thymically derived Treg's in Aire-deficient mice indeed may be a key player in driving autoimmunity in the model. Suggestion that such a mechanism may be in play came

from previous work which demonstrated that temporal knockout of Aire in mice after three weeks of age did not predispose to widespread autoimmunity [52]. Yang et al. used elaborate adoptive transfers of FoxP3+ Treg's from neonatal Aire wildtype or knockout donors into Treg ablated recipients to show that Aire wildtype Treg's could protect but not Aire knockout Treg's. In contrast to these findings, two independent studies demonstrated that co-transfer of an Aire-deficient thymic lobe and an Aire wildtype thymic lobe into a single nude recipient mouse did not protect against autoimmunity which favors a primary role for Aire in deletion rather than dominant tolerance [44, 53]. In addition, previous work has also demonstrated that genetic crossing of the Aire knockout and FoxP3 knockout mouse results in a more severe autoimmune syndrome than either single knockout alone [54]. These latter findings strongly argue that a defect in Treg selection is not the sole, critical immune tolerance defect in the Aire-deficient model. Further work will be needed to parse these issues out including more detailed characterization of the Treg TCR specificities that may be absent in the Aire-deficient model and how they potentially contribute to disease when missing.

### SEPARATE BOX

In addition to CD4+ FOXP3+ Tregs, CD8+ CD28low Tregs in Aire-deficient mice have also been shown to be defective in their ability to suppress autoimmune colitis in vivo [55]. CD8+ CD28low Tregs are a distinct population of T cells that can prevent the development of experimental autoimmune encephalomyelitis (EAE) and experimental colitis [56, 57]. CD8+ CD28low Tregs from $Aire^{o/o}$ mice have altered TCR repertoires and decreased suppressive function [55]. By immunoscope analysis, the T cell receptor (TCR) gene structure of CD8+ CD28low Tregs from $Aire^{o/o}$ mice was distinct from that of wildtype mice. Furthermore, in the colitis transfer model, cotransfer of CD8+ CD28low Tregs from wildtype mice prevented development of colitis, whereas cotransfer of CD8+ CD28low Tregs from $Aire^{o/o}$ mice did not. Aire thus appears to play an important role in shaping the repertoire of CD8+ CD28low Tregs and the ability of these cells to suppress colitis.

## Other Aire functions in mTECs

As noted above, Aire is best known for its role in upregulating mTEC TSA expression to prevent autoimmunity. By upregulating mTEC TSAs, Aire promotes negative selection of effector T cells and development of Tregs. It is now clear, however, that Aire has 1) functions that are independent of its promotion of TSA expression and 2) effects beyond autoimmune disease prevention. These extended roles for Aire are discussed below:

### TSA independent functions

An early indication that Aire has TSA-independent functions came from studies using the OTII RIP-mOVA double transgenic system[44]. In this system, membrane-bound ovalbumin (OVA) is expressed as a neo self-antigen in mTECs under the control of the rat insulin promoter (RIP), and OTII T cells expressing transgenic T cell receptor specific for OVA undergo thymic negative selection upon recognition of OVA. Although negative selection of OTII thymocytes was clearly Aire-dependent, Aire minimally affected mTEC expression of the OVA neo self-antigen [44]. Similar results were seen using the OTII RIP-$OVA^{hi}$ system, in

which soluble OVA is expressed under the control of RIP [58]. These findings collectively suggest that Aire my also regulate T cell negative selection and autoimmunity by TSA-independent mechanisms. Additionally, Aire-deficient mice develop organ specific autoimmunity of the salivary gland and pancreas, and pathogenic T cells appear to be directed against salivary and pancreatic antigens that, unexpectedly, are not Aire-regulated in the thymus [53, 59]. Thus, a mechanism distinct from Aire upregulation of TSAs in mTECs is likely to underlie these autoimmune manifestations.

Consistent with these initial observations, TSA-independent roles for Aire have now been delineated. Aire promotes mTEC expression of XCL1, a chemokine critical in recruitment of thymic dendritic cells[48], and XCL1 deficiency results in decreased thymic dendritic cell accumulation. Thymic dendritic cells have an important role in mediating thymocyte negative selection, and thymocytes from XCL-deficient mice are sufficient to transfer autoimmune lacrimal disease. As described above, XCL deficiency also impacts Treg development within the thymus[48], although Aire/XCL dependent mechanisms are unlikely to account for selection of all Treg TCRs [51].

In addition to upregulating XCL1, Aire also increases expression of ligands of CCR7 and CCR4, chemokines important in thymocyte migration [60]. Aire overexpression enhanced thymocyte chemotaxis and emigration via CCR7 and CCR4 chemokine action. In addition to modulating chemokine expression, Aire may also enhance self-tolerance through promoting mTEC apoptosis [61]. Aire is expressed in approximately 50% of $MHCII^{high}$ mTECs ($MEC^{hi}$) cells, and Aire deficiency is associated with increased $MHCII^{high}$ mTECs. One potential explanation for this is that Aire in $MEC^{hi}$ cells induces apoptosis, and this pro-apoptotic function may enhance self-tolerance by facilitating phagocytosis and cross-presentation of TSAs by thymic antigen-presenting cells [62]. In conflict with this interpretation is evidence using an inducible Cre mouse model that suggests that Aire does not affect mTEC lifespan [63]. Instead, Aire may be involved with physiologic downregulation of CD80 in mature mTECs[63]. Thus, Aire's role as a pro-apoptotic factor remains to be clarified.

Furthermore, three groups have independently reported the existence of post-Aire-expressing mTECs [63–65], a finding potentially at odds with Aire's role in inducing mTEC apoptosis. Prior to these reports, the most mature mTECs were considered to be $MEC^{hi}$ cells, a post-mitotic, Aire-expressing population that also express high levels of CD80 and MHCII. However, recent studies using lineage tracing approaches have delineated a population of post-Aire expressing cells that express low levels of CD80 and MHCII[63–65]. This population continues to express an array of TSAs, but at quantitatively reduced levels compared to Aire-expressing cells [64]. This population may also over-express a subset of TSAs, such as desmoglein, a pemphigus vulgaris-associated antigen [65]. Given its continued TSA expression, this post-Aire population likely continues to play a significant role in enforcing self-tolerance. Finally, Aire may play a role in Aire mTEC differentiation. Aire deficiency prevents the expression of epidermal markers of late mTEC maturation, such as CK6, CK10, involucrin, and LEKT1 [65], which suggests a role for Aire in end-stage mTEC differentiation. Moreover, Aire deficient mice may have changes in thymic architecture and mTEC morphology [66], which also lends support to Aire's function in mTEC differentiation.

## Anti-tumor immunity

Aire's role in preventing autoimmunity is evident by the spontaneous development of autoimmunity in mice and humans with Aire mutations. Recently, Aire's roles in modulating diseases outside of autoimmunity have been delineated. For instance, Aire's function in preventing effective anti-cancer immunity has recently been clarified (Figure 4). Aire's role in preventing cancer immunity may be regarded as an extension of its role in preventing autoimmunity. Because many Aire-regulated self-antigens expressed by mTECs are also expressed by tumors, the antigen-specific tolerance induced by Aire also prevents an effective anti-tumor response [67, 68]. mTECs express a number of well-known melanoma antigens [67, 68]. For instance, Aire upregulates mTEC expression of TRP-1 and consequently the negative selection of TRP-1 specific T cells in mice[68]. In the setting of Aire deficiency, TRP-1 specific T cells are rescued from negative selection and the T cell immune response against these melanoma antigens is enhanced. With the larger pool of melanoma specific T cells, Aire deficient mice demonstrate reduced melanoma growth and increased survival [67, 68]. These findings are likely to translate to patients, since human mTECs express a large number of known melanoma antigens [69]. Furthermore, case-control studies have associated melanoma protection with Aire polymorphisms that decrease Aire expression [70].

In addition to melanoma, other cancer types may also be modulated by Aire. In mouse models of primary and transplanted sarcoma, negative regulation of Aire-expressing mTECs enhanced anti-tumor immunity [71]. As described above, Aire also regulates Treg TCR clones that were originally isolated from prostate cancer, suggesting a role for Aire in regulating T cell immune response against prostate cancer [50]. Thus, Aire is likely to have a broad effect on multiple cancer types.

Recently, our group also provided evidence that modulation of Aire-expressing mTEC's may be a tractable approach for enhancing tumor-specific immune responses. Aire-expressing mTEC's have an interesting property of turning over quickly as a cell population. Studies with both genetic and BrdU labeling have demonstrated that in adult mice, mTEC's have a population half-life of approximately 12–14 days [61, 64]. The pool of new Aire-expressing mTEC's appears to be replenished through a process of mTEC maturation that involves RANK-RANK-Ligand signaling. Taking advantage of this knowledge, Khan et al. demonstrated that *in vivo* blockade of RANK-Ligand over a two week time window led to selective depletion of Aire-expressing mTEC's over other thymic epithelial cell populations [72]. Treated mice showed evidence of an aquired defect in thymic negative selection and in a TCR transgenic model, treated mice develop an enhanced anti-tumor response in a B16 melanoma model. Importantly, the loss of Aire-expressing cells was transient and the thymus could recover such cells after the removal of anti-RANK-Ligand antibody. Further work will be need to determine if this approach of altering thymic Aire-activity could complement the wide array of current treatments involving peripheral tolerance and checkpoint blockade [73] but this remains an open and exciting possibility.

## Aire in GVHD

Aire mediated TSA upregulation in mTECs also impacts the development of chronic graft versus host disease (cGVHD), a major complication of allogeneic hematopoietic stem cell

transplantation [74, 75]. Autoimmune-like manifestations are a part of cGVHD, and patients who have developed acute graft versus host disease (aGVHD) are strongly predisposed to cGVHD. In aGVHD patients, donor T cell mediate immune destruction of particular recipient tissues, including the thymus. Interestingly, donor T cells in aGVHD appear to selectively target Aire-expressing mTECs within the recipient thymus. As a consequence, mTEC expression of TSAs, especially those restricted to tissues affected in cGVHD, are decreased in aGVHD [75]. aGVHD is associated with impaired negative selection of self-reactive T cells, which allows the generation of T cells that can mediate autoimmunity associated with cGVHD[74]. Understanding that aGVHD induces an Aire-deficient state that predisposes to the generation of autoreactive T cells potentially allows for the development of interventions that may increase Aire expression to prevent cGVHD development.

### Aire in tumorogenic keratinocytes

Aire is induced at low levels in mouse and human tumor keratinocytes, a cell type involved in human skin squamous cell carcinoma, and Aire mRNA induction in keratinocytes (but not mTECs) is dependent on keratin 17 (K17) [76]. Furthermore, in keratinocytes, Aire protein interacts with K17 protein, and Aire mRNA interacts with ribonucleoprotein hnRNP K in a K17 dependent manner. Global K17 deficiency protected mice from skin tumorigenesis, and this protection was associated with decreased expression of neutrophil activation markers, dermal mast cell density, and expression of other proinflammatory genes. Thus, Aire's pro-inflammatory role in keratinocytes undergoing tumorigenesis appears to be entirely distinct from its immunoregulatory role in mTECs in dampening anti-tumor immunity, and opens up a new field of investigation.

## Conclusions

In the last few years, multiple, sometimes unexpected, roles for Aire beyond its best-known functions have been described. Aire's role in selecting Tregs, its multiple effects that do not involve upregulation of mTEC TSAs, and the multiple pathologic processes governed by Aire have now been established. Furthermore, molecular insights into Aire's function are now being clarified and account for some of the fascinating aspects by which it carries out its functional activity. Looking forward there is still much that needs to be learned about the biology by which this critical regulator of tolerance operates and how pathways it controls contribute to human disease.

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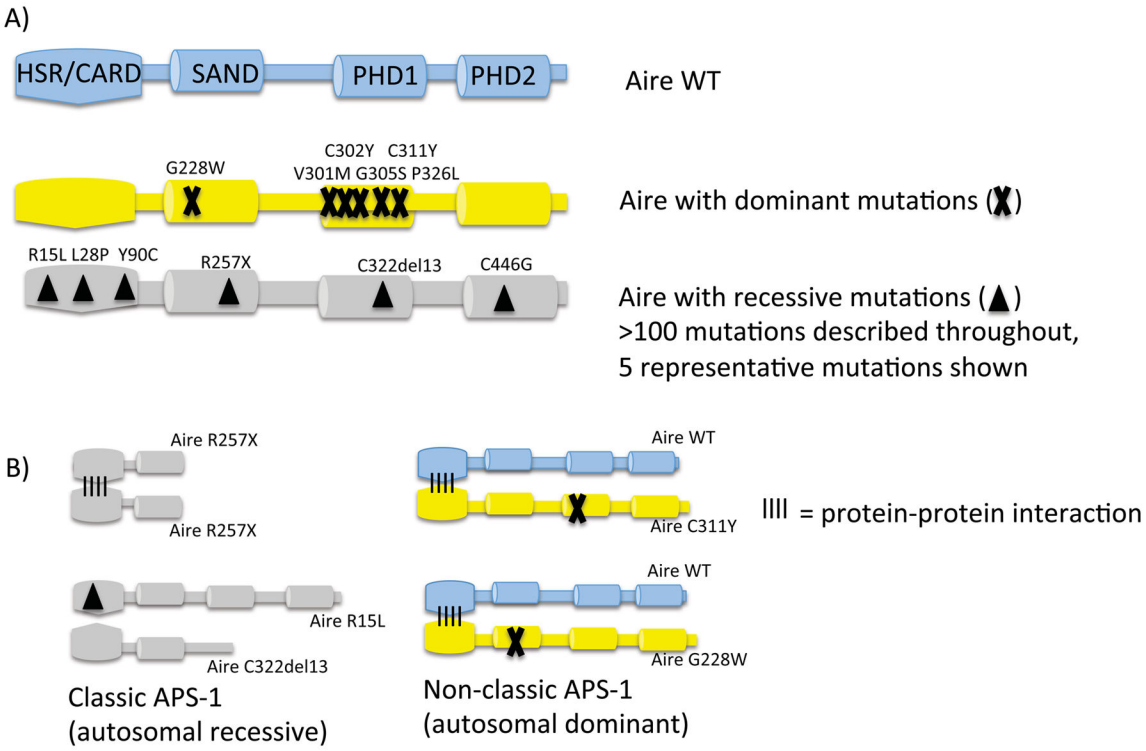


**Figure 1.**

Disease-causing Aire mutations in patients with classic and non-classic APS-1. A) The wildtype (WT) Aire protein with its domains (top). Dominant Aire mutations (X) and 5 representative recessive Aire mutations (triangle) from more than 100 recessive mutations described are shown below. Dominant mutations cluster in the PHD1 domain whereas recessive mutations are found throughout the Aire protein, including the HSR/CARD multimerization domain. B) Mutant Aire proteins and how they can cause disease. Classic APS-1 can occur with homozygous Aire R257X mutations (upper le]). These mutant Aire proteins can multimerize but lack critical Aire domains. Alternatively, compound heterozygotes with HSR/CARD domain mutant (R15L) that prevents multimerization and truncation mutant (C322del13) (lower left) can also lead to classic APS-1. Non-classic APS-1 occurs with one copy of an Aire C111Y or G228W mutation (right).

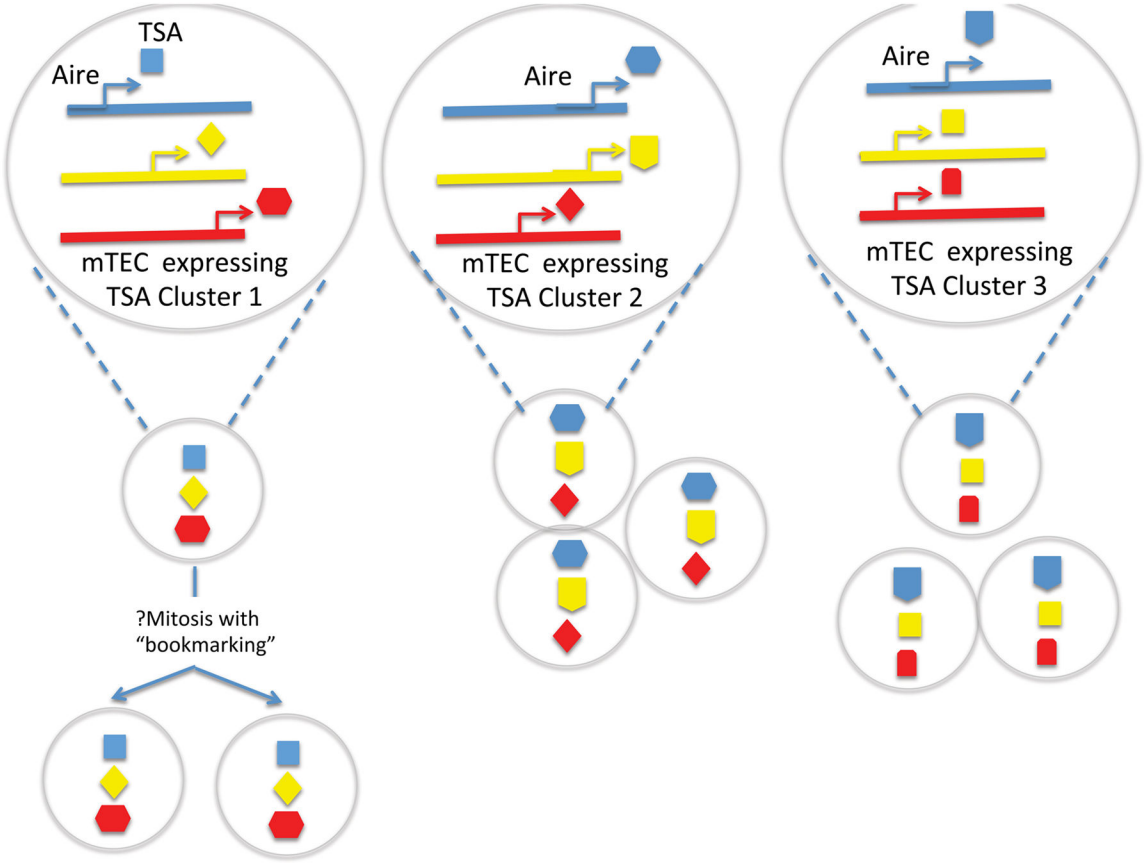


**Figure 2.**

Ordered stochasticasticity of Aire regulated gene expression. Aire-regulated TSA expression in single mTEC does not occur randomly but occur in microclusters of co-expression. Interchromosomal clusters of TSA expression are shown, although clustering of genes located in close proximity in the genome has also been demonstrated. "Bookmarking", or the maintenance of gene-expression programs after cell division, may result in the clonal expansion of mTECs expressing TSA microclusters (left).

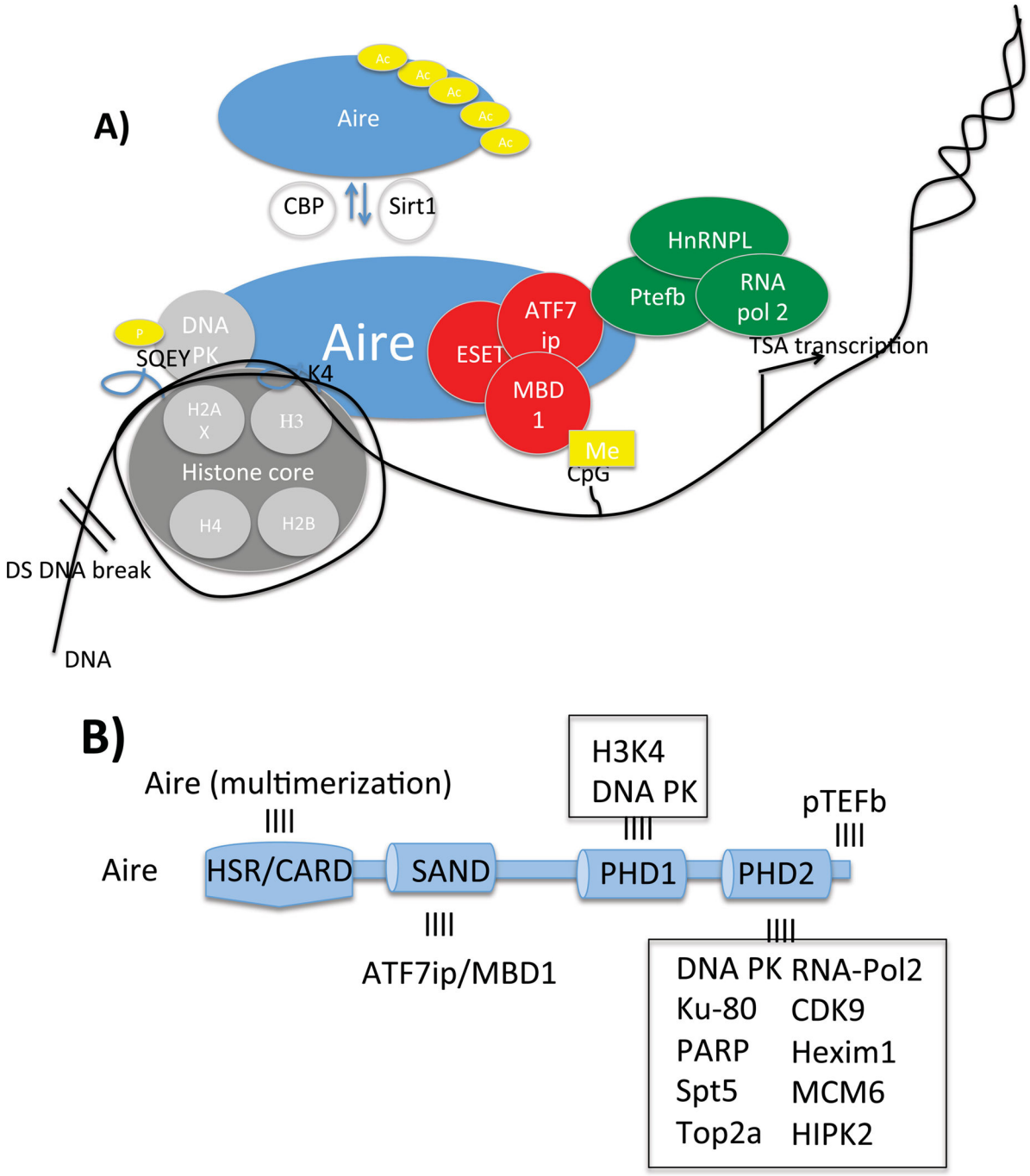


**Figure 3.**

Aire and it's binding partners. A) Schematic illustration of a partial set of Aire's interacting partners. Dozens of Aire interacting partners with diverse functions have been identified. Here is shown Aire's ineractions with histone core proteins either directly or through its interactions with DNA-PK; ATF7ip/MBDl/ESET complex that interacts with methylated DNA; and pTEFb, HnRNPL and RNA pol2 to release stalled polyermases. Aire also

interacts with Sirtl and CBP which control Aire aceytlation. B) Map of regions of Aire that interact with binding partners.

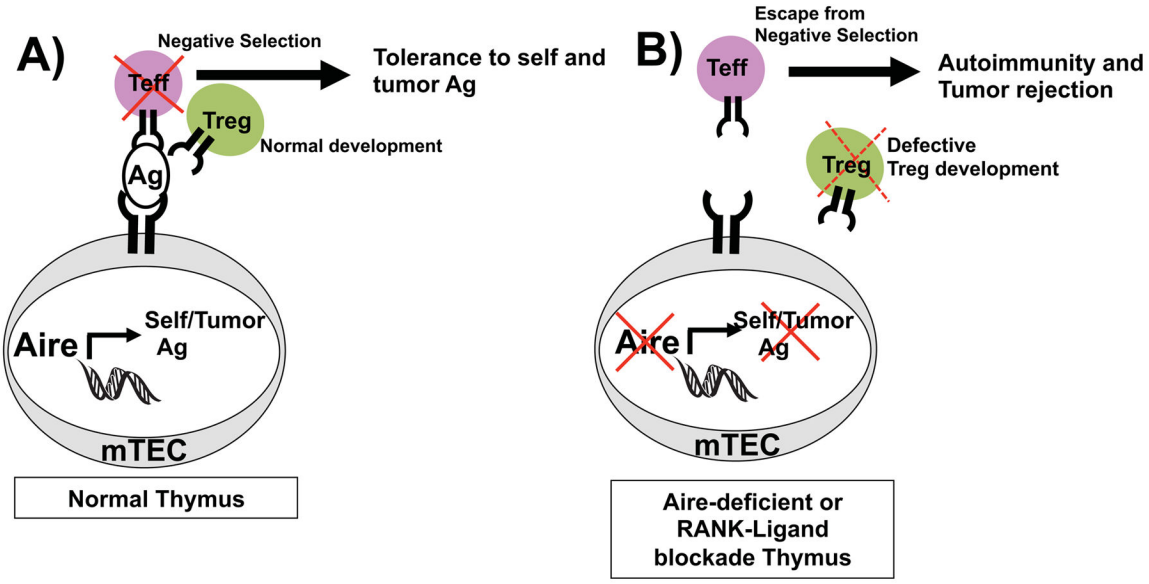


**Figure 4. Aire enforces central tolerance toward self-antigens**

A) In wildtype thymus, Aire in medullary thymic epithelial cells (mTECs), promotes ectopic expression of self-antigens (Ag), which include melanoma Ags. T cells recognizing these antigens undergo negative selection to enforce self-tolerance. B) In Aire deficienct thymus or thymus treated with anti-RANKL antibody, lack of self-antigen expression allows escape of self-reactive T cells from negative selection, which predisposes to autoimmunity.