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AIRE in the Thymus and Beyond

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Summary

The maintenance of immunologic self-tolerance requires the coordination of multiple complementary systems. Studies of the *Autoimmune Regulator (Aire)* gene have revealed that *Aire* promotes self-tolerance in part by inducing the transcription of a wide array of tissue-specific antigens (TSAs), particularly in the thymus. The importance of *Aire* is highlighted by the fact that patients and mice defective in *Aire* expression develop a multi-organ autoimmune syndrome. In this review we discuss recent progress in our understanding of *Aire's* control of immune tolerance at the cellular and molecular levels, and also address the potential importance of *Aire* expression both in the thymus and in the peripheral lymphoid organs. The detection of both *Aire* and TSA expression by cell populations outside of the thymus raises the possibility that such expression may play a relevant role in the maintenance of self-tolerance.

Introduction

Since its discovery as a master transcriptional regulator controlling expression of a wealth of genes within the thymus [1,2], *Aire* has significantly changed the face of immune tolerance theory. Named for its role as an Autoimmune Regulator, AIRE drives ectopic expression of many tissue-specific, sequestered, and otherwise "peripheral" proteins within the thymus.

Aire is primarily, but not exclusively, expressed by medullary thymic epithelial cells (mTECs), and ensures developing thymocytes are exposed to a comprehensive view of "self," permitting early deletion of autoreactive cells before acquisition of effector functions. Although its molecular mode of action remains somewhat elusive, its influence on immune tolerance continues to expand, encompassing a multitude of cells, tissues, diseases and molecular interactions. A number of purported functions for AIRE are still hotly debated, including a role in regulatory T-cell generation; molecular mechanisms controlling its putative transcriptional regulation; its genetic targets; and its peripheral expression and impact.

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AIRE is an important regulator of normal T cell development

Loss-of-function mutations in the human *AIRE* gene are the single causative defect in a rare, systemic autoimmune syndrome termed APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) or APS-1 (autoimmune polyendocrinopathy syndrome type I) [3]. Patients develop a severe, complex array of symptoms, with candidiasis and insufficiency of the parathyroid and adrenal glands serving as diagnostic hallmarks. However, APECED patients commonly develop multiple other endocrinopathies including type-1 diabetes, hypogonadism, and hypothyroidism [3–5]. The collective pathologies of APECED patients are highly varied, even between siblings [6], suggesting a contributive interplay of other genetic factors or environment.

Mouse strains made deficient in *Aire* have been an invaluable tool enabling researchers to dissect *Aire's* role in immune regulation, and more recently, to begin unraveling its somewhat elusive intracellular targets and mechanisms of action. The first described *Aire*-knockout mouse strain developed a relatively mild autoimmune syndrome featuring T-cell hyperactivity; production of autoantibodies to liver, testis, pancreas and adrenal glands; leukocyte infiltration of liver and ovaries; and adrenal or thymic atrophy [1]. A second knockout strain [2] revealed a more severe tolerance breakdown, featuring cellular infiltration of and autoantibody reactivity to the salivary gland, retina, stomach, pancreas and ovary.

Interestingly, the disease spectrum differs in severity and specificity of target organs depending on the genetic background of the knockout strain [1,7,8]. Non-Obese Diabetic (NOD) mice, for instance, surprisingly become resistant to type I diabetes when *Aire*-deficient—while insulin-producing pancreatic islets normally come under autoimmune attack in NOD mice, immune foci instead target exocrine tissue in the pancreas of *Aire*-knockout NODs [7,9].

Although studies of *Aire* knockout mice have irrefutably improved our understanding of T cell tolerance, it has been argued that differences between disease spectra of APECED patients and mice limit their relevance [4,5]. This argument centers on the generally milder murine pathology, where severe infiltration of endocrine organs does not equate to a lack of organ function, manifest as devastating polyendocrinopathy in patients [4,5]. Notable exceptions include hypogonadism and retinal autoimmunity, which occur in all *Aire* knockout mice generated to date, and disease severity in *Aire* knockout NOD mice, which waste and die within four months of birth [9]. One of the most striking similarities between murine and human pathologies paradoxically lies with the broad variability of symptoms and severity, even between first-degree relatives, or littermate inbred mice [1,4,6,10]. This suggests a conserved, probabilistic mode of action for AIRE and tells us that differences between mice and humans are a likely pairing of AIRE's stochastic intricacies and basic physiological differences between the species.

Mechanisms of Tolerance in the Thymus

Although the requirement for *Aire* in the maintenance of normal immunologic self-tolerance was established by cloning the gene from individuals with APECED [3,11], and further supported by *Aire*-knockout mice, the precise mechanism of *Aire*-mediated tolerance remained elusive. Indeed, many elements of the immune system are grossly normal in *Aire*-deficient animals [1,2]. The complementary discoveries that *Aire* was highly expressed by mTECs [12,13], and that the thymic medulla was a site of expression of otherwise tissue-specific antigens (TSAs) such as insulin and thyroglobulin [14,15], suggested that TSA expression might be essential for self-tolerance, and that *Aire* might play a role in this process. Indeed, in the absence of functional AIRE, mTECs express a severely restricted array of self-antigens [2], implicating a defect in negative selection which allows a broad set of autoreactive T cells to reach the periphery. Supporting this hypothesis, transplantation of *Aire*-deficient thymic

stroma is sufficient to induce multi-organ autoimmunity in Nude (*Foxn1*-knockout) mice [2], and transgenic expression of rat insulin promoter (RIP)-driven antigens like ovalbumin (OVA) and hen-egg lysozyme (HEL) induces their expression in the thymus, and tolerizes cognate TCR-transgenic thymocytes in a strictly *Aire*-dependent fashion [16,17].

One important and unanswered question involves the contribution of *Aire* to regulatory T-cell (Treg) generation. *Aire*-deficient mice have no gross defects in Treg number or function, and cotransplantation of *Aire*-replete and *Aire*-knockout thymi into Nude mice fails to rescue from disease [18], suggesting that dominant tolerance mediated by regulatory populations selected in an *Aire*-replete thymus is not sufficient to suppress autoreactive T cells exported from the cotransplant. However, transgenic expression of neo-self-antigens in thymic *Aire*-expressing cells also appears to skew some antigen-specific TCR-transgenic T cells toward a regulatory lineage [19]. Whether different APC populations play unique roles with regard to deletion versus regulatory T cell induction, and the role of AIRE-regulated antigens in the development of regulatory cells, remains an area of active inquiry.

Molecular Mechanisms of AIRE Function

The diversity of AIRE-regulated self-antigens incites strong interest into precisely how this single gene controls its transcriptional portfolio to represent so many tissues and organs. The predicted domain structure of the AIRE protein, particularly the putative DNA-binding SAND domain, initially suggested that AIRE might interact directly with DNA; indeed some studies have supported this through gel-shift and chromatography assays [20,21]. However AIRE's SAND domain lacks the critical KDWK motif required for DNA-binding in other SAND domain proteins, and while some studies have reported direct association of AIRE with TSA promoter regions using ChIP of *in vitro* transfection systems [22], it remains unclear whether this reflects direct DNA binding or simply association as part of a larger macromolecular complex, and whether such binding is relevant *in vivo*. Furthermore, genomic analysis to date has failed to identify a consensus AIRE-binding site among the vast number of AIRE-regulated genes in the thymus.

AIRE's genetic targets also provide clues to its function. In the thymus, AIRE-regulated genes show clear evidence of chromosomal clustering [23,24], suggesting that epigenetic mechanisms may play a role in their transcriptional regulation. Indeed, AIRE also appears to associate with the nuclear matrix [25], a scaffold for chromatin alteration, further supporting the idea that it may act as a broad-scale epigenetic modifier. AIRE localizes to nuclear bodies adjacent to, but distinct from, splicing factor-associated nuclear speckles. This distribution is disrupted in a dominant-negative, disease-causing G228W SAND domain mutation [26], suggesting that AIRE's function is associated with its appropriate distribution in nuclear bodies.

Closer examination of chromosomally clustered regions of AIRE regulation, however, paints a more complex picture. For example, while the casein locus contains a family of genes uniformly expressed in mammary epithelium, its thymic expression is differently regulated. In the thymus, AIRE-induced casein genes sit directly beside AIRE-repressed casein genes, which are in turn next to genes unresponsive to AIRE. Furthermore, single-cell sorting of mammary and thymic epithelium demonstrated that while all casein locus genes are coordinately upregulated in each mammary epithelial cell, any one thymic epithelial cell expresses only a few casein genes, and the distribution appears random with regard to clustering in the locus [27]. By extrapolation from this and other studies, it seems that only ~1–2% of thymic epithelial cells express any particular TSA, with a distribution that appears stochastic in nature. AIRE-mediated regulation of these large genetic loci may therefore be more complex than simply

opening the door to broad transcriptional de-repression, and may require both gene-specific interaction of specific cofactors and some stochastic element causing wide variation per cell.

Together, these results support a model in which AIRE appears to act through epigenetic mechanisms that are necessary, but not sufficient, for expression of individual genes within a locus, and that other cofactors may be involved in determining precisely which antigens are expressed within each cell. Indeed, AIRE's collaborative interactions with transcriptional coactivators like CBP [28], transcriptional elongation machinery such as pTEF-B [29], or DNA-specific kinases like DNA-PK [30] may play an important role in deciding which antigens are expressed in each cell (Figure 1).

In support of such epigenetic mechanisms, a number of recent studies have shown that the PHD1 domain of AIRE, a highly conserved Zn-finger domain [31] and a common site of causative mutation in APECED patients [32], binds to histone H3 in a methylation-sensitive manner [33,34]. Methylation of lysine 4 in the amino-terminus of histone H3 generally reflects the accessibility of a particular chromatin region, with unmethylated histones marking regions that are transcriptionally inactive, and trimethylated histones marking regions of high transcriptional activity. AIRE's PHD1 domain appears to have a selective affinity for binding unmethylated H3K4, as recent crystal structures have confirmed [35,36]. This suggests an attractive hypothesis: that AIRE may be built to selectively recognize and bind to transcriptionally inactive heterochromatic regions via their unmethylated H3K4 subunits, recruiting additional machinery to help facilitate site-specific transcription in these regions.

AIRE: Beyond Autoantigens

The kaleidoscope of genes regulated by AIRE was first revealed with microarrays of mTECs from wildtype and *Aire*-knockout mice [2,18]. Although *Aire* deficiency has a profound effect on expression of many genes in mTECs, its function may also extend beyond TSA expression. For example, using a sensitive negative selection system where the exogenous antigen OVA is transgenically expressed in the thymus, Anderson *et al.* [18] reported impaired deletion of OVA-specific thymocytes in *Aire* knockout mice despite the fact that OVA expression was not reduced. Indeed, *Aire*-deficient mTECs showed altered intrinsic interaction with thymocytes in this system despite the presence of OVA [18]. Similarly, thymic expression of the autoantigen α-fodrin was not impaired in *Aire*-knockout mice, despite demonstrable autorecognition in the periphery [37]. Furthermore, expression of various genes involved in antigen presentation and chemokine expression appears to be controlled by AIRE [18,24].

AIRE expression is also used to define end-stage matured mTECs [38,39] and an active role for AIRE in this maturation has been proposed [40,41]. Loss of *Aire* appears to alter thymic architecture [40] and skew mTEC development toward the proliferative MHC class II^{high} $CD80^{high}$ subset [39,42]. These thymic abnormalities may occur as a result of perturbed thymocyte migration and selection; a theory supported by several recent studies, which together showed that the number of mature mTECs is governed by direct interactions with positively selected, autoreactive CD4+ thymocytes [43–45]. The presence of autoreactive cells may therefore create a driving force to develop the very cell type capable of inducing their deletion. Expression of *Aire* speeds apoptosis in transfected cell lines and marks post-mitotoic mTECs that die quickly [39], suggesting that it may precipitate the death of mTECs and perhaps aid in the dispersal of tissue-specific antigens. This raises the important and unresolved question of whether *Aire*-expressing mTECs directly delete autoreactive T cells or simply serve as antigen reservoirs for other APC populations.

Extrathymic AIRE and Promiscuous Gene Expression

The precise distribution of *Aire* transcript and protein expression outside the thymus has remained controversial. All accounts concur on the high expression levels *of Aire* within mTECs, and nearly all on the presence of detectable transcript in secondary lymphoid organs and/or spermatogonia [2,46–48]. But whether such extrathymic transcripts are translated into functional protein, the identity of peripheral *Aire*+ cells, and the role and physiologic relevance of extrathymic *Aire* remain contentious. *Aire* transcript and protein are reportedly expressed by spermatogonia and spermatocytes, and loss of *Aire* in these cells appears to correlate with T-cell independent deficiencies in scheduled and sporadic apoptosis [46]. These defects are particularly interesting given recent reports of AIRE acting as a pro-apoptotic factor in mTECs [39].

In secondary lymphoid organs of mice and humans, *Aire* transcript has been detected in monocyte/dendritic cell lineages [47,48], and a cell-intrinsic effect on BAFF production from dendritic cells, independent of thymic *Aire* expression, has been reported [49]. Recent evidence also suggests that *Aire* is expressed in the lymphoid stroma. Lee *et al.* [50] and Gardner *et al.* [51] both reported detection of *Aire* transcript among CD45-negative lymphoid stroma, and AIRE protein has also been shown in nuclear bodies in secondary lymphoid organs of mice [51] and humans [52]; however, there are some conflicting data on this [42].

Recent evidence now suggests that the secondary lymphoid organs may also be a site of promiscuous expression for tissue-specific antigens, though the nature and relevance of this phenomenon remains contentious. By analogy, however, the discovery of TSA expression in the thymus provides a useful historical precedent. When tissue-specific antigen promoters such as insulin and elastase I were first exploited to drive expression of exogenous T antigens in target tissues, a number of these antigens were, surprisingly, found to be “promiscuously” expressed in the thymus. [14,53]. These observations led a number of groups to suggest that such promiscuous gene expression might be an important property of thymic stroma, and might promote negative selection of antigen-specific T cells.

Suggestively, a number of recent reports have observed similar phenomena mediated by radioresistant cells of the secondary lymphoid organs. Lee *et al.* [50], using a transgenic system to express a truncated form of cytosolic OVA under the control of the intestinal fatty-acid binding protein promoter, found that OVA was promiscuously expressed by lymph node stroma, and that adoptive transfer of OVA-specific CD8 T cells induced rapid proliferation and subsequent deletion mediated by a radioresistant population. Similarly, Nichols *et al.* [54] showed that endogenous tyrosinase, a melanocyte-specific antigen, was expressed by radioresistant cells in secondary lymphoid organs, inducing rapid proliferation and subsequent deletion of adoptively transferred tyrosinase-specific CD8 T cells. Finally Gardner *et al.* [51], using a transgenic *Aire* reporter, identified a population of radioresistant cells in secondary lymphoid organs expressing nuclear AIRE protein and a host of AIRE-regulated TSAs. Transgenic self-antigen expression in these cells induced a similarly rapid proliferation and subsequent deletion of cognate CD8 T cells. Together, these results suggest the lymphoid stroma as a site of immunologically relevant expression of both *Aire* and TSAs.

Aire Expression in Secondary Lymphoid Organs: The Debate Continues

While our groups and others have generated evidence suggesting that extrathymic AIRE and lymphoid stroma may play a role in ectopic TSA expression and immune tolerance, our respective approaches have thus far identified distinct cell populations. Lee *et al.* [50] characterized a population of $UEA1^{+}$ $gp38^{+}$ MHC class II^{+} LNSCs which ectopically expressed transgenic antigen, resulting in deletion of cognate T cells. In the same study, LNSCs were shown to ectopically express a number of TSAs relevant to autoimmunity, including those

from the eye, thyroid, pancreas and central nervous system [50,55]. These cells, which upregulated PD-L1 to prevent CD8 T cell activation during chronic viral infection [56], are presumably part of the lymph node fibroblastic reticular cell (FRC) network. Far from a static scaffold, LN FRC form a matchmaking network for immune responses, expressing chemokines to attract and guide T cells and DC along processes rich in extracellular matrix, to meet in the paracortex [57,58]. They also deliver cytokines, chemokines and soluble antigen from lymph along specialized conduits to the rest of the organ [59]. Importantly, presentation of antigen via MHC on stromal cells in these studies was tolerogenic without contribution from dendritic cells. The mechanisms involved are under investigation, but certainly a blockade of PD-L1 rescued autoreactive $CD8^+$ T cells, which became activated and caused disease [60].

By comparison, a recent study by Gardner *et al.* [51] using an *Aire* reporter construct showed expression of both *Aire* transcript and nuclear AIRE protein in a unique population of tolerogenic stromal cells, termed eTACs (extra-thymic *Aire*-expressing cells). These cells, which express the epithelial marker EpCAM, are UEA1- and gp38-negative and phenotypically distinct from FRC populations. While expressing some markers reminiscent of mTECs (MHC class II, EpCAM), these cells appear to lack expression of canonical costimulatory molecules CD80 and CD86. This study also described weak expression of the *Aire* reporter in a subset of CD11c+ dendritic cells, though AIRE protein was not specifically detected in this population, and deletional tolerance of cognate T cells did not depend on it. As in the thymus, comparison of eTACs from WT and *Aire*-knockout mice demonstrated that *Aire* regulates a set of TSAs that includes several important autoantigens, as well as genes important in antigen processing and presentation, suggesting that *Aire* expression has broad transcriptional consequences for TSA expression in the periphery. Surprisingly, the genes regulated by AIRE in eTACs had virtually no overlap with AIRE-regulated genes in the thymus, suggesting a complementary role in the maintenance of self-tolerance (Figure 1). In addition, this lack of overlap reinforces the idea that AIRE-regulation of transcription is complex and may vary between cell types because of changes in the array of transcriptional or epigenetic factors that differ between the two cell populations.

The relationship between these populations, and the physiologic relevance of extrathymic AIRE in a non-transgenic context, remain to be defined. The limited evidence available offers only a few clues. First, the immunologically relevant role of AIRE appears to be restricted to radioresistant populations [2]. Second, the fact that transplantation of *Aire*-deficient thymic stroma into Nude (*Foxn1* knockout) hosts is sufficient to induce *Aire*-like autoimmunity [2] suggests that peripheral *Aire* expression may not be sufficient to compensate for loss of thymic *Aire*. This is neither surprising nor inconsistent with the idea that central and peripheral AIRE may play unique and complementary roles, and that multiple overlapping systems are required to maintain normal self-tolerance. Furthermore, because we know nothing about the *Foxn1*-dependence of eTAC development, it is not clear whether the Nude mouse presents the best system for investigating these issues. Careful comparison of the relative spectra of autoimmune disease observed upon loss of central or peripheral *Aire*, particularly in autoimmune-prone strains, remains to be done. Such experiments are eagerly anticipated, though the proper experimental approach to test this has been challenging, moreso given the recent report by Guerau-de-Arellano and colleagues that the critical time for *Aire* expression may be early in perinatal development [61].

The study by Guerau-de-Arellano *et al.* also comments on *Aire* mRNA expression in secondary lymphoid stroma and its relevance [61]. Using a Tetracycline-responsive transgenic system designed to allow temporal control of *Aire* expression in an otherwise *Aire*-deficient mouse, the authors reported detecting transgene expression in lymph nodes and spleen, but argued that the absence of Tetracycline-responsiveness of this transgenic *Aire* in lymph nodes, despite the observed autoimmunity in these mice, suggests that peripheral *Aire* expression is not relevant

to disease progression. However, factors such as overexpression of the transgene, its imprecise tissue-specificity (which, for example, is also expressed in cortical epithelium), and the fact that splenic *Aire* levels do respond to Tetracycline administration, all cast doubt on the relevance of these conclusions to endogenous peripheral *Aire*. Ultimately, peripheral *Aire* must be assessed using experiments appropriate to the task.

Conclusions

The study of *Aire* continues to yield groundbreaking insights into the mechanisms of immunologic tolerance, though each step forward seems to raise as many questions as it answers. In the thymus, the relationship between deletion and generation of regulatory populations remains an active area of inquiry, and recent evidence that AIRE may also promote tolerance by means independent of TSA expression has raised important new questions. At the molecular level, recent work identified a role for AIRE in binding histones in a methylation-sensitive manner, suggesting an attractive mechanism by which AIRE may recognize and promote expression in otherwise transcriptionally inactive regions of the genome. Analysis of AIRE-regulated loci in single cells has shown that notions of broad epigenetic deregulation by AIRE are oversimplified, and that additional stochastic and regulatory mechanisms appear to be at play. Clearly, much exciting work remains to determine the mechanisms behind AIRE's role as a transcriptional regulator and mediator of self-tolerance.

In the periphery, the role of *Aire* remains incompletely understood, though increasing evidence suggests that secondary lymphoid stroma is the site of both ectopic TSA expression and *Aire* expression at transcript and protein levels. Growing evidence also suggests that a network of TSA-expressing stroma may play a role in the maintenance of immunologic tolerance, and that self-antigen expression in the periphery may complement self-antigen expression in the thymus, providing a safety net to eliminate autoreactive T cells that evade thymic negative selection. Further research is, of course, necessary to understand the relationship between peripheral AIRE and TSA expression, and between these identified populations, and the ultimate relevance of these phenomena. We look forward to the coming research into the respective roles and relevance of these populations in preventing autoimmunity, and anticipate rapid progress in this emerging field.

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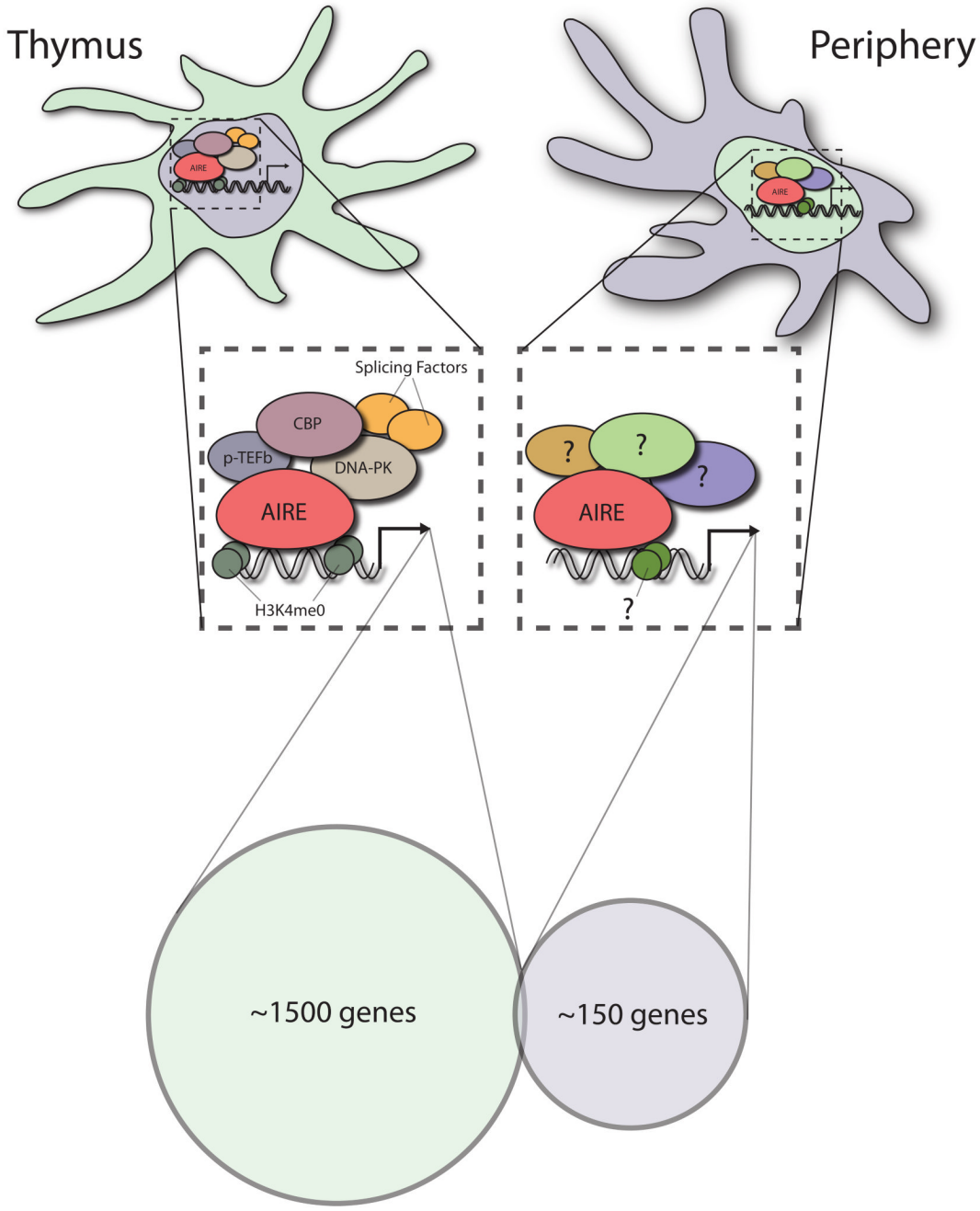

Figure 1. Transcriptional activity of AIRE in mTEC's and eTAC's

Shown are two of the major known *Aire*-expressing cell populations in the thymus (mTEC) and the periphery (eTAC). Recent work has demonstrated that AIRE promotes the transcription of an array of a complementary set of genes in the two cell populations. The number of *Aire*-induced genes in the periphery also appears to be smaller and may reflect a lower level of *Aire* expression within this cell population. As outlined in the text, AIRE has been shown to bind to and interact with a number of proteins that are involved in transcription, including CBP, P-TEFb, and DNA-PK. AIRE localizes intracellulary in mTEC's to nuclear speckles which are enriched for a variety of splicing factors that may also participate in its regulation of transcription. The PHD1 domain of AIRE has been shown to have specificity for the H3K4me0

mark on chromatin suggesting a mechanism by which AIRE may target repressed or inactive genes. Taken together, a picture is emerging in which AIRE may promote transcription through epigenetic mechanisms and a collaboration with a variety of co-factors. The exact identity and contribution of these interactions to the regulation of AIRE-dependent transcription in eTAC's and mTEC's remains to be determined.