Aire

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Abstract

Mutations in the transcriptional regulator, Aire, cause APECED, a polyglandular autoimmune disease with monogenic transmission. Animal models of APECED have revealed that Aire plays an important role in T cell tolerance induction in the thymus, mainly by promoting ectopic expression of a large repertoire of transcripts encoding proteins normally restricted to differentiated organs residing in the periphery. The absence of Aire results in impaired clonal deletion of self-reactive thymocytes, which escape into the periphery and attack a variety of organs. In addition, Aire is a proapoptotic factor, expressed at the final maturation stage of thymic medullary epithelial cells, a function that may promote cross-presentation of the antigens encoded by Aire-induced transcripts in these cells. Transcriptional regulation by Aire is unusual in being very broad, context-dependent, probabilistic, and noisy. Structure/function analyses and identification of its interaction partners suggest that Aire may impact transcription at several levels, including nucleosome displacement during elongation and transcript splicing or other aspects of maturation.

For decades, immunological tolerance has been one of the favorite enigmas of immunologists. How does the immune system exercise its primary function of dispensing with microbial invaders while remaining inert to the body's own constituents? The short answer is that the immune system—more specifically, the lymphocyte component of the adaptive immune system—is rendered tolerant to the latter but not to the former.

Over the years, it has become apparent that an intricate network of mechanisms establishes and maintains lymphocyte tolerance (reviewed in 1–3). These mechanisms have classically been grouped into two broad categories: central and peripheral. Central tolerance relates to immature lymphocytes as they differentiate in the primary lymphoid organs, the thymus for T cells and the fetal liver or bone marrow for B cells. Hence, the relevant antigens would be those synthesized specifically by stromal cells in these organs, specifically by hematopoietic cells that circulate through them or, ubiquitously, by all cells. The major mechanisms in operation in central tolerance are clonal deletion, inactivation, and diversion of self-reactive lymphocytes. Peripheral tolerance, on the other hand, concerns mature lymphocytes after they have exited the primary lymphoid organs and are circulating through the blood, lymph, and secondary lymphoid organs or are percolating through parenchymal tissues. Relevant antigens would be tissular substances not encountered previously, during lymphocyte differentiation. Clonal deletion, anergy, and diversion are operative in peripheral tolerance as well, but a variety of other mechanisms also come into play, including clonal ignorance and suppression of self-reactive lymphocytes.

Thus, it seems that evolution has furnished organisms with a comprehensive net of protection against potentially destructive self-directed—or autoimmune—responses. However, this impression has derived largely from results on genetically engineered mouse models. One is forced to ask how these different mechanisms integrate to enforce tolerance in unmanipulated mice and humans: Which of the diverse tolerization modes dominate? Which are experimental anomalies? To what extent are they interdependent or redundant? How do their roles vary with genetics, environment, or developmental stage? One approach to addressing such questions concerning how a tolerant state is achieved or maintained is to explore how it is lost, i.e., in contexts of autoimmunity. In this regard, the multiorgan autoimmune disease autoimmune polyendocrinopathy-candidiases-ectodermal dystrophy (APECED) has yielded important insights of late (reviewed in 4).

APECED REVEALS AIRE

APECED is a rare but devastating primary immunodeficiency disease. Classified as a "Disease of Immune Dysregulation" (Type IV PID) (5), APECED is characterized by a set of three abnormal features—chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency (6)—a triad first reported in 1946 (7). Classically, an individual must present with at least two of these three clinical abnormalities to be diagnosed with APECED, which usually occurs before the age of ten. However, more recently, identification of the causal genetic lesion has permitted extension of the diagnosis to patients with atypical presentations—for example, with one of the hallmark features in isolation (8–12). Most patients also routinely exhibit a variable number of other autoimmune manifestations, including thyroiditis, type 1 diabetes (T1D), ovarian failure, alopecia, or hepatitis. These secondary features differ widely from individual to individual, even between siblings with exactly the same genetic lesion and similar environmental exposures.

APECED has unusually simple genetics for an autoimmune disease (13). Almost always exhibiting an autosomal recessive mode of inheritance, it is generally considered to be a monogenic disorder, in striking contrast to most other autoimmune diseases, with their often knotty genetic influences. However, more recent analyses have revealed effects of additional genetic loci, in particular the human leukocyte antigen (HLA) complex, on certain

disease manifestations (e.g., 14, 15). APECED is a rare disorder, although there are pockets of elevated frequency in Finland, Sardinia, and Iran, where it can afflict as many as 1 in 10,000 people, probably reflecting particular founder mutations (16). Family studies permitted localization of the underlying gene to the q22 region of chromosome 21 (17).

A major leap forward in our understanding of APECED came in 1997 when two groups used heroic positional strategies to clone the responsible gene (18, 19). The protein encoded at this locus was termed Aire for autoimmune regulator. As detailed below (see section entitled Molecular Mechanisms of Aire), this 545 amino acid protein has several structural domains reminiscent of those found in known transcription factors, prompting the speculation that it is some sort of transcriptional regulator. Over 60 mutations have by now been localized in the *AIRE* genes of different APECED patients. Given their scatter throughout the protein sequence, they have not so far provided many insights into its function; in addition, save for one possible exception (8, 20), the different mutations have not to date been convincingly associated with particular disease manifestations. Just after Aire was identified, there was significant controversy over its pattern of expression, with some groups claiming a very broad organ distribution (18) and others a quite restricted localization to lymphoid organs, in particular the thymus (19, 21). This discrepancy almost certainly reflected differences in reagent specificity and technique reliability, and eventually resolved in favor of the latter view (22).

This new information on structure and expression of Aire prompted a number of early hypotheses on how it might operate to control autoimmunity: by driving the organization of thymic stroma (23); by somehow controlling thymocyte tolerization (19); by regulating peripheral B and T cell responses to antigenic stimuli (18); by provoking apoptosis of parenchymal cells and thereby enhancing cross-presentation of their antigens (24); or by promoting the differentiation of $CD4^+Foxp3^+$ regulatory T cells (Tregs) (24). Given the impossibility of rigorously evaluating such hypotheses in humans, investigators rapidly cloned the mouse equivalent of the *AIRE* gene, termed *Aire*, to develop an experimental model of APECED (25).

AIRE'S ROLE IN THE THYMUS

The Major Mechanism: Promotion of Clonal Deletion of Self-Reactive Thymocytes

Studies on mice have permitted extensive mechanistic dissection of how Aire operates to control autoimmunity. First, the ready availability of tissues and appropriate histological and cell-sorting reagents allowed a clearer delineation of just where it is expressed: primarily in lymphoid organs, above all in the thymus; and within the thymus, primarily in medullary epithelial cells (MECs) and secondarily in dendritic cells (DCs) (26–28). The location in MECs was particularly intriguing (*a*) because of suggestions that this cell type is involved in negative selection of mature $CD4^+8^-$ and $CD4^-8^+$ self-reactive thymocytes (29), and (*b*) because of the coincident emergence of a body of data establishing that transcripts encoding a diversity of peripheral-tissue antigens (PTAs) are ectopically expressed specifically in MECs (reviewed in 30). Thus, the hypothesis arose that Aire regulates the thymic expression and presentation of PTAs and thereby controls thymocyte tolerization and consequently autoimmunity.

This hypothesis could be directly evaluated in Aire-knockout (KO) mice (26, 31, 32). These animals have a rather normal immune system but are afflicted with multiorgan autoimmunity, manifested as both inflammatory infiltrates and serum autoantibodies. Although initial descriptions of the disease in Aire-KO mice on a mixed C57Bl/6(B6)X129 genetic background indicated that it is relatively mild, broader organ implication and greater severity were noted when the mutation was crossed onto different mouse strains (References 32–34;

and see below, Two Disease Peculiarities). The autoimmune manifestations in the KO animals tracked with Aire's absence from thymic epithelial cells, as demonstrated in experiments employing either (*a*) radiation/bone marrow chimeras (disease partitioned with Aire deficiency in the radio-resistant rather than radiosensitive cells), or (*b*) thymus transfers (disease associated with an Aire-deficient thymus rather than periphery) (26, 32, 35). Purification of MECs from Aire-KO and Aire-wild-type (WT) littermates followed by gene-expression profiling revealed that Aire does indeed promote the expression of a battery of PTA gene transcripts (e.g., encoding insulin, salivary protein 1, and fatty acid–binding protein); interestingly, there were also a number of PTA transcripts (e.g., encoding C-reactive protein and GAD67) whose expression appeared to be Aire independent, an observation later substantiated on a broader scale (36). Aire also regulates the expression of a range of non-PTA genes in MECs, either positively or negatively (37, 38). The significance of Aire control of PTA expression for the autoimmune manifestations in Aire-KO mice has been confirmed by linking loss of this control with development of particular T cell and autoantibody specificities, specifically for the eye (39) and stomach (40).

It remained to establish how Aire control of PTA expression in thymic MECs was translated into an effect on immunological tolerance. Through crossing of the Aire-KO mutation into T cell receptor (TCR)/neo-self-antigen double-transgenic mouse systems, investigators could demonstrate that, in the absence of Aire, self-reactive thymocytes escape the usual clonal deletion that keeps them from emerging into the periphery, resulting in some, but not all, cases in rapid and severe autoimmune disease (27, 35, 41). Interestingly, the *Aire* mutation in heterozygous state also provoked autoimmunity, though it was less aggressive than the disease of homozygous mutants.

Although these studies clearly linked Aire control of PTA expression to clonal deletion, several reports have emerged describing situations in which a lack of Aire compromised tolerance induction without affecting expression of the corresponding PTA (27, 32, 34). Thus, there is now considerable interest in defining roles for Aire in tolerance induction beyond the control of PTA transcript levels in MECs. Evidence has been reported for an effect on the presentation of antigens by MECs (27). Although such a deficiency would certainly be consistent with observations that transcripts of a number of genes involved in antigen processing and presentation are also regulated by Aire (27, 37, 38), so far the actual molecular defect remains undefined. In particular, it does not appear to reflect lower cell-surface display of major histocompatibility complex (MHC) or costimulatory molecules (27). The recent observation that Aire expression rapidly induces cells to die (42) has prompted the hypothesis that at least some of its influence on antigen presentation may reflect cross-presentation of Aire-induced apoptotic bodies (42), in line with the finding that thymic hematopoietic cells extend the range of clonal deletion by cross-presenting MEC-derived antigens (43).

Our current view of Aire's major function in the thymus is schematized in **Figure 1**.

An Additional Function in the Selection of Regulatory T Cells?

As argued above, Aire clearly operates through induction of T cell tolerance in the thymus. A number of investigators have been uncomfortable with this notion, raising the question of how a thymic stromal cell population as rare as MECs could possibly purge the entire emerging T cell repertoire of self-reactive specificities. This issue is exacerbated by recent reports, based on polymerase chain reaction (PCR) analyses of individual MECs, that a given PTA transcript is expressed by only a subset of MECs (44, 45). Nonetheless, such skepticism might be considered ill-founded because fully mature thymocytes spend almost two weeks in the medulla (46, 47); thymocytes are extremely motile (48); and the T cell repertoire can be effectively cleansed by as few as a hundred dendritic or other hematopoietic cells (49, 50). An

alternative or additional possibility is that Aire promotes positive selection of Tregs by MECs, on the basis of the same set or a subset of PTAs. Indeed, there are precedents that peptides expressed by thymic epithelial cells can drive the selection of Tregs (51, 52).

Aire does not seem to influence the Treg compartment at a global level. Numbers of $CD4^+Foxp3^+$ Tregs in Aire-KO mice are normal, and these cells are normally active in standard in vitro suppression assays (27, 32, 35, 53). In contrast, one group has reported a lower proportion of circulating $CD4^+Foxp3^+$ cells in some established APECED patients (53). However, it is important to solidify this contention by analyzing patients at an early stage of disease to rule out downstream effects of other immune system abnormalities or of any therapy received. For example, the observed reduction in Tregs might be secondary to the chronic fungal infection and autoimmune inflammation in these individuals, e.g., by their sequestration in inflamed sites, as has been observed in the HIV context (54). Another argument against a global defect in Tregs in Aire-deficient individuals stems from the very different nature and aggressivity of the autoimmune phenotypes in IPEX (immune dysregulation-polyendocrinopathy-enteropathy-X-linked inheritance) and APECED patients, or in *scurfy*- and Aire-KO mice. In addition, there is a clear synergy between mutations in Foxp3 and Aire: The disease in double-deficient animals was substantially worse than that of either single mutant (55), indicating that Aire can still have an impact when Tregs have been removed from the equation. Evidence from Aire-WT/Aire-KO dual-thymus transfer experiments also argues against primary perturbations of dominant tolerance. If the autoimmune disease imparted by maturation of T cells in an Aire-deficient thymus was due to defective Tregs, it should have been prevented by the regulatory cells generated by the cografted WT thymus, and this was not the case (27, 32).

The issue of Aire influences on Tregs was also examined at the level of individual self-reactive TCR specificities, with pairs of transgenes encoding a TCR and its cognate antigen crossed onto Aire-negative or Aire-positive backgrounds. Results from several systems demonstrated that clonal deletion was affected by the absence of Aire, but that there was no significant effect on the numbers of $Foxp3^+$ Tregs generated (27, 35, 41).

To address this issue in a different manner, Aschenbrenner et al. (56) generated a transgenic mouse line in which expression of an epitope from influenza hemagglutinin (HA) was directed to Aire-positive thymic MECs, relying on a transgene whose expression was driven by the *Aire* promoter/enhancer, and coupled it with an anti-HA TCR-transgenic line that can generate a robust population of Tregs in the presence of thymically expressed HA (52). They

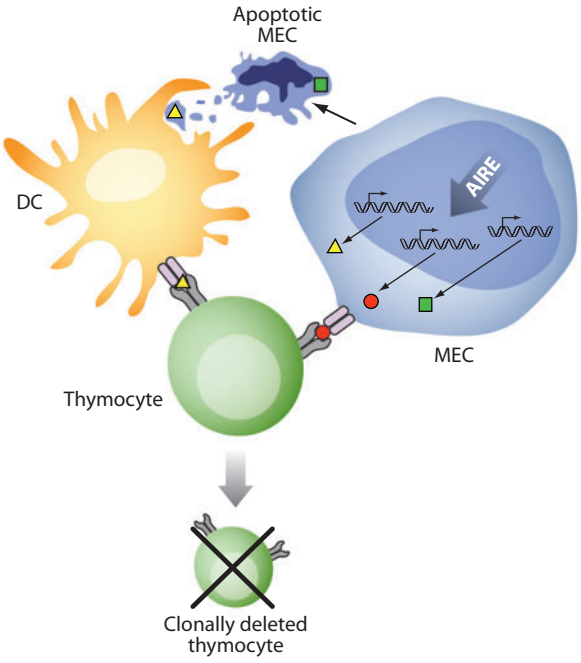

Figure 1

Aire promotes clonal deletion of self-reactive thymocytes. Aire induces MEC expression of a broad repertoire of peripheral tissue antigens (PTAs), which are processed and then presented on surface-displayed MHC/HLA molecules. Soon after the induction of Aire and PTAs, MECs die by apoptosis. Mature thymocytes percolate through the medulla, and, if their TCRs recognize an MHC:PTA complex in the appropriate affinity/avidity window, they will be overactivated and deleted from the repertoire. Thymocytes can recognize MHC:PTA complexes directly on MECs or indirectly on DCs that have engulfed apoptotic MECs or MEC fragments.

observed an augmentation in the representation of Tregs recognizing the HA epitope. Although this study showed that Aire-positive MECs can select Tregs, it fell short of demonstrating that either Aire or Aire-positive MECs have a critical function in molding the Treg repertoire because it failed to establish that the ability of Aire-positive MECs to select Tregs actually depends on Aire and that Aire-positive MECs are required for selection. Indeed, many cell types, for example thymic cortical epithelial cells, can promote the emergence of Tregs in the HA system (51, 52).

It would seem, then, that there has not yet been clear demonstration of an important effect of Aire on the selection of Tregs in the thymus, nor of a direct influence on the peripheral Treg compartment (free of any confounding autoimmunity, immunodeficiency, infection, or drug treatments). Nonetheless, because Aire does control PTA expression in thymic MECs, it remains an attractive idea that it might also drive clonal deviation of certain thymocytes into the Treg lineage, rather than their clonal deletion, depending, for example, on the affinity/avidity of the TCR-MHC/PTA interaction.

Some Function in MEC Differentiation?

Farr and coworkers (57, 58) have argued that Aire plays a role in the differentiation of thymic MECs. In their progressive restriction model, Aire and PTA expression are properties of immature MEC precursors, and turning *Aire* on drives differentiation of MECs into progressively restricted epithelial cell fates, with individual cells taking on different fates. The initial impetus for suggesting such a scenario was microscopic evidence of intriguing epithelial organoids in the thymus, e.g., structures resembling thyroid follicles (57). Alternatively, other investigators have championed a terminal differentiation model whereby increasingly broad PTA expression accompanies MEC maturation from the Aire^{-}MHC-II^{lo}CD80^{lo} to the Aire^{+}MHC-II^{hi}CD80^{hi} phenotype, and Aire does not serve a differentiative function (30). An early argument in favor of this scenario was that a more diverse repertoire of PTAs was expressed by what was assumed to be the more mature MEC subsets (36).

At present, the terminal differentiation model is generally considered to be the more accurate representation of MEC differentiation on the basis of results from several recent studies that weighed the major distinguishing features between the two models. First, it is now very clear that Aire-positive MECs represent a late stage of maturation—not only do MHC-II^{lo} and/or B7^{lo} cells give rise to them (42, 59–61), but also they are a postmitotic, terminal population (42, 61). Second, single-cell PCR analyses of PTA expression in individual MECs failed to reveal preferential coexpression of transcripts characteristic of particular epithelial lineages—rather, transcripts were expressed in a probabilistic fashion, and often monoallelically (References 44, 45; and see further discussion in Molecular Mechanisms of Aire). In addition, MEC expression of PTAs depended on transcription factors and transcriptional start sites different from those employed in the relevant peripheral cells (45).

AIRE'S ROLE IN THE PERIPHERY

Although Aire is primarily expressed in the thymus, *AIRE/Aire* gene transcripts have also been detected in peripheral tissues, in particular the peripheral lymphoid organs (e.g., 21, 26, 62–64). This finding has provoked speculation about an extrathymic role for Aire, either in antigen presentation or, more specifically, in peripheral-tolerance induction.

Peripheral Hematopoietic Cells

RNA transcripts encoding Aire have been found in monocytes and in an array of DC types (either ex vivo or derived in culture) from both mice and humans (26, 62, 64). Several lines of evidence support the argument that Aire in murine DCs is unlikely to contribute much to tolerance induction. First, although *Aire* gene expression was detectable with very sensitive

PCR assays, signals were very low in DCs compared with in MECs: Transcripts were reduced by a factor of 10 or more according to quantitative analyses, and protein was undetectable by flow cytometry using a sensitive and specific intracellular staining technique that gives a clear signal in MECs (26, 28; D. Gray, C. Benoist, and D. Mathis, unpublished results). In addition, Aire expression in mouse DCs seems to be of little consequence as far as PTA expression is concerned: There were minimal differences in the gene-expression profiles of Aire-WT and Aire-KO mice (65; E. Venanzi, C. Benoist, and D. Mathis, unpublished data), and transcripts that had a strong Aire dependence in MECs did not in DCs. Most directly, experiments on radiation/bone marrow chimeras demonstrated that the autoimmune manifestation characteristics of Aire-KO mice developed independently of an Aire defect in cells of the radio-sensitive hematopoietic lineage, instead partitioning with Aire-deficient radio-resistant stroma (26, 32, 35). There have been a few reports of alterations in the antigen-presenting capabilities of DCs or macrophages from Aire-KO mice—albeit, paradoxically, as an enhanced effectiveness (65, 66). However, data from other investigators were not able to confirm this conclusion (27).

On the other hand, the root of APECED patients' striking susceptibility to *Candida* infection, a disease hallmark, remains curiously obscure. Investigators have reported alterations in the antigen-presenting capabilities and transcriptional programs of DCs derived from APECED patients versus healthy controls, or of monocytes transfected or not with *AIRE* (67, 68), supporting the argument that Aire promotes human DC maturation or function. Again, it will be important to rule out any effects of confounding factors like those mentioned above (in the section entitled An Additional Function in the Selection of Regulatory T Cells?) before embracing the significance of these divergences, but they do have the potential of explaining the increased susceptibility of APECED patients to fungal infections. And, again, contradictory data, providing no evidence for a defect in antigen presentation, have been published (69).

Putting together the two sets of data, admittedly derived from two different species, we think it possible that Aire expression in peripheral DCs can affect the presentation of antigens in the periphery, especially under inflammatory conditions, and thereby can influence susceptibility to fungal infections. But so far, Aire appears to be of little consequence for the establishment and maintenance of tolerance to self.

Peripheral Stromal Cells

Two recent papers on the expression of Aire and PTA transcripts in the stromal cells of peripheral lymphoid organs have excited great interest. Lee et al. (70) described a population of lymph node stromal cells expressing a repertoire of PTA transcripts that overlaps quite a bit, but not perfectly, with that of thymic MECs. These stromal cells could directly present a transgenically targeted antigen to T cells, activating and eventually deleting them. Although the stromal cells also expressed low levels of *Aire* gene transcripts, the level of Aire protein was not assessed, nor was the Aire-dependence of endogenous-PTA expression. On the other hand, Gardner et al. (71) found PTA transcripts in stromal cells residing in both the lymph nodes and spleen. At least some of these cells also expressed the *Aire* gene at the protein level: Indeed, they were originally noticed as cells expressing a green fluorescent protein reporter under the dictates of *Aire* transcriptional control elements. The repertoire of PTA transcripts made by these stromal cells appeared to be of limited diversity and seemed rather distinct from that of thymic MECs. An antigen transgenically targeted to the peripheral stromal cells could entice T cells to make sustained contact and provoke full-blown activation followed by death.

Although they are potentially of substantial interest, it is important to keep in mind what these studies do and do not demonstrate. They do show that stromal cells in the peripheral

lymphoid organs have the capacity to present transgene-encoded antigens directly to T cells, provoking activation-induced cell death. They do not, however, establish that the endogenously encoded antigens ectopically expressed in these cells, often at lower levels, have the same capacities. Nor do they provide any information on how important this proposed mechanism really is in maintaining tolerance. In this regard, it should be kept in mind that thymus transplant experiments by multiple groups have provided no evidence for an important role for Aire-positive peripheral cells in warding off autoimmunity (26, 32, 35). Lastly, it is important to understand why the cells identified in the two studies appear to be so different: in the lymph nodes only versus in all peripheral lymphoid organs; UEA-1$^+$ERTR-7$^+$gp38$^+$ versus negative for all of these markers.

Peripheral Autoimmune Effector Mechanisms

Information on just how defective tolerance induction in mice lacking Aire translates into peripheral autoimmune disease is beginning to emerge. DeVoss et al. (72) found that Aire-KO mice with an additional genetic deficiency in T cells were devoid of autoimmune manifestations. The CD4$^+$, but not CD8$^+$, T cell compartment was critical for disease development. The CD4$^+$ T cells of Aire-KO mice were skewed to the T helper (Th) 1 phenotype, and Th1 cytokines were implicated in pathology. This group found minimal evidence for a role for B cells, but contradictory findings were reported by Gavanescu et al. (73), who found autoimmune pathology greatly muted in the absence of B cells, as well as a significant improvement in disease parameters after treatment with anti-CD20 monoclonal antibody. The most likely explanation for the divergent results is that the B cell–deficiency mutation relied on by DeVoss and colleagues ($\mu MT^{-/-}$) is leaky, with residual B cell numbers and antibody titers varying according to the particular genetic background and mouse colony (74). In neither study did autoantibodies appear to be directly pathogenic; rather, the impact of B cells was at the level of T cell priming and expansion. In both studies, however, the readouts for an effect of transferred autoantibodies on disease development were confined to an examination of organ infiltrates. Other influences remain possible, perhaps akin to the recently reported dampening of monocyte (and monocyte-derived DC) expression of genes responsive to type I interferons (IFNs) by the anti-IFN antibodies present at high titers in the serum of most APECED patients (75, 76).

Definition of the peripheral autoimmune effector mechanisms in Aire-KO mice has potential implications for the treatment of APECED patients. It is hoped that the demonstrated therapeutic effects of anti-CD4 (72) or anti-CD20 (73) monoclonal antibodies on the progression of multiorgan autoimmune disease in mice can be translated to patients. This would represent an important advance, especially given the limited treatment options available at the present time.

AIRE IN THE REPRODUCTIVE ORGANS

The testis and ovary stood out as parenchymal tissues with readily detectable *Aire* gene transcripts (26), and investigators have found Aire protein as well, specifically in spermatogonia and spermocytes (142). This is an intriguing finding because there has been a long history of the testis spuriously expressing transgene constructs. Might Aire promote promiscuous expression of batteries of proteins in the reproductive organs as well, and, if so, what might be their function? Mice lacking Aire are often infertile (26, 31), but this state likely reflects autoimmune attack on these organs. Schaller et al. (142) recently demonstrated that Aire-KO mice have perturbations in the waves of scheduled and sporadic germ cell apoptosis that normally take place during murine spermatogenesis. Given that such death is thought to eliminate cells with damaged DNA, this observation suggests that Aire might function somehow in enforcing germ-line stability. If this does prove to be an important function, one will be led to question whether Aire was "borrowed" from the reproductive system by the immune system during evolution, or vice versa.

TWO DISEASE PECULIARITIES

Target-Organ Heterogeneity

An intriguing feature of APECED has always been the heterogeneity of clinical manifestations in different patients, even in cohabitating siblings with exactly the same genetic lesion. Three explanations come to mind. First, even though APECED is classified as a monogenic disorder, there may be disease-modifying genes that alter target-organ specificity. Second, environmental factors may impact disease manifestations. And, third, stochastic elements may come into play, an obvious example being the randomly generated T and B cell repertoires: A particular organ may not be targeted in a given individual simply because he/she did not generate high-affinity T or B cells that recognize the relevant antigens. Given the small number of patients, distinguishing between these different explanations in the APECED context is not currently feasible. Fortunately, Aire-KO mice allow direct experimental evaluation of the different possibilities.

Backcrossing of the Aire-null mutation onto different genetic backgrounds revealed important genetic influences on the organs targeted by autoimmunity (32–34). For example, B6-KO mice developed a relatively mild autoimmune disorder, their Balb/c counterparts showed a predominant gastritis, and nonobese diabetic (NOD)-KO mice had very severe disease, dominated by pancreatitis. Interestingly, different genetic loci controlled targeting of different organs. Some of the disease-modifying loci mapped to the MHC, whereas others did not. Among the latter were genes within genetic intervals (*idd3*, *idd5*) that control susceptibility to T1D in NOD mice. In contrast, there has been essentially no evidence of environmental influences on the organs targeted by the autoimmunity of Aire-KO mice. Neither a battery of innate immune system stimulants, nor a defect in a critical element of the majority of Toll-like receptor pathways (MyD88), nor germ-free conditions could augment or diminish disease on the B6 or NOD background, respectively (77).

Experiments assessing the effects of stochastic elements like the TCR or autoantibody repertoires on the Aire-KO autoimmune disease have not yet been reported but promise to be of great interest.

Target-Organ Choice

Aire controls the expression of thousands of PTA transcripts in the thymus. Yet the autoimmunity that develops in Aire-deficient mice or humans appears circumscribed, targeting a limited number of organs and a restricted set of antigens within each organ. Why is disease not more rampant? Part of the explanation must lie in the fact that autoimmunity, like any immune response, is HLA/MHC-restricted, so that not every self-antigen can be presented to the potentially self-reactive T cell repertoire. Another factor that may confine the autoimmune attack is that peripheral mechanisms of T cell tolerance almost certainly keep many autoreactive T cells under control. However, surprisingly few additional organs were targeted when mutations in Aire and Foxp3 were combined (55), leaving open the possibility that regulatory cells other than Foxp3$^+$ Tregs play a dominant role. Lastly, one is led to consider the importance of B cell tolerance, which may provide an additional filter for which autoimmune responses are ultimately fruitful.

AIRE AND OTHER AUTOIMMUNE DISEASES

The multiorgan autoimmune manifestations of Aire-deficient APECED patients, and the particular target organs involved, raise the possibility that genetic variation in the *AIRE* locus might also play a role in more common organ-specific autoimmune diseases, such as T1D, thyroiditis, etc. First, full loss-of-function mutations, in the heterozygous state, might result in inefficient presentation of self-antigens in the thymus and borderline tolerance, a state that might favor the development of certain of the organ-specific autoimmune diseases with a different etiology. Indeed, heterozygote effects have been documented in both humans

and mice (27, 41, 78). Alternatively, sequence polymorphisms affecting the coding region of *AIRE* or its expression, changes too subtle to elicit full APECED on their own, might generally augment the propensity to develop autoimmune disease. Relevant in this context is an Italian APECED family with a high prevalence of autoimmune thyroiditis, thyroid manifestations cosegregating with a heterozygous, apparently dominant-negative, mutation of *AIRE* (8, 20).

Initial attempts to address this issue provided very limited evidence that genetic variation in *AIRE* has a significant impact on more common autoimmune diseases. In a large study of individuals presenting with Addison's disease, T1D, or autoimmune thyroiditis, Meyer et al. (79) searched for an overrepresentation of heterozygous carriers of two of the most common *AIRE* mutations (a 13–base pair deletion in exon 8, the R257X nonsense mutation). Neither mutation was overrepresented in cases relative to controls. Other investigators have looked for an association between autoimmune diseases and particular *AIRE* haplotypes, using tagging single nucleotide polymorphisms to distinguish variants segregating through the population. No significant association was observed with vitiligo (80), Addison's disease (81), or T1D (82). On the other hand, the situation with two autoimmune diseases, both skin disorders (alopecia and vitiligo), remains cloudy, with some studies arguing for a genetic association with certain *AIRE* alleles (73, 76, 83) and others against (80, 84). Divergent results such as these usually come from underpowered analyses and/or dissimilar test populations. Furthermore, the *AIRE* chromosomal region has not scored positively in any of the recent genome-wide association studies. Thus, if there is a contribution of *AIRE* variation to the genesis of common autoimmune diseases, it likely represents fairly rare cases, perhaps with family-specific mutations. This scenario awaits testing in large-scale resequencing studies.

Although genetic analyses have not so far provided very strong evidence of an association of most organ-specific autoimmune disorders with particular *AIRE* haplotypes, there are data arguing that a breakdown in other elements of the pathway of Aire-mediated tolerance induction may be involved in certain of these diseases. After the HLA complex, the second human T1D susceptibility locus to be identified was the *INS* gene, which encodes insulin. The number of tandem repeats (VNTRs) in the *INS* promoter region is variable in different individuals, and these polymorphisms have been associated both with levels of *INS* transcripts in the thymus and with diabetes incidence: The greater the number of VNTRs, the more thymic transcripts, and the less diabetes (85–87). Although these studies were only correlative, experiments on *INS*-transgenic mouse models have confirmed the validity of the overall conclusions (88).

Similarly, Aire, in conjunction with interferon regulatory factor 8 (IRF8), controls MEC expression of the *CHRNA1* gene, which encodes the α-subunit of the muscle acetylcholine receptor, implicated as an antigen in myasthenia gravis (89). *CHRNA1* has a biallelic functional variant in the promoter region that is associated with early onset of myasthenia gravis.

HUMAN VERSUS MURINE AIRE

Certain investigators have speculated that human Aire may serve a function different from, or in addition to, that of its murine counterpart. The most frequently cited argument in support of this notion is that the initially described autoimmune disease manifested by Aire-KO mice on the B6x129 mixed genetic background was milder than that of APECED patients and involved a dissimilar spectrum of organs (26, 31). In addition, one strain of mice lacking Aire (B6 Aire-KO) was found to have a profile of autoantibodies different from that of Aire-defective humans (90). However, these arguments were substantially weakened when it was reported that the identity and number of organs subject to autoimmune attack in Aire-KO mice vary strikingly according to the genetic background, and autoimmune attack is very severe on certain backgrounds, notably the NOD, where it

provokes rapid wasting and death by 15 weeks of age (32–34). Actually, it is not unexpected that mice and humans would show a different spectrum of target organs and antigens—after all, like essentially all autoimmune disorders, the Aire-KO disease results from an immune response, and, like any immune response, it is subject to MHC/HLA restriction, implying that any two mouse strains or human patients will not necessarily be able to respond to a given autoantigen. Indeed, as already mentioned, genetic analyses in both mice (33) and humans (14, 15) have been able to document MHC/HLA effects on the spectrum of organs targeted.

Another reported difference between the two species is that some established APECED patients, but not Aire-KO mice on a single genetic background, have a defect in Tregs (53). But, as argued above (An Additional Function in the Selection of Regulatory T Cells?), it will be important to substantiate this difference in the absence of any complicating treatments or of the confounding *Candida* infection and at comparable levels of background autoimmunity. The *Candida* infection itself represents an interesting and striking divergence between humans and mice lacking Aire, but we do not yet know whether the apparent dissimilarity is a reflection of Aire functioning in a different manner in the two species or whether it relates more to differences in physiology (e.g., of the skin) or in the environment. Of course, infection by *Candida* should not occur in the specific-pathogen-free environment in which most experimental mice live, so there is currently no real information on whether Aire-KO animals are or are not more susceptible to this (or any) fungal infection. We therefore suggest that, although it remains a theoretical possibility that there are significant species-dependent disparities in Aire function, to date no inarguable evidence of such has been published.

MOLECULAR MECHANISMS OF AIRE

As outlined above, Aire affects key aspects of the induction of immunological tolerance to PTAs: their ectopic expression in the thymus; the efficiency of their presentation by MECs; and MEC apoptosis and turnover, which may serve to facilitate their cross-presentation. Aire has usually been thought of as a transcription factor (91), a function certainly compatible with such a broad range of activities. A number of other findings argue for a role for Aire in the regulation of transcription: It is located predominantly in the nucleus, typically in punctate structures reminiscent of, but distinct from, PML (promyelocytic leukemia) bodies (64, 92–95); its domain structure and organization are highly evocative of transcriptional regulators; it demonstrably associates with other transcription factors and can modulate the transcription of a variety of reporter and endogenous genes in cotransfection assays (94, 96–100); and chromatin immunoprecipitation experiments in transfected cells show it to be associated with the loci it transactivates (99). Thus, at first glance, Aire appears to be a classical transcription factor.

Modalities of Aire-Mediated Gene Regulation: A Conventional Transcription Factor?

However, we must take into account several unusual features of Aire's influence on gene expression when considering its molecular mode of operation:

- Recent bioinformatic reevaluations of the impact of Aire on MEC transcription have revealed that it influences the expression of even more genes than previously thought—several thousand rather than several hundred—representing a substantial fraction of the total genome (36; E. Venanzi, C. Benoist, and D. Mathis, unpublished results). By several metrics, this impact is 5–8 times broader than that of classic transcription factors such as Foxp3 or Runx. This breadth is difficult to reconcile with the notion that Aire operates by interacting with specific DNA sequences, as it is hard to imagine

that its binding site occurs in thousands of promoters of such disparate structure and cell-type specificity (unless the bulk of transcriptional effects are actually secondary, e.g., through the mediation of more restricted sets of transcription factors or via microRNAs).

- Aire-regulated genes are clustered genomically. Such an organization is not unusual, as certain transcription factors are able to bind to promoters in arrayed members of duplicated gene families (e.g., the CIITA and MHC class II genes) or to activate the transcription of a whole chromatin loop through a locus-control region. However, with Aire, induced, repressed, and unaffected loci are interspersed within a given cluster (36, 38).
- The transcriptional footprint of Aire varies profoundly with the cell type in which it is expressed. Comparative gene-expression profiles of Aire-positive and -negative MECs, of lymph node stromal cells, and of pancreatic islet β cells showed the same overall scope of Aire activity (a plethora of loci implicated, roughly twice as many activated as repressed), but there was only very limited overlap in the identity of the genes affected in the different contexts (71, 101, 102). The same observation has been made with transfected tissue culture cells (J. Abramson, A. Koh, C. Benoist, and D. Mathis, unpublished observations). Such patterns would not be expected from a sequence-specific transcription factor, which would generally tend to transactivate the same set of promoters carrying its recognition motif, irrespective of cell type.
- The expression of PTA genes in MECs is independent of transcriptional regulators essential for activity of the same loci in their home cells in the periphery (45). In addition, the transcriptional start sites used in MECs often differ from those employed in peripheral tissues. For instance, initiation of *Ins* gene transcripts in MECs utilizes some of the start sites found in pancreatic β cells, but also a novel one located 30.9 kb upstream (45).
- Aire's influence on ectopic expression of PTA genes in thymic MECs is not that of a simple on/off switch. Rather, its effect is usually only quantitative, with most PTAs expressed to some level in the absence of Aire. (Notably, the *Ins* gene is one of the rare exceptions, its ectopic expression appearing fully dependent on Aire.) And a number of PTA transcripts are not affected by Aire at all (26, 36, 38, 101).
- It has been suspected for some time that individual MECs express only a subset of the Aire-dependent PTA repertoire (103). Recent experiments exploiting single-cell PCR of isolated MECs have shown that the expression of a given PTA transcript is probabilistic (44, 45). In addition, monoallelic transcription was often observed, with only one of the two copies of a gene transcribed in any given cell. A monoallelic expression pattern again points to a stochastic determinism, as the probability of expression from each chromosome is independent of expression from the other [ruling out that stochastic PTA transcription reflects stochastic expression of the transcription factors controlling them, as posited by earlier models (104)]. One question left unanswered by these single-cell studies concerns the dynamic nature of Aire's activity: Are the stochastic expression patterns stable for a single MEC, or do the snapshot views we have so far obtained correspond to a slice in time, the expression of Aire target genes in a chromosome or cell fluctuating over time? However, the rapid cell death provoked by Aire expression (42) does tend to downplay this issue.
- Ectopic expression of PTAs in the thymus is noisy, and Aire accentuates this noise (101, 105). A comparison of

gene-expression profiles from MECs of individual mice (strictly inbred and gender-matched littermates) revealed significantly more interindividual variability in Aire-responsive PTA transcripts than in the bulk of Aire-neutral transcripts. In contrast, this variability was not true of Aire-responsive PTA transcripts in their home cells in the periphery. The degree of variation in MECs was not subtle, with up to a tenfold difference between extremes. Comparable interindividual variability in PTA expression was observed in the human thymus (105), although this finding is more difficult to interpret because of the unavoidable genetic, age, and environmental diversity in the donors. From a mechanistic standpoint, this noise implies that the stochastic nature of ectopic gene expression at the single-cell level somehow extends to the entire thymus, a surprising concept as one would have expected cell-to-cell variation to be statistically smoothed out over the totality of MECs. Nonetheless, this variability may also play an important part in the stochastic element of susceptibility to autoimmune diseases.

These various findings are not easily reconcilable with the view of Aire as a conventional, sequence-specific transcription factor. They support the argument that Aire does not exert domineering control on the genes whose expression it regulates, but instead accentuates or biases programs already in place.

Aire Structural and Functional Domains

Aire contains several structural domains found in other transcription factors, most of which are well conserved in Aire proteins across phyla (106). The presence of APECED-causing mutations in these domains underscores their functional significance (**Figure 2**).

- The SAND domain (also known as the KDWK domain) is a component of several chromatin-associated transcriptional modifiers that have a wide range of functions across species: the Sp100 proteins (107); the glucocorticoid-modulatory-element-binding protein (108) [GMEB-1 and -2; also known as Pif (109)]; the Ski modifier of Smad signaling (110); the *Drosophila DEAF1* gene product, involved in homeotic regulation, and its mammalian equivalent DEAF-1 (previously known as NURD), which is involved in developmental neural patterning through interactions with LMO4 (111). SAND domains are also found in transcriptional regulators in plants (112). The DNA-binding properties of the SAND domain, which have been analyzed at the structural level, are centered around the conserved KDWK core motif (108, 113). However, the DNA-binding properties and resultant specificity of Aire's SAND domain remain unclear. It has been difficult to obtain convincing evidence of particular target sequences because reported experiments

Figure 2

Domain structure of the Aire protein. Aire contains several identifiable domains, which have homology with domains of transcriptional control proteins from diverse phyla. The CARD (caspase-recruitment domain) overlaps with the dimerization region, once referred to as the homogeneously staining region (HSR) (although investigators must still determine whether all functions previously mapped to the HSR can be attributed to the CARD motif). The four LXXLL (where X is any amino acid) are shown as light gray bars. Red bars indicate the position of missense mutations in APECED patients (see **http://bioinf.uta.fi/AIRE**); the dominant-negative G228W mutation is in black.

have lacked certain of the controls required for validation or have yielded internally inconsistent results. Indeed, the SAND domain of Aire does not contain the canonical KDKW motif (108, 113). Interestingly, the DNA-binding specificity of SAND domains in other factors seems to be rather relaxed, with short recognition motifs and little influence from flanking residues or from the exact spacing within the motifs: Sp100 recognizes repeats of unmethylated CpG dinucleotides (107), and GMEB binds loosely spaced PuCGPy motifs (109). Perhaps not surprisingly, then, recent data indicate that Aire binds to DNA in a rather nonspecific fashion (100).

- Two plant homeodomain (PHD) fingers are also found in Aire. PHDs, members of the huge family of zinc-finger proteins, are thought to be restricted to the nucleus and include transcriptional coactivators and chromatin-modulating elements (114). They are phylogenetically widespread, found from plants to mammals, and are fairly common: Computational searches have identified PHD structures in more than 100 mammalian proteins. PHDs are related to, but different from, the zinc fingers with E3-ubiquitin ligase activity characteristic of the RING subfamily. Indeed, this similarity was the impetus for a series of experiments that revealed that Aire's PHD1 functions as an E3-ubiquitin ligase (115). However, the nuclear magnetic resonance solution structure of PHD1 and further functional assessments did not support this notion (116). In general, PHDs are considered to be primarily protein-protein interaction domains (114), which, when analyzed at the structural level or by mutagenesis, were seen to involve their loop2 region (114). Interestingly, this loop is precisely the site affected by several of the point mutations of APECED patients, highlighting its functional role (**Figure 3**). The functional relevance of Aire's PHD domains is well established from transfection studies (96, 99, 100, 115, 117), although the relative importance of the two PHDs diverged markedly between the studies, likely reflecting the different transcriptional targets and other experimental conditions used.

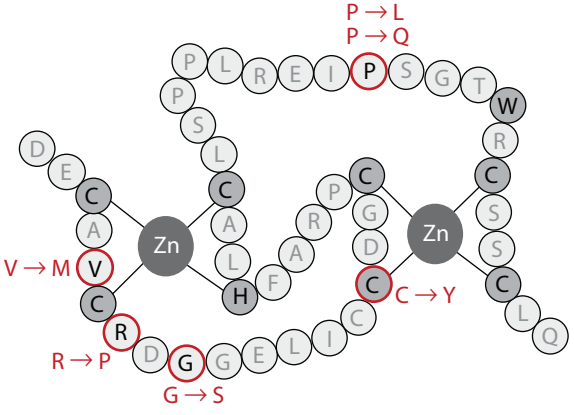

Figure 3

The PHD1 domain. The first of Aire's two PHDs (plant homeodomains), displayed in the typical structure of PHDs. Coordinating cysteines are in dark gray, and the positions of known APECED mutations are shown in red.

Certain of the interactions involving PHD domains occur between two transcription factors (118–120), but recently there have been several reports of liaisons with nucleosomal histones, in particular with their amino-terminal tails (121, 122). In some cases, PHDs bind methylated forms of histones, such as histone H3 trimethylated at lysine-4 (H3K4me3), one of the key marks associated with transcriptional activity. For example, PHDs in the proteins BPTF, Yng1, and ING2 have a marked preference for H3K4me3 (123–125). In other instances, PHDs specifically recognize the unmethylated form of the same histone stretch—for example, BHC80 binding to H3K4 is inhibited by methylation (126). In still other cases, the methylated and unmethylated histone forms are bound indiscriminately (127). Thus, PHD domains are important

in reading the histone code and thereby in targeting transcription factors to particular active or inactive regions of chromatin. Interestingly, Aire PHD1 binds the amino-terminal tail of H3, but only in its unmethylated K4m0 form; in contrast, binding tolerates modifications at K9 and K14 (99, 100). Such specificity is normally considered to be characteristic of repressive factors, serving to lock inactive chromatin in its silent state. Because its major function is to derepress genes that would normally be silent, the operational logic for Aire might be precisely the opposite: Binding of the PHD1 domain to H3K4m0 may help to concentrate Aire in regions of inactive chromatin, where it can then have an activating influence. As is true of other PHD/histone complexes, the binding of Aire PHD1 to H3K4m0 is of low affinity (in the 5–30 μM range). The fast off-rates implied by such affinities suggest dynamic interactions rather than a stable, long-term fixation.

- Aire contains four interspersed LXXLL sequences, a small motif found in many transcriptional coactivators, including CBP (CREB-binding protein) and STAT (signal transducers and activators of transcription) factors. This motif has been studied particularly well in the nuclear receptor family (128, 129), where it is thought to mediate protein-protein interactions. The importance and mode of action of the LXXLL sequences in Aire remain to be established.
- Finally, a CARD (caspase-recruitment domain) is found at the amino-terminus of Aire. This 100 amino acid stretch is the site of many APECED-causing mutations and was long referred to as a homogeneously staining region (HSR) domain. More recently, structure-based sequence analyses, which can detect similarities in the absence of primary sequence conservation, suggested that this region encompasses a CARD, as it harbors the key hydrophobic residues in proper spacing (130). This prediction was tested functionally, revealing that the point mutations most likely to disrupt proper folding of the CARD structure were those with the strongest impact on Aire's transactivation potential. Initially identified as key elements in the recruitment and activation of caspases during apoptosis, CARDs are now recognized to be involved in a wider variety of processes, many of them in the inflammatory realm, by mediating homo- or hetero-dimerization through homotypic interactions, as in the apoptosis cascade. Thus, Aire's CARD may participate in dimerization or in interactions with other transcriptional control proteins, perhaps CBP (130).

Aire's Partners

Under normal conditions, Aire is found predominantly in punctate nuclear bodies, where it is likely to partner with a number of other proteins. Indeed, such interactions can be detected by gel filtration of extracts from cultured cells, transfected Aire being incorporated into large complexes of >650 kD (131). Mutations in the SAND domain and PHD1 had little effect on such complexes, but alterations in the amino-terminal region (HSR or CARD) or truncation beyond PHD1 reduced their formation. Thus, distinct regions of Aire seem to participate in the protein-protein interactions subtending complex formation. The identification of Aire's partners promises to yield important clues about its mechanism of action. Certain putative interactors have been reproducibly identified, and others require confirmation. But the emerging notion is that Aire is a highly collaborative protein, apparently associated with a score of partners that may participate in regulating different facets of the expression of Aire's target loci. Currently thought to be of great interest are:

- CBP (CREB-binding protein). This ubiquitous transcriptional activator was the first Aire partner to be identified (96). CBP is a histone and nonhistone

acetylase that activates transcription. It can synergize with Aire to turn up expression of reporter constructs, colocalizing with it in nuclear bodies (95–97, 130). Formation of a complex with Aire may promote migration of CBP to the nucleus: RANK (receptor activator of NF-κB) induces Aire expression in the epithelial component of fetal thymic organ cultures, accompanied by migration of CBP from the cytoplasm into nuclear bodies (130). Aire seemed to be directly responsible for this translocation, as CBP remained largely cytoplasmic in RANK-treated Aire-deficient thymus cultures. Thus, one of Aire's functions may be to facilitate the import and/or retention of CBP in the nucleus of MECs.

- DNA-PK (DNA-dependent protein kinase). This protein is another Aire partner, initially identified in pull-down assays from Aire-transfected monocytes (132) and confirmed in the broad mass-spectrometry screen discussed below. DNA-PK is a serine/threonine kinase, composed of two regulatory subunits (Ku70 and Ku80) and a large catalytic subunit. It is activated by double-stranded DNA breaks and is therefore a key player in nonhomologous end joining and is essential for the VDJ recombination characteristic of immune receptor genes. DNA-PK phosphorylates a number of proteins implicated in transcription, in particular RNA polymerase-II (Pol-II), but also Fos, Jun, and TBP. It can phosphorylate Aire in vitro, at two different positions in the amino-terminal region (positions 68 and 156), and alanine replacements at these positions result in a marked decrease in Aire's transactivation activity in transfected cells. The role of DNA-PK in transcription is still incompletely understood, but one clue may lie in its involvement in resolution of the double-stranded breaks generated by DNA topoisomerase II beta (Topo IIβ) during transcriptional activation (133). Topo IIβ produces a transient double-stranded break in nucleosomal DNA, which appears necessary for nucleosome displacement during gene activation. DNA-PK might facilitate histone displacement, in line with its acknowledged role in fostering histone H2AX replacement during DNA damage repair (134). This release of nucleosomal inhibition of transcription could influence either the initiation or elongation steps.
- P-TEFb. In a similar vein, an effect of Aire on transcriptional elongation was recently suggested by the observation that it interacts, in coprecipitation and pull-down assays, with the P-TEFb complex (135), a heterodimer of the Cdk9 and Ccnt1 cyclin (CycT1). P-TEFb is a key element in transcriptional elongation (136, 137), permitting Pol-II to be released from the initiation complex established at the promoter region. Recently, investigators realized that a surprisingly large proportion of the eukaryotic genes inactive in a given cell type actually have initiation complexes formed on their promoter regions: Pol-II initiates the transcription of a short RNA (~30–50 bases) but is unable to proceed further (138, 139; reviewed in 137). For these loci, then, gene expression is regulated by release of Pol-II from the promoter region rather than by differential formation of the initiation complex. Negative elongation factors associated with the initiation complex can block Pol-II's progression, and/or positive factors such as P-TEFb are required to release Pol-II from its tight interactions within the initiation complex poised at the promoter region. Interestingly, Aire expression in cultured cells increased the proportion of Pol-II on the intragenic regions relative to the promoter region of its target genes and augmented the recruitment of P-TEFb, as determined by chromatin immunoprecipitation experiments (135). In addition, Aire expression increased the

proportion of long transcripts relative to the short ones typical of stalled polymerases. However, one caveat to all of the results concerning P-TEFb is that these experiments employed cells that had been treated with trichostatin-A, a deacetylase inhibitor, so we do not know the extent to which they will prove to be reflections of an abnormal state of histone modification.

- Many others. Most recently, a broad screen for protein-protein interactions involving Aire, relying on high-throughput mass spectrometry, yielded a large array of proteins, many of which were validated in subsequent coprecipitation or pull-down tests (J. Abramson, C. Benoist, and D. Mathis, unpublished data). About 20 independent proteins were found to interact, either directly or indirectly, with Aire. Some were previously identified interactors (e.g., DNA-PK), whereas others were newly revealed partners belonging to several biological pathways. Perhaps most striking was a set of proteins involved in the splicing and other processing of primary transcripts. Their functional relevance for Aire-mediated transcriptional activation was verified in RNAi-knockdown experiments. In keeping with this observation, Aire had a far more pronounced effect on the levels of spliced forms of transcripts than it did on levels of their unspliced nuclear precursors. These observations support the notion that Aire has a role in the maturation of primary transcripts.

Coda: A Speculative Perspective on Aire's Influence on Transcription

Given the known or suspected functions Aire's structural domains exert in other transcription factors, and the known or suspected activities of its identified partners, can one frame a coherent (if highly speculative) hypothesis as to Aire's mode of action (**Figure 4**)? Its recruitment to chromatin might involve rather non-specific binding to DNA by the SAND domain, sharing the low level of specificity characteristic of other SAND domains. In particular, binding of Aire to regions rich in CpG dinucleotides (CpG islands enriched in the promoter regions of many genes) could facilitate its recruitment to a large number of loci, consistent with its wide impact on transcription. PHD1 would also contribute importantly to this recruitment, favoring inactive chromatin through interaction with H3K4me0 residues. These basic interactions might be viewed in a dynamic fashion, not so much as Aire binding to static chromatin but rather as it ferrying the targeted regions to nuclear bodies, perhaps to exploit transcription and/or RNA processing factories therein.

From there, Aire might have several different but synergistic activities. Guiding the formation of initiation complexes is by no means ruled out by the existing data and is in line with the observation of distinct initiation sites for PTAs in MECs relative to their peripheral home cell types. More likely, by recruiting P-TEFb and other elongation factors, Aire might help in rescuing stalled Pol-II molecules, promoting their disengagement from otherwise sterile initiation complexes. By interacting with splicing complexes, Aire might favor the maturation and export of transcripts derived from its target genes. Thus, Aire's interactions and functional activities would be multifaceted, addressing several steps in the generation of mature mRNAs. Such a broad role is consistent with the currently held view that transcriptional initiation, elongation, termination, and splicing are not events that occur sequentially and disconnectedly, but rather are performed in concert by large multimolecular complexes, transcripts being spliced before they are even completed (140).

These effects seem rather nonspecific, as are the Aire interaction partners identified so far. How, then, might one account for Aire's preferential impact on PTAs, rather than on ubiquitously expressed housekeeping genes? The key is likely to be that Aire activates primarily genes that need help: inactive loci packaged in closed chromatin marked by H3K4me0 genes,

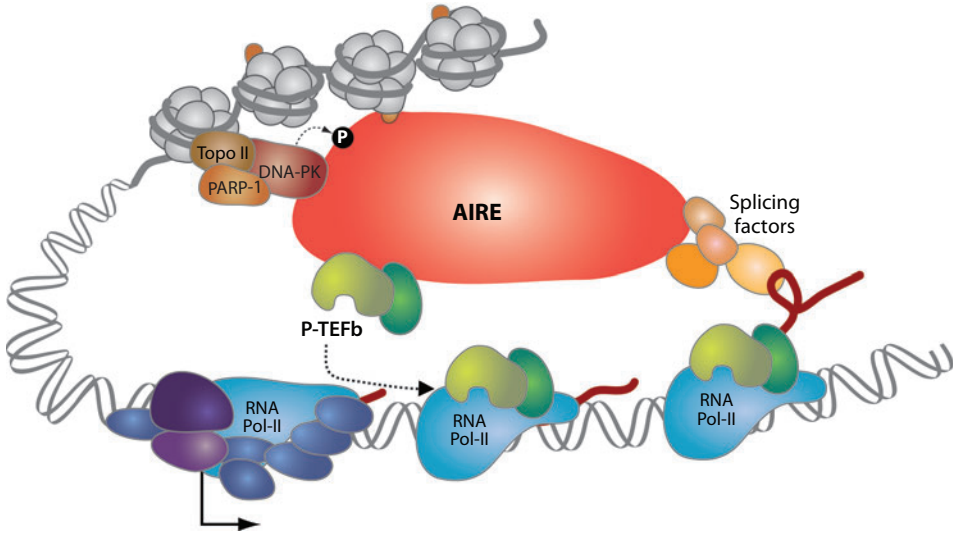

Figure 4

A speculative and incomplete model of Aire transcriptional activation. As outlined in the text, Aire interacts functionally with several partners that it may recruit to transcriptional centers; the nature and function of these partners suggest mechanisms and points of impact for Aire's activity. Interaction of Aire's PHD1 with the unmethylated tail of histone H3 (H3K4me0, orange tails on the gray nucleosomes) preferentially recruits it to regions of inactive chromatin. DNA-PK affects transcription by participating in the transient double-stranded breaks and resolution thereof, catalyzed by Topo II, that lead to nucleosomal opening/displacement and release of topological constraints during transcription. Recruitment of P-TEFb, a key complex in transcriptional elongation, would facilitate the release of stalled Pol-II from the initiation complex. Finally, Aire would increase the availability (or modify the composition and specificity?) of splicing complexes to facilitate maturation of the transcripts (*red strings*).

or with stalled Pol-II on their promoters that need derepression, or ineffectively spliced transcripts. Active genes, bristling with properly methylated histones and with rapidly processive Pol-II complexes, would not be affected. This interpretation might explain Aire's bewilderingly broad activity and the fact that its targets are almost completely different in different cell types. (One should note, however, that this interpretation of Aire as a generous helper of poor genes cannot be the whole story, as it does regulate some genes that are quite richly transcribed according to gene-expression profiles.)

In summary, although we are beginning to get glimpses of the unusual mode of Aire's effect on transcription, the precise mechanisms and interacting partners remain largely obscure. In addition, it seems unlikely that such a multitasking factor would not somehow exploit or influence the regulatory properties of noncoding RNAs. Although preliminary analyses showed no strong impact of Aire on the levels of a few miRNAs tested (M. Giraud, C. Benoist, and D. Mathis, unpublished observations), it is quite plausible that Aire would elicit derepression of PTAs by affecting the miRNAs that contribute to repress them.

WHAT REGULATES THE REGULATOR?

Rather little is known about the transcriptional controls impinging on the *Aire* locus itself. There is a striking contrast between the loose, noisy, promiscuous gene expression that Aire promotes and the very tight control of its own expression. Although it is rather abundant in Aire-positive cells, with high mRNA levels and easily detectable protein, this expression is largely confined to a single epithelial cell type

in the thymus, in rare cells in the spleen and lymph nodes, and in the gonads (21–23, 26, 70, 71, 141, 142). Even in MECs, Aire is turned on only at the latest stage of differentiation, a few days before a cell's demise (42, 61). Most likely, given its mode of action, Aire is too dangerous a protein to be expressed in a widespread manner or for extended periods of time.

The molecules responsible for controlling *Aire* gene expression have not been explored to any significant degree. The promoter regions of the human and mouse genes share stretches of conserved sequences (141), plausibly the target of similar transcriptional activators, but whose identity and importance remain to be established.

Several connections have been made between members of the tumor necrosis factor (TNF) family and Aire expression. Lymphotoxins (LTs) were first invoked as inducers of Aire, but data on this point have been conflicting. One group reported that signals through the LTβ receptor regulate *Aire* and PTA gene transcription, and not the composition or structure of the thymus epithelium (143, 144). In contradiction, Boehm et al. (145) concluded that LTβ receptor signals are required for proper MEC differentiation and organization but have no impact on Aire expression. The current consensus favors the second interpretation: A modest change in the number of MECs is observed in mice lacking the LTβ receptor, but the levels of Aire on a per-cell basis are not affected by this deficiency, nor are the levels and patterns of Aire-controlled PTA expression (60, 146, 147). Interestingly, LT may have a greater impact on the Aire-negative MEC population and PTA expression there (147).

However, the RANK/RANK-ligand pair (60, 148–150), and its downstream signal transducers TRAF6 (TNF receptor–associated factor 6) and NIK (NF-κB inducing kinase) (150, 151), have a much more profound effect on the generation of the Aire-positive MEC compartment, with a severe reduction in $Aire^+$ cells in the corresponding knockouts. Signaling through RANK also synergizes with CD40. The required soluble RANK ligand and surface-bound CD40 ligand signals needed for proper maturation of MECs are furnished by positively selected single-positive thymocytes in the adult, or by $CD4^+CD3^-$ lymphoid-inducer cells during the fetal period (60, 149, 150), providing a molecular basis for the long-recognized cellular cross talk between the epithelial and lymphoid lineages that establishes a normal thymus architecture (152). The signal from mature thymocytes is not absolutely indispensable, though, because Aire can be detected in the rudimentary medulla of $Rag^{-/-}$ mice (23, 26, 60, 148). Here, again, it is difficult to appreciate the direct impact of RANK signaling on the activation of Aire transcription, independently of the promotion of MEC differentiation.

CONCLUSIONS

Aire is a fascinating protein that the immune system may have co-opted—from what could be a more primordial role in genomic remodeling of the germ line—to promote tolerance to self constituents. There are many mysteries left to solve concerning both its impact on lymphocyte populations and its role in the few nonthymic locales where it appears to reside. New surprises will almost certainly be encountered in unraveling the mechanisms underlying Aire's unusual properties as a transcriptional regulator.

DISCLOSURE STATEMENT

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