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Dominant *Autoimmune Regulator* mutations associated with common organ-specific autoimmune diseases

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Summary

The autoimmune regulator (*AIRE*) gene is crucial for establishing central immunological tolerance and the prevention of autoimmunity. Mutations in *AIRE* cause a rare autosomal recessive disease, autoimmune polyendocrine syndrome type 1 (APS-1), distinguished by multi-organ autoimmunity. We here report multiple cases and families with mono-allelic mutations in the first plant homeodomain (PHD1) zinc finger of AIRE, which follow dominant inheritance, typically characterized by later onset, milder phenotypes, and reduced penetrance compared to classical APS-1. These missense PHD1-mutations suppress gene expression driven by wild type AIRE in a dominant negative manner, unlike CARD or truncated AIRE mutants, which lack such dominant capacity. Strikingly, exome array analysis revealed that the PHD1 dominant mutants are found with relatively high frequency (> 0.0008) in populations. Our results provide novel insight into the molecular action of AIRE and demonstrate that disease-causing mutations in the *AIRE* locus are more common and variable than previously appreciated.

INTRODUCTION

The autoimmune regulator (*AIRE*) is a key player in shaping central immunological tolerance to self. AIRE is mainly expressed in medullary thymic epithelial cells (mTECs), but to some extent also in rare hematopoietic populations of lymph nodes (Gardner et al., 2008). In mTECs, AIRE induces expression of thousands of tissue-restricted proteins, which are presented on major histocompatibility complex class I (MHC-I) and MHC-II molecules to developing T cells, percolating through the thymic medulla. This "projection of self" by mTECs is essential for the elimination of auto-reactive T cells, either via clonal deletion (Taniguchi and Anderson, 2011) or via their conversion into Foxp3+ regulatory T cells (Cowan et al., 2013); a critical step for the induction of functional immunological tolerance to self and prevention of autoimmunity (Taniguchi and Anderson, 2011).

In humans, mutations in the *AIRE* gene cause autoimmune polyendocrine syndrome type 1 (APS-1), also called autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), a rare autosomal recessive disease characterized by autoimmune attack against peripheral (mainly endocrine) tissues, as well as by generation of various autoantibodies, including interferon-specific autoantibodies (Meager et al., 2006). The majority of APS-1 patients develop at least two (diagnostic dyad) of the three main components, including adrenocortical insufficiency, hypoparathyroidism and chronic mucocutaneous candidiasis (Ahonen et al., 1990; Husebye and Anderson, 2010). In addition, premature ovarian insufficiency, pernicious anemia, vitiligo, alopecia, enamel hypoplasia, and keratitis are common components. The disease typically manifests in childhood, but milder forms with late debut are seen, which are not always recognized as APS-1 at first.

About 100 APS-1-causing mutations have been found throughout the *AIRE* gene (http://www.hgmd.cf.ac.uk) (Ferguson et al., 2008). All are assumed to be inherited in an autosomal recessive manner, except for one mutation in the SAND-domain, p.G228W, which follows a dominant inheritance pattern (Cetani et al., 2001). Since AIRE is known to operate as a homo-oligomer (Kumar et al., 2001; Pitkanen et al., 2000), it is rather surprising that only one mono-allelic mutation in the *AIRE* locus has been linked to APS-1 and/or other forms of organ-specific autoimmune disorders so far.

Based on analysis of human patients followed by biochemical and population analyses, we here report a group of novel mono-allelic *AIRE* mutations. These mutations cluster within the first plant homeodomain (PHD1) zinc finger domain, associate with organ-specific autoimmune diseases with varying penetrance and severity, sometimes, but often not matching the diagnostic criteria of APS-1. Furthermore, we delineate the molecular mode of action by which these unique mutations interfere with the function of wild type (WT) AIRE protein. Our results provide novel insights into the molecular action of the AIRE protein and indicate that disease-causing mutations in the *AIRE* locus are much more common than previously thought and can cause more variable autoimmune phenotypes.

RESULTS

Novel p.C311Y AIRE mutant exerts a dominant negative effect

The study was initiated by the discovery of a heterozygous c.932G>A (p.C311Y) mutation in *AIRE* in a North-African patient (I:2, **Figure 1A, Table 1 and Table S1**) diagnosed with adult-onset of chronic mucocutaneous candidiasis, adrenal insufficiency, enamel dysplasia, pernicious anemia, partial diabetes insipidus, and interferon omega autoantibodies (**Figure**

1A). Importantly, no other mutations or copy number variations were detected. His family history revealed a daughter (II:1, with partner 1) who had hypoparathyroidism, enamel dysplasia, primary ovarian insufficiency, autoimmune gastritis, pernicious anemia, and the same mono-allelic p.C311Y mutation indicating dominant inheritance. With his second partner (I:3), he had four children of whom three carried the mono-allelic p.C311Y mutation and developed various forms autoimmunity; one daughter (II:2) had alopecia areata and nail dystrophy on one of ten finger nails, another daughter (II:4) had hypoparathyroidism, and primary ovarian insufficiency, while a son (II:3) was diagnosed with autoantibodies against tyrosine hydroxylase (often associated with APS-1) (Hedstrand et al., 2000), but otherwise had no autoimmune manifestations (**Figure 1A, Table 1** and Table S1). To exclude autosomal recessive inheritance at the *AIRE* locus, we performed microsatellite markers analysis, which validated that the affected children had indeed inherited different maternal *AIRE* alleles (Figure S1).

We next analyzed if p.C311Y can repress the transcription-transactivation potential of WT AIRE in a dominant negative manner. To this end we utilized the human thymic epithelial 4D6 cell line, which was transfected with either WT-*AIRE* and/or mutated *AIRE* expression vectors. We then measured the mRNA expression of a panel of AIRE-dependent (*KRT14*, *S100A8* and *IGFL1*) and –independent genes (*CCNH* and *PRMT3*) (Giraud et al., 2012). As expected, the WT-AIRE induced strong expression of all analyzed AIRE-dependent genes, whereas p.C311Y, p.G228W, p.L28P and the deleterious major Finnish mutation p.R257* did not (**Figure 1B**, Figure S2). No differences among the *WT-AIRE* or *AIRE* mutants were seen for AIRE-independent genes (**Figure 1B**, Figure S2). Strikingly, when 4D6 cells were co-transfected with different ratios of WT-*AIRE* and the above mutants, p.C311Y

completely abolished the ability of WT-AIRE to induce expression of AIRE-dependent genes (**Figure 1B**, Figure S2), as did the previously reported SAND domain mutant p.G228W (dominant negative control) (Su et al., 2008). Conversely, neither p.R257* nor the p.L28P CARD mutation showed this inhibiting effect (recessive controls). Taken together, these data validate that the p.C311Y mutant exerts a dominant negative effect on WT AIRE function, both *in vitro* and in human patients.

Identification of dominant-negative variants of AIRE

As the phenotype in family A segregated with a heterozygous mutation in *AIRE* with an inhibitory effect on transcription of AIRE-dependent genes, we asked if there might be more dominant *AIRE* mutations. To test this hypothesis we generated a panel of expression vectors with reported disease-causing mutations including several located in the PHD1, CARD, and SAND domains (**Figure 2A**). First we tested the dominant negative effect of AIRE-mutants in co-transfection experiments with WT-AIRE in 4D6 cells. Similarly to the p.C311Y mutation, virtually all missense mutations in the PHD1 finger, including p.E298K, p.V301M, p.C302Y, p.R303P, p.G305S, p.D312N, and p.P326L revealed a dominant negative effect on AIRE-dependent genes (**Figure 2B**, Figure S3, Table S3). Interestingly, the dominant negative effect of p.V301M varied with the downstream gene tested (**Figure 2B**, Figure S3 in the Supplement), which was surprising but reproducible in several independent experiments. In contrast, most of AIRE's CARD mutants, as well as the truncated PHD1-mutant p.C311* revealed a clear recessive pattern, while the common p.C322del13, p.R328Q and p.C446G displayed only a partial dominant effect (**Figure 2B**, Figure S3, Table S3). Conversely, p.R471C (PHD2 domain) had no effect on AIRE-dependent gene transcription (**Figure 2B**, Figure S3,

Table S3). As expected, AIRE-independent transcriptional activity was not affected in any of these analyses (**Figure 2B**, Figure S3). This series of experiments demonstrated that the heterozygous mutations in *AIRE* can be segregated into three groups according to their potential to impact on the transcription-transactivation potential of WT AIRE in; (i) dominant negative, (ii) recessive, and (iii) partial dominant negative manners. Moreover, our data revealed that most of the mutations operating in a dominant negative manner are clustered within the PHD1 finger, while most recessive mutations were clustered within the CARD domain.

Dominant negative mutants physically co-localize with WT AIRE

To better understand the unique properties of the dominant mutants, we next analyzed their nuclear localization patterns. 4D6 cells were co-transfected with red fluorescent protein (RFP)-tagged WT AIRE plasmids together with expression vectors encoding individual AIRE mutants tagged with enhanced green fluorescent protein (EGFP). Importantly, all dominant mutants, including the PHD1 missense mutations, localized in nuclear speckles typical for WT-AIRE and co-localized with WT-AIRE protein (yellow overlay) (**Figure 3A** and Figure S4, Table S2 and S3). In contrast, recessive CARD mutants (p.L28P, p.LL28_29PP; p.Y90C; p.L97P) which are thought to disrupt AIRE homo-oligomerization (Kumar et al., 2001; Pitkanen et al., 2001), failed to provide the same speckles and stained diffusely throughout the nucleus when transfected alone. In co-transfections, however, all CARD mutants partly co-localized with WT-AIRE, indicating that when co-expressed some functional oligomers are able to form.

Since virtually all analyzed PHD1 mutants demonstrated a dominant negative effect, we sought to gain more insights about the impact of these mutants on molecular structure of this domain. Specifically, *in silico* analysis predicted that the p.C311 residue is crucial for chelating Zn^{2+}, and thereby is critical for correct folding of the PHD1 finger. Indeed, a substitution of the cysteine with tyrosine is predicted to disrupt PHD1 folding (Chakravarty et al., 2009) (**Figure 3B**). Additional structural analyses revealed that many of the reported missense mutations changed amino acids that are conserved among different species (Bjorses et al., 2000; Org et al., 2008; Spiliotopoulos et al., 2012) (Figure S5), and can similarly affect the Zn^{2+}-binding or folding of the domain.

Taken together, these data suggest that most of the PHD1 mutants can, unlike their CARD mutant counterparts, physically associate with WT AIRE in nuclear speckles and form a homo-oligomer, which is however not functional due to dysfunctional PHD1 fingers.

Proof of concept – additional PHD1 dominant-negative AIRE mutations segregate with organ-specific autoimmunity

Our *in-vitro* analyses predicted that in addition to the p.C311Y mutation, more dominant mutations are clustered within the PHD1 finger and may therefore similarly cause organ-specific autoimmunity in human patients. To validate this hypothesis, we performed a thorough analysis of patient cohorts available to us. First, we reinvestigated a previously described case, in which p.C311Y had been reported as a compound heterozygous mutation with p.R257* in two Finnish siblings with childhood-onset of APS-1 (Bjorses et al., 2000) (**Table 1**, (Family B, II:3 and II:4), **Figure 4A** and Table S1). Re-sequencing *AIRE* in this family confirmed the earlier report, but also revealed that one of the affected siblings' son (III:1)

had inherited p.C311Y, but not p.R257*. He manifested with vitiligo and severe pernicious anemia due to autoimmune gastritis at young age. Moreover, the maternal grandmother (I:2), also a heterozygous p.C311Y carrier, was diagnosed with pernicious anemia and several autoantibodies characteristic of APS-1 (**Table 1, Figure 4A** and Table S1). In contrast, the third daughter (II:1), a heterozygous carrier of p.R257*, was without detectable autoantibodies.

Next, we reinvestigated a woman with APS-2 characterized by adrenal insufficiency, autoimmune thyroid disease, primary ovarian insufficiency and autoantibodies characteristic of APS-I with a mono-allelic c.901G>A (p.V301M) mutation (**Table 1** (Family C), **Figure 4A** and Table S1) (Soderbergh et al., 2000). Her daughter also with a p.V301M mutation, had autoantibodies against IL-17F, which are often found in APS-1 patients. However, she did not present with any additional autoimmune manifestations at age 30 years. Finally, additional screening of a large cohort of 85 Russian APS-1 patients and some of their family members identified a young girl with a mono-allelic p.C302Y mutation, who developed hypoparathyroidism and autoantibodies against interferon omega, NALP-5 and 21-hydroxylase (**Table 1 (subject D**). Like p.C311Y, p.C302Y revealed dominant negative effects on AIRE-mediated transcription (**Figure 2B**, Figure S3 and Table S3). A very similar case with a *de novo* mono-allelic p.C302Y mutation was reported by us earlier (Oftedal et al., 2008) (**Table 1** (subject E)).

In summary, our data illustrate that individuals with bi-allelic disease-causing *AIRE* mutations develop classic early onset APS-1 phenotypes, while those carrying one of three different mono-allelic mutations in the PHD1 finger (p.C311Y, p.V301M and p.C302Y) segregate with clear, but varying autoimmune phenotypes, ranging from late-onset classical

APS-1 (e.g. I:3, Figure 1A), to APS-2 (**Table 1, Figure 4B** and Table S1), and isolated organ-specific autoimmunity (e.g. vitiligo, PA, and APS-1-specific auto-antibodies).

Increased frequency of dominant PHD1 mutations in various forms of organ-specific autoimmunity

The above findings raised the question whether dominant PHD1 mutations could generally cause organ-specific autoimmunity. To answer this question, we sequenced the full exon 8 (encoding the PHD1 finger) in several autoimmune patients and controls available to us from our national registry. We first analyzed the presence of PHD1 mutants in familial cases characterized by the presence of adrenal insufficiency, autoimmune thyroid disease and/or type 1 diabetes (i.e. APS-2 and /or APS-3). Indeed, among 41 such families, we identified one family with three family members bearing a mono-allelic c.977C>T (p.P326L) mutation (**Table 1** (Family F), **Figure 4A** and Table S1). The mother (II:3) was diagnosed with autoimmune thyroid disease, adrenal insufficiency, pernicious anemia and vitiligo. Her children both acquired vitiligo at 10 (III:1) and 7 (III:2) years of age, respectively.

Furthermore, since pernicious anemia, vitamin B12 deficiency, and/or vitiligo seemed to be often associated with heterozygous PHD1 mutations in previous cases (**Figure 4B**), we next screened large cohorts of patients with these conditions. Among 177 probands and 26 affected relatives with pernicious anemia, we identified several dominant negative PHD1 mutants; First, a patient with a heterozygous c.913G>A (p.G305S) mutation who was intrinsic factor (IF) antibody positive and developed severe anemia and neuropathy at age 43 (**Table 1,** (Family G), Figure 4A and Table S1). Her mother (II:2) and maternal grandmother (III:2) were reported to have pernicious anemia, the mother also suffered from

hypothyroidism and cirrhosis. p.G305S is close to the zinc binding site and predictably disrupts the zinc finger structure. Not surprisingly, the dominant negative effect on gene transcription was evident (**Figure 2B,** Figure S3 and Table S3). Another patient in this cohort developed intrinsic factor antibody positive pernicious anemia at age 81 years and was heterozygous for both c.946C>T (p.R316W) and the common c.967-979del13bp (p.C322del13) mutation on the same allele (**Table 1** and Table S1 (subject H)). Both p.R316W and (p.C322del13) were predicted to have a partial dominant negative effect.

Similarly, among 170 patients with isolated and familial (n=64) vitiligo, a female who developed acrofacial vitiligo at age 21 years, with gastric parietal cell autoantibodies, low normal serum vitamin B12 level , and a heterozygous mutation in c.983G>A (p.R328Q) (**Table 1** (subject I) and Table S1). Like p.P326L, a mutation in this C-terminal part of PHD1 does not disrupt the histone binding site, but still displays an incomplete inhibition of AIRE-dependent gene transcription (**Figure 2B,** Figure S3 and Table S3). *AIRE* sequencing revealed that the patient also had p.V484A; a sequence variant that has been described in a patient with alopecia and nail dystrophy (Buzi et al., 2003). We were unfortunately unable to perform an allele discrimination assay in this patient.

Importantly, sequencing of 450 control blood donors did not reveal presence of any of the dominant negative PHD1 mutations, demonstrating that dominant PHD1 mutations are clearly over-represented among patients suffering from various forms of organ-specific autoimmunity.

The frequency of dominant negative PHD1 AIRE mutations in populations

To better estimate the frequency of some of the dominant negative PHD1 AIRE mutations, we analyzed multiple exome chip datasets that were available, containing some of the PHD1 *AIRE* sequence variations. Specifically, sequence analysis from existing exome chip datasets from a total of 1670 Scandinavian individuals (healthy controls (n=637), and patients with attention deficit (n= 589) or movement disorders (n=444)), we determined the minor allele frequency of p.V301M to be 0.00089 (i.e 3 out of 1667 persons), while other covered mutations p.G303S, p.R303Q, and p.R257* were not found. The relatively high frequency of the p.V301M dominant mutant was further validated by additional datasets obtained from public databases, including the recently published data from The Broad Institute (covering over 60 thousand individuals) (Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org)), 1000 Genome database (http://www.1000genomes.org) and the Washington Database (~6 thousand individuals) (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/). All above databases confirmed and broadened these findings and demonstrated that dominant-negative PHD1-mutations are present with minor allele frequency reaching 0.0009 (mainly p.V301M and p.R303Q) (**Table 2**). It should be stressed however, that most of the dominant negative PHD1 variants were not covered on these exom chips, suggesting that the actual frequency may be even higher.

DISCUSSION

Molecular aspects of dominant-negative mutations of AIRE

Many proteins are active only in the form of a multimeric complex, composed of two or more copies of the same protein. It is well established that in many of these cases, mono-

allelic mutations can completely or partially disrupt the structure and thereby the activity of the entire multimeric complex in a dominant-negative manner. Since AIRE was shown to form a homo-tetramer *in vivo* (Kumar et al., 2001), it is rather surprising that only one mono-allelic mutation in the *AIRE* locus has been linked to APS-1 and/or other forms of organ-specific autoimmune disorders so far. We identify several novel heterozygous missense mutations in *AIRE*, primarily clustered within its PHD1 zinc finger (**Figure 4B**), which are characterized by dominant inheritance, later debut, milder phenotypes, and reduced penetrance. Interestingly, most autosomal recessive missense mutations causing APS-1 are predominantly found within the CARD domain (Bjorses et al., 2000), suggesting that the recessive or dominant character of the given mutation is, to a large extent, determined by its position within the AIRE protein. This likely reflects the different and unique roles of the individual domains of the AIRE protein. Specifically, while the CARD domain has been shown to be critical for AIRE homo-oligomerization and speckled nuclear localization (Bjorses et al., 1999; Kumar et al., 2001), the PHD domain of AIRE functions as an epigenetic reader, specifically recognizing unmethylated lysine 4 on histone 3 (H3K4me0) (Org et al., 2008). The PHD1 domain was shown to be absolutely critical for AIRE's transcription-transctivation activity, as well as for its capacity to prevent multiorgan autoimmunity in transgenic mouse models (Bjorses et al., 2000; Koh et al., 2010; Koh et al., 2008). *In silico* simulations revealed that the PHD1 residues N295-C310 are important in the intermolecular interactions with histone H3 residues (**Figure 2B,** Figure 3B and Table S3). PHD1 is unable to interact with H3K4me0 if the zinc chelating cysteines are mutated, as is the case for C311Y (Bottomley et al., 2005). The formation of salt-bridges between the side chains of H3 residue R2 and D312 was shown to be crucial for binding specificity (Koh et al., 2008), explaining why the

296 structure is highly conserved in AIRE among different species and also in PHD-zinc finger
297 domain-containing proteins (Figure S5).
298 Unlike the PHD1 mutants, mutations clustered within the CARD domain of AIRE do
299 not exert any dominant negative effect (**Figure 2B,** Figure S3 and Table S3). In homozygotes
300 these mutations impact on AIRE oligomerization and correct nuclear localization (Bjorses et
301 al., 1999; Kumar et al., 2001; Pitkanen et al., 2001), yet may be able to form oligomers when
302 expressed along with WT AIRE (**Figure 3A,** Figure S4 and Table S2). Interestingly, truncating
303 AIRE mutations such as p.R257* and p.C311* also behave in a recessive manner, in spite of
304 their ability to co-localize and interact with WT-AIRE (**Figure 3A**, Figure S4 and Table S2). This
305 suggests that the above truncations do not disrupt the core structure of the AIRE complex,
306 necessary for its biological activity. Such core structure likely involves formation of functional
307 dimers within the truncated tetramer (**Figure 5A**).
308 It is therefore not entirely surprising that mono-allelic and dominant negative
309 mutations in this domain will impact on the structure and thus the activity of the entire AIRE
310 tetramer. However, such dominant effect seems to follow incomplete inheritance, as most
311 of the patients develop milder phenotypes with later onset compared to patients with
312 classical, autosomal recessive APS1. This could be because the AIRE tetramers still have
313 some residual activity, and/or that some pure WT-AIRE tetramers are still formed and are
314 sufficient to induce some level of self-tolerance. Moreover, the extent of the dominant
315 effect seems to depend on which residue is mutated. Our results suggest that mutations in
316 residues 302 and 311 resemble more classical APS-1 than other mutations, although we
317 observed large diversity within the two families with p.C311Y studied here.
318

Clinical aspects of dominant-negative mutations of AIRE

The genetic contribution of *AIRE* to other autoimmune diseases than APS-1 has been studied by us and others, but in most cases only SNPs or a few common mutations have been analyzed, thereby overlooking rare mutations or large deletions (Jin et al., 2007; Pforr et al., 2006; Thomson et al., 2007; Torok et al., 2004; Turunen et al., 2006; Vaidya et al., 2000). Although some heterozygous mutations in *AIRE* have been associated with autoimmunity in single patients (Table S4), a dominant negative effect on AIRE function was not considered in these cases. Here, we demonstrate for the first time that the heterozygous variants observed in the families as well as other mutations analyzed within AIRE exon 8 have an inhibitory effect on AIRE-mediated transcription. This contrasts to classical APS-1 with recessive inheritance and early presentation (mean age 9.1 years (Wolff et al., 2007a)); 90% develops all three components by age 20 years (Wolff et al., 2007a), Organ-specific autoimmunity in the heterozygous cases presents later (mean age 24.4 years, n = 12), progresses more slowly, fewer patients develop the diagnostic dyad, and the penetrance is incomplete (**Figures 4B and 5B**). This is reminiscent of autoimmune lymphoproliferative syndrome, which shows 60 % penetrance among family members harboring the same heterozygous gene mutation (Price et al., 2014), or to the incomplete penetrance seen in families carrying heterozygous CTLA4 mutations (Kuehn et al., 2014). More importantly, the unusual heterozygous cases may not even be recognized as APS-1 as many patients masquerade as common types of organ-specific autoimmunity in one or several organs. Thus, the original classification of APS-1 as a strictly autosomal recessive disease (with one exception (Cetani et al., 2001)) is obsolete. Instead, we propose that APS-1 exists in two forms: (i) 'classical', characterized by recessive inheritance, presence of at least two of three

main components, and interferon antibodies; and (ii) 'non-classical', characterized by dominant heterozygous mutations mainly in AIRE's PHD1 zinc finger and a milder less penetrant autoimmune phenotype (**Figure 5B**). Families with dominant clustering of organ-specific autoimmunity, especially when pernicious anemia and / or vitiligo manifests at early age, might have such mutations, although the clinical phenotype might be expanded when larger materials are investigated. Furthermore, it is reasonable to assume that mutation carriers have a significant risk for polyendocrinopathy, which should be reflected in their follow-up programs. Moreover, autoantibodies against interferons, hallmarks of classical APS-1, are much less prevalent in the non-classical form probably reflecting some residual AIRE-function at least for some of the PHD1 mutations.

Since deep DNA sequencing of thousands of different patients was beyond the scope of the current study, we cannot provide accurate estimates of the prevalence of non-classical APS-1 since a population cohort with autoimmune phenotypes was not available. Based on our own data and publicly available databases representing patients with diverse conditions in different ethnic groups, a conservative estimate puts dominant *AIRE* mutations at a genotype frequency of 1-2 persons per thousand, not restricted to the Scandinavian population as also is underpinned by literature reports (Cervato et al., 2010; Ferrera et al., 2007; Stolarski et al., 2006; Vogel et al., 2001) (**Table 2** and Table S4). However, further studies are needed to establish the prevalence and risk associated with mutations in the PHD1 domain in larger populations.

In conclusion, this study represents the first demonstration that *AIRE* mutations associate with common organ-specific autoimmunity with a variable phenotype ranging from classical APS-1 to a non-classical form that mimics common organ-specific

autoimmunity. Finally, our study provides important insights into the molecular mode of action of the AIRE protein and highlights unique structural properties that are required for AIRE's biological activity.

EXPERIMENTAL PROCEDURES

Patients

Norwegian, Finnish, and Russian patients were recruited from the respective national patient registries and biobanks of patients with APS-1, adrenal insufficiency and polyendocrine syndromes. Vitiligo patients were recruited by the Sheffield Teaching Hospitals NHS Trust, Sheffield, UK; pernicious anaemia patients were recruited by Manchester Centre for Genomic Medicine, Central Manchester University Hospitals NHS Trust in collaboration with the Pernicious Anaemia Society of United Kingdom. For estimation of population frequencies of AIRE mutations, exome chip data from cohorts with healthy controls (n=637), and patients groups without known susceptibility for autoimmunity were available (for details, see Supplemental Methods). All participating patients signed an informed consent. Samples from blood donors were recruited from the Haukeland University Hospital blood bank. The study was approved by the Regional Ethics committees in each institution.

AIRE sequencing, copy number analysis and microsatellite typing

All 14 exons of the *AIRE* gene (EMBL acc. Number AJ009610) were amplified by PCR and sequenced as described previously (Wolff et al., 2007b). The PHD1 zinc finger is encoded by exon 8 (see Supplemental Methods). Copy number analysis was performed by duplex TaqMan real-time PCR assay (Boe Wolff et al., 2008). Microsatellite typing of the *AIRE* region was performed according to Myhre *et al* (Myhre et al., 2004). The samples used to estimate population frequencies for *AIRE* mutations were genotyped on the HumanExome 12v1_B (ADHD study) and HumanCoreExome 12v1-

1 (movement disorders study) Bead chips respectively (Illumina Inc, San Diego, CA). For further information and analysis of data see Supplemental Methods in the Supplement.

Assay of autoantibodies

Autoantibodies typical of APS-1, were assayed by radioligand binding assays as previously described (Husebye et al., 1997; Oftedal et al., 2008) (Supplemental Methods).

Assay of AIRE-regulated genes

The human 4D6 thymic epithelial cell line was transfected with AIRE-containing plasmid constructs using the Fugene HD transfection reagent (Promega Corporation, Madison, WI, USA) according to the manufacturers' protocol. Mutations in *AIRE* were engineered using site-directed mutagenesis (Supplemental Methods). Genes previously shown to be regulated by AIRE (Abramson et al., 2010) were analyzed by quantitative PCR, and the comparative Ct-method (Applied Biosystems, Carlsbad, CA, USA) (SupplementalMethods).

Immunofluorescence

4D6 cells were grown on sterile coverslips and transfected with EGFP-AIRE and/or RFP-AIRE fusion plasmids using Fugene HD transfection reagent, and analyzed under a Zeiss LSM 510 META Laser Scanning confocal microscope (Supplemental Methods).

Structure modelling

Sequence alignment was made using Clustal Omega Multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalo). Modelling of PHD1 was performed using PyMOL and the coordinates of the PDB entry 1XWH (Bottomley et al., 2005).

SUPPLEMENTAL INFORMATION

Supplemental information includes supplemental methods, Supplemental figures S1-S5, Supplemental table S1-S5.

AUTHOR CONTRIBUTIONS

BEO, AH, ASBW, EB and AV performed the experiments. MKV did the HLA genotyping and SJF did the in-silico analysis and structural modelling. TF and PMK provided the gen-analysis and microsatellite typing of the *AIRE* region, and KH, TZ and SJ provided the genetic frequency data. MMA, JP, EHK, APW, SB, WGN, WACS, LSS, EMO, KL, and ESH provided samples and clinical data for the patients. BEO, JA and EH coordinated the study and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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583

584 **Figure 1. APS-1 family with dominant inheritance**. (**A**) Pedigree showing the North-
585 African/Norwegian family with the dominantly inherited p.C311Y mutation. The lover panel
586 show the heterozygous mutation in exon 8 revealed by Sanger sequencing.
587 (**C**)Transcriptional regulation by *WT AIRE* and the different mutations. The AIRE-regulated
588 gene keratin 14 *(KRT14)* was tested together with the AIRE-independent gene cyclin H
589 (*CCNH*) and normalized against the endogenous control beta$_2$-microglobulin (*B2M*). Cells
590 were transfected with various amounts of WT AIRE and mutants, alone or in combinations.
591 The results are shown as fold difference (FD) compared to cells transfected only with WT
592 AIRE (dotted line), error bars are representing SEM.
593
594 **Figure 2. Heterozygous mutations in AIRE and the effect on gene regulation.** (**A**) Model of
595 the AIRE protein with domains and common mutations classified as recessive (black) and
596 dominant (red). (**B**). The AIRE-regulated gene *KRT14* (red bars), and *CCNH* not regulated by
597 AIRE (blue bars). Transcriptional regulation by *WT-AIRE* and mutants was performed as
598 described in Figure 1B. The results are shown as fold difference (FD) compared to cells
599 transfected only with WT AIRE (dotted line), error bars are representing SEM.
600
601 **Figure 3. Subcellular co-localization of the mono-allelic variants. (A)** Confocal fluorescence
602 images displaying the subcellular localization of WT-RFP-AIRE (red) and mutant-EGFP-AIRE
603 (green) constructs. Overlay images shows the degree of co-localization (yellow). Nuclei were
604 visualized with DAPI counterstain (blue). (B) The solution structure of the PHD1 domain of
605 AIRE, showing the Zn^{2+} –ligating residues. Zn^{2+} shown as sphere, and cysteines as sticks. The

606 C311 mutation hotspot is shown in cyan (right). Modelling shows that the C311Y mutation
607 would disrupt Zn^{2+} ligation.
608
609 **Figure 4. The AIRE PHD1-domain**. (**A**) Pedigrees of families with p.C311Y (Family B),
610 p.V301M (Family C) p.P326L (Family F) and p.G305S (Family G) AIRE mutations. (**B**) The AIRE
611 protein with its different domains. The mutations investigated in this study are shown, now
612 color-coded for dominant (red) and recessive (black). The AIRE PHD1 is shown, together with
613 cake diagrams each representing one patient depicting clinical manifestations and
614 autoantibodies.
615
616 **Figure 5. Dominant mutations in AIRE and organ-specific autoimmunity.** (**A**) Schematic
617 illustration of recessive and dominant *AIRE* mutations. The homozygous R257* truncated
618 protein can form oligomers, but they lack critical domains. In the heterozygous state R257*
619 does not interfere with WT-AIRE. PHD1 mutants can form oligomers but AIRE lack
620 transcriptional activity due to its putative interaction with WT-AIRE. Formation of a small
621 fraction of WT:WT oligomers may account for some induction of tolerance and a milder
622 autoimmune phenotype. (**B**) Manifestations and autoantibodies in patients with recessive
623 (from references (Meager et al., 2006; Perheentupa, 2006; Wolff et al., 2007a)) and
624 dominant (this study) mutations. AI, adrenocortical insufficiency; CMC, chronic
625 mucocutaneous candidiasis; HP, hypoparathyroidism; PA, pernicious anemia; V, vitiligo; n.a.,
626 data not available.

Table 1. Families with heterozygous mutations in the *AIRE* gene, their manifestations and autoantibodies

[a]Family	Patient	YoB	Mutation	[b]Manifestations	[c]Organ-specific autoantibodies	[d]Cytokine autoantibodies	[e]HLA class II genotypes stratified to AI risk
A	I:2	1951	p.C311Y;WT	**CMC**, **AI**, PA, PDI, EH	SCC	IFN-ω, IFN-α2	Neutral
	II:1	1971	p.C311Y;WT	**HP**, PA, EH, POI	NALP-5	IFN-ω, IFN-α2	Protective
	II:2	1988	p.C311Y;WT	AA, nail dystrophy		IFN-ω, IFN-α2	Neutral
	II:3	1990	p.C311Y;WT		TH		Protective
	II:4	1995	p.C311Y;WT	**HP**, POI	NALP-5	IFN-ω, IFN-α2	Neutral
	II:5	1998	WT;WT				Protective
B	I:2	1928	p.C311Y;WT	PA, Blind, T2D	21-OH, NALP-5, AADC, IF		Neutral
	II:1	1959	p.R257*;WT	L, oral cancer			Protective
	II:3	1961	p.C311Y;p.R257*	**CMC**, **AI**, POI, A	21-OH, SCC, 17-0H, AADC, TH	IFN-ω, IFN-α2, IL-17F, IL-22	Protective
	II:4	1965	p.C311Y;p.R257*	**HP**, **CMC**, **AI**, POI, A	21-OH, SCC, 17-OH, TPH-1, NALP-5	IFN-ω, IFN-α2, IL-17F, IL-22	Protective
	III:1	1984	p.C311Y;WT	PA, V	GPCA, IF		Neutral
C	I:2	1955	p.V301M;WT	**AI**, AT, POI	21-OH, AADC[f]	IL-17F	Very High

	II:1	1977	WT;WT		n.a	n.a	n.a.
	II:2	1980	p.V301M;WT			IL-17F	Neutral
D		2010	p.C302Y;WT	**HP**	21-OH, NALP-5	IFN- ω	n.a
E		2001	p.C302Y;WT	**HP**	NALP-5	IFN-ω	n.a.
F	I:1	1935	p.P326L;WT				Neutral
	I:2	1943	p.P326L;WT				Neutral
	I:3	1943	WT;WT				Intermediate
	I:4	1944	p.P326L;WT	Low B12	GPCA		High
	II:1	1967	p.P326L;WT				Intermediate
	II:3	1972	p.P326L;WT	**AI**, PA, V, hypothyroidism	21-OH		High
	II:4	1974	p.P326L;WT		TPH-1		High
	II:5	1984	WT;WT		GAD, TPH-1		Intermediate
	III:1	1992	p.P326L;WT	V	GPCA		Intermediate
	III:2	2005	p.P326L;WT	V	n.a.	n.a.	Intermediate
G	I:1		n.a	PA	n.a	n.a	n.a
	I:2		n.a	No autoimmunity	n.a	n.a	n.a

	II:1	1934	n.a	No autoimmunity	n.a	n.a	n.a
	II:2		n.a	PA, hypothyroidism, cirrhosis	n.a	n.a	n.a
	II:3		n.a	PA	n.a	n.a	n.a
	III:1	1959	WT;WT	No autoimmunity	n.a	n.a	n.a
	III:2		p.G305S;WT	PA	IF		n.a
	III:3	1972	p.G305S;WT	No autoimmunity	n.a	n.a	n.a
H			p.R316W, p.C322del13;WT	PA			Intermediate
I[g]		1975	p.R328Q;WT	V, low normal B12	GPCA, GAD	n.a.	High

[a]All members of families were analyzed for autoantibodies against 21-OH, 17-OH, GAD, SCC, AADC, TPH-1, TH, NALP-5, IFN-ω, IFN-α2, IL-17F and IL-22, unless otherwise stated.

[b]A, asplenia; AA, alopecia areata; AI, adrenocortical insufficiency; AT, autoimmune thyroid disease; CMC, chronic mucocutaneous candidiasis; EH, enamel hypoplasia; HP, hypoparathyroidism; L, lupus erythematosus disseminates; PA, pernicious anemia; PDI, partial diabetes insipidus; POI, primary ovarian insufficiency; T2D, type 2 diabetes; V, vitiligo. Main components of APS-1 are indicated in **bold**

[c]AADC, aromatic L-amino acid decarboxylase; GAD, glutamic acid decarboxylase; GPCA, gastric parietal cell antibody; ICA, islet cell antibody; IF, intrinsic factor; 17-OH, 17-hydroxylase; 21-OH, 21-hydroxylase; NALP-5, NACHT leucine-rich repeat protein 5; SCC, side-chain cleavage enzyme; TH, tyrosine hydroxylase; TMH, thyroid microsomal hemoagglutinating; TPH-1, tryptophan hydroxylase; n.a., data not available.

[d]IFN-α2, interferon-alpha 2; IFN-ω, interferon-omega; IL-17F; interleukin-17F; IL-22, interleukin-22; n.a., data not available.

[e]Risk assessment for HLA genotypes were defined as in Erichsen et al., JCEM 2009. Full HLA class II haplotypes are given in Supplemental table S5. The genotypes conferring "very high" and "high" risk of developing AI also confer increased risk of developing PA (Lahner et al., Dig Liver Dis 2010).

[f]Initially positive for autoantibodies against AADC, but negative in recent samples.

[g]Immunofluorescence testing for adrenal, ovarian and pituitary autoantibodies was negative, as were anti-mitochondrial, anti-smooth muscle and thyroid peroxidase (TPO) autoantibodies. The patient previously tested positive for autoantibodies against tyrosinase and tyrosinase-related protein 1 and 2.

Table 2. Minor allele frequency (MAF) of missense mutations within AIRE exon 8 (PHD 1 protein domain)

		Norwegian exome data			ExAC Browser			1000 Genomes			Genome Variant Server		
Variant	Protein effect	Allele count	Allele No	MAF	Allele Count	Allele No	MAF	Allele Count	Allele No	MAF	Allele Count	Allele No	MAF
21:45710990 G / A	p.E298K	n.a	n.a	n.a	1	121632	0.000008222			n.a	n.a	n.a	n.a
21:45710995 T / G	p.C299W	n.a	n.a	n.a	1	121584	0.000008225			n.a	n.a	n.a	n.a
21:45710999 G / A rs150634562	p.V301M	3	3340	0.00089	111	121496	0.0009136*	2	5006	0.00039	5	13001	0.00038
21:45711005 C / T	p.R303W	n.a	n.a	n.a	1	121256	0.000008247			n.d	2	13002	0.00015
21:45711006 G / A rs139808903	p.R303Q	n.d	n.d	n.d	22	121228	0.0001815**			n.a	n.a	n.a	n.a
21:45711014 G / A	p.G306R	n.a	n.a	n.a	1	121096	0.000008258			n.a	n.a	n.a	n.a
21:45711025 C / G rs74162062	p.I309M	n.a	n.a	n.a	14	120718	0.0001160^			n.d	n.a	n.a	n.a
21:45711044 C / T	p.R316W	n.a	n.a	n.a	4	119274	0.00003354	2	8596	0.00023	2	13002	0.00015

rs139874934													
21:45711044 C / G rs139874934	p.R316G	n.a	n.a	n.a	1	119274	0.000008384			n.a	n.a	n.a	n.a
21:45711045 G / A	p.R316Q	n.a	n.a	n.a	4	119214	0.00003355	1	760	0.0013	n.a	n.a	n.a
21:45711054 A / C	p.H319P	n.a	n.a	n.a	3	117232	0.00002559			n.a	n.a	n.a	n.a
21:45711075 C/A rs179363885	p.P326Q	n.a	n.a	n.a	n.a	n.a	n.a			n.d	n.a	n.a	n.a
21:45711075 C/T rs179363888	p.P326L	n.a	n.a	n.a	n.a	n.a	n.a			n.d	n.a	n.a	n.a
21:45711080 C / T rs74162063	p.R328W	n.d	n.d	n.d	21	116188	0.0001807^^			n.d	10	12982	0.00077
21:45711081 G / A	p.R328Q	n.a	n.a	n.a	4	116112	0.00003445			n.d	n.a	n.a	n.a
21:45711092 A / C	p.S332R	n.a	n.a	n.a	1	114898	0.000008703			n.a	n.a	n.a	n.a

n.a= not analysed in this dataset

n.d = no frequency determined

*The majority mutations are found in European (minus Finnish), followed by Finnish, South Asian and African populations

**The majority mutations are found in European (minus Finnish), followed by Latino populations

^The majority mutations are found in European (minus Finnish), followed by South Asian population

^^The majority mutations are found in European (minus Finnish), followed by Finnish population