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Homeodomain-Interacting Protein Kinase 2, a Novel Autoimmune Regulator Interaction Partner, Modulates Promiscuous Gene Expression in Medullary Thymic Epithelial Cells

Kristin Rattay,* Janine Claude,*1 Esmail Rezavandy,* Sonja Matt, † Thomas G. Hofmann, † Bruno Kyewski,* and Jens Derbinski,*2

Promiscuous expression of a plethora of tissue-restricted Ags (TRAs) by medullary thymic epithelial cells (mTECs) plays an essential role in T cell tolerance. Although the cellular mechanisms by which promiscuous gene expression (pGE) imposes T cell tolerance have been well characterized, the underlying molecular mechanisms remain poorly understood. The autoimmune regulator (AIRE) is to date the only validated molecule known to regulate pGE. AIRE is part of higher-order multiprotein complexes, which promote transcription, elongation, and splicing of a wide range of target genes. How AIRE and its partners mediate these various effects at the molecular level is still largely unclear. Using a yeast two-hybrid screen, we searched for novel AIRE-interacting proteins and identified the homeodomain-interacting protein kinase 2 (HIPK2) as a novel partner. HIPK2 partially colocalized with AIRE in nuclear bodies upon cotransfection and in human mTECs in situ. Moreover, HIPK2 phosphorylated AIRE in vitro and suppressed the coactivator activity of AIRE in a kinase-dependent manner. To evaluate the role of HIPK2 in modulating the function of AIRE in vivo, we compared whole-genome gene signatures of purified mTEC subsets from TEC-specific Hippk2 knockout mice with control mice and identified a small set of differentially expressed genes. Unexpectedly, most differentially expressed genes were confined to the CD80lo mTEC subset and preferentially included AIRE-independent TRAs. Thus, although it modulates gene expression in mTECs and in addition affects the size of the medullary compartment, TEC-specific HIPK2 deletion only mildly affects AIRE-directed pGE in vivo. The Journal of Immunology, 2015, 194: 000–000.

The thymus is the exquisite site of T cell development, a process that consists in the generation of a highly diverse TCR repertoire and subsequent selection (“quality control”) of those TCRs (i.e., thymocytes), which are both self-MHC restricted and self-tolerant (1). Intrathymic self-tolerance induction (central tolerance) entails the probing of a diverse array of self-MHC–restricted TCRs against a plethora of self-peptide/MHC complexes displayed by various APC lineages and subsets thereof. These include subsets of thymic dendritic cells (DCs), cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively), and thymic B cells (2). This diversity of thymic APCs with each subset presumably displaying partly nonoverlapping self-peptides (MHC ligandomes) ensures a maximal intrathymic representation of the immunological “self” of the body.

Intrathymically presented self-Ags are of intrathymic and extra-thymic origin. Extrathymic self-Ags enter the thymus via the blood or via immigrating DCs or are generated within the thymus by one particular APC type, mTECs, by a process known as promiscuous gene expression (pGE). MTECs transcribe and translate >3000 so-called tissue-restricted Ags (TRAs) (3–5). These TRAs are presented to thymocytes either autonomously by mTECs themselves or are handed over to neighboring DCs, which, in turn, process and/or present these transferred Ags. Both mTECs and DCs concurrently mediate the two major modes of self-tolerance imposition, namely, induction of apoptosis (recessive tolerance) or fate diversion into the regulatory T cell lineage (dominant tolerance) (2, 6). The scope of pGE represents essentially all tissues of the body, and thus ensures a maximal coverage of central tolerance. Failure of pGE results in...
a severe multiorgan autoimmune disease in mice and humans (7). How a specialized, terminally differentiated epithelial cell can override the tight regulation of tissue-specific gene expression is still poorly understood.

Progress in understanding the molecular regulation of such a diverse set of genes in one specialized cell type has been slow to come. The thymic transcriptional cofactor autoimmune regulator (Aire) is to date still the only identified molecular regulator specifically dedicated to control a sizeable fraction of the promiscuously expressed gene pool (7–9). It is currently thought that one major mode by which Aire targets such a wide array of genes is by relieving stalled polymerase II, thus allowing full-length transcription (10). Other general transcription factors like Myc (11) have been shown to also operate via elongation rather than initiation of transcription, although the target range of Aire seems to surpass that of other “classical” transcription factors (4, 5). Stalling polymerase II, which binds genomewide to promoters, would be one way to silence transcription of TRAs in mTECs (and other cell types), which are not supposed to be expressed in these cells. This mechanism would, at least in part, explain the significant enrichment of TRAs among the targets of Aire. In addition, Aire promotes induction of double-strand breaks and splicing of nascent mRNA transcripts (7–10, 12). Yet, there have to be other levels of Aire-dependent and -independent gene regulation, given the intricacies of pGE, that is, the mosaic expression pattern of TRAs at the population level and the TRA coexpression patterns at the single-cell level (13–15). Obviously any advance in understanding the molecular regulation of pGE by Aire and beyond would help in understanding how the mTEC-specific MHC ligandome is generated in the first place and importantly how dysregulation at the genetic and epigenetic levels could undermine central tolerance, and thus explain autoimmune pathologies.

Likewise, little is known about how Aire expression itself is regulated along mTEC differentiation and which posttranslational modifications ensure the function of Aire as part of a multiprotein transcriptional complex. One way to approach this complex issue is the identification of Aire’s partners within the nucleus either by biochemical or functional screens. A series of studies using either approach identified >40 factors binding either directly or indirectly to Aire or interfering with its transactivation (7–9, 12, 16, 17). Yet, the functional hierarchy of the various components through which Aire acts in such a complex remains completely unknown, and it is likely that the current list is still incomplete.

In this study, we used a yeast two-hybrid (Y2H) screen to identify additional potential Aire interaction partners. We identified homeodomain-interacting protein kinase 2 (HIPK2), a protein kinase involved in transcriptional regulation and cell fate decision as a novel partner. It directly binds to Aire, and the interaction has a functional impact on the cotranscriptional activity of Aire in vitro assays. Moreover, HIPK2 also influences pGE in mTECs in vivo, affecting transcription of both Aire-dependent and -independent genes.

**Materials and Methods**

**Yeast two-hybrid screen**

The Yeast two-hybrid screen was performed by the Genome and Proteome Core Facility at the German Cancer Research Center (DKFZ) as described previously in Albers et al. (18).

**GST pull-down assays**

The GST-AIRE expression vector was generated using standard PCR-based cloning procedure, and the AIRE cDNA sequence was confirmed by DNA sequencing. GST-AIRE fusion protein and GST control protein were expressed in *Escherichia coli* BL21 and purified as described using glutathione Sepharose beads (19). GST-pull-down assays were performed by incubation of GST and GST-AIRE with in vitro–translated, 35S-labeled HIPK2 or HIPK1. Subsequently, pull-downs were analyzed by reducing SDS-PAGE and autoradiography as reported previously (19).

**Immunofluorescence stainings**

H1299 cells were transfected to introduce cloned Myc-AIRE and Flag-HIPK2. For immunofluorescence analysis, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization for 5 min with 0.5% Triton X-100. The primary Abs anti-myc (1 μg/ml; mouse IgG1; Santa Cruz Biotech) and rabbit anti-flag (4 μg/ml; rabbit IgG; Sigma-Aldrich) were added and incubated for 1 h at room temperature. The secondary Ab staining with goat Alexa Fluor 488 anti-mouse (10 μg/ml; Molecular Probes) and goat Alexa Fluor 594 anti-rabbit (10 μg/ml; Molecular Probes) was performed for 40 min. Hoechst 33342 staining was used for nuclear staining.

On human thymus sections, all sections were performed, which were fixed in paraformaldehyde. Blocking was performed using goat serum. AIRE was stained by using the primary Ab clone 6.1; HIPK2 was stained using an affinity-purified HIPK2 Ab from rabbit (19). Goat anti-mouse IgG-Alexa 488 and goat anti-rabbit Cy3 were used as secondary reagents. Hoechst33342 served as a nuclear counterstain.

**In vitro phosphorylation assay**

In vitro phosphorylation of bacterially expressed and purified GST-AIRE was performed using 6×His-HIPK2 purified from *E. coli* essentially as described previously (20). In brief, 1.5 μg purified GST-AIRE was incubated with 100 ng 6×His-HIPK2 protein in kinase reaction buffer containing γ-[32P]-ATP as phosphate donor. In vitro phosphorylation reactions were analyzed by SDS-PAGE and autoradiography as described previously (20).

**Luciferase assay**

Gal4-reporter assays were essentially performed as described previously (21). In brief, 300 ng of an expression vector coding for the DNA binding domain of Gal4 or encoding a Gal4-AIRE fusion protein was transfected in 293 cells along with 500 ng Gal4-luciferase reporter gene, 50 ng RSV-lacZ (for normalization of the transcription efficiency), and either 750 ng Flag-HIPK2. Flag-HIPK2 K221A (kinase-deficient mutant), or empty Flag vector as indicated. Total DNA amounts were kept equal in all transfections by addition of empty Flag vector. Cells were harvested 24 h posttransfection, and analyzed and normalized using luminometric measurement as described previously (21).

**Mice**

Hipk2 conditional knockout (ko) mice (Hipk2floxflo) (*B6.Cg-Hipk2tm1Tgh*) were generated by Taconic Artemis GmbH (Köln, Germany). In brief, exons 3 and 4 of Hipk2 were targeted. The targeted region is flanked with loxP sites and a selection marker flanked with flippase recognition target (FRT) sites. C57BL/6Ntgic embryonic stem cells were electroporated with the targeting vector, and positive embryonic stem cell clones were validated by Southern blot. Validated clones were injected into mouse blastocysts to generate chimeric mice. To obtain germline transmission and to eliminate the selection marker, we bred the resulting chimeras to mice carrying a ubiquitously expressed Fpl transgene (C57BL/6-Tg(CAG-Fipe)-2Arte). Animals heterozygous for the transgene cassette were backcrossed onto the C57BL/6 background. To generate epithelial cell–specific Hipk2ko mice (*B6-Tg(Foxn1-cre)1Tbo Hipk2tm1Tgh*), we crossed Hipk2floxflo mice with Foxn1cre mice (*B6-Tg(Foxn1-cre)1Tbo*) (22). All breedings and cohort maintenance were performed in the central animal laboratory of the DKFZ under approved conditions in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Specific Purposes and the German Legislation.

**Genotyping of Hipk2 deletion**

The Hipk2 gene locus was analyzed in a three-primer PCR, using genomic DNA and the following primers: hipk2-1: 5′-GAAATTCTGGTGGACTCTT-CAGG-3′; hipk2-2: 5′-CTCATCCCAATGATCTTTGTCC-3′; and hipk2-3: 5′-CCCCAGCACAATAATGCTTCC-3′. The PCR conditions were as follows: initial activation of the polymerase for 3 min at 94°C, followed by 33 cycles of 30-s denaturation at 94°C, annealing for 30 s at 72°C and extension for 45 s at 72°C, and finally with a 5-min fill in step at 72°C. A band size of 174 bp was diagnostic for the wild-type allele, a band size of 402 bp for the floxed allele, and a band size of 295 bp for the deleted allele. Floxed Hipk2 mice were crossed with FoxN1Cre mice to generate a thymic epithelial cell–specific ko for Hipk2.
mTEC preparation

Primary mTECs were isolated by sequential fractionated enzyme digestion as previously described (14). Thymi were cut into pieces and digested in collagenase under magnetic stirring for 15 min at room temperature, followed by several digestion rounds in collagenase/dispase enzyme mix for 25 min at 37°C in a water bath under magnetic stirring. The collagenase/dispase enzyme mix was pooled and filtered through a 40-μm cell strainer. After digestion, the single-cell fraction was pre-enriched for thymic stromal cells by depleting CD45<sup>−</sup> cells with anti-CD45 magnetic beads and the AutoMACS (Miltenyi Biotec).

Pre-enriched stromal cell fractions were stained using the following Abs: 1) anti-CD45-PE-Cy5 (clone 30-F11; BD), anti-EP-CAM-A647 (G8.8 hybridoma), anti-CD80 (clone 16-10A1; BD Pharmingen), anti-Ly51-FITC (clone 6C3; BD Pharmingen); Dead cell staining was performed using propidium iodide in a final concentration of 0.2 μg/ml. Cells were sorted on an Aria II cell sorter from BD. mTECs were defined as CD45<sup>+</sup>Ly51<sup>−</sup>EpCAM<sup>+</sup>, of which the CD80<sup>−</sup> and the CD80<sup>+</sup> subsets were sorted separately; 2) Aire staining: anti-CD45-PerCP (clone 30-F11; BD Pharmingen), anti–EpCAM-A647 (G8.8 hybridoma), anti–CD80-PE (clone 16-10A1; BD Pharmingen), anti–CD1-Pacific Blue (cell culture supernatant (23), Pacific Blue Protein Labeling Kit from Life Technologies), anti–Aire-FITC (clone 5H12) (24), Fixable Viability Dye eFluor 780 (eBioscience).

Total RNA preparation

Total RNA of primary sorted mTECs was isolated and purified using the High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer’s protocol.

For quantitative PCR (qPCR), the isolated RNA was reverse transcribed into cDNA using random primers and Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer’s protocol.

Quantitative real-time PCR

Quantitative real-time PCR was performed in a total volume of 20-μl reactions using Power Sybr Green Mix (Applied Biosystem) and the GeneAmp 7300 (Applied Biosystem). Intron spanning primers were designed using Primer3 software. Reactions were performed in technical duplicates and normalized to total thymus cDNA using the 88 cycle threshold method.

Microarray analysis

Isolated purified RNA samples were run on Illumina MouseWG-6 v2.0 Expression Bead Chip Sentrix arrays. Labeling of the samples and hybridization were performed by the Genome and Proteome Core Facility of the DKFZ in Heidelberg.

The microarray values were quantile normalized followed by Limma analysis to identify differentially expressed genes between wild-type and HIPK2-deficient animals. Each microarray analysis was performed in biological duplicates for mTEC<sup>WT</sup> and mTEC<sup>KO</sup> fractions on the Hipk2<sup>+/−</sup> and Hipk2<sup>−/−</sup> background. Genes with a fold change of >2 or <0.5 and with a p value ≤0.0005 were considered to be differentially expressed. Statistical t test calculation was performed in R. Chipster software was used for further data analysis.

TRAs were defined using the public database (http://symatlas.gnf.org) (25). A gene was defined as a TRA if its expression was five times more than the median expression over all tissues in less than five tissues (26).

The significance of TRA enrichment (p value) was calculated using χ<sup>2</sup> tests.

Gene ontology (GO) analysis on the differentially expressed genes was performed using GeneCodis (27–29). The p values were computed using the hypergenomic distribution method (for further information, see http://genecodis.dacya.ucm.es) in combination with the false discovery rate p value correction (30).

Results

HIPK2 interacts with AIRE, and both factors colocalize at nuclear bodies

To identify novel AIRE-interacting proteins, we performed a Y2H screen. We used an AIRE deletion mutant lacking its potential auto-antitoxing PHD2 domain as bait to screen a murine testis and total mouse embryo (E17) cDNA library. Note that testis (i.e., spermatogonia and spermatocytes) is one of the few tissues expressing Aire at the mRNA and protein level and displaying pGE (31, 32). Using this approach, we identified cDNA clones encoding Ubc9 (also called Ube2i), protein inhibitor of activated STAT 1 (PIAS1), desumoylating isopeptidase-1 (DeSi-1), ZFP451, and HIPK1 as AIRE-interacting factors (Supplemental Fig. 1A–E).

Of note, PIAS1 (33) and Ubc9 (34) have been previously described as AIRE binding proteins, testifying to the specificity of our Y2H screen.

We decided to focus on the HIPK family kinases as potential novel AIRE binding proteins, because, similar to what has been reported for the AIRE protein (35), HIPKs localize to nuclear bodies (NBs), which overlap with promyelocytic leukemia (PML) NBs (36). Because HIPK family members are highly conserved showing a largely identical domain structure and amino acid composition (37), we analyzed potential interactions of AIRE with two HIPK family members, HIPK1 and HIPK2. GST pull-down assays revealed a weak interaction of AIRE with HIPK1 in vitro (Supplemental Fig. 1F). In contrast, a strong interaction of AIRE and the HIPK family member HIPK2 was detected, and this in-
teraction was comparable in strength with the interaction observed between HIPK2 and its known partner protein p53 (36) (Fig. 1A). Thus, we focused our analysis on a potential interplay between AIRE and HIPK2. Because AIRE and HIPK2 are known to localize to NBs, we asked whether both factors might colocalize in the cell nucleus. In fact, immunofluorescence staining and confocal microscopy of ectopically expressed AIRE and HIPK2 revealed a partial colocalization of both factors in the nucleus and at NBs (Fig. 1B). This subcellular localization is compatible with previous reports showing independently that each molecule localizes to PML NBs, a subtype of NBs (35, 36). To substantiate AIRE-HIPK2 colocalization under physiological conditions, we performed immunofluorescence staining of endogenous AIRE and HIPK2 proteins in sections from human thymus. Remarkably, confocal microscopy identified colocalization of AIRE and HIPK2 in part of the mTECs (Fig. 1C), compatible with a functional interplay between AIRE and HIPK2 under physiological conditions in the thymus. Taken together, these results indicate that HIPK2 is a novel AIRE-interacting protein that partially colocalizes with AIRE in the cell nucleus and in NBs.

**HIPK2 phosphorylates AIRE in vitro and represses its transcriptional coactivator function in a kinase-dependent manner**

We next analyzed whether AIRE might be a substrate for HIPK2. To this end, we performed in vitro kinase assays in the presence of γ-[32P]−ATP using bacterially expressed, recombinant His-HIPK2 and GST-AIRE as substrate. AIRE phosphorylation was analyzed by SDS-PAGE and subsequent autoradiography. His-HIPK2 was found to be strongly auto-phosphorylated (Fig. 2A), which is a prerequisite for its proper activity (20). Interestingly, the full-length AIRE protein (and truncated AIRE products) was clearly phosphorylated by HIPK2. These data indicate that HIPK2 directly phosphorylates AIRE in vitro.

We next addressed the functional consequence of HIPK2-mediated AIRE phosphorylation. AIRE is essential for regulating pGE in the thymus (8). To address a potential impact of HIPK2 on AIRE-mediated transcriptional activity, we fused AIRE to Gal4 and measured its transactivating activity using a Gal4-dependent luciferase reporter gene assay. Gal4-AIRE expression resulted in a profound increase in reporter gene activity, indicating that Gal4-AIRE functions as a transcriptional coactivator (Fig. 2B). Coexpression of wild-type HIPK2 resulted in a dose-dependent reduction of the AIRE-driven reporter gene activity, indicating that HIPK2 represses AIRE-controlled transactivation, as has been previously reported for DAXX (38). Of note, this repressive effect of HIPK2 required its kinase activity, as a kinase-deficient HIPK2 point mutant (HIPK2 K221A) failed to suppress the coactivator function of AIRE (Fig. 2B). These results indicate that HIPK2 modulates AIRE-regulated gene expression in a kinase-dependent fashion.

**Phenotype of TEC-specific HIPK2 deficiency in vivo**

To assess whether the AIRE–HIPK2 interaction had an in vivo effect on the regulation of gene expression in mTECs in general and the regulation of pGE in particular, we analyzed primary mouse mTECs. A conditional HIPK2 ko mouse line containing LoxP-flanked Hipk2 alleles was generated and crossed with a mouse strain expressing Cre recombinase under control of the TEC-specific promoter of the FoxN1 gene to inactivate Hipk2 in both TEC lineages (Fig. 3A). MTECs were isolated from HIPK2ko and control mice, and was analyzed by Illumina bead microarrays (deposited in the Gene Expression Omnibus under accession number GSE63432).

Within the biological duplicates of each experiment, the two different mTEC subsets, that is, mTECs CD80low versus mTECs CD80high, clustered together (Fig. 4A). Furthermore, within the CD80low populations, there was a close correlation between the wild-type and HIPK2-ko mTECs of the biological duplicates. In the CD80low fraction, the variation between the biological duplicates was found to be higher. The top 139 differentially regulated genes (filtered according to 3 SD = 99.7%) are displayed by the heat map (Fig. 4B). The biological significance cutoff was set to ≥2-fold differential expression in mTECs of ko mice compared with the controls. To compare changes in gene expression levels between enriched immature and mature mTECs in Hipk2ko and control mice, the differential expression was analyzed and represented using scatterplots. As previously reported, CD80low and CD80high control mTECs clearly differed in their mRNA transcriptome (20) (genes plotted outside the red lines, indicating a ≥2-fold differential ex-

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**FIGURE 2.** HIPK2 phosphorylates AIRE and regulates AIRE-dependent cotranscriptional activity. (A) AIRE serves as a HIPK2 substrate in vitro. In vitro phosphorylation assays were performed using recombinant His-HIPK2 and GST-AIRE proteins in the presence of radioactively labeled ATP. Phosphorylation was visualized by autoradiography. (Left panel) autoradiogram; (right panel) corresponding Coomassie Brilliant Blue staining. Asterisks indicate truncated Aire products. (B) HIPK2 regulates AIRE-controlled cotranscriptional activity in a kinase-dependent fashion. Luciferase reporter gene assays are shown. The luciferase activity of a 5xGal4-luciferase reporter was measured. The Gal4-Luciferase reporter was transfected as indicated with Gal4, Gal4-Aire, HIPK2, and a kinase-deficient HIPK2K221A point mutant. The means and SDs of three independent experiments are shown.
expression). This was also the case for Hipk2ko mice (Fig. 4C, 4D). A comparison of the CD80lo mTECs between Hipk2 ko and control mice showed differential expression of a limited set of genes between these two groups (Fig. 4E). In contrast, in the CD80hi mTECs, only seven genes were differentially expressed when comparing control versus Hipk2ko mice (Fig. 4F). Thus, HIPK2 deficiency had only a limited effect on the steady-state transcriptome of mTECs irrespective of their maturation stage (Supplemental Table I).

Interestingly, the array analysis revealed that HIPK2 absence resulted in downregulation of the majority of differentially expressed genes (not predicted by the suppressive effect of HIPK2 on the coactivating activity of Aire; Fig. 2). This observation was validated...
by quantitative real-time PCR analysis of the top 10 gene hits of the array list, with both assays showing highly concordant results (Fig. 5).

**HIPK2 deficiency affects the expression of TRAs in mTECs**

Next, we asked whether the differentially expressed gene pool displayed features of pGE, that is, enrichment for Aire dependency and TRA content as insinuated by the in vitro effect of HIPK2 on the function of AIRE (Fig. 2). The number of differentially expressed genes and TRAs were normalized to the relative representation of TRAs on Illumina bead array. We observed a significant increase (≥2-fold increase) in the representation of TRAs among the differentially downregulated genes when compared with the total gene pool (Fig. 6A).

Further, we investigated whether HIPK2 deficiency had a selective effect on Aire-dependent genes. We therefore compared the number of differentially downregulated Aire-dependent and -independent genes with their normal distribution on the Illumina bead array. This calculation gives the relative extent of Aire-dependently regulated genes affected by the Hipk2 ko phenotype (Fig. 6B). There was no specific influence of the Hipk2 ko on Aire-dependent gene expression observed. Thus, HIPK2 does not selectively affect the Aire-dependent gene pool.

To further characterize the HIPK2 target gene pool in mTECs, we performed a GO analysis using the GeneCodis3 software. The differentially expressed genes were tested for their annotation classification concerning the cellular component, biological process, and molecular function (Supplemental Fig. 2). The cellular component analysis provided mainly hits in transmembrane, membrane coupled, or associated hits, followed by the heterotrimeric G protein complex annotation. The biological process analysis identified ion transport and cation transport as main hits, followed by ATP biosynthetic processes. The most prominent hits of the molecular function annotation analysis were voltage-gated ion channel activity and ATPase activity coupled to transmembrane movements of ions. Taken together, the GO annotation analysis of the HIPK2 target gene pool implies selective enrichment for genes involved in signaling transduction pathways.

Interestingly, TEC-specific HIPK2 deficiency also resulted in a significant contraction of the medullary, but not of the cortical compartment. Thus, the number of mTECs was reduced by 62% (58% in CD80 hi; 70% in CD80 lo), whereas cTEC numbers remained unaltered (Supplemental Fig. 3A–C). The mTEC/cTEC ratio was reduced by 50%, whereas the mTEC/mTEC<sup>lo</sup> ratio was unaltered between Hipk2<sup>ko</sup> and control mice (Supplemental Fig. 3D). The frequency of Aire-positive mTECs was comparable in Hipk2<sup>ko</sup> compared with control mice (Supplemental Fig. 3E), analyzing 4-wk-old (control: 16.0%; Hipk2<sup>ko</sup>: 21.5%) and 7-wk-old mice (control: 12.6%; Hipk2<sup>ko</sup>: 11.4%). Taken together, thymic deletion of HIPK2 results in an overall reduction of the mTEC compartment without altering its major subset composition.

**Discussion**

In this study, we used a protein–protein interaction screening approach to identify novel AIRE binding factors. We identified five interaction partners, two of which, PIAS1 and Ubc9, had been previously reported to interact with Aire (33, 34). We provide evidence that HIPK2, a known regulator of cell fate and transcription, is a novel Aire binding protein, adding to the growing list of Aire binding partners and Aire “allies” (7–9, 12, 16, 17). In accordance with the physical interaction observed between both factors, AIRE colocalizes with HIPK2 in the cell nucleus in NBs both upon ectopic expression and also under physiological conditions in situ in thymic sections. In addition, HIPK2 regulates the transactivating function of AIRE in a kinase-dependent fashion. Because our data indicate that AIRE is a HIPK2 substrate, which is directly phosphorylated by HIPK2 in vitro, HIPK2 presumably regulates the transcriptional activity of AIRE through phosphorylation.

HIPK2 is known to be functionally modulated through covalent modification with small ubiquitin-like modifier (SUMO)-1 (39, 40). Interestingly, three other factors identified in our Y2H screens, namely, Ubc9, PIAS1, and DeSi-1, are known enzymatic regulators of the posttranslational SUMO modification pathway. The SUMO conjugating enzyme Ubc9 and the SUMO E3 ligase PIAS1 actively catalyze the SUMOylation of substrates, whereas DeSi-1 functions as an antagonist of SUMOylation by catalyzing deconjugation of SUMO from substrate proteins. Yet, PIAS, previously identified as an AIRE interacting protein, does not lead to SUMO modification of AIRE (33). However, ectopic expression of PIAS1 attracted AIRE to SUMO-1–containing nuclear complexes, which suggests that AIRE function may be controlled by the SUMO pathway, which can modulate protein localization and plays a prominent role in transcriptional regulation (41).

Notably, AIRE and HIPK2 share some of their interacting proteins including the transcriptional cofactors CBP and DAXX, two factors shown to be recruited, similar to AIRE and HIPK2, to PML NBs in response to cellular stress (36, 38, 40, 42–44). HIPK2 interacts with CBP and stimulates the acetylation activity of CBP/p300 (36, 45). Remarkably, acetylation has been shown to affect different aspects of the function of Aire, that is, the selection of target genes (46), protein stability, and nuclear localization (34). The tripartite interaction between Aire, HIPK2, and CBP may thus stimulate Aire acetylation and phosphorylation, with the latter leading to suppression of coactivator activity in vitro.

Immunohistochemical staining of sections from human thymus revealed colocalization of AIRE and HIPK2 in a subset of mTECs under physiological conditions. Of note, ectopic expression of AIRE has been shown to stimulate the induction of DNA double-strand breaks (12). Interestingly, DNA double-strand breaks are a well-known trigger of HIPK2 stabilization and activation by
stimulating activation of the DNA damage checkpoint kinase ATM (19, 47). Through engaging the DNA damage checkpoint kinase ATM, AIRE may provoke HIPK2 activation and stabilization, thereby leading to the observed coexpression/colocalization of AIRE and HIPK2 in mTECs.

Our results show that the genetic ablation of HIPK2 modulates the promiscuous expression of TRAs in mTECs, though the affected number of genes was relatively low. Moreover, analysis of the Aire dependency of the differentially expressed genes showed the Hipk2ko effect not to be restricted to Aire-dependent genes, but instead other genes were also regulated. Our in vitro data indicated that HIPK2 had a repressive effect on Aire’s transcriptional regulatory capability predicting that the removal of HIPK2 would thereby lead to an upregulation of target genes. Yet, we observed that the majority of differentially regulated genes in Hipk2ko mice were downregulated. Hence the effect of HIPK2 deficiency on Aire-dependent regulation of TRAs might be compensated for by another regulatory component. It is possible that another HIPK family member such as HIPK1 might instead phosphorylate Aire in place of HIPK2 (Fig. 7A).

Furthermore, we did not observe an exclusive effect on Aire-dependent, but rather Aire-independent genes were also affected by HIPK2 deficiency, implying that HIPK2 might modify another factor(s) regulating pGE in mTECs (Fig. 7B). We hypothesize that HIPK2 regulates this factor(s) by phosphorylation, thereby activating its transcriptional activity, which, in turn, would lead to promiscuous expression of certain TRAs. In the absence of HIPK2, these particular of TRAs would be downregulated.

Among the affected genes, we noted components of signaling cascades regulating cell migration, differentiation, or motility. Interestingly, we could also observe an effect of HIPK2 deficiency on the overall size of the mTEC compartment; that is, the number of CD80lo and the mature CD80 hi mTECs was substantially reduced, whereas their ratio (i.e., their developmental progression) was not altered. Given the heterogeneity of CD80lo mTECs with respect to CCL-21 expression and a pre- and post-Aire stage (48–50), we cannot exclude that the transcriptional changes observed in this study were confined to either of these subsets.

It is currently unclear why the medulla in contrast to the cortex was selectively affected by HIPK2 deficiency and how this relates to the aforementioned changes in gene expression of mTECs. In summary, our data add HIPK2 to the growing list of molecules that modify Aire posttranslationally and regulate pGE and mTECs development, two intricately connected processes.

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Disclosures

The authors have no financial conflicts of interest.

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