Keratin-dependent regulation of Aire and gene expression in skin tumor keratinocytes

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Expression of the intermediate filament protein keratin 17 (K17) is robustly upregulated in inflammatory skin diseases and in many tumors originating in stratified and pseudostratified epithelia1–3. We report that autoimmune regulator (Aire), a transcriptional regulator, is inductively expressed in human and mouse tumor keratinocytes in a K17-dependent manner and is required for timely onset of Gli2-induced skin tumorigenesis in mice. The induction of Aire mRNA in keratinocytes depends on a functional interaction between K17 and the heterogeneous nuclear ribonucleoprotein hnRNP K4. Further, K17 colocalizes with Aire protein in the nucleus of tumor-prone keratinocytes, and each factor is bound to a specific promoter region featuring an NF-κB consensus sequence in a relevant subset of K17- and Aire-dependent proinflammatory genes. These findings provide radically new insight into keratin intermediate filament and Aire function, along with a molecular basis for the K17-dependent amplification of inflammatory and immune responses in diseased epithelia.

High levels of expression of KRT17 (encoding K17) have been correlated with aggressive behavior and poor prognosis for several types of human tumors2. Genetic loss of Krt17 but not the related Krt14 delays tumor onset in a Gli2-transgenic mouse model of basaloid skin tumorigenesis, correlating with striking changes in the amplitude and character of inflammatory and immune responses2. Moreover, K17 positively regulates several effectors of mitogenic signaling associated with oncogenic transformation (for example, Akt-PKB6, mTOR7 and the Rac1 GTPase8) and is necessary to sustain normal Akt-PKB-mediated oncogenic transformation in Ewing sarcoma6. Whether K17 influences additional types of tumors in a similar manner and which mechanisms account for its effect are unknown.

Targeted expression of the early genes from human papillomavirus (HPV) type 16 (E1 to E7) to basal keratinocytes of the epidermis causes tumors in adult mouse skin that resemble human skin squa-
mous cell carcinoma9. Tumors arise with complete penetrance between postnatal day (P) 60 and P120 in HPV16Tg/+ ear skin (FVB/N strain) (Supplementary Fig. 1a), with no apparent discordance between the sexes. Although normally restricted to ectodermal appendages and glabrous skin10, expression of K17 is robustly upregulated in the interfollicular epidermis between P20 and P40 in HPV16Tg/+ mice (Supplementary Fig. 1b). In comparison to HPV16 Tg/+; Krt17−/− mice, HPV16 Tg/+; Krt17−/− mice exhibit delayed hyperplasia and tumorigenesis in ear skin (Fig. 1a,b and Supplementary Fig. 1c). As has been observed in Gli2Tg/+; Krt17−/− mice5, the delay correlated with profound reductions in key determinants of tumor growth, including mitotic activity (Fig. 1c), blood vessel expansion (Supplementary Fig. 1d), and inflammatory and immune response readouts (Supplementary Fig. 1e) such as myeloperoxidase activity reflecting neutrophil infiltration (Fig. 1d) and dermal mast cell density (Fig. 1e). Transgene expression, p53 and apoptosis levels were indistinguishable between the genotypes in ear skin (Supplementary Fig. 1f–h), and there was no indication of cell fragility in HPV16 Tg/+; Krt17−/− skin by electron microscopy (Supplementary Fig. 1i). Commercial quantitative RT-PCR (qRT-PCR) arrays identified many proinflammatory cytokines whose expression was significantly depressed in tumor-prone ear tissue from HPV16 Tg/+; Krt17−/− mice relative to the HPV16 Tg/+; Krt17+/− reference model (Supplementary Tables 1–5). Thus, the loss of Krt17 delays tumorigenesis in mouse skin and significantly attenuates the expression of several key proinflammatory signaling molecules in two oncogenic paradigms (Gli2Tg and HPV16 Tg) involving strains that are differentially sensitive to skin carcinogenesis (C57BL/6 and FVB/N, respectively)11.

To explore how proinflammatory gene expression might be regulated by K17, we devised a custom, 96-well plate–based qRT-PCR assay to quantify the mRNA levels for inflammation- and disease-relevant genes (Supplementary Table 6) in A431 cells (derived from a human epidermoid carcinoma and expressing K17; ref. 1).

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Twenty-two genes were consistently upregulated (>2-fold) in A431 cells after treatment with TPA (12-O-tetradecanoylphorbol-13-acetate) (Supplementary Fig. 2a), which elicits a robust inflammatory response in keratinocytes.12 Of these genes, 19 were similarly upregulated in P40 HPV16Tg/+ ear tissue relative to age-matched wild-type ear tissue (Fig. 2a). In comparison to A431 cells with non-silencing short hairpin RNA (shRNA) control, A431 cells with stable KRT17 knockdown13 exhibited a substantially attenuated response to TPA (Supplementary Fig. 2a). Reintroduction of K17 but not the highly homologous K42 protein14 largely restored TPA-dependent cytokine upregulation in A431 cells (Supplementary Fig. 2b). Therefore, the expression of multiple proinflammatory cytokines also depends on K17 in human skin tumor keratinocytes.

Several observations converged on Aire (autoimmune regulator), a transcriptional regulator with a well-known role in the medullary thymus in the establishment of tolerance15–17, as a component of the K17-mediated regulation of gene expression in skin keratinocytes. First, mutation in AIRE causes autoimmune polyendocrinopathy–candidiasis–ectodermal dysplasia (APECED)18, which often presents with candidiasis–ectodermal dystrophy (APECED)18, which often presents with cutaneous manifestations including alopecia, nail dystrophy, vitiligo and enamel hypoplasia10. Second, several of the proinflammatory genes determined to have expression dependence on K17 in tumor-prone keratinocytes (Supplementary Fig. 2a) have Aire-dependent expression in thymic epithelial cells (for example, Ccl19, Ccl22, Cxcl5, Ccr7, Il6 and Cda40)19–21. Third, qRT-PCR and/or RNA in situ hybridization showed that Aire mRNA expression was induced and dependent on K17 in several settings, including TPA-treated human A431 cells (Fig. 2b), ear tissue from P40 HPV16Tg/+ (Fig. 2b,d) and P70 Glit2Tg/+ (Fig. 2b) mice, and TPA-treated ear skin from C57BL/6 and FVB/N wild-type mice (Fig. 2c and Supplementary Fig. 2c). Sense-strand controls for RNA in situ hybridization yielded negative findings (Supplementary Fig. 2d,e). Aire expression was low in untreated or wild-type skin (Fig. 2c,d),22, and the observed levels of induced Aire mRNA in treated or diseased skin remained below constitutive levels in the thymus (Supplementary Fig. 2c). Fourth, there was a report of a physical interaction between the AIRE and K17 proteins23, which we confirmed (Supplementary Fig. 2f). Finally, we crossed Aire−/− mice with Glit2Tg/+ mice (both available in the C57BL/6 strain) to assess the impact of Aire loss on skin tumorigenesis (Fig. 2e). The average age of tumor onset was 79 ± 3.7 (s.e.m.) d in male Glit2Tg/+; Aire−/− mice, in comparison to 65 ± 3.9 d in Glit2Tg/+ mice (*P < 0.001). An average delay of 23 d was previously reported for Glit2Tg/+; Krt17−/− mice (Fig. 2f).

Notably, Aire expression was not dependent on K17 in the medullary thymus (Supplementary Fig. 3a,b), which expresses Krt17 (ref. 10). Further, loss of Krt17 did not induce a break in central tolerance leading to systemic autoimmunity, as is seen with Aire loss24 (Supplementary Fig. 3c–e). Our findings thus identify a new and major role for extrathymic Aire expression in keratinocytes, particularly during skin tumorigenesis, where its expression depends on K17.

We next investigated how Aire mRNA levels depend on K17. The ribonucleoprotein hnRNP K, a versatile regulator of gene expression with a known role during tumorigenesis25, physically and functionally interacts with K17 to influence expression of the CXCR3 ligands CXCL9, CXCL10 and CXCL11, along with several other cytokines, in tumor skin keratinocytes. We report here that Aire mRNA is also bound to hnRNP K, in a TPA- and K17-dependent manner, in human A431 and mouse Glit2Tg keratinocytes (Fig. 2g,h). The TPA-dependent induction of AIRE mRNA was markedly attenuated in A431 keratinocytes pretreated with small interfering RNA (siRNA) targeting hnRNP K (Fig. 2i). Relative to empty vector control, overexpression of hnRNP K was sufficient to increase AIRE levels in A431 cells, again in a K17-dependent manner (Fig. 2j). Thus, Aire mRNA transcripts are regulated in a K17- and hnRNP K-dependent manner in skin tumor keratinocytes.

We next assessed whether K17 also regulates Aire at the protein level. Consistent with previous reports26,27, AIRE fusion proteins expressed via transfection frequently localized to round and smooth-surfaced punctae in the nucleus and cytoplasm of human A431 cells (Fig. 3a) and mouse keratinocytes (data not shown) in the absence of stimulus. These AIRE-positive punctae were distinct from lysosomes and stress granules but occurred immediately adjacent to PML (promyelocytic leukemia) nuclear bodies (Supplementary Fig. 4a),
which are associated with the storage of proteins poised for transcriptional activation. Further, heat shock protein 70 (HSP70), previously identified to associate with AIRE and keratin, colocalized with the AIRE-positive nuclear punctae and did so in a K17-dependent manner (Supplementary Fig. 4b). Strikingly, within 1 h of TPA treatment, the nuclear localization pattern of AIRE shifted from punctate to diffuse (Fig. 3a). This shift was markedly hindered in A431 lines expressing K17, and K14 and K18, which were also present in A431 keratinocytes, did not yield the same fractionation pattern and were not observed in the nucleus under these assay conditions (Supplementary Fig. 4h).

We next asked whether K17 might also localize to the nucleus (along with Aire) in keratinocytes. In the presence of leptinomin B (LMB), which inhibits exportin 1–dependent nuclear export (thereby trapping low-abundance or rapidly shuttling proteins in the nucleus), K17 was present in the form of intranuclear punctae in human A431 keratinocytes (Fig. 3b), where it readily colocalized with AIRE protein expressed via transfection (Fig. 3c), and in HeLa cells (Supplementary Fig. 4c,d). Intranuclear K17 was also present in LMB-treated mouse epidermal keratinocytes in primary culture, where it colocalized with the type II partner K5 (Supplementary Fig. 4e). No evidence of nuclear (or any form of) K17 could be detected in LMB-treated Krt17−/− mouse keratinocytes (Supplementary Fig. 4f), establishing the specificity of our reagents. Presence of K17 and Aire in the nucleus was further supported by immunoblot analyses performed on subcellular fractions from cells not treated with LMB, including one enriched for nucleoplasmic proteins (for example, histone H3) and devoid of nuclear envelope markers (Supplementary Fig. 4g). By contrast, K14 and K18, which were also present in A431 keratinocytes, did not yield the same fractionation pattern and were not observed in the nucleus under these assay conditions (Supplementary Fig. 4h). Furthermore, nuclear K17 could be readily observed in human tumor keratinocytes in tissue sections prepared from biopsies of skin basal cell carcinomas from patients (Fig. 3d). Bioinformatic analyses identified a putative nuclear localization sequence (NLS) within the K17 sequence (Fig. 3e), a dilysine motif in the tail domain that is conserved across species for K17 but is not present in other type I intermediate filament proteins. In comparison to wild-type K17, GFP-K17 (with either the human or mouse sequence) harboring a single point substitution in the putative NLS (p.Lys399 Ala) when
Figure 3 K17 regulates Aire subnuclear distribution and localizes to the nucleus to promote gene expression. (a) Apotome-acquired images of the subnuclear distribution of mCherry-AIRE in A431 keratinocytes. The graph indicates the percentage of cells showing a punctate (blue) or diffuse (red) pattern after TPA treatment, relative to treatment with DMSO. shKRT17, cells stably expressing shRNA targeting KRT17; shControl, cells stably expressing empty shRNA vector. n is the number of cells analyzed. (b) Single-plane confocal images of vehicle- (70% methanol) or LMB-treated A431 keratinocytes immunostained for K17 and IκBα (positive control for LMB treatment). The images to the right highlight K17-positive nuclear punctae (arrows) and include the z-plane image. (c) Images as in b, except that the A431 cells were transfected with vector expressing mCherry-AIRE before treatment and K17 immunostaining. Images in a–c are representative of 15, 10 and 5 distinct experiments, respectively. Scale bars: 5 µm (a–c) and 1 µm (z planes). (d) Single-plane confocal images of K17 immunostaining (green; or staining with rabbit IgG control) in tissue sections of human skin basal cell carcinoma. Bottom frames, 3× digital magnification of the boxed regions in the top row. Arrows indicate K17-positive nuclear punctae. Scale bars, 20 µm. (e) Schematic of keratin protein highlighting a conserved predicted bipartite NLS (in bold letters). The asterisk indicates the position of Lys399. (f) Graph depicting the percentage of HeLa cells with nuclear punctae positive for GFP-K17, wild type (WT) or Lys399Ala (NLS mutant), as observed by confocal microscopy. n is the number of cells. (g) Graph depicting the percentage of A431 cells where mCherry-AIRE punctae colocalize with GFP fused to wild-type or Lys399Ala K17. n is the number of mCherry-AIRE–positive cells counted. (h) Normalized transcript levels of TPA-induced target genes in A431 K17-null keratinocytes transfected with vector expressing wild-type or Lys399Ala K17 fused to GFP, with either human (left) or mouse (right) K17 sequence. n = 3 biological replicates. Error bars, s.e.m.
expressed through transient transfection in K17-null A431 keratinocytes (Online Methods) showed attenuated ability to localize to the nucleus (Fig. 3f), colocalize with nuclear mCherry-AIRE punctae (Fig. 3g) and foster a full cytokine expression response after treatment with TPA (Fig. 3h). These findings establish that K17 can localize to the nucleus and suggest that the nuclear form of K17 might have a role in rapid dispersion of the normally quiescent Aire-containing nuclear punctae after a relevant stimulus.

Although not believed to directly bind DNA, Aire binds a plethora of transcriptional proteins to activate target gene expression. We conducted chromatin immunoprecipitation (ChIP) assays to assess whether Aire protein, along with K17, was present at the promoter of relevant inflammatory and immune response genes in tumor-prone keratinocytes (Supplementary Table 7). Using RFP-Trap beads in A431 cells transfected to express mCherry-AIRE (with vector encoding mCherry as a control), ChIP analysis showed enrichment for specific segments within the proximal 5′ upstream region for MMP9, CCL19, CXCL10 and CXCL11 but not control genes (Fig. 4a,b). Endogenous K17 could readily be immunoprecipitated from nuclear fractions prepared from A431 cells, whether TPA treated or not (Supplementary Fig. 5a). After TPA treatment, the same or adjacent segments within the MMP9, CCL19, CXCL10 and CXCL11 gene promoters were enriched in K17 immunoprecipitations (Fig. 4c,d). We observed no enrichment after DMSO treatment or with immunoprecipitation using preimmune serum. We verified that the PCR-amplified promoter fragments migrated at the expected size in gel electrophoresis (Supplementary Fig. 5b). These ChIP findings indicate that K17 and AIRE each associate with the same promoter regions of select target genes with K17-dependent expression.

Analysis of the promoter segments enriched in ChIP samples for mCherry-AIRE and K17 identified the presence, in all cases, of consensus binding sites for nuclear factor (NF)-κB (5′-GGGRNNYYCC-3′, where R is any purine, N is any base and Y is any pyrimidine) (Supplementary Fig. 5c,d). NF-κB has a keratinocyte-autonomous role in skin inflammatory conditions and thus represents an ideal candidate to mediate the recruitment of K17 to relevant gene promoters. Electrophoretic mobility shift assays (EMSAs) confirmed that a protein complex associated with an NF-κB consensus oligonucleotide
probe in a TPA-dependent manner in nuclear extracts prepared from A431 keratinocytes (Fig. 4e). Adding antisera against either K17 or the NF-kB p65 subunit yielded a supershift in the mobility of the NF-kB probe only in the presence of TPA (Fig. 4e). The use of pre-immune serum or IgG control antibodies (Fig. 4e), excess unlabeled oligonucleotide as a competitor (Fig. 4f) or Oct1 oligonucleotide to which binding was not induced by TPA (Supplementary Fig. 5e) confirmed specificity. Lastly, reciprocal coimmunoprecipitation indicated that endogenous K17 and p65 interact in A431 keratinocytes (Supplementary Fig. 5f). p65 (NF-kB) thus might act as a molecular bridge between K17 and Aire at promoter sequences for the specific set of genes that these factors co-regulate in tumor-prone skin keratinocytes.

In conclusion, the findings reported here establish a new role for K17 in regulating gene expression at the transcriptional level in skin keratinocytes, which involves a nucleus-localized form of this keratin protein (Fig. 4g). They provide a mechanistic basis for a possible feedback loop whereby K17 upregulation, an early event when inflammation sets in, would promote the maintenance of a specific type of proinflammatory and immune responses in skin. Lastly, they also establish the involvement of Aire in promoting gene expression in keratinocytes and skin undergoing acute inflammation or tumorigenesis, a newly defined role that requires a physical and functional partnership with K17.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.P.H. and D.J.D. jointly led the characterization of skin tumorigenesis in the HPV mouse model, with assistance from M.C.H. and A.S.B. R.P.H. led the studies performed in A431 cells and mouse keratinocytes in culture, with contributions from J.T.J., B.-M.C., B.G.P., Y.G. and J.H.S.O. and D.C. conducted central tolerance study with assistance from R.P.H. and A.S.B. J.M.T. provided human basal cell carcinoma samples. W.Z. and F.W. performed the flow cytometry analyses. F.W. provided guidance to R.P.H. for EMSA analyses. R.P.H., D.J.D. and P.A.C. contributed to experimental design and data analysis. R.P.H. and P.A.C. jointly wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mouse models. All protocols involving mice were approved by the Johns Hopkins Institutional Animal Care and Use Committee. C57BL/6 Krt17−/− mice were bred to HPV16/ S. transgenic mice (FVB/N strain; obtained from the National Cancer Institute) to create HPV16/ Tg+/- mice. The resulting HPV16/ mice were backcrossed with wild-type FVB/N mice for at least six generations. To generate Gl2Z/; Krt17−/− mice; C57BL/6 Aire−/− mice were obtained from Jackson Laboratories (stock 0006360) and bred with C57BL/6 Gl2Z/ mice; resulting Gl2Z/; Aire−/− mice were crossed with Gl2Z/; Krt17−/− littermates to generate the progeny of interest. All mice with tumorigenic transgenes were housed individually or with littermate controls upon weaning and were fed rodent chow and water ad libitum. Both male and female mice were used in this study. The sample size of mice required for this study was empirically determined from previous experience5. Mice deemed sick by veterinary staff were not used. Investigator blinding and randomization were not conducted. Genotyping protocols have been described previously for the Gl2Z (ref. 5), Krt17 (ref. 5), HPV16 (National Cancer Institute Mouse Repository, strain 01XT3) and Aire (Jackson Laboratories, stock 000630) alleles.

Cell lines. Mouse epidermal keratinocytes were isolated for primary culture or immortalized as described37. Parental A431 and HeLa cell lines (American Type Culture Collection) were confirmed to be mycoplasma free (data not shown). The generation of A431 cells stably expressing shRNA targeting KRT17 has been reported13. Generation of A431 cells null for KRT17 was conducted by CRISPR-Cas9–mediated genome engineering38. Specifically, a target sequence in the first exon of human KRT17 (5′-GCTCCCTCAGCCGCTTGGGGGGCGG3′ (PAM motif underlined)) was chosen, and a 20-nt guide sequence (5′-GCTCCCTCAGCCGCTTGGGGGGG-3′) was cloned into the BbsI site of pX458 (pSpCas9(BB)-2A-GFP; obtained from AddGene (plasmid 48138)) according to the cloning protocol38. For sequencing, the KRT17 regions targeted in the knockout cell line were PCR amplified and cloned into the pJET1.2/blunt cloning vector using the CloneJET PCR Cloning kit (Life Technologies, K1231). Sequencing results showed a framemash and premature stop codon formation for both alleles of KRT17 (data not shown).

Antibodies, reagents and plasmid constructs. The primary antibodies used in this study included rabbit polyclonal antibodies against Krt17 (ref. 10), phosphorylated histone H3 (Cell Signaling Technology, 9701), histone H3 (Cell Signaling Technology, 9715), m-Cherry (BioVision, 5993), p65 (Santa Cruz Biotechnology, sc-372X) and nesprin-3 (GeneTex, GTX87974); goat polyclonal antibodies against eIF3 (Santa Cruz Biotechnology, sc-13677) and p53 (Santa Cruz Biotechnology, sc-315); chicken polyclonal antibodies against Krt14 (Covance, AF64) and Krt5 (Covance, AF138); rat polyclonal antibodies against CD11b (eBioscience), CD32, eBioscience), CD4 and CD45 (eBioscience, 45-0451-80); hamster polyclonal antibody against CD11c (eBioscience, 17-0112-81), CD207 (eBioscience, 14-2075-82), CD4 (BD Biosciences) and analyzed using FlowJo software (Tree Star). monoclonal antibodies against PECA-1 (Chemicon, CBL1337), Hsp70 (StressGen, ADI-SPA-812), LAMP-1 (DSHB, University of Iowa, H4A3), actin (Sigma, A5414), GADPH (Santa Cruz Biotechnology, sc-365062), 1xBtx (Cell Signaling Technology, 4814), E7 (Invitrogen, 28-0006), hnRNPK (Santa Cruz Biotechnology, sc-32307) and PML (Santa Cruz Biotechnology, sc-966). Secondary antibodies used included ones conjugated to Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 647 (Invitrogen) for indirect immunofluorescence and horseradish peroxidase (HRP)–conjugated goat anti–mouse, goat anti–rabbit and rabbit anti–goat antibodies (Sigma) for chemiluminescence immunoblotting. DNA was stained using Hoechst (Sigma). All commercial antibodies were used according to the manufacturer’s recommendations. Staining with TUNEL (Roche, 11767291910) for apoptotic cells was carried out according to the manufacturer’s instructions. TPA (Sigma, P1835) was dissolved in DMSO (for cell treatments) or acetone (for ear tissue topical treatment) and used at a 200 nM or 25 ng/ml working concentration, respectively. LMB (Sigma, L2913) was dissolved in 70% methanol and used at a 40.7 nM working concentration.

A full-length human AIRE cDNA clone (a gift from P. Peterson, University of Tartu) was moved into the mCherry-C1 vector (Clontech) using HindIII and SacII restriction enzymes, generating the mCherry-AIRE construct. The mCherry and mCherry-AIRE plasmids were transiently transfected into A313 cells using FuGENE HD (Promega, E2311) at a 1:3 (DNA amount: FuGENE volume) ratio according to the manufacturer’s instructions. siRNA oligonucleotides targeting hnRNPK and their transfection protocol have previously been described. Overexpression of hnRNPK from plasmid has been described.14 The GFP-fused K7 Lys399Ala mutant was generated from pEGFP-C3 plasmid encoding wild-type K714 using the Phusion mutagenesis kit (Life Technologies, F-541).

Tissue collection and morphological analyses. Histological assays for all ear tissue sections5 and electron microscopy39 were conducted as described. Epidermal thickness measurements, image quantification and myeloperoxidase assays were carried out as described5,38. The basal cell aspect ratio was determined by dividing the length by the width of individual basal cells using ImageJ software. Immunohistochemistry and immunofluorescence images were acquired using a Zeiss fluorescence microscope with an Apotome attachment. Confocal immunofluorescence images were acquired using a Zeiss LSM 710 microscope. Images from similar experiments were equally brightened, contrasted and cropped using ImageJ software for optimal presentation.

For analysis of autoinflammation, all tissues were obtained from male mice from 4–10 months of age (n = 4–5 per genotype). Tissues were embedded longitudinally in paraffin, and 3-μm sections were cut and stained with hematoxylin and eosin or Mason’s trichrome blue (Histoserv) before microscopic evaluation and grading. Grading was performed by two independent investigators blinded to sample identity, and scores were averaged.

Deidentified archival samples from paraffin–embedded blocks of diagnostic biopsies for human basal cell carcinomas were used and thus qualified for a waiver of patient consent as approved under Johns Hopkins Institutional Review Board protocol NA_00072381. Tissue sections were immunostained for K17 and stained with Hoechst, and images were acquired with the Apotome attachment as stated above.

Flow cytometry. Mouse ears were collected, washed with PBS, split in half and placed in 0.4 mg/ml Liberase (Roche, 05-401-054-001) diluted in serum-free RPMI 1640 medium containing 5% penicillin-streptomycin at 1.5 h at 37°C. Liberase was inactivated by adding complete RPMI 1640 medium containing 5% FCS, and tissues were manually homogenized by syringe before passage through a 70-μm filter and centrifugation at 200g for 8 min. Cells were resuspended in PBS and centrifuged in a 30–70% Percoll gradient (GE Healthcare, 17-0891-01). Mononuclear cells at the gradient interface were collected, washed, resuspended in PBS, incubated with 5 μg/ml FcR blocker (anti-CD16/ CD32, eBioscience), washed with PBS and resuspended in staining buffer (1% FCS in PBS) before labeling with respective antibodies for 30 min on ice. After two washes with staining buffer, cells were collected on a FACScalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

RNA in situ hybridization. Aire sense and antisense probes were generated by PCR subcloning a 418-bp fragment from a plasmid encoding mouse Aire cDNA40 (provided by P. Peterson, Tartu University) into the pcr II-TOPO vector (forward primer, 5′-AAAGGCAGATGGCACAATT-3′; reverse primer, 5′-ACACGGCACACTCA TCCTCG-3′. In vitro transcription using T7 (Ambion, 2082) and Sp6 (Ambion, 2071) polymerases yielded the sense and antisense probes, which were labeled with DIG and purified using Illustra Microspin G-50 columns (GE Healthcare, 27-5330-01). We used 5 μl of probe per slide. For TPA treatment, one mouse ear was treated four times, with a 48-h interval between each treatment, with 20 ng/ml TPA, and the other ear of the same mouse was treated with acetone as a control. The in situ hybridization protocol was performed as previously reported. The duration of the alkaline phosphatase reaction was the same for all samples. Four sets of biological replicates were used, with one representative image being depicted.

Protein extraction, subcellular fractionation and coimmunoprecipitation. Protein lysates for immunoblotting were prepared in urea sample buffer (8 M deionized urea, 0.5% SDS, 30 mM Tris, pH 6.8, 5% glycerol and 5% β-mercaptoethanol) after homogenization (ear tissue) or PBS washing (cultured cells). All samples were sheared using progressively finer-gauged needles (22 1/2, 25 1/2 and 26½) and subjected to a Bradford assay and processed.
for immunoblotting analysis as previously described. Subcellular fractionation was conducted on the basis of correspondence with R. Foisner (Max F. Perutz Laboratories). After three rinses with PBS on ice, cells were scraped into hypotonic lysis buffer (10 mM Tris, pH 7.5, 1 mM MgCl₂ and 10 mM KCl plus protease inhibitors), incubated on ice for 10 min and sheared with a 20 1/2-gauge needle to release nuclei. After centrifugation at 800g for 5 min, the supernatant was removed (soluble fraction), and the pelleted nuclei were resuspended in nuclear envelope extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂ and 0.2 mM EDTA plus protease inhibitors) and incubated twice on ice for 10 min for each incubation. Samples were then vortexed for 1 min before centrifugation at 16,300g for 10 min. The supernatant containing the nuclear envelope was retained. The pellet containing histones was resuspended in urea sample buffer. The soluble fraction, nuclear extract and histone sample were separated by 10% SDS-PAGE and subjected to immunoblot analysis using relevant markers.

Immunoprecipitation for K17, p65 and hnrNPS K was conducted as previously described. mCherry and mCherry-AIRE were immunoprecipitated using 10 µl of RFP-Trap beads (Chromotek) per sample, and samples were incubated for 1 h at 4 °C with rotation. HPV16 E7 protein was immunoprecipitated from P2 mouse skin subjected to the Dynabeads comminoprecipitation kit (Invitrogen) supplemented with protease inhibitors. Protein content was determined by Bradford assay, and 1.5 mg of antibody to HPV16 E7 or IgG (Source) was coupled to Dynabeads, which, after clearing, were incubated with the extracted proteins for 1 h at 4 °C. After washing and elution, the immunoprecipitation samples were separated by SDS-PAGE and subjected to immunoblot analysis using relevant markers.

Quantitative RT-PCR. Commercial qRT-PCR plates (SA Biosciences, PAMM-011A, PAMM-025, PAMM-073, PAMM-014A and PAMM-052) were used to analyze ear tissue samples from P40 HPV16E7+ and HPV16E7−/−; Krt17−/− mice. Custom qRT-PCR assays were performed on ear tissue samples from P80 Gli2−/+ and Gli2−/−; Krt17−/−; P40 HPV16E7+ and HPV16E7−/−; Krt17−/− mice and A431 cells stably expressing control or KRT17 shRNA. Total RNA was isolated from ear tissue using TRIzol reagent (Invitrogen) and was treated with DNase I (Qiagen) and cleaned on an RNeasy Mini column (Qiagen). RNA was isolated from ear tissue using TRIzol reagent (Invitrogen) and was treated with DNase I (Qiagen) and cleaned on an RNeasy Mini column (Qiagen). RNA from A431 cells was isolated using the RNeasy kit (Qiagen). Concentration and purity for all RNA samples were assessed by spectrophotometry. RNA (1 µg) was reverse transcribed with the RT² First-Strand kit (Qiagen) or iScript (Bio-Rad). qRT-PCR was performed on the first-strand cDNA using the RT² Profiler PCR Array (Qiagen) or SSOAdvanced SYBR Green (Bio-Rad) as described by the manufacturer. The PCR parameters for the custom qRT-PCR screen included incubation at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s. Controls with no template or no reverse transcriptase, standard curves and a melt curve were included on every PCR plate. Data analysis for the commercial qRT-PCR arrays was performed using the template provided online by SA Biosciences. Normalized expression values from the custom qRT-PCR data were calculated using Microsoft Excel by first averaging the relative expression for each target gene (2^−(ΔCq target gene − Cq reference gene)) across all biological replicates and then dividing the relative expression value for the experimental condition by that for the control condition (2^−(ΔCq experimental − ΔCq control)). Error bars were derived from the standard error of the ΔCq values (Cq target − Cq reference) across all biological replicates. Actb, Gapdh and Rps18 were all used as reference genes. Normalized expression values for each target gene in all qRT-PCR experiments were derived from at least three biological replicates. A list of all custom qRT-PCR primers used is provided in Supplementary Table 6.

Chromatin immunoprecipitation. Cells were treated with DMSO or TPA for 3 h at 37 °C under 5% CO₂ before execution of the ChIP protocol as described. One microliter of antibody to Krt17 or preimmune serum was added per 1 mg of total protein for all K17 ChIP assays. Ten microliters of RFP-Trap beads was used for each immunoprecipitation condition for all mCherry-AIRE ChIP assays. Antibody incubations took place overnight at 4 °C. The eluates were precipitated and resuspended in 60 µl of sterile water, with 1 µl used per PCR (40 cycles) with SSOAdvanced SYBR Green mix and primers as outlined in Supplementary Table 7. PCR products were separated by electrophoresis on 1.5% agarose gels.

Electrophoretic mobility shift assays. EMSAs were conducted using the Gel Shift Assay System (Promega, E3300) as previously described with modifications. A431 keratinocytes were serum-starved for 24 h and then treated for 1 h with TPA (200 nM) before the isolation of nuclear extracts. For supershift analyses, nuclear extracts were incubated with 1 µl of either rabbit IgG (Source), rabbit antibody to p65 (Cell Signaling Technology), preimmune serum or antibody to K17 (ref. 10) for 15 min at room temperature before incubation with 32P-labeled NF-κB oligonucleotide. For competition binding analyses, 50-fold excess non-labeled NF-κB or Oct1 (control) oligonucleotide was incubated for 15 min at room temperature with nuclear extract before incubation with 32P-labeled NF-κB oligonucleotide. Oligonucleotide labeling and gel shift assays were conducted according to the manufacturer’s protocol (Promega, Gel Shift Assay System). Samples were resolved on a 6% DNA retardation gel (Invitrogen) in 0.5x TBE buffer. Autoradiography was carried out on dried gels using phosphor screens and a phosphorimager.

Description of statistical methods. All error bars represent the standard deviation across biological replicates divided by the square root of the sample size (s.e.m.). Where technical replicates were conducted, the corresponding values were averaged to yield a single value per biological replicate. All P values were obtained by simple t test with a two-sided distribution and equal variance (Microsoft Excel), except as stated in Supplementary Figure 3.

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