A prevalent Krt8-to-Krt5 cellular state transition in skin is co-opted by p63 for enamel organ development

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Abstract

Tooth enamel, the hardest tissue in vertebrates, is crucial for mastication and dental protection. Its formation depends on the enamel organ (EO), a specialized epithelial structure derived from oral epithelium. A fundamental question persists: how does uniform oral epithelium differentiate into diverse EO cell types? While p63, a master regulator of ectodermal development, coordinates multiple signaling pathways essential for dental placode formation, its specific roles in EO development remain unclear due to the early developmental arrest in *p63* knockout mice. Using single-cell RNA sequencing data from mouse incisors, we demonstrate that *p63* is expressed across all EO cell types, serving both shared and distinct functions. Through trajectory reconstruction, we identify the role of p63 in regulating both amelogenic (AmG) and non-AmG lineage commitment during EO development. Our comparative transcriptome analyses reveal that p63 regulates the Krt8-to-Krt5 transition during AmG cell differentiation, paralleling its function in skin development. This parallel is reinforced by comparative motif discovery, revealing shared transcription factor usage, particularly p63 and AP-2 family members, in both AmG and skin epidermal cells during this transition. Chromatin accessibility analysis further illustrate that p63 mediates this transition through chromatin landscape remodeling. Together, these findings demonstrate that p63 co-opts the Krt8-to-Krt5 transition mechanism from skin development for EO development, providing novel insights into the molecular mechanisms underlying EO development and potential therapeutic targets for enamel disorders.

Keywords: p63, Krt8-to-Krt5 transition, enamel organ development, scRNA-seq, ATAC-seq

Introduction

Tooth enamel, the hardest and most mineralized tissue in vertebrates, is essential for masticatory function and tooth protection (Lacruz et al. 2017). Enamel development is orchestrated by ameloblasts, specialized cells of the enamel organ (EO), which originate from the oral ectoderm (Liu et al. 2016). During EO development, the oral epithelium invades the dental mesenchyme and differentiates into four distinct cell types: inner enamel epithelium (IEE), stratum intermedium, stellate reticulum, and outer enamel epithelium (Smith and Nanci 1995). The IEE subsequently differentiates into ameloblasts, which form the enamel matrix. While these major EO cell types are well characterized, the mechanisms governing oral epithelial specification into diverse EO cell lineages remains poorly understood.

The oral epithelium shares characteristics with other surface epithelia, including the skin, with structural and functional variations in thickness and keratinization based on location and mechanical demands (Presland and Dale 2000; Romano et al. 2012). Like other stratified epithelia, oral epithelial cells undergo morphological, biochemical, and transcriptional changes, with keratins playing a key role in defining cell identity and differentiation (Cohen et al. 2022; Roberts and Horsley 2014). In skin development, keratin expression undergoes a critical transition: the single-layered surface ectoderm, initially expressing Keratin 8 (Krt8) transitions into stratified epidermal progenitor cells by downregulating Krt8 and upregulating Krt5 (Blanpain and Fuchs 2006).

The transcription factor p63, particularly its N-terminally truncated isoform ΔNp63, is a master regulator of stratified epithelial development (Barbieri and Pietenpol 2006; Soares and Zhou 2018). In skin, p63 plays essential roles in chromatin remodeling for epithelial lineage

commitment (Li et al. 2019; Yu et al. 2021) and terminal differentiation (Koster et al. 2007), including its regulation of the Krt8-to-Krt5 transition (Fan et al. 2018). Beyond the skin, p63 is also crucial for tooth development, with *ΔNp63* widely expressed throughout all stages EO morphogenesis (Rufini et al. 2011). Mice lacking *p63* (*p63*$^{-/-}$) exhibit arrested tooth development at the dental lamina stage (Laurikkala et al. 2006). Additionally, patients with *p63*-related ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome frequently present with enamel defects and dental anomalies (Kantaputra et al. 2012; Koul et al. 2014; Rinne et al. 2006). However, because *p63*$^{-/-}$ mice fail to initiate tooth development, the specific role of p63 in EO differentiation remains unknown. Furthermore, whether the cellular state transitions observed in skin contribute to EO development, and how p63 mediates these transitions, remains unexplored.

To address these knowledge gaps, we analyzed publicly available single-cell RNA sequencing (scRNA-seq) and chromatin accessibility datasets to investigate p63's specific cellular and molecular roles in EO development. Our findings demonstrate that p63 co-opts the Krt8-to-Krt5 cellular transition, previously identified in skin, to drive epithelial differentiation during EO development. This study uncovers a conserved p63-driven regulatory mechanism for epithelial keratinization across different tissues, providing new insights into epithelial plasticity during development.

Materials and Methods

Analysis of scRNA-Seq Data from rom Mouse Incisor

We analyzed a scRNA-seq dataset from adult mouse incisors (8–12 weeks, GSE131204) using the Seurat pipeline (Stuart et al. 2019). Quality control filtering (minimum 5 cells per gene, nFeature_RNA > 1,000, percent mitochondrial reads < 10%) yielded 3,171 high-quality cells. For enamel organ (EO) heterogeneity analysis, we excluded immune and mesenchymal cells based on established markers, retaining 2,341 EO cells. Following Seurat's standard workflow, we identified 2,000 highly variable genes after log normalization and performed principal component analysis. The top 10 principal components, selected using the ElbowPlot function, were used for downstream analysis. We constructed a shared nearest-neighbor graph using the FindNeighbors function and identified 15 main cell clusters (resolution = 1.2) using the FindClusters function. Cell types were classified using cluster-specific marker genes from previous research (Sharir et al. 2019) and visualized using Uniform Manifold Approximation and Projection (UMAP). For p63 expression analysis, cells with non-zero p63 counts were classified as p63-positive ($p63^+$). We identified differentially expressed genes (DEGs) between $p63^+$ and p63-negative cells (p63-DEGs) using the FindMarkers function. Similarly, we determined DEGs between Krt8-positive and Krt5-positive cells (Krt-DEGs). These DEGs were ranked by combining p-value and average log fold change, then analyzed using gene set enrichment analysis (GSEA) software (Subramanian et al. 2005). To analyze EO cell differentiation trajectories, we employed Monocle2 (Qiu et al. 2017). Cell embeddings and clusters from Seurat were converted into a CellDataSet object and ordered in pseudotime. We assigned the trajectory root to the node containing the majority of actively cycling cells. Branch-dependent expression

analysis was performed using the BEAM function (q-value < 0.01). Gene Ontology analysis was conducted using the clusterProfiler R package (Yu et al. 2012).

Analysis of ATAC-Seq Data from Mouse Incisor and Skin

We analyzed Assay for Transposase-Accessible Chromatin with sequencing (ATAC-seq) datasets from skin (E9 and E13, GSE97213) and incisor (E12 and E16, PRJNA668198) to investigate chromatin accessibility during the Krt8-to-Krt5 transition. For skin datasets, reads were aligned to the mouse mm10 reference genome using Bowtie2 (--local option) (Langmead and Salzberg 2012) and processed with SAMtools (Li et al. 2009). After excluding mitochondrial and unplaced/random contigs, peaks were called using MACS2 (Zhang et al. 2008). For visualization in the UCSC genome browser, filtered BAM files were converted to bigwig format and merged by developmental stage. Differentially accessible chromatin regions between E13 and E9 were identified using the DiffBind R package (Ross-Innes et al. 2012). Regions showing increased accessibility during the skin Krt8-to-Krt5 transition underwent motif enrichment analysis using HOMER (Heinz et al. 2010), followed by motif scanning of Krt-DEGs cis-regulatory elements with BETA (Wang et al. 2013). Incisor ATAC-seq data was processed using the ENCODE pipeline (Hitz et al. 2023). Merged BAM files from each developmental stage were converted to bigWig format using the deepTools2 package (Ramirez et al. 2016) for IGV visualization. Accessibility differences between E16 and E12 were calculated using IGV's subtract operation (Robinson et al. 2011).

Results

p63 Exerts Both Common and Distinct Functions in Various Cell Types During Enamel Organ Development

Genetic mutations in p63 cause ectodermal dysplasia, a group of rare hereditary disorders affecting ectodermal organs including skin, sweat glands, and teeth (Romano et al. 2012). Patients with these mutations often exhibit various dental abnormalities, including tooth agenesis, alterations in tooth shape and size, fused teeth, and enamel dysplasia (Kantaputra et al. 2012). Despite this clear link between p63 mutations and dental anomalies, p63's specific role in enamel organ (EO) development and amelogenesis remains poorly understood. This knowledge gap exists largely because germline p63 knockout mice exhibit tooth developmental arrest at the lamina stage, before the onset of amelogenesis (Laurikkala et al. 2006). The advent of scRNA-seq technique has revolutionized our ability to examine gene expressions at unprecedented resolution (Jovic et al. 2022). To understand p63's functions in EO development, we revisited a scRNA-seq dataset from continuously growing adult mouse incisor teeth, which contain all stages of EO cell differentiation from stem/progenitors to terminally differentiated ameloblasts (Sharir et al. 2019). Using Seurat (Stuart et al. 2019), we clustered EO cells into 15 subtypes and assigned putative cell identities based on their distinct gene expression signatures (Fig. 1A). As previously reported (Sharir et al. 2019), these 15 EO cell types formed a transcriptomic continuum in UMAP space and could be grouped into three classes, with 'cycling cells' giving rise to both amelogenic (AmG) and non-AmG EO cells (Fig. 1A, red dashed arrows). While p63 was broadly expressed across all 15 EO cell types (Fig. 1B), consistent with previous *in situ* hybridization results (Laurikkala et al. 2006), single-cell resolution revealed that not every

individual cell expressed p63 (Fig. 1B). Notably, p63 showed highest expression in the inner stellate reticulum/stratum intermedium (ISR/SI) cells (Fig. 1C), a subset of non-AmG EO cells that serve as a stem cell reservoir for ameloblast renewal (Liu et al. 2016). This finding suggests a possible role for p63 in dental stem/progenitor cell replenishment. To further elucidate p63's specific roles in different EO cell types, we identified the differentially expressed genes (DEGs) between p63 positive ($p63^{+}$) and p63 negative ($p63^{-}$) cells across all EO cell types. Functional classification of these DEGs revealed that p63 exerts distinct functions in different EO cell types, while maintaining shared roles in p53 signal transduction, chromatin remodeling and odontogenesis (Fig. 1D). Together, these results demonstrate p63's essential and diverse roles in regulating EO development.

p63 Participates in Both AmG and Non-AmG Lineage Commitment During EO Development

Given p63's crucial role in epidermal lineage commitment (Li et al. 2019), we investigated its functions in EO cell fate specification by reconstructing the developmental trajectory of EO cells using Monocle2 (Qiu et al. 2017). The analysis revealed a branched continuum with four branch points representing cellular fate decisions. At branch point 1 (Fig. 2A, circle 1), cycling cells bifurcated into AmG and non-AmG lineages. Notably, p63 showed pseudo-temporal expression in both AmG and non-AmG processes during EO cell fate specification (Fig. 2A). Analysis of genes with branch point-dependent expression revealed that p63, along with other EO stem cell marker genes (Gan et al. 2020; Yu et al. 2020), functions as a branching gene at all four branch points (Appendix Figs. 1A–D). We then focused specifically on the branching genes responsible for AmG and non-AmG lineage bifurcation at branch point 1 (Fig. 2B). Gene ontology (GO)

analysis of these genes demonstrated enrichment for epidermal cell-related biological processes, including odontogenesis (Fig. 2C). Importantly, p63 was found to be involved in odontogenesis-related biological processes (Fig. 2D). Together, these results suggest that p63 functions as a potential regulator in the temporal specification of both AmG and non-AmG EO cell fates during EO development, with particular importance in the bifurcation of AmG and non-AmG lineages (Appendix Fig. 1E).

Krt8-to-Krt5 Cellular State Transition Occurs in Both AmG and Non-AmG Cells During EO Development

The oral cavity's epithelial layer consists of stratified squamous epithelium with varying degrees of stratification and keratinization, reflecting the functional demands of specific anatomical regions (Presland and Dale 2000; Romano et al. 2012). Among epithelial-derived structures, the enamel organ is unique in its ability to form enamel, the hardest vertebrate tissue. Previous studies in mammalian skin have established p63's genome-wide effect on epidermal cell fate commitment (Li et al. 2019), particularly in governing the transformation of Krt8^{+} progenitor cells to Krt5^{+} epidermal progenitors, known as the Krt8-to-Krt5 transition (Fan et al. 2018). To investigate whether this p63-directed epidermal lineage formation mechanism applies to EO cell lineage specification, we examined *Krt8* and *Krt5* expression in EO cells (Figs. 3A, B). *Krt8* expression was higher at the root of the EO cell transcriptomic continuum (Fig. 3A, arrow-tail), while *Krt5* expression was elevated at the differentiation trajectory's end (Fig. 3B, arrowheads). Gene expression analysis along pseudotime revealed decreasing *Krt8* expression (Fig. 3E) and increasing *Krt5* expression (Fig. 3F) during EO cell differentiation, indicating a Krt8-to-Krt5 transition. This Krt8-to-Krt5 transition was observed in both AmG (Fig. 3C) and non-AmG EO

cell differentiation (Figs. 3G, H), as evidenced by the inverse expression trends of *Krt8* and *Krt5* along each cell lineage (Figs. 3C, G, H). Quantification analyses of expression percentage (Appendix Fig. 2A) and levels (Appendix Fig. 2B) of *Krt8*, *Krt5* and *p63* showed that *p63* expression coincides with the Krt8-to-Krt5 transition, evidenced by p63's positive correlation with Krt5 and negative correlation with Krt8 in both cell lineages. Notably, along the AmG cell differentiation trajectory, *p63* expression peaked at an intermediate stage during the Krt8-to-Krt5 transition (Fig. 3D). These results indicate that p63 may play a role in regulating the Krt8-to-Krt5 transition during EO development, particularly during AmG cell differentiation.

p63 Regulates Krt8-to-Krt5 Transition in AmG Cells During EO Development

To examine p63's role in regulating the Krt8-to-Krt5 transition during AmG cell differentiation, we conducted a detailed analysis of the AmG cell lineage. During AmG cell differentiation, p63 expression in major AmG cell types showed a gradual increase, accompanied by decreasing *Krt8* and increasing *Krt5* expression (Fig. 4A), characteristic of a Krt8-to-Krt5 transition. AmG cell trajectory analysis confirmed that p63 expression pseudo-temporally coincides with this Krt8-to-Krt5 transition (Fig. 4B). To further investigate p63's regulatory role, we performed differential gene expression analysis comparing two groups in the AmG cells: $p63^{+}$ vs. $p63^{-}$ (p63-DEGs) and $Krt5^{+}$ vs. $Krt8^{+}$ (Krt-DEGs). We identified 4,935 p63-DEGs and 4,987 Krt-DEGs, with substantial overlap of 4,753 shared DEGs (Fig. 4C). Analysis of transcriptomic changes between these groups, based on DEG ranking by fold change and p-value, revealed similar patterns (Fig. 4D). GO analysis of Krt-DEGs showed significant enrichment in ‘signal transduction by p53 class mediator’ (Fig. 4E). These findings suggest that p63 regulates the Krt8-to-Krt5 transition during AmG cell differentiation.

p63 Regulates Krt8-to-Krt5 Transition During AmG Cell Differentiation Through Chromatin Remodeling

Previous research showed that p63-governed chromatin accessibility changes drive the Krt8-to-Krt5 transition in the skin (Fan et al. 2018). To investigate whether p63 similarly regulates this transition during AmG cell differentiation, we analyzed the chromatin accessibility of Krt8 and Krt5 in incisor development at embryonic day 12 (E12) and E16 (Wang et al. 2022). Comparative analyses revealed opposing dynamics: the Krt8 locus showed decreased chromatin accessibility as incisor development progressed (Appendix Fig. 3A), while the Krt5 locus showed increased accessibility (Appendix Fig. 3B), indicating that chromatin remodeling also underlies the Krt8-to-Krt5 transition during AmG cell differentiation. To investigate p63's role in this process, we performed comparative motif analyses on the cis-regulatory elements of Krt-DEGs and on regions that gained chromatin accessibility during the skin Krt8-to-Krt5 transition (Fig. 5A). *De novo* motif discovery using HOMER (Heinz et al. 2010) identified p63 and TFAP2C as the two most enriched transcription factors (TFs) in regions gaining chromatin accessibility during skin transition (Fig. 5C). Interestingly, motif scan using BETA (Wang et al. 2013) identified AP-2 family and p53 class TFs as the most enriched TF groups in Krt-DEG cis-regulatory elements (Fig. 5B), suggesting conserved AP-2 and p53 family TF usage during Krt8-to-Krt5 transition in both AmG and skin cells. Furthermore, gene set enrichment analysis of ranked p63-DEGs from AmG cells showed strong enrichment for both genes associated with regions gaining chromatin accessibility during skin Krt8-to-Krt5 transition (Fig. 5D) and Krt5^{+} AmG cell signature gene set (Fig. 5E). More importantly, *p63* knockout (KO) inversely altered chromatin accessibility dynamics at the candidate cis-regulatory elements (cCREs) of *Krt8* and

Krt5 in the skin (Fig. 5F). Notably, these ENCODE-compiled cCREs contained p63 binding sites (Fig. 5F) that overlap with differentially accessible regions in incisor (Appendix Figs. 3A, B). These results indicate that p63 regulates the Krt8-to-Krt5 transition through chromatin landscape remodeling during AmG cell differentiation in the EO. Collectively, our study provides convincing evidence that p63 co-opts the prevalent Krt8-to-Krt5 transition in skin for EO development.

Discussion

Our study demonstrates that p63 co-opts the Krt8-to-Krt5 transition, previously established in skin development, to orchestrate EO development. Through scRNA-seq analysis, we show that p63 is expressed across all EO cell types and participates in both AmG and non-AmG lineage commitment. Importantly, we identify that p63 regulates the Krt8-to-Krt5 transition during AmG cell differentiation through chromatin landscape remodeling, similar to its role in skin development.

p63 is a Ubiquitous Player During EO Development

While major EO cell types are well documented (Smith and Nanci 1995), the mechanisms controlling their specification remain incompletely understood. Understanding these mechanisms is crucial since precise and proper EO cell coordination ensures functional enamel formation (Lacruz et al. 2017). As a master regulator of ectodermal development, p63 integrates key signaling pathways in early tooth development for dental placode formation (Laurikkala et al. 2006), but early dental arrest in p63$^{-/-}$ mice has limited our understanding of its later roles in EO development. Our scRNA-seq data reanalysis unravels that p63 regulates shared functions across EO cell types, including p53 signaling, chromatin remodeling, and odontogenesis, while also controlling cell-type-specific processes like amelogenesis and mineralization (Fig. 1). Trajectory analysis reveals that p63 participates in both AmG (Fig. 2) and non-AmG (Appendix Fig. 2) lineage commitment during EO development, acting as a branching gene. These results indicate that p63 orchestrates EO development by controlling EO cell lineage commitment and differentiation, aligning with previous study demonstrating p63 expression in both

undifferentiated EO cells and differentiated ameloblasts (Rufini et al. 2006). In the current study, we identified DEGs between p63^{+} and p63^{-} cells across different EO cell types to infer p63's role in EO development (Figs. 1, 4, 5). Given that enamel formation requires precise coordination between different EO cell types (Liu et al. 2016), future studies investigating cell-cell communications between these different cell populations would provide additional insights into how p63 orchestrates EO development through potential paracrine signaling mechanisms.

p63 Orchestrates a Conserved Regulatory Mechanism for Epithelial Keratinization in EO Development

Surface epithelia, including oral epithelium, form essential protective barriers against chemical, microbial, and physical challenges, making them indispensable for organismal viability (Presland and Dale 2000). To establish and maintain this protective function, surface epithelial cells undergo a tightly regulated differentiation program that results in the formation of structural proteins, particularly keratins (Roberts and Horsley 2014). These keratins serve as critical markers of cellular state transitions in epithelial biology, playing essential roles in maintaining tissue homeostasis and adapting to developmental cues (Cohen et al. 2022). Recent studies have revealed that mutations and genetic variants in keratins increase dental decay risk and susceptibility (Duverger et al. 2018; Duverger et al. 2014), highlighting the importance of understanding keratin regulation in EO development. Through reanalysis of incisor scRNA-seq data, we discovered that the Krt8-to-Krt5 transition, a hallmark of epidermal fate commitment in the skin (Fan et al. 2018), occurs during EO cell differentiation (Fig. 3). The AmG Krt8-to-Krt5 transition coincided with pseudo-temporal p63 expression (Fig. 4A, B), consistent with previous study showing that p63 colocalizes with Krt5 during EO development (Rufini et al. 2006).

Similar transcriptome changes between p63^{+} vs p63^{-} and Krt5^{+} vs Krt8^{+} EO cells suggested p63 regulation of the Krt8-to-Krt5 transition during AmG cell differentiation (Figs. 4C–E). Supporting this, gene set enrichment analyses of DEGs between p63^{+} vs p63^{-} cells showed strong enrichment of Krt5^{+} cell signature genes (Figs. 5D, E). Motif analyses of chromatin remodeling regions for Krt8 and Krt5 during both skin (Fig. 5F) and incisor (Appendix Fig. 3) development both revealed the presence of p63 binding sites. Together, these findings demonstrate that p63 co-opts the Krt8-to-Krt5 transition mechanism from skin development for EO development through chromatin landscape remodeling, revealing a conserved p63-driven regulatory mechanism for epithelial keratinization across different tissues. This finding is particularly significant as it establishes a molecular link between keratin regulation and enamel formation, potentially offering new therapeutic targets for enamel disorders.

Our multi-omics data re-analysis approach, combining single-cell transcriptomics and chromatin accessibility data, provides a powerful framework for understanding gene regulatory functions in contexts where traditional genetic approaches are limited by developmental arrest. Together, these findings not only reveal a conserved developmental mechanism across different epithelial tissues but also provide crucial insights into the molecular basis of enamel formation, opening new avenues for therapeutic interventions in enamel disorders.

Author Contributions

Q. Tang, contributed to conception, design, data acquisition and interpretation, performed statistical analyses, drafted, and critically revised the manuscript. J.-M. Lee, L. Li, C. Cai, and H. Jung, contributed to data interpretation, and critically revised the manuscript. H.-J.E. Kwon, contributed to conception, design, and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure Legends

Figure 1. *p63* exhibits diverse functional roles during enamel organ development. (**A**) DimPlot illustrating major dental epithelial cell types in the 8-week-old mouse incisor. (**B**) Dynamic distributions of p63-expressing ($p63^+$) cells across major dental epithelial cell types. (**C**) VlnPlot demonstrating different expression levels of p63 in major dental epithelial cell types. (**D**) Gene ontology (GO) analyses of differentially expressed genes (DEGs) between $p63^+$ vs $p63^-$ groups in each dental epithelial cell type. upper IEE, upper inner enamel epithelium; OEE, outer enamel epithelium; VEE, ventral enamel epithelium; DEEx, dental epithelial extensions; IEE–OEE, junction between the IEE and OEE; SI, stratum intermedium; ISR, inner stellate reticulum; OSR, outer stellate reticulum; pre-AmB, pre-ameloblasts; AmB prox, proximal ameloblasts; AmB dist, distal ameloblasts; G2/M, M/G1 and S, cycling dental epithelial cells.

Figure 2. p63 is a regulator of lineage bifurcation in enamel organ development. (**A**) Pseudotime analysis illustrating the divergence of amelogenic (AmG) and non-AmG lineages within dental epithelial cells. As per the updated model, a population of actively cycling progenitors give rise to both AmG and non-AmG epithelial cells during dental epithelial cell specification. Notably, p63 is consistently expressed throughout the entire pseudotime trajectory. The numbers 1–4 indicate the branching points along the pseudotime trajectory. (**B**) Branched heatmap depicting the expression patterns of representative branching genes, including p63, during the bifurcation of AmG and non-AmG epithelial cells at branching point 1. (**C**) GO analysis of the branching genes identified at branching point 1. (**D**) cnetplot demonstrating that p63 is a key player in odontogenesis-related biological processes.

Figure 3. The occurrence of Krt8-to-Krt5 cellular state transition during enamel organ development. (A, **B**) FeaturePlots showing the expressions of Krt8 (A) and Krt5 (B) in major dental epithelial cell types. (**C**) VlnPlot depicting dynamic expression changes of Krt8 and Krt5 during AmG lineage cell specification. (**D**) Branched heatmap showing p63 expression during the Krt8-to-Krt5 transition at the AmG and non-AmG bifurcation point. (**E**, **F**) Pseodotemporal expression of Krt8 (E) and Krt5 (F) during the AmG and non-AmG bifurcation. (**G**, **H**) VlnPlot showing dynamic expression changes of Krt8 and Krt5 during non-AmG lineage cell specification. upper IEE, upper inner enamel epithelium; DEEx, dental epithelial extensions; SI, stratum intermedium; VEE, ventral enamel epithelium; AmB dist, distal ameloblasts.

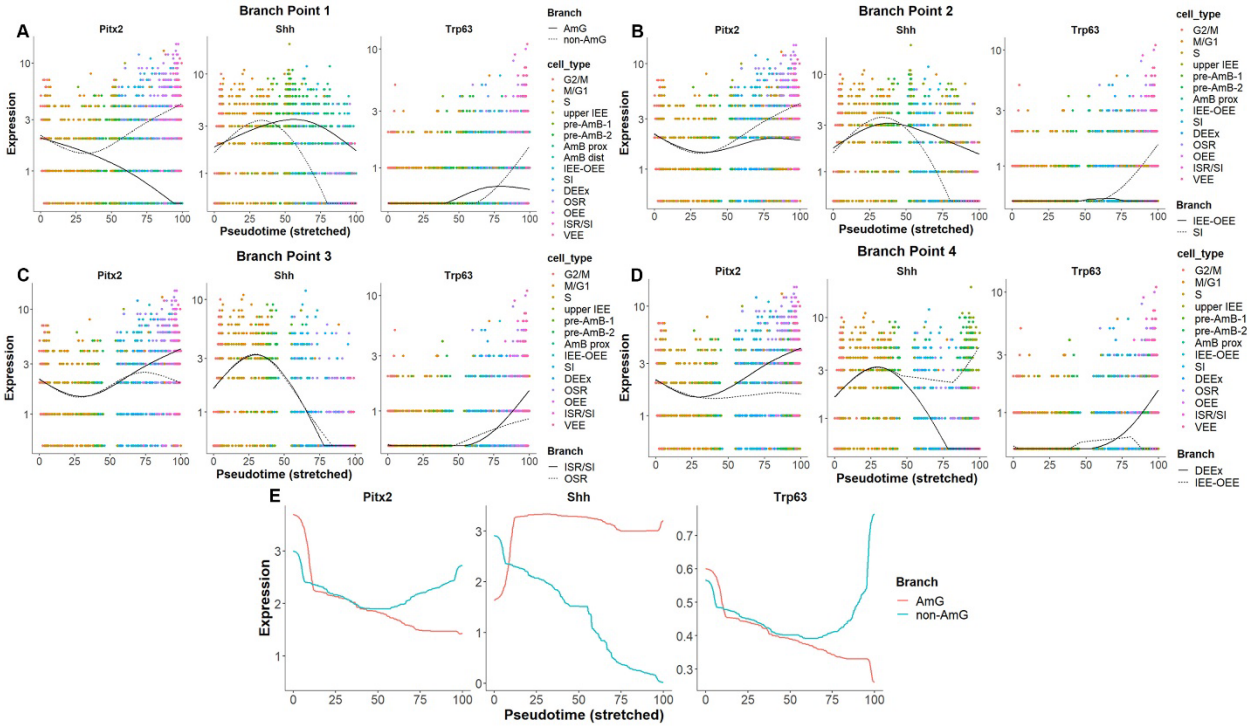
Figure 4. p63 regulates the Krt8-to-Krt5 transition during AmG cell differentiation. (A) Dot Plots showing the concurrent expression of p63 with Krt5 during the amelogenic Krt8-to-Krt5 transition. (**B**) Pseudotemporal expression changes of Krt8, Krt5 and p63 throughout amelogenesis. (**C**) Venn diagram illustrating the significant overlap (4753 genes) of differential expressed genes (DEGs) between the $p63^+$ vs. $p63^-$ and $Krt5^+$ vs. $Krt8^+$ dental epithelial cell groups. (**D**) Heatmap displaying the similarity of transcriptomic changes between group 1 ($p63^+$ vs. $p63^-$) and group 2 ($Krt5^+$ vs. $Krt8^+$). (**E**) GO analysis of DEGs in the $Krt5^+$ vs. $Krt8^+$ group.

Figure 5. p63 remodels open chromatin regions during the Krt8-to-Krt5 transition in AmG cell differentiation. (A) Schematic representation of the integrative analysis workflow comparing enriched motifs in $Krt8^+$ vs. $Krt5^+$ DEGs from dental epithelium and genomic loci (E13 vs. E9 ATAC-seq differential binding sites) that gained chromatin accessibility during the

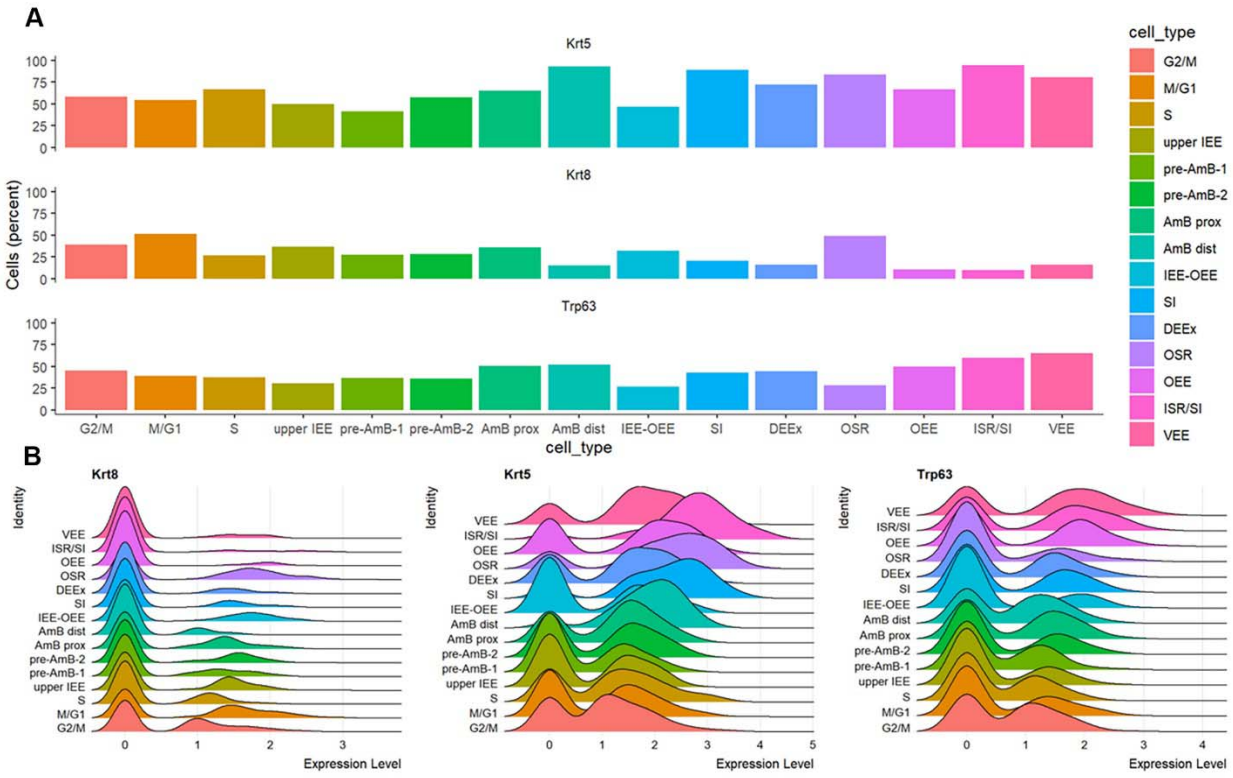
Krt8-to-Krt5 transition in skin. (**B**) Motif scan analysis revealing high enrichment of p53 and AP-2 family transcription factors (TFs) in DEGs between Krt8^{+} vs. Krt5^{+} dental epithelial cells. (**C**) Motif enrichment analysis demonstrating high enrichment of p63 and AP-2 family TFs in gained open chromatin regions during the Krt8-to-Krt5 transition in skin. (**D**-**E**) Gene set enrichment analysis (GSEA) of ranked DEGs between p63^{+} vs. p63^{-} dental epithelial cells against (D) genes associated with gained chromatin regions during the Krt8-to-Krt5 transition in skin and (E) signature genes of Krt5^{+} dental epithelial cells. (**F**) ATAC-seq tracks revealing that p63 knockout reverses chromatin accessibility dynamics in the candidate cis-regulatory elements (cCREs) of Krt8 (turquoise) and Krt5 (yellow). cCREs are combined from all cell types by ENCODE. Multiple p63 binding sites are present in the cCREs of Krt8 and Krt5, as visualized by the ReMap track. TRP63 binding sites with labeled chromosome coordinates indicate their presence in differentially accessible regions during incisor development.

SUPPLEMENTAL APPENDIX

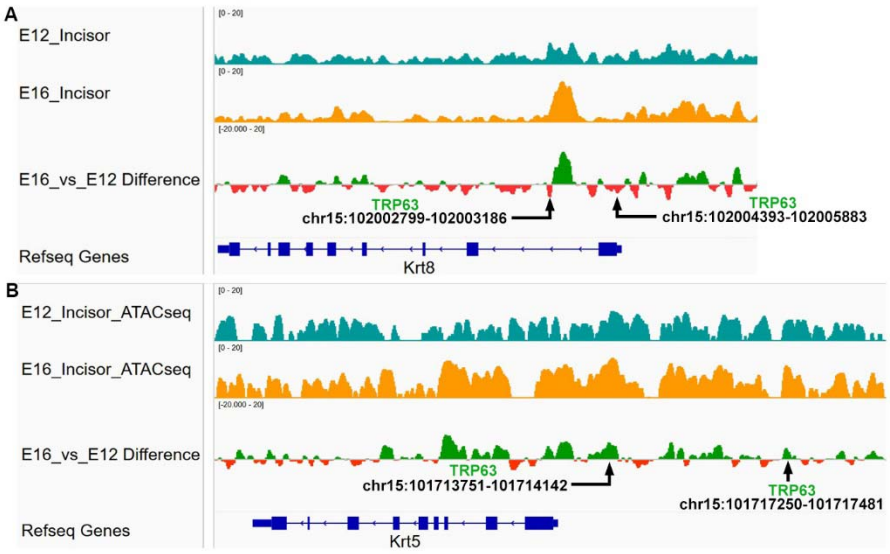
Supplemental Figures



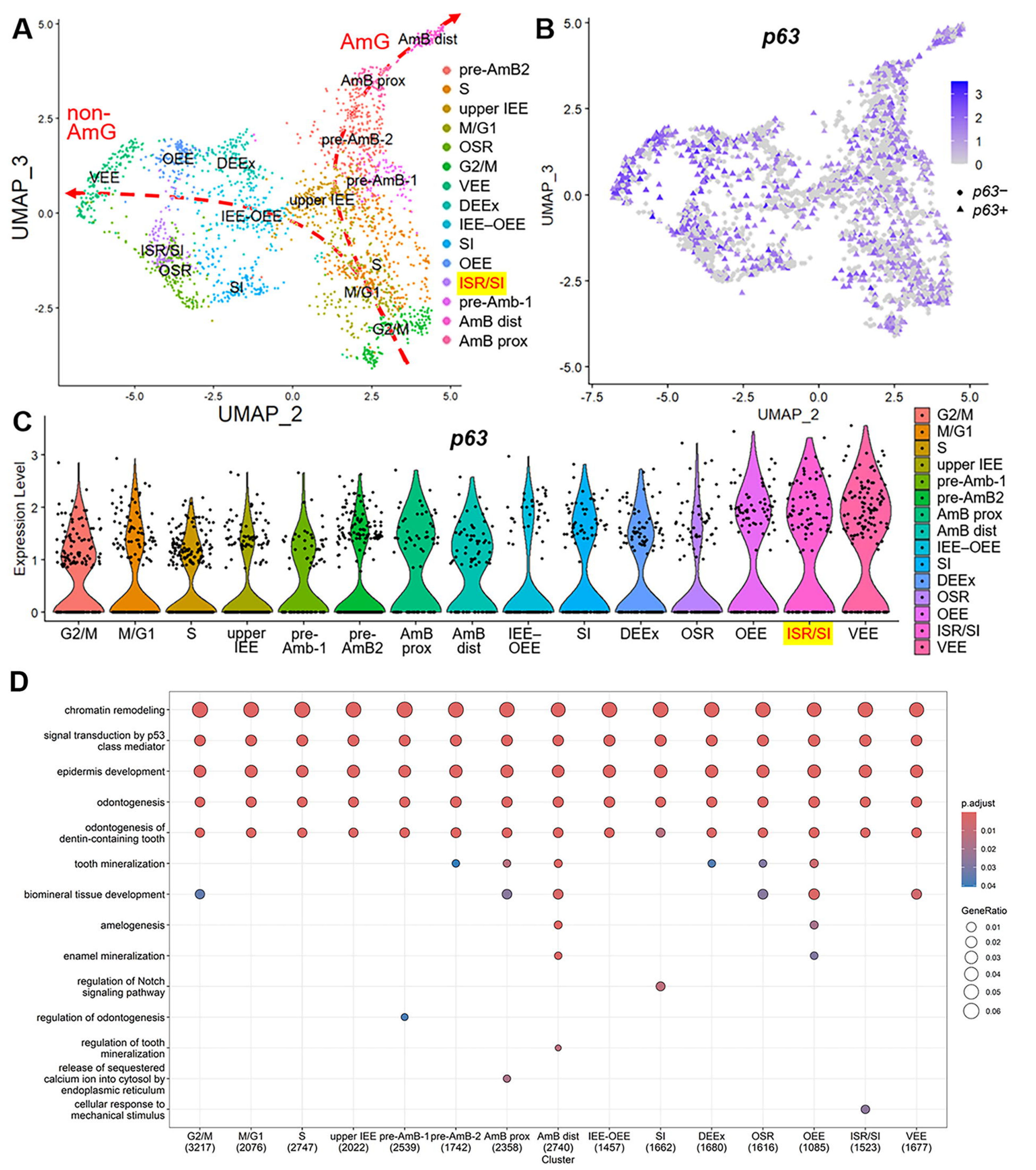
Appendix Figure 1. p63 functions as a branching gene throughout enamel organ development. (**A–D**) p63 acts as a branching gene at multiple branching points along the pseudotime trajectory of dental epithelial cell differentiation. (**E**) Kinetic curve along the pseudotime trajectory of dental epithelial cell differentiation demonstrating that p63 is a regulator of lineage bifurcation during enamel organ development.

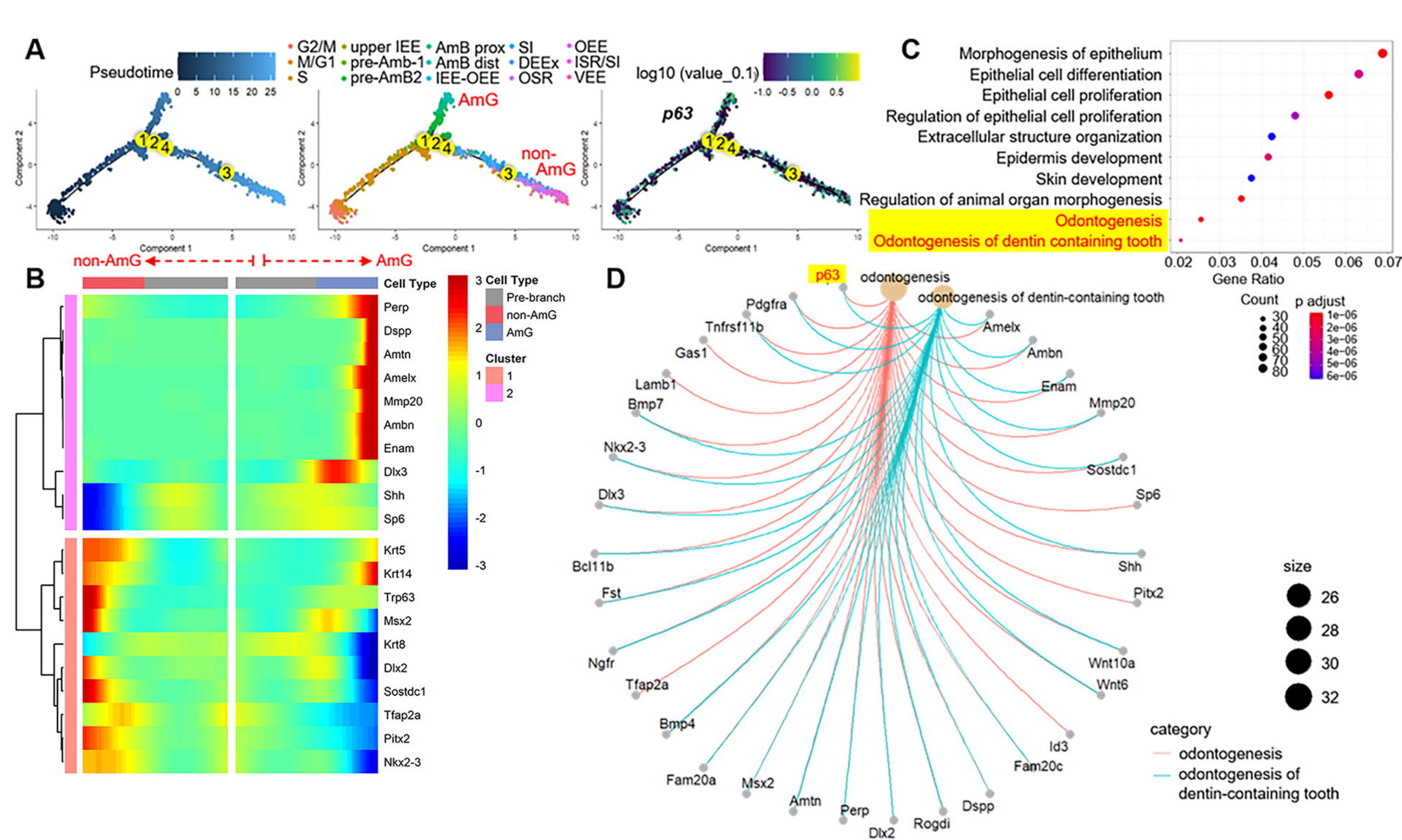


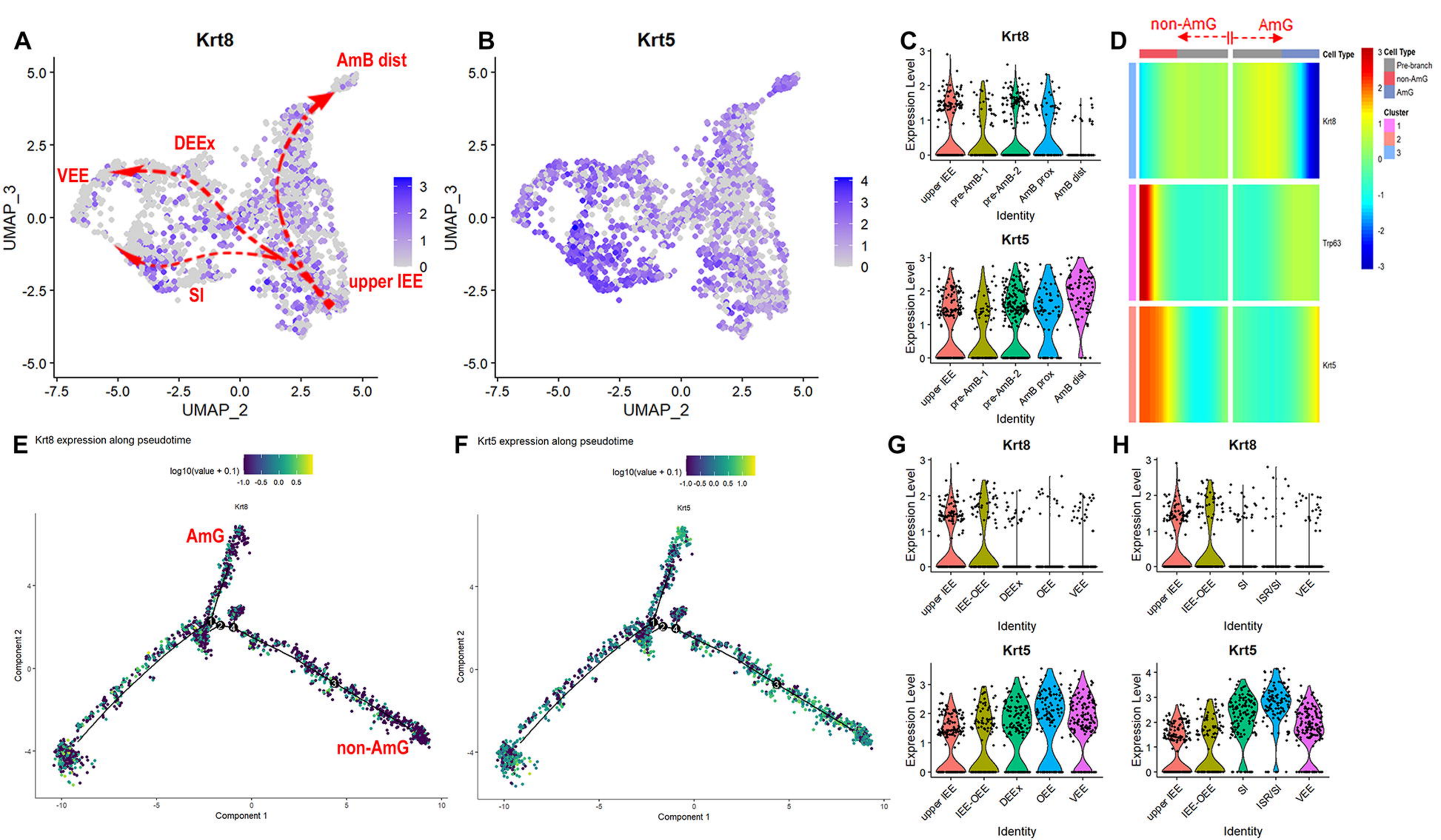
Appendix Figure 2. p63 expression coincides with Krt8-to-Krt5 transition during enamel organ development. (**A, B**) The percentage of p63-expression cells (A) and p63 expression level (B) are both positively correlated with the Krt8-to-Krt5 transition during enamel organ development.

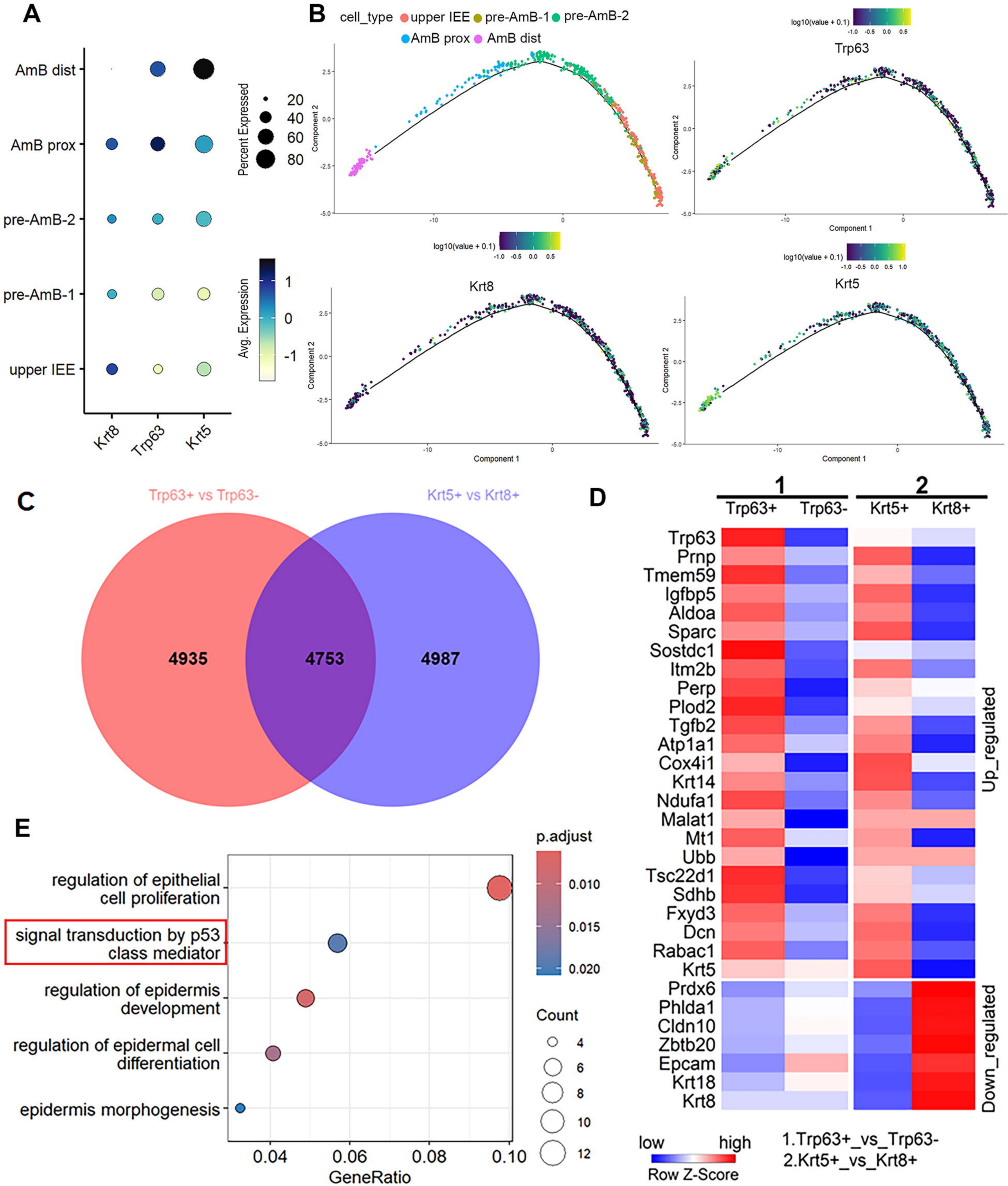


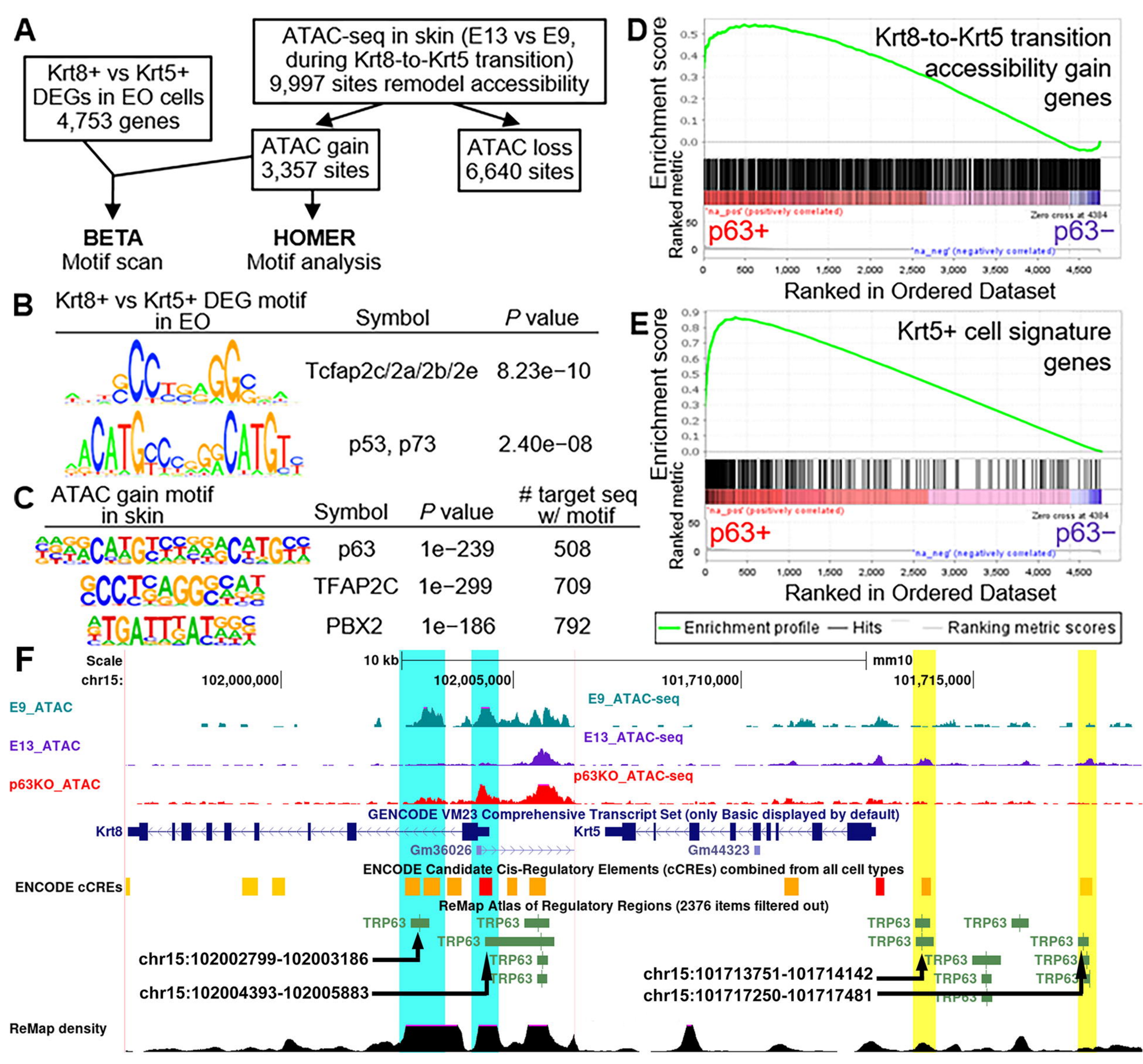
Appendix Figure 3. Dynamic chromatin accessibility of Krt8 and Krt5 during mouse incisor development. (A, B) ATAC-seq profiles demonstrating differential chromatin accessibility of Krt8 (A) and Krt5 (B) during mouse incisor development. The Krt8 locus showed decreased chromatin accessibility at E16 compared to E12, whereas the Krt5 locus exhibited increased chromatin accessibility. Red bars indicate regions of decreased chromatin accessibility at E16 relative to E12, while green bars highlight regions with increased chromatin accessibility. TRP63 binding sites with labeled chromosome coordinates indicate their presence in differentially accessible regions during the skin Krt8-to-Krt5 transition.


A
AmG
AmB dist
AmB prox
non-AmG
pre-AmB-2
pre-AmB-1
upper IEE
VEE
OEE
DEEx
IEE-OEE
ISR/SI
OSR
SI
S
M/G1
G2/M
UMAP_3
UMAP_2
pre-AmB2
S
upper IEE
M/G1
OSR
G2/M
VEE
DEEx
IEE–OEE
SI
OEE
ISR/SI
pre-Amb-1
AmB dist
AmB prox
B
p63
p63−
p63+
C
p63
Expression Level
G2/M
M/G1
S
upper IEE
pre-Amb-1
pre-AmB2
AmB prox
AmB dist
IEE–OEE
SI
DEEx
OSR
OEE
ISR/SI
VEE
D
chromatin remodeling
signal transduction by p53 class mediator
epidermis development
odontogenesis
odontogenesis of dentin-containing tooth
tooth mineralization
biomineral tissue development
amelogenesis
enamel mineralization
regulation of Notch signaling pathway
regulation of odontogenesis
regulation of tooth mineralization
release of sequestered calcium ion into cytosol by endoplasmic reticulum
cellular response to mechanical stimulus
G2/M (3217)
M/G1 (2076)
S (2747)
upper IEE (2022)
pre-AmB-1 (2539)
pre-AmB-2 (1742)
AmB prox (2358)
AmB dist (2740)
IEE-OEE (1457)
SI (1662)
DEEx (1680)
OSR (1616)
OEE (1085)
ISR/SI (1523)
VEE (1677)
Cluster
p.adjust
GeneRatio


A
Pseudotime
0 5 10 15 20 25
G2/M
M/G1
S
upper IEE
pre-Amb-1
pre-AmB2
AmB prox
AmB dist
IEE-OEE
SI
DEEx
OSR
OEE
ISR/SI
VEE
log10 (value_0.1)
-1.0 -0.5 0.0 0.5
AmG
non-AmG
p63
Component 1
Component 2
non-AmG
AmG
B
Cell Type
Perp
Dspp
Amtn
Amelx
Mmp20
Ambn
Enam
Dlx3
Shh
Sp6
Krt5
Krt14
Trp63
Msx2
Krt8
Dlx2
Sostdc1
Tfap2a
Pitx2
Nkx2-3
3 Cell Type
Pre-branch
non-AmG
AmG
2
Cluster
1
2
1
0
-1
-2
-3
C
Morphogenesis of epithelium
Epithelial cell differentiation
Epithelial cell proliferation
Regulation of epithelial cell proliferation
Extracellular structure organization
Epidermis development
Skin development
Regulation of animal organ morphogenesis
Odontogenesis
Odontogenesis of dentin containing tooth
0.02 0.03 0.04 0.05 0.06 0.07
Gene Ratio
Count
30
40
50
60
70
80
p adjust
1e-06
2e-06
3e-06
4e-06
5e-06
6e-06
D
p63
odontogenesis
odontogenesis of dentin-containing tooth
Pdgfra
Tnfrsf11b
Gas1
Lamb1
Bmp7
Nkx2-3
Dlx3
Bcl11b
Fst
Ngfr
Tfap2a
Bmp4
Fam20a
Msx2
Amtn
Perp
Dlx2
Rogdi
Dspp
Fam20c
Id3
Wnt6
Wnt10a
Pitx2
Shh
Sp6
Sostdc1
Mmp20
Enam
Ambn
Amelx
size
26
28
30
32
category
odontogenesis
odontogenesis of dentin-containing tooth


A
Krt8
AmB dist
DEEx
VEE
SI
upper IEE
UMAP_3
UMAP_2
B
Krt5
C
Krt8
Krt5
Expression Level
Identity
upper IEE
pre-AmB-1
pre-AmB-2
AmB prox
AmB dist
D
non-AmG
AmG
Cell Type
Pre-branch
non-AmG
AmG
Cluster
Krt8
Trp63
Krt5
E
Krt8 expression along pseudotime
log10(value + 0.1)
AmG
non-AmG
Component 1
Component 2
F
Krt5 expression along pseudotime
G
Krt8
Krt5
IEE-OEE
DEEx
OEE
VEE
H
SI
ISR/SI


A
AmB dist
AmB prox
pre-AmB-2
pre-AmB-1
upper IEE
Krt8
Trp63
Krt5
Percent Expressed
20
40
60
80
Avg. Expression
1
0
-1
B
cell_type
upper IEE
pre-AmB-1
pre-AmB-2
AmB prox
AmB dist
Component 1
Component 2
log10(value + 0.1)
Trp63
Krt8
Krt5
C
Trp63+ vs Trp63-
Krt5+ vs Krt8+
4935
4753
4987
D
1
2
Trp63+
Trp63-
Krt5+
Krt8+
Trp63
Prnp
Tmem59
Igfbp5
Aldoa
Sparc
Sostdc1
Itm2b
Perp
Plod2
Tgfb2
Atp1a1
Cox4i1
Krt14
Ndufa1
Malat1
Mt1
Ubb
Tsc22d1
Sdhb
Fxyd3
Dcn
Rabac1
Krt5
Prdx6
Phlda1
Cldn10
Zbtb20
Epcam
Krt18
Krt8
Up_regulated
Down_regulated
low
high
Row Z-Score
1.Trp63+_vs_Trp63-
2.Krt5+_vs_Krt8+
E
regulation of epithelial cell proliferation
signal transduction by p53 class mediator
regulation of epidermis development
regulation of epidermal cell differentiation
epidermis morphogenesis
0.04
0.06
0.08
0.10
GeneRatio
p.adjust
0.010
0.015
0.020
Count
4
6
8
10
12


A
Krt8+ vs Krt5+ DEGs in EO cells 4,753 genes
ATAC-seq in skin (E13 vs E9, during Krt8-to-Krt5 transition) 9,997 sites remodel accessibility
ATAC gain 3,357 sites
ATAC loss 6,640 sites
BETA Motif scan
HOMER Motif analysis
B
Krt8+ vs Krt5+ DEG motif in EO
Symbol
P value
Tcfap2c/2a/2b/2e 8.23e−10
p53, p73 2.40e−08
C
ATAC gain motif in skin
Symbol
P value
target seq w/ motif
p63 1e−239 508
TFAP2C 1e−299 709
PBX2 1e−186 792
D
Krt8-to-Krt5 transition accessibility gain genes
Enrichment score
Ranked metric
p63+
p63−
Ranked in Ordered Dataset
E
Krt5+ cell signature genes
Enrichment score
Ranked metric
p63+
p63−
Ranked in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
F
Scale
chr15:
10 kb
mm10
102,000,000
102,005,000
101,710,000
101,715,000
E9_ATAC
E9_ATAC-seq
E13_ATAC
E13_ATAC-seq
p63KO_ATAC
p63KO_ATAC-seq
GENCODE VM23 Comprehensive Transcript Set (only Basic displayed by default)
Krt8
Krt5
Gm36026
Gm44323
ENCODE Candidate Cis-Regulatory Elements (cCREs) combined from all cell types
ENCODE cCREs
ReMap Atlas of Regulatory Regions (2376 items filtered out)
TRP63
chr15:102002799-102003186
chr15:102004393-102005883
chr15:101713751-101714142
chr15:101717250-101717481
ReMap density