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.

References

Barbieri CE, Pietenpol JA. 2006. P63 and epithelial biology. Exp Cell Res. 312(6):695-706.

Blanpain C, Fuchs E. 2006. Epidermal stem cells of the skin. Annu Rev Cell Dev Biol. 22:339-373.

Cohen E, Johnson C, Redmond CJ, Nair RR, Coulombe PA. 2022. Revisiting the significance of keratin expression in complex epithelia. J Cell Sci. 135(20).

Duverger O, Carlson JC, Karacz CM, Schwartz ME, Cross MA, Marazita ML, Shaffer JR, Morasso MI. 2018. Genetic variants in pachyonychia congenita-associated keratins increase susceptibility to tooth decay. PLoS Genet. 14(1):e1007168.

Duverger O, Ohara T, Shaffer JR, Donahue D, Zerfas P, Dullnig A, Crecelius C, Beniash E, Marazita ML, Morasso MI. 2014. Hair keratin mutations in tooth enamel increase dental decay risk. J Clin Invest. 124(12):5219-5224.

Fan X, Wang D, Burgmaier JE, Teng Y, Romano RA, Sinha S, Yi R. 2018. Single cell and open chromatin analysis reveals molecular origin of epidermal cells of the skin. Dev Cell. 47(1):21-37 e25.

Gan L, Liu Y, Cui DX, Pan Y, Wan M. 2020. New insight into dental epithelial stem cells: Identification, regulation, and function in tooth homeostasis and repair. World J Stem Cells. 12(11):1327-1340.

Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and b cell identities. Mol Cell. 38(4):576-589.

Hitz BC, Lee JW, Jolanki O, Kagda MS, Graham K, Sud P, Gabdank I, Strattan JS, Sloan CA, Dreszer T et al. 2023. The encode uniform analysis pipelines. Res Sq.

Jovic D, Liang X, Zeng H, Lin L, Xu F, Luo Y. 2022. Single-cell rna sequencing technologies and applications: A brief overview. Clin Transl Med. 12(3):e694.

Kantaputra PN, Matangkasombut O, Sripathomsawat W. 2012. Split hand-split foot-ectodermal dysplasia and amelogenesis imperfecta with a tp63 mutation. Am J Med Genet A. 158A(1):188-192.

Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M, Roop DR. 2007. P63 induces key target genes required for epidermal morphogenesis. Proc Natl Acad Sci U S A. 104(9):3255-3260.

Koul M, Dwivedi R, Upadhyay V. 2014. Ectrodactyly-ectodermal dysplasia clefting syndrome (eec syndrome). J Oral Biol Craniofac Res. 4(2):135-139.

Lacruz RS, Habelitz S, Wright JT, Paine ML. 2017. Dental enamel formation and implications for oral health and disease. Physiol Rev. 97(3):939-993.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with bowtie 2. Nat Methods. 9(4):357-359.

Laurikkala J, Mikkola ML, James M, Tummers M, Mills AA, Thesleff I. 2006. P63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. Development. 133(8):1553-1563.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S. 2009. The sequence alignment/map format and samtools. Bioinformatics. 25(16):2078-2079.

Li L, Wang Y, Torkelson JL, Shankar G, Pattison JM, Zhen HH, Fang F, Duren Z, Xin J, Gaddam S et al. 2019. Tfap2c- and p63-dependent networks sequentially rearrange chromatin landscapes to drive human epidermal lineage commitment. Cell Stem Cell. 24(2):271-284 e278.

Liu H, Yan X, Pandya M, Luan X, Diekwisch TG. 2016. Daughters of the enamel organ: Development, fate, and function of the stratum intermedium, stellate reticulum, and outer enamel epithelium. Stem Cells Dev. 25(20):1580-1590.

Presland RB, Dale BA. 2000. Epithelial structural proteins of the skin and oral cavity: Function in health and disease. Crit Rev Oral Biol Med. 11(4):383-408.

Qiu X, Mao Q, Tang Y, Wang L, Chawla R, Pliner HA, Trapnell C. 2017. Reversed graph embedding resolves complex single-cell trajectories. Nat Methods. 14(10):979-982.

Ramirez F, Ryan DP, Gruning B, Bhardwaj V, Kilpert F, Richter AS, Heyne S, Dundar F, Manke T. 2016. Deeptools2: A next generation web server for deep-sequencing data analysis. Nucleic Acids Res. 44(W1):W160-165.

Rinne T, Hamel B, van Bokhoven H, Brunner HG. 2006. Pattern of p63 mutations and their phenotypes--update. Am J Med Genet A. 140(13):1396-1406.

Roberts N, Horsley V. 2014. Developing stratified epithelia: Lessons from the epidermis and thymus. Wiley Interdiscip Rev Dev Biol. 3(6):389-402.

Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. 2011. Integrative genomics viewer. Nat Biotechnol. 29(1):24-26.

Romano RA, Solomon LW, Sinha S. 2012. Tp63 in oral development, neoplasia, and autoimmunity. J Dent Res. 91(2):125-132.

Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, Dunning MJ, Brown GD, Gojis O, Ellis IO, Green AR et al. 2012. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature. 481(7381):389-393.

Rufini A, Barlattani A, Docimo R, Velletri T, Niklison-Chirou MV, Agostini M, Melino G. 2011. P63 in tooth development. Biochem Pharmacol. 82(10):1256-1261.

Rufini A, Weil M, McKeon F, Barlattani A, Melino G, Candi E. 2006. P63 protein is essential for the embryonic development of vibrissae and teeth. Biochem Biophys Res Commun. 340(3):737-741.

Sharir A, Marangoni P, Zilionis R, Wan M, Wald T, Hu JK, Kawaguchi K, Castillo-Azofeifa D, Epstein L, Harrington K et al. 2019. A large pool of actively cycling progenitors orchestrates self-renewal and injury repair of an ectodermal appendage. Nat Cell Biol. 21(9):1102-1112.

Smith CE, Nanci A. 1995. Overview of morphological changes in enamel organ cells associated with major events in amelogenesis. Int J Dev Biol. 39(1):153-161.

Soares E, Zhou H. 2018. Master regulatory role of p63 in epidermal development and disease. Cell Mol Life Sci. 75(7):1179-1190.

Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, 3rd, Hao Y, Stoeckius M, Smibert P, Satija R. 2019. Comprehensive integration of single-cell data. Cell. 177(7):1888-1902 e1821.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES et al. 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 102(43):15545-15550.

Wang S, Sun H, Ma J, Zang C, Wang C, Wang J, Tang Q, Meyer CA, Zhang Y, Liu XS. 2013. Target analysis by integration of transcriptome and chip-seq data with beta. Nat Protoc. 8(12):2502-2515.

Wang Y, Zhao Y, Chen S, Chen X, Zhang Y, Chen H, Liao Y, Zhang J, Wu D, Chu H et al. 2022. Single cell atlas of developing mouse dental germs reveals populations of cd24(+) and plac8(+) odontogenic cells. Sci Bull (Beijing). 67(11):1154-1169.

Yu G, Wang LG, Han Y, He QY. 2012. Clusterprofiler: An r package for comparing biological themes among gene clusters. OMICS. 16(5):284-287.

Yu W, Sun Z, Sweat Y, Sweat M, Venugopalan SR, Eliason S, Cao H, Paine ML, Amendt BA. 2020. Pitx2-sox2-lef1 interactions specify progenitor oral/dental epithelial cell signaling centers. Development. 147(11).

Yu X, Singh PK, Tabrejee S, Sinha S, Buck MJ. 2021. Deltanp63 is a pioneer factor that binds inaccessible chromatin and elicits chromatin remodeling. Epigenetics Chromatin. 14(1):20.

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W et al. 2008. Model-based analysis of chip-seq (macs). Genome Biol. 9(9):R137.

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.

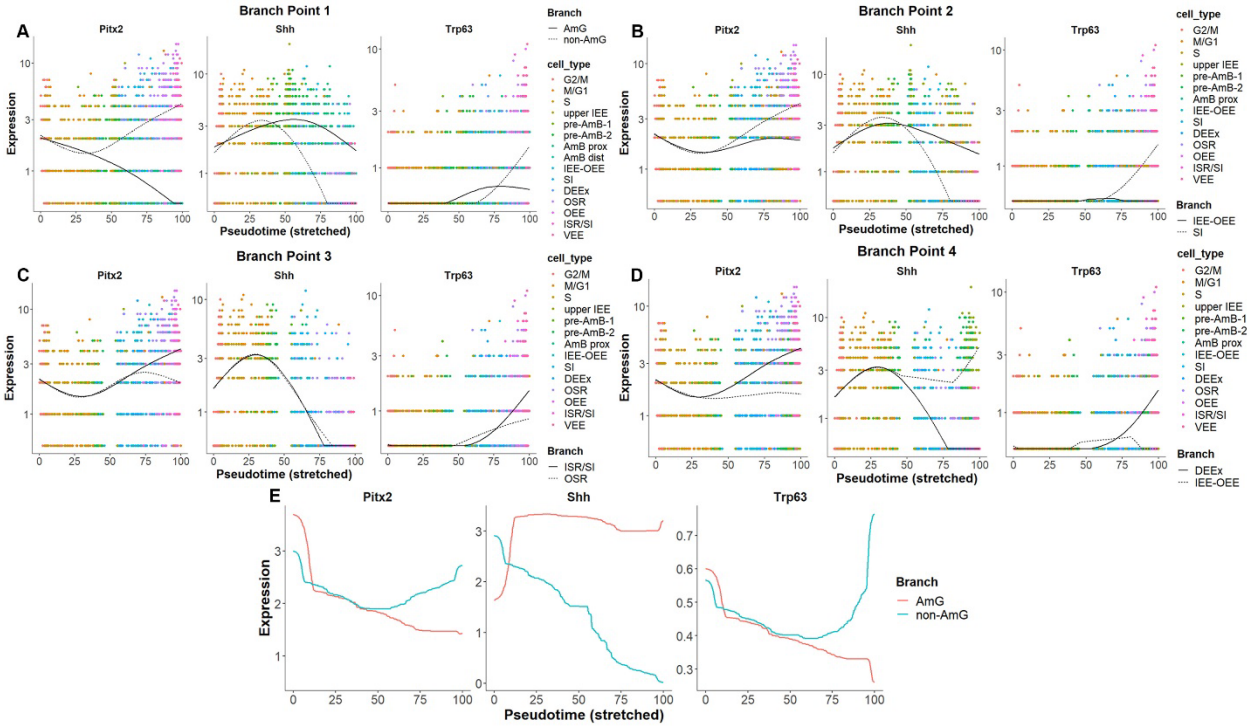
Figure 4. p63 regulates the Krt8-to-Krt5 transition during AmG cell differentiation. (A) Dot Plots showing the concurrent expression of p63 with Krt5 during the amelogenic Krt8-to-Krt5 transition. (**B**) Pseudotemporal expression changes of Krt8, Krt5 and p63 throughout amelogenesis. (**C**) Venn diagram 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

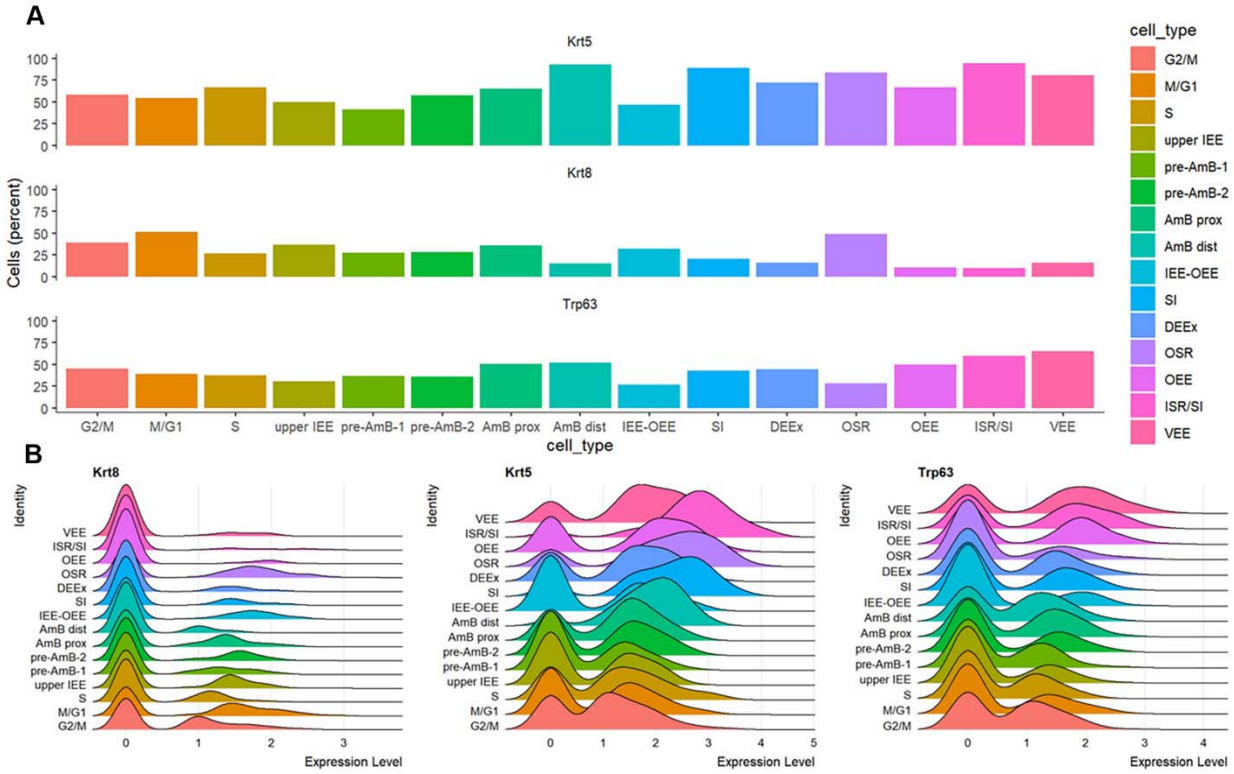
Krt8-to-Krt5 transition in skin. (**B**) Motif scan analysis revealing high enrichment of p53 and AP-2 family transcription factors (TFs) in DEGs between $Krt8^{+}$ vs. $Krt5^{+}$ dental epithelial cells. (**C**) Motif enrichment analysis demonstrating high enrichment of p63 and AP-2 family TFs in gained 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

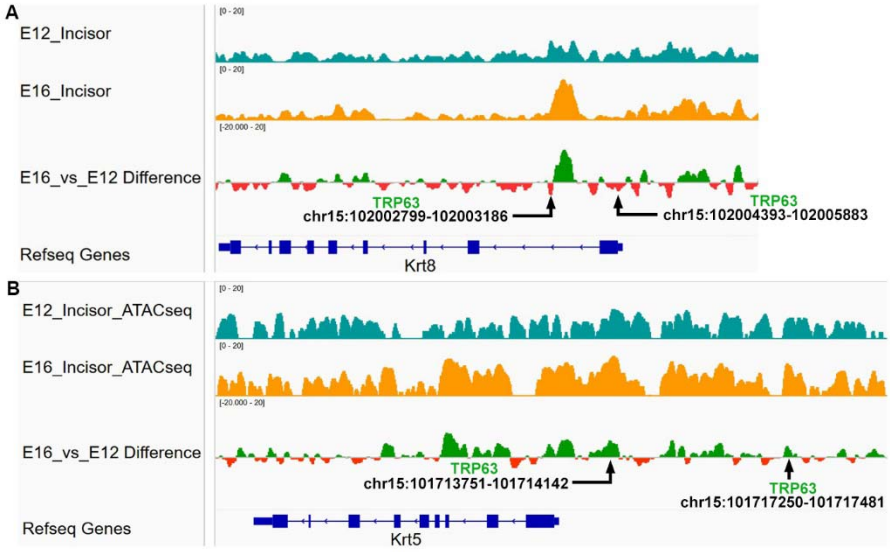
Supplemental Figures



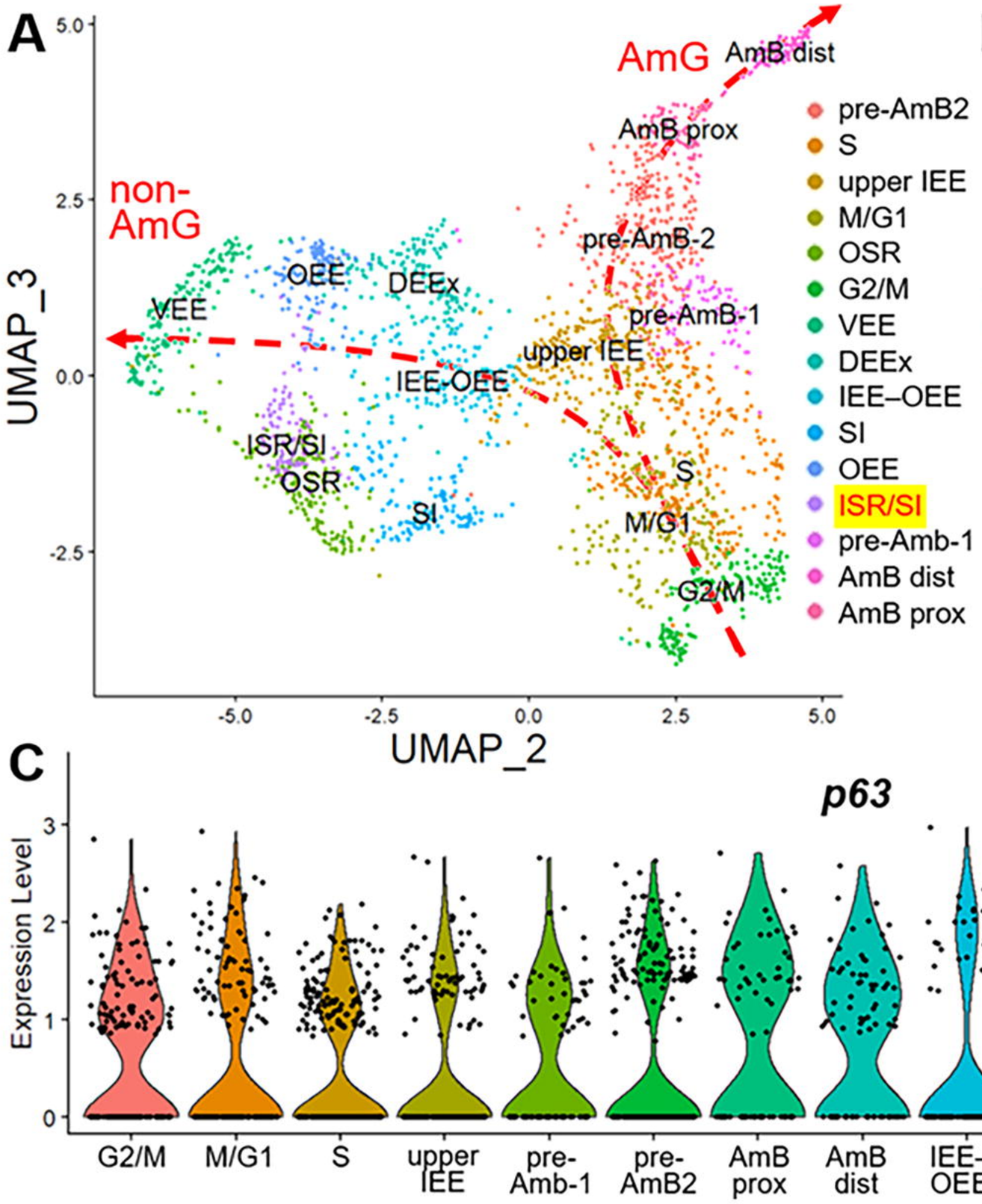
Appendix Figure 1. p63 functions as a branching gene throughout enamel organ development. (**A–D**) p63 acts as a branching gene at multiple branching points along the pseudotime trajectory of dental epithelial cell differentiation. (**E**) Kinetic curve along the pseudotime trajectory of dental epithelial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

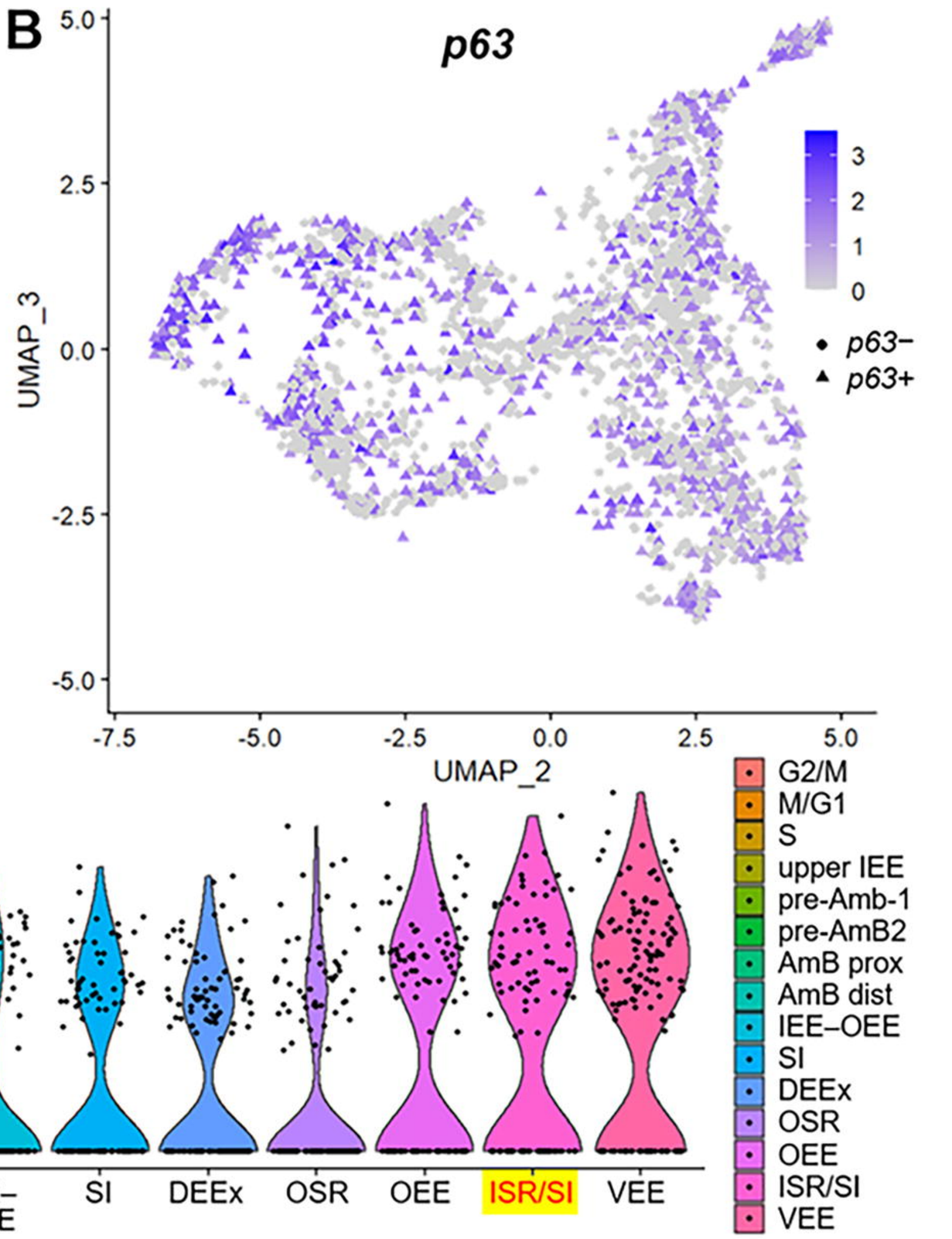


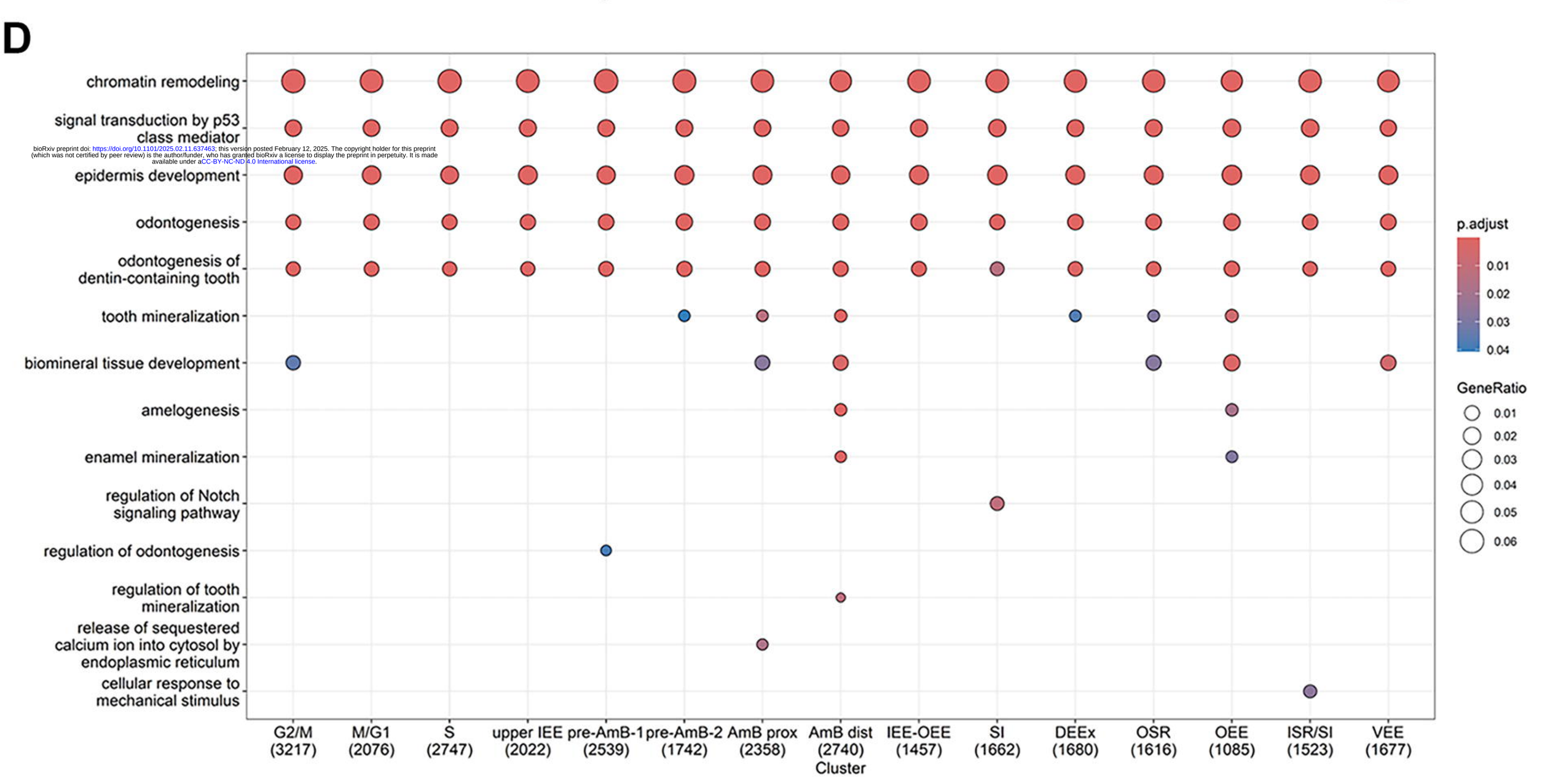
Appendix Figure 2. p63 expression coincides with Krt8-to-Krt5 transition during enamel organ development. (**A, B**) The percentage of p63-expression cells (A) and p63 expression level (B) are both positively correlated with the Krt8-to-Krt5 transition during enamel organ development.

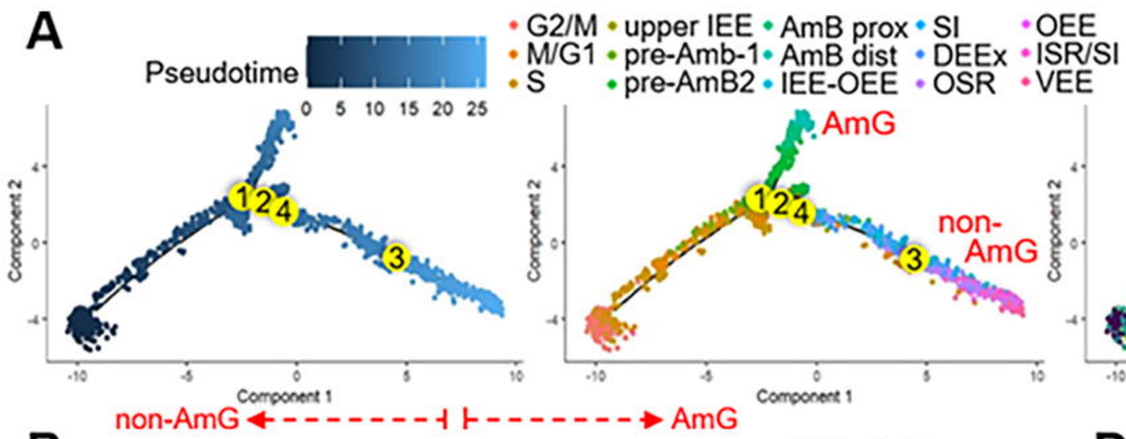


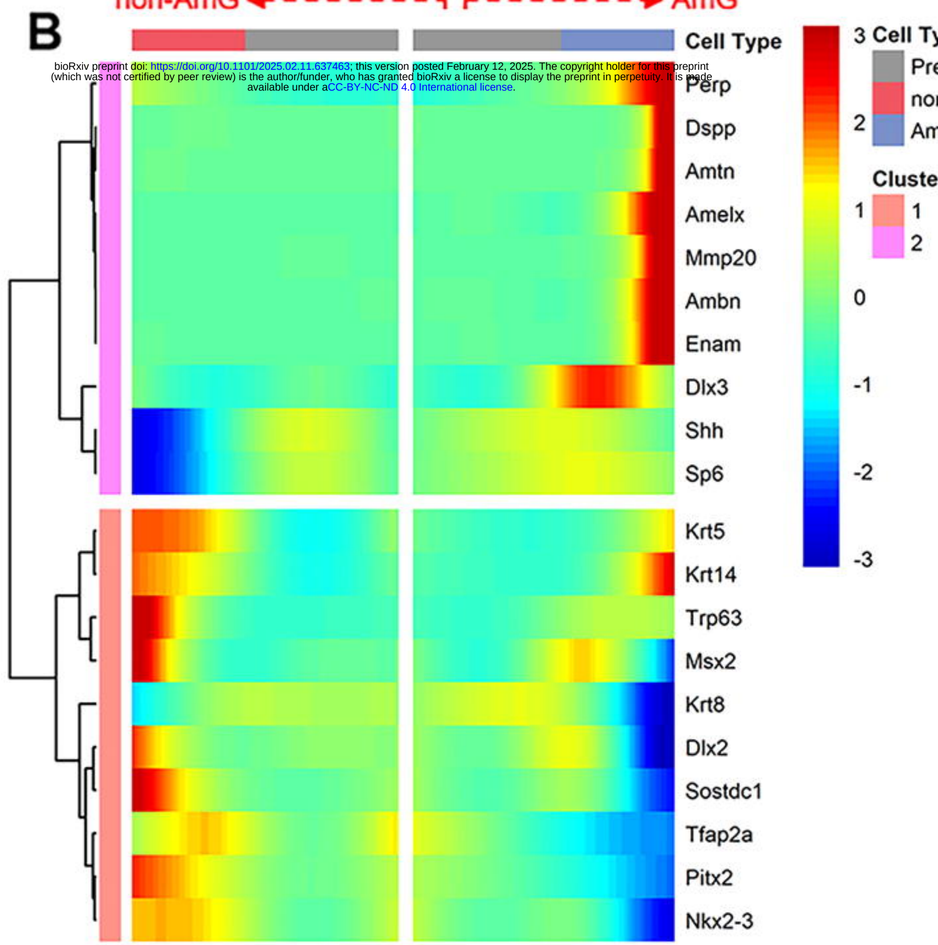
Appendix Figure 3. Dynamic chromatin accessibility of Krt8 and Krt5 during mouse incisor development. (A, B) ATAC-seq profiles demonstrating differential chromatin accessibility of Krt8 (A) and Krt5 (B) during mouse incisor development. The Krt8 locus showed decreased chromatin accessibility at E16 compared 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.

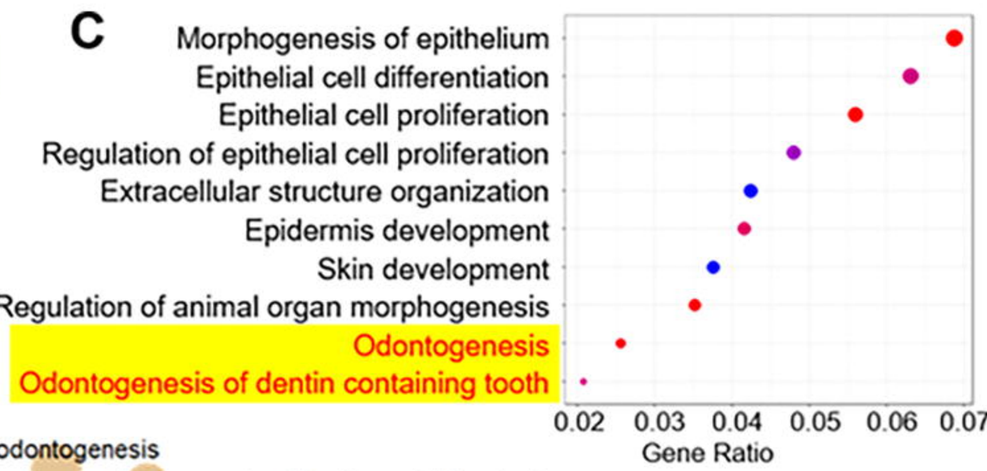


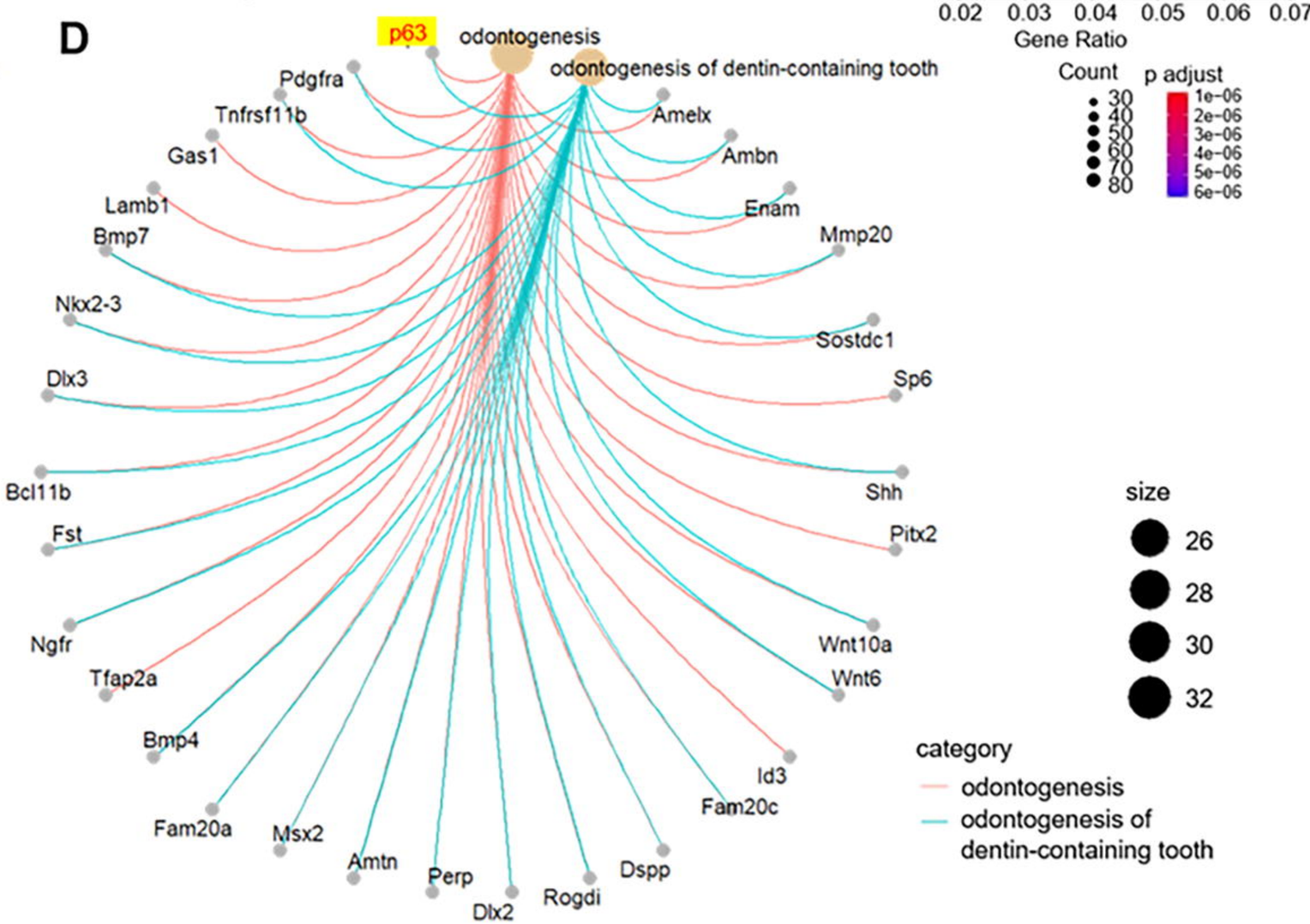


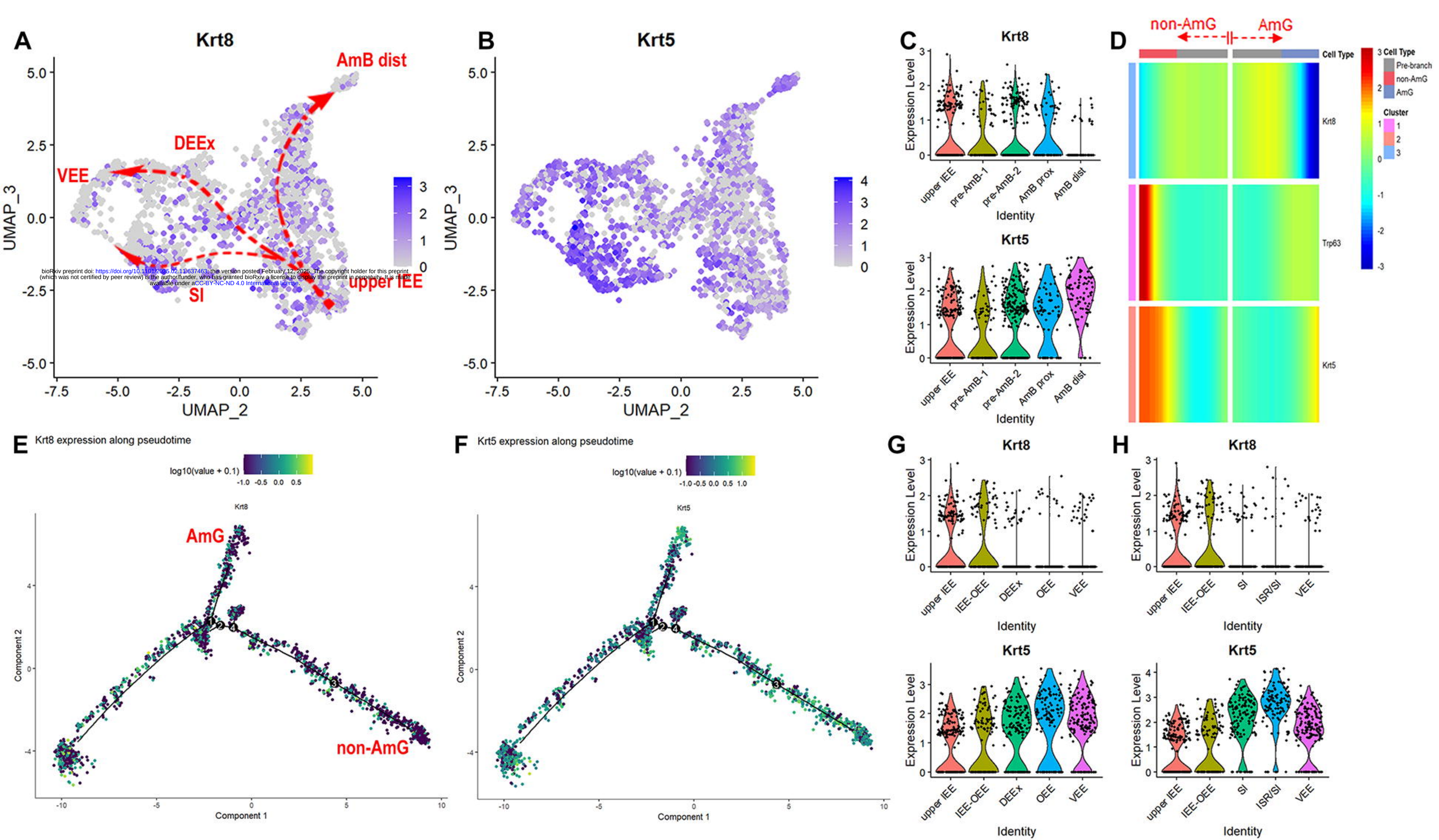




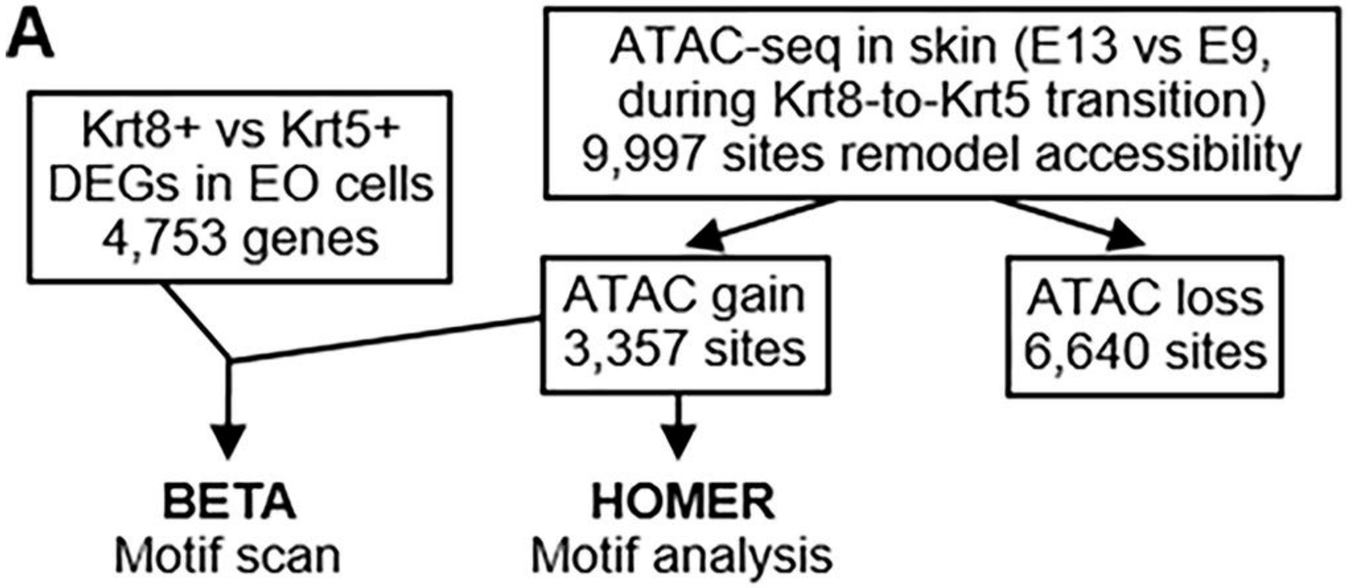






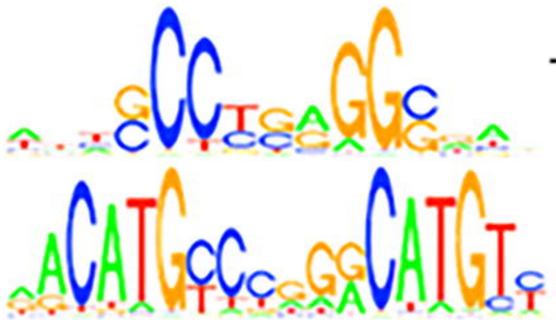


A



B

Krt8+ vs Krt5+ DEG motif in EO



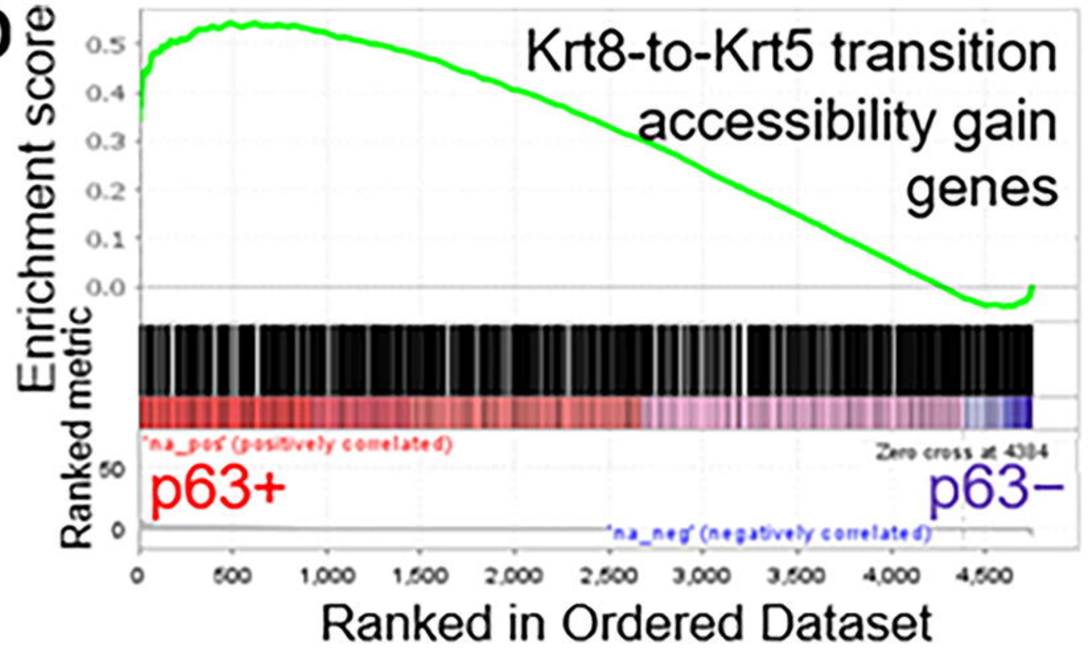
Symbol	*P* value
Tcfap2c/2a/2b/2e	8.23e−10
p53, p73	2.40e−08

C

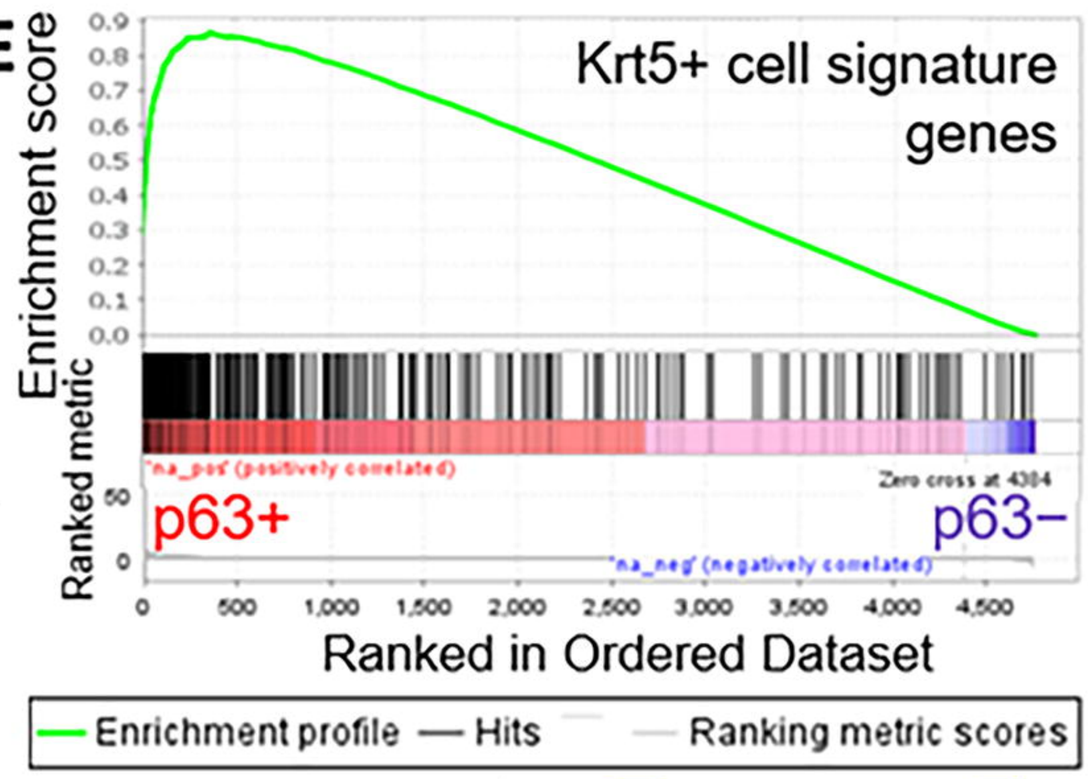
ATAC gain motif in skin

Symbol	*P* value	# target seq w/ motif
p63	1e−239	508
TFAP2C	1e−299	709
PBX2	1e−186	792

D



E



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