

A large pool of actively cycling progenitors orchestrates self-renewal and injury repair of an ectodermal appendage

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The classical model of tissue renewal posits that small numbers of quiescent stem cells (SCs) give rise to proliferating transit-amplifying cells before terminal differentiation. However, many organs house pools of SCs with proliferative and differentiation potentials that diverge from this template. Resolving SC identity and organization is therefore central to understanding tissue renewal. Here, using a combination of single-cell RNA sequencing (scRNA-seq), mouse genetics and tissue injury approaches, we uncover cellular hierarchies and mechanisms that underlie the maintenance and repair of the continuously growing mouse incisor. Our results reveal that, during homeostasis, a group of actively cycling epithelial progenitors generates enamel-producing ameloblasts and adjacent layers of non-ameloblast cells. After injury, tissue repair was achieved through transient increases in progenitor-cell proliferation and through direct conversion of Notch1-expressing cells to ameloblasts. We elucidate epithelial SC identity, position and function, providing a mechanistic basis for the homeostasis and repair of a fast-turnover ectodermal appendage.

Ectodermal appendages—such as teeth, hair follicles, mammary glands and nails—share many aspects of their development and adult renewal^{1–3}. The maintenance and repair of these appendages is enabled by resident adult SCs that have the capacity for both prolonged self-renewal and generation of differentiated cells. Adult tissue homeostasis and repair in these organs have been thought to involve a few long-lived quiescent SCs that generate rapidly dividing, short-lived transit-amplifying proliferating progenitors, which then produce differentiated cells^{4–6}. However, recent studies in several organs have used lineage tracing in combination with mathematical modelling and single-cell approaches to demonstrate that renewal capacity can be distributed over a large population of actively dividing progenitors or SCs⁷. Importantly, seemingly homogeneous SC and progenitor populations are in fact highly heterogeneous^{8,9}. Therefore, how SCs are utilized during tissue renewal and how such heterogeneous populations are regulated remain central questions in SC biology.

The mouse incisor provides a model system for understanding the renewal and regeneration of adult tissues. This organ continuously replaces materials that are lost as a result of abrasion from gnawing, and the entire tooth is replaced every 4 weeks¹⁰ (Supplementary Fig. 1a,b), making the mouse incisor one of the most rapidly renewing mineralized tissues in mammals. This continuous growth is

driven by epithelial and mesenchymal SCs that give rise to cells that produce calcified enamel or dentin and cementum, respectively^{11,12}.

Earlier studies proposed that the incisor follows a classical SC paradigm, similar to that proposed for the haematopoietic system¹³, with a few slow-cycling SCs residing in the proximal portion of the outer enamel epithelium (OEE) or stellate reticulum (SR) of the labial cervical loop (laCL) that give rise to transit-amplifying inner enamel epithelium (IEE) cells, which subsequently differentiate into all of the epithelial lineages¹⁴ (Fig. 1a–c). This model was based on the observation that a few putative SC markers chosen from a candidate-based approach, such as *Sox2*, *Bmi1*, *Gli1* and *Lrig1*, are expressed by long-lived (label-retaining) cells in the OEE of the laCL and can be lineage-traced^{15–18}. However, owing to the broad expression of these markers throughout both the quiescent and proliferating regions, as well as the limitations in the available genetic tools, our knowledge regarding the identity, hierarchies, heterogeneity and kinetics of the SCs remain poorly understood.

To address these questions, we examined cell identities using an unbiased scRNA-seq approach and then studied the kinetics and dynamics of the dental epithelial populations in incisors. By combining this information with genetic-lineage tracing and injury-repair studies, we have uncovered a highly dynamic SC model that is distinct from the traditional view of SCs in the mouse incisor and

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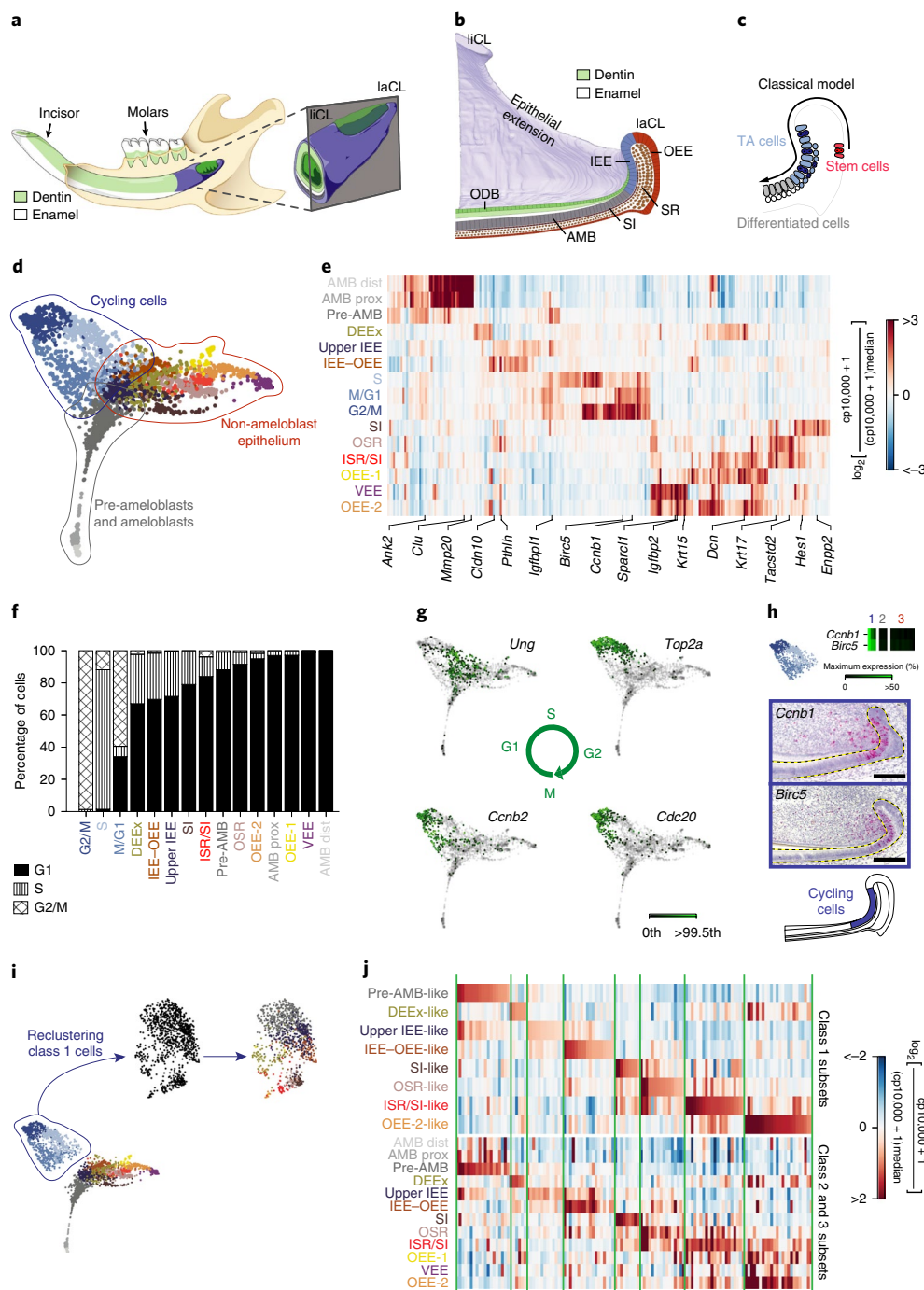


Fig. 1 | Identification of distinct cell types by single-cell transcriptomic analysis and delineation of their spatial organization in the incisor epithelium. **a**, Schematic of the mouse lower jaw and magnification of the incisor growth region showing enamel (white) and dentin (green). **b**, An illustration of a sagittal section through the incisor growth region showing epithelial regions. AMB, ameloblasts; ODB, odontoblasts. **c**, The current model posits that SCs in the OEE give rise to transit-amplifying (TA) cells in the IEE, which then differentiate into enamel-secreting ameloblasts. **d**, SPRING representation of the incisor epithelium dataset with 15 spectral clusters, grouped into 3 main classes as follows: cycling cells, pre-ameloblasts and ameloblasts, and non-ameloblast epithelial cells. **e**, A heat map showing the average expression of the top 20 enriched genes per cluster. Highlighted genes were used to further assess spatial expression patterns. AMB dist, distal ameloblasts; AMB prox, proximal ameloblasts; DEEx, dental epithelial extensions; IEE-OEE, junction between the IEE and OEE; ISR, inner stellate reticulum; OSR, outer stellate reticulum; VEE, ventral enamel epithelium; cp, counts per. **f**, The fraction of cells per cluster in each cell-cycle phase. **g**, SPRING plots showing gene expression of cell-cycle markers (*Ung*, *Top2a*, *Cdc20* and *Ccnb2*). Gene expression is plotted on a linear scale; the colour scale indicates levels of expression from grey (zero counts) to dark green (color saturated at 99.5th expression percentile). **h**, Expression of the cell-cycle markers *Ccnb1* and *Birc5* in the 15 spectral clusters (top) and their spatial localization using RNAscope (middle two panels). Bottom, a schematic highlighting the cycling region. **i**, Class 1 cells before (left) and after regressing out the cell cycle (middle). Right, class 1 cells are coloured according to the most similar profile among class 2 and 3 populations. **j**, The top 20 enriched genes for each of the cell populations identified in class 1 after regressing out the cell-cycle effect (top) show similar expression patterns (annotated in green vertical lines) to their counterparts in the class 2 and class 3 populations (bottom). For **h**, scale bars, 100 μ m. Dashed lines outline the epithelium.

that explains the tissue homeostasis and injury repair of a fast-turn-over ectodermal appendage.

Results

An unbiased analysis of cellular heterogeneity in the incisor epithelium. As previous experiments examined mice at various stages of development^{12,16,18}, we first determined when the incisor is in steady state (Supplementary Fig. 1c–h, Supplementary Note) and then reassessed the expression of the marker genes using high-resolution single-molecule *in situ* hybridization and genetic-lineage tracing. We discovered that cells of the posterior OEE, the presumed SC location, were not proliferative despite rapid epithelial turnover (Supplementary Fig. 1d,e), and that SC marker genes were more broadly expressed within the incisor growth region than previously appreciated (Supplementary Fig. 2), suggesting that the label-retaining OEE cells may not function as SCs during homeostasis. This prompted us to revisit the cellular hierarchies that underlie the homeostasis and repair of adult mouse incisors.

To address this question in an unbiased manner, we performed scRNA-seq of sorted epithelial cells (Supplementary Fig. 3a). We sequenced a total of 3,173 cells from 5 incisors of adult male mice and visualized the high-dimensional, whole-transcriptome data using SPRING¹⁹. This graph-based method is well suited for studying differentiation trajectories as it preserves the relationships between transcriptionally similar cells. Gene expression analysis identified 690 immune and 134 mesenchymal cell transcriptomes (Supplementary Fig. 3c–e), which were removed from the dataset to focus on the epithelium. The remaining cells were divided into 15 clusters that were characterized by distinct gene expression signatures (Fig. 1d); the clustering was unbiased and independent of any established markers of the incisor epithelium.

To assign putative biological identities to each cluster, we computed a heat map of the 20 most enriched genes per cluster (Fig. 1e, Supplementary Table 1), which revealed the existence of three major categories of cells (Fig. 1d). Class 1 was enriched in cycling cells (Fig. 1d, blue), class 2 contained pre-ameloblasts and ameloblasts (Fig. 1d, grey), and class 3 consisted of the remaining incisor epithelium or the non-ameloblast epithelium (Fig. 1d, brown and orange).

We next assessed the overall distribution of dividing cells and found that the vast majority were in class 1 (Fig. 1f). The transcriptomes of class 1 cells reflected successive phases of the cell cycle and formed a visible loop on the SPRING plot, implying that some cells at the end of the cycle return to their original state, reflecting self-renewal. We identified domains that are characteristic of S, G2/M and M/G1 phases, as exemplified by expression of *Ung* (G1 and S), *Top2a* (end of S phase to G2), *Cdc20* (end of G2 phase to M entry) and *Ccnb2* (end of M phase to G1 entry; Fig. 1g). Spatially, class 1 cells were localized to the IEE and the neighbouring stratum intermedium (SI) region of the incisor epithelium (Fig. 1h, Supplementary Fig. 3f). Given the dominance of the cell-cycle signature among the transcriptomes of class 1 cells, we further visualized class 1 cells using SPRING after regressing out the cell-cycle effect²⁰. We found that class 1 cells had signatures reflecting class 2 and 3 populations, presumably reflecting progenitors that are cycling but beginning to upregulate differentiation genes (Fig. 1i,j, Supplementary Table 2). Interestingly, even with the cell cycle regressed out, the expression of the putative SC marker genes (*Sox2*, *Gli1*, *Bmi1* and *Lrig1*) did not localize exclusively to any sub-region of the SPRING plot (Supplementary Fig. 2c,f,i,l).

Class 2 cells (Fig. 1d), which formed a simple linear continuum in the SPRING graph, were ordered in pseudotime using population-balance analysis²¹ (PBA; Fig. 2a). Changes in gene expression through pseudotime (Fig. 2b–g, grey arrow) revealed groups of genes, such as *Amelx*, *Ambn*, *Enam* and *Mmp20*, that are expressed by secretory-stage ameloblasts²² (Fig. 2d,e). Furthermore, we identified groups of genes that were upregulated (*Clu* and *Steap*;

Fig. 2d,e), downregulated (*Igfbpl1* and *Ank2*; Fig. 2g), or transiently upregulated and then downregulated (*Otop2* and *Dspp*; Fig. 2f), most of which have not previously been linked to the differentiation of ameloblasts (Supplementary Table 3). *In situ* hybridization validated the expected correlation between the spatial and pseudo-temporal organization of cells undergoing amelogenesis (Fig. 2c–g, Supplementary Fig. 3g).

The third major class of cells was composed of 9 clusters that formed a continuum of transcriptional states in the two-dimensional (2D) representation of the SPRING *k*-nearest neighbour (kNN) graph without displaying clear boundaries (Fig. 1d). Enriched genes in each cell type labelled distinct compartments within the non-ameloblast region of the incisor epithelium (Fig. 3a–h, Supplementary Fig. 3h, Supplementary Table 4). Gene set enrichment analysis (GSEA) highlighted active transcription and translation in the upper IEE and at the IEE–OEE junction, suggesting that these populations may be immediate descendants of the progenitors (Supplementary Table 5), as well as significant enrichment of the Notch pathway in the SI.

Proliferation dynamics of the incisor epithelium. Together, these observations suggested that the IEE (class 1) houses progenitor cells that give rise to the two other classes (classes 2 and 3) of cells. Indeed, RNA velocity analysis²³ confirmed these relationships (Fig. 4a), indicating class 1 cells as the root (Fig. 4b–e). We further computed the fate biases using FateID²⁴, which also suggested that certain class 1 cells may differentiate toward specific class 2 and/or class 3 cell states (Fig. 4f–i). As such a lineage hierarchy is inconsistent with the currently accepted model (Fig. 1c), we further examined the dynamics of the incisor growth region using green fluorescent protein (GFP)-tagged H2B label dilution (Supplementary Fig. 4a–e, Supplementary Note). Epithelial cells were isolated from the incisor growth region of adult *TetOn-H2B-GFP* mice²⁵, which were pulse treated with doxycycline, after various days of chase to dilute out the GFP label; GFP densities were measured using fluorescence-activated cell sorting (FACS; Fig. 5a,b). A simple Poisson model was used to fit the distribution of the GFP signal at day 7, with day 1 as the initial condition. The data indicated that, on average, the proliferating population divided 2.98 ± 0.20 times between day 1 and 7, and that $60 \pm 15\%$ of the cells were non-dividing (Fig. 5b).

Histological analysis revealed that H2B–GFP, which was initially present in proliferating IEE and adjacent SR cells (Fig. 5c, top), was completely diluted after 21 d, consistent with the rapidly cycling nature of this region and indicating that non-dividing cells were post-mitotic cells that left the IEE. By contrast, several GFP⁺ cells were detected in the SR–OEE region and the ameloblast layer at 21 d chase (Fig. 5c, middle), corresponding to a GFP peak (Fig. 5b, green line); these remained for up to 12 months (Supplementary Fig. 4f). Importantly, H2B–GFP labelling was absent in the SR–OEE region after a short pulse (Fig. 5c, top), suggesting that, similar to previous studies in the ear epidermis²⁶, the label-retaining cells in this region are post-mitotic.

To challenge these findings using an independent assay, we quantitatively analysed the spatial distribution of proliferating cells within the growth region at different timepoints (Fig. 5d–f, Supplementary Note). We focused on the initial 48 h following 5-ethynyl-2'-deoxyuridine (EdU) injection to study the first cell cycle, before labelled cells are lost due to label dilution (more than four cell cycles²⁷) or distal displacement. After 45 min, the highest concentration of EdU⁺ cells was in the centre of the IEE, with labelling also present in the immediately adjacent portion of the SR and the epithelial extension from the laCL towards the lingual cervical loop (liCL; Fig. 5f), confirming that these compartments are sites of active cell division. By 48 h, higher levels of EdU⁺ cells were found at the liCL and the ridge of the adjacent epithelium, reflecting a doubling in the proliferating cells, and in the more

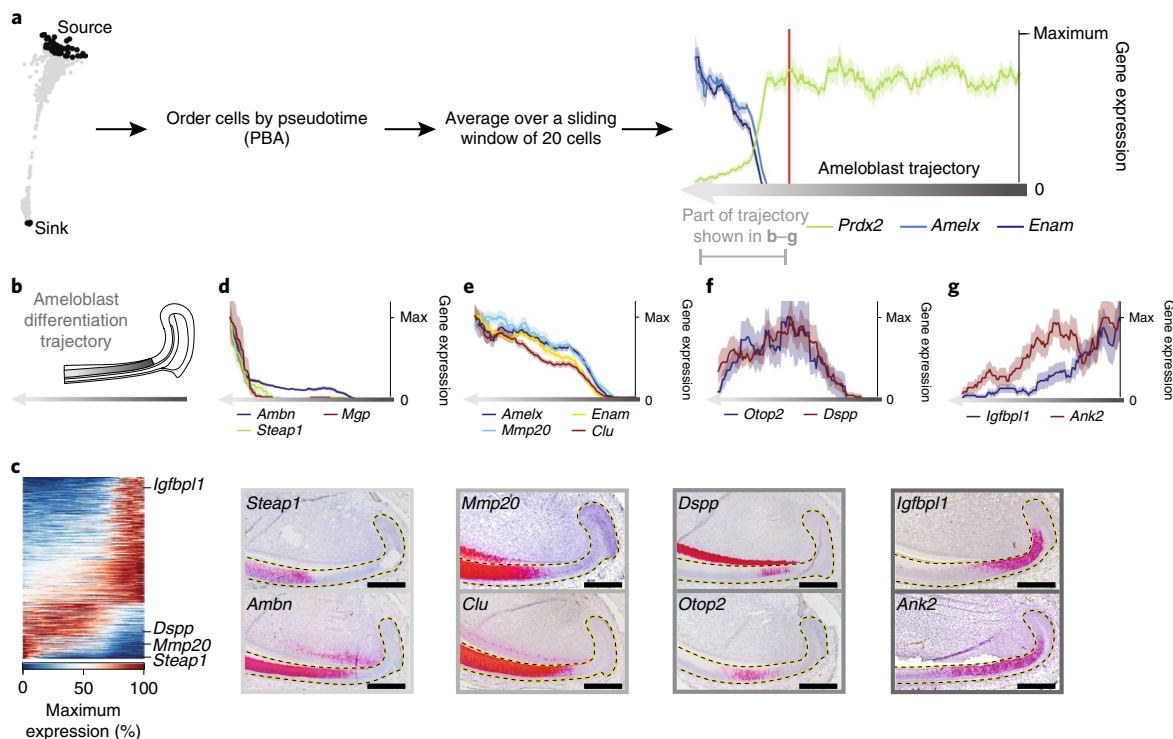


Fig. 2 | An unbiased catalogue of cell types during amelogenesis. **a**, PBA was used to order 431 cells through the pseudotime of their differentiation trajectory from pre-ameloblast to ameloblast. Black regions define the source and sink cells of the trajectory. Gene expression along the trajectory was smoothed by averaging over a sliding window of 20 cells along the trajectory. We centred the exploration of gene-expression changes along the ameloblast trajectory around the onset of the expression of known mature ameloblast genes by excluding the first 300 pre-ameloblast cells. The resulting ordering of averaged cells is referred to as the ameloblast differentiation trajectory. **b**, Schematic of the ameloblast differentiation trajectory; dark grey represents earlier cells in the trajectory. **c**, A heat map of 439 genes that showed statistically significant variability (false discovery rate (FDR)-adjusted $P < 0.05$) along the ameloblast differentiation trajectory. Rows are genes, columns are single cells ordered by pseudotime. **d–g**, Expression of selected markers of ameloblast differentiation along pseudotime (scRNA-seq; top) and in situ (RNAscope; bottom). The y axis shows the average expression of a gene within the window of $20 \pm \text{s.e.m.}$ cells (or $+1/(\text{window size})$ for mean values of zero), normalized to the maximum value. Dashed lines outline the epithelium. Scale bars, 100 μm .

distal portion of the IEE, corresponding to distal displacement of cells. Lastly, similar to the H2B–GFP results, EdU^+ cells were observed in the SR–OEE region, away from the IEE (Fig. 5f). We verified these patterns of cell proliferation and movement by counting EdU-labelled and 5-bromodeoxyuridine (BrdU)-labelled cells in incisor growth-region compartments following a double-pulse analysis (Fig. 5g–i).

Taken together, the kinetics results support the hypothesis generated from the scRNA-seq data that proliferating IEE progenitors give rise to two groups of cells—a larger population that populates the ameloblast layer and a smaller population that exits the IEE to become SR and OEE cells (Fig. 5j).

scRNA-seq reveals changes in cell cycle and differentiation program during recovery. Having elucidated the steady-state population hierarchy and kinetics, we next analysed the system under non-homeostatic conditions by treating wild-type mice with 5-fluorouracil (5FU)^{28,29} for 4 d. This eliminated the majority of proliferative epithelial cells, which resulted in tissue loss and severely perturbed the shape of the cervical loop, whereas the non-cycling regions were relatively unaffected (Fig. 6a–c, Supplementary Fig. 5a). Moreover, 5FU-treated mice exhibited abnormal ameloblast organization and delayed and disorganized enamel matrix formation (Fig. 6b,d,e). Then, 3 d after treatment with 5FU (that is, recovery day 3), the proliferating cell population burgeoned, without concomitant proliferation in the OEE (Fig. 6c,e). Furthermore, cell-cycle time in the IEE was shortened, as evidenced by the

increase in cells that re-entered the cell cycle after a 24 h chase ($23\text{--}47 \text{ BrdU}^+\text{EdU}^+$ cells per section; Fig. 6c, yellow box). The regeneration process peaked between 3–5 d after treatment with 5FU and, by recovery day 10, treated incisors were indistinguishable from the controls (Fig. 6b–e).

To determine the transcriptional signature during recovery, we performed scRNA-seq analysis on sorted incisor epithelial cells from 5FU-treated mice at recovery day 3 (Supplementary Fig. 3b). SPRING visualization of transcriptomes from the recovery conditions retained the three-class layout observed in the control (Supplementary Fig. 5b,c). We next visualized the relative density of cells in recovery versus control conditions (Fig. 6f). We also calculated the relative abundance of each of the 15 populations after annotating cells in recovery by the closest expression profile that was observed in the control (Fig. 6g). In the recovery sample, we detected counterparts to all of the control clusters with similar gene-expression profiles, except for the proximal ameloblast cluster (Supplementary Fig. 5d, Supplementary Table 6). We found an increase in class 1 cells at recovery day 3 (57.1% of the recovery cells versus 36.7% in control; Fig. 6g), which was also shown by increased and expanded expression domains of *Ccnb1* and *Birc5* (Fig. 6i, Supplementary Fig. 5e). The transcriptomic signature of class 1 cells remained very similar to their control counterparts as depicted by comparable GSEA profiles (Supplementary Table 7). By contrast, the class 2 population (pre-ameloblasts and ameloblasts) was rarely observed in the recovering epithelium (1.3% of the recovery cells versus 18.3% in control; Fig. 6g). This was also

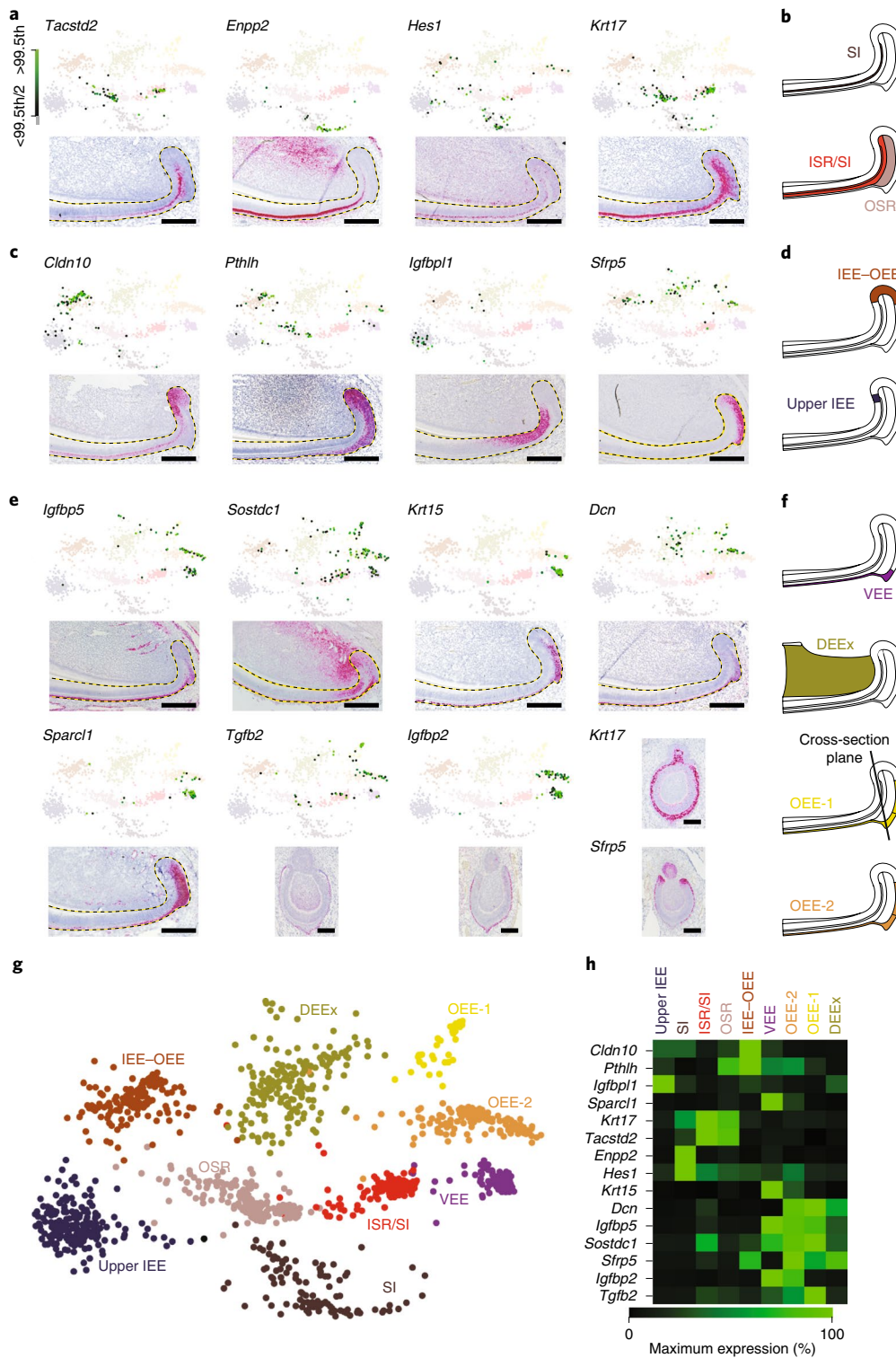


Fig. 3 | Mapping of class 3 cell types to spatial regions of the incisor-epithelium growth region. a,c,e, SPRING plots (top) and in situ detection by RNAscope (bottom) of marker transcript combinations for each cell type in class 3. Dashed lines outline the epithelium. Scale bars, 100 μ m. Color scale shows expression percentile. **b,d,f**, Schematics of the expression pattern for the cluster are shown. Candidate genes were selected from the top ten enriched genes per cluster. Ranking is provided in Supplementary Table 1. **g**, An expanded SPRING view of the 9 clusters composing class 3. **h**, A heatmap showing the expression profile of genes used to define each cell type in class 3.

demonstrated by the distal shift in the expression of two pre-ameloblast markers, *Igfbp1* and *Ank2* (Fig. 6j, Supplementary Fig. 5f), as well as ameloblastin (Fig. 6d), implying delayed differentiation of ameloblasts.

Owing to the large increase in proliferative cells, we next investigated the underlying subsets of class 1 cells using SPRING after regressing out the cell-cycle effect. Similar to what we observed in the control cells, class 1 cells showed a substructure that

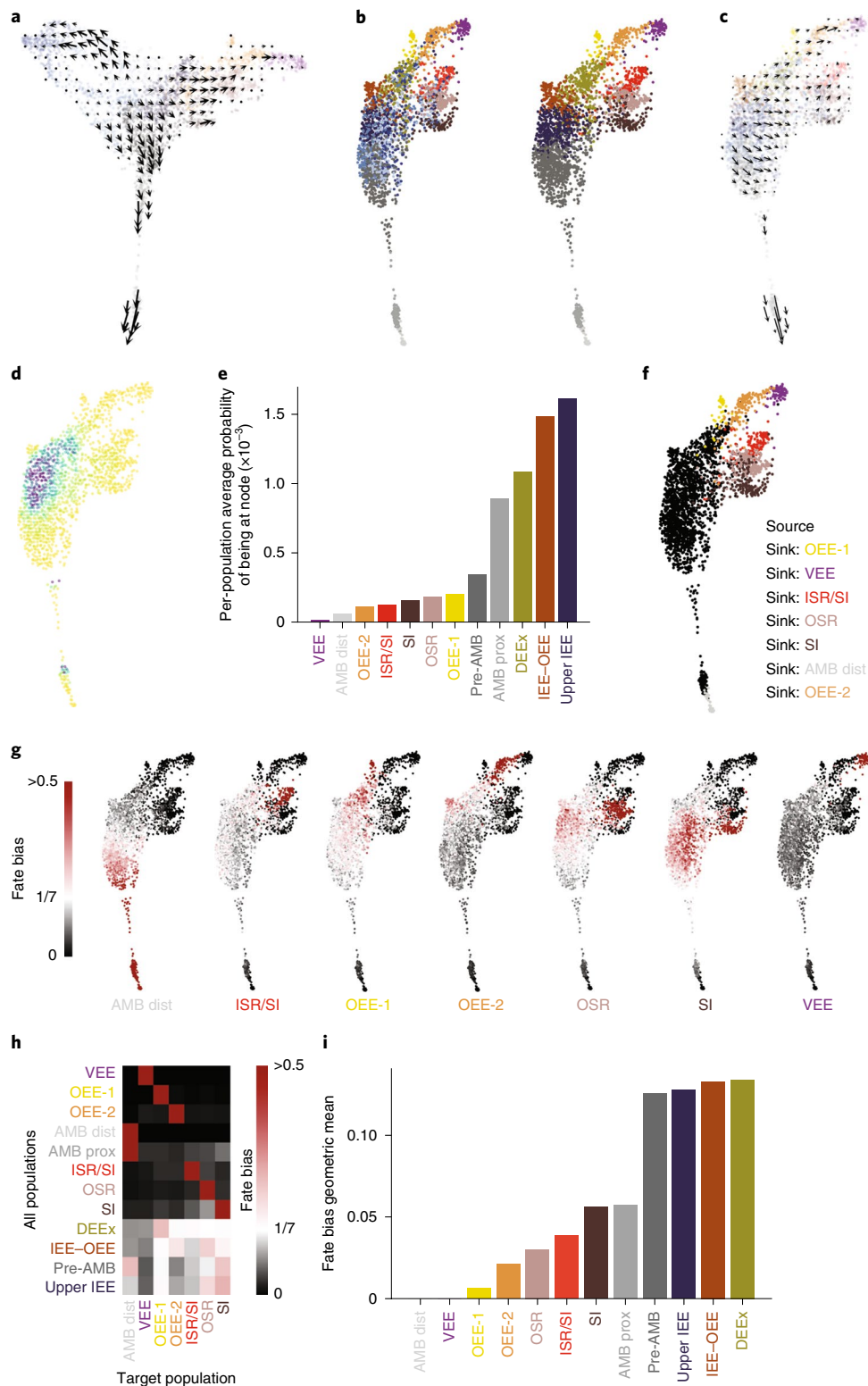


Fig. 4 | Class 1 cells house progenitor cells that give rise to class 2 and class 3 populations **a**, Velocity vectors projected onto the SPRING plot shown in Fig. 1d and smoothed over a regular grid. **b**, SPRING plot of all of the cells shown in Fig. 1d after regressing out the cell cycle (left). Right, the same plot as on the left after merging the cycling class 2–3-like cells with their non-cycling counterparts. **c**, Velocity vectors projected onto the SPRING plot from **b** and smoothed over a regular grid. **d**, The differentiation start point is represented as high-density regions on the 2D SPRING plot after a Markov process with transition probabilities on the basis of reversed velocity vectors. **e**, The data from **d** are shown as average density for each population shown in **b**, right. **f**, The seven target populations (fate) used to infer the fate biases of each cell using FateID. **g**, SPRING plots coloured by FateID with a fate bias towards a given target population. The colour ranges from white (equal probability of each fate) to red (>0.5 probability of a certain fate). **h**, The data from **g** with FateID fate biases averaged over the populations shown in **b**, right. **i**, Uniformity of the rows in **h** measured by calculating the geometric mean; the highest bars represent populations with the most uniform fate biases and, therefore, candidate multipotent states.

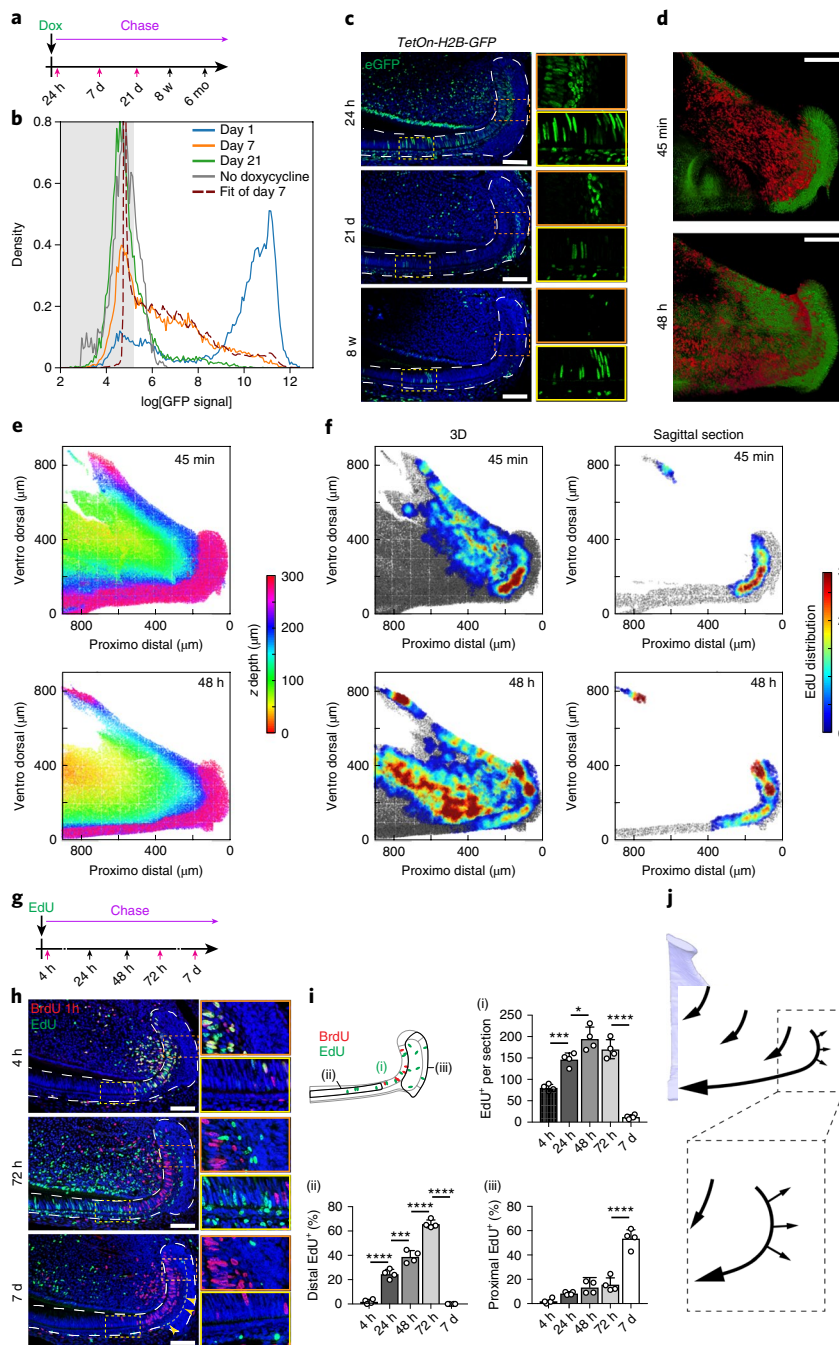


Fig. 5 | Proliferation dynamics of the incisor epithelium growth region. **a**, Schematic of doxycycline pulse treatment and timepoints of sample collection during the chase; five samples were collected per timepoint. Red arrows indicate timepoints of samples that were analysed by FACS in **b**. **b**, FACS histograms showing H2B-GFP dilution patterns at chase days 1, 7 and 21, and the best fit of the distribution of the GFP signal at day 7 using the distribution at day 1 as the initial condition and the proportion of non-dividing cells (r) and the number of divisions (λ) as the parameters. The best fit was for $r = 0.60 \pm 0.15$ and $\lambda = 2.98 \pm 0.20$, indicating that the proliferating population has divided three times on average between day 1 and 7 and the proportion of cycling cells is $1 - 0.60 = 0.40$. The experiment was performed once. **c**, GFP immunostaining in the incisor growth region of H2B-GFP expressing mice at the indicated time after pulse treatment with doxycycline. Magnifications of the areas indicated by orange and yellow dashed boxes are shown on the right. **d-f**, A whole-mount proliferation assay 45 min (top) and 48 h (bottom) after EdU injection. **d**, Two-photon micrographs of $K14^{CreER}; R26^{mT/mG}$ mice. The membrane of epithelial cells is labelled green and EdU is labelled red. **e**, A z-depth view of the incisor growth region calculated from the two-photon images. **f**, Heat maps showing the local average percentage of EdU⁺ cells in three dimensions (3D; left) and in sagittal section (right) of the incisor growth region. The experiment was performed once. **g**, EdU/BrdU labelling assay and timepoints of sample collection during the chase. The red arrows mark samples used in **h**; four samples were collected per timepoint. **h**, Immunostaining of mice killed at the indicated timepoints after EdU injection; BrdU was injected 1 h before organs were collected. **i**, EdU⁺ cells were counted in the entire incisor growth region (i), distal to the BrdU⁺ region (ii) and proximal to the BrdU⁺ region (iii). The percentage of total EdU⁺ cells present in distal or proximal region is shown in (ii) and (iii); $n = 4$ animals. For the bar charts, data are mean \pm s.d. Normally distributed data were analysed using one-way ANOVA with Tukey-Kramer post hoc test. Significance was taken as $P < 0.05$ with a confidence interval of 95%. **** $P < 0.0001$. **j**, Schematic of cell dynamics (outline by arrows) in the incisor growth region. For **c** and **h**, dashed lines outline the epithelium; magnifications ($\times 2.6$) of the areas indicated by orange and yellow dashed boxes are shown on the right; scale bars, 100 μ m.

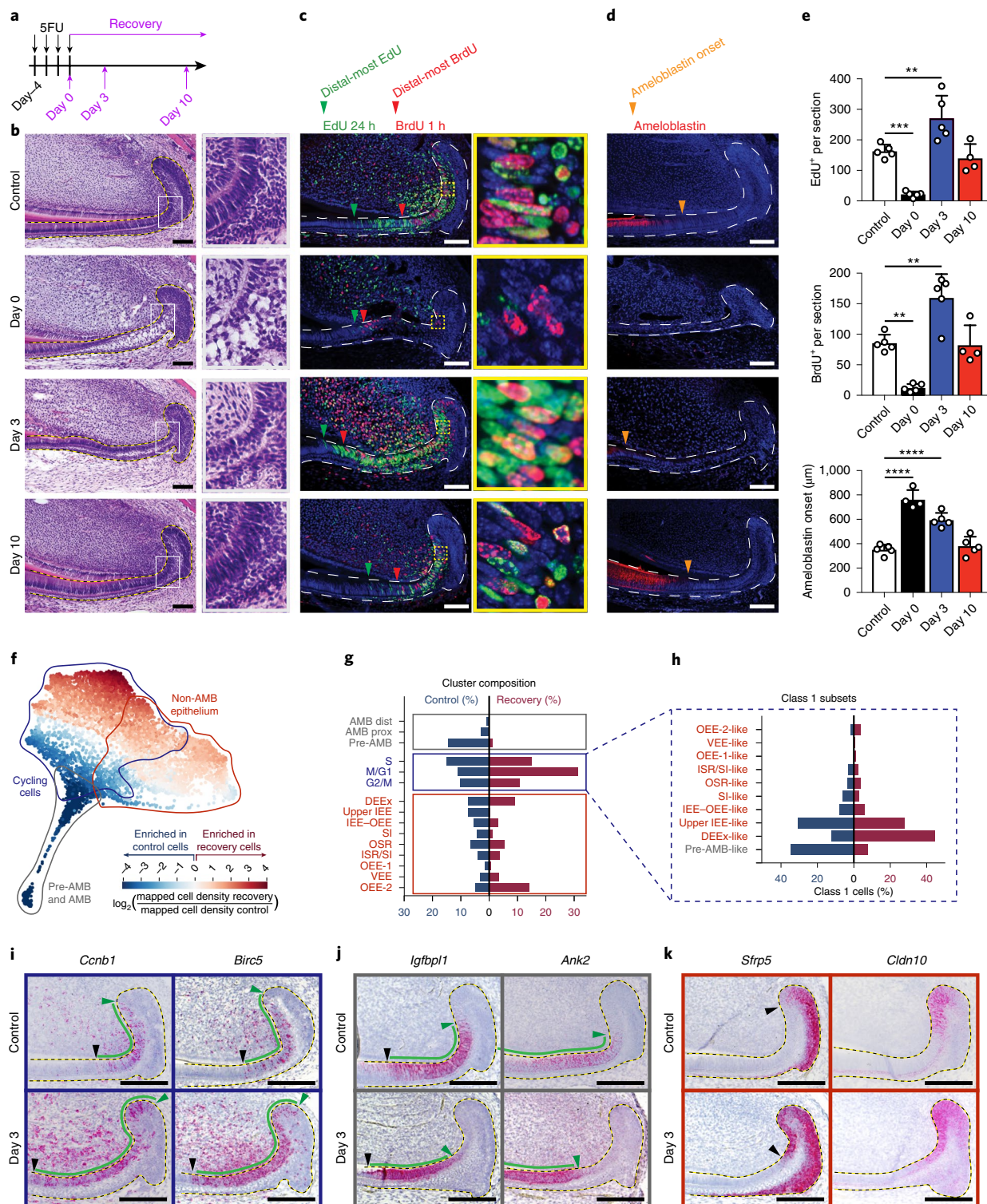


Fig. 6 | Single-cell transcriptomic analysis reveals changes in cell cycle and differentiation program after injury. **a**, Schematic of 5FU treatment and sample collection timepoints during recovery. **b–d**, Control and 5FU-treated mice were analysed at the indicated timepoints using haematoxylin and eosin (H&E) staining (**b**), EdU and BrdU labelling (**c**), or ameloblastin immunostaining (**d**). Green and red arrowheads indicate the most distal EdU-labelled and BrdU-labelled cells, respectively, and orange arrowheads indicate the onset of ameloblastin expression. Boxed areas (grey in **b**, yellow in **c**) are magnified (**b**, $\times 3.7$; **c**, $\times 9.1$) on the right. **e**, Quantification of EdU⁺ and BrdU⁺ cells per section and the distance of ameloblastin expression onset from the proximal edge of the laCL are quantified from control or 5FU-treated mice; $n = 5$ animals, except for ameloblastin at day 0 and EdU and BrdU at day 10, for which $n = 4$ mice. For the bar charts, data are mean \pm s.d. Normally distributed data were analysed using parametric tests including one-way ANOVA with Tukey–Kramer post hoc test. Significance was taken as $P < 0.05$ with a confidence interval of 95%. **f**, SPRING plot of injury and control cells coloured by the fold change in density between the two conditions. **g**, The proportion of cells in each population defined in control (blue) and recovery (red) conditions. **h**, Cycling-cell subset size for control and recovery conditions. **i–k**, RNAse staining for markers of class 1 cycling cells (**i**), class 2 pre-ameloblasts (**j**), and markers of class 3 cells in control (top) and recovery day 3 (bottom) samples (**k**). For **b** and **i–k**, dashed lines outline the epithelium. For **b–d** and **i–k**, scale bars, 100 μ m.

reflected the class 2 and 3 populations (Supplementary Fig. 5h,i, Supplementary Table 8). In class 1, there was a large decrease in the pre-ameloblast signature (from around 35% to 4% of cells), a marked increase in the epithelial extension signature (from 12% of cells in control to 43% of cells in injury) and a decrease in SI-like cells (from 8% to 4%; Fig. 6h). Recovery was also associated with the upregulation of several genes that are typically expressed in class 3, including *Sfrp5* and *Cldn10*, the expression domains of which expanded towards the proliferating regions (Fig. 6k, Supplementary Fig. 5g). The data from the recovery studies indicate that, after injury, the epithelium is repaired by recruiting more progenitors to cycle and by triggering changes in the proportions of progenitor cells, as well as by increasing the number of times progenitor cells replicate owing to a shortened cell cycle and a delayed onset of differentiation.

Injury induces Notch1-mediated conversion of SI cells into ameloblasts. To further study the mechanisms that underlie recovery, we focused on the SI cells. SI cells have been postulated to descend from the OEE–SR¹⁶ and to act as differentiated supporting cells for ameloblasts³⁰. However, our kinetic analyses indicated that SI cells are an intermediate population derived from the IEE, and that they are in fact progenitors of SR and OEE cells. To determine the role of the SI during homeostasis and injury, we studied our scRNA-seq results to identify markers that would enable the labelling of these cells and found that several components of the Notch signalling pathway were highly enriched in SI cells (Supplementary Fig. 6b, left panels). This was consistent with our observation of active Notch1 signalling in SI cells by immunostaining (Fig. 7a, Supplementary Fig. 6a) as well as with previous reports of *Notch1* expression in SI cells^{12,31,32}. Interestingly, during the initial days after injury, *Notch1* expression and activity were downregulated (Supplementary Fig. 6a–d, Supplementary Table 9).

To fate-map and examine the clonal capacity of SI cells during both homeostasis and repair, we generated *Notch1^{CreER}; R26^{tdTomato}* mice (Fig. 7b). Marked cells were present exclusively in the SI layer 24 h after a single dose of tamoxifen (Fig. 7c, top row). Then, 48 h after CreER activation, labelled cells were found in the SR and the OEE, confirming that SR and OEE cells are derived from SI cells. Notably, 8 d after induction, scattered labelled cells were observed among the overlying ameloblasts, suggesting that descendants of SI cells infrequently contribute to normal ameloblast turnover (Fig. 7c, top row). This prompted us to investigate whether SI cells could act as reserve progenitors; lineage tracing of *Notch1^{CreER}; R26^{tdTomato}* cells after treatment with 5FU led to labelling in the proliferative region of the IEE and ameloblast layer as early as 24 h after Cre induction (Fig. 7c, top row). When a higher dosage of tamoxifen was used, we observed labelling of almost every cell in the IEE–ameloblast layer of 5FU-treated mice; by contrast, in untreated mice, the IEE–ameloblast layer remained sparsely labelled (Supplementary Fig. 7a,b). In injured mice, the number of labelled IEE and ameloblast cells continued to increase in the first 5 d after CreER induction but then decreased thereafter as labelled cells moved distally while being displaced proximally by newly formed unlabelled cells (Fig. 7c, Supplementary Fig. 7b).

To distinguish between upregulated *Notch1* expression in injured IEE cells or ameloblasts and contribution to the IEE–ameloblast layer by *Notch1*-expressing SI cells, we performed live imaging of control and 5FU-treated incisor explants (Fig. 7d). In control explants, SI cells frequently sent small transient protrusions into the adjacent IEE–ameloblast layer; by contrast, in 5FU-treated explants, the persistent protrusions of SI cells extended further into the injured IEE–ameloblast layer, and the SI cells frequently migrated completely into these damaged layers (Fig. 7e,f, Supplementary Videos 1–4). The descendants of the *Notch1*-expressing cells in the IEE–ameloblast layer did not maintain Notch activity, as indicated by negative

staining of the Notch1 intracellular domain (NICD; Fig. 7g), such that they lost their original SI fate and few of the descendants were actively proliferating (Fig. 7h). Importantly, the contribution of SI cells to injury repair is not limited to 5FU-induced injury, as an increase in labelled ameloblasts derived from *Notch1*-expressing cells was similarly observed after mechanically severing the incisor tip (Supplementary Fig. 7c,d).

Lastly, we tested the functional importance of *Notch1*-expressing SI cells during regeneration by eliminating *Notch1⁺* cells through diphtheria toxin expression³³ (Supplementary Fig. 7e). Elimination of *Notch1⁺* cells resulted in marked reduction in proliferative *Notch1⁺* (mGFP⁺) cells in the IEE–ameloblast layer, impaired repair and delayed ameloblast differentiation at recovery day 7, compared with 5FU-treated samples in which the *Notch1*-expressing cells were not ablated (Fig. 7i–n). Together, these experiments indicate that *Notch1⁺* SI cells are critical for timely and proper recovery from injury.

Discussion

The continuously growing mouse incisor model has enabled the identification of a number of fundamental mechanisms in this renewing tissue^{34–36}, but the underlying SC biology has remained unclear. Here, through an unbiased interrogation of the incisor epithelium, we pinpointed the identity of the various populations that comprise the mouse incisor, and we uncovered the cellular hierarchy and division kinetics of this renewing structure. Together, our data highlight a mechanism by which incisor progenitors generate both the ameloblasts and the non-ameloblast epithelium (Supplementary Fig. 7f). Although homeostatic renewal from highly proliferative progenitors has been observed in other self-renewing tissues^{26,37,38}, it has not yet been documented in epithelial appendages, such as the hair follicle³⁹ or the nail⁴⁰. Our findings that the epithelial SCs give rise to the non-ameloblast cell layers also suggest that the SCs may produce components of their own niche.

SCs and their descendants exhibit a diverse repertoire of responses to meet the demands of injury-induced recovery^{41,42}. Over the past several years, the transcriptional programs that underlie these cellular behaviours have been dissected using scRNA-seq, revealing transcriptional events and cell states that are unique to injury-induced recovery, such as activation of progenitor reservoirs^{33,44}, transition of one cell state to another^{45,46} or initiation of a developmental program⁴⁷. Our findings indicate that the incisor epithelium recovers from cytotoxic injury using the same cell types that enable homeostatic renewal without the emergence of a cell type that is unique to injury. It is still possible that a distinct repair program exists and was not detected, either because we probed the injured epithelium at a single timepoint or owing to the type of injury that we used. Thus, future studies comparing diverse injury and damage models will be useful for determining the range of approaches that tissues can utilize for repair. Furthermore, the finding that the progenitor cells in the incisor epithelium are highly heterogeneous in their differentiation potentials suggests that they are biased to differentiate either into ameloblast or into one of the non-ameloblast cell subtypes. The mechanisms that control such lineage biases are becoming more recognized^{9,48}, but how such specification occurs in the incisor remains to be elucidated.

Our results provide an unbiased catalogue of cell types in the mouse dental epithelium at the single-cell level and offer a resource for functional studies of specific cell populations and genes during steady state and injury. Although the current study revealed the function of one of the non-ameloblast cell-layer populations, it will be important to determine the functional role of the other cell types of this class. Similarly, it will be of interest to use similar techniques to elucidate SC identity and position and the function of the mesenchymal compartment of the incisor niche to generate a broad and complete understanding of incisor renewal as a model for organ homeostasis.

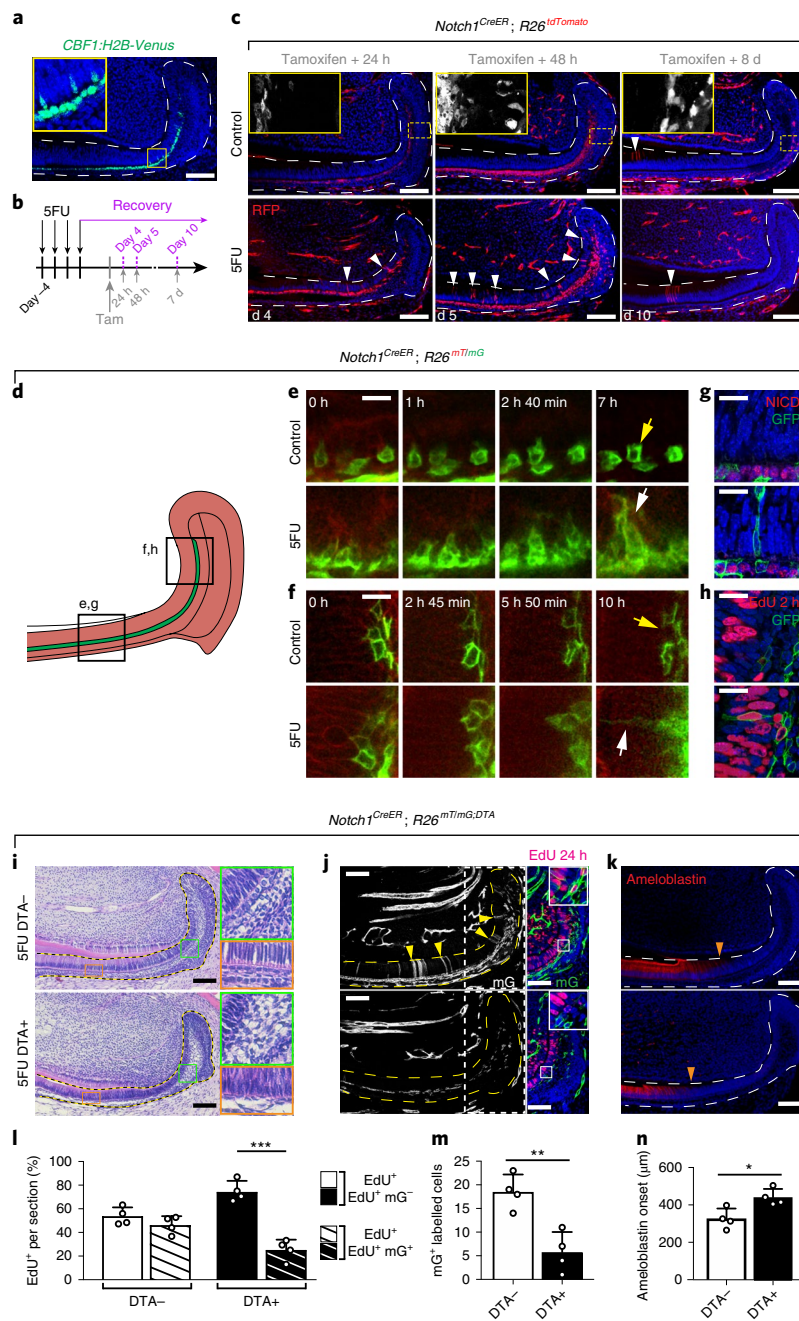


Fig. 7 | SI cells undergo direct conversion to ameloblasts to contribute to the recovery of injured incisor epithelium. a, *Notch1* pathway activity was assessed using the *CBF1:H2B-Venus* reporter. **b**, Schematic of 5FU treatment, tamoxifen induction and sample collection timepoints of *Notch1^{CreER}; R26^{tdTomato}* mice. **c**, During recovery, *Notch1*-expressing cells contribute to the regeneration of wounded dental epithelium. Incisors from control (top row) and 5FU-treated (bottom row) mice were analysed at the indicated timepoints after tamoxifen injection. The white arrowheads indicate traced cells that have entered the proliferating region of the IEE-ameloblast layer. Areas indicated by yellow boxed areas are magnified ($\times 4.1$) on the top-left, in which tdTomato⁺ cells are shown in white without DAPI staining. **d**, Schematic of explant time-lapse imaging. **e, f**, Two-photon time-lapse live imaging shows the movement of *Notch1*-expressing cells during 8 h (ameloblast region; **e**), or 10 h (IEE region; **f**) of culture (Supplementary Videos 1–4). **g**, NICD (red) and GFP (green) immunostaining in control *Notch1^{CreER}; R26^{mT/mG}* mice (top) and *Notch1^{CreER}; R26^{mT/mG}* mice at recovery day 5 (bottom), 48 h after tamoxifen induction. **h**, EdU (red) and GFP (green) immunostaining in control *Notch1^{CreER}; R26^{mT/mG}* mice (top) and in *Notch1^{CreER}; R26^{mT/mG}* mice at recovery day 5 (bottom), 48 h after tamoxifen induction. **i–n**, Ablation of *Notch1*-expressing cells impaired normal recovery after injury with 5FU. DTA⁺ (*Notch1^{CreER}; R26^{mT/mG}; Ros26^{DTA/+}*) and DTA⁻ mice were examined at recovery day 7 using H&E (**i**), EdU and GFP (**j**), and ameloblastin immunostaining (**k**). Areas indicated by green ($\times 4$), orange ($\times 4$) and yellow ($\times 3.5$) boxes are magnified. Areas indicated by white boxed on the left panels in **j**, are shown with EdU and GFP staining on the right panels. The yellow arrowheads indicate traced cells that have entered the proliferating region of the IEE or the ameloblast layer. The orange arrowheads indicate the onset of ameloblastin expression. The number of proliferating cells (**l**), cells descended from *Notch1*-expressing progenitors in the ameloblast or IEE layers (**m**) and the distance of ameloblastin expression onset from the proximal edge of the laCL (**n**) are quantified from DTA⁺ or DTA⁻ mice injured by treatment with 5FU; $n = 4$ animals. For bar charts, data are mean \pm s.d. Normally distributed data were analysed using parametric tests including one-way ANOVA with Tukey–Kramer post hoc test. Significance was taken as $P < 0.05$ with a confidence interval of 95%. For **a**, **c** and **i–k**, dashed lines outline the epithelium. For **a**, **c** and **g–k**, scale bars, 100 μ m. For **e** and **f**, scale bars, 10 μ m.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at <https://doi.org/10.1038/s41556-019-0378-2>.

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Author contributions

A.S. conceived the study, designed and performed experiments, and analysed data. P.M., D.C.-A., M.W., T.W., J.H., L.E., K.H. and P.P. performed experiments and analysed data. R.Z., K.K. and T.M. analysed data. C.W.S. contributed essential reagents. O.D.K. and A.M.K. directed the study and wrote the paper with A.S., P.M., R.Z., and J.H.

Competing interests

A.M.K. is a founder of 1CellBio. C.W.S. is employed by Genentech.

Additional information

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Methods

Mice. Mice were maintained in the University of California San Francisco (UCSF) specific pathogen-free animal facility in accordance with the guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center. All of the experimental procedures were approved by the Laboratory Animal Resource Center at UCSF. The incisor injury and kidney-capsule transplantation experiments were performed according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary Office, Zurich. Male mice aged 8–12 weeks were used for all of the experiments, except those in which mice were analysed at various ages to determine the steady state (as described in the main text). The following mouse lines were used: wild-type (C57BL/6J, Jax: 000664); *Gli1*^{CreER} (*Gli1*^{tm3(cre/ERT2)Alj}, Jax: 007913); *Sox2*^{CreER} (*Sox2*^{tm1(cre/ERT2)Hoch}, Jax: 017593); *Bmi1*^{CreER} (*Bmi1*^{tm1(cre/ESR1)Mrc}, Jax: 010531); *Lrig1*^{CreER} (*Lrig1*^{tm1(cre/ERT2)Rjc}, Jax: 018418); *Notch1*^{CreER} (*Notch1*^{tm5(cre/ERT2)Rko}, Jax: 027235); *K14*^{Cre} (*Tg*(KRT14-cre)1Amc, MGI: 2445832); *R26R*^{mt/mG} (*Gt*(ROSA)26Sor^{tm4(ACTB-tdTomato, EGFP)Loa}, Jax: 007576); *R26R*^{lacZ} (*Gt*(ROSA)26Sor^{tm1Sor}, Jax: 003474); *R26R*^{tdTomato} (*Gt*(ROSA)26Sor^{tm14(CAG-tdTomato)Hsc}, Jax: 007914); *Rag1*^{-/-} (*Rag1*^{tm1Mom}, Jax: 002216); *TetOff-H2B-GFP* (*Tg*(tetO-HIST1H2B)/GFP)47Efu, Jax: 005104); *K5tTA* (*Tg*(KRT5-tTA)1216Glk, MGI: 357575); *TetOn-H2B-GFP* (*Gt*(ROSA)26Sor^{tm1(rtTA-M2)lac}::*Coll1a1*^{tm7(tetO-HIST1H2B)/GFP}), Jax: 016836); *rtTA* (*Gt*(ROSA)26Sor^{tm1(rtTA-M2)lac}::*Coll1a1*^{tm1(tetO-cre)Haho}, Jax: 021025); *CBF1:H2B-Venus* (*Tg*(Cp-HIST1H2BB/Venus)47Hadj, Jax: 020942); and Rosa-DTA (B6.129P2-Gt(ROSA)26Sor^{tm1(DTA)Lky/J}, Jax: 009669).

Tissue preparation and histological analysis. Euthanized mice were perfused using PBS and then 4% paraformaldehyde (PFA) in PBS. Lower mandibles were dissected away from the rest of the cranium and fixed with 4% PFA in PBS overnight at 4 °C. Mandibles were then decalcified, paraffin sectioned and H&E stained as described previously¹⁵.

Immunofluorescence staining. For immunofluorescence, paraffin sections were rehydrated and antigen retrieval was performed by sub-boiling slides in a microwave for 15 min in a citrate buffer (pH 6.2) containing 10 mM citric acid, 2 mM EDTA and 0.05% Tween-20. For BrdU immunostaining, samples were additionally washed with 2 N HCl for 30 min. The following primary and secondary antibodies were used: anti-ameloblastin (sc50534, Santa Cruz Biotechnology); rat anti-BrdU (ab6326, Abcam); mouse anti-BrdU (B35128, Invitrogen); anti-GFP (GFP-1020, Aves; ab13790, Abcam); anti-NICD (cleaved Notch1, D388, Cell Signaling); anti-RFP (600-401-379, Rockland). EdU was detected using a Click-iT Plus EdU Alexa Fluor 488 Assay Kit (Invitrogen, C10637). Samples were blocked in 1× animal-free blocker (Vector Laboratories, SP-5030) supplemented with 2.5% heat-inactivated goat serum, 0.02% SDS and 0.1% Triton X-100. All of the antibodies were diluted in the same blocking solution without serum. For the detection of NICD, primary antibodies were first detected using biotinylated secondary antibodies, and then sequentially amplified using VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, PK-6100) and Tyramide Signal Amplification (PerkinElmer, SAT704B001EA). TUNEL staining (Roche, 12156792910) was performed according to the manufacturer's instructions. DAPI (Invitrogen, D1306) was used for nuclear staining and all of the images were acquired using a Leica-TCS SP5 confocal microscope.

X-gal staining. β-Galactosidase expression from *Gli1*^{CreER}; *R26*^{lacZ} transgenic mice was detected by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining as described previously¹⁸.

In situ hybridization. An RNAscope 2.5 HD Red (ACD, 310036, 322350) detection kit was used according to the manufacturer's instructions. Sections were boiled in the target retrieval solution at ~100 °C for 15 min and incubated in the Protease Plus solution at 40 °C for 15 min. DapB (ACD, 310043) was used as a negative control along with appropriate positive controls. Negative controls showed little to no background staining. Probes for the following genes in *Mus musculus* were used: *Ambn* (508241), *Ank2* (413221), *Birc5* (422701), *Bmi1* (312181), *Ccnb1* (316241, custom-ordered against E5-E7), *Cldn10* (320269 custom-ordered from 2–806 bp of NM_001160099.1), *Clu* (427191), *Dspp* (448301), *Dcn* (413281), *Enpp2* (402441), *Gli1* (311001), *Hes1* (417701), *Hey1* (319021), *Igf1bp2* (405951), *Igf1bp5* (425731), *Igf1bp1* (488851), *Krt15* (319091), *Krt17* (479911), *Lrig1* (310521), *Mmp20* (479051), *Notch1* (404641), *Nrarp* (411771), *Otop2* (534911), *Pthlh* (456521), *Sfrp5* (405001), *Sostdc1* (313151), *Sox2* (401041), *Sparcl1* (424641), *Steap1* (531061), *Tacstd2* (471751) and *Tgfb2* (406181).

Dual EdU and BrdU pulse-chase labelling. Mice were given an intraperitoneal injection of 1 mg/25 g body weight of EdU (Invitrogen, C10637) and then analysed at various timepoints after treatment (exact chase timepoints are provided in the main text). Mice were injected with 1 mg/25 g body weight BrdU 1 h before analysis.

H2B-GFP label dilution experiments. For the short H2B-GFP pulse experiments, mice were injected once intraperitoneally with 200 μl of doxycycline (2 mg ml⁻¹, Enzo Life Sciences, ALX-380-273-G005) and then analysed at various timepoints

after treatment (exact chase timepoints are provided in the main text). For the long pulse experiments (Supplementary Fig. 4f), mice were treated once with 200 μl of doxycycline (2 mg ml⁻¹) by intraperitoneal injection. Simultaneously, doxycycline was added continually to their drinking water (1 mg ml⁻¹ supplemented with 5% sucrose) for 3 weeks.

Incisor injury and kidney capsule transplantation. *Notch1*^{CreER}; *R26*^{mt/mG} adult mice were injected intraperitoneally with 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, H7904) dissolved in corn oil (100 mg kg⁻¹ body weight). Then, 24 h later, lower incisors were carefully dissected and the posterior part of the incisors was separated by cutting immediately anterior to the lingual cervical loop after the onset of mineralization using a surgical scalpel. The dissected incisors were transplanted under the kidney capsule of immunocompromized mice (*Rag1*^{-/-}). Intact uncut incisors were used as controls. To minimize postoperative pain, 1 h before intervention, *Rag1*^{-/-} mice were injected subcutaneously with buprenorphine (0.1 mg kg⁻¹ body weight, Temgesic, Reckitt Benckler, 3002981). The recipient mice were then anaesthetized by isoflurane inhalation (Attane Isoflurane, Piramal Healthcare, NDC 66794-014-25), laid on one side and an incision was made to enable the exposure of one kidney. The kidney capsule was then incised using forceps. The capsule was lifted and the incisors were grafted under the capsule. The mice were then sutured with an absorbable polyglycolic acid suture (Resorba, PA10242) and the skin incision was closed with a metal clip (Fine Science Tools, 12040-01). Buprenorphine was supplied in the drinking water (10 μg ml⁻¹) for 3 d. Then, 10 d after transplantation, mice were killed and kidneys were collected and dissected, and the regions hosting the grafted incisors were isolated. The samples were then fixed in paraformaldehyde 4% for 1 h, washed with PBS and cleared for whole-mount confocal imaging by immersion in Focus Clear (CelExplorer, FC-101) overnight at 4 °C.

Micro-computed tomography. For ex vivo imaging of the entire incisor, mandibles were collected and dehydrated through an ethanol series up to 70% ethanol. Samples were scanned using MicroXCT-200 (Carl Zeiss Microscopy) at 40 kV and 160 μA. We obtained 800 projection images, taken at a total integration time of 4 s with linear magnification of ×2 and a pixel size of 10 μm. To image the incisor growth region, samples were also soaked in phosphotungstic acid overnight to differentially stain soft tissues as described previously⁴⁹ and scanned at 60 kV and 200 μA. We obtained 1,200 projection images, taken at a total integration time of 6 s with a linear magnification of ×10 and a pixel size of 2.4 μm. Micro-computed tomography calibration and mineral-density determination were performed as described previously⁵⁰.

For in vivo assessment of the enamel eruption rate (Supplementary Fig. 1a,b), mice were anaesthetized and the cranial region spanning the length of the incisors was scanned using Scanco vivaCT 40 (Scanco Medical AG). Images were taken at 55 kV with an integration time of 250 ms and a pixel size of 10.5 μm. Images were analysed using Avizo (FEI).

Cell suspension. Proximal incisors (roughly 3 mm in length) were first severed away using a pair of scissors after removal of the surrounding jaw bones. The bulbous portion, as well as the lateral wing-shaped epithelium, and the surrounding mesenchyme were subsequently dissected from the rest of the incisor and collected in cold Ca²⁺- and Mg²⁺-free HBSS (pH 8.0, UCSF Cell Culture Facility, CCFJA005-16CT01) and digested enzymatically at 37 °C in collagenase type II (2 mg ml⁻¹; Gibco/BRL type II in DMEM) for 30 min, followed by collagenase inactivation with 2% FBS. The digested tissue was mechanically disrupted using a 1 ml pipette tip and then centrifuged at 400g for 3 min. Cells were resuspended in HBSS (pH 8.0) containing 2% FBS.

FACS analysis. To sort epithelial cells, proximal-incisor cell suspensions were stained with monoclonal antibodies that specifically recognize CD326 (G8.8, AF647 conjugate, Biolegend, AB_1134104) for 30 min on ice followed by centrifugation at 400g for 3 min. Before FACS analysis (BD FACSAria2 SORP), cells were filtered through a 40 μm mesh (BD Falcon, 22363547) and stained with DAPI.

Time-lapse live imaging. Time-lapse live imaging was performed as previously described⁵¹. In brief, mandibular incisors were dissected in PBS with 0.5% glucose⁵² and embedded in 0.65% low-melting agarose supplemented with 0.5% glucose and 1% penicillin–streptomycin in organ culture dishes (Becton Dickinson, 353037). Culturing medium (50% DMEM/F12, 50% rat serum, 0.5% glucose, 250 μg ml⁻¹ ascorbic acid and 1% penicillin–streptomycin) was then added and agarose was carefully removed from the top of the sample to expose the dental epithelium for imaging. Samples were maintained under constant medium perfusion using a Delta T pump (Bioptechs, 60319131616) at 37 °C, 95% O₂ and 5% CO₂, and were imaged at a wavelength of 920 nm using a Nikon A1R two-photon microscope equipped with a ×25 1.1 NA water-immersion lens to capture the movement of GFP⁺ Notch-expressing SI cells. Images were taken every 5 min for 8 h. Time-lapse videos were generated using ImageJ.

Single-cell RNA barcoding and sequencing. Epithelial cells from five controls and five 5FU-treated mice were isolated as described above. The resulting cell

suspensions (~20,000 cells each) were submitted as separate samples with targeted cell recovery of 3,000 per sample to be barcoded for scRNA-seq using a Chromium Controller (10X Genomics, 120263) and a Single Cell 3' Library Kit v2 (10X Genomics, PN-120236/37/62). The resulting libraries were sequenced on a HiSeq 4000 (Illumina, SY-401-4001) using a HiSeq 4000 PE Cluster Kit (Illumina, PE-410-1001) with a HiSeq 4000 SBS Kit (150 cycles, Illumina, FC-410-1002); one sample was loaded into each sequencing lane. About 3,500 control cells (~89,000 reads per cell) and about 5,000 5FU-treated cells (~62,000 reads per cell) were successfully barcoded and their transcriptomes sequenced. Raw sequencing data were processed for initial quality control analysis and alignment by the sequencing core of the Institute of Human Genetics (UCSF) using the Cell Ranger (v.2.0.0) pipeline.

scRNA-seq data clean-up and normalization. We performed extra steps to clean and normalize our data. We opted for thresholds of less than 1,000 total counts or more than 10% of counts coming from mitochondrial genes to filter out cells from the analysis, leaving 3,173 and 4,645 high-quality transcriptomes for control and recovery conditions, respectively. After initial visualization of the two conditions individually, we identified a fraction of cells (824 in control and 301 in recovery) that formed well-separated clusters and were characterized by distinct expression of either immune genes or mesenchymal cell markers (Supplementary Fig. 3d). These cells were excluded from further analysis. Data were rescaled to 10,000 total counts per cell.

Visualization of scRNA-seq data in 2D. The high-dimensional scRNA-seq data were visualized in 2D as a force-directed layout of an undirected kNN graph using SPRING¹⁹. Interactive explorers of resulting SPRING plots can be accessed using the hyperlinks provided in Supplementary Table 10.

Classification of cells by cell-cycle phase. Each transcriptome in the scRNA-seq data was classified as G1, G2/M, S (Fig. 1f) using cell-cycle scores calculated as part of the procedure for regressing out the cell cycle described in the next section.

Regressing out the cell-cycle effect. The cell-cycle effect was regressed out from the count data by using simple linear regression (implementation in Scanpy⁵³, which is equivalent to Seurat¹⁹). The procedure was performed solely for the purpose of SPRING visualization (Figs. 1i and 4b, Supplementary Fig. 5h).

Defining the cell populations observed in scRNA-seq data. In control epithelial cells, populations were defined as belonging to spectral clusters of the kNN graph shown (Fig. 1d). Spectral clustering requires predefining into how many clusters a kNN graph is to be divided. Given the continuous nature of the epithelial-cell data with no clear boundaries visible in the SPRING graph, clusters are a convenient but not entirely natural way of describing the data. Therefore, the number of clusters reported for control epithelial cells in this study reflects a chosen degree of granularity. We confirmed that clusters represented distinct states (at least 20 significantly differentially expressed genes between any pair of clusters). The SPRING graph in Fig. 1d was first divided into 12 clusters, a configuration that well-captured 3 clusters representing different phases of the cell cycle (among class 1 cells) as well as 3 clusters with distinct gene expression along the ameloblast differentiation trajectory (class 2). To gain a better resolution for the class 3 cells, which seemed to be more complex than class 1 and class 2 from analysis of the data, we built a separate SPRING graph for class 3 cells only and found that 9 spectral clusters best captured the complexity of these cells. The resulting 15 populations were given names on the basis of RNAscope staining patterns of population-enriched genes (Fig. 3, Supplementary Table 1). To define populations in the injury condition (Fig. 6h, Supplementary Tables 6 and 8), we used a classification-based approach, for which each cell in the injury conditions was given the label of the population in the control condition with the most similar expression profile. Likelihoods from the Bayesian classifier described in the next section were used as a similarity measure.

Bayesian classifier of cell populations. To annotate single-cell transcriptomes using a chosen set of reference expression profiles, we applied a Bayesian cell classifier on the basis of a multinomial model that was reported previously^{54,55} and adapted from ref. ⁵⁶; we describe the model here for completeness.

Given (1) raw counts u_j for gene $j = 1, \dots, N$ from a single cell and (2) a reference expression matrix X_{ij} (reference population $i = 1, \dots, M$; gene $j = 1, \dots, N$), the classifier returns the likelihood of cell type i given u , defined as $P(i|u) = \frac{1}{Z} P_o(i) \prod_{j=1}^N p_{ij}^{u_j}$ where Z is a constant ensuring $\sum_i P(i|u) = 1$ and $P_o(i)$ is a prior expectation on the abundance of reference cell population i (set to $\frac{1}{M}$ = constant for all applications in this paper, that is, a naive prior was used) and $p_{ij} = X_{ij} / \sum_k X_{ik}$ is the fraction of transcripts from gene j in reference cell population i .

To classify each cell in the recovery conditions as one of the 15 populations defined in the control condition (Fig. 1d), the reference matrix X contained the per-population average expression of all genes. To classify each class 1 cell by its most similar profile among class 2 and class 3 populations, we used a reduced list

of genes. For a gene to be included, it had to be among the top 100 most enriched genes in any class 2 or 3 population but not within the top 100 enriched genes in class 1 populations. Population-enriched genes were identified as described below. Such gene selection was used to exclude cell-cycle-related genes, which are uninformative in distinguishing between class 2 and class 3 subsets but could, in principle, confound the classification.

Identification of population-enriched genes. Population-enriched genes were assessed by considering only genes that were (1) detected in at least 3 cells and with at least 6 counts after normalization across all cells, and (2) statistically significantly higher in cluster i compared with all other cells (FDR-adjusted $P < 0.05$, Mann–Whitney U -test). The heat map in Fig. 1e was generated using genes filtered, as described above, that were found within the top 20 ordered by fold change in a cluster of i versus all other cells. Furthermore, a pseudo-value of 1 count per 10,000 was added to buffer against low expression and avoid division by zero. The table containing the top 20 per control cluster is provided in Supplementary Table 1.

RNA velocity analysis. RNA velocity analysis was performed as described previously²³ using the `velocyto.py` analysis pipeline (<http://www.velocyto.org>). In brief, the method distinguished between unspliced and spliced transcripts in scRNA-seq and uses this information to extrapolate the future transcriptional state of each cell. Extrapolated states are vectors in the high-dimensional gene-expression space, and Fig. 4a,c shows their projection onto the 2D SPRING representation.

FateID analysis. Fate biases were calculated using FateID²⁴; the analysis was based on the vignette provided at <https://github.com/dgrun/FateID>. The method requires the selection of target states that represent differentiated populations. Figure 4f shows the target states that were chosen. These target states served as the training set in an iterative classification in which the test set is composed of cells in the neighbourhood of the training set. Neighbours are determined on the basis of a distance matrix, which can be defined by the user. Here we used the same distance matrix that was used to generate the SPRING graph shown in Fig. 4b.

Ameloblast trajectory analysis. Class 2 cells, which form a simple linear continuum in the SPRING graph (Fig. 1d), were ordered through pseudotime using PBA²¹ as described previously¹⁶. The method requires manually selecting source and sink cells of the trajectory, which—in our case—were the pre-ameloblast and distal-ameloblast tips, respectively (Fig. 2a). Our choice of the source and sink was based on extensive previous knowledge of several key molecular markers of amelogenesis^{17,57,58}, and was supported by our RNA velocity analysis (Fig. 4a). Cells were ordered by the diffusion ‘potential’ parameter of PBA. Gene expression along the trajectory was smoothed by averaging over a sliding window of 20 cells along the trajectory. We centred the exploration of changes in gene expression along the ameloblast trajectory around the onset of the expression of known mature ameloblast genes by excluding the first 300 pre-ameloblast cells.

Genes variable along the ameloblast trajectory were identified as described previously⁵⁹ with minor changes (Supplementary Table 2). Before statistical testing, we prefiltered genes to be expressed at a rate of at least 3 counts per 10,000 in at least 2 cells, and have a variability statistic of $V > \text{mode}(V)$ where V is the corrected Fano Factor described previously⁶⁰. For every gene i among the surviving 2,453 genes, a statistic t was calculated as $t_{i,\text{observed}} = \mathbf{a}_{i,\text{max}} - \mathbf{a}_{i,\text{min}}$ where \mathbf{a}_i is a vector with the smoothed expression of i along the ameloblast trajectory. The procedure was repeated in a random ordering on class 2 cells for multiple rounds, each time yielding $t_{i,\text{random}}$. The one-sided P value of this permutation test was defined as the fraction of times that $t_{i,\text{observed}} > t_{i,\text{random}}$. Multiple-hypothesis testing was accounted for by controlling the FDR at 5% using the Benjamini–Hochberg procedure.

Cell-density changes on the kNN graph. The following was performed to estimate the density of cells from the control condition on the graph combining the control and recovery conditions (Fig. 6f). Every cell from the control condition was asked to vote for its five nearest neighbours (Euclidean distance in the same principal-component space as used for the SPRING visualization). Votes were counted for every cell resulting in a raw vote vector $\mathbf{v}_{\text{raw}} = (v_1, v_2, \dots, v_n)$ and smoothed votes were obtained as $\mathbf{v}_{\text{smoothed}} = O_L \mathbf{v}_{\text{raw}}$ where the smoothing operator is $O_L = e^{-L/\beta}$ where L is the Laplacian matrix of the kNN graph visualized using SPRING and β is the parameter that determines the extent of smoothing. In physical terms, this parameter is equivalent to diffusion time, with larger values reflecting broader diffusion. We used $\beta = 1$. Smoothed votes were normalized to a total count of 1 million. The same voting was applied on the recovering cells, resulting in a cell density map for which the enrichment in recovery versus control cells can be assessed.

GSEA. For the GSEA, we used the GSEA PreRanked tool^{61,62} on genes ranked by an enrichment score E_i obtained from comparing cells within a given population i with all other epithelial cells. The E_i was defined as:

$$E_i = -\log_{10}(P_i)sr_i^2$$

where P_i and r_i are the P value and fold change, respectively, obtained from comparing cells in population i to cells outside of i as described in the ‘Identification of population-enriched genes’ section; $s = \begin{cases} 1 & \text{if } r_i \geq 1 \\ -1 & \text{if } r_i < 1 \end{cases}$

The c2.cp.reactome.v6.2.symbols.gmt curated Molecular Signatures Database was probed and pathways for which $P < 0.05$ and FDR-adjusted $P < 0.25$ were considered to be significant.

Statistics and reproducibility. All of the data points are biological replicates that were randomly assigned without investigator blinding. All experiments, except for the scRNA-seq and the FACS histograms showing H2B-GFP dilution patterns (Fig. 5b), were replicated at least three times. Images in Fig. 5d,f were derived from two independent biological experiments that showed similar results. All of the other images are representative of at least three independent biological samples. No data were excluded. No statistical methods were used to predetermine sample size and differences in intrasample variances were present. Statistical significance was determined using Prism (GraphPad Software). Normally distributed data were analysed using parametric tests, including one-way ANOVA with Tukey–Kramer post hoc test, except in Supplementary Fig. 4b in which an unpaired t -test with Welch's correction was used. Bar charts indicate the mean of samples and error bars represent \pm s.d. of the mean. Significance was taken as $P < 0.05$ with a confidence interval of 95%. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The scRNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code [GSE131204](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131204). Source data for figures are provided in Supplementary Table 11. All other data supporting the findings of this study are available from the corresponding authors on reasonable request.

Code availability

The custom code developed for the whole-mount proliferation assay (Fig. 5d–f) is provided at <https://github.com/kephale/incisor-cell-segmentation>. The code for selected single cell RNAseq data analyses including SPRING visualizations is available as Jupyter notebooks at https://www.github.com/rapolaszilionis/Sharir_et_al_2019.

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Reporting Summary

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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Only common tests should be described solely by name; describe more complex techniques in the Methods section.
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- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
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Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated
- Clearly defined error bars
State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about [availability of computer code](#)

Data collection	AxioVision v4.8.2; Leica Applications Suite v4.9; Nikon A1R two-photon; MicroXCT-200; Scanco vivaCT 40; BD FACSDiva v8.0.1; Chromium Controller (10X Genomics, 120263); HiSeq4000 (Illumina, SY-401-4001); Cell Ranger (10X Genomics, v2.0.0).
Data analysis	FlowJo v9.9; Prism v7.0h; Excel v14.7.7; Photoshop CC2015.5; Illustrator CC2015.3; Avizo Light 9.1.1; ImageJ2; R version 3.4.3 (2017-11-30), Python – Jupyter Notebook; https://github.com/AllonKleinLab/SPRING/ ; https://github.com/theislab/scanpy ; https://github.com/dgrun/FateID ; GSEA Desktop v3.0. Custom code was developed to perform cell segmentation analysis (Figure 5). Custom code used open source libraries FunImageJ (version 0.2.5) and Mosaic toolsuite (version 1.0.8). Both libraries are based upon ImageJ, but were invoked directly through FunImageJ which is the key library for ensuring reproducibility of this study.

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- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Single-cell RNA sequencing data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE131204. Source data for figures have been provided as Supplementary Table 11. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	In most cases we assumed a minimum of 4 mice would be required to recognize differences between conditions, based upon published data in this system and in other contexts. To determine the steady state (Supplementary figure 1) 3 mice/developmental stage were analyzed. A minimum number of animals were used to conform with NIH guidelines.
Data exclusions	For the H2B dilution experiment, one mouse from the 1 day and one mouse from the 21 days chase were excluded due to very low of GFP coverage, probably due to error in Dox injection.
Replication	All experiments were replicated at least twice with similar findings, except 1) single cell RNAseq (due to prohibitive costs). 2) Modelling proliferation dynamics using H2B-GFP incisor (A cohort of 20 mice was used for FACS-sorting cells assay)
Randomization	Samples were randomly assigned.
Blinding	Investigators were not blinded to group allocation because treatments and data collection were performed by the same people.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> Unique biological materials
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
<input checked="" type="checkbox"/>	<input type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology
<input type="checkbox"/>	<input checked="" type="checkbox"/> Animals and other organisms
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Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used

The following primary antibodies were used: Ameloblastin (sc50534, Santa Cruz biotechnology, clone J2612, various lot #s); rat anti-BrdU antibody (ab6326, Abcam, clone BU1/75(ICR1), various lot #s); mouse BrdU antibody (B35128, Invitrogen, clone MO-BU-1, various lot #s); GFP (GFP-1020, Aves, Chicken IgY, various lot #s); ab13790, Abcam, clone GR3190550, various lot #s); NICD (Cleaved Notch1, 4147 (Val1744/D3B8), Cell signaling, various lot #s); RFP (600-401-379, Rockland, various lot #s).

The following secondary antibodies were used (1:500 dilution each): goat anti-rat IgG Alexa fluor 488 (Invitrogen; A-11006, AB_2534074), goat anti-rabbit IgG Alexa fluor 568 (Invitrogen; A-11011, AB_143157), goat anti-mouse IgG Alexa fluor 488

(Invitrogen; A-11001, AB_2534069), goat anti-rabbit IgG Alexa fluor 633 (Invitrogen; A-21070, AB_2535731), rabbit anti-rat IgG Alexa fluor 488 (Thermo Fisher Scientific; AB_2535796), and biotinylated goat anti-rabbit IgG (Vector Laboratories; AB_2313606).

EdU was detected using Click-iT Plus EdU Assay Kit (ThermoFisher, C10640).

Validation

Validation available from peer-reviewed publications: Ameloblastin (MacDougall, M., et al. 2000, Cloning characterization and immunolocalization of human Ameloblastin Eur. J. Oral. Sci. 108 303-310).

Validation statements available from manufacturers, and state the species and application for which each antibody has been validated:

- rat BrdU (Abcam #ab6326, <https://www.abcam.com/BrdU-antibody-BU175-ICR1-ab6326.pdf>)
- mouse BrdU (Invitrogen #B35128, <https://www.thermofisher.com/order/genome-database/generatePdf?productName=BrdU&assayType=PRANT&detailed=true&productId=B35128>)
- GFP (Aves #GFP-1020, <http://www.aveslab.com/wp-content/uploads/GFP-10201.pdf>)
- GFP (Abcam #ab13790, <http://www.abcam.com/gfp-antibody-ab13970.html>)
- NICD (Cell signaling #4147, <https://media.cellsignal.com/pdf/4147.pdf>)
- RFP (Rockland #600-401-379, https://rockland-inc.com/store/Antibodies-to-GFP-and-Antibodies-to-RFP-600-401-379-O4L_24299.aspx)
- Click-iT Plus EdU Assay Kit (ThermoFisher #C10640, <https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp10637.pdf>).

Animals and other organisms

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Laboratory animals

Male mice aged 8–12 weeks were used for all experiments, except those analyzing mice at various age to determine the steady state (Supplementary Figure 1). The following mouse lines were used: Wild-type (C57BL/6J, Jax: 000664); Gli1CreER [Gli1tm3(cre/ERT2)Alj, Jax: 007913], Sox2CreER [Sox2tm1(cre/ERT2)Hoch, Jax: 017593]; Bmi1CreER [Bmi1tm1(cre/ESR1)Mrc, Jax: 010531]; Lrig1CreER [Lrig1tm1.1(cre/ERT2)Rjc, Jax: 018418]; Notch1CreER [Notch1tm5(cre/ERT2)Rko, Jax: 027235]; K14Cre [Tg(KRT14-cre)1Amc, MGI: 2445832]; R26RmT/mG [Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo, Jax: 007576]; R26RlacZ [Gt(ROSA)26Sortm1Sor, Jax: 003474]; R26RtdTomato [Gt(ROSA)26Sortm14(CAG-tdTomato)Hze, Jax: 007914]; Rag1^{-/-} [Rag1tm1Mom; Jax: 002216]; TetOff-H2B-GFP [Tg(tetO-HIST1H2BJ/GFP)47Efu, Jax: 005104]; K5tTA [Tg(KRT5-tTA)1216Gik, MGI: 3575755]; TetOn-H2B-GFP [Gt(ROSA)26Sortm1(rtTA*M2)Jae::Col1a1tm7(tetO-HIST1H2BJ/GFP)Jae, Jax: 016836]; rtTA [Gt(ROSA)26Sortm1(rtTA*M2)Jae::Col1a1tm1(tetO-cre)Hah, Jax: 021025]; CBF1:H2B-Venus [Tg(Cp-HIST1H2BB/Venus)47Hadj, Jax: 020942]. Rosa-DTA [B6.129P2-Gt(ROSA)26Sortm1(DTA)Lky/l, Jax: 009669].

Wild animals

The study did not involve Wild Animals.

Field-collected samples

The study did not involve Field-Collected Samples.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Wild-type (C57BL/6J, Jax: 000664) mice were used as a source of incisor epithelial cells for single cells RNA sequencing (Figures 1,3,6). TetOn-H2B-EGFP mice were used as the source for incisor epithelial cells for the label dilution experiment (Figure 5).

Proximal incisors (roughly 3 mm in length) were first severed away using a pair of scissors after removal of the surrounding jaw bones. The bulbous portion, as well as the lateral wing-shaped epithelium, and the surrounding mesenchyme were subsequently dissected from the rest of the incisor and collected in cold HBSS (pH 8.0) and digested enzymatically at 37°C in collagenase type II at concentration 2 mg/ml (Gibco/BRL type II in DMEM) for 30 min, followed by collagenase inactivation with 2% FBS. The digested tissue was mechanically disrupted using 1 ml pipette tip and centrifuged at 400 x g for 3 min. Cells were resuspended in HBSS (pH 8.0) containing 2% FBS.

FACS plots are provided in Supplementary Figure 3.

Instrument

BD FACSAria2 SORP

Software

BD FACSDiva v8.0.1; FlowJo v10

Cell population abundance

In all cases, sorted cells were checked for purity by running a fraction of the sorted material on a cytometry, including fresh DAPI to exclude dead cells. Prior to determination of purity, events were gated for FSC x SSC to exclude bubbles and debris in the

sample collection tube, and only DAPI(lo) events were considered. Sorted samples generally had a purity >80%. Samples that were obviously outliers for the frequency of DAPI+ events or of obviously low purity were removed from downstream analysis, on a case-by-case basis.

Gating strategy

Single cell RNAseq sorting:

CELLS: x-axis:FSC-Area (linear scale); y: SSC-Area (log scale)

SINGLETs: x-axis: FSC-Area; y-axis: FSC-Width

SINGLETs: x-axis:SSC-Area; y-axis: SSC-Width

LIVE CELLS: x-axis: FSC-Area; y-axis: DAPI (DAPI- cells were gated)

EPCAM-APC+ CELLS: x-axis SSC-Area; y-axis: EPCAM-APC (EPCAM-APC+ cells were gated and collected)

label dilution experiments:

CELLS: x-axis:FSC-Area (linear scale); y: SSC-Area (log scale)

SINGLETs: x-axis: FSC-Area; y-axis: FSC-Width

SINGLETs: x-axis:SSC-Area; y-axis: SSC-Width

LIVE CELLS: x-axis: FSC-Area; y-axis: DAPI (DAPI- cells were gated)

EPCAM-APC+ CELLS: x-axis SSC-Area; y-axis: EPCAM-APC (EPCAM-APC+ cells were gated)

Analysis of GFP: histogram of GFP signal of EPCAM-APC+ cells (epithelial cells were analyzed for GFP signal)

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.