ADDITIONAL METHODS AND THEORY

Steady state

To ensure that we were studying self-renewal and differentiation dynamics during adult homeostasis and not during growth, we first determined when the incisor reaches steady state. Using 3D micro-computed tomography (μCT) images of the hemi-mandible and incisor growth region at different time points during postnatal development, we quantified the weekly rate of increase in incisor volume and volume of the incisor growth region. Next, we administered BrdU to mice and measured the amount of proliferating cells. To assess apoptosis, TUNEL staining was performed on incisor growth regions of 8- and 16-week-old mice.

We found that the mouse incisor continues to increase in volume during the entire first year of life, up to 52 weeks (Supplementary Fig. 1c, left column). The rate of growth increased during the first three weeks post-partum (0.4 to 0.6 mm/week) and then started to decrease, until it stabilized at 8 weeks at an average of 0.1 mm/ week (Supplementary Fig. 1g, left chart). Consistent with this, the incisor growth region expanded between 1 and 5 weeks of age, after which it maintained a constant size (Supplementary Fig. 1c, right column and 1g, right chart). The percentage of cycling cells (BrdU-positive) in the incisor growth region did not change significantly over the course of the experiment (Supplementary Fig. 1d, h). However, the distribution of these cells evolved over time. During puberty (until 5 weeks), the OEE and dense SR still incorporated BrdU. However, from 8 weeks onwards, when the incisor and its growth region reached adult size, proliferation was confined to the IEE and the epithelial extensions (Fig. 1b), and proliferating cells were not observed in the OEE and the adjacent SR (Supplementary Fig. 1d,e). This result supports the view that the OEE and the adjacent SR portion of the incisor growth region cease cycling once the tooth reaches maturity. Finally, consistent with previous reports[1], between 8 and 16 weeks of age, apoptosis in the incisor growth region was negligible (less than 2 cell/section) (Supplementary Fig. 1f). Thus, our data indicate that the mouse incisor maintains a steady state between 8 and 16 weeks of age, a period when the incisor significantly reduces its growth rate

and tissue homeostasis is maintained by stable proliferation with little apoptosis in the incisor growth region.

Enamel growth rate during steady state

To measure the rate of enamel growth during steady state, we notched incisors from 8-week-old mice and scanned them using *in vivo* µCT, first immediately after the notch was made and then 4 days later (Supplementary Fig.1a,b). The change in distance between the notch and the tooth's labial surface per day revealed enamel rate growth of 110 µm/day (n=3, range 82-115 µm). This is consistent with previously reported values[2].

Analysis of incisor growth region proliferation kinetics

To determine incisor growth region proliferation kinetics, we initially used the *K5tTa;tetOff-H2B-EGFP* (*tetOff-H2B-EGFP*) system[3], in which constitutive EGFP protein expression in the incisor epithelium can be shut off by treatment with doxycycline (DOX), enabling a label dilution assay. However, *tetOff-H2B-EGFP* incisors were chalky white and smaller than normal, and they crack and break spontaneously (Supplementary Fig. 4a). µCT analysis showed a significant decrease in incisor volume and enamel density (Supplementary Fig. 4b). These results indicate that the incisors of *tetOff-H2B-EGFP* lines are abnormal, as was also noted in other organs of this line[4,5], and that this line cannot be used to determine incisor cell kinetics.

To overcome this limitation, we utilized transgenic mice in which expression of inducible H2B-GFP (GFP), driven by a tet-response element (TRE), is controlled by a tetracycline (Tet)-transactivator (rtTA) *TetOn-H2B-EGFP*[6]. µCT analysis showed that the incisors of TetOn-H2B-GFP mice were indistinguishable from controls (Supplementary Fig. 4a, bottom row, 5b). Additionally, without Dox the incisor epithelial cells were GFP-negative (Supplementary Fig. 4c), demonstrating that the system was not leaky. Transient activation of H2B–GFP (pulse) by Dox injection to TetOn-H2B-GFP mice during the perinatal period (P2-P4), when the OEE and the

adjacent SR are still cycling, led to labelling throughout the growth region that persisted for at least 6 weeks (Supplementary Fig. 4d). We also examined the anterior tibialis muscle from the same mice and found ample labelling of Pax7+ cells, confirming that our pulse regimen is sufficient to label even quiescent skeletal muscle SCs (Supplementary Fig. 4e). Therefore, we used the TetOn-H2B-GFP system for a quantitative analysis of incisor epithelial kinetics.

Modelling proliferation dynamics using H2B-GFP incisor FACS-sorted cells

TetOn-H2B-EGFP mice were pulsed with doxycycline to induce expression of green fluorescence in the incisor epithelium. After the pulse, transcription of H2B-GFP ceases and, with each subsequent division, the H2B-GFP intensity is halved[18]. Epithelial cells (Epcam$^+$) from the incisor growth region of TetOn-H2B-EGFP mice after 24 hours, 7 and 21 day chases were isolated and GFP densities were measured by FACS. The distribution of H2B-GFP signal in the incisor epithelium cells changes over time due to cell division, which causes dilution of GFP signal and increase in population, and cell loss by distal transport; apoptosis is negligible in the incisor epithelium during homeostasis.

We fitted the distribution of the GFP signal at day 7 using the distribution at day 0 as the initial condition. We assumed that there are two populations within the sorted cells, one with stochastically dividing cells and the other with non-dividing cells. For simplicity, we further assumed that the dividing cells have a uniform rate of loss by distal transport and that the number of divisions n is sampled from a Poisson distribution,

$$R_\lambda(n) = \frac{\lambda^n e^{-\lambda}}{n!}.$$

We considered that the GFP signal x of a cell in the data is the sum of the real signal and the background x_b (=123, which we obtained as the average of the negative control data (Fig. 5b)), and that a cell with signal x will divide into two cells each with signal $(x - x_b)/2 + x_b$. Let y be the

log of the GFP signal and $P_{\mathrm{day0}}(y)$, $P_{\mathrm{day7}}(y)$ be the densities of y at day 0 and day 7, respectively, we have

$$P_{\mathrm{day7}}(y) = \frac{1}{r + (1-r)\sum_{n=0}\ 2^n R_\lambda(n)} \left[r P_{\mathrm{day0}}(y) + (1-r) \sum_{n=0}\ 2^n R_\lambda(n) P_{\mathrm{n}}(y) \right]. (1)$$

Here, r is the proportion of cells that do not divide, including both the quiescent and the post-mitotic populations, and

$$P_n(y) = [density\ of\ \ y = \log(2^{-n}(x - x_b) + x_b)\ \ where\ x\ is\ GFP\ signal\ from\ \ day\ 0].$$

For fitting, we calculated the right-hand side of equation (1) using the day 0 data with different parameters (r, λ), and compared it with the left-hand side (least mean squares difference). We set $y = 5.2$ as the lower bound for the fitting, since below this value (Fig. 5b, shaded area), the signal is comparable to the background noise and the fitting is meaningless.

Whole-mount proliferation assay

Mice (n=3 for each time point) were given an intraperitoneal injection of 1 mg/25g EdU and then analyzed at 45 min and 48 hours post-treatment. 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, collected in cold PBS, and imaged at a wavelength of 960 nm using a Nikon A1R two-photon microscope equipped with a 25X numerical aperture 1.1 water immersion lens. Images were analyzed using a custom bioimage analysis pipeline built upon the ImageJ2 framework[7] using FunImageJ[8] to facilitate the use of a high-performance computing cluster.

To preprocess images, a mask of the epithelium was computed from the membrane channel with a Gaussian filter, a triangle threshold, and morphological erosion. The membrane channel was then inverted and masked, such that membranes are dark and cytoplasm is light

within the epithelium only. Each image was then divided into 100 x 100 tiles for segmentation, taken along the full depth of the Z-stack. Each tile was segmented using a region competition[9] algorithm that uses local energetics derived from image intensity and region properties. The segmentation is initially seeded at local maxima taken over a radius of 2 microns. The segmentation iteratively minimizes the energy of image intensity, a piecewise-smooth energy function representing an elastic balloon energy term for a sphere with a 5 μm radius, and a curvature regularization term used to reduce concavity of regions. Region fusion was introduced using the Kullback-Leibler (KL) divergence to merge regions, where the sum of the KL divergence of merging each region into its complement is less than 0.005, and region fission is achieved by creating new regions when a given region bifurcates. Region competition was performed on each tile until convergence or 50 iterations was reached. Tiles were stitched to create a whole image of the segmentation results.

Per-cell statistics were computed on the resulting set of regions within the complete stitched segmentation. Regions were excluded using minimum and maximum size constraints to filter segments that labeled contiguous and fragmented membrane regions, as opposed to the desired cell bodies. The centers of the filtered regions were rendered into an image, which was inspected manually. Regions where cells were incorrectly excluded or included were annotated, and the mask that denotes valid epithelial tissue was updated with the annotations. The remaining regions were reported as cells. The cell centers were calculated as the center of mass of the respective segmented region, and cell intensity from the EdU channel was measured within a sphere of radius 3 microns about the cell center. To minimize false positive labels due to background signal, cells were thresholded based on the measured EdU signal by manual inspection of threshold values. The local average %EdU+ (shown in heat maps in Fig. 5e,f) was calculated as the fraction of 100 nearest neighbor image segments that were identified as EdU+.

References

1. Hu, J. K.-H. *et al.* An FAK-YAP-mTOR Signaling Axis Regulates Stem Cell-Based Tissue Renewal in Mice. *Cell Stem Cell* **21**, 91-106.e6 (2017).
2. Hu, J. C.-C. *et al.* Enamel Defects and Ameloblast-specific Expression in Enam Knock-out/lacZ Knock-in Mice. *J. Biol. Chem.* **283**, 10858–10871 (2008).
3. Tumbar, T. *et al.* Defining the epithelial stem cell niche in skin. *Science* **303**, 359–363 (2004).
4. Brennand, K., Huangfu, D. & Melton, D. All β Cells Contribute Equally to Islet Growth and Maintenance. *PLOS Biol.* **5**, e163 (2007).
5. Sartaj, R. *et al.* Characterization of slow cycling corneal limbal epithelial cells identifies putative stem cell markers. *Sci. Rep.* **7**, 3793 (2017).
6. Foudi, A. *et al.* Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat. Biotechnol.* **27**, 84–90 (2009).
7. Rueden, C. T. *et al.* ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* **18**, 529 (2017).
8. Harrington, K. I. S., Rueden, C. T. & Eliceiri, K. W. FunImageJ: a Lisp framework for scientific image processing. *Bioinforma. Oxf. Engl.* **34**, 899–900 (2018).
9. Cardinale, J., Paul, G. & Sbalzarini, I. F. Discrete region competition for unknown numbers of connected regions. *IEEE Trans. Image Process. Publ. IEEE Signal Process. Soc.* **21**, 3531–3545 (2012).