Spatial organization within a niche as a determinant of stem-cell fate

Panteleimon Rompolas¹, Kailin R. Mesa¹ & Valentina Greco¹

Stem-cell niches in mammalian tissues are often heterogeneous and compartmentalized; however, whether distinct niche locations determine different stem-cell fates remains unclear. To test this hypothesis, here we use the mouse hair follicle niche and combine intravital microscopy with genetic lineage tracing to re-visit the same stem-cell lineages, from their exact place of origin, throughout regeneration in live mice. Using this method, we show directly that the position of a stem cell within the hair follicle niche can predict whether it is likely to remain uncommitted, generate precursors or commit to a differentiated fate. Furthermore, using laser ablation we demonstrate that hair follicle stem cells are dispensable for regeneration, and that epithelial cells, which do not normally participate in hair growth, re-populate the lost stem-cell compartment and sustain hair regeneration. This study provides a general model for niche-induced fate determination in adult tissues.

Stem-cell niches in adult tissues constitute a spatially distinct micro-environment, including neighbouring cells, signals and extracellular material. Anatomical and molecular heterogeneity seems to be a common feature between mammalian stem-cell niches across different tissues; however, it is unclear whether the specific location that a stem cell occupies within the niche can influence its function.

Haematopoietic stem cells with divergent roles in homeostasis and pathophysiology are proposed to associate with distinct niche compartments, such as the endosteum or the vasculature in the central bone marrow, which may affect their behaviour and possibly their long-term fate. Moreover, in the intestinal crypt, fast-cycling stem cell and quiescent progenitor populations reside in distinct positions at the bottom of the crypt, whereas the transient amplified pool of precursors line the walls of the crypt, progressively differentiating as they reach the surface of the villi. A neutral competition model has been proposed for long-term homeostasis of the intestinal niche, but the short-term behaviour of individual stem cells in different positions at the bottom of the crypt has not been determined. The hair follicle in the skin represents another highly compartmentalized niche where stem cells reside in the bulge, while a pool of progenitors, called the hair germ, is clustered in a different niche location directly below the hair follicle. A common theme of niche compartmentalization in the above examples raises the question as to whether stem cells within their compartments are functionally equivalent. Specifically, it is not clear whether each stem cell can stochastically generate every lineage in a tissue, or whether the precise position within the niche can impose a distinct fate.

The mouse hair follicle is a self-contained mini-organ that represents a unique system for monitoring niche behaviour in vivo, because the location of stem cells and differentiated cell types is anatomically distinct and molecularly well defined. Hair follicles normally undergo stereotypic cycles of regeneration, which in the young mouse are highly synchronized across large areas of the skin and therefore the exact timing of rest, growth and regression phases can be accurately predicted. During hair growth, mesenchymal–epithelial crosstalk at the bottom of the hair follicle induces the formation and upwards expansion of seven concentric differentiated layers. These inner layers make up the hair shaft and supportive inner root sheath (IRS), whereas a relatively undifferentiated outer cell layer called the outer root sheath (ORS) grows downwards to envelop the elongating hair follicle fully. Taking advantage of the accessibility of the skin hair follicle, we previously established the ability to visualize these processes non-invasively, in vivo. Here, we have developed a new approach to mark single stem cells in different positions within the niche and re-visit the same lineages over a period of several weeks to months, in live mice. Furthermore, we use laser-induced cell ablation to test whether hair follicle stem cells are required for hair regeneration and to address how injury-induced cell mobility between different niches affects their fate.

Niche location predicts stem-cell fate

To explore the significance of specific niche positioning to stem-cell fate, we implemented an in vivo lineage tracing approach at single-cell resolution by live imaging. To mark hair follicle stem cells in the bulge and hair germ compartments genetically, we used mice containing either K19-CreER (expressing tamoxifen-inducible Cre; also known as Krt19-CreER) or Lgr5-CreER, in addition to Rosa-stop-IΔTomato reporter alleles (Extended Data Fig. 2a). Mice were induced with a single low dose of tamoxifen in the first rest phase of the hair cycle (first telogen, approximately postnatal day (P) 20) and stem cells were visualized in vivo three days later (P23), while the hair follicles were still quiescent (Extended Data Fig. 3a). We verified that marked cells did not translocate from their initial position within the niche and that no additional ectopic expression of the Cre reporter occurred owing to Cre recombinase leakage while hair follicles remained quiescent (Extended Data Fig. 3b). As hair regeneration commenced, we re-visited the same follicles in separate imaging sessions and the lineage progression of previously identified single stem cells was documented (Fig. 1).

Analysis of the in vivo lineage tracing data showed that during this process the fate of individual stem cells followed highly stereotypic patterns, which correlated with their original location within the niche.

¹Department of Genetics, Department of Dermatology, Yale Stem Cell Center, Yale Cancer Center, Yale School of Medicine, New Haven, Connecticut 06510, USA.
at the onset of a regeneration cycle (Fig. 1b–d). Specifically, most of the stem cells located within the bulge did not contribute to the subsequent hair cycle or were lost, and a smaller fraction of bulge stem cells produced lineages only in the relatively undifferentiated outer layer (ORS) (Fig. 1b, c, Extended Data Figs 4 and 5, and Supplementary Video 1). Conversely, cells located in the hair germ consistently contributed to hair follicle growth by generating differentiated lineages (Fig. 1b, c, Extended Data Figs 4 and 6, and Supplementary Video 1). Even within each niche compartment the precise location dictated different stem-cell behaviours. For example, within the bulge, stem cells situated in the lower half of the compartment were more likely to proliferate and generate ORS lineages than stem cells situated in the upper half, which were either quiescent or generated limited clones that remained in the bulge (Fig. 1d). These data show a direct correlation between a specific niche location and stem-cell fate.

To test the long-term fate of hair follicle stem cells, we traced bulge lineages over two consecutive hair cycles. Bulge stem cells that persisted in the upper portion of the bulge compartment after the first cycle remained there during the second cycle (Extended Data Fig. 7). However, lower bulge descendants that acquired an ORS fate were often found in the hair germ after hair follicle regression and entry into the next rest phase (second telogen; Fig. 1e and Extended Data Fig. 8). These ORS clones more frequently gave rise to differentiated ORS lineages after a full hair cycle (n = 128; error bars represent s.e.m.). For example, within the hair germ, stem cells originating from the bulge or hair germ (n = 23; error bars represent s.e.m.).

**ORS expansion is spatially regulated**

Our data suggest that the ORS represents an intermediate stage between quiescent bulge stem cells and hair germ cells. Notably, lineage tracing indicated that ORS clones often expanded discontinuously towards the bulb (Fig. 2a, Extended Data Fig. 8 and Supplementary Video 2). To understand how the niche influences this mode of ORS expansion, we collected several time-lapse recordings of hair follicles in advanced growth stages (anagen III–IV; Supplementary Videos 3 and 4). Analysis of cell
behaviour at this stage of growth showed that the ORS undergoes a spatially regulated mode of expansion. Specifically, cell proliferation was restricted to a narrow zone between the lower bulge and the bulb (Fig. 2b and Supplementary Videos 3 and 4). Cell divisions in this ‘proliferative zone’ were highly oriented, with a mitotic spindle perpendicular to the long axis of growth (Fig. 2b). These oriented cell divisions may not contribute directly to the longitudinal expansion of the ORS, and as a result this proliferative zone displayed higher cell density than other areas (Fig. 2c, d and Supplementary Videos 3 and 4). Further analysis of the time-lapse videos revealed that cells at the distal border of the proliferative zone became mobile and migrated rapidly towards the bulb, thus directly contributing to the downward expansion of the ORS. This previously uncharacterized mode of cell migration indicates highly dynamic cell–cell contacts and may partially explain the discontinuous appearance of the ORS clones observed by lineage tracing (Fig. 2e, f and Supplementary Video 5). This bimodal type of ORS growth and the spatially defined areas of proliferation and migration highlight the regional control that the niche exerts during growth.

**Bulge stem cells are dispensable**

Our lineage tracing experiments suggest a functional compartmentalization of the niche, in which stem cells positioned in the lower bulge may specify the ORS and those in the hair germ the differentiated hair lineages. To test the stringency of niche-imposed fates towards hair regeneration, we used laser-induced cell ablation to remove specifically either the bulge or the hair germ at the onset of hair growth (first telogen, P20; Fig. 3a). To recognize each targeted compartment we used reliable anatomical features of the niche (Extended Data Fig. 1a), because available genetic markers label overlapping populations that extend across both the bulge and hair germ (Fig. 3b). Notably, after ablation of either the bulge or the hair germ, the niche consistently recovered the lost cell populations (Fig. 3c). Overall, our data show that the bulge and hair germ populations are mutually dispensable for hair regeneration as long as a functional interaction between the epithelium and the mesenchymal dermal papilla is maintained (Fig. 3f). Furthermore, they suggest that the ability of the hair germ to initiate hair growth may occur independently of bulge input.

**Cell fate changes on niche injury**

To explore the cellular mechanisms of niche recovery, we performed time-lapse recordings shortly after bulge laser ablation. The hair germ became proliferative consistent with previous experiments that show hair germ contribution to the niche after stem-cell depletion due to plucking. Notably, distant epithelial cells above the bulge (infundibulum) were also observed to become proliferative, and some cells descended rapidly into the niche (Supplementary Videos 6 and 7). These findings raised the possibility that neighbouring epithelial cells situated above the bulge may contribute to the recovery of the niche. To test this hypothesis, we implemented our in vivo lineage tracing approach to monitor the behaviour of cells outside the hair follicle niche after bulge ablation. To mark the outermost epithelial layers located above the bulge exclusively we took advantage of the particular expression profile of K14-CreER/Rosa-stop-tdTomato mice, in which labelling is strongly biased towards the interfollicular epidermis, infundibulum and sebaceous glands (Fig. 4a and Supplementary Video 8). After induction, follicles that did not contain any labelled cells within the niche were targeted for bulge ablation (Fig. 4a, b). In the days after the ablation there was a significant influx of labelled epithelial cells into the niche, in contrast to neighbouring non-ablated follicles where no additional tdTomato+ cells appeared to enter the hair follicle (Fig. 4b).

We found that these ‘new’ niche cells not only contributed to re-establishing the lost bulge compartment but also participated in the subsequent hair growth, suggesting that they acquired a different fate on assuming their new position in the hair follicle niche (Fig. 4b). To

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![Figure 2](https://example.com/figure2.png)  
**Figure 2** | Mode of ORS growth. **a**, *In vivo* lineage tracing sequence (top) and corresponding three-dimensional renderings of the Cre reporter (bottom) showing ORS expansion during hair growth. Arrows denote cell lineages in different colours. **b**, Graphical representation of the location and axis of cell divisions in the ORS and matrix (bulb) in advanced hair follicle growth (anagen III–IV). **c**, ORS cell distribution during active hair growth. **d**, Quantification of cell density in different regions of the outer hair follicle epithelial layer (n = 9 per region; error bars represent s.e.m.). **e**, Individual traces of migrating ORS nuclei (see also Supplementary Video 3). **f**, Traces of ORS nuclei depicting their relative positions at two time-points 8 h apart (see also Supplementary Video 3). Scale bars, 50 μm.
test further whether the niche influences the same type of behaviour on the epithelial cells that re-populated the bulge, we used label retention to analyse for quiescence, a hallmark of bulge stem cells, during the second growth cycle after bulge ablation (Fig. 4c). At full growth (third anagen) the bulge of ablated hair follicles displayed significant label retention compared to the lower growing portion of the follicle, but similar to the bulge of non-ablated neighbouring follicles (Fig. 4d, e). Thus, these data provide direct evidence that loss of a stem cell pool due to injury can induce neighbouring epithelial cell populations that do not normally have a hair follicle fate to be mobilized and contribute to re-establishing the niche anatomically as well as functionally. Most importantly, once these cells enter the niche they display characteristics consistent with a hair follicle fate enacted on them in their new location.

Discussion

The relationship between niche position and stem-cell fate is a fundamental question in mammalian stem-cell biology that has remained unanswered. Current approaches to address this problem involve the use of genetic lineage tracing tools based on inducible Cre recombinase, driven by stem-cell-specific promoters. However, the mosaic expression of the Cre reporter within the stem-cell pool and the inability to follow individual stem cells over time has greatly limited our understanding of the fate of individual cells at precise locations within the niche. To overcome these limitations we have devised a system that combines genetic lineage tracing with intravital microscopy to monitor the progression of single stem-cell lineages from their initial position, by re-visiting the same undisturbed niche in separate experiments in live mice.

Using this approach, we found evidence that establishes a strong link between a specific niche location and stem-cell fate. Although a cell-autonomous model is plausible, our data support a model for fate determination in the hair follicle that is based on the spatial organization of the niche (Extended Data Fig. 9). According to this model, a cell in the upper half of the bulge is favoured to remain uncommitted to a specific fate and therefore more likely to remain quiescent or self-renew. By contrast, a cell situated in the lower bulge will be subject to activating stimuli from the niche driving it to undergo limited amplification as part of the still relatively undifferentiated ORS. The fraction of the ORS pool that survives the regression phase of the hair cycle will now be situated in the compartment that becomes the new hair germ. Once in
mice were created by H. Clevers’s laboratory and obtained from The Jackson Laboratory. This may also explain how certain hierarchies that exist between different tissue compartments but the overall structure and function of the tissue is maintained because cells are capable of adopting new fates as dictated by their new niche microenvironment (Extended Data Fig. 9).

that part of the niche these cells receive different stimuli pushing them to commit towards a differentiation pathway to support the subsequent hair cycle.

Our model is consistent with previous data from the hair follicle and other stem-cell niches but directly demonstrates the significance of the niche for stem-cell fate determination. Our results from the laser ablation experiments further support this notion, highlighting the fact that niche stem cells can be dispensable for tissue regeneration, provided that the overall integrity of the niche is maintained. In this context, injury can induce cell mobility between different tissue compartments but the overall structure and function of the tissue is maintained because cells are capable of adopting new fates as dictated by their new niche microenvironment (Extended Data Fig. 9). This may also explain how certain hierarchies that exist between different stem-cell pools under homeostatic conditions can be re-shuffled and new ones established after injury, as part of a wound healing process. Identifying the extrinsic factors that make up a particular niche microenvironment is paramount for understanding the mechanism of stem-cell fate determination and our ability to manipulate stem cells for therapeutic purposes.

**METHODS SUMMARY**

*K14-CreER mice were created and obtained from G. Gu’s laboratory and obtained from The Jackson Laboratory. Lgr5-CreER mice were created by H. Clevers’s laboratory and obtained from The Jackson Laboratory. K14-CreER mice were created by E. Fuchs’s laboratory and obtained from The Jackson Laboratory.*

Retained at the use of the laboratory. Mice were bred to a mixed albino background and males were preferentially used for experiments. All studies and procedures involving animal subjects were approved by the Institutional Animal Care and Use Committee at Yale School of Medicine and conducted in accordance with the approved animal handling protocol. Expression of the Cre fluorescent reporter for the lineage tracing experiments was induced with a single intraperitoneal injection of tamoxifen (20 μg g⁻¹ and 1 μg g⁻¹ in corn oil for *K14-CreER* and *Lgr5-CreER*, respectively) at P20. For lineage tracing of epithelial populations above the hair follicle, *K14-CreER/Rosa-stop-tdTomato* mice were given a single intraperitoneal injection of tamoxifen (0.2 mg g⁻¹ in corn oil). For the label retention experiment, *K5-ITAcTRE-H2BGFP* mice were given doxycycline (1 mg ml⁻¹) in potable water at times specified. Intravital microscopy and laser ablation procedures were carried out as described previously. All lineage tracing and ablation experiments were repeated at least in triplicates or otherwise indicated.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Scadden, D. T. The stem-cell niche as an entity of action. Nature **441**, 1075–1079 (2006).
2. Spradling, A. C. et al. Stem cells and their niches: integrated units that maintain Drosophila tissues. Cold Spring Harb. Symp. Quant. Biol. **73**, 49–57 (2008).
3. Fuchs, E. The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137, 811–819 (2009).
4. Li, L. & Clevers, H. Coexistence of quiescent and active adult stem cells in mammals. Science 327, 542–545 (2010).
5. Greco, V. & Guo, S. Compartmentalized organization: a common and required feature of stem cell niches? Development 137, 1586–1594 (2010).
6. Copley, M. R., Beer, P. A. & Eaves, C. J. Hematopoietic stem cell heterogeneity takes center stage. Cell Stem Cell 10, 690–697 (2012).
7. Wilson, A. et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118–1129 (2008).
8. Sato, T. et al. Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. Nature Med. 15, 696–700 (2009).
9. Kopp, H.-G., Aveitia, S. T., Hooper, A. T. & Rafi, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) 20, 349–356 (2005).
10. Celso, C. L. et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457, 92–96 (2009).
11. Xie, Y. et al. Detection of functional haematopoietic stem cell niche using real-time imaging. Nature 457, 97–101 (2009).
12. Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature 495, 231–235 (2013).
13. Greenbaum, A. et al. CXXCL2 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature 495, 227–230 (2013).
14. Sangiorgi, E. & Capecchi, M. R. Bmi1 is expressed in vivo in intestinal stem cells. Nature Genet. 40, 915–920 (2008).
15. Takeda, N. et al. Interconversion between intestinal stem cell populations in distinct niches. Science 334, 1420–1424 (2011).
16. Barker, N. et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 459, 1003–1007 (2009).
17. Buzzaci, S. J. A. et al. Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature 495, 65–69 (2013).
18. Lopez-Garcia, C., Klein, A. M., Simons, B. D. & Winton, D. J. Intestinal stem cell replacement follows a pattern of neutral drift. Science 330, 822–825 (2010).
19. Snippe, H. J. et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell 143, 134–144 (2010).
20. Cotsarelis, G., Sun, T. T. & Lavker, R. M. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61, 1329–1337 (1990).
21. Tumbar, T. et al. Defining the epithelial stem cell niche in skin. Science 303, 359–363 (2004).
22. Greco, V. et al. A two-step mechanism for stem cell activation during hair regeneration. Cell Stem Cell 4, 155–169 (2009).
23. Ito, M., Kizawa, K., Hamada, K. & Cotsarelis, G. Hair follicle stem cells in the lower bulge form the secondary germ, a biologically distinct but functionally equivalent progenitor cell population, at the termination of catagen. Differentiation 72, 548–554 (2007).
24. Rompolas, P. et al. Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. Nature 487, 496–499 (2012).
25. Trempeul, C. S. et al. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J. Invest. Dermatol. 120, 501–511 (2003).
26. Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L. & Fuchs, E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118, 635–648 (2004).
27. Claudinot, S., Nicolas, M., Oshima, H., Rochat, A. & Barrandon, Y. Long-term renewal of hair follicles from clonogenic multipotent cells. Proc. Natl. Acad. Sci. USA 102, 14677–14682 (2005).
28. Liu, Y., Lyle, S., Yang, Z. & Cotsarelis, G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. J. Invest. Dermatol. 121, 963–968 (2003).
29. Ito, M. et al. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nature Med. 11, 1351–1354 (2005).
30. Morris, R. J. et al. Capturing and profiling adult hair follicle stem cells. Nature Biotechnol. 22, 411–417 (2004).
31. Zhang, Y. V., Cheong, J., Ciaparno, N., McDermott, D. J. & Tumbar, T. Distinct self-renewal and differentiation phases in the niche of infrequently dividing hair follicle stem cells. Cell Stem Cell 5, 267–278 (2009).
32. Müller-Rover, S. et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J. Invest. Dermatol. 117, 3–15 (2001).
33. Jahoda, C. A., Horne, K. A. & Oliver, R. F. Induction of hair growth by implantation of cultured dermal papilla cells. Nature 311, 560–562 (1984).
34. Kaufman, C. K. et al. GATA-3: an unexpected regulator of cell lineage determination in skin. Genes Dev. 17, 2108–2122 (2003).
35. Leguei, E. & Nicolas, J.-F. Hair follicle renewal: organization of stem cells in the matrix and the role of stereotyped lineages and behaviors. Development 132, 4143–4154 (2005).
36. Hsu, Y.-C., Pasolli, H. A. & Fuchs, E. Dynamics between stem cells, niche, and progeny in the hair follicle. Cell 144, 92–105 (2011).
37. Sequeira, I. & Nicolas, J.-F. Redefining the structure of the hair follicle by 3D clonal analysis. Development 139, 3741–3751 (2012).
38. Means, A. L., Xu, Y., Zhao, A., Ray, K. C. & Gu, G. A. CK19CreERT2 knockin mouse line offers for conditional DNA recombination in epithelial cells in multiple endodermal organs. Genesis 46, 318–323 (2008).
39. Madisen, L. et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nature Neurosci. 13, 133–140 (2010).
40. Yousset, K. K. et al. Identification of the cell lineage at the origin of basal cell carcinoma. Nature Cell Biol. 12, 299–305 (2010).
41. Jaks, V. et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nature Genet. 40, 1291–1299 (2008).
42. Chi, W., Wu, E. & Morgan, B. A. Dermal papilla cell number specifies hair size, shape and cycling and its reduction causes follicular decline. Development 140, 1676–1683 (2013).
43. Van Keymeulen, A. & Blanpain, C. Tracing epithelial stem cells during development, homeostasis, and repair. J. Cell Biol. 197, 575–584 (2012).
44. Barrass, V. et al. Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nature Cell Biol. 11, 190–196 (2009).
45. Nystul, T. & Spradling, A. An epithelial niche in the Drosophila ovary underlying long-range stem cell replacement. Cell Stem Cell 1, 277–285 (2007).
46. Pikus, M. V. et al. Epithelial stem cells and implications for wound repair. Semin. Cell Dev. Biol. 23, 946–953 (2012).
47. Vasioukhin, V., Degenstein, L., Wise, B. & Fuchs, E. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc. Natl. Acad. Sci. USA 96, 8551–8556 (1999).

Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to V.G. (valentina.greco@yale.edu).
METHODS

Mice. K19-CreER<sup>T2</sup> mice were provided by G. Gu. K14-H2BGFP<sup>T1</sup>, Lef1-RFP<sup>T3</sup> and pTRE-H2BGFP<sup>T3</sup> mice were obtained from E. Fuchs. K5-tTA<sup>T4</sup> mice were provided by A. Glick. Lgr5-CreER<sup>T6</sup>, K14-CreER<sup>T2</sup> and Rosa-stop-tdTomato<sup>T8</sup> mice were obtained from The Jackson Laboratories. Mice were bred to a mixed albino and males were preferentially used for experiments. All studies and procedures involving animal subjects were approved by the Institutional Animal Care and Use Committee at Yale School of Medicine and conducted in accordance with the approved animal handling protocol.

**In vivo imaging.** Three-week-old mice were anaesthetized with intraperitoneal injection of ketamine and xylazine (15 mg ml<sup>−1</sup> and 1 mg ml<sup>−1</sup>, respectively in PBS). Anaesthesia was maintained throughout the course of the experiment with vaporized isoflurane delivered by a nose cone (1% in air). The area of the skin around the head region was shaved using an electrical shaver and depilatory cream (Nair). The skin connecting the ear with the head was then mounted on a custom-made stage and a glass coverslip was placed directly against it. Image stacks were acquired with a LaVision TriM Scope II (LaVision Biotec) microscope equipped with a Chameleon Vision II (Coherent) two-photon laser. For collection of serial optical sections a laser beam (940 nm for GFP and 1,040 nm for RFP and tdTomato) was focused through a water immersion lens (numerical aperture 1.0; Olympus) and scanned with a field of view of 0.5 mm<sup>2</sup> at 600 Hz. Z-stacks were acquired in 2–3-μm steps to image a total depth of 100 μm within the tissue. Laser power intensity was increased accordingly for each wavelength to normalize exposure and counteract the loss of signal in higher tissue depths.

**In vivo lineage tracing.** To mark hair follicle stem cells genetically we used an inducible Cre-mediated system, using mice containing either K19-CreER/Rosa-stop-tdTomato or Lgr5-CreER/Rosa-stop-tdTomato alleles. Krt19 and Lgr5 are expressed in distinct but overlapping cell populations located in the bulge and hair germ, respectively<sup>T1</sup>. To mark epithelial cells above the bulge we used K14-CreER, which displays a biased expression towards the interfollicular epidermis, infundibulum and sebaceous glands. K14-H2BGFP and Lef1-RFP were used as general epithelial and mesenchymal fluorescent reporters as described previously<sup>T4</sup>. For induction, mice were given a single intraperitoneal injection of tamoxifen (20 μg g<sup>−1</sup>, 1 μg g<sup>−1</sup> and 0.2 mg g<sup>−1</sup> in corn oil for K19-CreER, Lgr5-CreER and K14-CreER, respectively) at −P20 or times specified. Three days later (−P23), expression of the Cre reporter was assessed by in vivo imaging. Serial optical sections with a volume that included the epidermis and the entire volume of hair follicles were acquired, in a pattern of successive and overlapping x–y fields-of-view that spanned a skin area of ~2 mm<sup>2</sup>. In each of these serial sections individual hair follicles were evaluated and single labelled stem cells were identified. For re-visiting the same hair follicles in separate experiments, distinctive inherent landmarks of the skin were used to navigate back to the original region; including the vasculature and clustering of hair follicles in uniquely arranged groups. For time-lapse recordings serial optical sections were obtained at 5-min intervals.

**Label retention.** Mice hemizygous for the pTRE-H2BGFP and K5-tTA alleles were used. This combination confers expression of H2B–GFP in all the Krt5-positive epithelial populations, which can be repressed with the administration of doxycycline. Mice were given doxycycline (1 mg ml<sup>−1</sup>) in potable water at times specified.

**Laser ablation.** Laser ablation was carried out with the same optics as for acquisition. A 900-nm laser beam was used to scan the target area (10–50 μm<sup>2</sup>) and ablation was achieved using 20–40% laser power for ~1–5 s. Ablation parameters were adjusted according to the depth of the target (30–80 μm).

**Image analysis.** Raw image stacks were imported into ImageJ (National Institutes of Health) for further analysis. For visualizing single-labelled stem cells that expressed the tdTomato Cre reporter the brightness and contrast was adjusted accordingly for the green (GFP) and red (RFP and tdTomato) channels, and composite serial image sequences were assembled. In the red channel the signal from the tdTomato Cre reporter was significantly higher and could easily be distinguished from the Lef1-driven RFP. To account for every tdTomato labelled cell in the hair follicle, maximum intensity z-projections of sequential optical sections that included the entire volume of the hair follicle were obtained. To quantify H2B–GFP signal intensities for the label retention experiment, 16-bit serial optical sections were acquired for the green channel only. Pixel intensity levels were equalized and z-projections of the entire volume of the hair follicles were obtained. Mean grey values were measured in 20 μm<sup>2</sup> areas within the bulge and ORS regions of the follicles using ImageJ.

**Statistical analysis.** Data are expressed as percentages or mean ± s.e.m. Statistical calculations and graphical representation of the data were performed using the Prism software package (GraphPad).

48. Rendl, M., Lewis, L. & Fuchs, E. Molecular dissection of mesenchymal-epithelial interactions in the hair follicle. *PLoS Biol.* 3, e331 (2005).

49. Diamond, I., Owolabi, T., Marzo, M., Lam, C. & Glick, A. Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rTA linked to the keratin 5 promoter. *J. Invest. Dermatol.* 115, 788–794 (2000).
Extended Data Figure 1 | Hair follicle anatomy and physiology. a, Scheme of a mouse hair follicle in quiescence. Different cell populations reside in defined anatomical compartments. b, In homeostasis the hair follicle undergoes repeated cycles of regeneration. Hair growth is fuelled by stem cells in the niche that proliferate and differentiate to form the seven concentric layers of the mature hair shaft and inner root sheath (IRS), whereas a basal epithelial layer called the outer root sheath (ORS) surrounds the entire structure. Notice that the seven inner layers expand from the matrix, at the interphase with the mesenchymal dermal papilla, where they are generated, towards the surface of the skin, whereas the ORS has a different mode of growth and expands in the opposite direction. A complete hair cycle alternates between phases of rest (telogen), growth (anagen) and regression (catagen).
Extended Data Figure 2 | Method for single stem-cell lineage tracing in live mice. 
a. Single hair follicle stem-cell labelling is achieved using a combination of either K19-CreER/Rosa-stop-tdTomato or Lgr5-CreER/Rosa-stop-tdTomato alleles and administration of a single low dose of tamoxifen to achieve a low frequency of Cre-mediated loxP recombination and mosaic expression of the fluorescent tdTomato reporter within the stem-cell niche. 
b. The lineage of single labelled stem cells is traced in vivo during hair growth. 
c. Using two-photon laser scanning microscopy we can re-visit the same hair follicles, non-invasively in live mice at different stages of hair regeneration. Each panel depicts low (top) and high (bottom) magnification images of live hair follicles captured in first telogen, second anagen and second telogen, respectively.
Extended Data Figure 3 | Fluorescent proteins and kinetics of the inducible tdTomato-Cre reporter. **a**, Panels depicting the green (left) and red (middle) channel as well as a composite image (right) of a group of follicles in rest phase (telogen). *K14-H2BGFP* marks all the epithelial cells in the skin including the hair follicles. *Lef1-RFP* marks mesenchymal cells in the dermis including the dermal papilla at the bottom of the hair follicles. The tdTomato-Cre reporter (*K19*- or *Lgr5*-driven) displays mosaic expression in the stem-cell niche after administering a low dose of tamoxifen. Notice that the fluorescent intensity of the tdTomato is several-fold higher and easily distinguishable from RFP in the red channel. **b**, Individual channels and composite images of a group of follicles three (P23) and five (P25) days after administering a low dose of tamoxifen show a non-leaky expression of the Cre reporter (tdTomato) and a quiescent niche between these time points. Scale bar, 100 μm.
Extended Data Figure 4 | Classification of hair follicle cell types in vivo.

a, Single optical sections (top) or 3D volume renderings (bottom) of the outer (ORS; left) or inner (right) hair follicle cell layers as seen in vivo using a K14-driven H2B–GFP fluorescent reporter. b, Single optical sections showing cells marked with the tdTomato-Cre reporter (in addition to K14-driven H2B–GFP) in the outer (ORS; left) and inner (right) hair follicle layers. Notice the differences in morphology between cells located in different layers within the hair follicle.
Extended Data Figure 5 | Relocation of bulge stem cells and progeny over a hair cycle. Examples of in vivo lineage tracing of bulge cells in rest and growth phases of a full hair cycle. Arrows point to the location of the original cell and that of its progeny occupying different positions in the niche after a full hair cycle. Scale bar, 50 μm.
Extended Data Figure 6 | A single hair germ cell generates a spatially restricted differentiated lineage. *In vivo* lineage tracing of a single cell located in the hair germ in rest and growth phases over a full hair cycle. In advanced hair growth (anagen) an IRS differentiated lineage can be visualized and it is restricted to one side of the follicle as the original founder cell. Scale bar, 50 μm.
Extended Data Figure 7 | Long-term fate of bulge stem cells. Examples of in vivo lineage tracing of a single bulge cell in rest and growth phases over two consecutive hair cycles. Arrows point to the location of the stem cell that remains uncommitted in the niche during both hair cycles. Scale bar, 50 μm.
Extended Data Figure 8 | Origin of the hair germ. Examples of in vivo lineage tracing of a single bulge cell lineage in rest and growth phases over two consecutive hair cycles. A bulge cell positioned in the lower bulge undergoes limited and more extended amplification in the ORS over two consecutive hair cycles. After regression of the follicle at the end of the second hair cycle some surviving ORS clones form part of the hair germ before the third hair cycle begins. Arrows depict the clonal expansion and contraction of the bulge stem-cell lineage during the two hair cycles. Scale bar, 50 μm.
Extended Data Figure 9 | Model for determining stem-cell fate based on the spatial organization of the hair follicle niche in homeostasis and after injury. In homeostasis, a stem cell located in the upper bulge does not commit to a specific fate and is likely to remain quiescent, self-renew or become lost. By contrast, a cell positioned in the lower bulge is more likely to become activated and undergo limited amplification as part of the ORS, while still remaining relatively undifferentiated. ORS cells that survive the regression phase in one hair cycle can be situated in the niche compartment that becomes the new hair germ. Once positioned within the hair germ a cell will commit towards a differentiation pathway generating the cell types necessary to support hair growth. Loss of a stem-cell pool due to injury can induce an epithelial cell to enter the niche and contribute to its recovery. However, once a cell enters the niche it is subject to the same inputs as previous resident cells and as a result it will acquire a hair fate and actively contribute to hair regeneration.