Pioneer factors govern super-enhancer dynamics in stem cell plasticity and lineage choice

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Adult stem cells occur in niches that balance self-renewal with lineage selection and progression during tissue homeostasis. Following injury, culture or transplantation, stem cells outside their niche often display fate flexibility. Here we show that super-enhancers underlie the identity, lineage commitment and plasticity of adult stem cells in vivo. Using hair follicle as a model, we map the global chromatin domains of hair follicle stem cells and their committed progenitors in their native microenvironments. We show that super-enhancers and their dense clusters (epicentres) of transcription factor binding sites undergo remodelling upon lineage progression. New fate is acquired by decommissioning and establishing new super-enhancers and/or epicentres, an auto-regulatory process that abates one master regulator subset while enhancing another. We further show that when outside their niche, either in vitro or in wound-repair, hair follicle stem cells dynamically remodel super-enhancers in response to changes in their microenvironment. Intriguingly, some key super-enhancers shift epicentres, enabling their genes to remain active and maintain a transitional state in an ever-changing transcriptional landscape. Finally, we identify SOX9 as a crucial chromatin rheostat of hair follicle stem cell super-enhancers, and provide functional evidence that super-enhancers are dynamic, dense transcription-factor-binding platforms which are acutely sensitive to pioneer master regulators whose levels define not only spatial and temporal features of lineage-status but also stenness, plasticity in transitional states and differentiation.

Hair follicle stem cells fuel cyclical bouts of hair follicle regeneration and hair growth and also repair damaged epidermis. Hair follicle lineage progression is governed in part by dynamic regulation of Polycomb (PcG)-mediated repression/de-repression typified by a trimethylation mark on lysine 27 of histone H3 (H3K27me3). However, hair follicle stem cell identity and function are mainly independent of PcG-regulated genes, indicating that additional epigenetic mechanisms underlie the governance of critical cell identity genes.

Figure 1 | Dynamic super-enhancer remodelling facilitates lineage progression. a, Identification of H3K27ac super-enhancers in hair follicle stem cells. b, H3K27ac-marked enhancers at Cdk19 and Macf1 loci in hair follicle stem cells. Red bars denote enhancers. c, Occupancy of hair follicle stem cell TFs reveals high enrichment particularly within super-enhancers. HFSC, hair follicle stem cell. d, Clustering of hair follicle stem cell TFs occurs in epicentres (red arrows) within super-enhancers, as illustrated by the Rad51b locus. e, Dynamic remodelling of super-enhancers during lineage progression. Example shows that the super-enhancer of the Col17a1 locus, active in hair follicle stem cells, undergoes complete decommissioning in TACs. f, Enhancer remodelling correlates with gene expression changes. Boxplot displaying the full range of variation (min. to max.) shows that the changes that take place in super-enhancers have a profound effect on gene expression during lineage progression (n = 2). g, Cell fate determinants switch between super-enhancer activation and PcG repression, typified by a swap in H3K27 modifications. Representative example (Cux1) shows this switch upon hair follicle stem cell to TAC fate commitment. h, Motif analysis of TAC super-enhancers for putative TF binding sites reveals that they are regulated by a new cohort of transcriptional regulators. SE, super-enhancer; TE, typical-enhancer.
Figure 2 | Super-enhancer epicentres confer tissue, lineage and temporal specificity. a, Lentiviral super-enhancer reporter and analysis scheme. Tel: telogen (quiescent hair follicle stem cells; no TACs, no hair growth); Ana: anagen (active TACs, hair growth). b, H3K27ac occupancy at the Cxcl14 locus. Red box highlights the Cxcl14 super-enhancer epicentre (bound by MED1 and seven hair follicle stem cell TFs; absent in TACs) cloned for reporter assays. c, Cxcl14-SE-eGFP expression in H2B-mRFP7 epidermis is limited to hair follicle stem cells and early hair follicle stem cell progeny along upper ORS in anagen (right). Hatched lines denote the spliced out middle-region of the hair follicle. d, Temporal activation of Cxcl14-SE-eGFP in SOX97 cells, concomitant with hair follicle stem cell niche establishment at P2. e, Hair-follicle-stem-cell-specific targeting by mir205- and Nfatc1- super-enhancer epicentres, whereas the Cxcl14 promoter and Elovl5 typical-enhancer display broader activity. Atypical Cited2-TE binds all seven hair follicle stem cell TFs and drives hair-follicle-stem-cell-specific targeting. f, Cxcl14-SE-eGFP is silent in hair follicle stem cells, but activated during the hair cycle in TACs and differentiating IRS progeny. Dotted lines denote epidermal–dermal border; solid lines delineate DP (dermal papilla). Bu, bulge (hair follicle stem cell niche). Green dot denotes hair shaft autofluorescence.

Recent in vitro studies suggest that genes controlling unique cellular identities are driven by so-called ‘super-enhancers’5,6,11. Representing a small fraction of total enhancers, super-enhancers encompass large chromatin domains bountiful in cell-type specific transcription factor (TF) binding motifs that enable TFs to bind cooperatively. Their richness in H3K27 acetylation renders super-enhancers mutually exclusive for their committed progenitors, although the TE binds all seven hair follicle stem cell TFs. The hair follicle stem cell TF binding was not concomitant with hair follicle stem cell niche establishment at P2, their committed progenitors in vivo, and hair follicle stem cells in vitro (Supplementary Table 1; see below).

Whereas typical-enhancers (1–2 kb) governed >90% of hair follicle stem cell genes, super-enhancers marked genes transcribed selectively in hair follicle stem cells (Extended Data Fig. 2g, h). Unbiased gene ontology (GO) analysis further distinguished super-enhancer regulated genes by a preponderance of transcriptional regulators, including Med1, H3K4me1, and Mediator complex allies facilitate interactions with promoters to initiate transcription14.

To explore the in vivo importance of super-enhancers in stem cells, we first conducted chromatin immunoprecipitation followed by next-generation sequencing (ChiP-seq) on hair follicle stem cells purified directly from skin (Extended Data Fig. 1). H3K27ac, Mediator subunit MED1 and H3K4me1 peaks resided within promoters (± 2 kb of annotated genes) (40%) and distal elements, considered enhancers (60%) of hair follicle stem cell chromatin. A total of 377 super-enhancers were identified by size (>28 kb) and elevated H3K27ac occupancy with ≥5 H3K27ac-enriched clusters (Fig. 1a, b and Extended Data Fig. 1a–d).

A total of >80% accuracy in super-enhancer gene assignments can be achieved by applying optimized RNA-seq and proximity algorithms14. Most remaining ambiguities arise from multiple expressed genes in close proximity of a super-enhancer14. We resolved these by requiring that hair follicle stem cell super-enhancer genes must (1) exhibit H3K4me3/H3K79me2-activating and lack H3K27me3-repressive modifications; and (2) maintain strict correlation between super-enhancer and candidate expression in three different states: hair follicle stem cells, their committed progenitors in vivo, and hair follicle stem cells in vitro (Supplementary Table 1; see below).

Notably, >60% of super-enhancers were occupied by ≥5 different hair follicle stem cell TFs. The hair follicle stem cell TF binding was not similarly distributed within open chromatin of comparable cohorts of typical-enhancers, even when flanking sequences were included to normalize for their smaller size (Extended Data Fig. 3a, b). Thus, binding of hair-follicle-stem-cell-specific TFs was not dictated by open chromatin per se, but rather by super-enhancers, which controlled critical cell identity genes, including themselves, in this adult stem cell niche.
Scattered across each super-enhancer were smaller (1–2 kb) regions densely packed with hair follicle stem cell TF consensus binding motifs and which bound the cohort of hair follicle stem cell TFs (Fig. 1d). These epicentres resembled recently described ‘hotspots’ within super-enhancers of cultured adipocytes. Notably, 1% of typical-enhancers had even one such cluster of hair follicle stem cell TF motifs, whereas most hair follicle stem cell super-enhancers had ten (Extended Data Fig. 3).

An auto-regulatory and cooperative mechanism predicts that super-enhancer remodelling must occur to progress along a lineage typified by environmentally induced changes in TF landscape. We tested this hypothesis by characterizing the super-enhancers of short-lived hair follicle stem cell progeny (transit-amplifying cells, TACs) that progress to make hair (Extended Data Fig. 1). The 381 super-enhancer-marked TAC genes diverged considerably from those of hair follicle stem cells (Fig. 1e). Notably, hair follicle stem cell TF genes lost their super-enhancers in TACs, while TAC TF genes gained super-enhancers. Thus, our findings broaden the concept of super-enhancer dynamics observed in macrophages isolated from different tissues, and supported the notion that enhancers are activated or silenced in lineage-specific fashion. However, they contrasted with prior studies suggesting that chromatin remains broadly permissive as intestinal stem cells progress through a lineage.

Like hair follicle stem cells, TAC super-enhancers controlled TF, BMP and WNT signalling genes, but the presence of cell-cycle related and NOTCH pathway super-enhancer-marked genes appeared unique to features of TACs (Extended Data Fig. 4). Interestingly, only 32% of hair follicle stem cell super-enhancers persisted in TACs. Half were reduced to typical-enhancers, suggestive of more subordinate roles. Analogously, 54% of genes that gained a super-enhancer in TACs were driven by typical-enhancers in hair follicle stem cells (Fig. 1e). Typical-enhancer to super-enhancer shifts correlated with increased transcription and appeared to provide an epigenetic readout to gauge transcriptional levels during lineage progression (Fig. 1f).

Most super-enhancer genes involved in dictating hair follicle stem cell fate were decommissioned in TACs. For this cohort, H3K27ac loss was accompanied by H3K27me3 gain, suggestive of ‘super-silencing’ (Fig. 1g). Conversely, specific TAC fate determinants became de-repressed by losing PcG-catalysed H3K27me3 marks, while simultaneously gaining H3K27ac to expose a new super-enhancer. An unbiased analysis revealed that TAC super-enhancers were enriched for the binding motifs of many TAC TFs (Fig. 1h).

To address functionality, we tested the ability of super-enhancer epicentres to drive reporter gene expression in vivo. A 1.2 kb epicentre within the Cxcl14 super-enhancer was used to generate a high-titre lentivirus harbouring Cxcl14-SE-eGFP (SE, super-enhancer) and Pkg-H2B-mRFP1 and injected into the amniotic sacs of living E9.5 embryos (Fig. 2a, b). This results in random transgene integration into skin progenitor chromatin.

By the adult stage, H2B–mRFP1 was expressed throughout skin epithelium. Notably, however, eGFP was confined to hair follicle stem cells that reside in the outer layer of the resting (telogen) phase ‘bulge’ niche (Fig. 2c and Extended Data Fig. 5). As a new hair cycle began, Cxcl14-SE-eGFP activity persisted in hair follicle stem cells and early transitory progeny within the upper outer root sheath (ORS) of regenerating hair.
folicles. By contrast, the reporter was silenced in committed TACs, which lack hair follicle stem cell TFs altogether. The specificity of Cxcl14-SE-eGFP extended to development, where it became faithfully activated coincident with hair follicle stem cell TF and niche establishment44 (Fig. 2d).

Similar eGFP patterns were observed when hair follicle stem cell super-enhancer epicentres from Nfatc1 and mir205 were used as drivers. However, in contrast to its super-enhancer, the promoter of Cxcl14 drove broad skin epithelial expression, as did the typical-enhancer of Elov5. Despite some tissue-specific elements, these regions lacked clustered hair follicle stem cell TF binding sites. By contrast, the typical-enhancer of Cited2 uncharacteristically contained all hair follicle stem cell TF motifs and correspondingly exhibited hair follicle stem cell specificity. Summarized in Fig. 2e, these results provide compelling evidence that concentration of binding sites for a diverse array of hair follicle stem cell TFs is what confers lineage and stage-restricted specificity, whose activity is largely refractory to integration site.

Prior knowledge of master regulators was not necessary to tease apart these specialized regulatory elements from those driving broader expression. Thus, by identifying a MED1-bound, H3K27ac-intense epicentre of the TAC super-enhancer-controlled Cax1 gene29, we could generate a reporter with activity restricted to the hair follicle channel (inner root sheath, IRS) TACs (Fig. 2f and Extended Data Fig. 5). These findings illustrate the power of super-enhancers and their epicentres for developing genetic tools with unprecedented cell-type, temporal, lineage and stage specificity.

Intriguingly, Cxcl14-SE-eGFP activity was silent in cultured hair follicle stem cells, consistent with the complete decommissioning of hair follicle stem cell niche super-enhancers in vitro (Extended Data Fig. 6). Super-enhancer-associated hair follicle stem cell TF genes were also repressed in vitro, but upon engraftment26 were faithfully restored. This behaviour suggested that super-enhancer epicentres are reversibly sensitive to their microenvironment. Additionally, although new super-enhancers were acquired in vitro, few corresponded to ‘signature genes’ of hair follicle stem cells proliferating in vivo, suggesting environmental adaptation is more critical than proliferative status (Fig. 3a, b and Extended Data Fig. 6).

Curiously, some genes acquiring super-enhancers in vitro were epidermal genes, while others have been implicated in wound-repair. If these dynamics reflect a transitional state analogous to early stages of wound-repair, super-enhancer regulated genes should change quickly once hair follicle stem cells exit their niche and migrate into a wound bed. To test this possibility, we fluorescently tagged niche hair follicle stem cells, introduced a shallow wound, and monitored for proteins whose genes had changed super-enhancer status in vitro. Shortly after epidermal injury, YFP-marked hair follicle stem cells downregulated both super-enhancer-regulated TF genes and LV-transduced Cxcl14-SE-eGFP. F. Sustained SOX9 during hair follicle regeneration. Note prevention of swap in H3K27 modifications of key super-enhancers upon TAC fate commitment. NFATc1 atypically persists in lower ORS and TACs, and mininbugle-like structures occur along ORS (arrows).
exhibited no luciferase activity, while culture-based epicentres were restricted expression to the hair follicle lineage. However following injury, in vitro epicentres were induced in activated hair follicle cells undergoing epidermal repair, while epicentres of quiescent hair follicle stem cells became repressed (Fig. 3g).

While these results are intriguing, it remains unclear how hair follicle stem cells exploit super-enhancer dynamics to elicit the plasticity that allows them to regenerate hair follicles during homeostasis, repair damaged epithelium following injury, and adapt to culture. We were drawn to SOX9, as Sox9 was the only hair follicle stem cell TF gene that maintained a super-enhancer in vitro, where it was expressed at lower levels (Fig. 3a and Extended Data Fig. 8). It was also maintained at reduced levels in wound-activated hair follicle stem cells, suggesting a role for SOX9 in transitional states (Fig. 3c). Curiously, quantitative Sox9 ablation in adult hair follicle stem cells strikingly reduced colony-forming efficiency in vitro, and Sox9 ablation during skin embryogenesis blocked LHX2, TCF3 and TCF4 expression and formation of functional hair follicle stem cells (Fig. 4a, b).

Conversely, ectopic Sox9 in cultured hair follicle stem cells induced Lhx2, Tcf7l1 and Tcf7l2 transcription. Even more impressive were the effects of Sox9 on epidermal keratinocytes, which in vitro as in vivo, did not express hair follicle stem cell TFs. Lhx2 showed >80× elevation upon Sox9 induction in epidermal cultures and repression shortly after Sox9 ablation in hair follicle stem cells. Neither Sox9, Tcf7l1 nor Tcf7l2 showed such sensitivity to LHX2, indicating a special importance for SOX9 in regulating hair follicle stem cell super-enhancer activity (Fig. 4c and Extended Data Fig. 8g).

If SOX9 is a true pioneer factor whose levels dictate whether super-enhancers will be epigenetically active or silenced, then inducing SOX9 in skin epidermis should activate genes such as Tcf7l1 and Lhx2 whose super-enhancers are PcG-silenced (Fig. 4d). We tested this possibility with a doxycycline-inducible Sox9 lentivirus transduced in utero into K14-rTA animals and activated at P0 or P21. Notably, Sox9 expression in epidermis activated other super-enhancer controlled hair follicle stem cell TF genes. The ability of Sox9 to initiate H3K27 acetylation was exemplified by its activation of normally PcG-silenced Lhx2 and Tcf7l1 in epidermis. The activation of Ccxl14-SE-eGFP in Sox9-expressing epidermis explicitly traced the phenomenon to super-enhancers (Fig. 4e).

Finally, prolonging SOX9 in the hair follicle lineage generated equally striking perturbations. The lower ORS was riddled with bulge-like structures concomitantly with persistent LHX2, TCF3/4 and SOX9 in this transitory zone. NFATc1 was atypically sustained in lower ORS and TACs, while the switch to TAC super-enhancers was impaired (Fig. 4f and Extended Data Fig. 9). The failure of Nfatc1 to become PcG-silenced in Sox9+/− TACs shows that Sox9 protects against H3K27me3 silencing at super-enhancer regulated genes. In summary, by coupling a pioneer factor, SOX9, which senses local changes in microenvironment, to chromatin platforms optimized for sensing TF concentration, super-enhancers elicit the chromatin dynamics required for skin stem cells to pursue distinct lineages, repair wounds and exhibit plasticity in transitional states.

Online Content Methods, along with any additional 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. Spradling, A., Drummond-Barbosa, D. & Kai, T. Stem cells find their niche. Nature 414, 98–104 (2001).

2. 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).

3. van Es, J. H. et al. Dil1+ secretory progenitor cells revert to stem cells upon crypt damage. Nature Cell Biol. 14, 1099–1104 (2012).

4. Blanpain, C. & Fuchs, E. Stem cell plasticity. Plasticity of epithelial stem cells in chromatin regeneration. Science 344, 1242291 (2014).

5. Whyte, W. A. et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 153, 307–319 (2013).

6. Hsu, Y. C., Li, L. & Fuchs, E. Transit-amplifying cells orchestrate stem cell activity in epidermal regeneration. Cell 157, 935–949 (2014).

7. Ezkioa, E. et al. EZH1 and EZH2 acetylome histone H3K27 trimethylation and are essential for hair follicle homeostasis and wound repair. Genes Dev. 25, 485–498 (2011).

8. Li, W. et al. Genome-wide maps of histone modifications unwind in vivo chromatin states of the hair follicle lineage. Cell Stem Cell 9, 219–232 (2011).

9. Loven, J. et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell 153, 320–334 (2013).

10. Hnisz, D. et al. Super-enhancers in the control of cell identity and disease. Cell 155, 934–947 (2013).

11. Gosselin, D. et al. Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. Cell 159, 1327–1340 (2014).

12. Lavin, Y. et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell 159, 1312–1326 (2014).

13. Liu, Z. et al. Enhancer activation requires recruitment of a macrotranscription factor complex. Cell 159, 358–373 (2014).

14. Downen, J. M. et al. Control of cell identity genes occurs in insulated neighborhoods in mammalian chromatosomes. Cell 159, 374–387 (2014).

15. Folquen, C. R. et al. Architectural niche organization by LHX2 is linked to hair follicle stem cell function. Cell Stem Cell 13, 314–327 (2013).

16. Kajdaj, M. et al. Sox9: a stem cell transcriptional regulator of secreted niche signaling factors. Genes Dev. 28, 328–341 (2014).

17. Keyes, B. E. et al. Ntact1 orchestrates aging in hair follicle stem cells. Proc Natl Acad Sci USA 110, E4950–E4959 (2013).

18. Chang, C. Y. et al. NFIB is a governor of epithelial-mesenchyme cell stem behaviour in a shared niche. Nature 495, 98–102 (2013).

19. Lien, W. H. et al. In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. Nature Cell Biol. 16, 179–190 (2014).

20. Siersbaek, R. et al. Transcription factor cooperativity in early adiopogenic hotspots and super-enhancers. Cell Rep. 7, 1443–1455 (2014).

21. Luyten, A., Zang, C., Liu, X. S. & Shvidrasani, R. A. Active enhancers are delineated de novo during hematopoiesis, with limited lineage fidelity among specified primary blood cells. Genes Dev. 28, 1827–1839 (2014).

22. Kim, T. H. et al. Broadly permissive intestinal chromatin underlies lateral inhibition and cell plasticity. Nature 506, 511–515 (2014).

23. Beronja, S., Livshits, G., Williams, S. & Fuchs, E. Rapid functional dissection of genetic networks via tissue-specific transduction and RNAi in mouse embryos. Nature Med. 16, 821–827 (2010).

24. Nowak, J. A., Polak, L., Pasoli, H. A. & Fuchs, E. Hair follicle stem cells are specified and function in early skin morphogenesis. Cell Stem Cell 3, 33–43 (2008).

25. Ellis, T. et al. The transcriptional repressor CDP (Cutii) is essential for epithelial cell differentiation of the lung and the hair follicle. Genes Dev. 15, 2307–2319 (2001).

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. Chen, T. et al. An RNA interference screen uncovers a new molecule in stem cell self-renewal and long-term regeneration. Nature 485, 104–108 (2012).

28. Wu, X. et al. Skin stem cells orchestrate directional migration by regulating microbubble-ACF7 connections through GSK3β. Cell 144, 341–352 (2011).

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Author Contributions R.C.A. and E.F. conceived the project and designed the experiments. R.C.A. and N.H. carried out the experiments, including FACS purification, ChIP-seq assays, data analysis and in vivo reporter assays. D.S.O. and L.P. carried out in vitro lentiviral injections and wounding experiments with mice. S.B.L., M.N. and M.K. contributed to in vitro experiments. A.A., S.R. and D.Z. performed bioinformatics analyses. E.F. supervised the project. R.C.A. and E.F. wrote the manuscript.

Author Information ChIP-seq data are deposited in GEO under accession number GSE61316. 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 E.F. (fuchsib@rockefeller.edu).
METHODS

Mouse lines. Female CD1 mice (8 weeks old, Charles River) were used for the purification of hair follicle stem cells. Female CD-1 mice transgenic for Krt14-H2B-GFP^20 (30–32 days old) were used for the purification of TACs. Krt15-CrePGR, Sox9^30, R26YFP^31 mice have been described^39. Krt19-CreER mice have been described^39. CreER was activated by intraperitoneal injection of mice with 20 mg ml^−1 tamoxifen (Sigma) in corn oil (Sigma) to specifically label hair follicle stem cells. For the generation of K14-H2B-iRFP mice, iRFP was first amplified from pShuttle-CMV-irFP (Addgene plasmid 31856) and fused with H2B, before the H2B-iRFP construct was assembled with the Krt14 promoter, β-globin intron and poly(A) sequences^40. Transgenic mice were generated with standard pronuclear injections. For lentiviral injections, transduced mice were confirmed by genotyping with RFP primers: forward 5′-ATCCTGGCTTCCCTGACTGAC-3′, reverse 5′-TCCAGCAGGTGTGATTCCTCTGTCG-3′. For TRE-mycSox9 transduced mice, positive mice were fed with doxycycline-containing chow, starting either at P0 (newborn) or at P21 (adult). No formal randomization was performed, and studies were not blinded. Mice were maintained in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of The Rockefeller University (RU), and procedures were performed with Institutional Animal Care and Use Committee (IACUC)-approved protocols.

Flow cytometry. Preparation of adult mouse back skins for isolation of hair follicle stem cells and TACs were done as previously described^28. Briefly, for telogen skin, procedures were performed with Institutional Animal Care and Use Committee (IACUC)-approved protocols.

ChIP-seq. Immunoprecipitations were performed on FACS-sorted populations from female mice or on cultured hair follicle stem cells^44. For each ChIP-seq run, 7 × 10^10 to 2 × 10^11 cells were used. Antibodies used for ChIP-seq were anti-H3K27ac (abcam, ab5792) anti-H3K4me1 (abcam, ab8895), anti-Crsp1 Trap220 (Med1, Belyhl Laboratories, A300-793A) and anti-H3K2me3 (Millipore, 07-449). Briefly, cells were cross-linked in 1% (wt/vol) formaldehyde solution, resuspended, and lysed. To solubilize and shear cross-linked DNA, lysates were subjected to a Bio-ruptor Sonicator (Diagenode, UCD-200) according to a 30× regimen of 30 s sonication followed by 60 s rest. The resulting whole-cell extract was incubated overnight at 4°C with 10 μl of Dynabeads Protein G magnetic beads (Life Technologies) which had been pre-incubated with 5 μg of the appropriate Ab. After ChIP, samples were washed, and bound complexes were eluted and reverse cross-linked. ChIP DNA was prepared for sequencing by repairing sheared DNA and adding Adaptor Oligo Mix (illuminia) in the ligation step. A subsequent PCR step with 25 amplification cycles added the additional Solexa linker sequence to the fragments to prepare them for annealing to the Genome Analyzer flow cell. After amplification, a range of fragment sizes between 150–300 bp was selected and the DNA was gel-purified and diluted to 10 nM for loading on the flow cell. Sequencing was performed on the Illumina HiSeq 2500 Sequencer following manufacturer protocols. ChIP-seq reads were aligned to the mouse genome (mm9, build 37) using Bowtie aligner^35. ChIP-seq signal tracks were presented by Integrative Genomic Viewer (IGV) software.

Bioinformatics analysis. H3K27ac peaks were called by the program MACS^34 (v1.4.2, default parameters) from the ChIP-seq data with the input as controls. The peaks were associated to genes using the mouse RefSeq annotations; those located according to increasing H3K27ac signal intensity^5. Enhancer-gene assignments were determined by the software GREAT^31 using the list of super-enhancer coordinates and the default setting. For motif analysis of enhancers located in super-enhancers, 1-kb sequences under the H3K27ac peaks were searched for enriched motifs using the software HOMER (v4.6) with the default setting (PMID 20513432). Enrichments were defined as 1-kb regions flanking either side of the H3K27ac peaks. The 1-kb size was chosen based on our analysis of the distances of H3K27ac peaks to the nearest transcription factor ChIP-seq peaks in hair follicle stem cells in vivo (distance of the two peak centres, Extended Data Fig. 3e), which showed an enrichment of TF binding within 1-kb regions of H3K27ac peaks. Overlapping epicentres were merged during this analysis. To analyse epicentre shifting, for each of the overlapping super-enhancers between hair follicle stem cells in vivo and in vitro, we determined the number of epicentres that were not overlapping in the two samples and considered them as shifting epicentres. To generate the heatmap (Extended Data Fig. 3c), the program seqMiner^36 was used to calculate the ChIP-seq read densities, which were the maximal numbers of overlapping ChIP-seq reads in 50-bp bins from −5 kb to +5 kb of the H3K27ac peak summits. The density matrix was clustered based on the H3K27ac ChIP-seq signal and then used to generate a heatmap.

Antibodies. The following antibodies and dilutions were used: SOX9 (rabbit, 1:1,000, Millipore), NFIB (rabbit, 1:1,000, Active Motif), LHX2 (rabbit, 1:2,000, Fuchs lab), K6 (guinea pig, 1:5,000, Fuchs laboratory), K24 (rabbit, 1:5,000, Fuchs lab), CD34 (rat, 1:100, BD-PharMingen), LEF1 (rabbit, 1:100, Fuchs lab), NFATC1 (mouse, 1:100, Santa Cruz), TCF3 (guinea pig, 1:200; Fuchs laboratory), TCF4 (rabbit, 1:300; Cell Signaling Technology), FHL2 (rabbit, 1:100, Abcam), PRGR4 (rabbit, 1:100, Abcam), CUX1 (rabbit, 1:200, Santa Cruz), β4-integrin (rat, 1:100, BD-PharMingen), GFP (chicken, 1:2,000, Abcam), RFP (rabbit, 1:5,000, MBL; or guinea-pig, 1:3000, Fuchs laboratory). Secondary antibodies coupled to Alexa488, RRX, or Alexa647 were from Life Technologies. Nuclei were stained using 4,6'-diamidino-2-phenylindole (DAPI).

Histology, immunofluorescence and imaging. Back skins from mice were embedded in OCT (Tissue Tek), frozen, cryosectioned (10–20 μm) and fixed for 10 min in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). For lentivirally transduced mice, head and backskins were pre-fixed in 4% PFA for 4 h at 4°C, followed by washes in PBS and incubation in 30% sucrose, before embedding in OCT. Sections were blocked for 1 h in gelatin block (5% normal donkey serum, 1% BSA, 2% fish gelatin, 0.3% Triton X-100 in PBS). Primary antibodies were diluted in blocking buffer and incubated at 4°C overnight (O/N). MOMBasic kit (Vector Laboratories) was used for blocking when primary antibodies were generated from mouse. After washing with PBS, secondary antibodies, were added for 1 h at room temperature (RT). Slides were washed with PBS, counterstained with DAPI and mounted in Prolong Gold (Invitrogen). Images were acquired with an Axio Observer.Z1 epifluorescence microscope equipped with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics), and with an ApoTome.2 (Carl Zeiss) slider that reduces the light scatter in the fluorescent samples, using 20× objective, controlled by Zen software (Carl Zeiss). Z stacks were projected and RGB images were assembled using Adobe Photoshop CS5.

Lentiviral expression constructs. Lentiviral super-enhancer reporters were generated by PCR amplification of selected enhancer regions from BAC clones, followed by insertion into Kpn1 and BsaB1 restriction sites of the Hpi1-EGFP construct^27. To generate the Sox9 expression construct, Sox9c DNA was PCR amplified, and inserted into the LV-TRE-PGK-H2BmRFP^1 construct. The resulting LV-TRE-mycSox9-PGK-H2BmRFP was used for in utero injections.

Partial thickness wound (dermabration) and hair follicle stem cell transplantation. Animals were anesthetized with ketamine/xylazine and administered buprenorphine analgesia. Skin was shaved and remaining hair cleared with hair removal solution. A rectangular area was gently sectioned between two parallel lines removed by a small rotary drill (Dremel) with a polishing wheel attachment (model 520), to create a partial-thickness wound. Hair follicle stem cell transplantations were described previously^26.

Cell culture. Primary hair follicle stem cells were isolated from PS2-60 K14-H2B-iRFP mice and plated onto mitomycin C-treated dermal fibroblasts in E-media supplemented with 15% (vol/vol) serum and 0.3 mM calcium^27. For colony formation assays, equal numbers of Sox9-deficient live cells were plated. After 14 days in culture, cells were fixed and stained with 1% (wt/vol) Rhodamine B (Sigma). Colony diameter was measured from scanned images of plates using Imager and colony numbers were counted. For viral infections, hair follicle stem cells were spun with lentivirus for 30 min at 1,100g in the presence of polybrene (0.1 μg ml^−1). For Sox9 overexpression studies, the PKG-Sox9-PK2-H2BYFP construct was transfected into cultured hair follicle stem cells or epidermal keratinocytes. 72 h later, YFP^+ and YFP^− cells were purified by FACS. Luciferase assays were performed as described^26.

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RNA extraction and qRT–PCR. FACS-isolated cells were sorted directly into TRIzol LS (Invitrogen). Total RNA was purified using the Direct-zol RNA MiniPrep kit (Zymo Research) per manufacturer’s instructions. DNase treatment was performed to remove genomic DNA (RNase-free DNase Set, Qiagen). Equal amounts of RNA were reverse-transcribed using oligo-dT primers (Superscript III, Life Technologies). qRT–PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. cDNAs were normalized to equal amounts using primers against Ppib2.

Statistics. For all measurements, 3 biological replicates and 2 or more technical replicates were used. Experiments were independently replicated twice, and representative data are shown. To determine the significance between two groups, comparisons were made using unpaired two-tailed Student’s t-test in Prism6 (GraphPad software). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

Sample size was predetermined based on the following considerations: at E9.5, surface ectoderm contained \( \approx 120,000 \) cells per embryo, each of which undergo 5–6 divisions until birth. We therefore estimate that the majority of hair follicles are derived from individual clones. Assuming 50% lentiviral infection efficiency at E9.5, we estimate \( \approx 60,000 \) independent lentiviral integration sites per animal. Being able to analyse \( \approx 100 \) hair follicles per animal, we reasoned that transducing \( \approx 2 \) embryos from two separate litters would achieve the requisite coverage and control for any aberration due to a particular litter, independent of LV construct.

29. Tumbar, T. et al. Defining the epithelial stem cell niche in skin. Science 303, 359–363 (2004).
30. Means, A. L., Xu, Y., Zhao, A., Ray, K. C. & Gu, G. A. CK19(CreERT) knockin mouse line allows for conditional DNA recombination in epithelial cells in multiple endodermal organs. Genesis 46, 318–323 (2008).
31. 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).
32. Nowak, J. A. & Fuchs, E. Isolation and culture of epithelial stem cells. Methods Mol. Biol. 482, 215–232 (2009).
33. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).
34. Zhang, Y. et al. Model-based analysis of ChIP-seq (MACS). Genome Biol. 9, R137 (2008).
35. McLean, C. Y. et al. GREAT improves functional interpretation of cis-regulatory regions. Nature Biotechnol. 28, 495–501 (2010).
36. Ye, T. et al. seqMINER: an integrated ChIP-seq data interpretation platform. Nucleic Acids Res. 39, e35 (2011).
37. Williams, S. E., Beronja, S., Pasolli, H. A. & Fuchs, E. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. Nature 470, 353–358 (2011).
Extended Data Figure 1 | FACS purification strategy to isolate hair follicle stem cells and TACs. a, FACS purification of wild-type hair follicle stem cells for ChIP-seq according to established markers Sca1hi and CD34lo. Sca1 is used to remove basal epidermal cells. b, FACS purification of TACs from Krt14-H2B–GFP mice. TACs are GFPloSca1lo/CD34lo. c, Epifluorescence of Krt14-driven H2B–GFP. Hair follicle stem cells and epidermal cells are GFPhi, whereas TACs are GFPlo. d, q-PCR to verify the FACS sorting strategy and measure enrichment of cell-type-specific marker genes. Mean and standard deviation are shown (n = 3). P values from t-test: *P < 0.05; **P < 0.01; ***P < 0.001, relative to hair follicle stem cells.
Extended Data Figure 2 | Enhancer distribution, size and gene assignment in hair follicle stem cells. a, Distribution of H3K27ac occupancy at promoter and enhancers in hair follicle stem cells. b, Distribution of typical-enhancers and super-enhancers in hair follicle stem cells. c, Enhancer size distribution in hair follicle stem cells. d, Number of individual H3K27ac peaks per gene. Super-enhancers are clusters of H3K27ac peaks and mainly consist of $\geq 5$ peaks per gene. e, f, Enhancer-gene assignments, exemplified by hair follicle stem cell super-enhancers Fzd6 and Btg2. FPKM, fragments per kilobase of transcript per million mapped reads (RNA-seq). g, Differential expression for genes driven by hair follicle stem cell super-enhancers and typical-enhancers. P values from t-test: ***, ***, ***, P $<$ 0.001. h, Density plot, contrasting expression levels of typical-enhancer versus super-enhancer associated hair follicle stem cell genes in hair follicle stem cells compared to epidermal progenitors. Note cell type-specific differences in expression for hair follicle stem cell genes controlled by super-enhancers but not typical-enhancers. i, Gene Ontology analysis of genes controlled by hair follicle stem cell enhancers. j, List of selected super-enhancer regulated hair follicle stem cell genes. SE, super-enhancer; TE, typical-enhancer.
Extended Data Figure 3 | Hair follicle stem cell TFs are enriched within super-enhancers and cluster in epicentres. a, b, Enrichment of hair follicle stem cell TFs within chromatin of super-enhancers, but not typical-enhancers. Comparisons were made with 377 randomly selected typical-enhancers and their flanking sequence extended 5’ and 3’ to match the average length of super-enhancers (average of 3 analyses is shown). Each ‘TF event’ (a) represents one hair follicle stem cell TF bound within a super-enhancer. ‘TF peaks’ (b) refers to the absolute amount of TFs occupying the super-enhancer. c, Heatmap showing ChIP-seq read densities (from −50 kb to +150 kb of peak centre) across H3K27ac peaks located in super-enhancers. Note that hair follicle stem cell TFs frequently bound densely together with strong H3K27ac peaks. d, Motif analysis of hair follicle stem cell super-enhancers for putative TF binding sites. e, Analysis of distance of H3K27ac peaks to their nearest transcription factor ChIP-seq peaks in hair follicle stem cells in vivo (distance of the two peak centres). Note that enrichment of TF binding occurs within 1-kb regions of H3K27ac peaks ('epicentres'). f, Frequency and distribution of hair follicle stem cell super-enhancer epicentres. g, Rare ‘atypical’ enhancers co-bound by 7 hair follicle stem cell TFs are more highly expressed in hair follicle stem cells versus committed progenitors.
Extended Data Figure 4 | Identification of super-enhancers in TACs.
a. Distribution of H3K27ac ChIP-seq signal across all enhancers in transit-amplifying cells (TACs) reveals 381 super-enhancers of little overlap with hair follicle stem cell super-enhancers. b. Tracking the status of TAC super-enhancers in hair follicle stem cells indicates striking enhancer remodelling upon lineage progression. Example shows the appearance of a de novo super-enhancer for the Dlx3/4 locus as hair follicle stem cells commit to a TAC fate. c. Examples of super-enhancer associated genes in TACs. Genes in green have a reported functional role in the hair lineage.
Extended Data Figure 5 | Super-enhancer reporters drive cell-type specific expression. a, The lentiviral control (CTRL) reporter construct (containing no enhancer) is silent throughout all stages of the hair cycle, despite efficient infection (as evidenced by H2B–mRFP1). b, Immunofluorescence showing that Cxcl14-eGFP super-enhancer reporter activity co-localizes with Krt24 hair follicle stem cells. DP, dermal papilla; Bu, Bulge. White dashed lines denote the epidermal–dermal border; solid lines delineate the DP. c, H3K27ac and MED1 ChIP-seq occupancy at the Cux1 locus in TACs. Red box shows the super-enhancer epicentre that was cloned for reporter assays. Note that epicentres bound by MED1 are sufficient to identify cell-stage specific loci, even without prior information about lineage-specific TFs. d, CUX1 expression pattern in hair follicles.
Extended Data Figure 6 | Hair follicle stem cells adapt to microenvironmental changes by reversible remodelling of super-enhancers.

a, Absence of Cxcl14-SE-eGFP reporter activity in transduced cultured hair follicle stem cells.
b, Transplanted cultured hair follicle stem cells establish de novo hair follicles and regain expression of hair follicle stem cell TFs.
c, Note extensive hair follicle stem cell super-enhancer remodelling upon culture conditions.
d, Hair follicle stem cells in vitro are molecularly distinct from activated hair follicle stem cells (aHFSC) in vivo.
e-h, H3K27ac levels at the Cxcl14, Sfrp1, Lhx2 and Ehf loci in hair follicle stem cells in vivo and in vitro.
i, Selected list of super-enhancer associated genes in hair follicle stem cells in vitro.
j, Note hair follicle stem cell super-enhancer plasticity in vitro and during wound repair. Fhl2 and Prrg4 display super-enhancer-mediated activity in vitro. Upon transplantation, hair follicle stem cells silence in vitro induced genes concomitant with hair follicle regeneration. However, during wounding, hair follicle stem cells (lineage marked with K19-CreER/R26YFP) regain expression of Fhl2 and Prrg4.

Note the dynamic regulation of super-enhancers and the resulting changes in gene expression. i. Selected list of super-enhancer associated genes in hair follicle stem cells in vitro. j. Note hair follicle stem cell super-enhancer plasticity in vitro and during wound repair. Fhl2 and Prrg4 display super-enhancer-mediated activity in vitro. Upon transplantation, hair follicle stem cells silence in vitro induced genes concomitant with hair follicle regeneration. However, during wounding, hair follicle stem cells (lineage marked with K19-CreER/R26YFP) regain expression of Fhl2 and Prrg4.
Extended Data Figure 7 | Hair follicle stem cells activate different epicentres within super-enhancers to sustain expression of critical genes in different microenvironments. a, b, H3K27ac and hair follicle stem cell TF ChIP-seq occupancies at the Macf1 and Rad51b loci in hair follicle stem cells in vivo and in vitro. Regions C, E and F mark epicentres active in vivo, richly bound by hair follicle stem cell TFs; adjacent regions D and G are novel epicentres active in vitro. Relative luciferase activities were driven by the 1–1.5 kb encompassing these epicentres. Mean and standard deviation are shown (n = 3). P values from t-test: ***P < 0.001. Functional validation of epicentre shifts in vivo.

c, Motif analysis of Macf1 epicentres (regions A and B, Fig. 3e) for putative TF binding sites.

d, Number and distribution of hair follicle stem cell super-enhancer epicentres in vitro.

e, Frequency of epicentre shifts in hair follicle stem cell super-enhancers (in vivo versus in vitro). Note that corresponding to the loss of hair follicle stem cell TFs in vitro, many super-enhancers display epicentre shifts to maintain expression of critical genes (for example, Macf1) in different microenvironments.

eGFP-reporter activity of in vitro epicentres is highly active in the epidermis, while physiological hair follicle stem cell epicentres are restricted to the hair follicle niche.
Extended Data Figure 8 | Hair follicle stem cell TFs are reduced outside the niche but are sensitive to Sox9 levels.  

a, Sox9 is expressed and displays nuclear localization in hair follicle stem cells in vitro.  
b, Colony formation assays on wild-type and Sox9-cKO hair follicle stem cells. Sox9^{fl/lo} Rosa26-YFP^{fl/+} hair follicle stem cells were seeded at 2,000 and 4,000 and transduced with lentiviral-Cre to achieve Sox9 ablation in vitro. All yellow and green colonies were not effectively targeted and are still SOX9^{+}. All red colonies (SOX9-negative) aborted, as revealed by quantifications of colony numbers and sizes shown at right.  
c, Sox9-overexpression in cultured hair follicle stem cells. SOX9 induces the expression of Tle4, Tcf7l1, Tcf7l2 and Lhx2.  
d, e, Hair follicle stem cell TFs are expressed at substantially lower levels in basal epidermal progenitors in vivo or in cultured epidermal keratinocytes relative to hair follicle stem cells.  
f, Downregulation of hair follicle stem cell TFs in Sox9-cKO hair follicle stem cells in vivo before hair follicle stem cells are lost.  
g, Doxycycline-inducible overexpression of Lhx2 in cultured epidermal keratinocytes does not induce hair follicle stem cell TFs. For b–g, mean and standard deviation are shown (n = 3). P values from t-test: *P < 0.05; **P < 0.01; ***P < 0.001; n.d., not detected; n.s., not significant.

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Extended Data Figure 9 | Sustained Sox9 expression in committed progenitors perturbs lineage progression. a, Sustained Sox9 in adult mice (doxycycline for 3 weeks in adult mice, starting at P21) leads to de novo formation of minibulge-like structures along the ORS. b, Immunofluorescence showing that Lef1 (normally H3K27me3 repressed in hair follicle stem cells, but H3K27ac super-enhancer induced in TACs) remains repressed in mycSOX9^+ hair follicles.