Oncogenic activation of PI3K induces progenitor cell differentiation to suppress epidermal growth

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Oncogenic lesions are surprisingly common in morphologically and functionally normal human skin. However, the cellular and molecular mechanisms that suppress their cancer-driving potential to maintain tissue homeostasis are unknown. By employing assays for the direct and quantitative assessment of cell fate choices in vivo, we show that oncogenic activation of PI3K–AKT, the most commonly activated oncogenic pathway in cancer, promotes the differentiation and cell cycle exit of epidermal progenitors. As a result, oncogenic PI3K–AKT-activated epidermis exhibits a growth disadvantage even though its cells are more proliferative. We then sought to uncover the underlying mechanism behind oncogene-induced differentiation via a series of genetic screens in vivo. An AKT substrate, SH3RF1, is identified as a specific promoter of epidermal differentiation that has no effect on proliferation. Our study provides evidence for a direct, cell autonomous mechanism that can suppress progenitor cell renewal and block clonal expansion of epidermal cells bearing a common and activating mutation in Pik3ca.
in AKT-mediated suppression of SH3RF1 scaffold function in supporting pro-renewal JNK signalling.

Results

Oncogenic activation in the PI3K pathway inhibits clonal expansion. We selected 35 known drivers of SCCs1 (Supplementary Fig. 1a–c) and generated open-reading frame (ORF)- and short hairpin RNA (shRNA)-expressing constructs to model their gain- and loss-of-function lesions. To test how these cancer drivers affect epithelial growth, we transduced epidermis of Cre-reporter mice across epithelial cancers13,28,29 (Supplementary Fig. 1d).

We reasoned that constructs that affect growth would be enriched or depleted in the epidermis over time, which we could measure by sequencing27 (Fig. 1a). Consistent with previous findings, lesions associated with clonal expansion in human skin3 were among the top promoters of epidermal growth (Fig. 1b; Supplementary Fig. 2a,b). We next measured progenitor cell proliferation and observed a significant reduction in clone size relative to wild-type. Statistics were derived from n = 91 wild-type and 90 PIK3CAH1047R clones pooled from 3 animals for each condition. Two-tailed Student’s t-tests were used, P < 0.0001. The centre line represents the mean, and error bars the s.d. Statistical source data for e are shown in Supplementary Table 4.

Oncogenic activation promotes the differentiation of epidermal progenitor cells. To probe how oncogenic Pik3ca inhibits clonal expansion, we used a conditional knock-in mouse31–33 in which activated Pik3ca (Pik3caH1047R) is expressed at physiological levels and under endogenous control (Fig. 2a). We established that Cre-recombinase efficiently replaces the wild-type protein with its oncogenic form, and results in the activation of AKT in Pik3caH1047R/H1047R (termed Pik3ca 2X from hereon) epidermis (Supplementary Fig. 2a,b). We next measured progenitor cell proliferation and observed a significant increase in their differentiation compared to wild-type during perinatal and adult stages (Fig. 2b; Supplementary Fig. 2c,d). We also tested for apoptosis and senescence, but detected no change in either of these events (Fig. 2c,d). Increased proliferation not balanced by apoptosis or senescence stood in contrast to our observation that oncogenic Pik3ca inhibited clonal growth, and suggested that there were additional mechanisms of growth inhibition.

Proliferation in wild-type and Pik3ca 2X epidermis was restricted to keratin 10 (K10)-negative basal progenitors (Supplementary Fig. 3a,b). We reasoned that an increase in their differentiation...
Fig. 2 | Oncogenic PI3K activation promotes epidermal differentiation but not apoptosis or senescence. a, Schematic of Pik3ca

\(^{loxP/loxP}\) knock-in mouse. b, Quantification of the rate of cell division, measured by EdU incorporation and average division interval (see Supplementary Fig. 2d and Methods), showing that basal progenitors in Pik3ca 2x epidermis divide faster than wild-type (WT) at P21. c, Quantification of activated caspase 3 staining shows no significant difference between Pik3ca 2x and WT epidermis at P21. d, Senescence-associated β-galactosidase (SA-βGal) staining and Cdkn2a expression failed to detect any senescent cells in WT and Pik3ca 2x epidermis at P21. ND, not detected. e, Schematic of the EdU–BrdU pulse–chase differentiation assay. EdU-only progeny cells (red), labelled by the initial 2-h pulse of EdU, are assessed for expression (brown) or absence (blue) of the differentiation marker K10, 6 h after completing the S phase. The rate of renewal is expressed as the fraction of new K10-negative progenitors out of the total number of progeny cells. f, Oncogenic Pik3ca promotes differentiation in epidermis at embryonic, perinatal and adult stages. g−i, GSEA (g) showing that oncogenic Pik3ca biases the basal progenitor cell transcriptome towards differentiation. Enrichment score (ES), normalized enrichment score (NES), false discovery rate (FDR) generated by GSEA2 program. Hair follicle stem cells (HFSC). Gene ontology (GO) enrichment (h) in genes upregulated in Pik3ca 2x epidermis (i). Statistics are based on RNA-seq performed in n = 3 animals for each condition. Panels g−i represent data pooled from three RNA-seq replicates. For b−d,f, statistics were based on n = 3 animals for each condition; the centre line represents the mean, and errors bars the s.d. Two-tailed Student’s t-tests were used. P values are as indicated (source data are provided in Supplementary Table 4).
enabled >95% of EdU-positive-only progenitors to complete mitosis (~3% remain in G2). This protocol also enabled the tracing of their daughter cell fate based on the expression of the differentiation marker K10 in both wild-type and Pik3ca 2x epidermis (Supplementary Fig. 3c,d). We readily detected EdU-positive-only differentiation marker K10 in both wild-type and Pik3ca 2x epidermis. A total of 96 wild-type and 102 Pik3ca 2x divisions were scored. e, Schematic (e) and representative image (f) of live imaging of single CreER-transduced mT/mG cells activated by tamoxifen at P19 produced three modes of fate choice 48 h after activation. Scale bar, 25 μm. g, Progenitor cell renewal is significantly suppressed in activated Pik3ca 2x epidermis. A total of 296 wild-type and 317 Pik3ca 2x doublets were scored. For d, g, statistics were based on n = 3 animals for each condition; the centre line represents the mean, and errors bars the s.d. Two-tailed Student’s t-tests were used, P values are as indicated (source data are provided in Supplementary Table 4).
progenitors could balance the loss of oncogene-expressing progenitors (Supplementary Fig. 4a–c).

Next, we performed RNA sequencing (RNA-seq) on basal progenitors expressing integrin α6 (α6, Itg6), isolated by fluorescence-activated cell sorting (FACS), from wild-type and Pik3ca 2× epidermis. We found that oncogenic Pik3ca shifts the progenitor expression profile towards differentiation in both mouse and human (Fig. 2f; Supplementary Fig. 4f). Gene ontology analysis further revealed an increased expression of cell cycle regulators and downregulation of negative regulators of differentiation in basal progenitors in Pik3ca 2× epidermis (Fig. 2f). The increased differentiation in Pik3ca 2× progenitors was also evidenced by the expression of differentiation markers and regulators, including the suppression of Ld27 and Sox928 and the elevation of Notch1, Notch3,39, Krt1 and Krt1130 (Fig. 2f). Together, our data demonstrate that oncogenic Pik3ca promotes the increased differentiation and cell cycle exit of progenitor cells in the epidermis.

**Pik3ca activation directly modifies cell fate choice in epidermis to inhibit renewal.** In epidermis, cell fate choice is associated with progenitor cell divisions, which give rise to two mitotically active daughters (symmetric renewal), two post-mitotic daughters (symmetric differentiation) or divide asymmetrically to maintain one progenitor cell and generate one differentiated daughter. To probe whether oncogenic Pik3ca promotes differentiation by regulating progenitor cell fate choice, we used two-photon intravital microscopy (Supplementary Fig. 5a). To visualize dividing progenitors and to follow the fate of their progeny over time, we crossed mice with an epidermal-specific photoactivatable H2B-mCherry transgene (K14-H2B-PAmCherry) to Pik3ca 2× mice. We then broadly activated oncogene expression using high-titre transduction with LV-Cre co-expressing membrane-associated green fluorescent protein (GFP) (LV-Cre-mGFP; Fig. 3a; Supplementary Fig. 5b). At P21, we photolabelled individual GFP-positive progenitors (Fig. 3b,c) and followed them as they divided (division 1). We tracked their daughter cells, which we scored as progenitors if they maintained mitotic potential, as evidenced by cell cycle re-entry (division 2), or as differentiated progeny if they left the basal layer and assumed flattened morphology (Fig. 3c,b). We observed that epidermal progenitors in wild-type skin undergo all three types of cell division, with asymmetric being more common (~50%) followed by similar rates of symmetric renewal (~25%) and differentiation (~25%, Fig. 3d). In contrast, symmetric renewal was significantly suppressed in oncogenic Pik3ca-expressing epidermis, whereas asymmetric division and symmetric differentiation were increased (Fig. 3d).
To investigate whether oncogene-driven differentiation is a cell autonomous process, we transduced Cre-reporter mice with LV-CreER and treated them with tamoxifen at P19 (Fig. 3e). We confirmed that a low dose of the drug resulted in sporadic labelling of epidermal progenitors and efficient activation of oncogenic Pik3ca expression (Supplementary Fig. 5c–h). At 48 h, we observed that most labelled progenitors had divided, and we scored their daughter cell fates (Fig. 3f). As with ubiquitous oncogene activation, symmetric renewal was significantly reduced, and both symmetric differentiation and asymmetric divisions significantly increased in Pik3ca 2x cells compared with wild-type (Fig. 3g).

Using three independent differentiation assays, we established that the progenitor renewal rate in Pik3ca 2x epidermis is significantly lower than in the wild-type epidermis (0.52 versus 0.43;
Supplementary Fig. 5i), and we expected this difference to have a profound effect on tissue growth41. Indeed, clonal expansion in epidermis under constant proliferation and tissue turnover rate (based on a long-term EdU chase assay and on previous results40) is predicted to vary between homeostasis and rapid expansion or loss when renewal rates change by as little as 5–10% (Supplementary Fig. 6a,b). To test whether oncogenic Pik3ca-driven differentiation is sufficient to promote the loss of clonal expansion, we generated a model of clonal growth using proliferation and renewal rates observed in wild-type and Pik3ca 2x epidermis (Supplementary Fig. 6c). Our model predicted that single cell activation of oncogenic Pik3ca in the adult epidermis would result in clone loss, which was consistent with our long-term lineage tracing analysis (Fig. 4a,b). To test whether oncogene-induced differentiation can affect tumour initiation, we transduced wild-type and Pik3ca 2x epidermis with lentivirus expressing the human papillomavirus (HPV) E7 oncogene. We found that progenitor renewal and clonal expansion were significantly increased in HPV E7 transduced wild-type epidermis, and that tumour growth was initiated as early as 1 month (Fig. 4c–f). Importantly, oncogenic Pik3ca significantly suppressed progenitor renewal and clonal expansion and delayed tumour initiation driven by HPV E7 (Fig. 4c–f). This result suggests that constitutively activated PI3K signalling can act as a tumour suppressor during epidermal tumour initiation.

The AKT substrate SH3RF1 mediates PI3K-driven differentiation. To functionally dissect the molecular mechanism of oncogenic Pik3ca-driven differentiation, we focused on AKT as the dominant effector kinase of PI3K signalling, which is activated in Pik3ca 2x epidermis (Supplementary Fig. 2b). We selected shRNAs that efficiently deplete Akt1, Akt2 or Akt3 transcripts, and showed that silencing of Akt1 and Akt2, but not Akt3, significantly promoted progenitor renewal in both wild-type and Pik3ca 2x epidermis (Supplementary Fig. 7a–c). Akt1 and Akt2 were similarly required for oncogenic Pik3ca-driven proliferation (Supplementary Fig. 7d,e). To test whether progenitor differentiation is regulated by a specific AKT substrate and independently of proliferation, we pooled 1,062 shRNAs targeting 242 validated AKT substrates42, and performed two genetic screens in vivo (Fig. 5a). For the differentiation screen, we collected basal (α, Ig<sup>hb</sup>) and suprabasal (α, Ig<sup>rm</sup>) epidermal cells (Fig. 5a). For the proliferation screen, we isolated dividing (EdU-positive) and non-dividing (EdU-negative) basal cells following an 8-h EdU pulse (Fig. 5a). We expected the following results: (1) shRNAs that inhibit differentiation drivers will be enriched in basal cells (pro-renewal; Fig. 5a,b); (2) shRNAs against drivers of progenitor renewal will be enriched in suprabasal cells (pro-differentiation; Fig. 5a,b); and (3) shRNAs that are dispensable for proliferation will be equally present in EdU-positive and EdU-negative progenitors (proliferation neutral; Fig. 5a,c).

We quantified the shRNA representation in each group of cells40 and identified Sh3rf1 as the top candidate whose silencing promoted differentiation without an effect on proliferation (Fig. 5b–d; Supplementary Tables 2, 3). To test whether Sh3rf1 is an AKT substrate in the epidermis, we pulled down endogenous protein and probed it using an antibody against the phospho-AKT substrate. We detected AKT phosphorylation of Sh3rf1 in wild-type epidermis that was further increased in Pik3ca 2x tissue (Fig. 5e). We observed the same result when full-length Sh3rf1 (SH3RF1WT) was expressed from a lentivirus (Fig. 5f); these results confirm that AKT phosphorylates SH3RF1 in vivo. To functionally test SH3RF1 as a specific differentiation regulator, we performed a series of gain- and loss-of-function experiments in vivo. To model the AKT phosphorylation-mediated suppression of SH3RF1, we transduced wild-type epidermis with shRNA constructs that most efficiently depleted its transcript (Supplementary Fig. 7f). Sh3rf1 depletion promoted progenitor differentiation without a significant effect on cell division,
Fig. 7 | SH3RF1 is a scaffold for JNK signalling in oncogenic Pik3ca-driven differentiation. a, Model of how AKT signalling can promote epidermal progenitor cell differentiation. b, Transcriptome of Pik3ca 2x progenitors showing significant suppression of JNK signature genes. ES, NES and FDR generated by the GSEA2 program. Statistics are based on RNA-seq performed in n = 3 animals for each condition. c, Phospho-JNK (p-JNK) staining in wild-type and Pik3ca 2x epidermis. The significant reduction of the phospho-JNK signal in Pik3ca 2x epidermis is rescued by SH3RF1 expression. e, SH3RF1(WT) and SH3RF1(WT) but not SH3RF1(ΔRing) rescues phospho-JNK suppression in Pik3ca 2x epidermis, while SH3RF1(ΔRing) can further enhance it. f, g, MLK1 rescues oncogenic Pik3ca-induced suppression of phospho-JNK (f) and differentiation (g; statistics are based on n = 3 animals for each condition). h, i, Knockdown of Jnk1 blocks SH3RF1(ΔRing)-enhanced phospho-JNK (h) and progenitor renewal (i; statistics are based on n = 3 animals for each condition). For d, e, f, h, experiments were repeated in n = 3 biological replicates each, with similar results obtained; unprocessed blots are provided in Supplementary Fig. 7g). These observations suggest that clonal expansion and the resultant clones represent the mean, and errors bars the s.d. Two-tailed Student’s t-tests were used; P values are as indicated (source data are provided in Supplementary Table 4).

thus validating our genetic screens (Fig. 5g). To counter the effect of AKT phosphorylation, we overexpressed SH3RF1(WT) in Pik3ca 2x epidermis (Fig. 5h). To control for a possible reduction in AKT phosphorylation that may be caused by substrate overexpression, we also expressed a known AKT substrate, H3F3B. We observed that SH3RF1 expression, but not H3F3B, suppressed oncogenic Pik3ca-driven differentiation while proliferation was unchanged (Fig. 5h). Finally, to test whether SH3RF1 can regulate oncogene-driven clonal expansion, we overexpressed SH3RF1(WT) in wild-type and Pik3ca 2x epidermis and observed that the resultant clones were significantly larger and were maintained long-term compared with Pik3ca 2x epidermis alone (Fig. 5i)). Together, our data suggest that SH3RF1 is an AKT substrate in the epidermis, where it acts as a specific mediator of oncogenic Pik3ca-driven differentiation and clonal growth.

Activated PI3K–AKT inhibits SH3RF1-mediated support of JNK signalling to promote progenitor differentiation. SH3RF1 is a SH3 domain-containing scaffold molecule necessary for JNK signalling43. SH3RF1 can also function as an E3 ubiquitin ligase to regulate additional pathways44,45. Importantly, the scaffold function of SH3RF1 is independent of its E3 ligase activity, and SH3RF1 with a deleted RING finger domain (SH3RF1(ΔRing)) can still support JNK signalling46,47. In addition, AKT-mediated phosphorylation of SH3RF1 only disrupts its scaffold function and not its ability to activate JNKs48,49. Therefore, we hypothesized that SH3RF1 function in oncogene-driven differentiation depends on its scaffold function in the regulation of JNK signalling. We generated an allelic series of SH3RF1 constructs (Fig. 6a,b) and expressed them in wild-type and Pik3ca 2x epidermis. We found that expression of SH3RF1(ΔRing) was equal to that of SH3RF1(WT) in promoting progenitor renewal and clonal expansion independently of proliferation (Fig. 6c–e, Supplementary Fig. 7g), confirming that the E3 ubiquitin ligase function of SH3RF1 is dispensable in oncogene-induced differentiation.

We next expressed AKT phosphorylation site (S304) mutated SH3RF1(ΔRing) as well as phosphomimetic SH3RF1(WT) in wild-type and Pik3ca 2x epidermis (Fig. 6a,b). We observed that SH3RF1(WT) had no effect on progenitor renewal or clonal growth, while SH3RF1(ΔRing) significantly promoted both processes (Fig. 6c–e; Supplementary Fig. 7g). These observations suggest that clonal expansion and the
progenitor renewal-promoting ability of SH3RF1 is dependent on, and suppressed by, AKT-mediated phosphorylation.

SH3RF1 promotes JNK signalling as a scaffold for assembly of the MLK–MKK complex6,49. This process has been demonstrated to be negatively regulated by AKT-mediated phosphorylation on S304 of SH3RF14,49. Meanwhile, JNK has been implicated as a positive regulator of progenitor renewal in skin6,51. Together, they suggest a molecular pathway whereby AKT phosphorylation and suppression of SH3RF1 inhibits the JNK signalling-mediated maintenance of progenitor cell fate (Fig. 7a). Consistent with our model, a gene set enrichment analysis (GSEA) of the oncogenic Pik3ca transcriptome demonstrated a significant suppression of JNK signature genes compared with the wild-type transcriptome (Fig. 7b). Moreover, immunofluorescence staining of epithelial tissues showed that phospho-JNK was significantly reduced in basal progenitors expressing oncogenic Pik3ca, and that the staining is largely recovered upon SH3RF1 WT expression (Fig. 7c; Supplementary Fig. 7h). To directly test whether PI3K–AKT-mediated phosphorylation of SH3RF1 can modify epidermal JNK signalling, we performed a series of experiments. Western blot analyses showed that JNK phosphorylation is suppressed in Pik3ca 2x epidermis relative to wild-type, and that this suppression can be rescued by the overexpression of SH3RF1 WT (Fig. 7d). SH3RF1 WT also rescued JNK phosphorylation, and expression of SH3RF1 WT but not SH3RF1 WT rescued and further enhanced phospho-JNK level in Pik3ca 2x epidermis. These results suggest that SH3RF1 promotes JNK signalling independently of its E3 ubiquitin ligase function, and that this positive regulation can be suppressed by AKT phosphorylation (Fig. 7e).

Last, we tested whether suppression of JNK signalling is necessary and/or sufficient for oncogenic Pik3ca-induced differentiation. We overexpressed the JNK activator, MLK1 (also known as MAP3K9) in Pik3ca 2x epidermis and observed increased phospho-JNK levels (Fig. 7f) and suppression of oncogenic Pik3ca-induced differentiation with no effect on proliferation (Fig. 7g; Supplementary Fig. 7i). This supports a model whereby JNK signalling is downstream of oncogenic Pik3ca in regulating epidermal progenitor renewal, and that its activation is sufficient to overcome oncogene-induced differentiation. To test whether the suppression of JNK is necessary for oncogene-induced differentiation, we expressed SH3RF1 WT in oncogenic Pik3ca 2x epidermis, while at the same time we depleted Jnk1 using shRNA knockdown (Fig. 7h). We observed that silencing of Jnk1 significantly abrogated the rescue effect of SH3RF1 WT on oncogenic Pik3ca-induced differentiation without affecting cell division (Fig. 7i; Supplementary Fig. 7j), confirming that JNK signalling is not only required for but is the dominant mediator of SH3RF1 function in regulating differentiation. Together, we demonstrate that the AKT-mediated phosphorylation of SH3RF1 and the subsequent suppression of JNK signalling is a critical pathway in oncogenic Pik3ca-induced differentiation, and provide a mechanism for oncogenic tolerance without triggering cell cycle arrest or cell death in the skin epithelium.

Discussion
How tissues respond to oncogenic mutations to prevent clonal expansion? Scenarios ranging from complete tumorigenesis to self-renewal and tissue replacement, and to no overt phenotypic consequence, have been described in normal and tumour settings. In this study, we add to this experimental toolbox by describing a simple paradigm that we describe here fits these requirements, as it allows the epidermis to manage oncogenic stress by driving excess oncogene-activated cells out of the mitotic progenitor pool. Even though lineage choice in epidermis is relatively simple, and differentiation is accompanied by observable and well-documented changes in cell morphology and marker expression, methods to directly assess cell fate choice in vivo have been slow to emerge. Indeed, investigations of how oncogenes affect cell fate choices and our discovery of oncogenic Pik3ca-induced differentiation were made possible only recently through the development of direct and quantitative assays, including intravital imaging7. In the current study, we add to this experimental toolbox by describing a simple EdU–BrdU pulse–chase assay as a sensitive method to quantify the progenitor cell renewal rate in skin epithelium.

Animal studies have already shown that activation of the PI3K–AKT pathway in mice through oncogenic mutations in Pik3ca WT, or loss of Pten WT, does not result in epidermal tumorigenesis63, even though tumours are initiated in multiple other epithelial organs. These observations suggest that the ability of oncogenic Pik3ca to promote tumorigenesis may be stringently restricted by a tumour-suppressive mechanism in distinct epithelia. Curiously, differentiation that is accompanied by a loss of proliferative potential is not seen across all epithelia, but seems specific for highly proliferative ones, such as skin epidermis64,65, intestinal epithelium66 and oral mucosa1. Future studies should explore whether these tissues employ differentiation to block clonal expansion and to counter oncogenic stress.

Our identification of differentiation as the dominant growth-suppressive mechanism in skin epithelium further suggests that its loss in the context of oncogenic lesions would provide oncogene-activated clones with the ability to expand while retaining higher proliferation rates. Supporting this notion, mutations identified as prominent clonal expansion promoters in both human tissue and our genetic screen are always gained earlier in SCC progression than in oncogenic lesions for PIK3CA67.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41556-018-0218-9.

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Author contributions
Z.Y. and S.B. conceived the study and designed the experiments. Z.Y. performed all the experiments and data analyses. M.S. developed the Edu–BrdU differentiation assay. Z.Y. and M.S. performed the intravital mouse imaging. Z.Y. and S.B. wrote the paper. All authors provided intellectual input, vetted and approved the final manuscript.
Competing interests
The authors declare no competing interests.

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Methods

Animals. We used equal numbers of male and female animals throughout the study. Pik3ca-lost/shRNA mice (donated by W. A. Phillips14,15), K14-H2B-Pan-Cherry mice (donated by V. Greco6), and K26(-/−)/K26-Cre reporter mice (Jackson Laboratory) were genotyped on the C57BL/6 or C37Bl/6-Tyr(−/−) background. Mice were housed and cared for in a AAALAC-accredited facility at the Fred Hutchinson Cancer Research Center, and all animal experiments were conducted in accordance with ethical regulations of the Fred Hutchinson Cancer Research Center and IACUC-approved protocols (project license number 50814).

Lentivirus production and transduction. Large-scale production and concentration of lentivirus were performed as previously described26,27. Detailed descriptions of the lentiviral transduction of 293T cells (Invitrogen) and primary keratinocytes in culture, and of in utero-geared lentiviral transduction in vivo can be found elsewhere26,27.

Lentiviral constructs. ORF overexpression was achieved using a pLX EF1 Barcode vector (Supplementary Fig. 1b). The pLX EF1 Barcode vector was modified from pLX302 (Addgene), in which the following were replaced: (1) the PGK-uroC cassette between Ppud/M1/Xba1 sites with a randomized 10-bp barcode; (2) the CMV promoter between Xho1/Nde1 sites with an EF1α promoter from pfEF-BOS (Addgene). RNA interference-mediated gene depletion was achieved using plKO1 shRNA vectors from the mouse TRC1.0 shRNA promoter from pEF-BOS (Addgene). ORF expression was achieved using plKO1-Cre vectors as previously described26,27. Cre-shRNA expression was achieved using pLKO-Cre vectors as previously described26,27. Cre-reporter mice were generated using pLX EF1 Cre vectors. This vector was modified from pLX302 (Addgene) by inserting Cre downstream of the PGK promoter using Kpn1/Xba1 sites. Expression of Cre-recombinase in tandem with ORF expression was achieved using plKO1-Cre-reporter mice.

Lentiviral constructs were modified from pLX302 (Addgene) by inserting Cre downstream of the PGK promoter using Kpn1/Xba1 sites. Cre-shRNA expression was achieved using plKO-Cre vectors as previously described26,27.

In vivo genetic screens. To quantify construct abundance in the screen for clonal expansion, head skin of mice at P21 was collected and digested in 0.25% collagenase at 37 °C for 1 h to release mesenchymal cells from the epithelium. Head skins from two animals were pooled and treated as a biological replicate to achieve ~200-fold coverage. To quantify construct abundance in the renewal and cell division screens, head skin of mice at embryonic day 18.5 (E18.5) was digested in 2 mg ml−1 dispase at 37 °C for 1 h to separate epidermis from dermis. Epidermal tissue was further digested with 0.25% trypsin for 30 min into single cells. Head skins from four animals were pooled to make one biological replicate, thus achieving an ~45-fold coverage. For the renewal screening, single epidermal progenitor cells were stained with CD26/PepCAM-APC (G8.8, 1:50; BD Biosciences) and CD49f/α2-integrin-PerCP (G0H13, 1:50; BioLegend). For the cell division screening, single epidermal cells were subjected to Click-iT EdU detection (C10388, Invitrogen) followed by CD49f/α2-integrin-PerCP staining. Cell populations of interest were isolated using a BD FACSaria II machine (BD Biosciences). Genomic DNA from all samples was extracted using a QiaAmp DNA tissue mini kit (Qiagen). Barcode pre-amplification, sequencing and data processing using the Deseq2 program28 were performed as previously described26,27.

Immunoprecipitation. E18.5 epidermis was snap-frozen using liquid nitrogen, mechanically pulverized and lysed in lysis buffer (150 mM NaCl, 10 mM HEPES buffer, pH 7.4, and 1% Nonidet P-40). Lysates were incubated with anti-V5 affinity beads (A7345, Sigma-Aldrich) of 100 μl per lysate (Invitrogen) pre-incubated with anti-SH3RF1 antibody (3H3, Abnova) overnight at 4 °C. Beads with affinity-bound proteins were washed six times with immunoprecipitation wash buffer (200 mM NaCl, 10 mM HEPES, pH 7.4, and 0.1% Nonidet P-40) before direct loading onto a 4–12% gel and subjected to western blot analyses.

Immunofluorescence and western blot analyses. The following primary antibodies were used: chicken anti-GFP (1:1,000 for immunofluorescence; ab13970, Abcam); mouse anti-BrdU (1:100 for immunofluorescence; MoBu-1, Invitrogen); mouse anti-b-actin (1:30,000 for western blotting; 60099, Proteintech); mouse anti-V5 (1:10,000 for western blotting; V5-10, Sigma-Aldrich); rabbit anti-Ki67 (1:1,000 for immunofluorescence; ab15580, Abcam); rabbit anti-phospho-AKT (1:1,000 for western blotting; D9E, Cell Signaling); rabbit anti-total AKT (1:500 for western blotting; C67E7, Cell Signaling); mouse anti-SH3RF1 antibody (1:400 for western blotting; 3H3, Abnova); rabbit anti-phospho-AKT substrate (1:1,000 for western blotting; 11087E, Cell Signaling); rabbit anti-phospho-SAPK/JNK (1:500 for western blotting; 2293, Cell Signaling), and rabbit anti-total SAPK/JNK (1:1,000 for western blottting; 9252, Cell Signaling). Tissues were processed for immunostaining as previously described26,27 and mounted in ProLong Gold with or without 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). For the immunoprecipitation and western blotting of cell cycle phase differentiation assays, tissue sections were first processed for EdU Click-iT following the manufacturer's instructions. Next, tissues were fully processed for K10 and GFP immunofluorescence detection. Last, tissue sections were incubated in 2 N HCl at 37 °C for 30 min to denature DNA, quenched with 0.1 M sodium borate, pH 8.5, and twice, and processed for BrdU immunofluorescence detection. Confocal images were taken on a Zeiss LSM780 system using a Plan-Apochromat ×10/1.4 oil objective. Images were processed using Zeiss Zen and Imago software. Western blotting was performed using a Novex system (Invitrogen), and chemiluminescent signals were captured using an Odyssey FC system (LI-COR).

EdU–BrdU cell division assay and quantification. To measure the average cell cycle interval in epidermis, we developed a cell cycle assay based on the specific labelling of cells that have completed cell division within a defined time frame. By using such a method, we minimized the potential artefact of conventional nucleotide incorporation-based proliferation assays; that is, tissues with a longer S phase but not an essentially faster division rate can appear to have more nucleotide incorporation (Supplementary Fig. 2d). To ensure the specificity of the assay, we tested the BrdU-specific antibody MoBU-1 and confirmed that animals injected with BrdU were readily labelled, while EdU (or sham)-injected animals showed no staining. Similarly, we detected EdU using Click-iT chemistry only in animals injected with EdU.

To perform the assay, as illustrated in Supplementary Fig. 2d, we first administered EdU to animals followed by BrdU injection 2 h later. We expected that cells that have completed the S phase during the first 2 h would incorporate EdU only, while cells subsequently going through S phase would have incorporated both EdU and BrdU (Supplementary Fig. 2d). Six hours later, we collected epidermis and detected EdU and BrdU signals using immunofluorescence. At this time point, we expected that cells that have completed the S phase during the initial 2 h and subsequently divided would give rise to two EdU–positive progeny cells, while cells that stalled in G2 would result in one EdU-positive-only cell (Supplementary Fig. 2d). However, we found that over our time frame, most cells divide (~97%) and that only a minority in both wild-type and Pik3ca 2x epidermis remains in G2 phase (Supplementary Fig. 3c,d).

To calculate the cell division interval, we defined the number of EdU-positive-only cells as E. As one cell division gives rise to two daughter cells, the number of cell divisions that give rise to EdU–positive-only cells is E/2. As we have demonstrated, only basal cells have the potential to divide in the epidermis (Supplementary Fig. 3a,b); therefore, the fraction (F) of basal cells that divide within 2 h to give rise to EdU-positive-only cells is F = (E/2)/B, where B is the total number of basal cells. We extrapolated this measure to the entire basal cell population by using the following ratio: D1 = 2 h/F, where D is the average cell division interval (in hours).

EdU–BrdU pulse–chase differentiation assay and quantification. Since we can uniquely label a population of cells (EdU-positive) that had undergone S phase within the initial 2 h and subsequently divided as described above, we next set out to determine how long after cell division an average daughter cell takes to express the differentiation marker K10. We administered EdU and then BrdU and collected tissues every 2 h. Tissues were processed to detect EdU–BrdU pulse–chase differentiation assays, tissue sections were first processed for EdU Click-iT detection (C10388, Invitrogen) followed by 4,6-diamidino-2-phenylindole (DAPI; Life Technologies). For EdU–BrdU pulse–chase labeling of cells that have completed cell division within a defined time frame, we first analyzed epidermis 6 h after the start of BrdU labeling. We can calculate the rate of progenitor cell renewal of the EdU-positive-only cells using the following equation: rate of renewal = number of EdU–positive-only K10-negative cells/total number of EdU–positive-only cells.

RNA-seq and GSEA analyses. We isolated inter-follicular epidermis from dermis in both mice and hair follicles using dispase digestion. Single cells were obtained following 15 min of trypsin digestion and then stained for basal integrin expression26. RNA from FACS-isolated α6 integrin-positive progenitors were extracted using TRIzol LS (Invitrogen) and a phenol–chloroform protocol. The extracted RNA was then purified using a QIAamp RNA mini kit (Qiagen) as per the manufacturer’s instructions. RNA quality was assessed using an Agilent 2100 Bioanalyzer, with all samples passing the quality threshold of RNA integrity number (RIN) score of >8. The library was prepared using an Illumina TrueSeq mRNA sample preparation kit at the Fred Hutchinson Cancer Research Center Genomic Core Facility, and complementary DNA was sequenced on an Illumina HiSeq 2000. Gene and transcript signatures were obtained from the following previously published expression profiles: HFSC; mouse basal/suprabasal cells; human basal/suprabasal cells; JNK activation signature26.
Statistics and reproducibility. All quantitative data are expressed as the mean ± s.d. Differences between groups were assayed by two-tailed Student’s t-test or one-way analysis of variance using Prism 5 (GraphPad software). Differences were considered significant when \( P < 0.05 \). All quantitative data were collected from experiments performed in at least three samples or biological replicates. The sample size was not predetermined and the experiments were not randomized.

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

Data availability
RNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE99659. Previously published microarray data that were re-analysed here are available under accession codes GSE41704\(^a\), GSE48859\(^b\), GSE26059\(^c\), and GSE50530\(^d\). Source data for Figs. 1e, 2b–d, f, 3d, g, 4a, c, e, 5g, h, j, 6c, e, 7g, i and Supplementary Figs. 2c, 3a, d, 4a–c, e, 5c–e, i, 7a–g, i, j are provided in Supplementary Table 4. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | BWA, (LI H. and Durbin R. 2009) ; FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) |
| Data analysis   | Deseq2 (Love et al., 2014); GSEA (Subramanian et al., 2005); R (https://www.r-project.org/); Flowjo; Prism5; Tophat2 (Kim et al., 2013); Zen (Zeiss); ImageJ (Schindelin et al., 2012) |

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

Data availability. RNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE99659. Previously published microarray data that were re-analysed here are available under accession code GSE41704, GSE48859, GSE26059, GSE50530.
Source data for Fig. 1e, 2b-d, 2f, 3d, 4a, 4c, 4e, 5g, 5h, 5j, 6e, 7g, 7i and Supplementary Fig. 2c, 3a, 3d, 4a-c, 4e, 5c-e, 5i, 7a-g, 7i, 7j have been provided as Supplementary Table 4. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Life sciences study design

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

- Sample size: No sample-size calculation was performed. All quantitative data were collected from experiments performed in at least a triplicate, all results obtained are highly significant thus no need for large cohort experiments.
- Data exclusions: No samples from experiments were excluded.
- Replication: All attempts at replication were successful. For general statistics at least 3 individual animals were used; for in vivo screen animal number were determined to ensure a >30 fold screen coverage, detail described in Method section.
- Randomization: Control and experimental male and female animals were randomly assigned into experimental groups.
- Blinding: No blinding were used in this study since since phenotypes were too apparent.

Reporting for specific materials, systems and methods

Materials & experimental systems

- n/a Involved in the study
- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

- n/a Involved in the study
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

The following primary antibodies were used: chicken anti-GFP (ab13970, 1:1000 for IF; Abcam); mouse anti-BrdU (MoBU-1, 1:100 for IF; Invitrogen); mouse anti-β-Actin (66009, 1:3000 for WB; Proteintech); mouse anti-V5 (V5-10, 1:3000 for WB; Sigma-Aldrich); rabbit anti-Keratin 10 (Poly19054, 1:1000 for IF; BioLegend); rabbit anti-Phospho-AKT (D9E, 1:1000 for WB; Cell Signaling); rabbit anti-total AKT (C67E7, 1:100 for WB; Cell Signaling); rabbit anti-Phospho-AKT Substrate (110B7E, 1:1000 for WB; Cell Signaling); rabbit anti-Phospho-SAPK/JNK (81E11, 1:500 for WB, 1:100 for IF; Cell Signaling); rabbit anti-total SAPK/JNK (9252, 1:1000 for WB; Cell Signaling).

Validation

All antibodies are validated by manufacture and previous publications we cited in the manuscript (Ref 43-49) using positive and/or negative controls such as protein knockdown, knockout, overexpression.

Eukaryotic cell lines

Policy information about cell lines

- Cell line source(s): 293T
- Authentication: Obtained from Invitrogen with certificate.
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Animals and other organisms

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Laboratory animals
All mice used in the current study are Mus Musculus. We used equal numbers of male and female animals throughout the study. Pik3caH1047R/H1047R (donated by Wayne A. Phillips31-33), K14-H2B-PAmCherry (donated by Valentina Greco41), and R26mT/mG and R26yfp/yfp Cre-reporter mice (Jackson Laboratories) were on the C57BL/6 or C57BL/6-Tyrc-2J/J background. Animals age from E18.5-12 month, all comparisons are done using animals of the same age as indicated in the manuscript.
Mice were housed and cared for in an AAALAC-accredited facility at Fred Hutchinson Cancer Research Center, and all animal experiments were conducted in accordance with Fred Hutchinson Cancer Research Center IACUC-approved protocols, project license number 50814.

Wild animals
No wild animal was used in this study.

Field-collected samples
No wild field-collected sample was used in this study.

Flow Cytometry

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

Methodology

Sample preparation
Isolation of epidermal cells was described previously (Beronja et al., 2013).

Instrument
BD LSR2, BD Aria2

Software
FlowJo 10

Cell population abundance
~1X10^6 cells were processed for each EdU/BrdU assay, ~2X10^6 cells were collected for each screen replicate, ~2X10^5 cells were collected for Pik3ca H1047R genotyping.

Gating strategy
First gate: FSC-A vs SSC-A
Second gate: SSC-W vs SSC-H
Third gate: FSC-A vs DAPI
Fourth gate: FSC-A vs GFP
Fifth gates: a6 Itg vs EpCAM (renewal screen); EdU vs EpCAM (proliferation screen); EdU vs BrdU (EdU-BrdU assay).
Gating FACS plots are provided in supplementary Fig 3 and 5.

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