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Authors

Kandyba, Eve
Jabouille, Arnaud
Calvet, Ferriol
[et al.](#)

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Chemically induced skin tumors arise from long-lived stem cells of the upper hair follicle

Eve Kandyba¹, Arnaud Jabouille¹, Ferriol Calvet², Yun Rose Li^{1,3,4}, Andrea Curtabbi¹, Diana Cristea¹, Joyce Shin¹, Reyno Delrosario¹, Jonathan Anzules³, Di Wu¹, David Quigley¹, Mark Taylor¹, Camila Zanette⁵, Fang Yin Lo^{5,6}, Jacob Higgins⁵, Jesse Salk⁵, Nuria Lopez-Bigas^{2,7,8,9}, Allan Balmain^{1*}

¹Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA. ²Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain. ³Department of Radiation Oncology and Cancer Genetics and Epigenetics, City of Hope Comprehensive Cancer Center, Duarte, CA, USA. ⁴Division of Bioinnovation and Genome Sciences, Translational Genomics Research Institute, Phoenix, AZ, USA. ⁵TwinStrand Biosciences, Seattle, WA, USA. ⁶Generate Biomedicines, Somerville, MA, USA. ⁷Centro de Investigación Biomédica en Red en Cáncer (CIBERONC), Instituto de Salud Carlos III, Madrid, Spain. ⁸Department of Medicine and Life Sciences, Universitat Pompeu Fabra, Barcelona, Spain. ⁹Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain.

*Corresponding author. Email: Allan.Balmain@ucsf.edu

The identification of the cancer cell of origin is a fundamental question in cancer biology. We used fluorescent lineage tracing of independent mouse skin stem cell populations, single cell transcriptomics, and Duplex sequencing, to identify the origin of chemically induced skin tumors. Tumors arose predominantly from *Lgr6*+ and / or *Lrig1*+ stem cells of the upper hair follicle, but only very rarely from the *Lgr5*+ and *Krt19*+ hair follicle bulge. *Lgr6*+ stem cells initiated by dimethylbenzanthracene responded to tumor promoter treatment resulting in clonal expansion of initiated cells carrying the canonical *Hras* Q61L mutation. Spontaneous mutations in *Kras* also clonally expanded, but did not generate tumors unless the *Hras* gene was deleted, thus revealing a competitive interaction between *Hras* and *Kras* pathways that influences clonal selection.

Mouse dorsal skin is composed of discrete morphological compartments including hair follicles (HF), sebaceous glands (SG), and interfollicular epidermis (IFE), each associated with distinct populations of stem and progenitor cells (1, 2). Genetically engineered mouse models have been used to demonstrate that tumors can arise from multiple different skin cell types, including those in the HF bulge, IFE, and even from differentiating cells (3–11). These genetic models however do not address the question of which cells give rise to tumors induced by environmental mutagens and tumor-promoting factors, both of which are important contributors to cancer risk in mouse as well as human populations (12, 13). Initiation of carcinogenesis by mutagen treatment gives rise to rare, mutated cells which persist in normal tissue for long periods but remain responsive to tumor-promoting factors (14–16). The relevance of these models to human cancer etiology has been highlighted by several recent studies showing that histologically normal human tissues harbor many cells carrying oncogenic mutations (17–24). These cells may lie dormant until they are exposed to exogenous or endogenous factors that stimulate early neoplastic growth. In agreement with this possibility, sequencing studies have shown that the majority of known or suspected human carcinogens lack activity as mutagens and may function as promoters of cells with pre-existing mutations in cancer driver genes (25–27). The importance of environmental promoting factors in human cancer etiology has also been emphasized by a worldwide analysis of how air pollution causes human lung cancer,

concluding that the most likely mechanism is tumor promotion rather than mutation induction (28). For these reasons, we sought to carry out an unbiased analysis of the locations of the cells in which the first initiating mutations arise, their persistence in normal skin, and their potential to respond to tumor promoters after periods ranging from two weeks to one year after initiation.

In mouse models that are dependent on exposure to mutagens and tumor-promoting factors, such as the commonly used dimethylbenzanthracene / 12-O-tetradecanoylphorbol-13-acetate (DMBA / TPA) mouse skin model, cells capable of acting as the cell of origin (COO) should satisfy several stringent criteria: 1) they must be capable of enzymatically metabolizing DMBA to its carcinogenic form to induce the signature *Hras* Q61L driver mutation (16); 2) they should acquire mutations after exposure to different mutagens which do not require metabolic activation, such as methyl-nitrosourea (MNU); 3) the target cells must be long-lived cells carrying *Ras* mutations that persist for up to one year (29, 30), but still respond to tumor promotion to generate benign papillomas that subsequently progress to malignancy.

Results

Chemical initiation predominantly takes place in upper HF SCs in vivo

We identified skin stem cells (SCs) capable of becoming initiated and giving rise to chemically induced tumors using the strategy outlined in Fig. 1A. Skin SC populations were

permanently labeled with a fluorescent reporter using mice with skin SC-specific promoters to drive tamoxifen (TAM)-inducible Cre recombinase activity. At 8 weeks of age, topical TAM was applied to activate Cre and induce irreversible tdTomato expression. One week later, mice were initiated with the mutagen, DMBA, and subsequently promoted with 20 weeks of TPA treatment to generate benign papillomas and malignant carcinomas (Fig. 1A). Lineage involvement of each fluorescently labeled (and potentially initiated) skin SC population was assessed by examining tdTomato+ expression within the resulting epithelial tissue, with fully tdTomato+ (red) labeled tumors indicative of a SC-derived, monoclonal COO (31).

Mice with SC-driven promoters for *Krt19*+ (bulge), *Lgr5*+ (bulge), *Lgr6*+ (isthmus, IFE, SG), and *Lrig1* (32) (infundibulum) were used to label each SC population in vivo (Fig. 1B). Fluorescent tdTomato+ SC populations were readily observed after the initial topical TAM treatment by flow cytometry (fig. S1A) and in skin cryosections (fig. S1, B and C) with *Krt19*+ and *Lgr5*+ tdTomato+ labeled cells localized to the lower HF bulge region (Fig. 1C and fig. S1C), *Lgr6*+ tdTomato+ labeled cells observed in the mid HF isthmus and overlying basal IFE (fig. S1, C and D), and *Lrig1*+ derived cells found mainly in the upper infundibulum (Fig. 1C and fig. S1C) and very occasionally in the IFE directly above the labeled infundibulum (fig. S1E). Following DMBA / TPA mediated tumorigenesis, papillomas presented as either predominantly uncolored, tdTomato- papillomas of unknown origin or, fully labeled with red fluorescence, indicative of a monoclonal SC origin (Fig. 1D). In fully labeled papillomas, the entire tumor epithelium displayed tdTomato+ fluorescence (Fig. 1D, lower left panel), however, the bulk epithelium of papillomas of unknown origin was primarily composed of unlabeled, tdTomato- cells, often with an overlying layer of tdTomato+ epithelial cells (Fig. 1D, lower right panel). These tdTomato+ cell contributions resembled previously reported epithelial “streak” cell populations (31), originating from the base and continuing radially up the sides of the tumor (Fig. 1D, right panel, and fig. S2A). These streaks most probably arose from incorporation of surrounding normal cells, as identified from previous sequence analysis of this recruited epithelium which showed a lack of the canonical *Hras* Q61L driver mutation in these cells (31). Immunofluorescence staining determined that the SC-derived, tdTomato+ streak cells displayed typical basal, KRT14 (fig. S2B), and suprabasal KRT10 (fig. S2C) differentiation status, however, the underlying tdTomato- bulk papilloma epithelium maintained KRT14 expression but displayed mosaic suprabasal KRT10 staining and abundant suprabasal KRT13 staining (fig. S2D), reflecting deviation toward a more mucosal differentiation lineage (33).

Comparison of the proportion of fully tdTomato+ papillomas per mouse observed across the different SC-specific

lineage tracing experiments revealed that the vast majority of tdTomato+ papillomas were generated by SCs of the upper HF which expressed either *Lgr6* (~57% tdTomato+ papillomas / mouse) and / or *Lrig1* (~85% tdTomato+ papillomas / mouse) (Fig. 1E). Notably, the HF bulge SC compartment very rarely gave rise to skin tumors, with only ~5% of papillomas found to originate from *Lgr5*+ SCs and none from *Krt19*+ bulge SCs (Fig. 1, E and F). Further, we observed that skin SC-derived, tdTomato+ benign tumors which eventually progressed to carcinomas (fig. S3, A and B) predominantly arose from *Lgr6* or *Lrig1* lineage-traced papillomas (fig. S3C). SCs expressing either *Lgr6* (34) or *Lrig1* (35) have been linked to wound healing responses, suggesting that both SC populations may respond to tumor promoters and could give rise to early lesions after DMBA / TPA treatment. However, based on the proximity of expression of these two markers within the upper HF, it is not presently clear whether they act independently, or are co-expressed in the same initiated SC population. In order to comprehensively answer this question, further studies would be needed using animals carrying independent lineage tracing markers for both genes. Therefore, for the present study, we focused mainly on functional and mechanistic analysis of *Lgr6*+ SCs as a major COO of chemically-induced skin tumors.

***Lgr6* knockdown perturbs skin tumor development**

We previously described the generation of *Lgr6* knockdown (KD) mice, which carry an inducible *Lgr6* shRNA construct inserted at the *Rosa26* locus (36). Eight week old *Lgr6* KD mice and controls (*Lgr6* WT) were treated with doxycycline (DOX) in water transiently for 10 days, which resulted in KD of *Lgr6* expression in the dorsal skin in vivo (36) prior to beginning DMBA-TPA treatment (Fig. 2A). Papilloma development was markedly reduced in *Lgr6* KD mice compared to control mice, suggesting that *Lgr6*+ cells are required for initiation of skin tumors (Fig. 2B). Expression of KRT13 in benign skin tumors has been associated with a higher risk for malignant conversion (37) and we observed KRT13 expression in the suprabasal layers of both WT (fig. S4A) and *Lgr6* KD tumor epithelium (fig. S4B). KRT13 and Ki67 co-immunofluorescence of WT (fig. S4C) and *Lgr6* KD papillomas (fig. S4D) detected fewer KRT13 positive cells in *Lgr6* KD tumors (fig. S4E) but comparable numbers of proliferating, Ki67+ cells (fig. S4F). A notable decrease was observed in carcinoma number in *Lgr6* KD mice compared to controls (Fig. 2C), and longer carcinoma-free survival (Fig. 2D), identifying *Lgr6*+ SCs as functional targets for initiation and promotion of skin carcinogenesis in vivo.

To investigate the expression of *Lgr6* and its carcinoma metagene (38) in human squamous carcinomas, we performed analysis of human single cell RNA-seq (scRNA-seq) datasets (fig. S5, A to I) from head and neck squamous

carcinomas (fig. S5, A to C) (39) and human cutaneous SCCs (fig. S5, F to I) (40). The gene set in the mouse *Lgr6* carcinoma network metagene (fig. S5E) (38) was expressed at the single cell level in cutaneous squamous carcinomas (fig. S5G), where it overlapped with expression of a population of Tumor-specific keratinocytes (Tsk) identified from scRNA-seq analysis of the human normal and tumor keratinocytes (fig. S5I).

Skin tumors originate from Lgr6+ SCs but not their direct progeny

To address the possibility that some of the tdTomato+ papillomas could have arisen directly from the fluorescently labeled progeny cells rather than from the parental *Lgr6*+ SCs, we initiated the skin with DMBA only two days after TAM labeling, to minimize the time during which the labeled *Lgr6*+ SCs could generate progeny that exit the SC niche (fig. S6A). Flow cytometry analysis revealed that seven days after TAM treatment, ~20% of dorsal skin cells (representing *Lgr6*-derived progeny) were present, whereas after only two days, less than 1% of *Lgr6*-derived tdTomato+ progeny were detectable in the skin (fig. S6B). Nevertheless, the total number of papillomas generated (fig. S6C) and the average percentage of fully labeled tdTomato+ papillomas per mouse after chronic TPA promotion in both lineage tracing approaches were comparable (~57% tdTomato+ papillomas per mouse using the regular approach and ~59% tdTomato+ papillomas per mouse in the two day approach (fig. S6, D and E). These data support the conclusion that *Lgr6*+ SCs, and not their immediate progeny, are the most likely target cells of DMBA initiation in vivo.

Long-lived, initiated Lgr6+ SCs give rise to skin tumors

Initiated cells can persist in normal mouse skin without generating skin tumors (29, 30, 41). We sought to determine whether the DMBA-initiated *Lgr6*+ SCs could persist long term in dorsal skin and, upon delayed exposure to TPA promotion, still give rise to skin tumors in aged mice. We topically exposed the *Lgr6*GFP / tdTomato reporter mice to DMBA at eight weeks old and delayed six months before administering TAM to label aged and, potentially, initiated *Lgr6*+ SCs in the skin (Fig. 3A). After chronic TPA treatment, both uncolored and fully tdTomato+ papillomas were observed, with almost half (48.6%) of the papillomas fully tdTomato+ labeled, indicative of a *Lgr6*-derived COO (Fig. 3B). The similarity in the proportions of tdTomato+ papillomas arising from *Lgr6*+ SCs that were exposed to promoter treatment either one week or six months after initiation (57% vs 48%, respectively) demonstrated that the majority of initiated *Lgr6*+ SCs persist in a dormant state (42). These cells do not give rise to tumors, but can do so when exposed to a tumor promoter. Mutation analysis of *Lgr6*-derived, tdTomato+

papillomas from regular and delayed DMBA / TPA carcinogenesis approaches revealed that the DMBA-signature *Hras* Q61L mutation was predominant in papillomas generated from both approaches (Fig. 3C).

A prediction from these findings was that cells carrying the genome wide mutations induced by DMBA in mouse skin, including the potent *Hras* Q61L mutation, could persist over long periods of time without being removed by the immune system or other cell death mechanisms. To test this, we used error-corrected Duplex sequencing (Duplex-seq) of genomic DNA from viable mouse skin cells (Fig. 3D) and FACS-purified *Lgr6*GFP+ SCs (Fig. 3E) and demonstrated that a single DMBA treatment induced the characteristic A > T / T > A trinucleotide mutational signature (43, 44) within two weeks of exposure (Fig. 3, D and E, left panels). This signature was maintained for up to one year after DMBA treatment (Fig. 3, D and E, right panels), both in total epithelial DNA (Fig. 3D) and in *Lgr6*GFP+ SCs (Fig. 3E) which still showed the presence of the DMBA-associated *Hras* Q61L mutation (fig. S7). The DMBA signature mutations were not detected in unexposed control DNA samples but, normal aging led to an increase in C > T / T > C mutations related to the “clock” signature SBS1 in both control and DMBA-treated skin (45). Finally, histological analysis of the early stages of papilloma development further supported the origins of papillomas from the upper HF (fig. S8, A to D), rather than from the classical HF bulge SC region (46). After initiation and short term TPA promotion of *Lgr6*GFP / tdTomato reporter mice (Fig. 3F) we observed expansion of *Lgr6*+ SCs with the *Hras* Q61L mutation and demonstrated with lineage tracing that these *Lgr6*+ SCs generate localized regions of tdTomato+ hyperplasia in the upper HFs directly beneath developing lesions after six weeks of promotion (Fig. 3G). Notably, classical histological studies of sequential stages of early papilloma development in this model also concluded that lesions arise from the upper HF rather than the bulge (47, 48). Collectively, our targeted lineage tracing and mutational signature analysis studies lend strong support to the identification of long-lived *Lgr6*+ SCs as a major COO of chemically-induced skin tumors, fulfilling the major criteria for cancer initiation originally proposed by Berenblum and Shubik (30).

HF Lgr6+ HF SCs display enriched expression of DMBA metabolism enzymes

Lgr6+ SC populations are found in the upper region of the HF as well as in the IFE. To address the question of which *Lgr6*+ population contained the initiated cells, we used flow cytometry to purify viable cells from unexposed, young dorsal mouse skin (10 weeks old) and performed scRNA-seq to help resolve transcriptional features responsible for the underlying susceptibility of *Lgr6*+ SCs to chemical initiation. A single cell transcriptional atlas of normal mouse skin was

constructed by exploiting common and unique gene expression profiles, which enabled the identification of 16 distinct skin cell sub-populations (or clusters) (Fig. 4A and fig. S9). By exploiting the expression of known skin differentiation (fig. S10A) and SC markers (fig. S10B), we could then determine the clusters designated to different SC populations to further probe the transcriptional characteristics which could influence chemical initiation events. We examined *Hras* expression within the normal skin cells (fig. S10C) and reanalyzed all *Hras*⁺ cells to determine the skin SC populations which would potentially express mutated *Hras* Q61L protein following DMBA initiation in vivo (Fig. 4, B and C, and figs. S10, D and E, and S11). This analysis showed that *Cd34*⁺ and *Lgr5*⁺ bulge SCs, both IFE and HF *Lgr6*⁺ SCs and also *Lrig1*⁺ SCs expressed *Hras* in vivo.

We then explored the underlying mutagenic effect of DMBA, which requires a two-step mechanism involving the action of two enzymes, EPHX1 and CYP1B1, which metabolize DMBA to a carcinogenic dihydrodiol-epoxide metabolite (49). Mis-repair of the resulting adducts with adenosine residues in DNA (49, 50) induces the signature *Hras* Q61L mutation (16). We therefore examined the expression of *Ephx1* and *Cyp1b1* in SCs of the HF bulge (*Lgr5*⁺ and *Lgr5*⁻), isthmus (*Lgr6*⁺), infundibulum (*Lrig1*⁺) and basal IFE (*Lgr6*⁺) using our single cell atlas (Fig. 4), with additional independent qPCR validation of FACS-purified skin SCs populations. Distinct SC populations were purified by exploiting GFP reporter expression (*Lgr5*GFP and *Lgr6*GFP) and antibody-based FACS sorting to specifically enrich each SC population from single cell suspensions prepared from normal and DMBA-exposed mouse skin (fig. S12A). GFP reporter expression was used to enrich for *Lgr5*GFP⁺ (from *Lgr5*GFPCreER mice) and *Lgr6*GFP⁺ (from *Lgr6*GFPCreER mice) cells, and bulge SCs (hfSCs, isolated from *Lgr5*GFPCreER mice) were purified and collected by the absence of *Lgr5*GFP expression and co-expression of α 6-integrin and CD34 antibody staining (fig. S12B). The sub-fractionated *Lgr6*GFP⁺ HF and IFE SC populations were collected by using SCA1 antibody staining, where *Lgr6*GFP⁺SCA1⁻ cells reflected *Lgr6*⁺ cells of the HF isthmus region and *Lgr6*GFP⁺SCA1⁺ cells represented *Lgr6*⁺ cells of the overlying IFE (fig. S12C). Bulge *Lgr5*⁺ hfSCs and both IFE and HF *Lgr6*⁺ SC populations expressed *Ephx1* in vivo (Fig. 4D). Comparable basal expression of *Ephx1* was observed in HF bulge cells that were either negative (*Lgr5*GFP⁻ hfSCs) or positive (*Lgr5*GFP⁺ SCs) for *Lgr5* (Fig. 4E), and this did not change following DMBA exposure.

Analysis of the whole HF *Lgr6*⁺ SC population (comprising both IFE and HF *Lgr6*⁺ cells) and enriched IFE and HF *Lgr6*⁺ SC populations of non-exposed skin revealed similar low expression of *Ephx1*, which did not increase following DMBA treatment (Fig. 4F). Although this analysis of *Ephx1* expression did not reveal any differences that could account

for the susceptibility of *Lgr6*⁺ cells to DMBA initiation, a different pattern was seen for *Cyp1b1*, which is essential for DMBA-induced mutations (49, 51). *Cyp1b1* expression was largely absent from the HF bulge and basal IFE, but it was expressed in abundance by *Hras*⁺ isthmus HF *Lgr6*⁺ SCs (Fig. 4G). Furthermore, qPCR analysis revealed that non-exposed *Lgr5*GFP⁻ hfSCs showed moderate *Cyp1b1* mRNA expression, unlike *Lgr5*GFP⁺ SCs, which displayed lower expression more comparable with the matched global skin cell population (Fig. 4H). HF *Lgr6*GFP⁺ SCs showed elevated *Cyp1b1* expression following DMBA exposure (Fig. 4I), whereas *Lgr6*GFP⁺ IFE cells displayed very low *Cyp1b1* mRNA levels in vivo. Analysis of CYP1B1 protein expression in FACS purified skin SC populations revealed that cell populations with the highest percentage of CYP1B1⁺ cells following DMBA exposure were derived from the upper HF region, namely *Lgr6*GFP⁺ HF and *Lrig1*⁺ SCs (Fig. 4J and fig. S12D). Mechanistically, these data suggest a model where upper HF SCs (rather than bulge SCs or IFE *Lgr6*⁺ SCs) are the primary target cells for DMBA initiation due to elevated levels of DMBA-metabolism enzymes (Fig. 4K).

Our conclusions differ from those of two other studies that investigated the nature of the initiation event and COO in the same DMBA/TPA carcinogenesis model. First, Li and colleagues demonstrated that progeny of KRT15⁺ HF bulge could contribute to papilloma development, suggesting the involvement of bulge SCs in tumor initiation (46). However, the majority of the KRT15⁺ progeny cells did not carry the signature *Hras* Q61L mutation, in spite of the fact that papillomas are clonal in origin (31). These bulge progeny cells may therefore correspond to recruited epithelial “streak” cells that were previously described (31), rather than acting as COO for papillomas. Secondly, although Langerhans cells (LCs) have been implicated as the site of DMBA metabolism in mouse skin (52, 53) and therefore essential for carcinogenesis, we found that LCs (fig. S13A) did not express either of the enzymes, *Ephx1* and *Cyp1b1*, required to metabolize DMBA to a carcinogenic form (fig. S13, B and C), however, isthmus *Lgr6*⁺ SCs expressed both enzymes required for DMBA metabolism (fig. S14, A to D). In support of this interpretation, others (54) have failed to replicate the results of the Modi study, attributing the discrepancy to the use of mice with constitutive rather than inducible depletion of LCs in skin.

***Lgr6*⁺ SCs are targets for tumor initiation by the alkylating agent, MNU**

We next tested whether the susceptibility of *Lgr6*⁺ SCs to initiation is specific to the carcinogen DMBA or extends to other mutagens that do not require metabolic activation. MNU and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) are potent mutagens that do not require enzymatic metabolism to elicit carcinogenic effects, and act as initiators of

mouse skin tumors (55). We carried out an analogous experiment to that described above for DMBA, but using MNU to initiate skin carcinogenesis in *Lgr6*GFP-tdTomato reporter mice (fig. S15A). All MNU-initiated, TPA-promoted mice generated tdTomato+ labeled papillomas (fig. S15B), with an average of ~60% labeled papillomas per mouse (fig. S15, C and D), similar to results described above for initiation by DMBA (Fig. 1E). MNU-initiated and TPA promoted, *Lgr6*+ SCs and their direct lineage traced tdTomato+ progeny also harbored both MNU-associated (G > A) *Kras* G12D and *Hras* G12E mutations (fig. S15E), which are the predominant driver mutations selected by tumors generated using this approach. Together, these data suggest that tumor initiating capacity is an intrinsic feature of *Lgr6*+ SCs and is not simply due to their high capacity to metabolize DMBA.

Clonal competition and selection in response to exogenous factors or endogenous processes.

Although DMBA induces thousands of mutations genome-wide, over 90% of papillomas carry the same initiating *Hras* Q61L mutation (16, 43). We tested the hypothesis that treatment with the strong skin tumor promoter, TPA, specifically selects this mutation from among a range of possible oncogenic mutations induced by DMBA (or those that may have arisen spontaneously). To investigate this question, we used Duplex-seq to detect spontaneously arising mutations in normal, untreated mouse skin and any new mutations induced by a single treatment with DMBA and examined the changes in clonal architecture induced by short term TPA exposure (Fig. 5A). We focused on the presence of hotspot mutations at codons 12, 13, and 61 of the three *Ras* gene family members (*Hras*, *Kras*, and *Nras*) in normal skin initiated with DMBA, with or without short term exposure to TPA. In control, unexposed skin samples (Fig. 5B) we observed infrequent, single mutant cells with a range of *Ras* hotspot mutations, predominantly in the *Kras* gene. Overall, spontaneous mutations in the *Hras* gene were rare – only a single skin sample from the ten control animals examined harbored a *Hras* Q61L mutation. Following DMBA initiation alone (Fig. 5C), we observed a considerable increase in the number of singlet *Ras* Q61 mutations, including Q61L and Q61H, which were found in all 3 *Ras* gene family members. Treatment of DMBA-initiated skin with TPA for 4 weeks resulted in a dramatic increase in the abundance of *Ras* Q61 mutations, most notably the *Hras* Q61L allele (Fig. 5D), which is the common driver mutation in tumors from this model.

In addition to the classical tumor promoter, TPA, a range of other molecules have been tested for activity as tumor promoters, although most are much less potent than TPA (55, 56). We next asked if weaker tumor promoter activity would be reflected in changes in mutant allele frequency using this short-term assay. A commonly used alternative promoter is

mezeirein, a tumor-promoting agent that has been classified as a weak or “second stage” promoter (55, 57). We first tested the possible selection of different *Ras* mutations by mezeirein using a long-term, 20-week promotion assay by comparing tumors generated from DMBA-mezeirein or DMBA-TPA exposure (fig. S16, A to D). As expected, after 20 weeks of treatment, fewer papillomas were observed in DMBA-initiated skin exposed to mezeirein promotion compared with TPA treatment (fig. S16B). Sequence analysis showed that the majority of papillomas generated by either TPA or mezeirein had activating mutations at *Hras* Q61L, although around 30% of the mezeirein-promoted papillomas had no detectable mutations in any of the major *Ras* target genes (fig. S16C). We next tested the utility of our short-term topical promotion assay (fig. S17A) to evaluate the extent of clonal expansion of *Ras* mutant cells in DMBA-initiated (fig. S17B) or untreated skin promoted by mezeirein (fig. S17C). We found that with DMBA initiation and mezeirein promotion, *Hras* Q61L clones were preferentially expanded (fig. S17B) but with a considerably lower response than observed after TPA exposure (Fig. 5D). In non-initiated skin, rare spontaneous singlet *Hras* mutations at codons 12 and 13 were observed with mezeirein promotion alone (fig. S17C), both findings in agreement with the weak promoting effect of mezeirein in generating skin tumors (fig. S16B). We next performed a wider survey of common hotspot mutations in other known cancer target genes in addition to *H-*, *K-*, and *Nras*. Mutations in *Pik3ca* at codon 1047 and *Trp53* at codons 133, 176, and 190 are detected in human and mouse tumors (43) and mutations in both genes were increased by DMBA treatment (fig. S17D). After DMBA plus TPA promotion, we observed an unexpected reduction in the presence of *Pik3ca* hotspot mutations, but a similar reduction was not seen after mezeirein treatment. In contrast, the DMBA-induced *Trp53* hotspot mutations increased in response to both TPA and mezeirein exposure. These data demonstrate the high complexity of changes in clonal architecture that are induced by exogenous exposures to different promoting factors.

Clonal competition between cells with spontaneous *Kras* and chemically-induced *Hras* mutations

An observation that arose from repeat analysis of rare mutant clones in untreated young mice was that while spontaneous mutations in the *Hras* gene were infrequent (present in only 1 / 10 samples), *Kras* G13R (GGC > CGC) transversions or *Kras* G12D (GGT > GAT) transitions were detected in multiple samples and at a frequency suggesting they are under positive selection in comparison to random mutational patterns (fig. S17D). In studies of control mice treated with TPA alone, in the absence of any initiating mutagen, we found that cells carrying either *Kras* G12D or *Kras* G13R mutations clearly respond to tumor promotion (Fig. 6A), although clones carrying

these *Kras* mutations almost never progress to form papillomas or carcinomas (16). In order to better understand underlying cell competition dynamics between spontaneous and DMBA-induced *Ras* mutant clones, we carried out a comprehensive analysis of the relative cell numbers and clonal expansion of different *Ras* hotspot mutations after 4 weeks or 8 weeks of treatment with TPA, and after 4 weeks of withdrawal from TPA (Fig. 6B: non-initiated, left panel, and DMBA-initiated skin, right panel). The frequency of mutations at codon 61 of all 3 *Ras* genes showed expected patterns of clonal expansion, with the highest frequency observed for *Hras* Q61L mutations (Fig. 6B). In DMBA-initiated samples, *Hras* Q61L mutation frequency was increased after 4 weeks of TPA and continued to increase with prolonged TPA exposure (8 weeks). After withdrawal of TPA promotion for 4 weeks, *Hras* Q61L mutation frequency remained relatively stable, demonstrating the persistence of these mutant cells within the skin epithelium during the withdrawal period (Fig. 6, C and D).

In non-initiated mice, the number of *Kras* G12D mutations increased after 4 weeks of TPA treatment, but these mutant cells did not persist after further promoter exposure (Fig. 6B). In contrast, the frequency of *Kras* G13R mutations increased after short term TPA promotion from around 7 mutant cells per million cells to approximately 60 mutant cells per million, with mutant cell outgrowth continuing to around 350 cells per million after 8 weeks of TPA exposure (Fig. 6B, left panel, and D and E, left panel). Cessation of promoter exposure after 4 weeks induced an even larger expansion to almost 900 mutant *Kras* G13R cells per million, indicating that continued exposure to TPA was not necessary for clonal expansion of these cells. A similar trend was seen following DMBA initiation (Fig. 6, D and E, right panel): while *Kras* G13R mutation frequency increased only minimally after the combination of induction of *Hras* mutations by DMBA and 4 weeks TPA (approximately 18 mutant cells per million), withdrawal of TPA for an additional 4 weeks, or continued TPA during this time, led to a substantial increase in *Kras* G13R mutant cells (over 1800 cells per million after withdrawal and > 2800 cells per million after DMBA plus 8 weeks of TPA). These levels are comparable to those seen for the *Hras* Q61L mutations after DMBA plus 8 weeks of TPA exposure (~2800 mutant cells per million). These findings raised the question of why, in wild type FVB mice, no *Kras* G13R mutant papillomas arise with or without DMBA and extended TPA exposure. Subsequent experiments involving the withdrawal of TPA for 4 weeks (after the initial DMBA and 8 weeks TPA) showed that while TPA withdrawal after 8 weeks of promotion had little effect on the frequency of *Hras* Q61L mutations, there was a strong decrease in cells carrying the *Kras* G13R mutation (from > 2800 mutant cells per million to < 125 mutant cells per million) (Fig. 6, B, D, and E), indicating that these

Kras mutant cells are removed from the skin epithelium by an as yet unknown mechanism.

We hypothesized that cells with chemically-induced, *Hras* driver mutations may interact in some way with an alternative cell population that carries the spontaneous *Kras* G13R mutations, possibly located in the lower follicle region which undergoes cyclical periods of growth and degeneration during hair cycling. To explore this possibility of interaction between *Hras* and *Kras* mutant cells, we investigated the selection of *Ras* driver mutations in tumors generated from DMBA-initiation and TPA promotion from wild type FVB mice (Fig. 6F and fig. S18) and animals in which the *Hras* gene has been deleted (Fig. 6G). While tumors initiated with DMBA and varying exposures to TPA promotion in wild type mice almost always carried exactly the same DMBA A > T signature mutation in *Hras* (*Hras* Q61L; Fig. 6F), benign skin papillomas generated from *Hras* knockout (KO) mouse skin following DMBA plus TPA promotion rarely carried the equivalent *Kras* Q61L mutation (13.6%, 3 / 22 tumors), despite an identical target sequence at codon 61 in both *Hras* and *Kras* (Fig. 6G) (43). Instead, the most common *Ras* driver mutations selected in *Hras* KO papillomas were either *Kras* Q61H (45.5%, 10 / 22 tumors) or *Kras* G13R (seen in 31.8%, 7 / 22 tumors). These findings are compatible with analysis of the original *Hras* knock-out mice by Ise *et al.*, who demonstrated a range of *Kras* mutations in skin tumors, with approximately 30% of tumors harboring *Kras* G13R mutations (58). Furthermore, analysis of *Hras* KO benign papilloma progression to carcinoma demonstrated that 80% (4 / 5 carcinomas) of malignant tumors carried the *Kras* G13R mutation (59). These data suggest a model whereby spontaneous *Kras* mutant cell number may increase in response to tissue damage caused by TPA exposure, and these mutant cells are capable only of transient clonal outgrowth but persist in the absence of competition from cells carrying a mutation in the *Hras* gene.

Discussion

Tumors induced by exposure to exogenous mutagens and tumor promoters, in contrast to those induced by germline genetic alterations, display a high mutational burden and thus reflect the complex genomic landscapes of human cancer (43, 60). The cells of tumors induced by exogenous carcinogens, which arise clonally from single initiated cells (31), may differ from those targeted in the majority of genetic models, as they must satisfy several stringent criteria including the ability to permit metabolic activation of carcinogens, and to respond to tumor promoters or wound healing factors. Here, we identify *Lgr6*⁺ and *Lrig*⁺ cells in the upper HF, rather than alternative cell populations in the lower bulge or the IFE, as major cell targets for initiation and promotion by chemical carcinogens and tumor promoters. These cells express the key

DMBA-metabolizing enzymes, *Cyp1b1* and *Ephx1*, whereas *Lgr6*⁺ cells residing in the IFE show minimal *Cyp1b1* expression. Finally, LRIG1⁺ SCs in the upper follicle express high levels of CYP1B1 and generate a high proportion of labeled tdTomato⁺ papillomas, but do not reside in the IFE. Taken together, these results provide evidence that the majority of papillomas arise in the upper follicle region rather than the IFE or the bulge region, in agreement with early histological analyses by Binder and colleagues (47, 48).

Our analysis was focused primarily on *Lgr6*⁺ cells, as these SCs are highly clonogenic in skin tumors (36), necessary for efficient wound healing responses (34), and their inhibition results in a strong decrease in efficiency of skin tumor initiation. Moreover, extensive lineage tracing and scRNA-seq analysis showed that *Lgr6*⁺ cells in papillomas and squamous carcinomas generate progeny that express a wide range of alternative SC markers in response to the stress of early neoplastic growth and are thus able to give rise to a high plasticity state that is associated with tumor development and responses to therapy (38). These results also have implications for cancer initiation in other tissues and in human patients, as *Lgr6* has been reported to play an oncogenic role in cancers of the mouse mammary gland (61), human breast (62, 63), human lung (64, 65), esophagus (66), and ovary (67).

Chemically-initiated, *Lgr6*⁺ SCs carrying strong *Hras* Q61L mutations can remain dormant in mouse dorsal skin for up to one year but still give rise to papillomas after exposure to a tumor promoter (30, 41). These observations confirm and extend studies showing that chemically-initiated cells (30, 68) or cells expressing strong *Hras* or *Kras* viral oncogenes (15) can remain dormant in skin for many months but generate tumors upon delayed challenge with promoting agents. The nature of these dormant mutant cells that persist over time remains unclear. It is possible that the original mutated cell may lie dormant for long periods, or that quiescent progeny cells that no longer express *Lgr6* can be reactivated by promoter treatment to undergo clonal selection. Collectively, our data emphasize the role of SCs in the upper HF, rather than classical bulge SCs or IFE cells, as the primary targets for initiation and promotion of skin carcinogenesis.

Our observation that rare, initiated cells expressing mutant *Ras* genes persist and are not removed by cell-cell competition with normal surrounding cells, contrasts with the results obtained using some mouse genetic models that involve activation of *Ras* mutations in different cell populations within the skin, generating extensive fields of mutant cells that compete with their normal neighbors. While some of these elegant genetic models demonstrated that normal cells may outcompete their mutant neighbors, restoring normal tissue architecture (69–72), others showed that the *Ras* mutant cells can be the winners, leading to early stage tumors after wounding or tumor promoter treatment (8–10, 69). The

reasons for these differences are unclear but may be related to the ratio of engineered mutant to normal cells in different models, or to the nature of the gene promoter used to drive oncogenic signaling in distinct cell types.

Our data on the role of spontaneously arising mutations in the *Kras* gene, particularly *Kras* G13R, may help to reconcile different models of clonal competition involving normal epithelial cells and those carrying different oncogenic *Ras* mutations. New mutations induced by DMBA are primarily in codon 61 of the various *Ras* gene family members, and these expand after treatment with a tumor promoter. Upon withdrawal of the promoter, cells with *Hras* Q61L mutations persist and go on to generate tumors carrying this mutation. A very different scenario is seen for cells with *Kras* G13R mutations, which arise spontaneously in normal, uninitiated mouse skin (fig. S19A). At early stages after promoter exposure, or even when promotion is halted, these mutations clonally expand to levels comparable with *Hras* Q61L (fig. S19B). However, while *Hras* Q61L mutations persist and ultimately give rise to papillomas after 8 weeks of TPA exposure, over 95% of *Kras* G13R mutations disappear during the same period and do not generate papillomas (fig. S19B). These data suggest that different cell populations within the same tissue are responding in opposite ways to exogenous chemical treatment. This situation is reversed in *Hras* KO mice, in which mutant cells with a *Kras* G13R mutation survive and generate papillomas (fig. S19C), indicating that the *Hras* pathway plays an inhibitory role in preventing clonal expansion of cells with spontaneous *Kras* G13R mutations.

The mechanisms that generate spontaneous mutations in the *Kras* and other genes remain unclear, as do the processes that underlie differential clonal selection in the presence or absence of *Hras*. The cells that harbor these *Kras* mutations are also unknown, but their transient activation by tissue damage and subsequent disappearance mimics the behavior of lower follicle *Lgr5*⁺ SCs, which respond to wounding and migrate into the IFE, but do not make a permanent contribution to the healed tissue (73). This observation of separate cell clones that expand in response to distinct selective pressures may also reflect the mechanisms underlying the repopulation of damaged lung tissue by alternative SC populations in patients undergoing smoking cessation (22). Our data have revealed dynamic competitive interactions between different cell clones that are influenced by exogenous exposures as well as by genetic background effects. An important goal for cancer prevention will be to alter these selective constraints in favor of clones that have little or no probability of malignant progression.

Materials and methods

Mouse husbandry

Animals were housed under standard conditions and treated in accordance with the rules and protocols stipulated by the UCSF Institutional Animal Care and Use Committee (IACUC). All mouse experiments were approved by the University of California San Francisco Laboratory Animal Resource Center. *Lgr5*, *Lgr6* and *Krt19* CreER mice (Jackson Laboratories, USA) and *Lrig1*CreER (32) mice were each crossed with tdTomato reporter mice from Jackson Laboratories with all mice maintained upon the FVB background. The following reporter mouse strains were then used for lineage tracing experiments: *Lgr5*GFP-CreER-tdTomato, *Lgr6*GFP-CreER-tdTomato, *Krt19*CreER-tdTomato, and *Lrig1*CreER-tdTomato. The *Hras* KO strain (58) was also maintained on the FVB background for DMBA/TPA carcinogenesis experiments.

Lgr6 knockdown experiments

Lgr6 knockdown mice were generated in a similar fashion to previously reported doxycycline-inducible knockdown mice, using the *Lgr6* shRNA sequence GCATGACACTGGAAGACAACT. Briefly, recombinant ES cells were injected into blastocysts, and chimeric animals were obtained at the UCSF Cancer Center Transgenic and Targeted Mutagenesis Core Facility (36). Sterile DOX water was prepared by dissolving 2 g of DOX (RPI Research Products International, USA) in 1 L of water containing 50 g of sucrose (EMD Millipore, USA).

Chemical carcinogenesis experiments

For chemical carcinogenesis experiments, 8 – 10 week-old male and female mice were randomly assigned to control or treatment groups. Initiation was carried out using a single dose of DMBA (25 µg per mouse in acetone; Sigma-Aldrich) or MNU (4 mg / ml in acetone, 200 µl dose; Spectrum, USA) applied to shaved dorsal skin – initiation refers to the process whereby a normal cell acquires an irreversible genetic alteration, such as a point mutation, following exposure to a mutagen such as DMBA or MNU. One week later, the mice were given biweekly topical treatments of TPA (200 µl of a 10⁻⁴ M solution in acetone; Sigma-Aldrich) for 20 weeks. The animals were then monitored for papilloma number (and carcinoma progression) over time during the treatment regime and visible skin tumors were collected at endpoint (either immediately after 20 weeks of promoter treatment or following observed carcinoma or lymph node metastasis development according to our IACUC protocol guidelines). For TPA versus mezerein promotion experiments, FVB mice were initiated with DMBA at 8 weeks of age, then two weeks later the mice were topically treated bi-weekly for 4 weeks (short term, for Duplex sequencing analysis) or 20 weeks (long term, for

papilloma development) with vehicle alone (acetone), TPA (as above), or mezerein (4 µg per dose, SC-202707, Santa Cruz Biotechnology). In *Lgr6* knockdown experiments, carcinoma-free survival was defined as the time point - recorded as weeks after the first TPA treatment was administered - at which the first carcinoma was observed on the dorsal skin of a mouse. Promoter cessation experiments involved the complete withdrawal of topical TPA treatment in experimental mice.

In vivo fluorescent lineage tracing (regular 7 day, 2 day, and delay experiments)

To permanently fluorescently label skin SC populations and all subsequent progeny for lineage tracing experiments, one dose of TAM (25 mg / ml in 100% ethanol; 200 µl dose; Sigma-Aldrich) was administered to shaved back skin. Upon sample collection, to preserve *Lgr5*GFP and *Lgr6*GFP reporter expression within the tissue, prior to embedding, excised skin or tumors were fixed in 4% paraformaldehyde (Thermo Scientific) for 2 hours and then placed in 30% sucrose overnight at 4°C before freezing the tissue in OCT (Fisher HealthCare) for cryosectioning. Embedded OCT skin samples were stored at -80°C.

Tissue collection and examination of tdTomato labeling

To quantify tdTomato labeling in the skin, a strip of back skin was collected from 3 independent mice 24 or 48 hours after TAM treatment to allow recombination events to take place in the skin. To quantify tdTomato labeling in tumors, tumors were collected after 20 weeks of TPA treatment or at endpoint. Tissues were harvested and embedded in OCT as described above, and 10 µm sections were collected for analysis. Following fixation, tdTomato+ fluorescence was examined in resected tumors using a Zeiss fluorescent stereoscope. Images were taken to record observations, and the number of distinctly tdTomato labeled tumors and unlabeled tumors (of unknown origin) was recorded. It was also possible to identify tdTomato+ tumors by eye by comparison due to vivid red color presentation (and by comparison with unlabeled tumors) but tdTomato+ positivity was also confirmed by microscopy and results from imaging sections of tumors correlated well with whole-mount observations.

Preparation of single cell keratinocyte suspensions for FACS sorting

Single cell suspensions of disaggregated dorsal back skin were prepared by removing the subcutaneous fat layer and floating the skin in warmed trypsin-EDTA (Gibco Life Technologies) at 37°C for 1 hour with gentle agitation before carefully scraping the epithelial cells into 10% fetal bovine serum (FBS; Biowest) diluted in Ca²⁺ free PBS (HyClone) and filtering through a 40 µm cell filter (Griener Bio-one). The cell

suspensions were then centrifuged at 1200 rpm, resuspended in 10% FBS and kept on ice prior to antibody staining for flow cytometry. FACS sorted cells for gene expression analysis were collected in RNA buffer (Qiagen) and were then centrifuged at 1200 rpm to pellet the cells before being resuspended in 250 μ l Tri-reagent (Invitrogen) and stored at -80°C for subsequent RNA isolation. Cells were stained with PE-conjugated anti-CD49f (integrin- α 6; 1:200, BD Pharmingen), Alexafluor 700-conjugated anti-CD34 (1:50, eBioscience), PE Cy7-conjugated SCA1 (1:100, eBioscience). Viability staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, ThermoFisher) was used to exclude dead cells. Keratinocyte cell populations were enriched and collected using a FACS Aria III (BD Biosciences). *Lgr6*GFP+ IFE cells were defined as GFP+ SCA1+ and *Lgr6*GFP+ HF cells as GFP+ SCA1- cells, respectively (total *Lgr6*GFP+ parental cells were also collected from each sample).

Immunofluorescent staining

Slides with 10 μ m sections were brought to room temperature and incubated in PBS for 5 min. For GFP staining, slides were blocked in 10% donkey serum (Jackson ImmunoResearch) diluted in 0.1% Triton-X100 (Sigma-Aldrich) for 30 min. A GFP antibody (1:3000, ab13970, Abcam) diluted in 10% donkey serum / 0.1% Triton-X100 was incubated on slides overnight at 4°C. The following day, slides were washed three times in PBS, incubated with a goat anti-chicken Alexa Fluor 488 (1:500, ThermoFisher) secondary antibody for 90 min and then washed twice with 0.1% Triton X-100 / Ca²⁺ free PBS. Immunostaining using primary antibodies to KRT14 (1:2000, #905301, Biolegend), LRIG1 (1:200, R&D), KRT13 (1:100, Progen), SCA1 (1:100, eBioscience) and Ki67 (1:1000, ab15580, Abcam) was performed for 2 hours at room temperature, slides were then incubated with an appropriate Alexa Fluor 488 or 594 secondary antibody at 1:500 for 1 hour and washed three times with PBS before counterstaining with DAPI and mounting.

RNA isolation and RT-PCR for Taqman Analysis

RNA was extracted from FACS sorted cells using the Qiagen RNeasy minikit, following manufacturer's instructions, and RNA concentration and quality were determined by Nanodrop spectrophotometry. RNA was reverse transcribed to cDNA using the Superscript III kit (ThermoFisher) according to the manufacturer's instructions. For expression analyses, the following Taqman gene expression assays were used: *Epha1*, *Cyp1b1*, *Gapdh* (ThermoFisher). For each sample, *Gapdh* was used as a reference to calculate delta CT values for genes of interest. For each qPCR experiment, the data presented show the mean and s.e. amongst the biological replicates (n = 3 independent replicates of each SC population for each treatment group).

scRNA-seq experiments

The scRNA-seq dataset was prepared as previously described (38). Briefly, single cell suspensions from three independent female *Lgr6*CreERGFP mice were prepared for FACS analysis. Cell samples were then pooled, FACS-enriched for DAPI- viable cells and the scRNA-seq library was processed by the Gladstone Genomics Core according to the 10x Genomics library preparation protocol before sequencing on a Novaseq S4 sequencer (Illumina). Raw sequencing data were processed as described (74). Sequences from the microfluidic droplet platform were de-multiplexed and aligned to mouse genome build mm10 using CellRanger version 3.1. Aligned sequences were then processed using Seurat version 3.2. Low-quality cells were filtered out from the data set based on a threshold for the number of genes detected (retaining cells with a minimum of 200 unique and maximum of 3000 genes per cell with fewer than 5% mitochondrial transcripts), leaving 3163 cells for further analysis. All genes that were not detected in at least three independent single cells were discarded, leaving 12,768 genes for all remaining analyses. Data were log-transformed [$\log(\text{TPM} + 1)$] for all downstream analyses, most of which were performed using the R software package Seurat. scRNA-seq cells were clustered by first identifying the top 2000 highly variable genes (using selection method vst), scaling and centering the expression of these genes, computing PCA on the scaled expression values. Uniform Manifold Approximation and Projection (UMAP) was used for dimension reduction using the top 15 principal components with the Louvain algorithm for community detection. We annotated clusters as cell types according to the expression of canonical marker genes. Figures including heatmaps, violin plots, and feature plots were created using Seurat. For *LGR6* gene and *Lgr6* mouse carcinoma metagene expression analysis (38) in human scRNA-seq experiments, two previously published datasets were analyzed: (1) A Head and Neck Squamous Cell Carcinoma (HNSCC, with matched normal tissues, GSE181919) dataset (39) comprising samples collected from 23 HNSCC patients. Briefly, the expression matrix was converted into a Seurat object maintaining the original metadata and was then log-normalized (*NormalizeData*, scale factor = 10000), variable features were found with *FindVariableFeatures* (method = "vst", nfeatures = 2000) and dimensionality reduction was performed using standard Seurat workflow (*RunPCA*, *FindNeighbors*, dims = 1:30, *RunTSNE*, dims = 1:30). The top 100 genes of the *Lgr6* carcinoma metagene were used (38) and gene names were converted into human orthologs using the Mouse Genome Informatics (MGI) report (downloaded from https://informatics.jax.org/downloads/reports/HMD_HumanPhenotype.rpt). Metagene expression was calculated using the *AddModuleScore* function; (2) scRNA-seq count matrices and accompanying metadata were imported to Monocle3 for human cutaneous squamous

cell carcinoma (cSCC; GEO GSE144236) and the mouse cSCC dataset (GEO GSE261766). Human cells were preprocessed by PCA (100 components) and embedded with UMAP (cosine metric; min_dist = 0.1, n_neighbors = 15), then clustered with Leiden on the UMAP graph. To minimize inter-patient effects, human manifolds were aligned by patient identity (align_cds) prior to recomputing UMAP and clustering; the aligned UMAP was used for all feature overlays. Metagene scores were computed as integrated expression across curated gene sets and visualized using Monocle3 multi-gene overlays. A human tumor-specific keratinocyte (Tsk) signature defined by the top 100 Tsk-enriched genes identified from Ji and colleagues (40) was similarly scored and projected onto the human cSCC UMAP.

Duplex-seq custom panel design

A hybrid selection panel optimized for Duplex-seq was designed by TwinStrand Biosciences to target 18 genomic hotspot regions and 48 kilobases (kb) of neutral genomic regions not predicted to be involved in positive selection in the mm10 mouse genome assembly. The hotspot genomic regions included the mouse *Trp53* coding sequence and mutation hotspots in *Hras*, *Nras*, *Kras*, and *Pik3ca*. 48 5-prime biotinylated probes (Integrated DNA Technologies, Inc.) were ordered against the hotspot genomic regions. The custom hotspot probes were blended with the TwinStrand Duplex Sequencing Mutagenesis Panel (Mouse-50) v1.0 (TwinStrand Biosciences, Inc.). Hybrid selection probes with predicted off-target effects, predicted secondary structure, and low mappability were removed. Panel quality control was performed with control DNA using the TwinStrand DuplexSeq Mutagenesis Library Preparation Kit and data processing protocols confirmed sequencing requirements for sample processing, analysis, and adequate panel coverage over the targeted regions.

Duplex sequencing library preparation

Duplex-seq libraries were prepared as described previously (75). In brief, 1.5 µg genomic DNA (gDNA) was fragmented to 300 bp enzymatically or with a Covaris ME220 ultrasonicator (if a sample had less than 1.5 µg gDNA available, then all available DNA was fragmented). DNA fragments were end-repaired, A-tailed, and ligated to Duplex-seq adapters. Whole genome libraries were then PCR-amplified with indexed primers. Libraries were hybridized overnight with the custom hybrid selection panel described above. Hybridized fragments were captured with streptavidin magnetic beads, beads were washed three times, and libraries were amplified with P5/P7 primers. To further enrich the targets of interest an additional round of hybridization, capture, washes, and PCR were performed (75). Duplex-seq libraries were sequenced on a NovaSeq 6000 with 2x150 bp paired-end reads.

325 million clusters were targeted for libraries with 1.5 µg gDNA input mass and clusters were scaled down proportionally for libraries with lower input mass.

Duplex-seq data processing

Primary and secondary analysis of Duplex-seq data were performed using TwinStrand's bioinformatics pipeline as previously described (75). In short, raw basecalls were demultiplexed to yield per-library unmapped reads. Duplex-seq adapters and their associated unique molecular identifiers (UMI) were extracted from unmapped reads and then bwa (compiled against commit 8c92aef) was used to align raw reads to the mm10 mouse reference assembly (76). UMIs and strand-defining elements were used to group raw read pairs. Raw read groups were pre-processed and then used to create duplex consensus sequences. Duplex consensus sequences were re-aligned similarly to the raw reads. Species-level classification of all duplex consensus was performed using the metagenomic sequence classification tool Kraken (77). Duplex consensus that were flagged as off-taxon by Kraken were manually reviewed, and any that were confirmed as true interspecies contaminants were excluded from the final duplex consensus alignments. Finally, duplex consensus alignments were further post-processed with balanced overlap hard-clipping to prevent double counting of bases at the 3-prime ends of overlapping paired-end consensus and 5-prime hard-clipping of 15 bases to eliminate artifacts resulting from mechanical shearing and end-repair. Overall assay performance for each sample was judged based on several metrics that were generated during the bioinformatics analysis pipeline, including demultiplexed reads per library, percent selected bases (indicating the rate of reads mapping to on-target locations), peak tag family size (indicating sufficient reads-per-source-strand for effective strand consensus generation), mean duplex molecular depth, and total duplex consensus bases. Somatic and germline variants were called from the post-processed duplex consensus sequences using VarDictJava (78) in tumor-only mode with parameters optimized for error-corrected data. Mutation frequency, simple base substitution spectra, and trinucleotide mutational spectra were all calculated as previously described (75). The number of mutated genomes per million genomes sequenced, captured the frequency of a given mutation in a group of samples, taking into account the size of the clones in each of the samples and correcting for differences of sequencing depth. Frequencies of specific amino acid mutations were computed by dividing the total number of molecules with mutations leading to that specific change by the total number of times the position was sequenced. When computing for an experimental group, the counts of each individual sample were aggregated and then the quotient computed, then multiplied by one million. The data and analysis for the mutations per

million was processed using deepUMicaller and deepCSA methods as previously described (24).

Statistical analysis

Statistical analyses were carried out using the Prism software package (GraphPad), and significance of differences between experimental groups was labeled as follows: ns, non-significant; * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S19

MDAR Reproducibility Checklist

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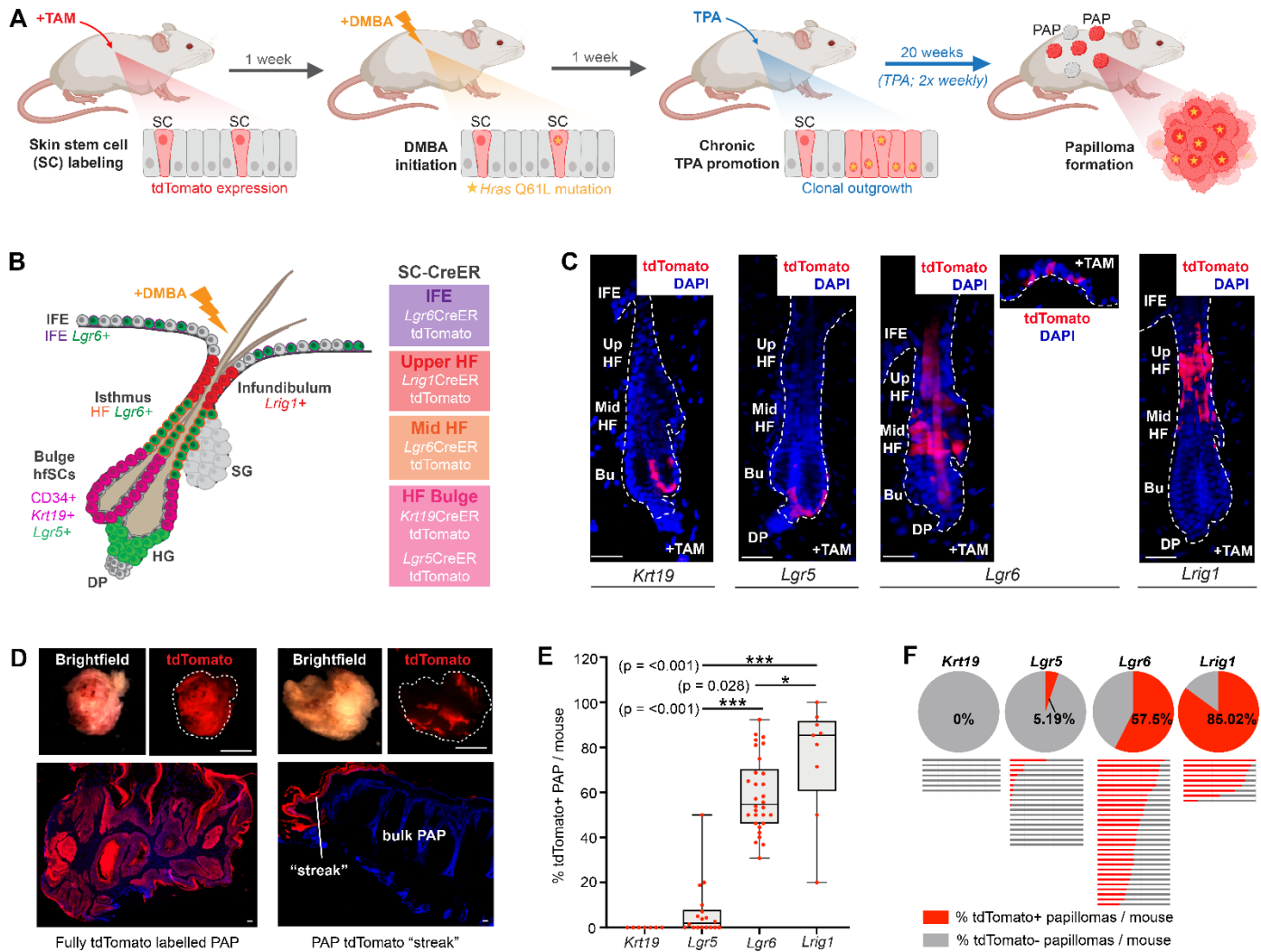
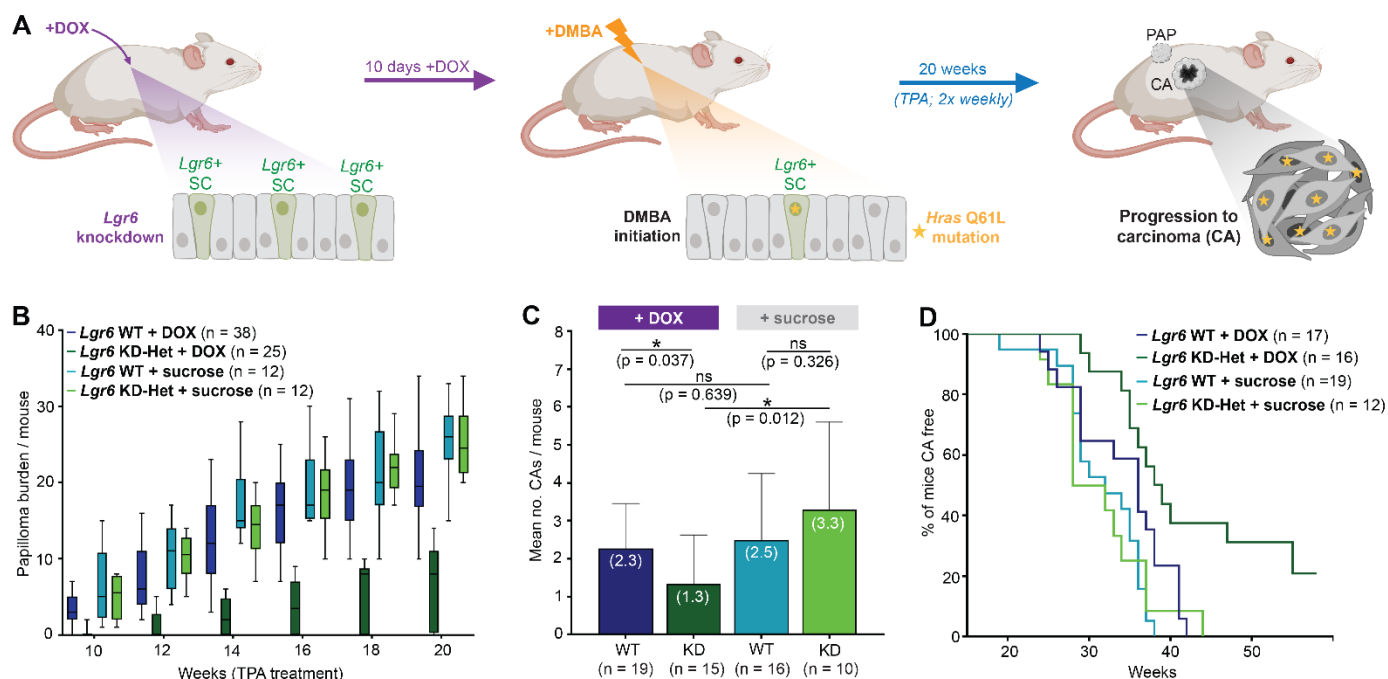


Fig. 1. Upper HF SCs are the predominant COO for DMBA-initiated skin tumors. (A) Illustration showing the skin SC-based lineage tracing approach used in combination with DMBA / TPA carcinogenesis. (B) Diagram of the location of specific skin SC markers of the HF and IFE and corresponding CreER mouse strains used for lineage tracing. (C) Representative images of the initial tdTomato labeling (red) of *Krt19* and *Lgr5* bulge hfSCs, *Lgr6*GFP+ SCs in the Mid HF and overlying IFE and *Lgr1* SCs of the Up HF, one day after TAM treatment. (D) Representative brightfield and fluorescence images of tdTomato fluorescence (red) in a fully labeled (left panel) and a largely unlabeled PAP of unknown origin (right panel) after chronic TPA treatment. Lower panels show representative cryosections of abundant tdTomato+ fluorescence within fully labeled PAP epithelium (left, lower) and red epithelial "streaks" (right, below), respectively. (E) Quantification of the percentage of PAPs labeled with tdTomato+ fluorescence from each SC compartment after chronic TPA promotion (number of mice per group: *Krt19*, n = 7; *Lgr5*, n = 18, *Lgr6*, n = 30, *Lgr1*, n = 9). The unpaired, two-tailed Student's *t* test was used to calculate p values with * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001. (F) Percentage of fully labeled tdTomato+ PAPs derived from each SC compartment after chronic TPA treatment with corresponding bars showing the percentage of labeled PAPs per individual mouse. DAPI counterstaining (blue) was used to label cell nuclei [(C) and (D)]. Abbreviations – Bu: bulge, DP: dermal papillae, HG: hair germ, DAPI: 4',6-diamidino-2-phenylindole, SG: sebaceous gland, IFE: interfollicular epidermis, Up HF: upper hair follicle, Mid HF: mid-hair follicle, +TAM: tamoxifen treated, hfSCs: hair follicle stem cells, PAP: papilloma, SC: stem cell. Scale bar = 50 μm. Elements of images were created using BioRender.



60

Fig. 2. *Lgr6*KD in vivo perturbs skin tumor development. (A) Schematic showing the DOX-inducible, *Lgr6*-targeted KD approach during skin carcinogenesis. (B) PAP development over time in WT and *Lgr6* KD-Het mice. (C) Mean CA burden in WT and *Lgr6* KD-Het mice after chronic TPA promotion (total number of CAs present per treatment group: *Lgr6* WT + DOX = 43 / 19 mice; *Lgr6* KD-Het + DOX = 20 CAs / 15 mice; *Lgr6* WT + sucrose = 40 CAs / 16 mice and *Lgr6* KD-Het + sucrose = 33 CAs / 10 mice). The unpaired, two-tailed Student's *t* test was used to calculate *p* values with ns: non-significant, * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001. (D) Graph representing the percentage of WT and *Lgr6* KD-Het mice lacking CA development over time (CA-free survival - defined as the time point in weeks after the first TPA treatment was administered at which the 1st CA was observed on the dorsal skin). Abbreviations - CA: carcinoma, DOX: doxycycline, KD: knockdown, Het: heterozygous, PAP: papilloma, SC: stem cell, WT: wild type. Elements of images were created using BioRender.

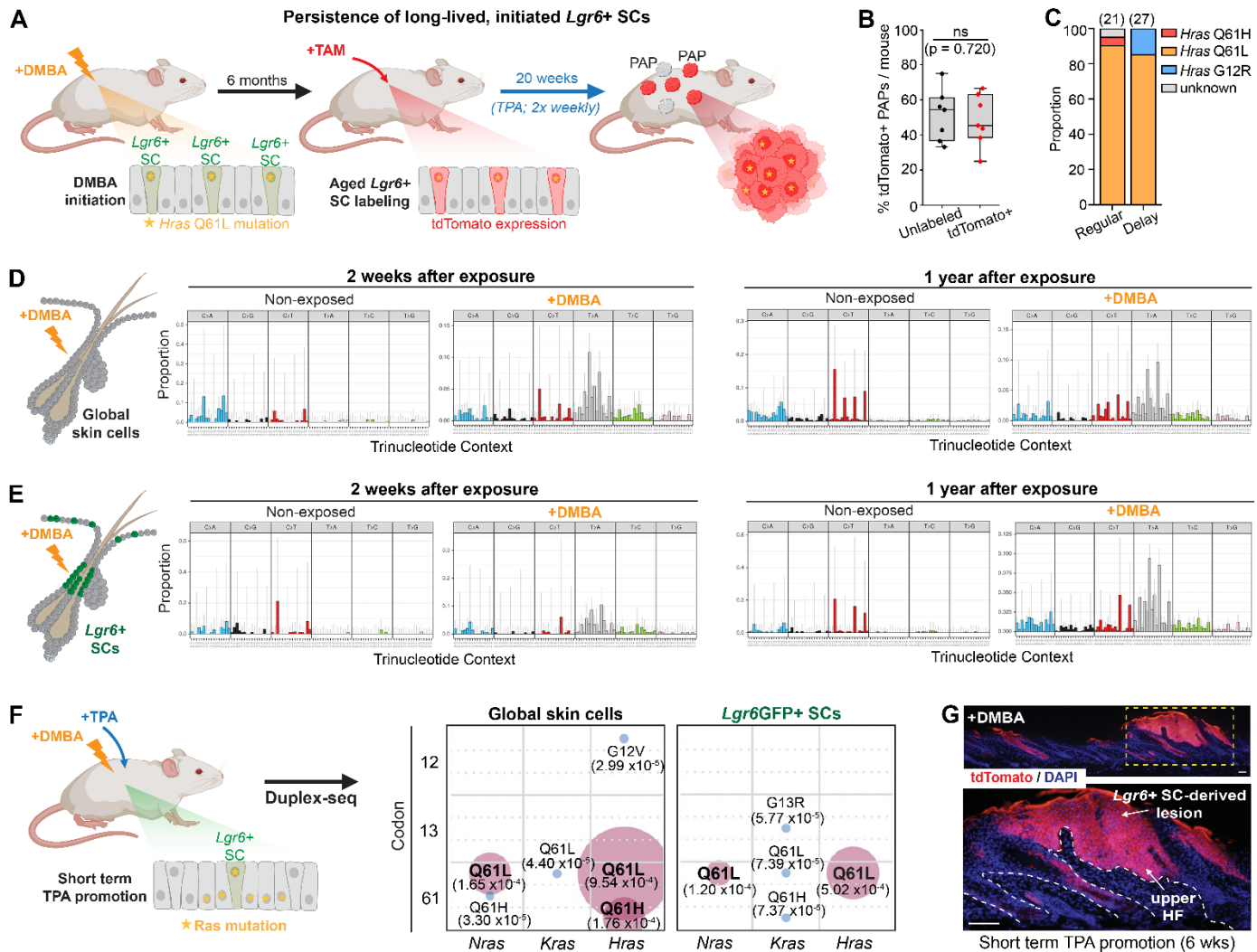


Fig. 3. Long-lived, initiated *Lgr6*⁺ SCs generate tumors during DMBA-mediated skin tumorigenesis. (A) Representation of the *Lgr6*⁺ SC-specific six month delay, lineage tracing approach used with DMBA / TPA carcinogenesis. (B) The percentage of fully labeled tdTomato⁺ PAPs per mouse following delayed chronic TPA treatment of DMBA-initiated, TAM-labeled *Lgr6*GFP / tdTomato mice (n = 7). (C) *Ras* mutation analysis of tdTomato⁺ PAPs generated from regular DMBA / TPA (n = 21) and delayed DMBA / TPA treatment (n = 27). (D) Trinucleotide signature of FACS-purified, non-exposed and DMBA-initiated, viable skin cell populations two weeks and one year after the initial exposure. The trinucleotide mutational signature induced by DMBA (25, 41) (grey bars; A > T / T > A) persisted for at least one year after treatment. (E) Trinucleotide signature analysis of non-exposed and DMBA-initiated, FACS purified, viable *Lgr6*GFP⁺ SCs two weeks and one year after the initial exposure. Red bars indicate the C > T trinucleotide signature corresponding to the classical SBS1 “clock” signature that increased over one year, independent of treatment with DMBA [(D) and (E)]. (F) Duplex-seq of DMBA-initiated, viable skin cells and *Lgr6*GFP⁺ SCs following four weeks of TPA promotion. A blue circle indicates the presence of a rare “singlet” mutation (single mutant cell) within the sample, a pink circle indicates a “multiplet” mutation indicating two or more cells with a particular mutation (potential clonal expansion) and the observed VAF value is displayed in parentheses below each mutation. Multiplet DMBA-associated mutations are highlighted in bold. (G) Representative cryosection of an early skin lesion labeled by *Lgr6*-derived, tdTomato⁺ cells (red) after six weeks of TPA treatment (DAPI, blue, was used to label cell nuclei; scale bar = 100 μm). Abbreviations – HF: hair follicle, DAPI: 4',6-diamidino-2-phenylindole, +TAM: tamoxifen treated, PAP: papilloma, SC: stem cell, VAF: variant allele frequency. In (B) the unpaired, two-tailed Student's *t* test was used to calculate p values with ns: non-significant, * p < 0.05, ** p < 0.01 and *** p < 0.001. Elements of images were created using BioRender.

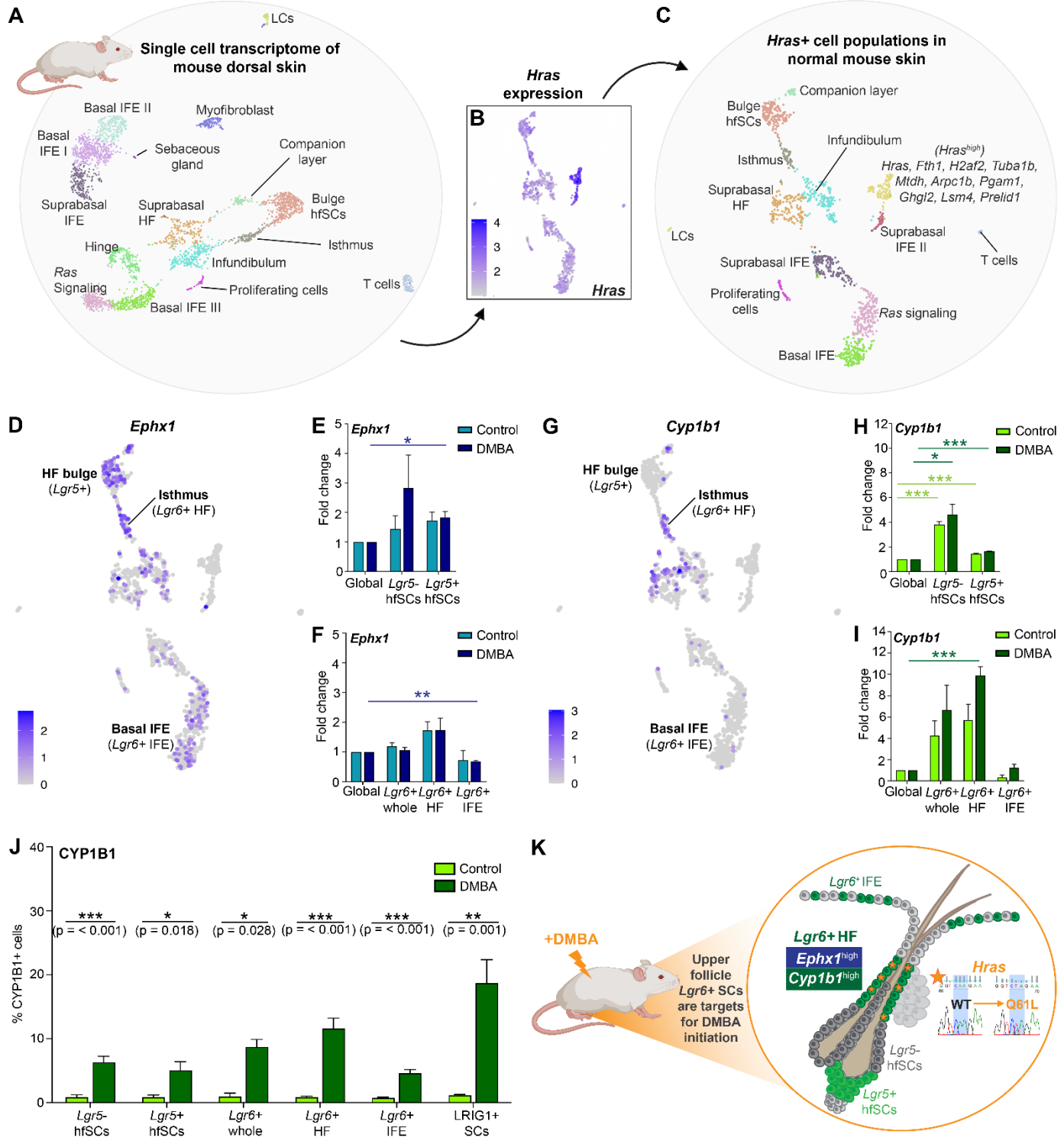


Fig. 4. *Lgr6*⁺ HF SCs are enriched with DMBA metabolism enzymes predisposing susceptibility for the acquisition of the DMBA-associated, *Hras* mutations in vivo. (A) Normal dorsal mouse skin transcriptome clustering using Seurat. (B) Enrichment and replotting of *Hras*⁺ cells from normal mouse skin. (C) Clustering of *Hras*⁺ cell populations using Seurat. (D) UMAP plot of *Hras*⁺ skin cells with subcluster expression of a DMBA metabolism enzyme, *Ephx1*. (E) qPCR of *Ephx1* expression in viable global skin cells, *Lgr5*GFP⁻ bulge hfSCs and *Lgr5*GFP⁺ SCs isolated from unexposed skin (pale blue) and skin 24 hours after DMBA exposure (dark blue) in vivo. (F) qPCR of *Ephx1* expression in viable global skin cells, parental *Lgr6*GFP⁺ SCs and sub-fractionated *Lgr6*GFP⁺ SCA1⁻ HF SCs and *Lgr6*GFP⁺ SCA1⁺ IFE SCs isolated from unexposed skin (pale blue) and skin 24 hours after DMBA exposure (dark blue). (G) UMAP of *Hras*⁺ cells isolated from unexposed mouse skin with subcluster expression of a DMBA metabolism enzyme, *Cyp1b1*. (H) qPCR of *Cyp1b1* expression in viable global skin cells, *Lgr5*GFP⁻ bulge hfSCs and *Lgr5*GFP⁺ SCs isolated from unexposed skin (pale green) and skin 24 hours after DMBA exposure (dark green). (I) qPCR of *Cyp1b1* expression in viable global skin cells, parental *Lgr6*GFP⁺ SCs and sub-fractionated *Lgr6*GFP⁺ SCA1⁻ HF SCs and *Lgr6*GFP⁺ SCA1⁺ IFE SCs isolated from unexposed skin (pale green) and skin 24 hours after DMBA exposure (dark green). (J) Bar chart showing the percentage of cells displaying CYP1B1 protein expression in purified, *Lgr5*GFP⁻ bulge hfSCs, *Lgr5*GFP⁺ SCs, the whole parental *Lgr6*GFP⁺ SC fraction, sub-fractionated *Lgr6*⁺ SCA1⁻ HF SCs, *Lgr6*GFP⁺ SCA1⁺ IFE SCs and LRIG1⁺ SCs isolated from unexposed skin (pale green) and skin 24 hours following DMBA exposure (dark green) in vivo (n = 5 independent biological replicates per SC type and exposure). (K) Illustration representing the in vivo expression of DMBA metabolism enzymes, *Ephx1* and *Cyp1b1*, in HF *Lgr6*GFP⁺ HF SCs mouse skin. Abbreviations – SG: sebaceous gland, Bu: bulge, HF: hair follicle, hfSCs: hair follicle stem cells, IFE: interfollicular epidermis, LCs: Langerhans cells. The unpaired, two-tailed Student's *t* test was used to calculate *p* values with * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001. Elements of images were created using BioRender.



Fig. 5. Targeted Duplex-seq to detect *Ras* hotspot mutations in unexposed and DMBA-initiated mouse skin. (A) Illustration showing the approach used to collect carcinogen-exposed mouse skin samples for targeted, Duplex-seq to detect the presence of *Ras* codon G12, G13, and Q61 mutations. (B) Duplex-seq detection of *Ras* hotspot mutations in individual control, unexposed skin samples (n = 10, red boxes highlight the presence of spontaneous codon 12 and 13 mutations). (C) Detection of *Ras* hotspot mutations in individual DMBA-initiated skin samples (n = 10, red boxes highlight the presence of codon 61 mutations). (D) Duplex-seq detection of *Ras* hotspot mutations in DMBA-initiated and TPA-promoted skin samples (n = 10, red boxes highlight the presence of codon 61 mutations). In (B) to (D), a blue circle indicates the presence of a rare “singlet” mutation within the sample (a single mutant cell) and a pink circle indicates a “multiplet” mutation, indicating two or more cells with a particular mutation. Note: in a single control animal [(B), top right], mutations were observed in *Hras* Q61L, *Kras* G12D, and *Kras* G13R, each as very small multiplets revealing minimal clonal expansion without any treatment - this was the only animal to display this pattern, which was not typical. Elements of images were created using BioRender.

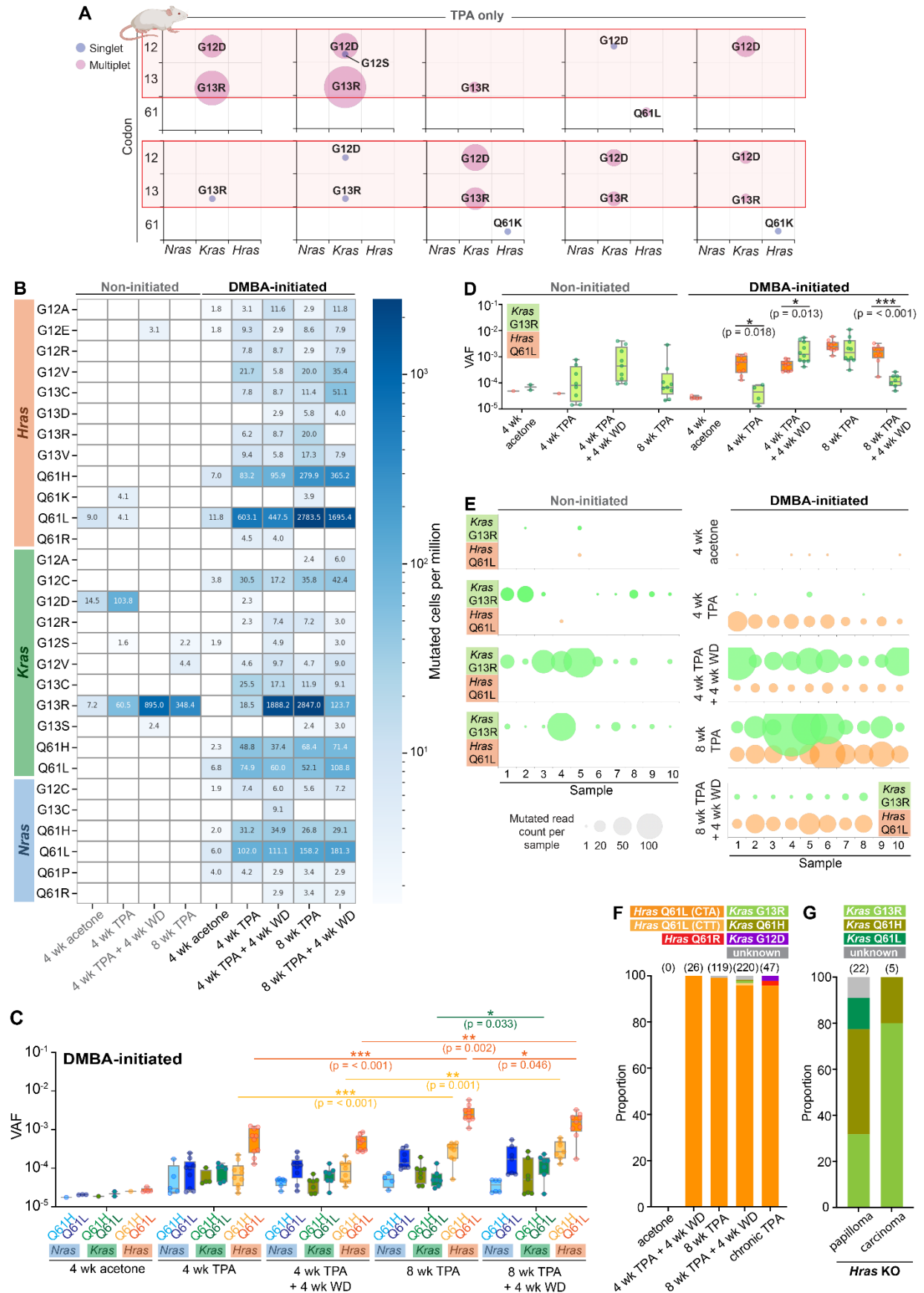


Fig. 6. Dynamic cell competition exists between spontaneous and DMBA-initiated *Ras* mutants in unexposed and DMBA-treated mouse skin. (A) Targeted Duplex-seq reveals the outgrowth of cells with spontaneous *Kras* G12D and *Kras* G13R mutations in response to short term TPA promotion in vivo. Each plot represents the presence of hotspot *Ras* mutations observed in an individual TPA-treated mouse skin sample (n = 10 biological replicates, a blue circle indicates the presence of a rare “singlet” mutation and a pink circle indicates the presence of a “multiplet” mutation (red boxes highlight codon 12 and 13 mutations). (B) Plot representing the estimated number of mutant cells per million for the most common *Ras* hotspot mutations observed in uninitiated and DMBA-initiated skin in the absence or presence of TPA promotion, and the effect observed after withdrawal of TPA promotion for four weeks. (C) Comparison of the frequency of Q61H and Q61L mutations in *Nras* (light and dark blue, respectively), *Kras* (olive and dark green, respectively) and *Hras* (yellow and orange, respectively) in each of the treatment groups. (D and E) Duplex-seq detection of the frequency (D) and mutated reads per sample (E) for *Hras* Q61L (orange) and *Kras* G13R (light green) mutations in each of the treatment groups. (F) *Ras* driver mutations observed in tumors generated from each of the DMBA-initiated and TPA-promoted groups (the number of tumors analyzed per group is displayed above each bar in parentheses). (G) Detection of *Kras* mutations observed in *Hras* KO papillomas (n = 22 tumors) and carcinomas (n = 5 tumors) generated from DMBA + TPA treatment. The unpaired, two-tailed Student’s *t* test was used to calculate p values with * p < 0.05, ** p < 0.01 and *** p < 0.001. Abbreviations – VAF, variant allele frequency, KO: knockout, WD: withdrawal. Elements of images were created using BioRender.



Chemically induced skin tumors arise from long-lived stem cells of the upper hair follicle

Eve Kandyba, Arnaud Jabouille, Ferriol Calvet, Yun Rose Li, Andrea Curtabbi, Diana Cristea, Joyce Shin, Reyno Delrosario, Jonathan Anzules, Di Wu, David Quigley, Mark Taylor, Camila Zanette, Fang Yin Lo, Jacob Higgins, Jesse Salk, Nuria Lopez-Bigas, and Allan Balmain

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