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Meso-scale spatial analysis of papilloma formation and clonal expansion in cancerized skin

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Abstract

Somatic driver mutations pervade phenotypically normal epithelia, yet only a minority of clones progress to tumors. To understand how tissue context modulates clonal selection, we apply the classical two-stage DMBA/TPA model of carcinogenesis to mouse back skin. We collect coordinate-registered, meso-scale grids spanning multiple centimeters with 1-mm punches and profile genotype and transcriptome from the same positions (WES, $n = 473$; bulk RNA-seq, $n = 690$), complemented by matched single-cell RNA-seq. This paired, spatially resolved design enables direct coupling of clonal selection metrics to local transcriptional programs. All sequenced papillomas ($n = 6$) harbor *Hras*^{Q61L} mutation events, but expected frequency versus observed papillomas indicate that *Hras*^{Q61L} is necessary but insufficient to drive tumorigenesis within the observation period. To quantify how field-level signaling constrains driver potency, we compute pathway activities and introduce a Laplacian-based “ruggedness” score to capture mesoscale heterogeneity. We find that field-level signaling is rewired before visible tumorigenesis: TNF α program-associated epithelial necroptosis rises broadly across promoted skin, while TGF β signaling activity becomes spatially “rugged,” with papillomas at local TGF β signaling minima. Selection mapping reveals a context shift: in untreated skin, positive selection corresponds with cell-cycle deregulation; in the cancerized field, it correlates with impaired differentiation. Moreover, the coupling between WNT signaling and selection evident in untreated tissue is abrogated for DMBA-initiated mutations. These data show that programs such as differentiation regulation, necroptosis, and TGF β topology reshape the selective landscape, constraining the effective potency of driver mutations and explaining why clones differ in their capacity to expand and progress to tumors.

Introduction

Recent perspectives move beyond the somatic mutation theory of carcinogenesis, which centers on cell-intrinsic changes, toward models that frame cancer as a breakdown of tissue-level organization (Sonnenschein and Soto 2020). Phenotypically normal human skin carries abundant somatic mutations that often expand into clones spanning multiple millimeters (Martincorena et al. 2015; Fowler et al. 2021). In sun-exposed human skin, thousands of clones are detectable, and ~25% of cells harbor oncogenic driver mutations by old age, despite normal histology (Martincorena et al. 2015; Jonason et al. 1996). It is plausible that such regions could leave

whole epithelial regions predisposed to transformation, a process termed field cancerization (Braakhuis et al. 2003; Slaughter et al. 1953). Because proliferation is a default metazoan program, tumor emergence might reflect the release from contextual constraints rather than the acquisition of novel proliferative capacity. In skin, the niche defined by tissue architecture, including follicular units and basement membranes, restricts clonal spread (Rompolas et al. 2016; Watt et al. 1988). Perturbations such as wounding or chronic irritation can relax these constraints, permitting expansion and sometimes formation of papillomas, benign, pre-cancerous epithelial lesions. Indeed, surveys of human skin, together with mouse studies, indicate strong homeostatic restraints on mutant clones until local conditions shift to favor their expansion (Li et al. 2025; Berenblum and Shubik 1949; Stenbäck et al. 1981; Fowler et al. 2021).

Many putative oncogenic clones expand within normal epithelia, yet only a subset forms tumors, indicating selection by competition and microenvironment context (Martincorena et al. 2015, 2018; Renz et al. 2024; Murai et al. 2018; Colom et al. 2021; Alcolea et al. 2014). In human skin, expanded clones are enriched for mutations in genes such as NOTCH1, TP53, and RBM10, suggesting that altered activity of these genes confers a selective advantage (Fowler et al. 2021; Martincorena et al. 2015; Renz et al. 2024). However, mutational overrepresentation is an indirect proxy. A more general interpretation is that selection acts on functional programs—stable, phenotype-defining cell states composed of transcriptional, epigenetic, and signaling activities—rather than on specific mutations per se. Targeted-perturbation transcriptomics identify TNF signaling as a key driver of epithelial clonal expansion (Renz et al. 2024). Other pathways, including TGF β , MAPK, PI3K, and WNT, regulate basal keratinocyte proliferation and differentiation in normal skin (Liarte et al. 2020; Cursons et al. 2015; Dainichi et al. 2016; Ghahramani et al. 2018). Tumors repurpose these pathways, rewiring networks during papilloma formation and progression (Renz et al. 2024; Taylor et al. 2024; Batlle and Massagué 2019). These results highlight the necessity for an unbiased, context-preserving comparison of transcriptomic determinants of clonal selection and field cancerization across normal, cancerized, and papillomatous skin.

Prior work does not retain meso-scale ($> \text{cm}^2$) coordinate information of genotype and transcriptome across cancerized fields. This gap is partly methodological. Single-cell RNA-seq exposes rich cell states but offers limited genotype capture and discontinuous spatial context (Taylor et al. 2024). Bulk DNA sequencing and bulk RNA-seq provide mutation and gene

expression readouts but no spatial resolution or cell-type specificity (McCreery et al. 2015; Nassar et al. 2015). Current spatial transcriptomics yields expression maps and can be leveraged to infer copy-number variants (CNV), yet typically lacks genome-wide mutation calls and is constrained in area and depth (Erickson et al. 2022; Ji et al. 2020). Though available skin grids maintain meso-scale coordinate information, they encompass whole-exome sequencing data in the absence of transcriptional information (Fowler et al. 2021; Martincorena et al. 2015). Consequently, micro-scale methods illuminate cellular interfaces, whereas macro-scale analyses average away local selection; the meso-scale—the biopsy-relevant ecological scale at which millimeter-sized clones compete within their immediate niche—remains underexplored.

Here, we couple genotype and transcriptome at the meso-scale to characterize skin field cancerization and papilloma emergence. To do so, we apply the classical two-stage mouse skin carcinogenesis model, in which initiation is induced by 7,12-dimethylbenz[a]anthracene (DMBA) and promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Abel et al. 2009). The separability of these steps is critical: DMBA introduces a distinctive T>A transversion signature, which provides a temporal marker for the clonal origin of lesions, while TPA acts later to expand initiated clones (Li et al. 2025; McCreery et al. 2015; Loehrke et al. 1983). Using coordinate-registered grids across back skin, we integrate paired whole-exome and bulk RNA-seq to map mutations and signaling programs at 1-mm resolution and complement analyses with a parallel single-cell RNA-seq dataset. This enables comparison of lesion-localized versus field-diffuse signaling within cancerized grids and stratification of transcriptional programs by mutation class (DMBA-induced vs. background) across treatments. We also introduce a Laplacian-based “ruggedness” metric to quantify spatial heterogeneity and estimate the expected frequency of initiating events from sequencing coverage and basal-cell geometry. Through these methods, we connect clonal selection to spatial signaling context, refining our understanding of how transcriptomic regulation gates progression to papillomas.

Results

Meso-scale genomic and transcriptomic profiling of mouse back skin confirms treatment efficacy

We generated meso-scale grids from mouse back skin by collecting paired whole-exome sequencing (WES) and bulk RNA sequencing (bulk RNA), retaining coordinate information to

preserve spatial context across the grids (Fig. 1A, S1A). Grids were derived from untreated mice (UN, $n = 4$), mice treated with the cell-cycle activator TPA for twelve weeks (TO, $n = 3$), and mice treated with the carcinogen DMBA followed by repeated TPA until the first visible papilloma (DT, $n = 3$). Cumulatively, we collected 690 bulk RNA and 473 WES samples, the majority of which are paired (Fig. S1A). All DT animals developed papillomas, whereas no macroscopic skin alterations were observed in UN or TO mice (Fig. 1B, C).

To complement the bulk RNA data, we collected back skin single-cell RNA sequencing (scRNA) from UN, TO, and DT mice after twelve weeks of treatment (Behm et al. 2023). All major skin cell types were identified (Fig. 1D), and we further annotated epithelial and immune compartments at fine resolution (Fig. S1B,C). Histology and scRNA cell type proportions confirmed skin thickening in TPA-treated conditions compared to control (Fig. 1E, F) (Abel et al. 2009). We performed deconvolution of bulk RNA data using scRNA-derived cell type annotations and expression signatures to infer cell type proportions in a treatment-blind manner. This confirmed epithelial thickening in DT grids, with significantly higher predicted epithelial cell proportions compared to UN (Fig. 1G). TO grids showed a similar trend, but the increase was not statistically significant.

Within the epithelial compartment, TPA treatment increased the relative abundance of cycling keratinocytes and K10/K14 double-positive keratinocytes, while the proportions of K10+ and K14+ single-positive keratinocytes decreased (Fig. S1D). These shifts are consistent with accelerated epidermal turnover (Cockburn et al. 2022). Furthermore, epithelial cell types associated with the hair follicle increased in relative abundance, aligning with the observed re-entry of hair follicles to anagen upon TPA treatment (Qiu et al. 2017).

Among immune cells, TPA treatment increased the proportions of innate lymphoid cells (ILC), natural killer (NK) cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, regulatory T cells (T reg), and conventional alpha-beta T cells (T ab) (Fig. 1H), consistent with previous reports of enhanced immune infiltration (Ortner et al. 2016; Nasti et al. 2021). The increase in overall lymphoid proportion calculated from bulk RNA deconvolution was not significant in TO or DT grids relative to controls (Fig. S1E), likely because the increase in some immune subtypes was offset by the stable abundance of other subtypes (Fig. S1F) and dilution of immune signals by epithelial thickening (Fig. 1F,G).

In summary, the scRNA and bulk RNA datasets provide complementary evidence confirming the efficacy of DMBA and TPA treatment in our data.

Exome analysis of papilloma clones demonstrates scope of expansion event

To investigate the mutational landscape underlying papilloma formation, we analyzed WES data from six papillomas that formed within the exome grids. A single dose of DMBA seeds the epidermis with thousands of mutations carrying a characteristic T>A transversion signature (Li et al. 2025; McCreery et al. 2015), which was apparent in papilloma-containing positions (Fig. 2A). Variant allele frequencies (VAFs) were expanded in papillomas compared to UN, TO, and non-papilloma positions from DT grids (Fig. 2B, Tables S1, S2). Small (<1%) VAFs were broadly detectable, since most samples exhibit $\geq 100\times$ coverage for >8.5% of all aligned positions (Table S3). In five of the six papilloma-containing positions, the VAF distribution was dominated by an expanded population of SNVs associated with the papilloma (Fig. S2A).

We leveraged the spatial context of our data to ask whether papilloma clones were confined to the visible lesion or embedded within broader clonal fields. We isolated single nucleotide variants (SNVs) defining each papilloma clone (Fig. 2C) and queried for their presence in neighboring positions. Most papillomas showed limited spatial extent; for example, the DT grid 2 a7 papilloma shared variants with only one adjacent coordinate (Fig. 2D). In contrast, the DT grid 1 f12 papilloma shared numerous SNVs with g14, a macroscopically normal biopsy >1 mm away that also harbored an expanded clone (Fig. 2E; Fig. S2B). This degree of shared mutation burden was not observed elsewhere in the sampled field, supporting the presence of a shared ancestral clone.

The VAF structure of DT1 f12 and g14 was consistent with subclonal diversification within the field. In f12, shared and f12-specific SNVs occupied similar VAF ranges, whereas in g14, g14-specific SNVs were generally larger than the f12-shared SNVs (Fig. 2F). Thus, the f12 papilloma appears to represent a papilloma-forming branch of a larger clone, while g14 contains a phenotypically normal expanded lineage. Within g14, both g14-specific and f12-shared SNVs showed T>A enrichment, including weaker but detectable DMBA signature contribution among higher-VAF mutations (Fig. S2C,D), indicating that the ancestral clone likely arose upon DMBA treatment. Interrogation of nonsynonymous mutations did not identify an obvious driver of the

expanded g14 lineage, suggesting that its growth may reflect an uncharacterized intrinsic alteration or local microenvironmental support rather than a canonical papilloma driver.

Across the dataset, most papilloma-specific SNVs were highly localized (Fig. 2G), consistent with each papilloma originating from a single dominant clone, though additional cell populations may be recruited during progression (Reeves et al. 2018). Our results reflect how papillomas arise independently and that their clonal expansion remains largely restricted to within a millimeter within the time frame of the experiment. This broader pattern reveals the rarity of a single DMBA/TPA-associated ancestral clone extending across millimeter-scale distances within the treated field, producing divergent local outcomes: a visible papilloma at one site and a driver-unresolved, phenotypically normal expansion at another.

We next asked which mutations drive papilloma formation. Only one non-synonymous mutation was recurrent in papillomas, and it was present in all six: *Hras*^{Q61L} (Fig. 2H). Remarkably, this mutation was not detectable in any non-papilloma position, including across UN and TO grids. This finding supports previous work identifying *Hras*^{Q61L} as the canonical driver of DMBA-induced papillomas and strongly suggests that it is necessary for papilloma development within twelve weeks (Quintanilla et al. 1986; Aldaz et al. 1989; Reeves et al. 2018; McCreery et al. 2015; Nassar et al. 2015; Balmain and Pragnell 1983).

We further tested whether all *Hras*^{Q61L} mutations lead to papilloma formation, or whether some remain non-expanded and undetectable. To estimate the expected number of *Hras*^{Q61L} events, we counted T>A mutations in the relevant trinucleotide context in each papilloma clone, assuming that each clone originates from a single basal cell and reflects a representative DMBA mutational burden. Using WES coverage, trinucleotide frequency, and histological estimates of basal cell density, we estimated that each WES DT grid should contain 9.6 ± 4.8 independent *Hras*^{Q61L} mutation events (Methods). Yet each of the three grids yielded only two papillomas within the observation period (Fig. S1A). Under a Poisson model using the calculated expectation, the probability of obtaining ≤ 2 papillomas in all three grids is only 2.86×10^{-4} . Papilloma formation is therefore not simply limited by acquisition of the canonical driver mutation but requires additional permissive conditions within the cancerized field.

Spatial analysis of signaling pathways discerns localized and diffuse papilloma shifts

On their own, DMBA-mutation-initiated cells can remain phenotypically silent for extended periods (Li et al. 2025; Loehrke et al. 1983). This suggests papilloma formation hinges not only on mutational burden but on environmental cues that permit clonal expansion. We therefore assessed the spatial distribution of signaling pathway activity across DT grids at meso-scale. We found that biopsies containing papillomas exhibit elevated MAPK, PI3K, VEGF, and TNF α signaling and reduced TRAIL, TGF β , and NF κ B signaling relative to all non-papilloma DT positions (Fig. 2I), consistent with prior reports (Cui et al. 1995; Glick et al. 1993; Chun et al. 2009; Larcher et al. 1996; Schioppa et al. 2011; Park et al. 2007; Xia et al. 2013; O’Leary et al. 2016). WNT signaling, a canonical skin architecture regulator (Lim et al. 2013; Ghahramani et al. 2018), was unchanged between papilloma and all other DT positions.

To determine the spatial extent of papilloma-induced signaling shifts, we compared pathway activity inside biopsies containing papillomas with adjacent skin (context). Several signaling pathways, including MAPK, PI3K, TRAIL, TGF β , and VEGF, showed significant differences between papilloma and context positions (Fig. 2J), reflecting highly localized signaling shifts detectable at the scale of a 1-mm punch. Comparisons of papilloma-adjacent positions with more distant non-papilloma DT positions revealed no further gradient of signaling (Fig. S2D). In contrast, TNF α pathway upregulation and NF κ B pathway downregulation were more diffuse, extending across the cancerized field (Fig. 2J). Thus, some pathways exhibit acutely localized papilloma-associated alterations, while others reflect broader field-level effects with papillomas as focal maxima or minima.

TNF α program activates necroptosis in epithelial cells of the cancerized field

To assess how differences in signaling pathway activity contribute to papilloma formation, we compared UN and non-papilloma DT positions. TNF α signaling inferred from bulk RNA was significantly elevated in a TPA-dependent manner, as expected (Fig. 3A, S3A) (Murakawa et al. 2006; Schioppa et al. 2011). NF κ B signaling, however, was downregulated specifically across the cancerized field, which was unexpected since one mechanism of NF κ B activation is via TNF receptors (Atretkhany et al. 2020).

We therefore complemented the targeted PROGENy analysis with comparative GSVA across Gene Ontology signatures to identify biological processes associated with papillomas and the surrounding cancerized field without restricting the analysis to predefined signaling pathways.

This broader analysis recapitulated several major pathway-level findings from the targeted analysis, including increased TNF α program activity, suppressed NF κ B signaling, and reduced TGF β -associated programs in papillomas. In addition to expected features of papilloma tissue, including increased mitotic activity, immune-response programs, and cellular stress responses, it revealed broader tissue-level remodeling involving extracellular matrix organization, epithelial structure and organization, and metabolic and nutrient-associated processes (Fig. S3B).

Because the TNF α pathway can activate multiple downstream processes, and because the GSVA analysis independently supported altered TNF α -associated activity, we examined known downstream pathways to understand what is driving this signal. We observed increased expression of necroptosis-associated genes and necroptosis-responsive damage-associated molecular patterns (DAMPs) (Fig. 3B; Fig. S3C). In contrast, apoptotic factors were unchanged, and survival pathways such as PI3K and NF κ B were unchanged or downregulated.

To assess whether these findings were accompanied by more comprehensive transcriptional evidence, we calculated a necroptosis activity score from bulk RNA (Zhong et al. 2023), which was significantly increased in a DT-dependent manner (Fig. 3C), and an apoptosis score (Zou et al. 2022), which showed no change between UN and DT (Fig. S3D). We also assessed expression of Gene Ontology pathways associated with cation channel and transmembrane transport, which are often disrupted during necroptosis (Grootjans et al. 2017), and found they were also dysregulated in a DT-dependent manner (Fig. S3E and F).

To dissect which cell types are responsible for the TNF α -associated necroptosis signal, we examined scRNA data from UN and non-papilloma DT positions. Epithelial cells were the only population with increased necroptosis signal (Fig. 3D), consistent with their established responsiveness to TNF α signaling (Kumari et al. 2013). Because several genes contributing to the necroptosis score (*Panx1*, *Myc*, *Egfr*, *Gata3*) are also involved in keratinocyte differentiation (Penuela et al. 2014; Gandarillas et al. 2000; Joly-Tonetti et al. 2021; Masse et al. 2014), we considered the possibility that the score might partially capture differentiation status rather than necroptotic activity per se. After controlling for subtype, necroptosis activity remained broadly elevated across DT epithelial cells, with the most significant differences observed in differentiated keratinocytes (K10+ and granular) (Fig. 3D, S3G). The data suggest that TNF α signaling engages a necroptosis-like transcriptional program coupled to keratinocyte

differentiation, revealing a potential intersection between cell death-associated signaling and epithelial maturation in the cancerized field.

TGF β signaling increases spatial heterogeneity in the cancerized field

Because we observed abrupt localized changes in pathway activity surrounding papillomas, we hypothesized that cancerized tissue may contain spatially restricted signaling microdomains that are obscured by average pathway activity. To quantify the local breakdown of signaling coherence preceding papilloma formation within the cancerized field, we calculated Laplacian scores for pathway activities for each non-papilloma position in DT and UN grids. This metric captures the extent to which pathway activity at a given position differs from that of its immediate neighborhood. Grids were scaled to account for signaling amplitude differences, and each position was modeled with contributions from neighboring positions (direct neighbors weighted 1, diagonals weighted $1/\sqrt{2}$). Higher absolute Laplacian values indicate greater dissimilarity with neighbors, corresponding to a more rugged signaling landscape (Fig. 4A). Among the pathways analyzed, only TGF β and VEGF signaling exhibited significant increases in ruggedness following DMBA/TPA treatment, with the effect being particularly pronounced for TGF β .

To identify which cell types drive this effect, we calculated per-cell TGF β activity in scRNA data from untreated and non-papilloma DMBA/TPA treated positions. Even in the absence of precise spatial positioning, the standard deviation among single cells from across the larger 5 mm punch provides a reasonable proxy for signaling ruggedness. DMBA-treated epithelial cells showed significantly greater dispersion of TGF β activity compared to untreated (Fig. 4B). Next, we asked which epithelial subtype contributes to the TGF β dispersion. Granular keratinocytes displayed the largest increase in dispersion, suggesting they substantially contribute to the increased ruggedness observed in bulk RNA data (Fig. 4B; Fig. S4A). Increased dispersion was observed across most pathway-driving genes in granular keratinocytes, arguing against an outlier-driven effect and supporting broad dysregulation of the TGF β transcriptional program rather than disproportionate contribution from a small subset of potentially non-specific genes (Fig. S4B).

TGF β is known to deliver anti-proliferative cues to basal cells (Kane et al. 1991; Chalmers et al. 2022) so we tested if DT treatment affects TGF β 's inhibition of proliferation. We generated a

proliferation score (Methods) from the bulk RNA of the DT and UN positions and found that TGF β activity negatively correlated with proliferation and that this relationship is unchanged between treatments (Fig. 4C).

Although the epithelial compartment demonstrates the greatest increase in TGF β dispersion, we wondered whether this was cell-autonomous and so evaluated canonical TGF β suppressor expression across compartments. We found negligible to modest increases in expression between UN and DT or pap across known TGF β suppressors in epithelial, lymphoid, and myeloid compartments, but consistent increases in the stromal compartment (Fig 4D). This is in accordance with a model in which the microenvironment contributes to tumor formation and progression, rather than being a consequence of papilloma presence; however it is possible that both are true. In contrast, the upregulation of TNF α program activators and downregulation of NF κ B program activators seems to occur across compartments, suggesting a possible mix of cell-autonomous and non-autonomous signals driving field cancerization and tumorigenesis.

In sum, these results suggest that the increased heterogeneity in keratinocyte activity produces a rugged signaling landscape where local cold spots in the cancerized field can nucleate papilloma formation. Because papillomas consistently show reduced TGF β signaling (Fig. 2I,J), such cold spots may explain why only a subset of *Hras*^{Q61L}-positive cells overcome local anti-proliferative TGF β signals to form papillomas.

DMBA treatment alters clonal selection dynamics in phenotypically normal skin

We considered how and to what extent DMBA treatment alters clonal dynamics in phenotypically normal skin, a process thought to underlie field-cancerization (Braakhuis et al. 2003). We first confirmed that DMBA introduces a signature of T>A mutations in the DT non-papilloma positions. Deconvolution of trinucleotide SNV composition into three mutational signatures across all positions revealed a signature that strongly resembles the canonical DMBA pattern (Fig. 5A, signature 2). The proportion of mutations explained by this signature was indeed greatest among DT positions (Fig. 5B). Clones from untreated positions uniformly lacked the DMBA signature, regardless of size (Fig. 5C).

In treated skin, small clones were enriched for the DMBA signature, whereas larger clones had signature weights more similar to untreated skin. This pattern is consistent with most DMBA-induced SNVs appearing in recently mutagenized cells that expanded only modestly

during the 10–12 weeks between treatment and tissue collection, rather than reaching the clone sizes of older background somatic mutations present in UN and TO skin. Clone-size estimate modeling supports this interpretation: TPA alone is predicted to increase neutral clone size, but over this interval the expected shift remains below our sequencing-based detection threshold. By contrast, DMBA introduces a large burst of mutational labels, allowing repeated TPA-driven proliferation to make a subset of otherwise neutral DMBA-labeled clones detectable at low VAF (Fig. 2B; Table S2).

To quantify deviations from neutral drift, we calculated a selection score for each position using a model of neutral clone-size distributions ((Simons 2016); Methods; Fig. S5A). Under neutral evolution, clone sizes follow an approximately linear incomplete-moment relationship, whereas positive selection produces deviations from this expectation; higher scores therefore indicate stronger evidence for non-neutral clonal expansion. When scores were calculated from T>A mutations, the hallmark class induced by DMBA, papillomas harboring driver *Hras*^{Q61L} mutations showed high selection scores, consistent with strong positive selection. In contrast, non-papilloma DT positions had lower T>A selection scores than UN or TO positions (Fig. 5D). Because most newly induced DMBA mutations will have negligible growth effects, these lower scores suggest that, outside papillomas, the 12-week experimental interval was insufficient for most DMBA-mutagenized cells to measurably depart from neutral expansion. The higher T>A selection scores in UN and TO positions likely reflect older background T>A mutations that had a longer window to expand before sampling. Selection scores calculated from non-T>A mutations were indistinguishable across treatments (Fig. 5E), consistent with background expansion in all positions.

Prior studies have shown exposure-induced clones can spread multiple millimeters in naturally aged human skin (Martincorena et al. 2015; Fowler et al. 2021); thus, we reasoned that our mutagenesis treatment could alter selection patterns at millimeter scale. Indeed, many SNVs were shared across neighboring positions across treatments (Fig. S5B and Fig 2D-G). However, simulating larger biopsies by pooling adjacent 2 × 2 positions abolished the differences in T>A selection between groups (Fig. S5C), meaning the 1 mm size of our meso-scale samples is well suited to analyse clonal selection following DMBA mutagenesis.

By leveraging our paired collection of DNA and RNA data for each biopsy, we next examined which transcriptional programs likely influence clonal selection and how they change upon

treatment. To do so, we correlated gene expression with T>A and non-T>A selection scores. WNT signaling, a known regulator of clonal growth in skin (Ghahramani et al. 2018), was positively associated with selection in UN positions and in DT positions when calculated from non-T>A mutations (Fig. 5F). In contrast, no association was seen for DT selection scores derived from T>A mutations (Fig. 5G; Fig. S5D). That is, the relationship between WNT signaling and clonal selection appears attenuated upon DMBA treatment, suggesting that treatment-induced mutational or microenvironmental effects may dominate selection drivers and obscure the more modest contribution of WNT-associated transcriptional programs. By leveraging the DMBA-induced T>A mutations, we are thereby able to see how field cancerization reshapes the transcriptional and signaling context that governs clonal dynamics. This pattern of altered dynamics was common across the whole transcriptome. In untreated positions, individual genes whose expression correlated with selection were consistent across both mutation classes. After DMBA/TPA treatment, the transcriptional programs associated with expansion of T>A mutations significantly diverged from those associated with the background mutations (Fig. 5H); in other words, DMBA/TPA globally altered the transcriptional programs of clonal expansion.

We wanted to understand the underlying biological mechanisms enabling positive selection and how they change between treatments. Two major processes can support clonal expansion in skin: increased proliferation or attenuated differentiation (Renz et al. 2024; Alcolea et al. 2014; Roshan et al. 2016). In UN positions, higher selection scores correlated with reduced expression of cyclin-dependent kinase (CDK) inhibitors, consistent with cell-cycle deregulation driving clonal advantage (Fig. 5I). In DT positions, however, no such correlation was observed. Instead, selection scores negatively correlated with keratinocyte differentiation programs (Fig. 5J). Mutations in differentiation and epigenetic genes were infrequent and unlikely to drive this phenomenon (Fig. S5E). These findings suggest that DMBA shifts the basis of positive selection: in untreated skin, selection is linked to cell cycle control, whereas in the cancerized field it is linked to dysregulated differentiation.

Lastly, we asked how the microenvironment modulates selection across treatments. Negative regulation of IFN- β was associated with neutral selection, consistent with previous evidence that IFN- β regulates keratinocyte growth and differentiation (Bielenberg et al. 1999) (Fig. S5F). This association was similar across treatments. By contrast, negative regulation of fibroblast

proliferation was associated with neutral selection only in UN positions (Fig. S5G). Given the complex crosstalk between fibroblasts and keratinocytes, our data cannot distinguish whether fibroblast proliferation influences keratinocyte clonal dynamics differently across treatments, or whether DMBA and/or TPA perturb fibroblast proliferation and thereby decouple it from keratinocyte clonal expansion.

Discussion

Our meso-scale analyses of clonal expansions at millimeter resolution supports a model in which the DMBA/TPA-induced cancerized field is shaped by both the mutational spectrum and the cellular context of clonal expansion. Whereas positive selection in untreated skin is largely governed by release from cell-cycle restraint, in the cancerized field, impaired differentiation is the dominant selective force. This shift, together with localized signaling heterogeneity, creates a tissue environment in which only a subset of driver-bearing clones are able to overcome anti-proliferative barriers to quickly progress to papillomas.

Our dataset connects single-cell and histological resolutions, enabling us to evaluate which transcriptional programs underlie clonal selection in a spatially resolved manner.

All papillomas harbored *Hras*^{Q61L}, consistent with its necessity for dysplasia (Balmain et al. 1984; Quintanilla et al. 1986). However, the presence of *Hras*^{Q61L} alone is insufficient (Aldaz et al. 1989; McCreery et al. 2015): from DMBA mutation burden, coverage, and basal-cell geometry we expected 9.6 ± 4.8 *Hras*^{Q61L} events per grid but observed only two papillomas, indicating that some *Hras*^{Q61L}-bearing basal cells are relatively indolent. Although other Ras-family drivers have been reported (Nassar et al. 2015; McCreery et al. 2015; Cataisson et al. 2022), we detect only *Hras*^{Q61L}, likely reflecting strain-dependent differences in papilloma incidence and driver spectra (Woodworth et al. 2004; Reiners et al. 1984; Nagase et al. 2003). Mechanistically, the Q61L mutation fixes HRAS in a GTP-bound, MAPK-activating state (Newlaczyl et al. 2014), matching the localized MAPK upregulation in papillomas we observe. HRAS potency is dose-dependent: increased *Hras* copy number or expression amplifies MAPK output. Papillomas emerge only when signaling crosses a threshold; sub-threshold activation yields only hyperplasia (Cataisson et al. 2022). These data expose a limit of mutation-centric models: initiating lesions arise frequently, but progression requires a permissive context. As in germline driver syndromes—where ubiquitous drivers cause tumors only in select tissues—

outcome reflects the interaction between mutation identity, dosage, and microenvironment (Zocchi et al. 2021).

Papilloma-defining SNVs remain confined to 1 mm punches within the observation period, rarely spilling over to neighboring punches. Though prior work in human skin has shown clones can encompass multiple millimeters, these clones develop across a substantially longer lifespan and under different exposures than in mouse (Martincorena et al. 2015; Fowler et al. 2021). Thus, the millimeter-scale tissue unit emerges as the operative arena in mouse skin where clones either expand or are suppressed (Rompolas et al. 2016; Watt et al. 1988), showcasing a model wherein tumorigenesis is more like a punctuated and context-dependent process than a driver-driven steady expansion.

Two cancerized field-level programs—TNF and TGF β —further reshape clonal outcomes before overt tumorigenesis. First, TNF α program-associated epithelial necroptosis appears to be specifically induced across TPA promoted skin, particularly within differentiated keratinocytes. Necroptosis releases DAMPs, perturbs barrier function, and amplifies inflammation—mechanistically consistent with TPA’s rapid induction of keratinocytic TNF signaling and with the resistance of TNF-deficient mice to DMBA/TPA promotion (Moore et al. 1999). In contrast, NF κ B signaling and apoptosis, two pathways canonically triggered by TNF, are downregulated and unchanged, respectively (Kalliolias and Ivashkiv 2015). Reduction in NF κ B activity has been shown to provide a selective growth advantage and cooperate with *Hras* mutations to promote papillogenesis (Park et al. 2007). NF κ B normally protects keratinocytes from TNF-induced death, and its inhibition unleashes necroptosis (Kumari et al. 2021), providing a mechanistic bridge between NF κ B suppression and the necroptotic elevation we observe. These events represent field-wide perturbations that erode homeostatic restraint, raising the likelihood that initiated clones achieve sustained expansion.

In parallel, TGF β signaling exhibits increased topological ruggedness rather than global suppression, with keratinocytes contributing strongly to this dispersion. Because TGF β signaling is a well-established anti-proliferative cue for basal keratinocytes (Chalmers et al. 2022), altered spatial patterning may create microdomains of weakened restraint. Proliferation inversely tracks TGF β signaling, and papillomas consistently occur at local TGF β signaling minima. Consistently, partial loss of TGF β signaling increases papilloma susceptibility in two-stage models (Cui et al. 1995; Hoot et al. 2008; Glick et al. 1993; Go et al. 2000), supporting a

permissive role for local TGF β program minima. These “cold spots” may provide permissive niches in which *Hras*^{Q61L}-bearing clones can overcome differentiation-linked brakes. Genetic models showing increased papilloma susceptibility under partial TGF β signaling loss support this interpretation, while later pro-oncogenic roles of TGF β lie outside our temporal scope.

Some pathways display papilloma-restricted changes whereas others diffuse across the cancerized field, illustrating how papillomas emerge as focal extrema superimposed on a promoter-primed field. Localized increases in MAPK/PI3K and VEGF signaling with concomitant reductions in TRAIL and TGF β signaling create microdomains consistent with niche construction, permissive proliferation, and early angiogenic induction. In contrast, TNF α program elevation and NF κ B program suppression in papillomas relative to the field extend broadly into surrounding tissue, showing a gradual spatial gradient rather than an abrupt boundary.

In untreated skin, perturbation studies show that clones can expand through either impaired differentiation or enhanced proliferation (Renz et al. 2024; Alcolea et al. 2014; Roshan et al. 2016). We demonstrate that field effects rewrite the selective landscape, shifting fitness from proliferative acceleration in untreated skin to resistance to differentiation in cancerized regions. This is interesting because *Notch1*, one of the most recurrently-mutated genes in expanded clones of normal skin, is a negative regulator of WNT-mediated differentiation, but its inactivation has also been shown to impair tumor growth (Abby et al. 2023; Martincorena et al. 2015; Renz et al. 2024; Alcolea et al. 2014). Although WNT signaling influences clonal expansion in phenotypically normal skin (Ghahramani et al. 2018), it was not significantly altered across treatments, contradicting reports of TPA-induced WNT elevation (Qiu et al. 2017; Su et al. 2018). This discrepancy may reflect methodological differences between bulk transcriptomic and protein-based measurements or sample timing. Our cell-type-resolved analyses suggest that observed field effects are cell non-autonomous: the microenvironment surrounding a keratinocyte poised to form a papilloma may fail to support—and may even actively repress—its unrestrained expansion. This model explains why most initiated clones remain quiescent, with only a minority expanding under permissive conditions.

Clonal competition imposes an additional barrier for positively-selected clones. Expanded clones can suppress neighbors, and only a minority achieve sustained growth (Martincorena et al. 2018; Renz et al. 2024; Colom et al. 2021; Alcolea et al. 2014). Clinically, many *Hras*-mutant

papillomas regress, consistent with the restoration of homeostatic control. Initiated cells may persist but fail to maintain dominance once environmental constraints are re-established (Li et al. 2025; Lesko et al. 2024; Iversen 1981). Papilloma emergence is thus best understood not as the inevitable outcome of oncogenic initiation, but as the rare convergence of mutation, ecological scale, and field-level signaling states that collectively permit outgrowth.

Our temporal window ends at the first papilloma, so we cannot assess malignant progression, such as the canonical switch in TGF β function (Cui et al. 1995). While bulk RNA sequencing limits cell-type resolution, comparison with single-cell RNA-sequencing data supports our main inferences. This work elaborates on known field cancerization mechanisms and generates hypotheses for others; targeted perturbations of necroptosis and TGF β signaling within defined microdomains, combined with barcoded initiated clones, would test whether altering field topology reroutes clonal fate.

Our observations are constrained by the resolution of the data. For example, we cannot detect negative selection after DMBA treatment because we cannot resolve the growth or absence of growth of individual cells, which represents the functional clone size immediately after mutagenesis. Deeper sequencing and higher-accuracy approaches, such as Duplex-seq, could lower this limit of detection and enable reliable capture of smaller clones. Similarly, using a grid of punches smaller than 1 mm would provide a more granular view of clonal dynamics, particularly when paired with transcriptomic profiling.

The dynamics we observe refine broader theoretical models: rather than tumorigenesis reflecting the acquisition of a novel proliferative program, our data support the model wherein proliferation is a latent cellular default re-enabled when contextual restraints are eroded. Similarly, while attractor-state models posit transcriptomic landscapes governing fate, in this system mutation-specific effects (e.g., *Hras*^{Q61L}) interact with ecological context to dictate outcome, underscoring that neither paradigm alone suffices. In sum, meso-scale, mutation-aware cartography offers a framework for understanding why only a subset of driver-bearing keratinocytes form tumors. DMBA/TPA creates a field in which inflammation and necroptotic perturbations partner with anti-proliferative signaling ruggedness to reshape selection, favoring impaired differentiation over cell-cycle deregulation and confining effective expansion to permissive microdomains. This research links genotype to spatial state to outcome and offers

actionable levers to modulate the potency of tumor driver mutations and skin cancerization programs.

Methods

Chemical skin carcinogenesis model

Female C57Bl6/J mice were shaved and treated with a single initiating dose of 7,12-dimethylbenz[a]anthracene (DMBA; 100 µg in 100 µl acetone; Sigma-Aldrich) applied to the dorsal skin. One week later, promotion was initiated by topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA; 10 µg in 100 µl acetone; Sigma-Aldrich) three times weekly. Mice were monitored for papilloma formation and sacrificed one week after the first lesion appeared (10–12 weeks total). A control cohort received TPA alone under identical conditions for 10 weeks. All animal work complied with institutional ethical guidelines (licenses G-59/17, G-96/21, Regierungspräsidium Karlsruhe; Procedure Project Licence PPL80/2619, Animal Welfare and Ethical Review Body [AWERB]).

Tissue collection of skin grid and livers

Dorsal skin from untreated, TPA-, and DMBA/TPA-treated mice was dissected, and a 2 × 2 cm spatial grid of 1 mm full-thickness punch biopsies was collected. Samples for nucleic acid extraction were placed in 96-well plates on dry ice, while adjacent biopsies from the grid margin were fixed for histology. Livers were rinsed in PBS, minced, snap-frozen in liquid nitrogen, and stored at –80 °C.

Nucleic acid isolation

DNA and RNA were co-extracted from skin biopsies and liver using a hybrid protocol combining the Qiagen AllPrep DNA/RNA Micro Kit and DNeasy Blood & Tissue Kit. Tissues were homogenized in 350 µl RLT buffer with 1% β-mercaptoethanol using a TissueLyser II (30 Hz, 2 × 3 min). Lysates were clarified, mixed with 12 µl Proteinase K in 688 µl nuclease-free water, and incubated at 56 °C for 10 min (final volume = 1038 µl). Each lysate was divided equally for RNA and DNA isolation (519 µl each).

RNA isolation

519 μ l lysate was combined with 260 μ l ethanol and processed on RNeasy MinElute spin columns. After one RW1 wash, on-column DNase digestion (15 min) and standard RW1/RPE/ethanol washes were performed. RNA was eluted in 14 μ l RNase-free water and quality-checked using Agilent TapeStation High Sensitivity RNA ScreenTape.

DNA isolation

The remaining 519 μ l lysate was mixed with an equal volume of Buffer AL, incubated at 56 °C for 30 min, then ethanol-precipitated and applied to DNeasy Mini spin columns. Columns were washed with Buffers AW1 and AW2, dried ($4200 \times g$, 10 min), and DNA was eluted twice with 25 μ l pre-warmed (70 °C) Buffer AE. DNA concentration and integrity were assessed using Qubit dsDNA HS and TapeStation Genomic DNA ScreenTape assays.

Histology

Skin biopsies were fixed overnight in 4% paraformaldehyde (Santa Cruz Biotechnology), transferred to 70% ethanol, paraffin-embedded, and sectioned at 5 μ m. Sections were deparaffinized, rehydrated through graded ethanol, stained with hematoxylin and eosin, and imaged using a Zeiss Axio Imager M2 microscope with an Axiocam 506 color camera.

Whole-exome sequencing

Dual-indexed whole-exome capture libraries were generated from mouse skin biopsy and liver DNA using the SureSelect XT HS and XT Low Input Enzymatic Fragmentation protocols (Agilent Technologies). Approximately 10–20 ng genomic DNA per position was fragmented at 37 °C for 20 min in SureSelect Fragmentation Buffer with Enzyme Mix, followed by end repair and dA-tailing in a thermal cycler. Indexed adaptors were ligated and purified with AMPure XP beads (Beckman Coulter). Libraries were PCR-amplified for 11 cycles, purified again with AMPure XP beads, and evaluated for quality and fragment size using the Agilent TapeStation High Sensitivity D1000 ScreenTape assay (Agilent Technologies).

For target enrichment, ~1000 ng of each library was hybridized with the SureSelect Mouse All Exon capture library (49.6 Mb; Agilent Technologies) following the manufacturer's instructions. Captured DNA underwent stringent heated washes, a second PCR amplification (10

cycles), and final AMPure XP bead purification. Libraries were quantified using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific) and re-assessed for size distribution with the TapeStation High Sensitivity D1000 ScreenTape. Equimolar pools were sequenced on Illumina NextSeq 2000 and NovaSeq 6000 platforms using a 100 bp paired-end protocol.

Bulk RNA-Sequencing

Total RNA was isolated from half of each lysate (519 µl) by adding 260 µl absolute ethanol and applying the mixture to RNeasy MinElute spin columns (Qiagen). Columns were washed once with Buffer RW1, followed by on-column DNase digestion for 15 min at room temperature. Subsequent washes were performed with Buffer RW1, Buffer RPE, and 80% ethanol. Residual wash buffer was removed by centrifugation at maximum speed for 5 min. RNA was eluted in 14 µl RNase-free water and stored at -80 °C.

RNA concentration and integrity were assessed using the Agilent TapeStation High Sensitivity RNA ScreenTape assay (Agilent Technologies). RNA libraries were prepared according to the manufacturer's protocol and sequenced on Illumina NextSeq 2000 and NovaSeq 6000 platforms with 100 bp paired-end reads.

WES mutation signature and selection score

Whole exome sequencing was demultiplexed and aligned to GRCm38mm10_PhiX using the DKFZ/ODCF Roddy pipeline (<https://github.com/DKFZ-ODCF/AlignmentAndQCWorkflows>). Somatic variants were called using GATK v4.0.9.0 Mutect2 with liver samples from the same animal as a matched normal, and filtered using FilterMutectCalls (DePristo et al. 2011). Somatic variants were annotated using VEP v110.1 and were further filtered to only retain SNPs with probability_no_artifact < 0.9 (McLaren et al. 2016). Summary statistics per sample, individual, and treatment are available in Tables S1-3.

The fraction of variants for each of the 96 possible trinucleotide contexts was calculated using the R package SomaticSignatures function mutationContext for all positions with at least 30 identified variants (Gehring et al. 2015). We then used non-negative matrix factorization via the function identifySignatures to extract three mutational signatures. One signature has a strong preference for T>A transversions in C.G, G.G, and T.G contexts, and we term this the "DMBA

signature” because it resembles the DMBA-induced signature reported in literature (McCreery et al. 2015; Nassar et al. 2015; Li et al. 2025).

Variants were filtered to retain only those with read depth ≥ 30 and VAF < 0.5 . For each biopsy containing at least 10 such variants, clone sizes were estimated as twice the VAF and used to compute the first incomplete moment of the clone size distribution (Klein et al. 2010; Simons 2016). Linearity of the log–linear incomplete moment plot was quantified by singular value decomposition (SVD), and a *selection score* was defined as $\log_{10}(1/\text{singular_value_ratio})$, such that higher scores indicate greater deviation from neutrality and emphasize expanded clones. For pooled analyses, all variants detected within a 2×2 grid window were aggregated by summing reference and alternate read counts across positions, after which the selection score was recalculated on the pooled data. Expanded clones were identified from SNVs using MOBSTER (Caravagna et al. 2020).

Single-cell RNA-seq preprocessing, integration, and cell type annotation

Raw mouse skin scRNA sequencing reads from Behm et al 2023 (E-MTAB-13556, E-MTAB-12454, and E-MTAB-12456) were processed with Cell Ranger v9.0.1 (10x Genomics) using the mouse reference genome (refdata-gex-GRCm39-2024-A). These animals are the same strain and underwent the same treatment protocols as the current manuscript. Empty droplets were excluded with DropletUtils (Lun et al. 2019), and cells were retained if they contained ≥ 200 expressed genes, $\geq 1,000$ unique molecular identifiers (UMIs), and $< 10\%$ mitochondrial reads. Doublets were predicted with Scrublet (Wolock et al. 2019) at an expected rate of 20% per sample and removed. The following samples, which contained $> 3,000$ cells after all filtering steps, were used in this work: TO1_COL, TO1_TRY, TO2_COL, TO2_TRY, TO3_COL, TO3_TRY, ana_c9_3, ana_c9_4, tel_c10_1, tel_c10_2, tel_c9_1, tel_c9_2, ana_dt2_1, ana_dt2_2, ana_dt2_3, ana_dt3_1, ana_dt3_2, ana_dt3_3, ana_dt4_1, ana_dt4_2. This corresponds to 6 UN samples across two individuals; 6 TO samples across three individuals collected at 10 weeks; and 8 DT samples, 2 of which contain papillomas, across three individuals collected at 12 weeks.

After quality control, count matrices across all samples were aggregated, normalized, scaled, and subjected to principal component analysis (PCA) in Seurat (Hao et al. 2021). Batch effects were corrected with Harmony (Korsunsky et al. 2019), and clustering was performed on

Harmony-corrected embeddings followed by UMAP visualization (Becht et al. 2019). Final cell type identities were assigned based on canonical marker expression, with ambiguous or low-quality clusters designated as doublets and excluded.

Differential cell type enrichment between conditions was tested using the mixed-effects association of single cells (MASC) (Fonseka et al. 2018)), modeling condition as a fixed effect and sample as a random effect.

Bulk RNA analysis

Bulk RNA sequencing was demultiplexed, aligned to GRCm38mm10_PhiX, and read count was calculated using the DKFZ/ODCF Roddy pipeline (<https://github.com/DKFZ-ODCF/RNAseqWorkflow>). Raw read counts generated by featureCounts were imported into DESeq2 (Love et al. 2014) using position metadata as design input. Variance stabilizing transformation was applied to normalized counts for exploratory analysis. Sample quality was assessed by principal component analysis (PCA) of all expressed genes, and outliers were identified and excluded prior to downstream analyses.

Bulk RNA-seq deconvolution

Cell type proportions in bulk RNA-seq were estimated with BisqueRNA (Jew et al. 2020) using cell types derived from scRNA-seq under the same treatment conditions as reference. Marker genes uniquely enriched in each compartment were identified with Seurat (3884 genes total) (Hao et al. 2021) and used for reference-based decomposition of bulk counts.

Signaling pathway activity scoring

Bulk count matrices were filtered to retain genes with >5 counts in $\geq 50\%$ of samples and variance-stabilized with DESeq2 (vst on size-factor-normalized counts) (Love et al. 2014). Positions were scored with PROGENy's mouse model (model_mouse_full) using decoupleR's multivariate linear model (run_mlm) to infer pathway activity weights from gene-level expression (Schubert et al. 2018; Badia-i-Mompel et al. 2022).

To compare papilloma versus non-papilloma signaling, positions were restricted to DT grids and PROGENy activity weights were z-scored within animal and pathway. Differences were

tested with a linear mixed-effects model with animal as random effect, reporting Satterthwaite *p*-values via lmerTest (Kuznetsova et al. 2017).

Tumor, adjacent, and distant positions were classified by spatial proximity. For each pathway, local contrasts were calculated as the difference in pathway activity between tumor and neighboring non-tumor spots, and between adjacent and distant spots. Distributions of these differences were summarized per animal, and group-level significance was tested with linear mixed-effects models (lmerTest) as above.

KEGG pathway analysis

Differential expression contrasts were performed with DESeq2 (Love et al. 2014) between DT and UN conditions, and gene-level fold changes were visualized in KEGG pathways of interest (e.g., necroptosis, TNF signaling) using pathview (Luo and Brouwer 2013). For heatmap visualization, variance-stabilized counts of necroptosis- and NFκB/TNF-related genes were averaged by animal and scaled across positions.

Cell death/proliferation scoring

Necroptosis and apoptosis activities were quantified by composite scores calculated from curated up- and downregulated gene sets (Zhong et al. 2023; Zou et al. 2022). For each sample, gene expression values were variance-stabilized, Z-transformed, sign-adjusted according to expected regulation, and averaged to yield a single score per pathway. Proliferation activity was calculated in the same manner using a curated set of cell-cycle genes, with *Mki67*, *Pcna*, *Top2a*, *Cdk1*, *Ccna2*, *Ccnb1*, *Ccnb2*, *Cdc20*, *Aurkb*, *Birc5*, and *Mcm2–7* weighted positively and *Cdkn1a*, *Cdkn1b*, and *Rb1* weighted negatively.

Gene Set Variation Analysis (GSVA)

Variance-stabilized bulk RNA expression (DESeq2 vst on size-factor-normalized counts; genes retained if >5 counts in ≥50% samples) was used for single-position gene set variation analysis (GSVA) with mouse GO gene sets from msigdb (C5: BP/MF/CC). The R package GSVA (Hänzelmann et al. 2013) was run with a Gaussian kernel, which is suitable for log-scale data, and scores were z-standardized across positions. To compare pathway weights between treatments, GSVA z-scores were modeled by linear mixed effects with treatment as fixed effect

and animal as random intercept. Associations between pathway activity and clonal selection were tested separately within UN and DT by mixed models of GSVA z-score on selection score with animal as random intercept.

Spatial Laplacian analysis

For each pathway and animal, PROGENy activity weights were mapped to 2D grids by biopsy coordinates. Spatial roughness was quantified as the discrete 2D Laplacian using a 3×3 kernel with weights of 1 for orthogonal neighbors and $1/\sqrt{2}$ for diagonals, and the center weight set to the negative sum of neighbors to enforce zero-sum. At grid edges or missing positions, neighbors were masked and the kernel re-normalized. Grids were z-scored prior to convolution to control for treatment-specific differences in signal amplitude. Absolute Laplacian magnitudes were used as the response, and treatment effects were tested with generalized linear mixed models (gamma family, log link) including animal as a random intercept.

Clone size estimate modeling

To estimate whether neutral clone expansion would be detectable within the experimental interval, we simulated clone growth under a single-progenitor neutral-drift model. Each simulation was initialized with 5,209 founder cells, corresponding to approximately half the estimated number of basal cells in a 1-mm² circular punch biopsy based on estimates from literature (Behm et al. 2023; Aragona et al. 2017; Piedrafita et al. 2020). Clones were propagated for 12 weeks using a basal division interval of 5.8 days and a symmetric division probability of 0.04 (Piedrafita et al. 2020). For TPA-exposed conditions, we modeled a six-fold increase in division rate based on published estimates of TPA-induced epidermal proliferation (Behm et al. 2023; Aldaz et al. 1989). TPA was assumed to affect only the division rate; all other model parameters were held constant.

Simulated clone-size distributions for untreated (UN) and TPA-only (TO) skin were compared with sequencing-based detection limits. For each treatment group, the lower detectable clone-size limit was estimated as a product of the estimated basal cells per punch $\times$ median SNV limit of detection across detected SNVs in each animal $\times$ 2, where the factor of two accounts for diploidy, assuming heterozygous SNVs. These median lower detectable clone-size limits and percent of positions with at least 100 $\times$ coverage are reported in Table S2. Under these

assumptions, neutral drift over 12 weeks produced small clones: the largest simulated clones reached 6 cells in UN skin and 18 cells in TO skin, both well below the median detectable clone-size threshold and therefore sparsely detectable. Thus, although TPA is expected to shift the neutral clone-size distribution upward, this effect alone is not predicted to be resolvable by sequencing at the used depth over the experimental time scale. In DMBA/TPA-treated skin, the same proliferative expansion occurs in the context of orders of magnitude more newly induced mutations (Randerath et al. 1998; Behm et al. 2023; Nasar et al. 2015; McCreery et al. 2015; Li et al. 2025) increasing the number of neutrally expanding clones that are detected at low VAF.

Estimation of *Hras*^{Q61L} mutation events

Assuming papillomas arise from single basal cells, we counted the number of MOBSTER-identified SNVs clonally expanded within each papilloma in the T[T>A]G or reverse complement context, which we denote k , because this is the context in which the *Hras*^{Q61L} mutation arises (Caravagna et al. 2020). Next, we defined N as the total number of TTG/CAA center sites in the mouse reference genome with $\geq 5\times$ coverage, identified using SAMtools depth and BEDtools intersect. From this, we divided k by N to obtain μ , the T[T>A]G mutation rate per callable TTG/CAA center base. We estimated the number of cells per mm^2 by averaging basal cell area from literature (Piedrafita et al. 2020; Aragona et al. 2017; Behm et al. 2023) and scaled up to the total sampleable area to estimate the number of cells per grid. Multiplying μ by the estimated cell count yielded an expected number of cells with a given T[T>A]G mutation. This analysis predicted 5.8–17.2 independent *Hras*^{Q61L}-capable events per grid (9.6 ± 4.8). Under a Poisson model, observing ≤ 2 events in each of three grids had a probability of 2.86×10^{-4} when incorporating gamma-distributed uncertainty in the rate estimate.

Data access

All raw and processed WES and bulk RNA sequencing data generated in this study are available in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) under accession numbers E-MTAB-15745 and E-MTAB-15743.

Competing interest statement

Authors declare no conflicts of interest.

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 Bioinformatic pipelines and analysis: VB, PBC, JL
 Statistics and modeling: VB, AG, PBC
 Visualization: VB
 Data curation: VB
 Supervision and funding acquisition: MF, AG, DTO
 Ethics and compliance: MF, MB, DS, SDP
 Writing: VB, DTO, AG
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Figure Legends

Figure 1: Multi-omic meso-scale profiling demonstrates epithelial expansion and immune remodeling

- A. Schematic of 1 mm punch grid profiled by bulk RNA and whole-exome sequencing across untreated (UN), TPA-only (TO), and DMBA + TPA (DT) cohorts.
- B. Representative dorsal images at week 12 (grids UN 3 and DT 3); red arrows mark papillomas.
- C. Tumor-free survival of UN (n = 4), TO (n = 3) and DT (n = 3) mice.
- D. UMAP of all scRNA colored by cell types.

- E. H&E sections of UN (Untr.), TO (TPA), and DT (DMBA/TPA) skin; scale = 50 μ m.
- F. scRNA-derived epithelial proportion per position; boxplots show median $\pm$ IQR; compared to UN using mixed-effects association of single cells (MASC) (Fonseka et al. 2018). UN n = 6, TO n = 6, DT n = 6
- G. Bulk RNA epithelial fraction per position predicted by BisqueRNA (Jew et al. 2020) using the scRNA dataset as reference; each horizontal line represents a mouse mean; mixed-effects model with random intercepts for animal. UN n = 4 (480 positions), TO n = 3 (87 positions), DT n = 3 (480 positions)
- H. scRNA immune subtype proportions as a fraction of all immune cells; statistics as in panel F. Innate lymphoid cells (ILC), natural killer (NK) cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, conventional alpha-beta T cells (T ab), regulatory T cells (T reg). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001

Figure 2: Papillomas arise from independent clones with highly localized signaling changes

- A. Somatic SNV trinucleotide spectra from WES across all papillomas; mean $\pm$ SD.
- B. VAF density for UN (n = 2 in 123 positions), TO (n = 3 in 17 positions), non-papilloma DT (field, n = 3 in 304 positions) and papilloma (n = 3 in 6 positions), pooled.
- C. MOBSTER (Caravagna et al. 2020) decomposition for grid DT2 position a7 distinguishes a high-VAF expanded papilloma clone from a low VAF background tail.
- D. Grid map of the percentage of DT2 a7 papilloma clone SNVs found in adjacent positions. Papilloma position is marked with an asterisk.
- E. As in panel D but for the DT1 f12 papilloma clone.
- F. Left: VAF distributions in DT1 papilloma-containing position f12 for SNVs that are position-specific (red) or shared with nearby position g14 (grey). Right: SNV VAF distributions in DT1 g14 that are position-specific (grey) or shared with f12 (grey).
- G. As in panel D but for papilloma clones from DT1 b12, DT2 e8, and DT3 f14.
- H. Nonsynonymous somatic mutations per papilloma. Numbers above points denote total nonsynonymous SNVs per papilloma; *Hras*^{Q61L} (red) is present in every tumor.
- I. PROGENy-derived (Schubert et al. 2018) pathway activity scores for field (470 positions) and papilloma (10 positions) within DT grids (n = 3); grids were internally scaled and tested via a linear mixed model; horizontal lines represent field or papilloma means.
- J. Pairwise differences between papilloma and immediate-adjacent (context) pathway activity scores (positive values denote stronger signaling in the papilloma). Each horizontal line denotes a mouse mean; mixed-effects intercept test with animal as random effect; ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3: Dysregulated TNF α program-derived pathways in the cancerized field

- A. PROGENy-derived (Schubert et al. 2018) signaling pathway activity scores in bulk RNA UN (n = 2 in 123 positions) and non-papilloma DT (n = 3 in 470 positions). Horizontal lines denote the mean activity per animal; ns = not significant, *p < 0.05 linear mixed model with animal as random effect.
- B. Heat map of average gene expression per grid for processes downstream of TNF signaling such as necroptosis (necro), damage-associated molecular patterns (DAMPs), apoptosis (apop), PI3K, and NF κ B signaling. *p < 1×10^{-5} differential gene expression via DESeq2, which does not accommodate random effects (Love et al. 2014).

- C. Necroptosis score (Zhong et al. 2023) for non-papilloma bulk RNA positions across treatments. Horizontal lines denote the mean score per animal; ns = not significant, * $p < 0.05$, *** $p < 0.001$ linear mixed model with animal as random effect.
- D. Necroptosis score for scRNA UN and non-papilloma DT positions, separated by cell type (left) or keratinocyte subtype (right). Boxes show median $\pm$ IQR. ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ linear mixed model with animal as random effect.

Figure 4: Increased spatial heterogeneity of TGF β signaling define the cancerized field

- A. Schematic of the biological interpretation of the Laplacian distribution (left) and absolute Laplacian values (log scale) for PROGENy-derived (Schubert et al. 2018) signaling pathways in bulk RNA UN (n = 2 in 123 positions) and non-papilloma DT (n = 3 in 470 positions) (right). Horizontal lines denote mean score per animal; ns = not significant, * $p < 0.05$, *** $p < 0.001$ linear mixed model with animal as random effect.
- B. Standard deviation differences in inferred TGF β pathway activity between UN (n = 6) and non-papilloma (n = 6) DT, stratified by cell type (left) or epithelial subtype (right). Positive values indicate greater dispersion in DT. Bar shading reflects statistical significance (darker = more significant) from a Levene test applied to residuals from a linear mixed-effects model.
- C. Correlation between TGF β signaling and cell proliferation score (Methods) in UN (light gray) and non-papilloma DT (dark gray) positions. Thick lines show best-fit regressions with 95% confidence intervals; thin lines indicate data point density. *** $p < 0.001$, mixed-effects model with animal as random effect.
- D. Expression levels of TGF β program suppressors (supp.), TNF α program activators (act.), and NF κ B program activators averaged across samples and scaled per gene across compartments.

Figure 5: DMBA reshapes clonal selection and shifts the transcriptional basis of clonal expansion

- A. Somatic SNV trinucleotide spectra from WES across all non-papilloma positions deconvolved into three signatures. Signature 2 (sig 2) resembles available DMBA mutation profiles (McCreery et al. 2015; Li et al. 2025). UN n = 2 (147 positions), TO n = 3 (17 positions), DT n = 3 (298 positions)
- B. The proportion of SNVs within each position that is explained by signature 2 from panel A. Horizontal lines represent the mean score per animal; linear mixed model with animal as random effect.
- C. The proportion of SNVs within binned VAF intervals across all UN (left) and non-papilloma DT (right) positions explained by signature 2 from panel A.
- D. Selection scores calculated from T>A mutations (Methods) across treatments. Horizontal lines represent the mean score per animal; linear mixed model with animal as random effect.
- E. As in panel D, but for selection scores calculated from non-T>A mutations.
- F. PROGENy-derived (Schubert et al. 2018) WNT signaling activity versus selection scores in UN positions. Thick lines show best-fit regressions with 95% confidence intervals; thin lines indicate data point density. Linear mixed model with animal as random effect.
- G. As in panel G, but for WNT signaling activity in non-papilloma DT positions.
- H. Differences in effect size (β) rank between T>A and non-T>A associations across all genes in linear mixed model with animal as random effect. Horizontal line denotes median; one-sided Wilcoxon rank-sum test.

- I. Cyclin-dependent kinase (CDK) inhibitor activity GSVA score (Hänzelmann et al. 2013) versus T>A selection score. Statistics as in panel F.
- J. As in panel I, but for regulation of keratinocyte differentiation. ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

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