Regulation of 5-Hydroxymethylcytosine by TET2 Contributes to Squamous Cell Carcinoma Tumorigenesis

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DNA methylation is a key regulatory event controlling a variety of physiological processes and can have dramatic effects on gene transcription. Methylated cytosine (5-methylcytosine) can be oxidized by the TET family of enzymes to 5-hydroxymethylcytosine (5-hmC), a key intermediate in the demethylation cycle, and 5-hmC levels are reduced in malignancies such as acute myeloid leukemia and melanoma. We constructed a tissue microarray of human cutaneous squamous cell carcinoma tumors and found a global reduction in 5-hmC levels compared with that in the adjacent skin. Using a murine *K14-CreER* system, we have found that loss of *Tet2* promotes carcinogen-induced squamous cell carcinoma and cooperates with loss of *Tp53* to drive spontaneous squamous cell carcinoma tumors in epithelial tissues. Analysis of changes in 5-hmC and gene expression after loss of *Tet2* in the epidermis revealed focal alterations in 5-hmC levels and an increase in hair follicle transient amplifying cell genes along with a reduction in epidermal differentiation genes. These results show a role for TET2 in epidermal lineage specification, consistent with reported roles for TET enzymes in controlling lineage commitment in hematopoietic stem cells and embryonic stem cells and establishing TET2 as a bone fide tumor suppressor in squamous cell carcinoma.

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INTRODUCTION

Covalent modification of cytosine residues by methylation is an important regulator of gene transcription (Kohli and Zhang, 2013; Pastor et al., 2013). The DNA methylation–demethylation cycle proceeds through a series of intermediates, with 5-methylcytosine (5-mC) being converted first to 5-hydroxymethylcytosine (5-hmC), then to 5-formylcytosine, to 5-carboxycytosine, and finally to unmethylated cytosine (Kohli and Zhang, 2013; Pastor et al., 2013). Conversion of 5-mC to 5-hmC is carried out by the TET family of enzymes (TET1/2/3) (Ito et al., 2011, 2010; Tahiliani et al., 2009), and these enzymes can also contribute to subsequent oxidation steps (He et al., 2011; Ito et al., 2011). Oxidation of 5-mC to 5-hmC is essential for proper differentiation because embryonic cells from mice lacking *Tet1/2/3* are unable to develop into all germ layers (Ficz et al., 2011; Ito et al., 2010). Regulation of DNA methylation is also important for maintaining stem cells pools in adult tissues, such as the epidermis (Li et al., 2020) and hematopoietic cells (Moran-Crusio et al., 2011; Quivoron et al., 2011).

Reductions in 5-hmC are found in many tumor types (Haffner et al., 2011), including acute myeloid leukemia (AML) (Konstandin et al., 2011), melanoma (Bonvin et al., 2019; Lian et al., 2012), oral squamous cell carcinoma (SCC) (Cuevas-Nunez et al., 2018; Jäwert et al., 2013), and esophageal SCC (Murata et al., 2015), compared with those in normal cells, suggesting global dysregulation of the methylation cycle in cancer. Studies in AML have suggested that TET proteins may function as tumor suppressors responsible for the loss of 5-hmC (Delhommeau et al., 2009; Jankowska et al., 2009; Moran-Crusio et al., 2011; Tefferi et al., 2009). Across all tumor types, *TET2* is mutated at a much higher frequency than *TET1* or *TET3* (Jeschke et al., 2016), and *TET2* inactivating mutations have been correlated with reductions in 5-hmC levels in AML (Ko et al., 2010). Further support for a key role for TET2 in tumorigenesis comes from studies in melanoma, where overexpression of TET2 in melanoma cells with low 5-hmC can suppress tumor growth in vivo (Lian et al., 2012). This tumor suppressor ability depends on the catalytic activity of TET2, which is also required to re-establish the 5-hmC epigenetic landscape (Lian et al., 2012), suggesting that 5-hmC loss in melanoma is indeed due to reduced TET activity. In addition, expression of *TET2* but not *TET1* or *TET3* is significantly correlated with 5-hmC levels in esophageal SCC (Murata

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Abbreviations: 4-NQO, 4-nitroquinoline-N-oxide; 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; AML, acute myeloid leukemia; KC, keratinocyte; SCC, squamous cell carcinoma

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et al., 2015). However, the role of *TET2* loss in causing 5-hmC reductions and promoting SCC tumorigenesis has not been tested.

RESULTS

Global 5-hmC levels are reduced in human cutaneous SCC

The skin is the most frequent site of SCC tumor formation, but the level of 5-hmC in cutaneous SCC has not been examined. We have constructed a human tissue microarray containing 98 cutaneous primary SCC samples and 83 adjacent normal skin samples. These include T1 (n = 10), T2a (n = 20), and T2b (n = 68) tumors evaluated according to the Brigham and Women's Hospital staging system (Jambusaria-Pahlajani et al., 2013; Karia et al., 2014). We have performed immunohistochemistry on the tissue microarray using antibodies against 5-hmC and quantified results by counting the number of positive tumor cells and scoring for relative intensity of staining (Figure 1a). Consistent with previous results in melanoma (Lian et al., 2012), oral SCC (Cuevas-Nunez et al., 2018), and esophageal SCC (Murata et al., 2015), we have found that global 5-hmC levels are reduced in cutaneous SCC compared with those in normal skin; however, we saw no clear association between reductions in 5-hmC and tumor stage (Figure 1b). TET family enzymes have been found to be mutated in many different tumor types (Jeschke et al., 2016), and we sought to examine alterations in SCC across tissues (Han et al., 2018; Hoadley et al., 2018; Li et al., 2015; Lin et al., 2014; Pickering et al., 2014; Song et al., 2014) using the cBio cancer genomics portal (Cerami et al., 2012). Although *TET2* is mutated at a much higher frequency than *TET1* or *TET3* across all tumor types (Jeschke et al., 2016), genomic alterations in *TET1*, *TET2*, and *TET3* were found in 3–4% of SCC tumors, and some samples had alterations in more than one *TET* gene (Figure 1c). Aggressive and metastatic cutaneous SCC tumors (Li et al., 2015; Pickering et al., 2014) had the highest frequency of *TET* gene alterations, and alterations reported were mostly point mutations (Figure 1d). Previous studies in esophageal SCC showed loss of 5-hmC in tumors, which correlated strongly with reductions in *TET2* but not with *TET1* or *TET3* mRNA levels (Murata et al., 2015). Consistent with these data, only point mutations and deletions were found in *TET2* across SCC tumors from all sites, whereas *TET1* and *TET3* were found to be amplified in some tumors. We therefore hypothesized that TET2 may play an important tumor suppressor role in SCC.

TET2 has tumor suppressor functions in SCC

Stratified epithelial tissues function as a barrier from the external environment, and a major driver of human SCC is exposure to carcinogens (Dotto and Rustgi, 2016). We have previously found that oral SCC tumors demonstrate loss of 5-hmC (Cuevas-Nunez et al., 2018), but it was unclear whether *TET2* could function as a tumor suppressor in this context. We employed an oral carcinogenesis model where mice were subjected to 100 μg/ml 4-nitroquinoline-N-oxide (4-NQO) in their drinking water for 8 consecutive weeks and then were analyzed 16 weeks later for tumor formation (Kanojia and Vaidya, 2006). *K14-CreER* mice, which express an inducible Cre recombinase in basal keratinocytes (KCs) (Vasioukhin et al., 1999), were crossed to *Tet2* conditional knockout mice ($Tet2^{L/L}$) (Moran-Crusio et al., 2011). Treatment with 100 mg/kg tamoxifen for 5 consecutive days resulted in dramatically reduced TET2 protein and a modest reduction in 5-hmC levels in the oral mucosa of *K14-CreER* $Tet2^{L/L}$ mice compared with that in *K14-CreER* $Tet2^{+/+}$ mice (Figure 2a). Tamoxifen-treated *K14-CreER* $Tet2^{L/L}$ (n = 16) and $Tet2^{L/L}$ (n = 5) mice were then given 4-NQO in their drinking water. In addition, *K14-CreER* $Tet2^{L/L}$ (n = 6) and $Tet2^{L/L}$ (n = 3) mice that did not receive tamoxifen were also given 4-NQO in their drinking water. After treatment with 4-NQO, mice developed dysplasia, papillomas, and SCC (Figure 2b), and oral SCC tumors showed loss of TET2 protein (Figure 2c). We observed a significant increase in the number of total lesions on loss of *Tet2* in the oral mucosa (Figure 2d). In addition, a significantly higher percentage of lesions that formed in $Tet2^{-/-}$ mice were oral SCC tumors, whereas lesions that developed in $Tet2^{+/+}$ mice were mostly dysplasia and benign papillomas (Figure 2e).

Germline loss of *Tet2* leads to myeloid transformation with long latency (Moran-Crusio et al., 2011), and no phenotypes were reported in stratified epithelial tissues despite expression of TET2 in the oral cavity and the skin (Figures 2a, 3a and b), suggesting the need for cooperating events to drive tumorigenesis. The *TP53* tumor suppressor is inactivated in most SCC tumors (Cancer Genome Atlas Network, 2015; Cancer Genome Atlas Research Network, 2012) and can promote genome stability (Lane, 1992) and help to regulate DNA methylation (Tovy et al., 2017). We hypothesized that loss of *Tp53* would cooperate with *Tet2* loss to drive SCC tumorigenesis. We crossed $p53^{Flox}$ mice (Marino et al., 2000) with *K14-CreER* $Tet2^{L/L}$ mice and treated mice aged 6 weeks with 100 mg/kg tamoxifen for 5 consecutive days, resulting in loss of TET2 protein and reduced 5-hmC levels in the epidermis (Figure 3b). As expected, no tumors developed in control $Tet2^{+/+,\ L/+,\ or\ L/L}$ $p53^{+/+,\ L/+,\ or\ L/L}$ mice lacking the *K14-CreER* transgene after tamoxifen treatment (Figure 3c). Consistent with studies of germline $Tet2^{-/-}$ mice (Moran-Crusio et al., 2011; Quivoron et al., 2011), no SCC tumors developed in *K14-CreER* $Tet2^{L/+}$ $p53^{+/+}$ or *K14-CreER* $Tet2^{L/L}$ $p53^{+/+}$ mice (Figure 3c). SCC tumors developed at low frequency in mice lacking one allele of *p53*; however, there was no significant difference in tumor penetrance at 500 days between *K14-CreER* $Tet2^{+/+}$ $p53^{L/+}$, *K14-CreER* $Tet2^{L/+}$ $p53^{L/+}$, or *K14-CreER* $Tet2^{L/L}$ $p53^{L/+}$ mice, as assessed by Fisher's exact test (Table 1). There was also no significant difference between these groups in SCC tumor–free survival assessed by Log-rank test (Figure 3c). In contrast to mice lacking one allele of *p53*, *K14-CreER* $Tet2^{L/L}$ $p53^{L/L}$ mice had a significantly shorter SCC-free survival than *K14-CreER* $Tet2^{L/+}$ $p53^{L/L}$ mice ($P = 0.0127$; Log-rank test) and *K14-CreER* $Tet2^{+/+}$ $p53^{L/L}$ mice ($P = 0.0308$; Log-rank test). *K14-CreER* $Tet2^{L/L}$ $p53^{L/L}$ mice also had a significantly higher tumor penetrance at 500 days than *K14-CreER* $Tet2^{+/+}$ $p53^{L/L}$ mice (83.3% vs. 33.3%; $P = 0.0106$, Fisher's exact test) (Table 1). Finally, although *K14-CreER* $Tet2^{L/L}$ $p53^{+/+}$ mice did not develop tumors, SCC lesions formed in *K14-CreER* $Tet2^{L/L}$ $p53^{L/+}$ mice at low penetrance at 500 days, which was significantly lower than that in *K14-CreER* $Tet2^{L/L}$ $p53^{L/L}$ mice (4.6% vs. 83.3%; $P < 0.0001$, Fisher's exact test)

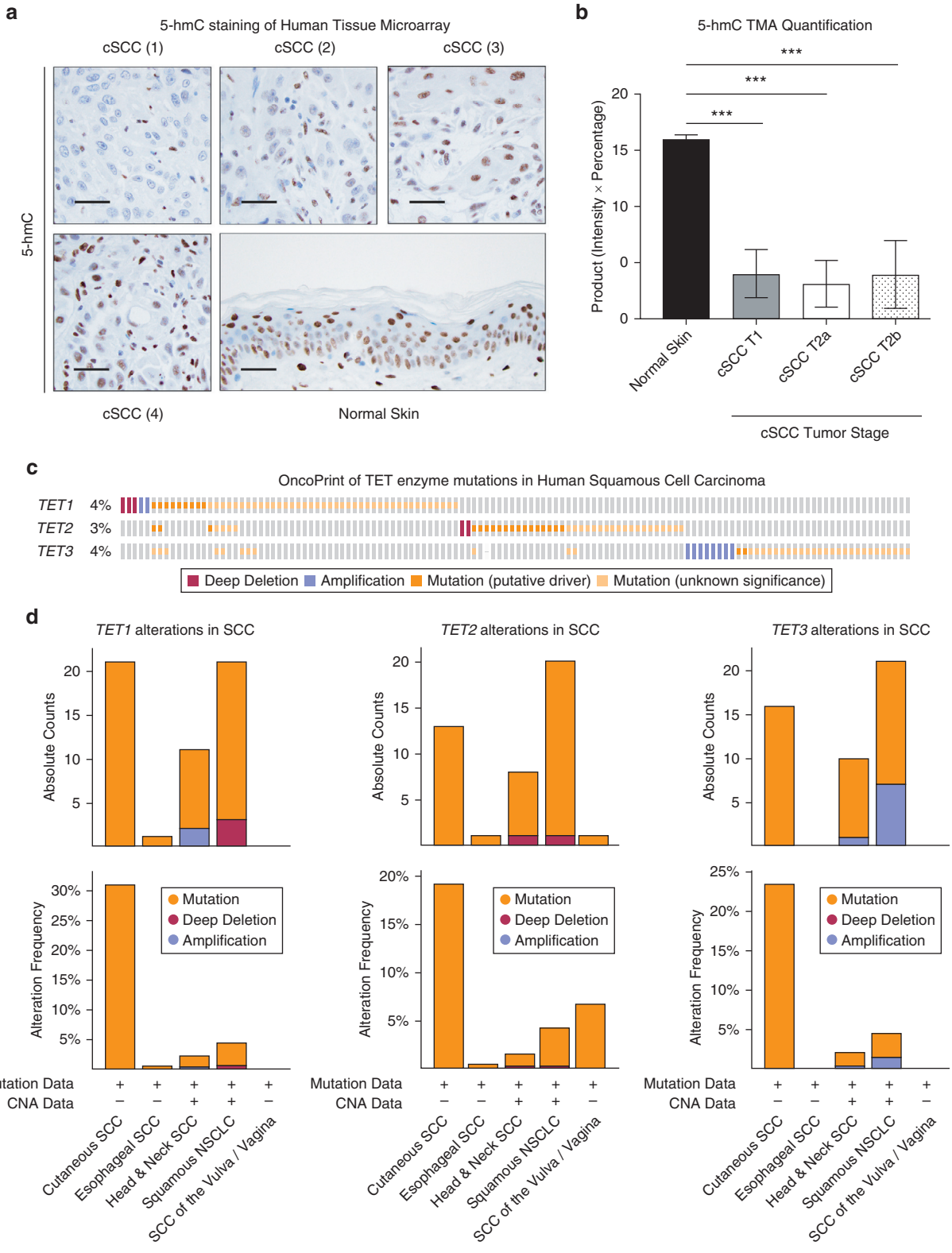


Figure 1. Altered regulation of 5-hmC in human SCC. (**a**) Representative samples of immunohistochemical staining of global levels of 5-hmC (brown) in the human cutaneous SCC tumors and normal skin from the tissue microarray. Numbers indicate the scoring of percentage and intensity. Bar = 100 μm. (**b**) Quantification of tumor-adjacent skin (n = 83) and stage T1 (n = 10), T2a (n = 20), and T2b (n = 68) cutaneous SCC tumors. ***$P < 0.001$ assessed by Student's unpaired *t*-test. (**c**) Percentage and type of genomic alterations in *TET1*, *TET2*, and *TET3* reported in SCC tumors across tissues (Han et al., 2018; Hoadley et al., 2018; Li et al., 2015; Lin et al., 2014; Pickering et al., 2014; Song et al., 2014) assessed using the cBio cancer genomics portal (Cerami et al., 2012). Only samples with alterations are shown. (**d**) Absolute counts (top) and frequency (bottom) of genomic alterations in *TET1*, *TET2*, and *TET3* found in the indicated SCC tumor type. 5-hmC, 5-hydroxymethylcytosine; CNA, copy number alterations; cSCC, cutaneous squamous cell carcinoma; NSCLC, nonsmall cell lung cancer; SCC, squamous cell carcinoma; TMA, tissue microarray.

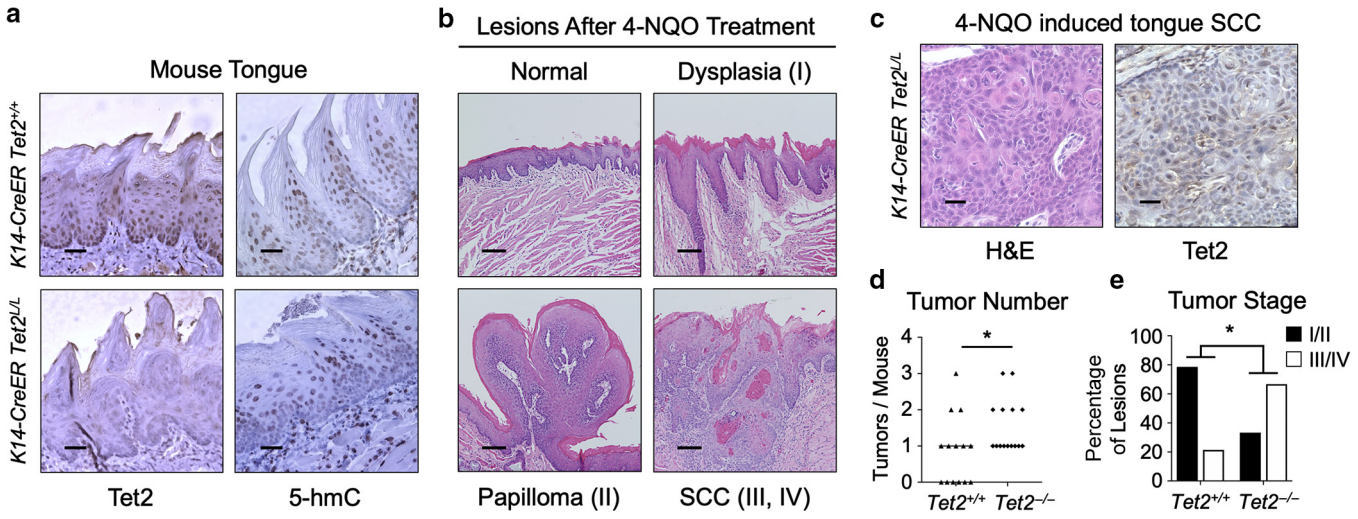


Figure 2. TET2 functions as a tumor suppressor in carcinogen-induced oral SCC. (**a**) Immunohistochemical staining of TET2 (left) and 5-hmC (right) in the tongue of mice of indicated genotype after tamoxifen treatment. Bar = 50 μm. (**b**) Representative images of each tumor grade in the tongue after 4-NQO treatment. Note that invasive SCC tumors were designated grade IV. Bar = 500 μm. (**c**) Histology (left) and immunohistochemical staining of TET2 (right) in an SCC tumor from a *K14-CreER Tet2*$^{L/L}$ mouse treated with tamoxifen and 4-NQO. Bar = 100 μm. (**d**) Quantification of oral lesions from *Tet2*$^{+/+}$ (*Tet2*$^{L/L}$ + tamoxifen, n = 5; *Tet2*$^{L/L}$ + vehicle, n = 3; *K14-CreER Tet2*$^{L/L}$ + vehicle, n = 6) or *Tet2*$^{-/-}$ (*K14-CreER Tet2*$^{L/L}$ + tamoxifen, n = 15) mice after treatment with 4-NQO. **P* < 0.05 by Mann–Whitney test. (**e**) Quantification of highest lesion grade in *Tet2*$^{+/+}$ (*Tet2*$^{L/L}$ + tamoxifen, n = 5; *Tet2*$^{L/L}$ + vehicle, n = 3; *K14-CreER Tet2*$^{L/L}$ + vehicle, n = 6) or *Tet2*$^{-/-}$ (*K14-CreER Tet2*$^{L/L}$ + tamoxifen, n = 16) mice after treatment with 4-NQO. **P* < 0.05 by Fisher's exact test. 4-NQO, 4-nitroquinoline-N-oxide; 5-hmC, 5-hydroxymethylcytosine; SCC, squamous cell carcinoma.

Figure 3. Loss of *Tet2* cooperates with *p53* loss in murine cutaneous SCC tumorigenesis. (**a**) Immunohistochemical staining of TET2 in normal human skin. Bar = 100 μm. (**b**) Immunohistochemical staining of TET2 (left) and 5-hmC (right) in the skin of mice of indicated genotype after tamoxifen treatment. Bar = 50 μm. (**c**) Kaplan–Meyer survival curve of SCC formation in mice of indicated genotype after treatment with 100 mg/kg tamoxifen for 5 days. **P* < 0.05 and ****P* < 0.05 by multiple-measures ANOVA. (**d**) H&E and immunohistochemical staining for p63, 5-hmC, and TET2 in a cSCC tumor from a tamoxifen-treated *K14-CreER Tet2*$^{L/L}$ *p53*$^{L/L}$ mouse. Immunohistochemical staining for TET2 in the normal skin from the same tumor-bearing mouse (right). Bar = 50 μm. 5-hmC, 5-hydroxymethylcytosine; cSCC, cutaneous squamous cell carcinoma; SCC, squamous cell carcinoma.

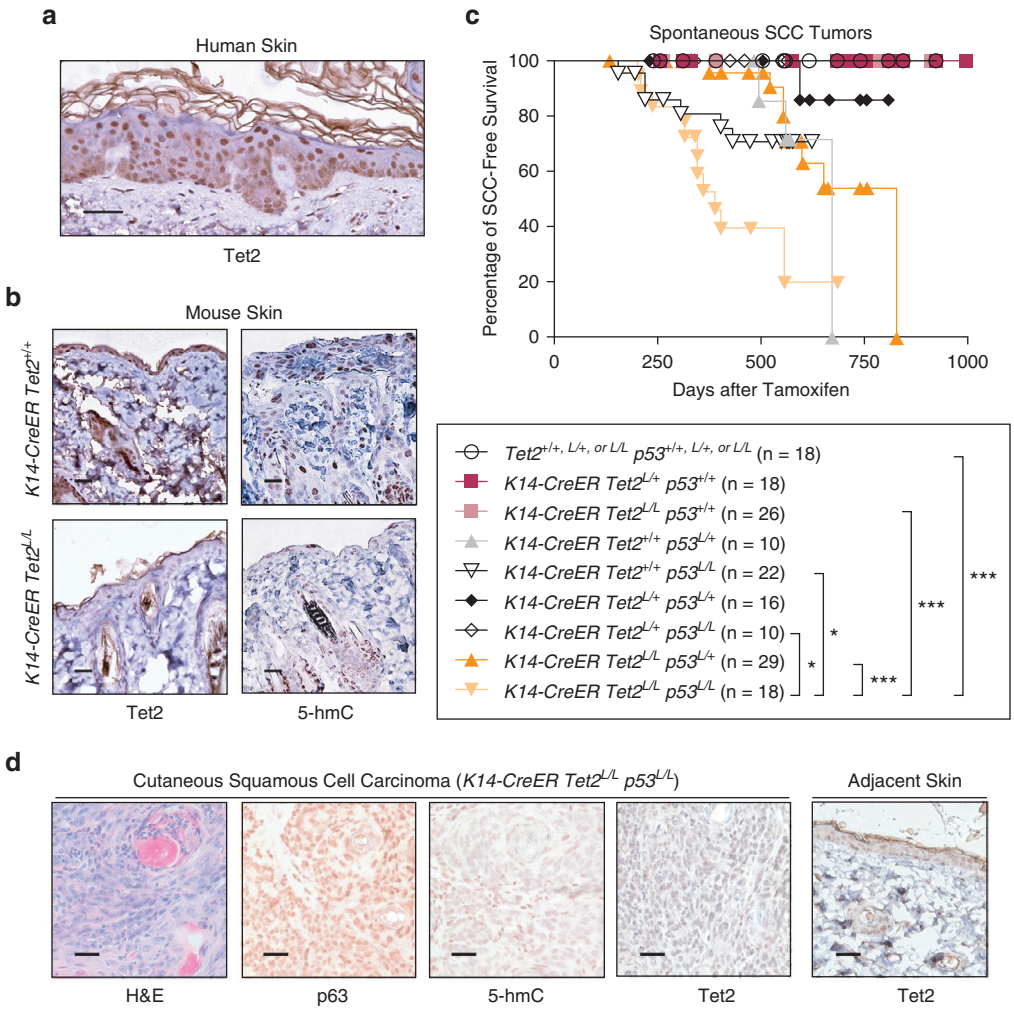


Table 1. Tumor Penetrance of Spontaneous Squamous Cell Carcinoma

Genotype	Number of SCC Tumor–Bearing Mice at 500 d	Number of SCC Tumor–Free Mice at 500 d
Tet2$^{+/+,\ L/+,\ or\ L/L}$ *p53*$^{+/+,\ L/+,\ or\ L/L}$	0	14
K14-CreER Tet2$^{L/+}$ *p53*$^{+/+}$	0	15
K14-CreER Tet2$^{L/L}$ *p53*$^{+/+}$	0	16
K14-CreER Tet2$^{+/+}$ *p53*$^{L/+}$	1	8
K14-CreER Tet2$^{L/+}$ *p53*$^{L/+}$	0	10
K14-CreER Tet2$^{L/L}$ *p53*$^{L/+}$	1	21
K14-CreER Tet2$^{+/+}$ *p53*$^{L/L}$	6	16
K14-CreER Tet2$^{L/+}$ *p53*$^{L/L}$	0	5
K14-CreER Tet2$^{L/L}$ *p53*$^{L/L}$	10	3

Abbreviation: SCC, squamous cell carcinoma.

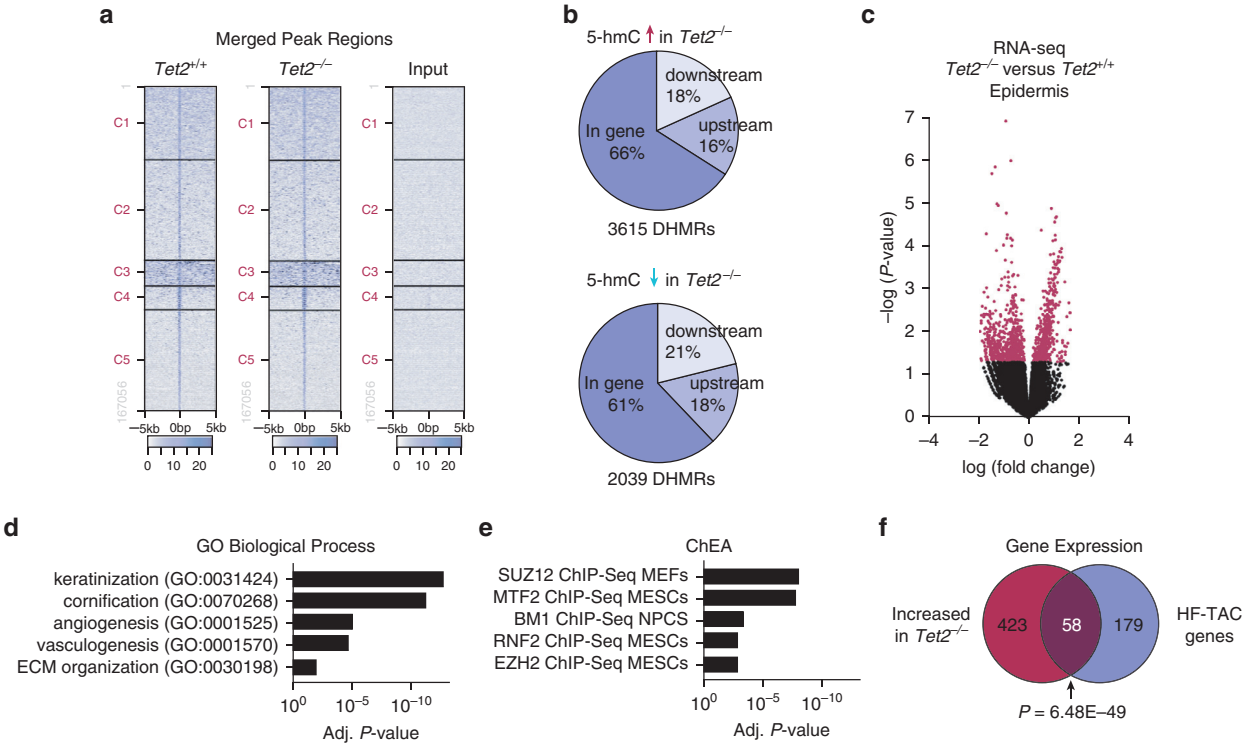
(Table 1). In addition, *K14-CreER Tet2*$^{L/L}$ *p53*$^{L/L}$ mice had a significantly shorter SCC tumor–free survival than *K14-CreER Tet2*$^{L/L}$ *p53*$^{L/+}$ mice ($P = 0.0001$; Log-rank test). Tumors developed in various locations, including the back, the legs, and the head, and developed from the epidermis. Cutaneous SCC tumors that developed in *K14-CreER Tet2*$^{L/L}$ *p53*$^{L/L}$ mice showed keratinization and stained strongly for p63, confirming their squamous identity (Figure 3d). Consistent with our human cSCC data (Figure 1a), we observed low global 5-hmC staining in SCC tumors with loss of TET2 protein (Figure 3d). These data show that loss of *Tet2* cooperates with loss of *Tp53* in stratified epithelial tissues to drive the formation of SCC.

Loss of *Tet2* in the epidermis alters the patterns of 5-hmC

Although the regulation of DNA hydroxymethylation has been examined in cultured KCs (Rinaldi et al., 2016), the function of TET2-dependent regulation of 5-hmC in KCs in vivo is unclear. To identify site-specific changes in 5-hmC patterns in the epidermis, *K14-CreER Tet2*$^{L/L}$ mice were treated with 100 mg/kg tamoxifen or vehicle control daily for 5 consecutive days, and then the epidermis was isolated from mice after 2 weeks. Samples from *Tet2*$^{+/+}$ or *Tet2*$^{-/-}$ skin were pooled, DNA was isolated, and genome-wide levels of 5-hmC were assessed by 5-hmC DNA immunoprecipitation followed by sequencing. Short-term loss of *Tet2* in KCs had minimal effects on global 5-hmC levels, and overall patterns of 5-hmC were similar between *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ epidermal KCs (Figure 4a). However, analysis of site-specific changes revealed 3,615 regions that had significantly increased 5-hmC in *Tet2*$^{-/-}$ epidermis compared with the epidermis in *Tet2*$^{+/+}$ mice, whereas 2,039 regions in the genome had significantly reduced 5-hmC (Figure 4b and Supplementary Table S1). Analysis of the location of these differentially hydroxymethylated regions revealed that the majority were found within gene bodies (Figure 4b). Changes in 5-hmC can alter gene expression through both proximal and long-range effects (Pastor et al., 2013). To link 5-hmC changes to gene expression, we performed RNA sequencing on the isolated epidermis of *K14-CreER Tet2*$^{L/L}$ mice treated with tamoxifen (*Tet2*$^{-/-}$) or vehicle control (*Tet2*$^{+/+}$) and identified 791 significant genes with >1.5-fold change (Figure 4c and Supplementary Table S2). Gene Ontology analysis (Ashburner et al., 2000) found enrichment in genes related to keratinization, cornification, and extracellular matrix organization (Figure 4d). Interestingly, examination of enriched transcription factor–binding sites in our gene set using the Chromatin Immunoprecipitation Enrichment Analysis database (Lachmann et al., 2010) found enrichment for PcG proteins (Figure 4e), which can interact with DNMT1 to regulate DNA methylation (Viré et al., 2006) and have been found to control lineage selection in the epidermis (Dauber et al., 2016; Lien et al., 2011). Previous studies have defined gene signatures for various cell populations in the mouse skin (Rezza et al., 2016), and we compared them with the gene expression changes in the epidermis after loss of *Tet2*. Interestingly, there was significant overlap between the genes increased after *Tet2* excision and hair follicle transient amplifying cell genes (Figure 4f), whereas there was minimal overlap with hair follicle stem cells, outer root sheath cells, hair matrix cells, or interfollicular epidermis cells (Supplementary Figure S1a). Consistent with this, we noted an increase in extracellular matrix genes *Lama2*, *Col15a1*, *Fbn1*, and *Spon1* expressed in the hair germ (Tsutsui et al., 2021) as well as key regulators of hair follicle transient amplifying cells (*Wnt5a, Lef, Msx1*) reported to be regulated by PcG proteins (Lien et al., 2011) (Figure 4g). In addition, we noted a reduction in epidermal (*Flg, Sprr1b*) and sebocyte (*Krt7, Scd3*) differentiation markers, consistent with the acquisition of a less differentiated hair follicle transient amplifying cell state. We noted increased transcription and significant increases in 5-hmC levels in the gene body of the *Msx1* and *Wnt5a* genes after *Tet2* excision (Figure 4h), consistent with previously reported associations between high 5-hmC levels in genes and transcriptional activation (Ficz et al., 2011). We also observed increased 5-hmC levels at Encyclopedia of DNA Elements–identified enhancer regions (ENCODE Project Consortium et al., 2020) distal to the *Krt7* gene (Supplementary Figure S1b), many of which have been shown to have long-range interactions with the *Krt7* gene (Dixon et al., 2012). In total, these data suggest that TET2 modulates 5-hmC levels to control both proximal and long-range chromatin interactions regulating KC lineage choice.

DISCUSSION

Reduced global levels of 5-hmC are found in many tumor types, including AML (Konstandin et al., 2011), melanoma (Lian et al., 2012; Saldanha et al., 2017), and SCC (Cuevas-Nunez et al., 2018; Jäwert et al., 2013; Murata et al., 2015) when compared with that in normal cells. Our data show that cutaneous SCC, which results in a similar number of deaths per year to that for melanoma (Karia et al., 2013; Siegel et al., 2021), exhibits globally reduced levels of 5-hmC (Figure 1a and b). Interestingly, unlike melanoma, we did not see a difference between low-stage and high-stage tumors (Figure 1b), although it should be noted that lower-stage tumor numbers were limited (T1: n = 10 and T2a: n = 20). The TET family of enzymes are the main regulators of cytosine methylation (Ito et al., 2011), leading to great interest in their role in controlling 5-hmC levels in tumors. Mutational inactivation of *TET2* was first described in human myeloid cancers (Delhommeau et al., 2009; Jankowska



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Genes upregulated in *Tet2*$^{-/-}$ epidermis	
Hair structural proteins	***Krt25, Krt26, Krt27, Krt28, Krt31,*** *Krt33a, Krt33b, Krt34,* ***Krt35, Krt36, Krt71,*** *Krt72,* ***Krt73,Krt81, Krt82, Krt83,*** *Krt86,* ***Krtap8-1,*** *Nes,* ***Tchh, Crnn, S100A3***
ECM/adhesion	*Lama2, Lamb2, Lamc1, Itga1, Itgb3, Collla1, Col15a1, Col6a3, Col8a1, Egfl6, Ntn4, Fbn1, Tnn, Spon1*
Transcription factors	***Hoxc13, Msx1, Msx2, Lef1, Ascl4,*** *Foxe1, Hes2, Hey2, Klf2, Dlx1, Tcf15, Sox17, Lhx6*
Hedgehog signaling	***Shh, Ptch2,*** *Hhip, Dhh*
Bmp signaling	***Bambi,*** *Bmp5, Acvrl1, Smad6*
Wnt signaling	*Wif1, Wnt5a, Wnt5b, Wnt11, Fzd9, Rspo4, Ror2*
Genes downregulated in *Tet2*$^{-/-}$ epidermis	
Epidermal differentiation	*Flg, Lce1g, Rptn, Sprr1b, Sprr2a1, Sprr2a3, Krt7, Scd3*

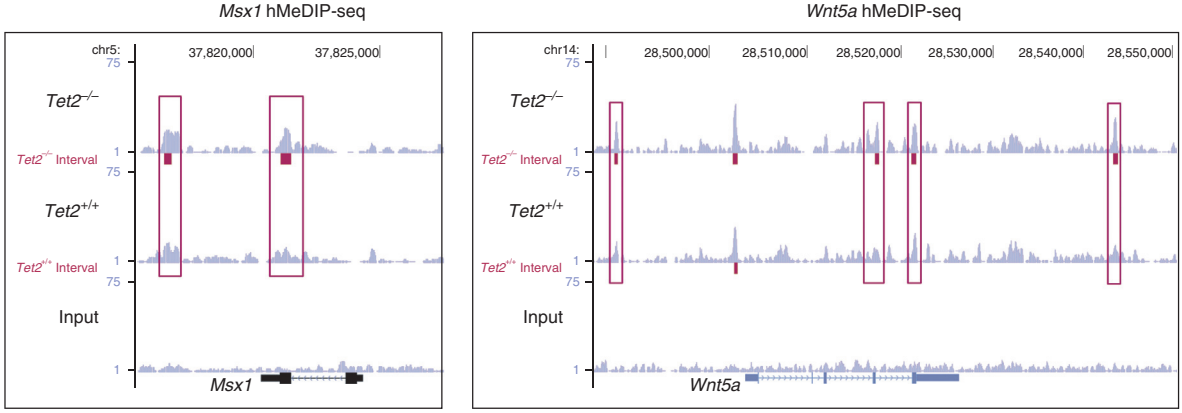

Figure 4. TET2 regulates an epidermal identity program in the skin. (**a**) Comparison of merged peak regions from hMeDIP-seq in *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ epidermis. (**b**) Location of DHMRs where 5-hmC is increased in *Tet2*$^{-/-}$ epidermis (top) or decreased in *Tet2*$^{-/-}$ epidermis (bottom) compared with those in *Tet2*$^{+/+}$ epidermis. (**c**) Volcano plot of transcript levels in *Tet2*$^{-/-}$ compared with those in *Tet2*$^{+/+}$ epidermis determined by RNA-seq. (**d**) Top five most significant GO terms in RNA-seq dataset. (**e**) Top five most significant ChEA terms in RNA-seq dataset. (**f**) Overlap between genes upregulated after *Tet2* loss in the epidermis and HF-TAC genes as defined by Rezza et al. (2016). (**g**) Significantly altered genes in the epidermis of mice after *Tet2* excision. Bold indicates the gene is part of the HF-TAC signature. (**h**) hMeDIP-seq peaks in *Msx1* and *Wnt5a* genes from the murine epidermis of the indicated genotype. Boxes highlight significant merged peak regions found in only one sample. 5-hmC, 5-hydroxymethylcytosine; Adj, adjusted; ChEA, Chromatin Immunoprecipitation Enrichment Analysis; DHMR, differentially hydroxymethylated region; ECM, extracellular matrix; GO, Gene Ontogeny; HF-TAC, Hair follicle transient amplifying cell; hMeDIP-seq, 5-hydroxymethylcytosine DNA immunoprecipitation followed by sequencing; RNA-seq, RNA sequencing.

et al., 2009; Tefferi et al., 2009), and all the three *TET* genes have since been shown to be altered in a wide range of tumor types (Jeschke et al., 2016), including SCC (Figure 1c and d). Previous work in melanoma has shown that the global dysregulation of 5-hmC can be reversed by overexpression of TET2 (Lian et al., 2012), and recent work in murine model systems has shown a tumor-suppressor role for *Tet2* in melanoma (Bonvin et al., 2019). We have previously shown reduced levels of 5-hmC in a 4-NQO–induced oral carcinogenesis model (Cuevas-Nunez et al., 2018), and it has recently been shown that TET2 levels are reduced in higher-grade 4-NQO–induced SCC lesions (Huang et al., 2020), but it was unclear whether TET2 could in fact act as a tumor suppressor. In this study, we show that *TET2* loss can indeed lead to a reduction in 5-hmC levels in epithelial cells (Figures 2a and 3b) and function as a tumor suppressor in carcinogen-induced and spontaneous SCC models (Figures 2d and e and 3c).

Similar to many tumor suppressors, inactivation of *Tet2* alone is insufficient to cause SCC tumorigenesis, and there were no reported skin phenotypes in germline $Tet2^{-/-}$ mice (Moran-Crusio et al., 2011; Quivoron et al., 2011). Tumor formation in $Tet2^{-/-}$ mice requires a second hit such as $Flt3^{ITD}$ mutation (Shih et al., 2015) or $N\text{-}Ras^{G12D}$ mutation (Kunimoto et al., 2018) to drive AML or $N\text{-}Ras^{Q61K}$ mutation to promote melanoma (Bonvin et al., 2019). Indeed, we find that loss of *Tet2* in KCs does not induce SCC tumors, but when combined with a loss of *Tp53*, it results in a very high spontaneous tumor incidence and dramatically decreased median survival. Interestingly, TET2 appears to function as a phenotypic modifier of *TP53* loss in epithelial tissues. Inactivation of *TP53* alone leads to the formation of SCC tumors, but an additional loss of *Tet2* increases tumor penetrance and reduces tumor-free survival, which contrasts with the lack of phenotype seen with only *TET2* loss (Figure 3c and Table 1). This result is also consistent with human genetic data, where a mutation in *TP53* is thought to be an early event in SCC formation (Inman et al., 2018; Yilmaz et al., 2017), whereas *TET2* mutations appear to occur at higher frequency in late-stage SCC tumors (Inman et al., 2018; Li et al., 2015).

Consistent with previous results in melanoma (Lian et al., 2012), oral SCC (Cuevas-Nunez et al., 2018), and esophageal SCC (Murata et al., 2015), we have found that global levels of 5-hmC are reduced in cutaneous SCC compared with that in adjacent skin (Figure 1a and b). Despite these profound changes in tumors, changes in 5-hmC levels in normal skin on the loss of *Tet2* were modest. These data could be explained by compensation by TET1 and TET3 in the regulation of 5-hmC in the normal epidermis, which may become more dysregulated as cells progress toward malignancy. This compensation model is supported by the fact that some human SCC tumors have mutations in more than one *TET* gene (Figure 1c). Alternatively, loss of *TET2* may offer a competitive advantage to cells during tumor formation, and a small population of cells in the epidermis with highly reduced 5-hmC may out compete others. It is worth noting that cSCC tumors do not generally have a loss of 5-hmC in all cells but have a heterogeneous mixture of cells with differing levels of 5-hmC expression. We have found that loss of *TET2* in the epidermis resulted both in site-specific losses and gains in 5-hmC levels (Figure 3). This is consistent with results seen in melanoma (Bonvin et al., 2019), where comparisons between benign nevi and melanoma also showed site-specific gains and losses of 5-hmC. TET2 has been shown not only to convert 5-mC to 5-hmC but also to facilitate the subsequent enzymatic reactions converting 5-hmC to 5-formylcytosine and then to 5-carboxycytosine (Ito et al., 2010). This suggests that TET2 may play a functional role both in the conversion of 5-mC to 5-hmC as well in the subsequent enzymatic reaction to convert 5-hmC to 5-formylcytosine in the normal epidermis in vivo. However, it is important to note that these results may be specific to the epidermis because there is a wide variation in 5-hmC levels across tissue types (Nestor et al., 2012).

TET enzymes are essential for proper lineage commitment and differentiation in embryonic stem cells (Dawlaty et al., 2014; Ficz et al., 2011), and loss of *TET2* has been shown to increase hematopoietic stem cell self-renewal (Moran-Crusio et al., 2011; Quivoron et al., 2011) and alter the differentiation of hematopoietic precursors (Ko et al., 2010). In the human epidermis, increased global 5-hmC levels (Lian et al., 2012) and high TET2 expression (Figure 3a) are found in more differentiated layers of the epidermis. We have found that *TET2* loss reduces differentiation genes while increasing the expression of genes associated with hair follicle transient amplifying cells (Rezza et al., 2016). Consistent with these findings, *K14-CreER* $Dnmt1^{-/-}$ mice were reported to have decreased proliferation and reduced upward migration in transit-amplifying cells in the hair matrix (Li et al., 2012), supporting a role for DNA methylation–dependent regulation of this cell population. Given the links between epidermal lineage choice and regulation by PcG proteins (Dauber et al., 2016; Lien et al., 2011), it is interesting to note the enrichment for PcG-regulated genes that are activated after the loss of *TET2* in the epidermis (Figure 4e) because DNMT1, DNMT3A, and DNMT3B as well as TET1 have been found to interact with PRC2 (Neri et al., 2013; Viré et al., 2006). TET1, TET2, and TET3 have both unique and overlapping roles (Putiri et al., 2014), and important future studies will be needed to dissect the functions of TET1 and TET3 in regulating epidermal lineage choice and their interactions with PcG proteins in epidermal lineages.

In total, our data support the hypothesis that loss of *TET2* in KCs leads to skewing of epidermal lineages, creating an expanded pool of cells that are susceptible to oncogenic transformation. We find that *TET2* loss resulted in a larger number and more advanced lesions after chemical carcinogenesis (Figure 2b). Furthermore, combined loss of *TET2* and *TP53* increased spontaneous SCC tumor development compared with *TP53* loss alone (Figure 3b and Table 1), similar to reports of $N\text{-}Ras^{Q61K}$-driven melanomas (Ito et al., 2011). After exposure to environmental carcinogens, this progenitor population may be more likely to progress toward malignant transformation, especially when combined with loss of *p53*, which compromises repair of DNA damage.

MATERIALS AND METHODS

Experimental animals

Standard breeding procedures of previously described *K14-CreER* (stock #005107; The Jackson Laboratory, Bar Harbor, ME) (Vasioukhin et al., 1999), $p53^{Flox}$ (stock # 008462; The Jackson Laboratory) (Marino et al., 2000), and $Tet2^{Flox}$ (stock #017573; The Jackson Laboratory) (Moran-Crusio et al., 2011) mice were used to generate all cohorts, which were a mixed C57Bl6/J-CD-1 background. To excise conditional alleles, mice were injected intraperitoneally with 100 mg/kg tamoxifen (#T5648; Sigma-Aldrich, St. Louis, MO) dissolved in sunflower seed oil (#S5007; Sigma-Aldrich) daily for 5 consecutive days. Oral tumors were induced by the addition of 100 μg/ml 4-Nitroquinoline-N-oxide (#N8141; Sigma-Aldrich) to the drinking water, which was changed weekly. After 8 weeks of treatment, mice were given normal drinking water for 16 weeks and were then assessed for tumor numbers (Kanojia and Vaidya, 2006). Tumor grades for 4-NQO lesions were assessed by a double-blind reading of two pathologists (DW and CGL). For 5-hmC DNA immunoprecipitation followed by sequencing and RNA sequencing analysis, *K14-CreER* $Tet2^{L/L}$ mice were treated with 100 mg/kg tamoxifen or vehicle control daily for 5 consecutive days. Two weeks after the first dose of tamoxifen, the epidermis was isolated by soaking samples in 0.2% dispase at 4 °C for 1 hour and then manually separating the epidermis from the dermis.

5-hmC DNA immunoprecipitation followed by sequencing

K14-CreER $Tet2^{L/L}$ mice were treated with tamoxifen (n = 2) or vehicle control (n = 2), and samples from each group were pooled. Genomic DNA from murine epithelial preps was purified, sonicated, and then ligated to Illumina barcode adaptors. Ligated DNA was denatured and incubated with a 5-hmC antibody (#39769; Active Motif, Carlsbad, CA) overnight at 4 °C, and then immunoprecipitated DNA was purified and sequenced using standard Illumina protocols (Xu et al., 2011). 75-nt sequence reads were aligned to the mouse genome (mm10) using the Burrow–Wheeler Aligner algorithm with default settings, and only reads that pass Illumina's purity filter, align with no more than two mismatches, and map uniquely to the genome were used in the subsequent analysis. Differentially hydroxymethylated regions were identified using MACS2 (Zhang et al., 2008).

Tissue microarray

The tissue microarray consisting of cutaneous SCC specimens and adjacent skin from Mohs surgery performed at Brigham and Women's Hospital (Boston, MA) was constructed upon appropriate approval from the institutional review board. Stains were manually scored by a pathologist (CGL) who was blinded to sample identity. Scores were derived by multiplying the percentage of immunoreactive cells (0% = 0, 1–9% = 1, 10–24% = 2, 25–74% = 3, 75–100% = 4) by staining intensity (negative = 1, weak = 2, moderate = 3, strong = 4) as described previously (Lian et al., 2012).

Study approvals

All mice were housed and treated in accordance with protocols approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee. The Mass General Brigham Institutional Review Board approved the use of tissues for this study. All human tissue studies used exclusively deidentified and discarded material collected in the course of routine clinical care, for which the Mass General Brigham Institutional Review Board has determined that signed informed consent was not required.

Data availability statement

The sequence datasets generated and analyzed during this study are available in the National Center for Biotechnology Information Gene Expression Omnibus repository under accession numbers GSE167949 (5-hmC DNA immunoprecipitation followed by sequencing and RNA sequencing).

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CONFLICT OF INTEREST

CDS is a steering committee member for Castle Biosciences; is a steering committee member and consultant for Regeneron Pharmaceuticals; is a consultant for Sanofi; has received research funding from Castle Biosciences, Regeneron Pharmaceuticals, Novartis, Genentech, and Merck; and is a chair for National Comprehensive Cancer Network. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MRR, CGL; Data Curation: MRR; Formal Analysis: MRR; Methodology: MRR, CGL; Project Administration: MRR; Resources: CDS; Supervision: MRR; Validation: RB, YW, DW, SX, MW; Visualization: MRR; Writing - Original Draft Preparation: MRR; Writing - Review and Editing: MRR, RB, YW, DW, CGL

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.09.026.

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SUPPLEMENTARY MATERIALS AND METHODS

Immunohistochemistry

Immunohistochemistry studies were performed on formalin-fixed, paraffin-embedded mouse tissue. Sections were deparaffinized, rehydrated, and blocked with 3% hydrogen peroxide. Antigen retrieval was performed by heating slides in a pressure cooker for 45 minutes with Target Antigen Retrieval Solution (#S1699; Dako, Carpinteria, CA). Sections were blocked with 10% goat serum for 30 minutes, and the following primary antibodies were incubated at 4 °C overnight: rabbit anti–5-hydroxymethylcytosine antibody (#39769; Active Motif, Carlsbad, CA), TET1 (#GTX124207; GeneTex, Irvine, CA), TET2 (#21207-1-AP; Thermo Fisher Scientific, Waltham, MA), or mouse anti-p63 (#CM163; Biocare, Brookline, MA). Sections were then incubated with the following horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature: goat anti-mouse IgG (H+L) (#626520; Invitrogen, Carlsbad, CA) and goat anti-rabbit IgG (H+L) (#PI-1000; Vector Laboratories, Burlingame, CA). Color development was performed using a DAB chromogen solution (#K3468; Dako, Santa Clara, CA) within an incubation time of 5 minutes. Sections were counterstained with Harris Hematoxylin (#245-697, Thermo Fisher Scientific, Kalamazoo, MI) and treated with Defining Solution (#310-350; Thermo Fisher Scientific) and followed by Bluing Solution (#HXB00242E; American MasterTech Scientific, McKinney, TX).

RNA sequencing

K14-CreER Tet2$^{L/L}$ mice were treated with tamoxifen (n = 3) or vehicle control (n = 3), and mRNA was isolated from murine epithelial preps, followed by cDNA synthesis, adaptor ligation, PCR enrichment, and paired-end sequencing on Illumina HiSeq. Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic (version 0.36), then mapped to the Mus musculus GRCm38 reference genome available on ENSEMBL using the STAR aligner, version 2.5.2b. Unique gene hit counts were calculated using featureCounts from the Subread package, version 1.5.2. Only unique reads that fell within exon regions were counted. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between groups of samples was performed. The Wald test was used to generate *P*-values and $\log_2$ fold changes.

SUPPLEMENTARY REFERENCE

Rezza A, Wang Z, Sennett R, Qiao W, Wang D, Heitman N, et al. Signaling networks among stem cell precursors, transit-amplifying progenitors, and their niche in developing hair follicles. Cell Rep 2016;14:3001–18.

Supplementary Figure S1. TET2 regulation of keratinocytes.
(**a**) Overlap between the genes upregulated after *Tet2* loss in the epidermis and HF-TAC genes as defined by Rezza et al. (2016). (**b**) hMeDIP-seq peaks associated with enhancer regions of the *Krt7* gene from the murine epidermis of the indicated genotype. HF-SC, XXX; HF-TAC, hair follicle transient amplifying cell; hMeDIP-seq, 5-hydroxymethylcytosine DNA immunoprecipitation followed by sequencing; ORS, outer root sheath.