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Combined *Kdm6a* and *Trp53* deficiency drives the development of squamous cell skin cancer in mice

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Abstract

Cutaneous squamous cell carcinoma (cSCC) has among the highest mutation burdens of all cancers, reflecting its pathogenic association with the mutagenic effects of ultraviolet light exposure. Although mutations in cancer-relevant genes like *TP53* and *NOTCH1* are common in cSCC, they are also tolerated in normal skin and suggest that other events are required for transformation; it is not yet clear whether epigenetic regulators cooperate in the pathogenesis of cSCC. *KDM6A* encodes a histone H3K27me2/me3 demethylase that is frequently mutated in cSCC and other cancers. Prior sequencing studies indicate that roughly 7% of cSCC samples harbor *KDM6A* mutations, including frequent truncating mutations suggesting a role for this gene as a tumor suppressor in cSCC. Mice with epidermal deficiency of both *Kdm6a* and *Trp53* exhibited 100% penetrant, spontaneous cSCC development within a year, and exome sequencing of resulting tumors reveals recurrent mutations in *Ncstn* and *Vcan*. Four of sixteen tumors exhibited deletions in large portions of chromosome 1 involving *Ncstn*, while another 25% of tumors harbored deletions in chromosome 19 involving *Pten*, implicating the loss of other tumor suppressors as cooperating events for combined Kdm6a- and Trp53-dependent tumorigenesis. This study suggests that *KDM6A* acts as an important tumor suppressor for cSCC pathogenesis.

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AUTHOR CONTRIBUTIONS

Conceptualization (LDW); Supervision (LDW and DYC); Investigation (LKS, NSA, LAS, CY, and DYC); Formal analysis (LKS, NSA, LAS, LAC, SMR, CAM, LDW, and DYC); Writing-original draft (LKS and DYC); Writing-review and editing (LKS, NSA, LAS, LAC, SMR, CAM, LDW, and DYC).

COMPETING INTERESTS

The authors declare that no competing interests exist.

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer in humans. Together with basal cell carcinoma, these keratinocyte carcinomas are estimated to have generated over 5.4 million cases affecting 3.3 million people in the United States, based on Medicare utilization data from 2012, which exceeds the aggregate incidence of all other human malignancies by a factor of three (Rogers et al. 2015; Siegel et al. 2020). Though curable in most instances by surgical intervention, mortality from advanced cSCC has risen over the past decade, while mortality from most other human malignancies has declined (Cronin et al. 2018; Henley et al. 2020; Karia et al. 2013; Nehal and Bichakjian 2018). Efforts to comprehensively characterize the drivers of cSCC have been hampered by the high mutational burden in these tumors, coupled with relatively few sequenced samples; power calculations demonstrate that even the largest study to date can nominate fewer than 50% of driver mutations (Chang and Shain 2021). As of this writing, data from 151 cSCC samples with panel or exome sequencing have been deposited in cBioPortal (Cerami et al. 2012; Gao et al. 2013) representing the major cSCC sequencing studies to date (Cammareri et al. 2016; Chitsazzadeh et al. 2016; Durinck et al. 2011; Inman et al. 2018; Ji et al. 2020; Li et al. 2015; Pickering et al. 2014; South et al. 2013; Wang et al. 2011; Yilmaz et al. 2017; Zheng et al. 2014). The most common recurrent mutations occur in “core” cancer-relevant pathway genes, like *NOTCH* family genes, *TP53*, *CDKN2A*, and MAPK pathway genes which have been experimentally defined and clearly implicated in the pathogenesis of multiple cancers including cSCC (Chang and Shain 2021), though alterations in many of these genes have been found to be clonally expanded in clinically normal skin (Fowler et al. 2021; Martincorena et al. 2015).

Frequent genetic lesions in epigenetic regulators have been identified in cSCC, including *KMT2D/MLL4* (41%), *KMT2C/MLL3* (32%), and *KDM6A/UTX* (7%), which encode components of the chromatin modifying COMPASS complex (Cerami et al. 2012; Gao et al. 2013; Shilatifard 2012). Though none of these factors have been demonstrated to drive cutaneous malignancy, a recent report demonstrated that *Kmt2d* deficiency in murine keratinocytes was associated with the development of keratinocyte hyperplasia and cytologic atypia as well as deregulated ferroptosis, which is thought to be a form of tumor suppressive programmed cell death (Egolf et al.). Although members of the COMPASS complex are frequently mutated in cSCC, these genes also have tumor suppressive activity in many other cancer types, including hematopoietic malignances and solid tumors (including bladder, lung, and others), suggesting that their dysregulation may play a role in cutaneous tumorigenesis (Campbell et al. 2018; Meeks and Shilatifard 2017).

KDM6A is a ubiquitously expressed histone demethylase located on chromosome Xp11.2, which has been demonstrated to regulate multiple developmental pathways, including *HOX* gene expression (Agger et al. 2007; Lan et al. 2007; Lee et al. 2007). Homozygous loss of *Kdm6a* in mice results in embryonic lethality in mid-gestation due to cardiac and neural tube defects; a quarter of *Kdm6a* hemizygous male mice are viable but have stunted growth and a reduced lifespan (Shpargel et al. 2012; Wang et al. 2012; Welstead et al. 2012). *KDM6A* is mutated in many cancers, including bladder, esophageal adenocarcinoma, lung squamous carcinoma, HNSCC, breast, colon carcinoma, as well as myeloid leukemia, myeloma, renal

cell carcinoma, and glioblastoma (Haaften et al. 2009; Wang and Shilatifard 2019); *KDM6A* escapes X inactivation, resulting in a gene dosage imbalance between males and females, which is thought to contribute to cancer sex bias (Dunford et al. 2017; Greenfield et al. 1998). Pan-cancer studies have suggested that *KDM6A* and COMPASS complex mutations are enriched particularly in squamous differentiation tumors (Campbell et al. 2018; Hoadley et al. 2014). In this report, we evaluate the role of *Kdm6a* in the development of cSCCs in mice.

RESULTS

KDM6A expression is dysregulated in human cSCC compared to normal skin.

The role of KDM6A expression in cSCC pathogenesis is unknown. Immunohistochemistry analysis of tissue microarrays and archived resection samples demonstrated moderate to robust expression of KDM6A in 15/15 normal skin specimens, with low or absent expression in 68/112 (60.7%) cSCC samples (Figure 1a). In addition to alterations in staining intensity, KDM6A expression patterns also varied between normal epidermis and cSCC. Immunofluorescence imaging of KDM6A demonstrated a nuclear distribution in normal epidermal keratinocytes in 12/15 (80%) samples, while the remaining exhibited both nuclear and cytoplasmic expression (Figure 1b). In contrast, 49/112 (43.8%) and 20/112 (17.9%) of cSCC samples exhibited cytoplasmic and nucleocytoplasmic KDM6A localization, respectively, while only 35/112 (31.3%) exhibited predominantly nuclear KDM6A expression. In this cohort, fewer higher stage tumors exhibited nuclear localization of KDM6A (6.7% of T3/T4 vs 35% of T1/T2, $p = 0.034$), and expression levels tended to be diminished with higher tumor stage (T1-T3, Figure S1a).

KDM6A is infrequently deleted in human cSCC.

In the first description of *KDM6A* as a relevant tumor suppressor in a survey of human cancers, deletions constituted 41% of genomic alterations in *KDM6A* (Haaften et al. 2009). Pan-cancer genomic analyses (Campbell et al. 2020) subsequently demonstrated that deletions constitute a large fraction of *KDM6A* alterations for cancers in which *KDM6A* is frequently mutated (Figure S1b). Comparatively few genomic studies of cSCC have been performed to date. Data from prior cSCC sequencing studies (Chang and Shain 2021; Li et al. 2015; Pickering et al. 2014) curated by CbioPortal indicate that 7% (11/151) of human cSCC contain non-synonymous *KDM6A* mutations, while 76% of tumors have *TP53* mutations, with all *KDM6A* mutations occurring in tumors with *TP53* mutations (Figure S1c). Variant allele frequencies suggest that *KDM6A* mutations occur in the dominant cSCC clone, not subclones (Figure S1d). Copy number analyses, however, were not described from these cases (Figure S1b). A cytogenetic analysis of cutaneous SCCs revealed loss of the X chromosome in 23% (3/13) cases analyzed; combined with a review of the literature, 23% (5/22) of examined cases demonstrated loss of the X chromosome (Jin et al. 1999).

To evaluate the frequency of deletions of *KDM6A* in human cSCC tumors, we analyzed publicly available genomic sequencing data (Cammareri et al. 2016; Durinck et al. 2011; Inman et al. 2018; Lee et al. 2014). After quality control checks (see Methods), there were 51 evaluable cSCC exome samples from 13 female and 38 male patients. Non-synonymous

mutations in *KDM6A* were demonstrated in 3/51 (5.9%) cases, and *TP53* mutations were found in 33/51 (64.7%) samples. Copy number analysis did not reveal losses in chromosome X or *KDM6A* in this dataset (Figure S2, Table S1).

Epidermal *Kdm6a* and *Trp53* deficiency results in the spontaneous development of cutaneous squamous cell carcinoma in mice.

To test whether *Kdm6a* is a relevant tumor suppressor in cSCC, we generated a model with keratinocyte-specific deficiency of *Kdm6a* and *Trp53*, since human *KDM6A* mutations have been found exclusively in the context of *TP53*-mutated cSCC (Figure S1c). We achieved *Kdm6a* and *Trp53* deficiency in keratinocytes by crossing mice bearing *Kdm6a*^{flox} and *Trp53*^{flox} alleles to mice with tamoxifen-inducible Cre recombinase under the control of the human keratin 14 promoter (*KRT14*-Cre^{ERT}), then treating adult mice with tamoxifen by oral gavage to induce recombination. Mice with epidermal *Kdm6a* and *Trp53* deficiency (DKO) typically developed ulcerated nodules reminiscent of keratoacanthoma type SCC in humans (Figure 2a). All female mice deficient in both *Kdm6a* and *Trp53* (*KRT14*-Cre;*Kdm6a*$^{fl/fl}$;*Trp53*$^{fl/fl}$, "DKO-f") developed tumors with a median latency of 337 days, whereas mice with homozygous *Kdm6a* deficiency alone (*KRT14*-Cre;*Kdm6a*$^{fl/fl}$) did not develop skin tumors. Homozygous epidermal *Trp53* deficiency caused a single, spontaneous skin tumor after over 1 year of observation (Figure 2b).

Since *Kdm6a* is located on chromosome X, male mice are hemizygous (*Kdm6a*$^{fl/y}$), though the *Kdm6a* paralog *Kdm6c* is encoded on the Y chromosome; these genes are known as *Utx* and *Uty*, respectively. Male DKO mice (*KRT14*-Cre;*Kdm6a*$^{fl/y}$;*Trp53*$^{fl/fl}$, "DKO-m") developed tumors more rapidly than female *Kdm6a* heterozygous (*KRT14*-Cre;*Kdm6a*$^{fl/+}$;*Trp53*$^{fl/fl}$) mice, which both lose only one copy of *Kdm6a* after induction. Both strains developed spontaneous skin tumors with complete penetrance, though male mice developed tumors with a median of 276 days, vs. 343 days in female mice ($p = 0.007$, Figure 2c). Though DKO-m mice tended to exhibit more rapid cSCC development than DKO-f mice, this difference was not statistically significant (median 276 vs 337 days, $p = 0.12$, Figure 2d).

Histopathologic examination of tumors revealed all stages of atypical squamous proliferations, including epidermal acanthosis, full thickness distribution of atypical cells that typify *in situ* SCC, and invasive SCC (Figure 3a). Tumors were predominantly crateriform squamous proliferations with cytologic atypia, frequent mitoses, and abundant keratinization reminiscent of keratoacanthoma type of squamous cell carcinomas in humans (Figure 3b), though some were moderately- or poorly-differentiated SCC (Figure 3c). While normal mouse skin in telogen exhibited robust, nuclear Kdm6a expression (Figure S3a), cSCC tumors were negative for Kdm6a by immunofluorescence (Figure 3e) and Trp53 by PCR, indicating a DKO cell-of-origin (Figure S3b–c). Clinically, some tumors appeared to be dermal proliferations, primarily located behind the ears of induced mice, where the histology did not clearly demonstrate epidermal connection in most cases. In one case, however an epidermal connection was present and was focal, suggesting that sampling variability might underlie cases where overt connections with the epidermis were not observed. In all cases, dermal or subcutaneous tumors resembled well-differentiated,

keratinizing SCC. In aggregate, the most common pattern was well-differentiated SCC, while the minority were moderately- to poorly-differentiated (Figure 3d). Two tumors exhibited aggressive, muscle-invasive disease, including one with osseus metaplasia that was confirmed to be keratinocyte origin by positive Trp63 and Krt14 immunofluorescence (Figure S3e). A single case of muscle-invasive spindle cell carcinoma demonstrated Krt14 positive immunofluorescence and did not express Kdm6a, indicating that a Kdm6a-deficient keratinocyte was the cell of origin (Figure 3e). Lung and regional lymph node examination did not reveal metastatic disease, with the caveat that mice were typically sacrificed early in the course of disease, with tumor diameters between 0.5–1cm.

We evaluated Kdm6a expression in female *Kdm6a* heterozygous tumors, because compared to DKO-f mice, there is no difference in latency or tumor penetrance. One tumor exhibited complete absence of Kdm6a staining, while 5/7 demonstrated mild or moderate reduction, and one tumor had no change in Kdm6a staining intensity (Figure S3d), suggesting that reduced expression of the wild type *Kdm6a* allele is a common feature in *Kdm6a* heterozygous tumors.

Genomic sequencing identifies potential cooperating events in skin tumorigenesis.

To determine potential cooperating genetic alterations in tumors arising in *Kdm6a* and *Trp53* deficient skin, we performed whole exome sequencing on 16 tumors from seven mice (DKO-m and DKO-f) with paired normal tissue from blood. Overall, single base substitutions or small insertions and deletions were infrequent, with a mean of 5.6 (range 1–18) non-synonymous mutations per tumor in protein coding regions (Figure 4a, Table S2). Despite low mutation burden, we identified non-synonymous mutations in *Ncstn* and *Vcan* in multiple mice. Versican, encoded by *VCAN/Vcan*, is an extracellular matrix proteoglycan that is mutated in 26% (32/122) of human cSCC cases in cBioportal (Figure S1c), though the significance of these alterations has not been established. Tumors from two independent mice had somatic mutations in *Vcan*, of which one caused a substitution mutation ($Vcan^{E498D}$) and the other a nonsense mutation ($Vcan^{K697*}$). Similarly, *Ncstn* frameshift mutations were found in tumors from two independent mice ($Ncstn^{353fs*}$ and $Ncstn^{637fs*}$). Nicastrin, encoded by *NCSTN/Ncstn*, is an integral member of the γ-secretase complex, which is responsible for proteolytic maturation of amyloid precursor protein (APP) and intramembranous proteolysis of Notch family receptors (Kopan and Ilagan 2004). Additionally, we found a $Kdm6a^{R612G}$ mutation in a male mouse with a variant allele frequency of 0.452, placing it in the dominant clone of this mouse, though the significance of this is unclear.

Copy number analysis of whole exome data revealed frequent amplifications and deletions in tumors arising in this mouse model. Notably, a region of chromosome 19qC1, encompassing the tumor suppressor *Pten*, was deleted in 4/16 tumors (Figure 4b, Table S3). Portions of chromosome 1 encompassing *Ncstn* were deleted in 4/16 tumors, including a tumor in a female mouse with the $Ncstn^{353fs*}$ allele, resulting in a total of 5/16 (31.3%) tumors with *Ncstn* alterations (Figure 4c). Finally, genomic analysis of four *Kdm6a* heterozygous tumors from three mice similarly yielded rare single nucleotide variants, but many copy number alterations (Figure S4 and table S4, S5). One tumor exhibited

copy loss of *Pten*, while another tumor lost the non-targeted X chromosome, resulting in biallelic *Kdm6a* loss. There were no single nucleotide variants in *Kdm6a*, suggesting that its decreased protein expression by immunohistochemistry in *Kdm6a* heterozygous tumors may be due to other factors, like epigenetic silencing, in addition to genetic mutation.

Discussion

Despite being the second most common human malignancy, the drivers of squamous cell skin cancer are not well understood because of the currently limited sequencing data for this tumor type (Chang and Shain 2021; Martincorena and Campbell 2015). A recent metanalysis of 1,048 melanoma whole exome sequencing samples revealed molecular subtype-specific, secondary genomic alterations that have implications for immunotherapy response that were not apparent in smaller sequencing studies (Conway et al. 2020). With an order of magnitude fewer samples sequenced, our understanding of cSCC driver mutations is far from complete. Development of *in vivo* models to study relevant driving mechanisms in cSCC has correspondingly lagged. Here, we demonstrate that KDM6A expression patterns in human cSCC are different from normal skin keratinocytes, suggesting that at least half of cSCC may exhibit altered KDM6A function. Complete penetrance of cSCC in *Kdm6a* and *Trp53* DKO mice demonstrate that these mutations are sufficient to prime cSCC development. Together, this study supports the establishment of *KDM6A* as a relevant tumor suppressor gene in cSCC pathogenesis.

The significant proportion of cSCC with cytoplasmic KDM6A localization suggests a disruption KDM6A nuclear function, although the causes and consequences are currently unclear. One study demonstrated that *KDM6A* mutated cancers uniformly had decreased expression of KDM6A (Ler et al. 2017) while another showed that cancer-related TPR domain mutations decreased expression of KDM6A in cell lines and disrupted binding to core COMPASS components causing aberrant KDM6A localization to the cytoplasm (Kato et al. 2020); however, the number of cases we observed with altered KDM6A expression greatly exceeded the number of inactivating *KDM6A* mutations detected in cSCC. This suggests that KDM6A is a tumor suppressor that, in addition to gene mutations, may be inactivated by epigenetic or other mechanisms that remain to be defined. Environmental factors like hypoxia have been described to directly modify KDM6A histone demethylase activity (Chakraborty et al. 2019), and a variety of post-translational modifications have been described, with at least four ubiquitination sites and 17 phosphorylation sites in the PhosphoSitePlus database (Hornbeck et al. 2015).

The long latency of cSCC in DKO mice suggests that additional events are required for full transformation, while the loss of residual Kdm6a expression in heterozygous tumors implies selective pressure favoring Kdm6a loss of function. The preponderance of copy number alterations in these cSCC tumors may be caused by *Trp53* loss in our model, consistent with studies demonstrating a significant association between copy number alterations and *TP53* mutations in many human cancer types (Ciriello et al. 2013). We indeed found that additional, recurrent alterations in *Pten*, *Ncstn*, and *Vcan* occurred in many mouse tumors, and importantly, the same genes are altered in *KDM6A*-mutated human cSCC (Figure S1c). Previous studies showed that mice with *Pten* deficiency can spontaneously

develop squamous papillomas which can transform to squamous cell carcinoma, while Pten haploinsufficient mice are more susceptible to chemical carcinogenesis (Suzuki et al. 2003). Several tumors had deletions or truncating mutations involving *Ncstn*, suggesting that this may be a key cooperating factor. Nicastrin haploinsufficiency results in spontaneous squamous tumors with a median latency of around 60 weeks, which is longer than observed in *Kdm6a*;*Trp53* DKO mice (Li et al. 2007a; Li et al. 2007b). *Ncstn*$^{+/-}$ mice exhibit defective Notch signaling and potentiate Egfr signaling, which may be relevant for cSCC pathogenesis (Li et al. 2007a). Notch1 is a well-established tumor suppressor in mouse skin, which acts through both cell autonomous and microenvironmental tumor suppressive mechanisms, making it a highly plausible cooperating event (Demehri et al. 2009; Hu et al. 2012; Nicolas et al. 2003). Finally, *Vcan* was recurrently mutated in our DKO tumors, and is also the most significant differentially expressed gene in the bone marrow cells of *Kdm6a* deficient mice (Tian et al. 2021), suggesting that Vcan may also influence Kdm6a tumor suppressive function.

We observed a trend towards earlier tumor development in male mice, which suggests that Kdm6a may be relevant for the sex disparity observed in human skin cancer development. Global non-melanoma skin cancer incidence statistics reveal a 2:1 male to female predominance in 2020 (Sung et al. 2021). One epidemiologic study suggested that non-melanoma skin cancers represent an “enigmatic” sex disparity—where there is a bias in incidence towards male sex that does not vary by geography (which is often used as a surrogate for sun exposure rate and skin type) or economic conditions, indicating that there may be intrinsic biological, rather than environmental, reasons for this disparity (Edgren et al. 2012). Male mice (and humans) have the Y chromosome gene *Kdm6c* (*Uty*), a *Kdm6a* (*Utx*) paralogue. Despite sharing 88% sequence identity between their JmjC catalytic domains, Kdm6c has 40 fold less histone lysine demethylase activity *in vitro* (Walport et al. 2014). Based on the observations in this study, Kdm6c does not effectively compensate for the tumor suppressive function of Kdm6a; however, it seems unlikely that Kdm6a deficiency alone causes the sex differences observed here, given the trend towards shorter tumor latency in DKO-male compared to DKO-female mice (median 276 vs 337 days, p=0.12, Figure 2d). This contrasts from a model in which Kdm6a-deficient male mice remained risk-neutral to chemically induced bladder cancer whereas female mice deficient for Kdm6a were at increased risk, despite overall greater risk of bladder cancer in males (Kaneko and Li 2018). In another model, loss of Kdm6a caused an aggressive, squamous-differentiated pancreatic cancer specifically in female mice (Andricovich et al. 2018), suggesting that there are likely sex- and cell type-specific Kdm6a functions that remain to be studied in cSCC. Finally, it is important to consider the emerging evidence of sex-discrepant effects of *TP53/Trp53* tumor suppression and its role in this model (Delbridge et al. 2019; Haupt et al. 2019; Haupt and Haupt 2021).

In summary, this study establishes *KDM6A/Kdm6a* as a relevant tumor suppressor gene for cSCC pathogenesis. Although the interplay between Kdm6a and Trp53 is not yet clear in keratinocytes, we demonstrate their deficiency in mice creates a premalignant state that results in spontaneous, fully penetrant cSCC with features consistent with human disease, including sex disparity and relevant, potentially cooperative genomic events. This model

should be valuable for mechanistic, pre-clinical studies designed to understand and prevent the progression from premalignant states to the development of invasive cSCC.

MATERIALS AND METHODS

Animals and tumor induction.

All mouse work was performed in accordance with institutional guidelines and approved by the Animal Studies Committee at Washington University. Generation of the conditional Kdm6a knockout was previously described (Tian et al. 2021). Mice were treated with tamoxifen at median 65 days from date of birth (range, 44–117 days). Tamoxifen (Cayman 13258) was dissolved at 20 mg/mL in corn oil (Sigma C8267) and was administered by oral gavage at 3 mg/dose for a total of 9 doses (3 doses per week). Tumor latency was calculated from the start of induction until tumor formation. Mice were euthanized for analysis when tumors reached a minimum size of 0.5cm. Mouse strain and analysis details are in the supplemental methods.

Tumor histopathology.

Human tissue microarrays were purchased from US Biomax, Inc (Sk801c and Sk802c) and subjected to immunohistochemistry or immunofluorescence studies. Cases that could not be evaluated (e.g. missing or folded tissue) were excluded. Human de-identified archival histology sections were analyzed with approval of the Human Research Protection Office (202111126). Murine tumors were harvested and fixed for at least 16 hours in 10% neutral buffered formalin before paraffin embedding and sectioning. Routine hematoxylin and eosin stained slides were interpreted by a board certified dermatopathologist (LAC). Kdm6a expression in keratinocyte lineage (by Krt14 and Trp63) was evaluated by immunofluorescence. Tissue staining details are in the supplemental methods.

Genomic sequencing.

Analysis of publicly available human genomic sequencing data was approved by the Washington University Human Research Protection Office (202104098). Data from prior studies was downloaded from NCBI dbGaP (accessions phs000418.v2.p1, phs000830.v1.p1, phs000785.v1.p1, and phs002019.v1.p1) and from the European Genome-Phenome Archive (accession EGA000010000516). Mouse tumor sequencing details are in the supplemental methods. Oncoprint data representation was generated at cBioPortal (Cerami et al. 2012; Gao et al. 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA821933, hosted at NCBI under the BioProject accession PRJNA821933.

Abbreviations:

UTX	Ubiquitously transcribed tetratricopeptide repeat on chromosome X
cSCC	Cutaneous squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma

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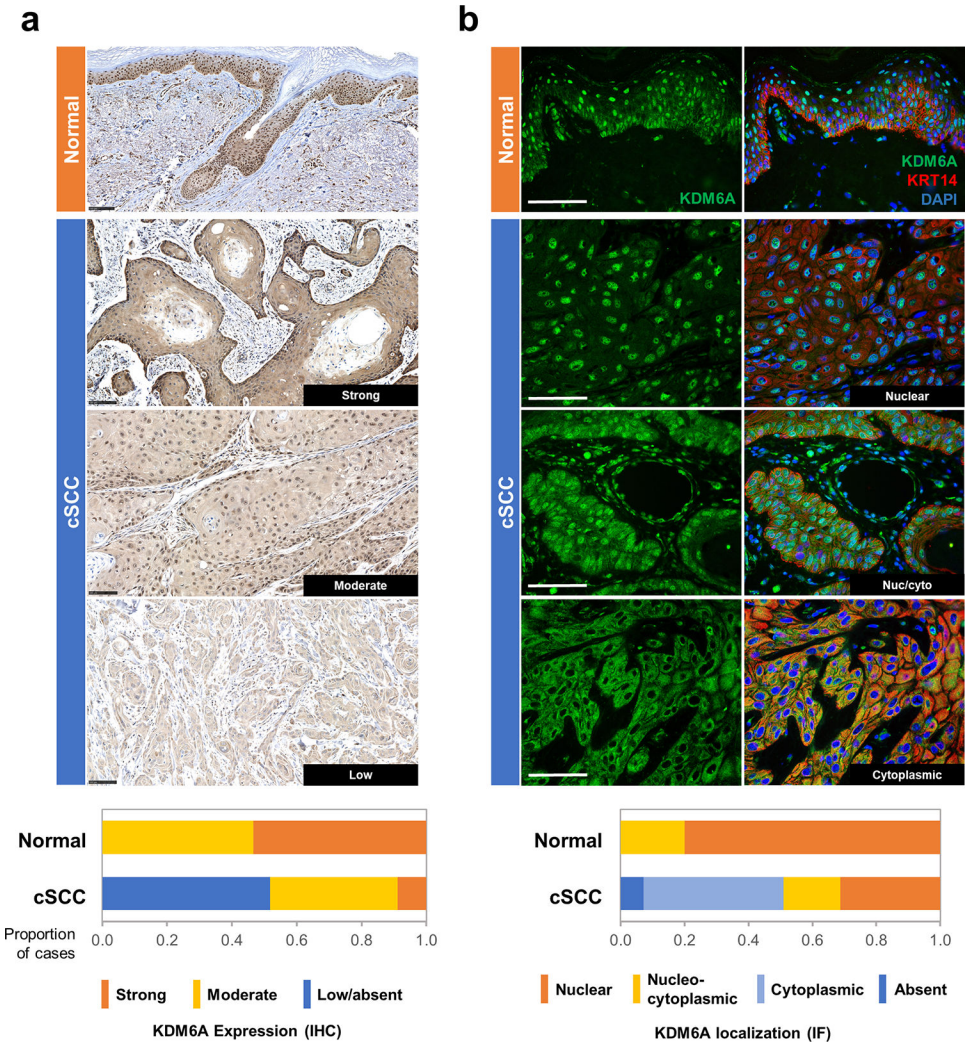

Figure 1: Human squamous cell carcinoma exhibits altered *KDM6A* expression

a. Immunohistochemical analysis of squamous cell carcinoma, representative images from normal skin and cSCC with strong (top), moderate (middle), and weak (bottom panel) KDM6A staining (scale=100μm). Summary of normal (n=15) and cSCC (n=112) KDM6A staining intensity.

b. Immunofluorescence analysis of KDM6A subcellular localization. Normal skin exhibits primarily nuclear KDM6A (green). Basal keratinocytes marked by KRT14 (red), while nucleus marked by DAPI (blue). cSCC exhibits multiple patterns, including strong nuclear staining of KDM6A (top, dotted line epidermal-dermal junction, scale = 100μm).

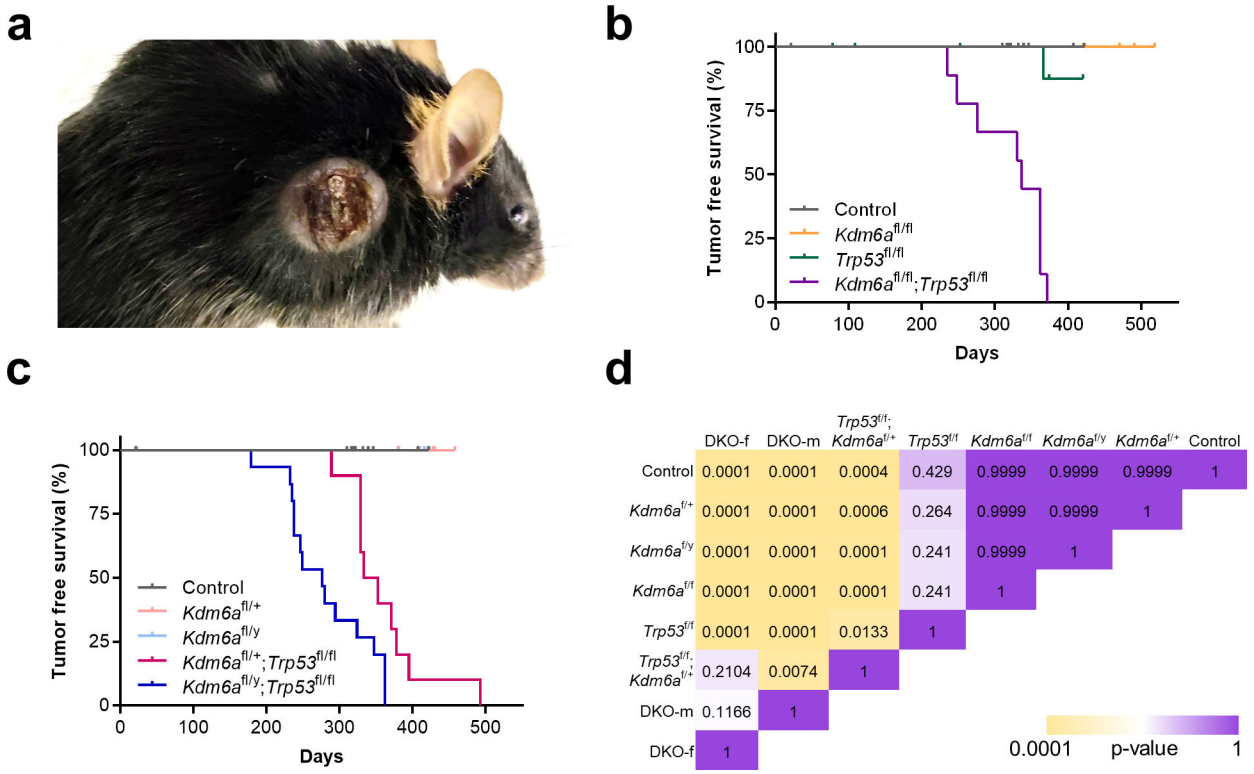

Figure 2: Epidermal Kdm6a and Trp53 deficiency drive squamous cell carcinoma development

a. Typical keratotic epidermal tumor on the dorsolateral skin of a DKO-m (*KRT14*-Cre^{ERT}+;*Kdm6a*$^{fl/y}$;*Trp53*$^{fl/fl}$) mouse

b. Female mice with *KRT14*-Cre^{ERT}+;*Kdm6a*$^{fl/fl}$ (n=12) or *KRT14*-Cre^{ERT}+;*Kdm6a*$^{fl/fl}$;*Trp53*$^{fl/fl}$ deficiency (DKO-f, n=9) compared to male and female control *KRT14*-Cre^{ERT} negative (n=20) or *KRT14*-Cre^{ERT}+;*Trp53*$^{fl/fl}$ (n=14) mice

c. Comparison between *Kdm6a*$^{fl/+}$ female (n=10) and *Kdm6a*$^{fl/y}$ male (n=13) mice, to mice with combination Kdm6a and Trp53 deficiency, DKO-m (n=15) and female *Kdm6a*$^{fl/+}$;*Trp53*$^{fl/fl}$ (n= 10). All mice express *KRT14*-Cre^{ERT} and were induced by oral tamoxifen.

d. Statistical significance of pairwise comparisons of tumor-free survival between indicated cohorts of mice, determined by log-rank test.

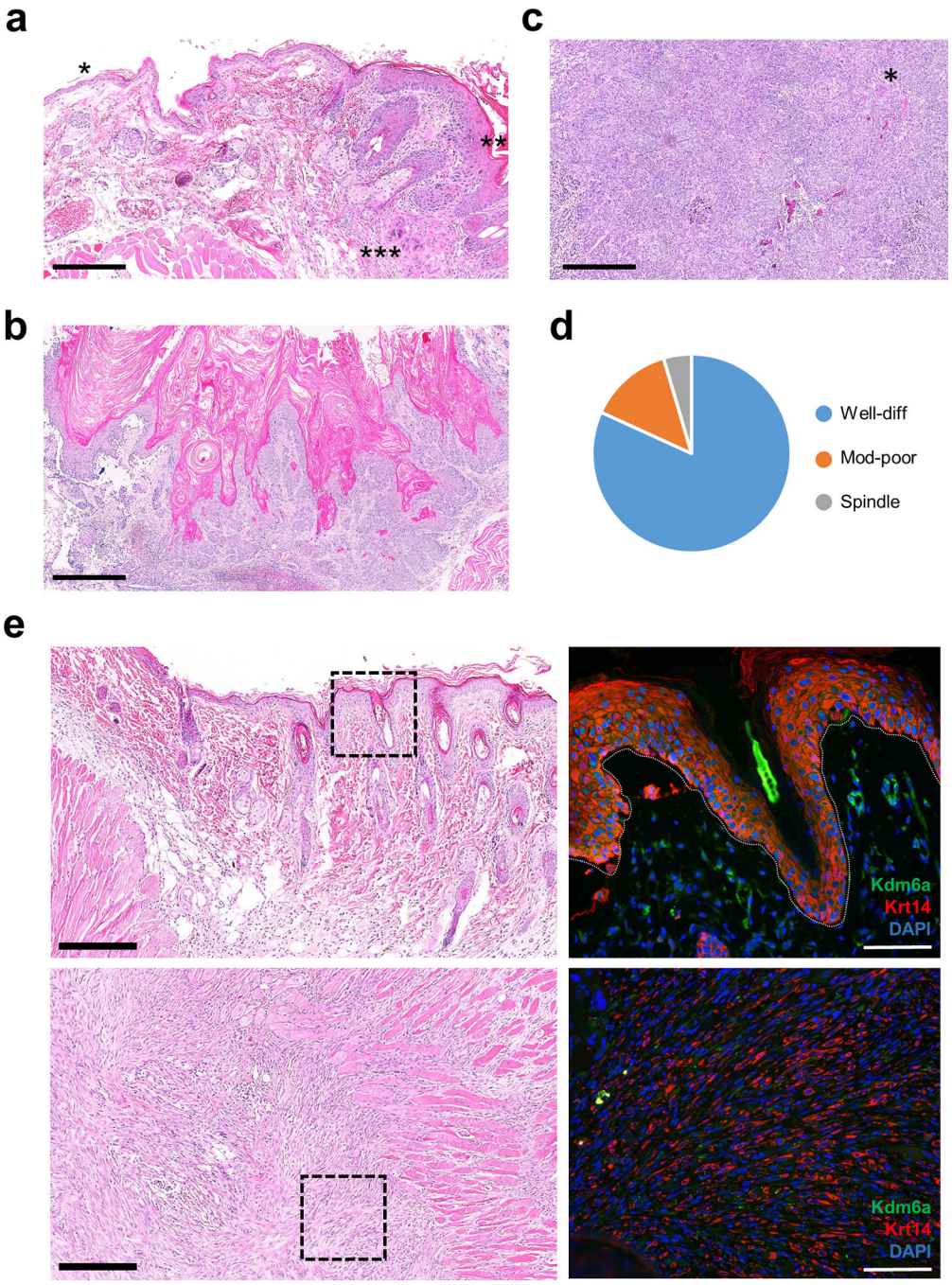

Figure 3: Epidermal Kdm6a deficiency drives squamous cell carcinoma formation

a. Hematoxylin and eosin (H/E) stained normal appearing epidermis (*) contiguous with hyperplastic epidermis with full thickness atypia (**) and invasive squamous carcinoma (***) in a DKO-f mouse (scale=250μm

b. Histopathology of a well-differentiated tumor from a DKO-f mouse (scale=500μm

c. Histopathology of a moderate/poorly-differentiated tumor with areas of dystrophic calcification (*) as well as squamous eddies (**) from a DKO-f mouse (scale=500μm

d. Distribution of well-differentiated (n=18), moderately-differentiated (n=3), and spindle-cell/poorly-differentiated (n=1) squamous tumors.
e. Epidermal component of squamous cell carcinoma (top panels) and muscle invasive spindle cell component (bottom panels) from a DKO-m mouse. Left panels are H/E stain (scale=500μm), where boxed areas examined by immunofluorescence (right panels) detecting Kdm6a (green), Krt14 (red), and DAPI (blue, scale=100μm).

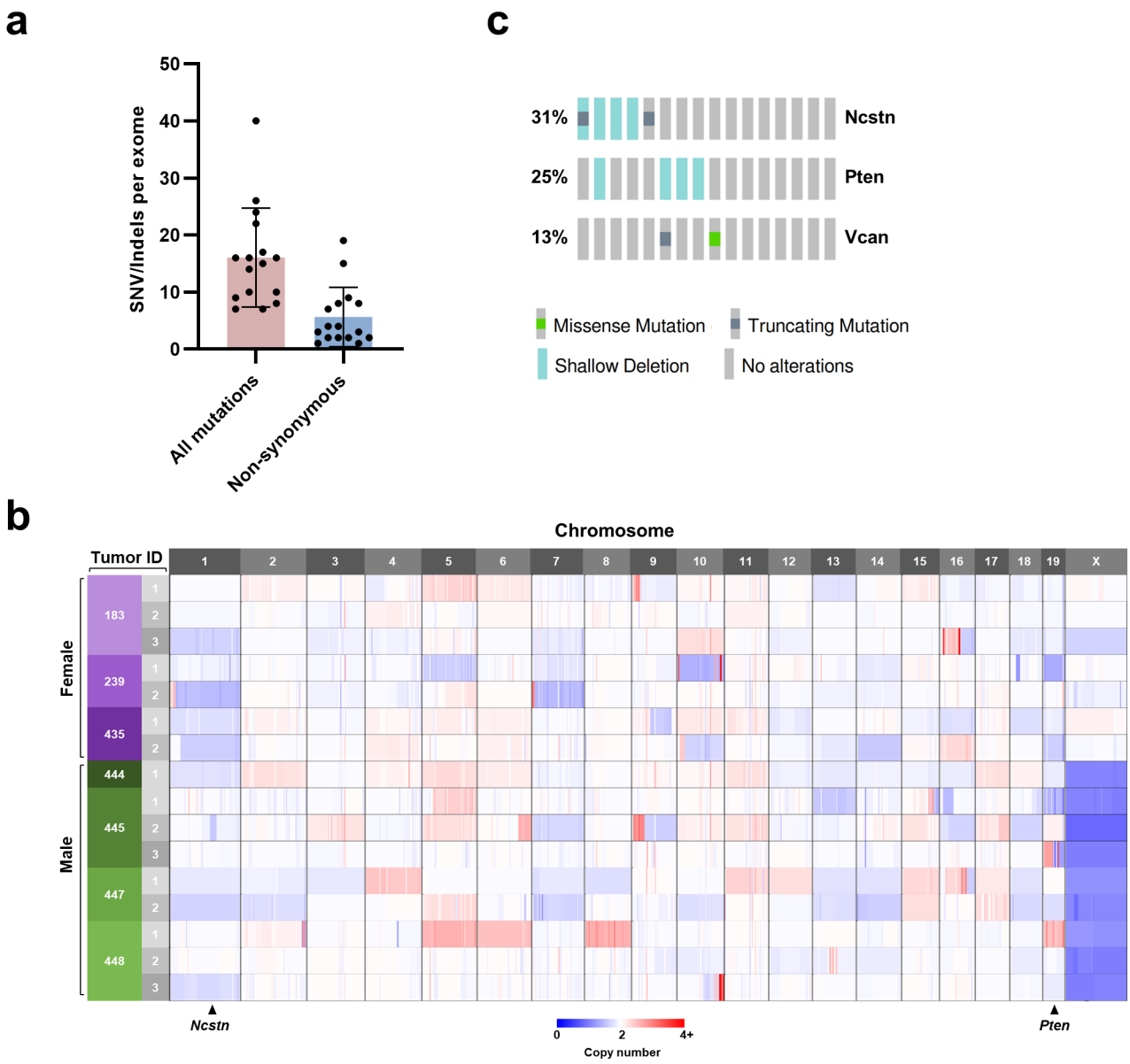

Figure 4: Exome sequencing squamous tumors reveals potential cooperating mutations

a. Mutation burden of single base substitutions and small insertions and deletions is relatively low, with mean of 16.1 (range 7–40) total variants per tumor and 5.6 (range 1–18) non-synonymous variants per tumor.

b. Copy number analysis for each tumor, with independent mice labeled by an identifying number and colored by sex (female in purple, male in green). Each unique tumor from one mouse labeled 1–3 in grey boxes.

c. Recurrently mutated genes in DKO tumors.