Oncogenic $\text{ALK}^{F1174L}$ drives tumorigenesis in cutaneous squamous cell carcinoma

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Cutaneous squamous cell carcinoma (cSCC) is the second most common skin cancer characterized by increased mortality. Here, we show for the first time that anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase of the insulin receptor superfamily, plays a pivotal role in the pathogenesis of cSCC. Our data demonstrate that the overexpression of the constitutively active, mutated ALK, $\text{ALK}^{F1174L}$, is sufficient to initiate the development of cSCC and is 100% penetrant. Moreover, we show that cSCC development upon $\text{ALK}^{F1174L}$ overexpression is independent of the cell-of-origin. Molecularly, our data demonstrate that $\text{ALK}^{F1174L}$ cooperates with oncogenic $\text{Kras}^{G12D}$ and loss of $\text{p53}$, well-established events in the biology of cSCC. This cooperation results in a more aggressive cSCC type associated with a higher grade histological morphology. Finally, we demonstrate that Stat3 is a key downstream effector of $\text{ALK}^{F1174L}$ and likely plays a role in $\text{ALK}^{F1174L}$-driven cSCC tumorigenesis. In sum, these findings reveal that ALK can exert its tumorigenic potential via cooperation with multiple pathways crucial in the pathogenesis of cSCC. Finally, we show that human cSCCs contain mutations in the ALK gene. Taken together, our data identify ALK as a new key player in the pathogenesis of cSCC, and this knowledge suggests that oncogenic ALK signaling can be a target for future clinical trials.

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Introduction

Anaplastic lymphoma receptor kinase (ALK) alterations have been identified in several human cancers, including neuroblastoma, glioblastoma, lung cancer, anaplastic large cell lymphoma, and renal cell carcinoma (Hallberg & Palmer, 2013). Most cancer-associated rearrangements in the ALK gene are associated with fusions, copy-gain number, or activating ALK mutations (Hallberg & Palmer, 2013). In mice, overexpression of the mutated ALK$^{F1174L}$ gene results in neuroblastoma development (Heukamp et al, 2012). An elevated expression of phosphorylated ALK as well as its ligands, midkine, and pleiotrophin has been found in patients with basal cell carcinoma (BCC) and cSCC (Ning et al, 2013). To investigate the possible role of ALK in the pathogenesis of skin tumors, we overexpressed $\text{ALK}^{F1174L}$ in the epithelial cells in the skin. A number of studies has addressed the cell-of-origin of BCC and cSCC. BCC can arise from the progenitor cells of the interfollicular epidermis, cells in the infundibulum of the hair follicle (HF) (Youssef et al, 2010), and HF stem cells (Grachtchouk et al, 2011). Similarly, compelling evidence suggests that cSCC can also arise not only from interfollicular epidermis but also from the HF stem cells (Lapouge et al, 2011; White et al, 2011; Sanchez-Danes & Blanpain, 2018). Based on these studies, we have decided to overexpress $\text{ALK}^{F1174L}$ in HF stem cells using $\text{Lgr5-CreERT2}$ (Barker et al, 2007) and $\text{K15-CreERT2}$ (Morris et al, 2004) mouse lines, and in all basal cells taking advantage of $\text{K5-CrePR1}$ (Zhou et al, 2002) and $\text{K14-CreERT2}$ (Vasioukhin et al, 1999) transgenic strains.

Results and Discussion

We induced the expression of $\text{ALK}^{F1174L}$ via topical administration of 4-hydroxytamoxifen (4-OHT) on the shaved back skin as well as on the ears and tails (Fig 1A). 100% of $\text{ALK}^{F1174L}$ Lgr5-CreERT2 mice developed skin lesions and had to be euthanized at the latest 4 mo after 4-OHT induction (Figs 1B and S1A). Skin lesions became apparent after 3 wk after transgene activation. Whereas 83% (11/13 mice) of $\text{ALK}^{F1174L}$ Lgr5-CreERT2 mice developed lesions in the ears and 69% (9/13 mice) in the tail, no abnormalities were seen in the back skin (Fig 1C). However, skin lesions on the back skin were occasionally observed in several $\text{ALK}^{F1174L}$ overexpressing mice carrying fight wounds (Fig S1B). It is widely accepted that epithelial cancers arise as a result of a multistep process involving tumor initiation, promotion, and progression (Hennings & Boutwell, 1970; Abel et al, 2009). The fact that skin wounding in combination with other inducing agents has been previously
demonstrated to promote skin carcinogenesis (Hoste et al, 2015) suggests that whereas ALKF1174L overexpression alone is sufficient to drive tumor formation in ear and tail skin, it might require an additional promoting treatment in the back skin. We nevertheless excluded those mice from further analysis because our study focused on the dissection of the role of ALKF1174L overexpression in the context of skin homeostasis. ALK overexpression was confirmed using Western blot with anti-pALK antibodies (Fig S1C). The presence of the luciferase (luc) reporter gene in the ALK transgene allowed us to monitor tumor development using IVIS imaging system (Heukamp et al, 2012). Analysis of the LSL-ALK<sup>11474L</sup> transgene expression shows strong luminescent signal from the tumors on ears and tail. (G) Pie chart representing the number of mice that developed the listed skin lesions out of total skin lesions diagnosed. (F) Representative hematoxylin and eosin (H&E) staining of such lesions from the ears. Scale bars = 100 µm. To note, each mouse developed several tumors. (G, H, I) Top to bottom. Genotypes, representative pictures of the tumors marked by pan-cytokeratin immune-labeling and tumor distribution per location. To note, because of leakage of the Cre expression, tumor formation was observed when no specific topical administration was performed. Scale bars = 250 µm.

Figure 1. Expression of ALK<sup>11474L</sup> in different skin compartments induces skin lesions and cSCC. (A) Graphical representation of Lgr5-CreERT<sup>2</sup>;LSL-ALK<sup>11474L</sup> genotype and experimental design. Topical application of 4OH-tamoxifen (+-OHT) in ears, back, and tail skin of mice resulted in skin lesion development. Then, mice were euthanized when termination criteria were observed (tumor size and ulceration). (B) Tumor-free survival of Lgr5-CreERT<sup>2</sup>;LSL-ALK<sup>11474L</sup> mice (n = 15, median 47 d) and controls (n = 12). Log-rank (Mantel–Cox) Test P < 0.0001, HR 28.12. All mice developed tumors. (C) From left to right. Topical administration of 4-OHT and after tumor formation per location. (D) Representative picture of in vivo imaging system (IVIS). Analysis of the LSL-ALK<sup>11474L</sup> transgene expression shows strong luminescent signal from the tumors on ears and tail. (E) Pie chart representing the number of mice that developed the listed skin lesions out of total skin lesions diagnosed. (F) Representative hematoxylin and eosin (H&E) staining of such lesions from the ears. Scale bars = 100 µm. To note, each mouse developed several tumors. (G, H, I) Top to bottom. Genotypes, representative pictures of the tumors marked by pan-cytokeratin immune-labeling and tumor distribution per location. To note, because of leakage of the Cre expression, tumor formation was observed when no specific topical administration was performed. Scale bars = 250 µm.

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Next, we examined the expression of ALK protein in the normal human skin. ALK expression was readily detected in virtually all basal keratinocytes (Fig S2A). Given the fact that ALK expression in humans has been described in both tumor types, BCC and cSCC, it was surprising that we did not observe any signs of BCC development in our mouse model. To investigate whether mutations in ALK gene are present in the human cSCC samples, we performed the analysis of publicly accessible data resources. Cohen et al (2015) described the landscape of mutations detected in cSCC diagnosed in patients undergoing BRAF inhibitor therapy for advanced metastatic melanoma and among other mutations, they have detected the ALK**F1174L** mutation (1/29 patients). This prompted us to investigate the frequency of ALK mutations in primary and metastatic human cSCC, and we focused on analysis of human cSCC using exome sequencing (Fig S2B) (Durinck et al, 2011; Lee et al, 2014; Pickering et al, 2014; Li et al, 2015; Yilmaz et al, 2017; Inman et al, 2018). Of 161 human cSCC cases analyzed, we have identified 32 cases (20%) carrying mutations in the ALK gene. PolyPhen-2 software was used to predict damaging effects of identified ALK mutations (Fig S2C) (Adzhubei et al, 2010). Whereas nearly 50% of cases predicted no effect on the ALK gene function, others were characterized as potentially harmful mutations. Of note, in addition to the p.F1174L mutation identified in cSCC occurring in the melanoma patient (Cohen et al, 2015), two additional cases harbored the p.G1201E (Murugan & Xing, 2011) and p.G1286R (Bresler et al, 2014) mutations, which are known ALK gain-of-function mutations (Fig S2C). We also checked for the presence of mutations in the Alk gene in the previously described mouse models of cSCC (Nassar et al, 2015). Two mutations were described, namely, the p.C787S and the p.C1012S, which, however, did not correlate with ALK expression (Nassar et al, 2015).

To directly assess whether the ALK**F1174L** (orthologue to ALK**F1174L**) mutation is present in mouse cSCC, we have sequenced exons 23 of the *Alk* gene from six independent cSCC induced by a combination of DMBA (7,12-dimethylbenzanthracene) and TPA (12-O-tetradecanoylphorbol-13-acetate) treatment (Fig S2D). None of the tumors exhibited mutations in the *Alk* gene. This could be due to the fact that all mouse DMBA/TPA tumors are induced on the back skin, a location devoid of skin lesions in ALK**F1174L**-expressing mice. Because DMBA/TPA–induced tumors are predominantly driven by oncogenic mutations in RAS signaling, an alternative explanation may be that ALK and RAS are mutually exclusive drivers of skin tumorigenesis. To test this hypothesis, we have analyzed the co-occurrence and overall frequency of ALK and RAS mutations in human cSCC using cBio Portal (Fig S2E). Among all RAS genes analyzed, we have observed that mutations in ALK gene appear to be mutually exclusive with mutations in KRAS gene (68 cases analyzed and 0 cases display co-occurrence). Mutations in HRAS and NRAS genes co-occurred with mutations in ALK in some cases (68 cases analyzed, 1 case co-occurrence of NRAS and ALK in the same patient, and 3 cases show co-occurrence of ALK and HRAS mutations).

Furthermore, we ruled out the possibility that the back skin of ALK**F1174L** Lgr5-CreERT2 mice contained early lesions, yet not visible to the naked eye. To this aim, we performed thorough histological and immunohistochemical examination with anti-Ki67 antibodies and quantified the proliferation index of keratinocytes identified by pan-Ck staining (Fig S3B). Even though some HF appeared dysplastic, no increased proliferation was observed.

To dissect the molecular mechanisms underlying the ALK**F1174L**-driven cSCC in the skin, we isolated tumor epithelial cells (TECs) from ALK**F1174L** lesions and normal keratinocytes from their wild-type littermates by FACS using a combination of EpCam+CD31+CD45+CD140a+ markers as previously described (Nassar et al, 2015) and independent triplicates were subjected to RNA sequencing (Fig 2A). Analysis of differentially expressed keratinocytes revealed a pronounced decrease in the expression level of Krt7 (Krt 1b) and Krt15, indicative of an impaired epithelial differentiation (Fig 2C). On the other hand, several other keratins, including Krt6, Krt7, Krt17, and Krt18 were significantly increased. Krt6, Krt16, and Krt17 are associated with aberrant proliferation, and Krt18 is a marker of embryonal keratinocytes and is often associated with poor differentiation and epithelial-to-mesenchymal transition (EMT) in cSCC (Fig 2C) (Watanabe et al, 1995). Gene ontology (GO) term analysis provided further insights into biological processes enriched in ALK**F1174L** TECs. Among significantly up-regulated processes were cell adhesion/migration and proliferation, along with alterations in metabolic processes (Fig 2B and E). The latter overall indicated a shift towards an anabolic and glycolytic (Warburg-like) metabolic phenotype, an additional hallmark that ALK**F1174L** cells would gain in support of transformation. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed ALK**F1174L**–associated signature including alterations in several signaling pathways including FAK and ECM, PI3K-AKT, and JAK-STAT3 (Fig 2B and D). Moreover, ALK**F1174L** expression resulted in an increase of Mek1 (Map2k1) expression (Fig 2D and F). Interestingly to note is that this signaling signature has been described as characteristic of cSCC development (Ratushy et al, 2012). The expression profile of ALK**F1174L**-driven tumorogenesis reveals the lack of Sonic Hedgehog (Shh) signaling pathway, which is characteristic of the typical BCC, demonstrating that ALK**F1174L** expression leads to cSCC formation without any cellular and molecular signs of concomitant BCC development.

Similarly to the DMBA/TPA model of mouse carcinogenesis, human SCCs are often associated with mutations in the RAS genes (van der Schroeff et al, 1990; Spencer et al, 1995). Overexpression of either Hras or Kras in mouse skin initiates tumorigenesis (Brown et al, 1998; Vitale-Cross et al, 2004; Caulin et al, 2007). The analysis of RNA-seq data obtained from the comparison of WT ear keratinocytes and ALK**F1174L** TECs did not reveal any significant deregulation in the Ras signature. Moreover, our data on the co-occurrence of mutations in RAS and ALK genes in human patients did not reveal any strong evidence suggesting that ALK and RAS are mutually exclusive drivers of cSCC (Fig S2E). Thus, we sought to functionally determine whether ALK**F1174L** can cooperate with Kras in driving SCC development. To answer this question, we have crossed ALK**F1174L** Lgr5-CreERT2 mice with Kras**G12D** strain (Fig 3A) (Jackson et al, 2001). Within less than 40 d, all mice developed ulcerative lesions on the ears and tails (n = 13) (Fig 3B and E). As shown in Fig 3B, the tumor penetrance/incidence remained as high as in the ALK**F1174L** transgene and reached 92% in ear and tail areas. However, similarly to ALK**F1174L** overexpression, no tumors were seen in the back skin of ALK**F1174L**Kras**G12D** Lgr5-CreERT2 mice. In analogy to ALK**F1174L** Lgr5-CreERT2 mice, the histological analysis revealed the presence of
Figure 2. ALK\(^{TTL4L}\)–driven tumorigenesis relied in bona fide, via PI3K-AKT/focal adhesion–ECM receptor interaction pathways. 

(A) Ear tumor from Lgr5-Cre\(^{ERT2}\);LSL-ALK\(^{TTL4L}\) mice and ears from control mice were prepared as a single-cell suspension. Tumor cells and keratinocytes were isolated by FACS for EpCAM expression e-negative selected for CD31/CD45/CD140a. RNA was extracted from sorted cells and used for RNA sequencing (RNA-seq).

(B) Top 15 biological processes and KEGG signaling pathways of up- and down-regulated genes from RNA-seq transcriptional profiling of sorted tumor cells versus normal keratinocytes. (C) Significantly deregulated keratins. (D) Genes significantly altered that cluster in the PI3K-AKT, Jak-Stat, and focal adhesion–ECM receptor interaction KEGG signaling pathways. (E) Clustering of biological processes using the MSigDB c5.bp.v6.2 gene set. Red nodes represent up-regulated gene sets and blue nodes
suggest that examine the degree of vascularization within individual tumors, we vascularization. induction of EMT, increase in proliferation and enhanced ALK in cutaneous squamous cell carcinoma Gualandi et al. on RNA-seq data. previously described FACS protocol (Nassar et al, 2015) and per-

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indication. Lines between the nodes represent association of the gene sets within the nodes. (f) Graphical representation of genes/pathways regulated by ALK\(^{IT74L} \) based on RNA-seq data.

represent gene sets down-regulated in the tumor cells. Node size shows the size of gene sets. Nodes that clustered together are classes with same or similar function indication. Lines between the nodes represent association of the gene sets within the nodes. (f) Graphical representation of genes/pathways regulated by ALK\(^{IT74L} \) based on RNA-seq data.

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Figure 3. Kras<sup>G12D</sup> synergizes with ALK<sup>T584L</sup> increasing tumorigenicity, epithelial-to-mesenchymal transition properties, and vascularization and proliferation.

(A) Graphical representation of Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup>;LSL-Kras<sup>G12D</sup> genotype. (B) Distribution of tumor formation per location in Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup>;LSL-Kras<sup>G12D</sup> mice. (C) Pie chart representing the number of mice that developed the listed skin lesions out of total lesions diagnosed. (D) Number of tumors per mouse in Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup> and Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup>;LSL-Kras<sup>G12D</sup> mice. Each dot represents the number of tumors per mouse. Mean ± standard error of the mean (5.917 ± 1.026; n = 12; 10.67 ± 1.280 n = 9; two-tailed t test P = 0.0086). (E) Tumor-free survival of Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup> (n = 15, median 47 d) and Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup>;LSL-Kras<sup>G12D</sup> (n = 13, median 29 d) mice. Log-rank (Mantel–Cox) test; P = 0.0002, HR = 0.1461. (F) Progression-free survival of Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup> (n = 15, median 37 d) and Lgr5-CreER<sup>T2</sup>;LSL-ALK<sup>T584L</sup>;LSL-Kras<sup>G12D</sup> (n = 15, median 37 d) and
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activator of transcription 3 (Stat3) were altered. We have observed down-regulation of the protein inhibitor of activated STAT, Pias3, a specific inhibitor of Stat3, which prevents DNA-binding activity of Stat3 and thereby abolished Stat3-mediated transcription (Chung et al, 1997). Moreover, the expression of another inhibitor of Stat3, Socs5 (Martens et al, 2005), was also decreased (Fig 2D). In addition, the expression of the tyrosine phosphatase Ptpn6 (Shp1), which was shown to dephosphorylate Stat3 but not Stat1 or Stat5 (Demosthenous et al, 2015), was also changed based on our RNA-seq results. Previous studies showed that the expression of constitutively active form of Stat3 driven by the K5 promoter (K5.Stat3C mice) resulted in an increased number of tumors as well as a shorter latency period after DMBA/TPA treatment (Chan et al, 2008) and Stat3-deficient mice are resistant to chemically induced carcinogenesis (Chan et al, 2004).

Based on these observations, we hypothesized that expression of ALK<sup>F1174L</sup> may trigger Stat3. To test this, we analyzed the protein homogenates from several ALK<sup>F1174L</sup> and ALK<sup>F1174L, Kras<sup>G12D</sup></sup> tumors with anti-Y705-Stat3 and anti-S727-Stat3 antibodies using Western blot (Fig 5A). The vast majority of tumors showed phosphorylation of Stat3, whereas Stat3 KO ES cells were devoid of any signal. To evaluate whether ALK<sup>F1174L</sup> overexpression can lead to phosphorylation of Stat3, we compared HEK293T cells transiently over-expressing the mutated ALK (pCMV-ALK<sup>F1174L</sup>) with their corresponding mock control (pCMV6-entry). As shown in Fig 5B, the overexpression of ALK<sup>F1174L</sup> in HEK293T cells resulted in Stat3 phosphorylation. On the basis of these results, we reasoned that Stat3 might play an essential role in mediating ALK<sup>F1174L</sup>-driven SCC formation. To test this possibility, we have crossed ALK<sup>F1174L</sup> Lgr5-CreERT2 mice to conditional Stat3 knockout mice (Jacobly et al, 2003) (Fig 5C). ALK<sup>F1174L</sup>; Stat3<sup>fl<sup/>/</sup>fl</sup> Lgr5-CreERT2 showed prolonged tumor-free survival and progression-free survival as compared with ALK<sup>F1174L</sup> Lgr5-CreERT2 mice. However, tumor formation still occurred (Fig 5D and E). We verified the presence of the recombined Stat3 floxed allele using PCR strategy (Mok et al, 2007) (Fig 5F). The resulting data revealed that 3/6 analyzed tumors from ALK<sup>F1174L</sup>; Stat3<sup>fl<sup/>/</sup>fl</sup> Lgr5-CreERT2 mice were partially recombined and 3/6 tumors revealed the lack of the Stat3 recombined allele (Fig 5F), suggesting that the recombination was partial. To test whether the resulting tumors were still expressing Stat3, we have performed immunostaining for p-Stat3 and observed that 100% of tumors still contained Stat3-positive cells (Fig 5G). This observation is in alignment with the data obtained from the analysis of the recombination efficiency (Fig 5F). We next evaluated the percentage of the recombination and counted the number of HF either positive or negative for p-Stat3 protein (all WT HF were devoid of p-Stat3). Whereas the vast majority of HFs in ALK<sup>F1174L</sup> Lgr5-CreERT2 mice showed readily detectable p-Stat3 protein, less than 50% of HF were positive for p-Stat3 in ALK<sup>F1174L</sup>; Stat3<sup>fl<sup/>/</sup>fl</sup> Lgr5-CreERT2 mice (Figs 5H and S6D). It is plausible that these p-Stat3-positive HFs occurred because of the low recombination efficiency and likely give rise to the tumors observed.

Although our data suggest that ALK<sup>F1174L</sup>-mediated tumorigenesis, at least in part, might occur via its downstream effector STAT3, further experimental evidence is required to determine the significance of these findings.

Our findings reveal a previously unknown role of oncogenic ALK signaling in cSCC. We show that the expression of a constitutively active ALK<sup>F1174L</sup> in mice can lead to the development of aggressive forms of cSCC (Fig 6). On the molecular level, ALK<sup>F1174L</sup> can cooperate with known cSCC drivers, including Kras<sup>G12D</sup> as well as with the loss of tumor suppressor p53. Moreover, our data demonstrate that Stat3 is essential for mediating the oncogenic effect of ALK<sup>F1174L</sup>. In addition to our data uncovering the essential role of oncogenic ALK<sup>F1174L</sup> in mouse cSCC, we show that human samples of cSCC contain ALK mutations as well. Therefore, our data provide a rational for oncogenic ALK as a novel therapeutic target and can serve as a basis for the design of future clinical trials.

Materials and Methods

Transgenic mice

All animal experiments have been approved by the cantonal veterinary authorities of Zurich, in accordance with Swiss laws. For DNA isolation, mouse biopsies were lysed using lysis buffer (5M NaCl, 2M Tris, pH 88.5, 0.5M EDTA, and 20% SDS) and proteinase K. DNA was

**Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup>/Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup>/ (n = 13, median 19 d) mice. Log-rank (Mantel–Cox) test; P < 0.0001, HR = 0.07945. (G) Ear tumor from Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup>/ mice were prepared as a single-cell suspension, and tumor cells were isolated by FACS for EpCam expression e-negative selected for CD31/CD45/CD140a. RNA from sorted cells was used for RNA-seq. (H) Above: Venn diagram representing deregulated genes in tumor cells over controls. Below: significantly altered genes in Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors. Horizontal columns represent the P-value of the analysis of the up-regulated genes from RNA-seq transcriptional profiling of sorted tumor cells versus normal keratinocytes. Processes and pathways marked by * are significantly changed also in the analysis of transcriptions of Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> versus Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumor cells. (I) Genes significantly regulated from RNA-seq analysis, between Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors that cluster in the indicated KEGG signaling pathways. (J) Representative immunofluorescences of Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors. Light or dark biopsies were immunohistochemically stained with DAPI, pan-cytokeratin (Pan-Ck), and vimentin antibodies. (K) Cells that co-expressed Pan-Ck and vimentin were counted as cells in epithelial-to-mesenchymal transition. Every tumor arising in the mice was analyzed and relative quantification is represented by a dot. Analysis of tumors from five Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> mice showed an increased number of relative vimentin* cells in Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors (4,907 ± 2,906, 8347 ± 3,885, Mann–Whitney test P = 0.0177). Scale bars = 50 µm. (M) Representative immunohistochemistry of Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors immuno-labelled with DAPI, pan-cytokeratin (Pan-Ck), and vimentin antibodies. (N) Analysis of tumors from five Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> mice showed an increased relative number of proliferating cells in the Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors (5,786 ± 4,958, 6,724 ± 6,482, Mann–Whitney test P = 0.0043). Scale bars = 100 µm. (O) Representative immunohistochemistry of Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumor immuno-labelled with Pan-Ck and with CD31 antibodies, to identify tumors and vessels. (P) Every tumor arising in the mice was analyzed and relative quantification is represented by a dot. Analysis of tumors from eight Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> mice showed an increased relative area occupied by vessels within the Lgr5-CreERT2<sup>fl</sup>/LSL-ALKF1174L<sup>fl</sup>/LSL-KrasG12D<sup>fl</sup> tumors (30.75 ± 34.34, 46.10 ± 35.24, Mann–Whitney test P = 0.0035). Scale bars = 100 µm. (Q, K, L) Data showed as mean ± SD.
Figure 4. p53 conditional KO increased tumorigenicity driven by ALK

(A) Graphical representation of Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ genotype. (B) Tumor-free survival of Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ (n = 20, median 49 d) and Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ (n = 20, median 49 d) mice. Log-rank (Mantel–Cox) test; P = 0.4998, HR = 0.7834. (C) Progression-free survival of Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ (n = 15, median 47 d) and Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ (n = 15, median 47 d) mice. Log-rank (Mantel–Cox) test; P = 0.4998, HR = 0.7834. (D) Number of tumors per mouse in Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ and Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ mice. Each dot represents the number of tumors in a specific mouse. Mean ± standard error of the mean (5.917 ± 1.026; n = 12; 10.12 ± 1.3 n = 17; Two-tailed t test P = 0.0254). (E) Pie chart representing the number of mice that developed the listed skin lesions out of total lesions diagnosed. (F) Representative hematoxylin and eosin (H&E) staining of acanthopapilloma 3 and multiple SCC2 from ear skin of Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ mice. In the magnification, it is possible to appreciate mesenchymal-like features of tumor cells and pronounced nuclear atypia. Scale bars = 100 µm. (G) Representative immunofluoresces of Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ ear tumor immuno-labelled with DAPI, pan-cytokeratin (Pan-Ck), and vimentin antibodies. Scale bar = 50 µm. Cells that co-expressed Pan-Ck and vimentin were counted as cells in epithelial-to-mesenchymal transition (EMT). All tumors diagnosed as cSCC type 1 or 2 from different mice were analyzed, and quantification of the relative number of cells in EMT per tumor is represented by a dot. Analysis of tumors showed that no significant changes were observed between SCC1 tumors of three Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ or three Lgr5-CreER$^{+}$:LSL-ALK$^{TIR}$:p53$^{fl/fl}$ mice (4.380 ± 1.425; 7.360 ± 1.996. Mann–Whitney test P = 0.3277). Strong increase in the EMT rate was instead noted in SCC2 tumors when compared with SCC1 tumors of the same cohort of mice (7.360 ± 1.996; 17.10 ± 1.875. Mann–Whitney test P = 0.0056) or to Lgr5-CreERT2:LSL-ALKF1174L mice (4.380 ± 1.425; 17.10 ± 1.875. Mann–Whitney test P = 0.0008). All values are showed as median with 95% confidence interval. (H) Above: the schematic structure of the p53 floxed allele and graphical representation of PCR strategy to determine the recombination efficiency of LoxP sites that drives the removal of exons 2–10 of the p53 gene. White boxes represent exons, arrowheads represent the LoxP sites, and purple lines indicate
precipitated in 50% lystate and 50% isopropanol and centrifuged at 20,817g for 30 min. After washing in 70% ethanol, 2 µl of DNA were mixed with 10 µl KAPA Taq ReadyMix with dye (KK1024; Kapa Biosystems) and with 0.8 µl of each primer (10 µM) and finally brought to 20 µl with MilliPore water. The following primers with according melting temperatures were used: 60°C, LSL-ALK_7083_Fw (CCATCAGTCATGTGACGAGG) LSL-ALK_7083_Rv (CAGCTGCAAGG TCCAGC), 60°C, Cre_Fw (CTATCAGCAACATTGTGGCCAGC) Cre_Rv (CC AGGTGAACATGATTGCGTAC), 60°C, LSL-KRAS_Fw1 (TGCTTCCTC CACAGCAT), LSL-KRAS_Rv (CTGTATAGTCGGTATACCCGT) KRAS_Fw2 (GCAGGTGCGAGGACCTAATA), 55°C, Stat3_floxed_Fw (TTGACCTGT GTCCTCAGAAA) Stat3_floxed_Rv (CCCTAGATTAGGCCAGACA), 60°C, p53-oIMR8543_Fw (GTTGAAAAAGGCTGACCA) p53-oIMR8544_Rv (GGAGGACAGAAGTGGAG), 58°C, iCre-Ks_Fw (CTTGCAAGATGCA GACA) iCre-Ks_Rv (TCTCTGGCAAGTCCATCCT), 60°C, K14-CreErt2_T_Fw (CGCATCCTTCCAGTTACAT) K14-CreErt2_T_Rv (GGGTCCATGGTGATACAAGG) K14-CreErt2_C_Fw (CTGGGAACATGACTTACGTAAC) K14-CreErt2_C_Rv (GTGACCTGAGGACCTAATA), 55°C, Stat3 (Tyr705) antibody D3A7 (9145S; Cell Signaling Technology). 500 anti-vimentin antibody (ab92547; Abcam), and 1:100 Phospho-p53 antibody (ab29547; Abcam), and 1:100 Phospho-Stat3 (Tyr705) antibody D3A7 (9145S; Cell Signaling Technology). After washing in PBS, Alexa Fluor 555 goat antirabbit IgG (A21428; Life Technologies) was added. The sections were washed in PBS and mounted with medium containing 1:10 Hoechst 33342 (H1399; Molecular Probes by Life Technologies). Images were captured with Leica DMi6000 B and analyzed using the LAS X software or with Zeiss Axio Scan.Z1 and processed with Zen software. Immunohistochemistry was performed following the instruction of Mouse on Mouse (M.O.M.) Basic Kit (BMK-2202; Vector Laboratories) combined with the protocol of ImmPRESS-AP Anti-Rabbit Ig Reagent–Alkaline Phosphatase (MP-5401-15; Vector Laboratories). Briefly, deparaffinization and antigen retrieval were performed as previously described. M.O.M. kit was used 1:1,000 monoclonal anti–pan-cytokeratin–FITC (F0397; Sigma-Aldrich), 1:500 anti-vimentin antibody (ab92547; Abcam), and 1:100 Phospho-Stat3 (Tyr705) antibody D3A7 (9145S; Cell Signaling Technology). After washing in PBS, Alexa Fluor 550 goat antirabbit IgG (A21248; Invitrogen) diluted 1:250 in PBS was applied for 1 h at room temperature. The sections were washed in PBS and mounted with DAKO Fluorescent Mounting Medium (S3023; DAKO) containing 1:100 Hoechst 33342 (H1399; Molecular probes by Life Technologies). Immunofluorescence and immunohistochemistry and hematoxylin & eosin analysis (H&E)

Induction of Cre activity, termination criteria, and in vivo imaging system

For activation of Cre activity, 6–12-wk-old mice were shaved on the back skin and 4-Hydroxytamoxifen (4-OHT) 25 mg/ml (±70% Z isomer H7904; Sigma-Aldrich) was topically administered for three consecutive days, using a small paint brush on both ears (inside and outside), on the tail, and on the shaved patch of the back skin. The mice were euthanized when termination criteria was reached. In most cases, size of tumors (≥ 0.5 cm of diameter, n = 2) was the adopted termination criteria. Secondary, mice were euthanized if developed one tumor bigger than 1 cm of diameter or if tumor was ulcerative or bleeding. Mice that showed wounds (signs of fights) were withdrawn from experiment. On the day of euthanizing, the animals were anesthetized with isoflurane (B506; Abbott) and shaved with a waxing cream (Veet). Then mice have been injected with 150 mg/kg body weight of Xenolight D-Luciferin-K· Salt Bioluminescent Substrate (122799; PerkinElmer) and imaged with IVIS Lumina III (PerkinElmer). In vivo and ex vivo analysis was performed, and tissue was collected according with the signal acquired for histological analysis. Skin, organs, and tumors were embedded in paraffin for immunohistochemistry and hematoxylin and eosin staining.

Immunofluorescence and immunohistochemistry and hematoxylin & eosin analysis (H&E)

Skin and mouse tumor samples were fixed in Roti-Histoxy (P078.3; Roth) for 2 h. Then washed in PBS and embedded in paraffin (MEDITE PURE Paraffin, 40-0020-00; MEDITE). Sections of 5-μm thickness were deparaffinized, rehydrated using the Automated Staining System AS-2 (SN: 180.001.1019.19; Pathistain), and antigen retrieval was performed in citrate buffer (pH 6.0, 82% 10-mM sodium citrate plus 18% of 10 mM citric acid) or in EDTA buffer (pH 8.0, 1 mM EDTA, 0.05% Tween 20) for 25 min at 110°C using Decloaking Chamber (SN: DG12-200-0134; Biocare Medical). At least 20 independent sections per genotype were histologically scored. For immunofluorescence assay, the sections were washed with PBS and incubated in blocking solution (5% horse serum in PBS) for 1 h. Subsequently, the slides were incubated overnight at 4°C with the following primary antibodies diluted in PBS: 1:500 monoclonal anti–pan-cytokeratin–FITC (F0397; Sigma-Aldrich), 1:500 anti-vimentin antibody (ab92547; Abcam), and 1:100 Phospho-Stat3 (Tyr705) antibody D3A7 (9145S; Cell Signaling Technology). Images were captured with Leica DMi6000 B and analyzed using the LAS X software or with Zeiss Axio Scan.Z1 and processed with Zen software. Immunohistochemistry was performed following the instruction of Mouse on Mouse (M.O.M.) Basic Kit (BMK-2202; Vector Laboratories) combined with the protocol of ImmPRESS-AP Anti-Rabbit Ig Reagent–Alkaline Phosphatase (MP-5401-15; Vector Laboratories). Briefly, deparaffinization and antigen retrieval were performed as previously described. M.O.M. kit was used 1:100 monoclonal anti–pan-cytokeratin–FITC (F0397; Sigma-Aldrich), Anti-Ki67 antibody (15580; Abcam), and 1:100 Anti-CD31 antibody (ab182981; Abcam). Detection of the signal was performed with the Vector Blue Substrate Kit (SK5300; Vector...
Figure 5. Stat3 is indispensable for ALK\textsuperscript{TIT14L} driven tumorigenicity. 

(A, B) Western blot analysis of phosphorylation status of Stat3 in (A) Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L} and Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L};LSL-Kras\textsuperscript{G12D} tumors and in (B) HEK293T cell line transiently expressing the ALK\textsuperscript{TIT14L} transcript or control.

(C) Graphical representation of Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L};Stat3\textsuperscript{fl/fl} genotype.

(D) Tumor-free survival of Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L} (\(n = 20\), median 51 d) and Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L};Stat3\textsuperscript{fl/fl} (\(n = 11\), median 63 d) mice. Log-rank (Mantel–Cox) test; \(P = 0.0202\), HR = 2.540.

(E) Progression-free survival of Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L} (\(n = 20\), median 37 d) and Lgr5-Cre\textsuperscript{ERT2};LSL-ALK\textsuperscript{TIT14L};Stat3\textsuperscript{fl/fl} mice (\(n = 11\), median 43 d). Log-rank (Mantel–Cox) test; \(P = 0.0616\), HR = 2.066.

(F) Above: structure of the Stat3 floxed allele and graphical representation of PCR strategy to analyze recombination efficiency.
Lgr5-CreERT2;LSL-ALKF1174L

Stat3 phosphorylation within the tumors (above) and in the hyperplastic skin and in the hair follicles (HF) adjacent to the tumor masses (below). To note, all hyperplastic HFs were being considered for the analysis. We analyzed show a mixed expression of full-length and truncated Stat3.

6660-00; MEDITE) containing three drops of CH₃COOH. Subsequently, the slides were dehydrated and finally placed for 20 min in xylol (103746; EBIS). Then, mounting was performed using Eukitt Quick hardening mounting medium (03989; Fluka). Images were captured with Zeiss Axio Scan.Z1 and processed with ZEN software.

Statistical analysis

A schematic illustration summarizing main findings.

Expression of ALK1174L in Lgr5/ hair follicle stem cells results in formation of different skin lesions as Cysts, acanthopapilloma (AP), keratoacanthoma (KA), or squamous cell carcinoma type 1 (SCC1). Additional KrasG12D expression leads to increased epithelial to mesenchymal transition, proliferation, and vascularization. Loss of p53 primes to formation of a more aggressive tumor: squamous cell carcinoma type 2 (SCC2). Finally, loss of Stat3 prevents the tumor formation ALK1174L-induced.

Figure 6. A schematic illustration summarizing main findings.

Quantifications

Quantification of Vimentin and pan-cytokeratin+ cells was performed using a machine learning bio-image analysis software: QuPath (Bankhead et al, 2017). The software was trained to identify tumor cells, stromal cells, EMT cells, and false positives. Quantification of CD31 signal and ki67+ cells over pan-cytokeratin area was performed with CellProfiler (www.cellprofiler.org), a cell image analysis software. Proliferation rate was calculated by identification of tumor area and subsequent count of proliferating nuclei within the mask made out of the tumor. Relative enrichment of vessels within the tumors was performed by identification of tumor and vessel areas and subsequent quantification with an inverse mask out of tumor area for CD31 signal. The correlation of the number of vessels with the CD31 signal was statistically significant, out of three tumors quantified (Pearson r = 0.9990; two-tailed P = 0.0289). For the quantification of vimentin staining, we have used five different Lgr5-CreER72;LSL-ALKF1174L;LSL-KrasG12D mice from which we analyzed 21 independent tumors (one section per tumor) and five different Lgr5-CreER72;LSL-ALKF1174L mice from which we analyzed 12 independent tumors. For CD31 quantification, we used 60 independent Lgr5-CreER72;LSL-ALKF1174L;LSL-KrasG12D tumors (one section per tumor) and 28 independent Lgr5-CreER72;LSL-ALKF1174L tumors (one section per tumor). For Ki67 staining, we used 70 independent Lgr5-CreER72;LSL-ALKF1174L;LSL-KrasG12D tumors (one section each) and 46 independent Lgr5-CreER72;LSL-ALKF1174L tumors (one section per tumor).

DNA extraction from paraffin tissue and sequencing of Alk exon 23

Paraffin-embedded cSCC from DMBA/TPA-treated mice were kindly gifted by the Laboratory of Prof. Sabine Werner. A few sections of tumors were collected in an Eppendorf tube together with 500 µl lysis buffer, which was incubated at 95°C for 10 min, and then centrifuged at 4°C, 20,817g for 5 min and the paraffin was removed. DNA extraction was performed as described above. Exome 23 of Alk was then amplified using KAPA Taq ReadyMix with dye (KK1024; Kapa Biosystems) with specific primers (msAlk-ex23_fw: CTATG-CATCGCCCCAGGAAG, msAlk-ex23_Rv: GGCCTGACTCCAGGAGCCCA; MT = 60°C), and amplicons were sent for Sanger sequencing to Hier (www.cellpro.org), a cell image analysis software.

Statistical analysis and graphical representation were performed with GraphPad Prism 5 software. The n reported in the figures or figure legends always refers to the number of mice used for each analysis. Column analysis was always performed with t test or Mann–Whitney test accordingly if the group of data considered in examination had a normal distribution or not (D’Agostino & Pearson omnibus normality test). Log-rank (Mantel–Cox) test was performed for statistical analysis of survivals.

Will produce a shorter mRNA missing exons 18–19–20. White boxes represent exons, arrowheads represent the LoxP sites, and purple lines represent the primers. Below: RT-PCR analysis of different tissues. Whereas wild-type ear skin shows no detectable expression of Stat3, only 3/6 of the Lgr5-CreER72;LSL-ALKF1174L Stat3+/– tumors analyzed show a mixed expression of full-length and truncated Stat3. Number of tumors per mouse in Lgr5-CreER72;LSL-ALKF1174L and Lgr5-CreER72;LSL-ALKF1174L Stat3+/– mice. Mean ± standard error of the mean (5.917 ± 1.026 n = 12, 5.727 ± 1.280 n = 11; two-tailed t test P = 0.9084). (G) Representative immunofluorescence images of Lgr5-CreER72;LSL-ALKF1174L;LSL-KrasG12D;Stat3+/– ear tumors immuno-labelled with DAPI, pan-cytokeratin (Pan-Ck), and p-Stat3Y705 antibodies show phosphorylation of Stat3 within the tumors (above) and in the hyperplastic skin and in the hair follicles (HF) adjacent to the tumor masses (below). To note, all Lgr5-CreER72;LSL-ALKF1174L;LSL-KrasG12D;Stat3+/– tumors analyzed (n = 8) showed p-Stat3Y705 expression. Scale bars = 50 µm. (H) From left to right, wild-type ears HFs, and non-hyperplastic Lgr5-CreER72;LSL-ALKF1174L and Lgr5-CreER72;LSL-ALKF1174L Stat3+/– ears HFs adjacent to the tumor masses have been immuno-labelled with DAPI, pan-cytokeratin (Pan-Ck), and p-Stat3Y705 antibodies. Insets display strong p-Stat3Y705 expression within the HFs of Lgr5-CreER72;LSL-ALKF1174L mice, whereas wild-type skin is devoid of p-Stat3Y705 expression. Scale bars = 25 µm. Quantification of the recombination was measured as relative number of HFs expressing or not expressing p-Stat3Y705. Only non-hyperplastic HFs were being considered for the analysis.
FACS sorting and RNA sequencing

FACS isolation of ear tumor cells and normal ear keratinocytes was performed as previously described (Nassar et al., 2015). mRNA from sorted cells was extracted using the RNeasy Plus Micro Kit (74034; Qiagen) according to the manufacturers’ instructions. Biological replicates from nine independent mice, three Lgr5-CreERT2;LSL-ALKF1174L, three Lgr5-CreERT2;LSL-ALKF1174L;KrasG12D, and three Cre−ittermates, were sent to the Functional Genomic Center Zurich for RNA sequencing. RNA-seq-poly-A strategy was used to build the libraries, and Illumina Novaseq 6000 (Illumina) Center Zurich for RNA sequencing. RNA-seq-poly-A strategy was performed as previously described (Nassar et al., 2015). mRNA was used for sequencing. For following analysis, genes were selected based on P-value less than 0.01, and Log, expression fold change is either above 0.5 or less than −0.5. GO and KEGG signaling pathways analysis was performed by using Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics web tool. Gene Set Enrichment Analysis was performed against the MSigDB c5.bp.v6.2 gene set and clustering of enriched biological processes was performed using Cyto-scape’s EnrichmentMap tool (version 3.2.0). Graphical visualization of RNA sequencing data was performed using R (version 3.5.0) and displayed in terms of Z-score values. RNA-seq data were deposited and made publicly available on the Gene Expression Omnibus (GSE147642).

Cell culture

HEK293T cell line (ATCC CRL-3216) was cultured in DMEM Low Glucose w/L-Glutamine w/Sodium Pyruvate medium (L0060-500; Dominique Deutscher), 10% FCS (S181B-500; Dominique Deutscher), 1% L-glutamine (25300024; Gibco), and 1% sodium pyruvate (11360-039; Gibco) and maintained in a humidified incubator at 37°C, 5% CO2. Western blot was performed. For following analysis, genes were selected based on P-value less than 0.01, and Log, expression fold change is either above 0.5 or less than −0.5. GO and KEGG signaling pathways analysis was performed by using Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics web tool. Gene Set Enrichment Analysis was performed against the MSigDB c5.bp.v6.2 gene set and clustering of enriched biological processes was performed using Cyber-scape’s EnrichmentMap tool (version 3.2.0). Graphical visualization of RNA sequencing data was performed using R (version 3.5.0) and displayed in terms of Z-score values. RNA-seq data were deposited and made publicly available on the Gene Expression Omnibus (GSE147642).

Western blot

Proteins were extracted using radio-immunoprecipitation assay (RIPA) lysis buffer (89900; Thermo Fisher Scientific) containing C omplete ULTRA protease inhibitor (05892970001; Roche) and PhosSTOP (4906837001; Roche) and Phosphatase Inhibitor Cocktail 2 and 3 (P5726, P0044; Sigma-Aldrich). Cell lysates were centrifuged for 30 min at 20,817g at 4°C and supernatant was collected. Protein quantification was performed by Pierce BCA assay according to the manufacturers’ instructions (23227; Thermo Fisher Scientific). 30 μg of protein lysates mixed with Laemmli sample buffer (161-0747; Bio-Rad) was loaded into Precast Tris–HCl gels, 4–20% (456-8093; Bio-Rad). Trans-Blot Turbo Transfer System (690BR013492; Bio-Rad) was used to blot the gel onto polyvinylidene fluoride (PVDF) membranes (170-4156; Bio-Rad) and subsequently blocked for 1 h in 5% milk in TBS-T (TBS with 0.1% Tween [P4780-500ML; Sigma-Aldrich]). Overnight application of the following antibodies diluted in TBS-T was used: 1:500 Phospho-ALK (Tyr1604) Antibody (3341; Cell Signaling Technology), 1:1,000 Phospho-STAT3 (T705) Antibody (9131; Cell Signaling Technology), 1:1,000 Phospho-STAT3 (T527) Antibody (9134; Cell Signaling Technology), 1:2,000 in GAPDH Loading Control Monoclonal Antibody (MAS-15738; thermo Fisher Scientific), and 1:5,000 Monoclonal anti-α-Tubulin (T-5168; Sigma-Aldrich). The membranes were washed in TBS-T and then incubated for 1 h at room temperature with the following secondary antibodies (1:2,000 in 5% milk in TBS-T): HRP anti-rabbit IgG (410406; BioLegend) and goat anti-mouse IgG HRP (405306; BioLegend). The signal was detected by application of Clarity Western ECL Substrate (170-5060; Bio-Rad) using Fusion FX VILBER LOURMAT (12-200168).

At least four independent tumors per genotype were subjected to Western blot analysis.

Supplementary Information

Supplementary information is available at https://doi.org/10.26508/lsa.201900601.

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Author Contribution

M Gualandi: conceptualization, data curation, formal analysis, investigation, and methodology.
M Iorio: data curation, formal analysis, investigation, and methodology.
O Engeler: data curation, formal analysis, investigation, and methodology.
A Serra-Roma: data curation, formal analysis, investigation, and methodology.
G Gasparre: data curation, formal analysis, investigation, and methodology.
D Hohl: investigation.
O Shakhova: conceptualization, data curation, and methodology.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.
References

Abel EL, Angel JM, Kiguchi K, Digiovanni J (2009) Multi-stage chemical carcinogenesis in mouse skin: Fundamentals and applications. Nat Protoc 4: 1350–1362. doi:10.1038/nprot.2009.120

Adzhubei IA, Schmidt S, Peshkin L, Ramsenky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR (2010) A method and server for predicting damaging missense mutations. Nat Methods 7: 248–249. doi:10.1038/nmeth.0410-248

Bankhead P, Loughrey MB, Fernandez JA, Mcart DG, Dunne PD, Mcquaid S, Gray RT, Murray LJ, Coleman HG, et al (2017) QuPath: Open source software for digital pathology image analysis. Sci Rep 7: 16878. doi:10.1038/s41598-017-17204-5

Barker N, Van Es JH, Kuipers J, Kujala P, Van Den Born M, Cozijnsen M, Patil RM, Ferris J, Diener J, et al (2011) Basal cell carcinomas in mice transgenic for a mutant H-ras transgene depends on the cell type targeted. Curr Biol 21: 516–524. doi:10.1016/j.cub.2011.03.042

Caulin C, Ncuoy T, Lang GA, Goepfert TM, Brinkley BR, Cai WW, Lozano G, Roop DR (2007) An inducible mouse model for skin cancer reveals distinct roles for gain- and loss-of-function p53 mutations. J Clin Invest 117: 1893–1901. doi:10.1172/JCI31721

Chan KS, Sano S, Kataoka K, Abel E, Carbalaj S, Beltran L, Clifford J, Peavey M, Shen J, Digiovanni J (2008) Forced expression of a constitutively active kinase in human cancer biology. Nat Rev Cancer 8: 1087–1094. doi:10.1038/nrc2479

Chung CC, Liao J, Liu B, Rao X, Jay P, Berta P, Shuai K (1997) Specific inhibition of Stat3 signal transduction by Pias3. Science 278: 1803–1805. doi:10.1126/science.278.5344.1803

Cohen DN, Lawson SK, Shaver AC, Du L, Nguyen HP, He Q, Johnson DB, Lumbang WA, Moody BR, Prescott JL, et al (2015) Contribution of beta-HPV infection and UV damage to rapid-onset cutaneous squamous cell carcinoma during BRAF-inhibition therapy. Clin Cancer Res 21: 2624–2634. doi:10.1158/1078-0432.ccr-14-2667

Demosthenous H, Han JJ, Hu G, Stenson M, Gupta M (2015) Loss of function mutations in PTPN6 promote STAT3 deregulation via JAK3 kinase in diffuse large B-cell lymphoma. Oncotarget 6: 44703–44713. doi:10.18632/oncotarget.6300

Durinc H, Ho C, Wang NL, Liao W, Jakkula LR, Collisson EA, Poms J, Chan SW, Lam ET, Chu C, et al (2011) Temporal dissection of tumorigenesis in primary cancers. Cancer Discov 1: 137–143. doi:10.1158/2159-8290.cd-11-0028

Gleich T, Chiticariu E, Huber M, Hohi D (2016) Keratoacanthoma: A distinct entity? Exp Dermatol 25: 85–91. doi:10.1111/exd.12880

Grachtchouk M, Pero J, Yang SH, Ermilov AN, Michael LE, Wang A, Wilbert D, Patel RM, Ferris J, Diener J, et al (2011) Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. J Clin Invest 121: 1768–1781. doi:10.1172/JCI6307

Hallberg B, Palmer RH (2013) Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. Nat Rev Cancer 13: 685–700. doi:10.1038/nrc3580

Hennings H, Boutwell RK (1970) Studies on the mechanism of skin tumor promotion. Cancer Res 30: 312–320.

Heukamp LC, Thor T, Schramm A, De Preter K, Kumps C, de Wilde B, Odersky A, Peifer M, Lindner S, Spruessel A, et al (2012) Targeted expression of mutated ALK induces neuroblastoma in transgenic mice. Sci Transl Med 4: 141ra91. doi:10.1126/scitranslmed.3003967

Hoste E, Arwert EN, Lal R, South AP, Salas-Alanis JC, Murrell DF, Donati G, Watt FM (2015) Innate sensing of microbial products promotes wound-induced skin cancer. Nat Commun 6: 5932. doi:10.1038/ncomms6932

Inman GI, Wang J, Nagano A, Alexandrov LB, Purdie KJ, Taylor RG, sherwood V, Thomson J, Hogan S, Spender LC, et al (2018) The genomic landscape of cutaneous SCC reveals drivers and a novel azathioprine associated mutational signature. Nat Commun 9: 3667. doi:10.1038/s41467-018-06027-5

Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Mantoja R, Jacks T, Tooves DA (2001) Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes Dev 15: 3243–3248. doi:10.1101/gad.943001

Jacoby JI, Kalinowski A, Liu MG, Zhang SS, Gao Q, Chai GJ, Li L, Iwamoto Y, Li E, Schneider M, et al (2003) Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. Proc Natl Acad Sci U S A 100: 12929–12934. doi:10.1073/pnas.1007939

Lague P, Yousseff K, Vokaer B, Achouri Y, Michaux C, Sotiropoulou PA, Blanpain C (2011) Identifying the cellular origin of squamous skin tumors. Proc Natl Acad Sci U S A 108: 7431–7436. doi:10.1073/pnas.101270108

Lee CS, Bhaduri A, Mah A, Johnson WL, Ungewickell A, Rios EI, Sipriavelli Z, Straight A, et al (2014) Recurrent point mutations in the kinetochore gene KNSR1 in cutaneous squamous cell carcinoma. Nat Genet 46: 1060–1062. doi:10.1038/ng.3091

Li YI, Hanna GJ, Laga AC, Haddad R, Lorch JH, Hammeeman PS (2015) Genomic analysis of metastatic cutaneous squamous cell carcinoma. Clin Cancer Res 21: 1447–1456. doi:10.1158/1078-0432.crr-14-1773

Marino S, Voojis M, Van der Gulden H, Jankers J, Berns A (2000) Induction of medulloblastomas in p53-null mouse mutants by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 14: 994–1004. doi:10.1101/gad.14.8.994

Martens N, Uzan G, Wery M, Hooghe R, Hooghe-Peters EL, Gelttler A (2005) Suppressor of cytokine signaling 7 inhibits prolactin, growth hormone, and leptin signaling by interacting with STAT5 or STAT3 and attenuating their nuclear translocation. J Biol Chem 280: 13817–13823. doi:10.1074/jbc.m411962000

Moh A, Iwamoto Y, Chai GJ, Zhang SS, Kano A, Yang DD, Zhang W, Wang J, Jacoby JI, Gao B, et al (2007) Role of STAT3 in liver regeneration: Survival, DNA synthesis, inflammatory reaction and liver mass recovery. Lab Invest 87: 1018–1028. doi:10.1038/layinvent.3700630

Morris RI, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G (2004) Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 22: 411–417. doi:10.1038/nbt950

Murugan AK, Xing M (2011) Anaplastic thyroid cancers harbor novel oncogenic mutations of the ALK gene. Cancer Res 71: 4403–4411. doi:10.1158/0008-5472.can-10-4041

Nassar D, Latil M, Boeckx B, Lambrechts D, Blanpain C (2015) Genomic landscape of carcinogen-induced and genetically induced mouse skin squamous cell carcinoma. Nat Med 21: 946–954. doi:10.1038/nm.3878

Ning H, Mitsui H, Wang CQ, Suarez-Farinhas M, Gonzalez J, Shah KR, Chen I, Coats I, Felsen D, Carucci JA, et al (2013) Identification of anaplastic lymphoma kinase as a potential therapeutic target in basal cell carcinoma. Oncotarget 4: 2237–2248. doi:10.18632/oncotarget.1357
Pickering CR, Zhou JH, Lee JJ, Drummond JA, Peng SA, Saade RE, Tsai KY, Curry JL, Tetzlaff MT, Lai SY, et al (2014) Mutational landscape of aggressive cutaneous squamous cell carcinoma. Clin Cancer Res 20: 6582–6592. doi:10.1158/1078-0432.ccr-14-1768

Ratushny V, Gober MD, Hick R, Riddky TW, Seykora JT (2012) From keratinocyte to cancer: The pathogenesis and modeling of cutaneous squamous cell carcinoma. J Clin Invest 122: 464–472. doi:10.1172/jci57415

Sanchez-Danes A, Blanpain C (2018) Deciphering the cells of origin of squamous cell carcinomas. Nat Rev Cancer 18: 549–561. doi:10.1038/s41568-018-0024-5

Spencer JM, Kahn SM, Jiang W, Deleo VA, Weinstein IB (1995) Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. Arch Dermatol 131: 796–800. doi:10.1001/archderm.131.7.796

Van der Schroeff JG, Evers LM, Boot AJ, Bos JL (1990) Ras oncogene mutations in basal cell carcinomas and squamous cell carcinomas of human skin. J Invest Dermatol 94: 423–425. doi:10.1111/1523-1747.ep12874504

Van Kranen HJ, De Gruij FR, De Vries A, Sontag Y, Wester PW,SENDEN HC, ROZEMULLER E, Van Kreijl CF (1995) Frequent p53 alterations but low incidence of ras mutations in UV-B-induced skin tumors of hairless mice. Carcinogenesis 16: 1141–1147. doi:10.1093/carcin/16.5.1141

Vasioukhin V, Degenstein L, Wise B, Fuchs E (1999) The magical touch: Genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc Natl Acad Sci U S A 96: 8551–8556. doi:10.1073/pnas.96.15.8551

Vitale-Cross L, Amphornphimoltham P, Fisher G, Molinolo AA, Gutkind JS (2004) Conditional expression of K-ras in an epithelial compartment that includes the stem cells is sufficient to promote squamous cell carcinogenesis. Cancer Res 64: 8804–8807. doi:10.1158/0008-5472.CAN-04-2623

Watanabe S, Ichikawa E, Tachakashi H, Otaka F (1995) Changes of cytokeratin and involucrin expression in squamous cell carcinomas of the skin during progression to malignancy. Br J Dermatol 132: 730–739. doi:10.1111/j.1365-2133.1995.tb00716.x

White AC, Khuu JK, Dang CY, Hu J, Tran KV, Liu A, Gomez S, Zhang Z, Yi R, Scumpia P, et al (2014) Stem cell quiescence acts as a tumour suppressor in squamous tumours. Nat Cell Biol 16: 99–107. doi:10.1038/ncb2889

White AC, Lowry WE (2011) Exploiting the origins of Ras mediated squamous cell carcinoma to develop novel therapeutic interventions. Small GTPases 2: 318–321. doi:10.4161/sgtp.18088

White AC, Tran K, Khuu J, Dang C, Cui Y, Binder SW, Lowry WE (2011) Defining the origins of Ras/p53-mediated squamous cell carcinoma. Proc Natl Acad Sci U S A 108: 7425–7430. doi:10.1073/pnas.1012670108

Yilmaz AS, Ozer HG, Gillespie JL, Allain DC, Bernhardt MN, Furlan KC, Castro LT, Peters SB, Nagarajan P, Kang SY, et al (2017) Differential mutation frequencies in metastatic cutaneous squamous cell carcinomas versus primary tumors. Cancer 123: 1184–1193. doi:10.1002/cncr.30459

Youssef KK, Van Kreijlouen A, Lapouge G, Beck B, Michaux C, Achouri Y, Sotropoulou PA, Blanpain C (2010) Identification of the cell lineage at the origin of basal cell carcinoma. Nat Cell Biol 12: 299–305. doi:10.1038/ncb2031

Zhou Z, Wang D, Wang XJ, Roop DR (2002) In utero activation of K5.CrePR1 induces gene deletion. Genesis 32: 191–192. doi:10.1002/gene.10064

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