Lkb1 inactivation drives lung cancer lineage switching governed by Polycomb Repressive Complex 2

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Adenosquamous lung tumours, which are extremely poor prognosis, may result from cellular plasticity. Here, we demonstrate lineage switching of KRAS+ lung adenocarcinomas (ADC) to squamous cell carcinoma (SCC) through deletion of Lkb1 (Stk11) in autochthonous and transplant models. Chromatin analysis reveals loss of H3K27me3 and gain of H3K27ac and H3K4me3 at squamous lineage genes, including Sox2, Dnlp63 and Ngfr. SCC lesions have higher levels of the H3K27 methyltransferase EZH2 than the ADC lesions, but there is a clear lack of the essential Polycomb Repressive Complex 2 (PRC2) subunit EED in the SCC lesions. The pattern of high EZH2, but low H3K27me3 mark, is also prevalent in human lung SCC and SCC regions within ADSCC tumours. Using FACS-isolated populations, we demonstrate that bronchioalveolar stem cells and club cells are the likely cells-of-origin for SCC transitioned tumours. These findings shed light on the epigenetics and cellular origins of lineage-specific lung tumours.

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ung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) are regarded as segregated entities of non-small-cell lung cancer. These two tumour types largely have unique genetic profiles, with KRAS and EGFR activation common in ADC, while PTEN inactivation, PI3KCA activation and NOTCH2 activation are common in SCC tumours\(^\text{1-2}\). However, there are cases of mixed histology tumours, where at least 10% of the biopsied tumour cells have one histology (either ADC or SCC) and the remainder are the other histology\(^\text{3}\). Adenosquamous (ADSCC) disease accounts for 2–3% of all diagnoses, though this diagnosis can only be made with wedge biopsy or tumour resection, which could lead to underestimation of its prevalence in patient populations\(^\text{3,4}\). ADSCC is a particularly poor prognosis tumour type\(^\text{5,6}\), and genetic studies have demonstrated that both histological components share mutations, suggesting a monoclonal tumour origin\(^\text{7}\). Notably, in many cases of disease progression after targeted therapy for EGFR mutation, when it is clinically justifiable to take a second biopsy, conversion of ADC to SCC has been observed\(^\text{8}\). Given these data, a better understanding of lung cancer lineage relationships could shed light on both the origins of lung cancer and how to overcome therapeutic resistance.

SCCs have long been proposed to arise from tracheal basal cells and ADCs have been proposed to arise from alveolar type II (AT2) cells or club (Clara) cells, due to markers of these cell types being present in the malignant lesions\(^\text{9,9}\). However, given the shared genetics of ADC and SCC lesions in ADSCC tumours, it must be possible for certain lung cells to drive both histologies. Basal cells, which express nerve growth factor receptor (NGFR), p63 and cytokeratin 5 (KRT5), serve as stem cells for the trachea, main bronchi and upper airways. Basal cells can replace the pseudostratified epithelium including secretory club cells, mucus-producing goblet cells and ciliated cells\(^\text{10-12}\). In more distal airways, club cells are a self-renewing population that maintain the ciliated cells\(^\text{13}\); subsets of club cells can give rise to ciliated and club cell lineages after injury\(^\text{14,15}\). In the alveolar space where gas exchange is carried out by alveolar type I cells, the surfactant-expressing AT2 cells act as stem cells\(^\text{16,17}\). Cells expressing club cell secretory protein (CCSP), including bronchoalveolar stem cells (BASCs), can give rise to AT2 cells\(^\text{18-22}\). There is also extensive plasticity in the lung and tracheal epithelium, as club cells can give rise to basal cells\(^\text{23}\), and may give rise to KRT5\(^+\)/p63\(^+\) cells or alveolar cells under certain injury conditions\(^\text{24,25}\). Cellular lineage switching, either in the normal situation or in cancer, could be modulated by epigenetic mechanisms, including histone modification governed in part by the Polycystic Recessive Complex 2 (PRC2).

Genetically engineered mouse models are unparalleled in their capacity to allow the study lung tumour origins and evolution. Using a LSL:Kras\(^{G12D/-}\); Lkb1\(^{flox/flox}\) (LSL = Lox-stop-Lox) mouse model of lung cancer, we demonstrated previously that Lkb1 inactivation dramatically accelerated KRAS-driven lung cancer progression and changed the tumour spectrum from purely ADC to ADC and SCC\(^\text{26}\). While KRAS is a common oncogene in lung ADC, LKB1/STK11, encoding a serine-threonine kinase implicated in energy sensing and cell polarity, is notable as being among the most commonly mutated tumour suppressors in ADC\(^\text{2}\). Additionally, mutations in LKB1 predominantly co-occur with KRAS activating mutations\(^\text{27,28}\). Subsequent studies with the LSL:Kras\(^{G12D/-}\); Lkb1\(^{flox/flox}\) mouse model demonstrated that the SCC tumours arise later during tumour progression than ADC and that SCCs are characterized by decreased lysyl oxidases and increased reactive oxygen species\(^\text{29-31}\). However, because of the simultaneous activation of KRAS and inactivation of Lkb1, it remained unclear if a unique pool of SCC competent cells were transformed only when Lkb1 was deleted, or if existing KRAS-induced ADC could convert to a squamous fate in response to Lkb1 deletion. Furthermore, due to the intranasal inhalation method to introduce Cre to drive the genetics, the cell-of-origin of this tumour type was unknown.

Here, we describe a stepwise mouse model of lung tumorigenesis that strongly supports the theory that established ADC cells can transition to SCC fate upon additional genetic perturbations, such as Lkb1 deletion. Using this model, we found that de-repression of squamous genes through loss of Polycomb-mediated gene repression accompanies the squamous transition. We also show that club cells and BASCs are the most fit populations to give rise to adenosquamous tumours. Together these data add to our understanding of the underlying epigenetic programmes and cellular origins of lineage-specific lung tumours.

**Results**

**Lkb1 deletion drives SCC transition of established KRAS ADCs.** Previously, we showed that Stk11 (Lkb1) deletion concomitant with induction of oncogenic KRAS drove acquisition of aggressive tumour characteristics, including SCC transition, not observed in KRAS tumours when Lkb1 is intact\(^\text{26}\). These data were confounded by the fact that Lkb1 mutations are relatively infrequent in pure SCC tumours (2%, see ref. 1). However, the model of KRAS and Lkb1 is actually a mixed histology model, containing ADC, SCC and mixed ADSCC tumours. Thus, we hypothesized that Lkb1 mutations may be more frequent in patient lung ADSCC samples. Data from a published study\(^\text{32}\) and from a cohort of ADSCC tumours at DFCI showed that of 23 ADSCC tumour cases, 6 harboured Lkb1 inactivation. These data suggest that Lkb1 mutations may be more frequent (26.1%), or at least as frequent, in ADSCC tumours as in ADC tumours (15.6%, 111 of 602 ADC tumours, \(P = 0.24\)). Together with data demonstrating KRAS is often activated in ADSCC tumours\(^\text{33}\), we propose that ADSCC in KRAS/Lkb1 mice is a clinically relevant recapitulation of the genetics found in human lung adenosquamous patients.

To dissect the contributions of KRAS and Lkb1 mutations during lung tumorigenesis, we used the dual recombinase model to activate oncogenic KRAS and vary the time-point of Lkb1 deletion. Our model includes that alleles Fse:Kras\(^{G12D/-}\); Fse:R26:CreERT2, Lkb1\(^{flox/flox}\) (Fse = Fret-Stop-Fret) and tamoxifen (Tam) inducible Cre-loxP recombination to make KRAS\(^{G12D}\) activation and Lkb1 inactivation separable (Fig. 1a). Mice were randomized into four arms: (1) KRAS activation with Lkb1 deletion at the same time (concomitant), (2) KRAS activation followed by Lkb1 deletion after 2 weeks, (3) KRAS activation followed by Lkb1 deletion after 10 weeks and (4) KRAS activation alone (Fig. 1a). Compared to mice with KRAS activation alone, overall survival of the cohorts was shortest when Lkb1 was deleted concomitant with KRAS activation, and was significantly shortened when Lkb1 was deleted either 2 weeks or 10 weeks after KRAS activation (\(P = 0.0081\) KRAS alone versus 10 weeks, Fig. 1b). We confirmed that tamoxifen administration was causing bi-allelic deletion of Lkb1 by performing western blot on whole tumour extracts (Supplementary Fig. 1a). In addition to accelerated tumour growth and reduced survival, lymph node and distant tumour metastases were present in all three cohorts in which Lkb1 was deleted (Supplementary Fig. 1b). We next sought to determine if deletion of Lkb1 long after KRAS activation could drive the increased histology spectrum observed previously\(^\text{26}\). In the two cohorts where Lkb1 was deleted after induction of KRAS, we observed acquisition of squamous characteristics to the same degree as observed with the concomitant model, with
approximately 40% of mice harbouring at least one tumour with purely squamous characteristics at the end of the study ($P = 0.96$ concomitant versus 10 week; Fig. 1c). Consistent with previous observations,34 tumours that develop when KRAS is activated by FlpO were uniformly low-grade lung tumours that always present as glandular adenomas and ADCs (Fig. 1d). In Lkb1-deleted tumours, purely squamous and transitioning tumours with areas of both ADC and SCC histology, as determined by immunohistochemistry (IHC) for the squamous markers KRT5, p63 and SOX2, were present (Fig. 1e,f). By contrast, the distal lung marker TTF1 (NKX2.1) was highest in the ADC lesions, and decreased during squamous transition.

Figure 1 | Lkb1 deletion drives SCC transition and tumour progression in established KRAS tumours. (a) Schematic of the four cohorts of mice used in this study: (1) KRAS activation with Lkb1 deletion at the same time (FlpO + Tam), (2) KRAS activation followed by Lkb1 deletion after 2 weeks (FlpO-2weeks-Tam), (3) KRAS activation followed by Lkb1 deletion after 10 weeks (FlpO-10 weeks-Tam) and (4) KRAS activation alone (FlpO). (b) Kaplan–Meier survival of the four cohorts, $n$ indicated in the figure, $P < 0.0001$ between FlpO + Tam and 2-week Tam, $P = 0.0081$ between 10-week Tam and FlpO. $P$ values in b represent log-rank test. (c) Percentage of mice with at least one purely squamous lesion as determined by histology at end point in the four cohorts, $n$ and $P$-values indicated in the figure, $P$-values represent $\chi^2$-test. (d) Haematoxylin and eosin staining of tumours at 10 weeks post FlpO show only adenocarcinoma histology. Scale bar, 50 μm. (e) Haematoxylin and eosin staining of tumours at 20 weeks post tamoxifen shows tumours that are undergoing transition from adenocarcinoma to squamous cell carcinoma histology, scale bar, 50 μm. (f) Validation of tumour subtyping by immunohistochemistry for TTF1 (NKX2.1), KRT5, p63, and SOX2. Scale bar, 50 μm. See also Supplementary Fig. 1a–c.
Consistent with the microenvironment also being affected\textsuperscript{35,36}, we observed that the tumour-associated myeloid cells changed during transition from ADC to SCC. As previously observed\textsuperscript{37}, ADC lesions stained positively for the macrophage marker F4/80, while the SCC lesions stained positively for MPO, a marker of tumour-associated neutrophils (Supplementary Fig. 1c).

Our first set of experiments demonstrated that Lkb1 deletion in cells that had KRAS activation long before was sufficient to produce tumours with squamous characteristics. It was tempting to speculate that this was evidence of a true transition from a KRAS +/- ADC cellular state to a KRAS +/- Lkb1-null squamous state. However, because the two genetic manipulations were performed on cells that remained within the same mouse during the entirety of the process, the possibility remained that Lkb1 deletion was allowing growth of a latent population of squamous-predisposed KRAS + cells. To address this issue, we isolated KRAS + lung ADC cells prior to tamoxifen treatment and transplanted them into immunocompromised mouse recipients (Fig. 2a). Cohorts of mice received either tamoxifen or placebo were aged until signs of tumour distress, and were then assessed for tumour histology. The tamoxifen-naïve cohort uniformly presented with ADC, which could be serially transplanted and never transitioned to SCC (Fig. 2b). In contrast, upon tamoxifen administration 2 weeks after transplant, the tumours in mice treated with tamoxifen took on squamous characteristics, with 40% of mice having at least one lesion that was completely SCC at the time of killing (P = 0.0225 Tam versus no Tam, Fig. 2c). In our previous studies, we observed that KRAS tumour propagating cells (TPCs) were present in both Sca1 + and Sca1- compartments, and a purely squamous model generated by biallelic inactivation of Lkb1 and Pten (Lkb1/Pten) harboured Sca1 + and NGFR + TPCs\textsuperscript{37}. To explore these populations in the transplanted tumours, we collected tumours from each mouse, dissociated and analysed EpCAM + /CD31 - /CD45 - cells for expression of NGFR and Sca1. We observed that KRAS transplanted tumours had very few Sca1 + /NGFR + cells and were indistinguishable from adeno-Cre induced KRAS tumours (P = 0.368; Fig. 2d and Supplementary Fig. 2a,b). By contrast, tamoxifen-treated mice harboured tumours with high levels of Sca1 and NGFR, and the population of Sca1 + /NGFR + cells was the same proportion of tumour as found in the pure Lkb1/Pten SCC tumours (P = 0.004 Tam versus no Tam, P = 0.720 Tam versus Lkb1/Pten). We also confirmed cell surface NGFR staining in the tamoxifen-treated mouse tumours by IHC (Supplementary Fig. 2c). Lastly, we isolated cells from squamous transitioned lesions for transplant, and found that both Sca1\textsuperscript{High}/NGFR\textsuperscript{High} and Sca1\textsuperscript{High}/NGFR\textsuperscript{Low} tumour cells could successfully transplant squamous disease (Fig. 2e). Together these data demonstrate that purely ADC lesions derived from transplanted KRAS + cells can transition to SCC, and that tumours can acquire the SCC TPC expression profile of Sca1 + /NGFR + upon Lkb1 deletion. Once the squamous transition has happened, the phenotype perpetuates.

**Loss of PRC2 activity accompanies SCC transition.** We hypothesized that the switch from ADC to SCC could be controlled in part through epigenetic mechanisms. The decreased, yet not absent, expression of TTF1 in the SCC lesions could be indicative of epigenetic memory of prior TTF1 positivity before the squamous differentiation process occurred, as was observed in other studies\textsuperscript{38}. To examine the possibility that an epigenetic mechanism such as PRC gene repression was involved in the ADC-SCC transition, we performed western blots for the common methylation marks catalysed by PRC, H3K27me1, H3K27me2 and H3K27me3, on whole tumour extracts from KRAS-induced ADCs (no tamoxifen), KRAS/Lkb1 ADCs and KRAS/Lkb1 SCCs (both tamoxifen at 10 weeks post Flp0). When compared to KRAS ADC or KRAS/Lkb1 ADC, there was a marked increase in histone H3 lysine 27 mono-methylation (H3K27me1) accompanied by a decrease in H3K27me3 in the KRAS/Lkb1 SCC lesions (Fig. 3a). We next examined expression of the components of the PRC2, the protein complex that catalyses the H3K27me3 mark, by western blotting. Interestingly, although SCC lesions have higher levels of the methyltransferase EZH2 than the ADC lesions, there is a clear lack of the essential PRC2 subunit EED in the SCC lesions (Fig. 3b). In a published data set from KRAS ADC, KRAS/Lkb1 ADC and KRAS/Lkb1 SCC\textsuperscript{31}, transcriptional levels of EED were not significantly different, suggesting regulation of protein stability. To explore this inverse correlation between EZH2 and H3K27me3 expression, we performed immunostaining for the two markers on serial tumour sections from KRAS/Lkb1 adenosquamous tumours, Pten/Lkb1/p53 adenosquamous tumours and Pten/Lkb1 SCC tumours (Fig. 3c). Consistently, we observed that ADC regions had sparse EZH2 staining and robust H3K27me3 staining. In contrast, squamous lesions on the same slides showed higher levels of EZH2 staining, and lower, but not absent, H3K27me3 staining (Fig. 3d and Supplementary Fig. 3a). We performed similar staining on human tumours, including six confirmed cases of adenosquamous cancer (Fig. 3e). Again, the pattern of higher EZH2 and slightly lower, but not completely absent, H3K27me3 was present in squamous lesions as compared to ADC lesions, which were H3K27me3 high and had lower levels of EZH2 (Fig. 3f and Supplementary Fig. 3b). While analysing these sections, we noted that normal airway and alveolar epithelium shared the ADC staining pattern of high H3K27me3 and low EZH2, while the oesophagus, which is the closest stratified squamous organ to the lung, had increased EZH2 staining and lower H3K27me3 (Supplementary Fig. 3b). This observation implies that normal lung epithelium may be maintained in a glandular state by PRC2 gene repression, and that loss of H3K27me3 gene repression may de-repress a programme predisposed to the squamous cell state.

Despite the obvious lack of EZH2’s catalytic mark, higher levels of EZH2 in the squamous tumours suggested that EZH2 may be required for squamous tumour growth and could represent a therapeutic target. Therefore, we bred the Ezh2 floxed alleles\textsuperscript{38} to the Cre-based LSL:KrasG12D; Lkb1\textsuperscript{flox/flox} model. We generated three genotypes with the Kras/Lkb1 alleles in the setting of Ezh2 +/-, Ezh2\textsuperscript{flox/+} or Ezh2\textsuperscript{flox/flox} alleles (Fig. 4a). Cohorts of these genotypes were analysed for tumour histology 11 weeks post Adeno-Cre virus administration. While the Ezh2\textsuperscript{flox/+} and Ezh2\textsuperscript{flox/flox} cohorts both had ~40% of mice with at least one purely squamous lesion at this time-point, all of the mice from the Ezh2\textsuperscript{flox/flox} cohorts had squamous lesions (Fig. 4b,c). We confirmed that both H3K27me3 and EZH2 were decreased in these Ezh2\textsuperscript{flox/flox} tumours by IHC (Supplementary Fig. 4a,b). This result suggests that EZH2 is dispensable for the squamous state, and that loss of the PRC2 complex activity could potentiate the switch to SCC. This result is unique from those observed when the PRC2 complex was depleted in KRAS or KRAS/p53 tumours—without Lkb1 deletion the squamous phenotype does not appear\textsuperscript{39}.

The genetic results described above suggest that loss of EZH2 function can perpetuate the squamous fate over several weeks of tumour development. We next wanted to test if inhibiting the catalytic function of EZH2 with a small molecule could drive the acquisition of squamous markers in short-term *in vitro* cultures. We first treated the human ADC cell line A549, which has the genotype of KRASG12S, LKB1Q37 with the inhibitor
GSK126 (ref. 40). As measured by flow cytometry, the NGFR⁺ cells within this cell line increased from 4.9 to 20.4% after 6 days in 10 μM GSK126 (P < 0.0001, Fig. 4d). RNA from the treated A549 cells likewise showed a fourfold increase in NGFR transcript level in response to GSK126 relative to vehicle-treated cultures, consistent with a de-repression of EZH2-mediated silencing of the locus (P = 0.0004; Fig. 4e). Although the LSL:KrasG12D cells that are WT for p53 are not able to grow as two-dimensional adherent cultures, they can be grown as three-dimensional (3D) cultures37. We confirmed that growing 3D tumour organoids derived from FlpO induced KRAS lesions in 100 nM 4-hydroxy tamoxifen was able to drive deletion of the Lkb1 allele, making this a tractable system in which to observe the early changes associated with Lkb1 loss (Supplementary Fig. 4c).

Firstly, we observed an increase in organoid re-seeding ability with tamoxifen treatment, consistent with the acquisition of more aggressive tumour characteristics upon Lkb1 deletion in vivo (P = 0.04; Fig. 4f and Supplementary Fig. 4d). Whereas KRAS tumour organoids were significantly decreased when passaged in continued 5 μM GSK126, organoids grown in the presence of tamoxifen (and therefore Lkb1 null) were equally passaged in continued presence of the EZH2 inhibitor (P = 0.0113 for Kras, P = 0.132 for KRAS/Lkb1; Supplementary Fig. 4d). Lastly, we observed that treating cultures with tamoxifen for 9–12 days (primary organoids) and re-seeding the cells in drug for an additional 9–12 days (secondary organoids) led a marked

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**Figure 2 | Lkb1 deletion after transplant of KRAS adenocarcinoma cells drives SCC transition.** (a) Schematic of adenocarcinoma induction, transplantation and deletion of Lkb1 in recipient mice. (b) Haematoxylin and eosin staining of transplanted KRAS tumours show only adenocarcinoma histology, while ADSCC and SCC were visible in tamoxifen-treated mice, scale bar, 50 μm. (c) Percentage of mice with at least one purely squamous lesion as determined by histology at end point in the four cohorts, n and P values indicated in the figure, P values represent χ²-test. (d) Representative flow cytometric plot of dissociated tumours from untreated (no tamoxifen) transplanted KRAS tumours (top) and tamoxifen-treated mice (bottom) gated on DAPI⁻/CD31⁻/CD45⁻/EpCAM⁺ cells and showing NGFR and Sca1 staining. (e) Haematoxylin and eosin staining tumours derived from transplanted Sca1HighNGFRHigh or Sca1HighNGFRLow cells from an SCC KRAS/Lkb1 lesion demonstrates that the switch to the squamous phenotype is stable, scale bar, 50 μm. See also Supplementary Fig. 2a–c.
decrease in Lkb1 expression and an increase in Sox2, Ngfr and Sca1 expression (Fig. 4g and Supplementary Fig. 4e). Interestingly, GSK126 treatment potentiated the increase in both Sca1 and Sox2, consistent with loss of H3K27me3 transcriptional repression allowing the SCC transition process.

Chromatin landscapes reveal de-repression of squamous genes.

To characterize the chromatin landscapes of KRAS/Lkb1 tumours, we performed chromatin immunoprecipitation (ChIP) on microdissected tumours confirmed to be either ADC or SCC by histology and qPCR (Supplementary Fig. 5a). The two activating marks, histone H3 lysine 4 tri-methylation (H3K4me3) and histone H3 lysine 27 acetylation (H3K27ac), and the PRC2-derived silencing mark, histone H3 lysine 27 tri-methylation (H3K27me3) were immunoprecipitated, followed by sequencing the chromatin bound DNA (ChIP-seq). ADC and SCC tumours could be clearly distinguished by differential H3K4me3 enrichment (Fig. 5a). Of the most significantly differential enrichment on H3K4me3 and/or H3K27ac marks between SCC and ADC tumours (Supplementary Fig. 5c and Supplementary Tables 1–4), we found much higher load of H3K27ac and H3K4me3 marks in the SCC tumour compared to the ADC tumour on the squamous genes Sox2, Np63, Ngfr and Krt5/6 (Fig. 5b and Supplementary Fig. 5a). Furthermore, we observed a significantly lower level of H3K27me3 mark in Sox2, H3K27me3.

Figure 3 | Loss of histone H3 lysine 27 trimethylation accompanies SCC transition. (a) Western blotting analysis performed on whole-cell extracts from tumours of the indicated genotypes and histologies. Histone H3 lysine 27 tri-methylation (H3K27me3) is markedly lower in SCC lesions; total histone H3 is the loading control. (b) Western blotting analysis performed on whole-cell extracts from tumours of the indicated genotypes and histologies. p63 and LKB1 confirm the histologies and genotypes of the lysates, and while EZH2 is more highly expressed in SCC tumours, the essential PRC2 subunit EED is absent in the SCC lesions. β–Actin is the loading control. (c) Immunohistochemistry for H3K27me3 on a panel of mouse lung tumours of the indicated histologies from KRAS/Lkb1, Pten/Lkb1/p53, and Pten/Lkb1 mice, scale bar, 50 μm. (d) Quantification of nuclear staining by dot analysis with Nikon software; data are mean ± s.e.m. measured on serially stained sections, n = 6–10. (e) Immunohistochemistry for H3K27me3 on a panel of human mixed adenocarcinomas, pure ADC and pure SCs. Scale Bar, 50 μm. (f) Quantification of nuclear staining, for H3K27me3 n = 6 ADSCC, 14 ADC, 9 SCC, for EZH2 n = 6 ADSCC, 7 ADC, 5 SCC, data are mean ± s.e.m. measured on serially stained sections. P values represent 2 tailed t-test. See also Supplementary Fig. 3a–c.
Ngfr and Krt5/6 loci compared to those in ADC tumours, consistent with de-repression of these squamous loci in SCC tumours (Fig. 5b). The differentially enriched regions for active histone marks also included the loci for the neutrophil chemoattractants Cxcl3/5, which were higher in the SCC tumours (Supplementary Fig. 5a). For ADC, the known expressed genes Scgb1a1, Foxa2 and SftpB had activating marks which were lost in SCC (Supplementary Fig. 5b). We also used the ROSE algorithm to call super-enhancers, many of which were shared between ADC and SCC, indicating that the tumours share some epigenetic memory (Supplementary Tables 5 and 6). For ADC, a unique super-enhancer was called at Scgb1a1, while Ifitm3 was a unique super-enhancer for SCC (Supplementary Tables 5 and 6). We next ran GREAT on the significantly enriched H3K4me3 loci in SCC tumours and found that the genes adjacent to these loci are enriched for H3K27me3, methylated CpG islands and PRC2 components in human embryonic stem cells and murine embryonic fibroblasts, suggesting that the activated loci in SCC tumours are normally repressed by PRC2 in other cell types (Fig. 5c).

A closer examination of the H3K27ac-enriched regions in each tumour type indicated that unlike the H3K27ac-marked loci that were common to all samples, which were devoid of H3K27me3, the uniquely SCC- or ADC-enriched loci were very often bivalently marked with H3K27me3 and H3K37ac in ADC, and became monovally marked with H3K27ac in SCC due to a loss of PRC2-mediated gene repression (Sox2 is an example) (Fig. 5d).
BASCs and club cells are cells-of-origin for SCC transition. We reasoned that the ‘choice’ of cells to transition to the squamous fate upon Lkb1 deletion may be predetermined by the tumour cell-of-origin. To test this theory, we used a fluorescence activated cell sorting (FACS) approach to enrich for populations of lung cells that are likely tumour cells-of-origin and which can be easily enriched using cell surface markers, including tracheal basal cells (enriched using positive selection for NGFR), non-basal cells of the trachea (the NGFR- fraction from the trachea that contains club, ciliated and goblet cells23,43), distal lung BASCs (enriched in the EpCAM$^+$Sca1$^+$ fraction) and distal lung alveolar type II (AT2) cells (enriched in the EpCAM$^+$Sca1$^-$ fraction)$^{12,21}$ (Fig. 6a and Supplementary Fig. 6a). To directly test the fitness of each population to propagate after activation of oncogenic KRAS we incubated each FACS-isolated population with no virus, adeno-GFP virus or adeno-FlpO virus before plating for organoid cultures$^{12,20}$ (Fig. 6b and Supplementary Fig. 6b). Strikingly, while the non-basal cells, BASCs and AT2 populations were all able to give rise to organoids after KRAS activation (Fig. 6c–d and Supplementary Fig. 6c), the basal cell population was consistently unable to produce any organoid after adeno-FlpO virus (Fig. 6c,d, $n = 4$). Visual inspection of the basal cell cultures showed single non-dividing cells several days after plating. The non-basal cells, despite having a dramatically decreased organoid forming efficiency compared to basal cells, were consistently able to grow robust KRAS$^+$ organoids (Fig. 6c–e). Both non-basal and BASC-derived organoids were predominantly bronchiolar in phenotype, while AT2-derived organoids were alveolar (Supplementary Fig. 7a).

Having formed KRAS$^+$ organoids with our in vitro culture system, we next sought to delete Lkb1 in these cultures and assess if organoids could take on squamous characteristics. To accomplish this, we serially passaged the cultures and added 100 nM 4-hydroxy tamoxifen to the transwell culture media (Fig. 7a). The non-basal cell-derived and BASC-derived cultures continued robust growth in tamoxifen, but the AT2-derived cultures reproducibly failed to grow in tamoxifen (Fig. 7b and Supplementary Fig. 7a). Tamoxifen administration to AT2-derived cultures from WT mice continued to grow normally

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**Figure 5 | Chromatin landscapes of ADC and SCCs reveal de-repression of squamous genes.** (a) Heatmap depicting global analysis of H3K4me3 marked chromatin in ADC and SCC from KRAS/Lkb1 mice. (b) Genome browser snapshots of the squamous loci Sox2, Ngfr and Krt6b/6a/5 with the indicated ChIP-sequencing peaks for H3K27ac, H3K27me3 and H3K4me3 in the ADC (blue) and SCC (red) KRAS/Lkb1 tumour samples. (c) GREAT analysis of genes adjacent to H3K4me3-enriched sites specific to SCC showed that this gene set is enriched for genes (from MSigDB) that are normally repressed by Polycomb in other cell types. Graphed as -log10 ($P$ value represents hypergeometric probability test.) Level of H3K27me3 marks at all loci enriched for H3K27ac (black), loci with H3K27ac unique to ADC (blue) or loci with H3K27ac unique to SCC (red) in either ADC (left panel) or SCC (right panel) from KRAS/Lkb1 mice. See also Supplementary Fig. 5a–c.
(data not shown), suggesting that tamoxifen itself was not detrimental and that KRAS+ AT2 cells cannot tolerate loss of Lkb1. We confirmed Lkb1 mRNA levels were decreased by tamoxifen administration in non-basal cell and BASC-derived cultures, and also that Sox2 gene expression was increasing in these cultures, though Sox2 expression was variable (Fig. 7c). The variability of Sox2 increase fit with the observation that only some organoids in each culture took on squamous characteristics (Supplementary Fig. 7b). Lastly, to demonstrate that we had taken a normal cell population and transformed it to a fully malignant state in vitro, we subcutaneously transplanted cells from the non-basal cell-derived cultures into immunocompromised mice. Large tumours formed (>500mm³), and upon histological examination, these tumours had regions of squamous differentiation right next to regions of ADC differentiation \( (n=2; \text{Fig. 7d}). \) We also transplanted cells orthotopically from BASC-derived cultures, while one mouse had solely squamous differentiated tumour, the other had solely mucinous ADC \( (n=2, \text{Fig. 7d and Supplementary Fig. 7c}). \) Therefore, our experiments rule out the basal cells and AT2 cells as likely cells-of-origin for adenosquamous tumours, whereas both non-basal tracheal cells and distal lung BASCs could drive squamous disease.

**Discussion**

Lung cancer lineage plasticity is an emerging concept that could influence the diagnosis and treatment of this devastating disease. In this manuscript, we used a unique model system to study lineage switching in KRAS-driven tumours in response to Lkb1 deletion. To validate the clinical utility of our model, we first examined a cohort of ADSCC lung cancer, finding that...
The LKB1 mutation was present in more than one quarter of the patients. We next demonstrated that KRAS-driven ADCs, either in the autochthonous setting or after transplant, retained the ability to transition into squamous tumours when Lkb1 was deleted many weeks or months after KRAS induction. We further demonstrated that a loss of PRC2-mediated gene repression was a hallmark of the lineage switched SCC tumours. Lastly, our lung organoid data suggest that a combination genetic alterations, including deletions of Lkb1 and alterations in the Polycomb Repressive Complex 2 (PRC2), are key drivers of this lineage switch.

Figure 7 | Lkb1 deletion drives squamous transition of club cell- and BASC-derived KRAS+ populations. (a) Schematic of Lkb1 deletion in three-dimensional KRAS+ cultures derived from non-basal tracheal cells, bronchioalveolar stem cells (BASCs) or alveolar type II (AT2) cells. (b) Representative brightfield images of KRAS+ colonies from alveolar type II cells treated with placebo or 4-hydroxy tamoxifen for 7 days, scale bar, 200 μm. (c) RT-qPCR for Lkb1 and Sox2 in tumour organoid 3D cultures treated with 100 nM tamoxifen for 9–12 days, mean ± s.e.m. on log2 scale is graphed, n varies by sample. (d) Representative haematoxylin and eosin staining from non-basal cell derived subcutaneous tumour (left) and BASC-derived orthotopic tumours (right), tumour histologies are indicated, scale bar, 100 μm. (e) Model: Here we combined Cre and FlpO recombinase technologies to temporally delete Lkb1 in established KRAS-driven lung adenocarcinomas. Serially transplanted KRAS+ adenocarcinoma could transdifferentiate into squamous disease when Lkb1 was deleted in the transplanted tumours. The Polycomb Repressive Complex 2 (PRC2), which represses genes through the histone H3K27me3 mark, was abrogated in the KRAS/Lkb1 squamous tumours through loss of expression of the EED subunit. This led to derepression of key squamous genes including Sox2, ΔNp63 and Ngfr. See also Supplementary Fig. 7a–c.
epigenetic state and cell-of-origin ultimately determine tumour phenotype (Fig. 7e).

Our data indicate that after Lkb1 is deleted in established KRAS ADCs, epigenetic reprogramming drives cells to take on squamous characteristics, ultimately resulting in full squamous transition of some tumours. These transitioned SCC tumours are characterized in both the mouse and human by a loss of the PRC2 H3K27me3 repressive chromatin mark, leading to activation of previously bivalently marked chromatin regions. Loss of PRC2-mediated gene repression leads to activation of squamous transcriptional programme, including the key squamous-associated genes Ngr, Sox2, ANP63 and Krt5/6. Sox2 and ANP63 are both well known to drive the squamous fate44–47. Thus, it is likely derepression of these genes that allows perpetuation of the squamous fate after the initial transition has occurred.

EZH2, which is the PRC2 methyltransferase that catalyses H3K27me3, has been proposed to be an oncogene in both lung ADC and SCC because its expression is correlated with poor prognosis of both histologies and its overexpression can drive ADC in mouse models39,48–50. Intriguingly, we and others have observed a decoupling of EZH2 expression levels from H3K27me3 levels in cancers51–53. In ADC regions, high and uniform H3K27me3 was found with relatively low expression of EZH2. It may be possible that EZH2 is only expressed in proliferating cells to replace the H3K27me3 marks lost during division. In SCC regions, there was often high EZH2, but lower levels of H3K27me3 mark than in ADC regions. We determined that reduction of H3K27me3 in SCCs was likely due to downregulation of the PRC2 component EED. EED loss may be the key to maintain an EZH2 high/H3K27me3 low state in other tumour types, or even in normal cells such as the oesophageal epithelium, and will warrant more study in the future. Because EZH2 was overexpressed in SCC tumours, it remained possible that EZH2 was critical for maintenance of the squamous fate with roles outside of the PRC2 complex, as has been observed in other tumour types54–56. However, genetic deletion of Ezh2 actually potentiated KRAS/Lkb1 SCC tumour growth, and EZH2 inhibition had no effect on KRAS/Lkb1 3D cultures. These data are supported by a recent study that demonstrated that Eed deletion accelerates KRAS/p53 tumour development by driving a switch to mucinous ADC39. Both of these studies suggest that the PRC2 complex may act as a tumour suppressor in lung cancer by limiting lineage identity.

There has been much controversy over the possible cells-of-origin of subtype and genotype-specific lung tumours. After using intranasal adenovirus-Cre to induce oncogenic KRAS, the first hyperproliferative cells observed were BASCs, implicating this population as possible ADC cells-of-origin57. More recent studies with lineage-specific KRAS activation in vivo suggested that AT2 cells appeared to be the only cells capable of giving rise to advanced ADC in the alveolar space, while club cells and BASCs appeared to be limited to driving bronchiolar hyperplasia within the same time frame57. Importantly, results varied when the genotype was altered to include SOX2 activation or deletion by downregulation EED and ultimate loss of PRC2-mediated gene repression of the squamous transcriptional programme. Because LKB1 affects many metabolic processes, one possibility is that metabolism of mammalian PRC2 class H3K27me3, has been proposed to be an oncogene in both lung cancer, including ADC, SCC and ADSCC, with the ultimate goal of identifying unique vulnerabilities of each that can be used in new therapeutic approaches.

Methods

Mouse cohorts. Mouse cohorts of Lkb1flox/flox, LSL:KrasG12D+/+, Lkb1flox/flox and LSL:KrasG12D+/+; Lkb1flox/flox, Ezh2floxed were all maintained in virus-free conditions on a mixed 129/FVB background. FSEKrasG12D/+ (Krstam37) mice were purchased from The Jackson Laboratory; Foxn1/Nu/Nu mice were purchased from Charles River Laboratories International Inc. All care and treatment of experimental animals were in strict accordance with Good Animal Practice as defined by the US Office of Laboratory Animal Welfare and approved by the Dana-Farber Cancer Institute Institutional Animal Care and Use Committee. Mice were given 2 × 10^5 pfu of Adeno-FipO virus via intranasal infections at 6–8 weeks old. Mice were administered daily by intraperitoneal injection of tamoxifen-free base (SIGMA T3948) in corn oil (12 µg µl^-1) at 132 µg tamoxifen per gram of body weight per day for five consecutive days. Magnetic resonance imaging scan was used for mouse lung tumour burden measurement as previously described58. Intratracheal transplants were performed as described23. Mice were monitored for signs of lung tumour onset and killed for gross and histological analysis and tumour isolation upon signs of distress.

Flow cytometry analysis and sorting. Tumours were dissected from the lungs of primary mice and tumour tissue was prepared as described59. Single-cell suspensions were stained using rat-anti-mouse antibodies. Antibodies for tumour cell analysis included anti-human-NGFR (EMD Millipore 05-446) coupled with goat-anti-mouse-PECy7 (Biolegend 403515), anti-mouse-NGFR (ABCam ab8875) coupled with donkey-anti-rabbit-PE (Ebiosciences 12-4739-81), anti-mouse-APC (AbD22582), anti-mouse-APC-Cy7 (Biolegend 118216), anti-mouse-CD31-APC (Fisher Scientific BDB551262) and anti-mouse-CD45-APC (Fisher Scientific BDB559864). Live cells were gated by exclusion of 4,6-diamidino-2-phenylindole (DAPI)-positive cells (SIGMA). Immune cell analysis is described in a previous study58. All antibodies were incubated for 10-15 min at 1:100 dilutions for primaries and 1:200 for secondary antibodies. Cell sorting was performed with a BD FACS Aria II with an 85 mm nozzle, and flow cytometric analysis was performed with a BD Fortessa and data were analysed with FlowJo software (Tree Star).

In vitro FipO and stem cell organoid culture. Cells isolated by flow cytometry as described above were split into equal aliquots, pelleted by pulse spin and resuspended in 100 µl MTEC plus media with 6 × 10^5 PFU per ml of Adeno-FipO or Adeno-GEF or no virus. Cell numbers for each condition/well ranged from 1,000–10,000 basal cells, 2,000–10,000 non-basal cells, 33,000–100,000 AT2 cells and 2,000–20,000 BASCs. These suspensions were incubated at 37°C, 5% CO₂ for 2 h in 1.5 ml tubes. Mock-infected cells (no virus) were also incubated
Western blot. Whole-cell extracts were made in RIPA buffer (0.5% deoxycholate, 1% IGEFA-Ca630, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris-8.1), lysates were cleared by centrifugation and protein content quantified with the Pierce BCA Protein Assay Kit (Thermo). For western blotting, 25 µg of protein extract per sample was denatured with heat and reducing agents, separated on a 4–12% acrylamide gel (BioRad) and transferred to nitrocellulose (GE Healthcare). Antibodies used for western blotting were EZH2 (BD Transduction Laboratories 612666, 1:1000), LKB1 (Cell Signaling 3947S, 1:1000), SUZ12 (AbCam ab12073, 1:1000), EED (Millipore 09-774, 1:500), JIMD3 (Aviva Systems Biology ARP40100500, 1:500), UTX (Genex GTX2121246, 1:1000), total Erk1,2 (Cell Signaling Technology 4695S, 1:2000), pERK1,2 (Cell Signaling Technology 3778S, 1:1000), pAMPK (Thir72) (4019P) (Cell Signaling 2535S, 1:1000), Histone H3 (AbCam ab1791, 1:20000), H3K27me3 (Millipore 07-448 1:2000) and H3K27me3 (Millipore 07-449 1:1000) all incubated overnight at 4 °C. β-Actin-HRP (SIGMA A8354, 1:20000) was used as a loading control. All antibodies have detailed species validation available online from vendors. Secondary antibody Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling 7074), 1:20000) or Anti-mouse IgG, HRP-linked Antibody (Cell Signaling 7076, 1:2000) were incubated for 1 h at room temperature. After washing, chemiluminescence was visualized with Western Lightning Plus-ECL (PerkinElmer) and exposed onto Kodak BioMax XAR film.

Immunofluorescence on organoid cultures. Colonies were fixed with 10% neutral-buffered formalin in overnight at room temperature. After rinsing with 70% ethanol, fixed colonies were immobilized with Histogel (Thermo Scientific) for paraffin embedding. Sectioned embedded colonies were stained with haematoxylin and eosin and immunostained with antibodies for CCSP (Corning 25-800-CR), 12.5 µg/ml bovine pituitary extract (Invitrogen 13028-014), 0.1 µg/ml cholera toxin (SIGMA C-8052), 25 ng/ml MEFG (Invitrogen 53000318) and 25 ng/ml rmFGF2 (R+D systems 3139-FB/CF), mixed 1:1 with growth factor-reduced Matrigel (Fisher Scientific), and pipetted into a 12-well 0.4 mm chamber and refreshed every other day. GSK126 was purchased from Xcess Bio as a 10 mM solution in DMSO, and 4-hydroxy-Tamoxifen (SIGMA) was resuspended in 1:1,000–1:2,000 into tissue culture media for use.

Patient lung tumours. Between July 2013 and April 2016, 9616 patients underwent targeted next-generation sequencing (NGS) at the Dana-Farber Cancer Institute/B Brigham and Women’s Hospital with informed consent under an IRB-approved research protocol. Among these population, 14 patients with adenocarcinoma non-small-cell lung cancer and 84 patients with an LKB1 mutation and ADC were identified using the Oncology Data Retrieval System (OncDRS). OncDRS is an internal system developed at the Dana-Farber Cancer Institute to integrate clinical and genomic data. All patients provided written informed consent, and no germline sequencing was performed. Tumour cell sequencing was performed on tumour DNA extracted from fresh, frozen or formalin-fixed paraffin-embedded samples and evaluated for single-nucleotide variants and genomic rearrangements67. The initial gene panel surveyed all exons of the 275 genes on the panel, along with 91 introns across 20 genes for rearrangement detection. DNA was isolated from tissue containing at least 20% tumour nuclei and analysed by massively parallel sequencing using a solution-phase Agilent SureSelect SitePro module in Cistrome analysis pipeline (http://cistrome.org). Wiggle files were also visualized in the Integrative Genomics Viewer (Robinson et al. 201167).

To correlate ChIP-Seq and gene set enrichment, peaks were associated with genes using GREAT42, allowing for a maximum distance of 10 kb between peak and associated gene, irrespective of directionality. Data are available at GEO repository (accession number GSE94363).
hybrid capture kit and an Illumina HiSeq 2500 sequencer. Data were analysed by an internally developed bioinformatics pipeline composed of reconfigured publicly available tools and internally developed algorithms including Indelocator, http://www.broadinstitute.org/cancer/pga/indelocator and Oncotator.76,77 Samples with a mean target coverage of <50 × were excluded from further analysis. Individual variants present at <10% allele fraction or in regions with <50% coverage were flagged for manual review and interpreted by the reviewing laboratory scientists and molecular pathologists based on overall tumour percentage, read depth, complexity of alteration and evidence for associated copy number alterations.

Data availability. Supporting data are available upon request from the corresponding authors.

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H.Z., C.F.B., S.K., H.W., C.F.K. and K.K.W conceived the ideas and designed the experiments, acquired the data and performed the analyses and interpretations. M.G., D.J.K, J.A.D., A.J.B., N.E.S., G.D., P.S.H., H.J., N.B. and D.S. provided materials and technical assistance. T.C. and S.L. assisted with mouse experiments. A.J.R., L.M.S. and A.J.K. performed cell and tissue culture experiments, acquired the data and performed the analyses and interpretations. M.Z. provided clinical samples and contributed to clinical data analyses. S.P., A.K.R., D.J.K, J.A.D., A.J.B., N.E.S., G.D., P.S.H., H.J., N.B. and D.S. provided materials and contributed to data interpretation and manuscript preparations. L.M.S. contributed to pathological examination. H.Z., C.F.B., C.F.K. and K.K.W wrote the manuscript.

Additional information

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Erratum: Lkb1 inactivation drives lung cancer lineage switching governed by Polycomb Repressive Complex 2

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