RB constrains lineage fidelity and multiple stages of tumour progression and metastasis

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Mutations in the retinoblastoma (RB) tumour suppressor pathway are a hallmark of cancer and a prevalent feature of lung adenocarcinoma1–3. Although RB was the first tumour suppressor to be identified, the molecular and cellular basis that underlies selection for persistent RB loss in cancer remains unclear4–6. Methods that reactivate the RB pathway using inhibitors of cyclin-dependent kinases CDK4 and CDK6 are effective in some cancer types and are currently under evaluation for the treatment of lung adenocarcinoma7–9. Whether RB pathway reactivation will have therapeutic effects and whether targeting CDK4 and CDK6 is sufficient to reactivate RB pathway activity in lung cancer remains unknown. Here we model RB loss during lung adenocarcinoma progression and pathway reactivation in established oncogenic KRAS-driven tumours in mice. We show that RB loss enables cancer cells to bypass two distinct barriers during tumour progression. First, RB loss abrogates the requirement for amplification of the MAPK signal during malignant progression. We identify CDK2-dependent phosphorylation of RB as an effector of MAPK signalling and critical mediator of resistance to inhibition of CDK4 and CDK6. Second, RB inactivation deregulates the expression of cell-state-determining factors, facilitates lineage infidelity and accelerates the acquisition of metastatic competency. By contrast, reactivation of RB reprograms advanced tumours towards a less metastatic cell state, but is nevertheless unable to halt cancer cell proliferation and tumour growth due to adaptive rewiring of MAPK pathway signalling, which restores a CDK-dependent suppression of RB. Our study demonstrates the power of reversible gene perturbation approaches to identify molecular mechanisms of tumour progression, causal relationships between genes and the tumour suppressive programs that they control and critical determinants of successful cancer therapy.

Inactivation of the RB pathway is prevalent in lung adenocarcinoma and decreases overall survival of patients2,3 (Extended Data Fig. 1). Despite the selective pressure to inactivate the RB pathway in lung adenocarcinoma, the consequences of RB1 inactivation remain unclear4–6. To model RB loss and therapeutic restoration of the RB pathway in lung tumours in vivo, we developed a mouse Rb1XTR allele that enables Cre-dependent inactivation of Rb1 (which encodes RB) and temporally controlled, FlpO-dependent restoration of the endogenous locus10 (Extended Data Fig. 2). We crossed mice that expressed the Rb1XTR allele with the KrasLSL-G12D+/+;Trp53lox/lox (hereafter KP) mouse model of lung adenocarcinoma11. Endotracheal transduction of KP and KrasLSL-G12D+/+;Trp53lox/lox;Rb1XTR/lox (hereafter KP;Rb1XTR/lox) mice with a Cre-expressing virus induces expression of oncogenic KrasG12D, deletes Trp53 and converts Rb1TR into its trapped Rb1TR state in lung epithelial cells (Fig. 1a, b). KP tumours robustly expressed RB, whereas KP;Rb1TR/lox tumours lacked RB expression (Fig. 1c and Extended Data Fig. 2b). Most KP lesions are slowly proliferating adenomas 8 weeks after tumour initiation, although a subset (around 15%) show early signs of carcinomatous progression that is marked by higher MAPK signalling and proliferation11–14 (Fig. 1d, e). Notably, at this time more than 60% of KP;Rb1TR/lox tumours were already carcinomas, had more proliferating cells and were larger than corresponding KP tumours (Fig. 1e–g and Extended Data Fig. 3a–c). However, the frequent KP;Rb1TR/lox carcinomas did not have high MAPK signalling, marked by expression of phosphorylated MEK1/2 (p-MEK) and phosphorylated ERK1/2 (p-ERK) (Fig. 1d, h, i and Extended Data Fig. 3a). By 14 weeks after tumour initiation, the fraction of KP and KP;Rb1TR/lox tumours that were carcinomas was similar. However, despite a high rate of proliferation in both, KP carcinomas had high levels of p-MEK and p-ERK whereas KP;Rb1TR/lox tumours did not (Fig. 1d–i and Extended Data Fig. 3d). Thus, although loss of RB strongly accelerates the transition to carcinoma, it largely abrogates the requirement for amplification of the MAPK signal to promote malignant progression.

MAPK-dependent signalling induces expression of D-type cyclins that bind to CDK4 and CDK6 (hereafter CDK4/6), which phosphorylate RB to drive cells out of quiescence and promote G1 progression. The cyclin A/E–CDK2 complex subsequently hyperphosphorylates RB, which allows progression through the G1/S restriction point12. Thus, we determined the phosphorylation status of RB and ERK1/2 in mouse and human lung adenocarcinoma tissues. Tumours with predominately p-ERK+ cells also had high RB phosphorylation (p-RB), which correlated with progression to higher tumour grades (Fig. 2a–d and Extended Data Fig. 3e–h). Human tumours with undetectable expression of RB or p16INK4a had lower p-ERK, similar to that observed in RB-deficient mouse tumours (Fig. 2e and Extended Data Fig. 3e–h). KP tumours that either naturally developed high levels of MAPK signalling or were pharmacologically induced to increase MAPK signalling, concurrently had high levels of p-ERK and p-RB807/S811 (Extended Data Fig. 4a–c). Additionally, these tumours had low p27, a negative regulator of CDK2, and high p-p27 T187, a CDK2-dependent modification that promotes p27 degradation14–20 (Extended Data Fig. 4d–f). Conversely, untreated KP;Rb1TR/lox tumours and KP tumours treated with a MEK1/2 inhibitor had low levels of p-ERK, p-RB807/S811 and p-p27 T187 and higher levels of total p27 (Extended Data Fig. 4c, g–k). These data suggest that amplification of MAPK signalling drives tumour progression by promoting CDK2-dependent suppression of RB.

Although current strategies to reactivate the RB pathway rely on inhibition of CDK4/6 in tumours in which Rb1 is intact, we found that palbociclib—a potent inhibitor of CDK4/6—had minimal effects on tumour growth or were pharmacologically induced to increase MAPK signalling, concurrently had high levels of p-ERK and p-RB807/S811 (Extended Data Fig. 4a–c). Additionally, these tumours had low p27, a negative regulator of CDK2, and high p-p27 T187, a CDK2-dependent modification that promotes p27 degradation14–20 (Extended Data Fig. 4d–f). Conversely, untreated KP;Rb1TR/lox tumours and KP tumours treated with a MEK1/2 inhibitor had low levels of p-ERK, p-RB807/S811 and p-p27 T187 and higher levels of total p27 (Extended Data Fig. 4c, g–k). These data suggest that amplification of MAPK signalling drives tumour progression by promoting CDK2-dependent suppression of RB.
cells that have wild-type Rb1 and sensitized these cells to palbociclib (Fig. 2g and Extended Data Fig. 5a–h). Cdk2 inactivation reduced p-RB S807/S811 levels in KP cells, but also led to increased CDK6 and p-RB S780 levels (Extended Data Fig. 5i). Thus, in KP cells, RB is primarily inactivated by Cdk2-dependent phosphorylation marked by p-RB S807/S811; however, in the absence of Cdk2, the persistent comparisons. n = 2 biological replicates, n = 3 technical replicates. P values were calculated from technical replicates. h, Clonogenic growth of human cell lines with non-genome targeting (hereafter inert) sgRNA or sgRNA against Cdk2, with or without palbociclib treatment. i, Area covered by cells using data from h. Data are mean ± s.d. Significance was determined by ANOVA with Sidak’s correction for multiple comparisons. n = 3 technical replicates. j, Xenograft growth of A549 (top) and H838 (bottom) cells that express inert or Cdk2-targeting sgRNAs. The day on which palpable masses had formed was set to day 0 and on this day treatments were initiated (t = 0). Data are mean ± s.d. Tumour volume significance was determined by unpaired two-tailed Student’s t-test on day 16. Number of mice is shown. Combination of Cdk2 sgRNA and palbociclib treatment is synergistic (A549 and H838 cells, combination index = 0.274 and 0.000002237, respectively).
requirement for RB suppression leads to a compensatory increase in CDK4/6-dependent RB suppression and a de novo vulnerability to inhibition of CDK4/6.

Many human lung adenocarcinoma cell lines are refractory to CDK4/6 inhibition despite expressing wild-type RB36 (Extended Data Fig. 5g). To determine whether loss of CDK2 activity sensitizes human lung adenocarcinoma cells to CDK4/6 inhibition, we inactivated CDK2 using CRISPR in four human Rb1 wild-type lines (Extended Data Fig. 5k). In A549 and EKVX cells—which are sensitive to the CDK4/6 inhibitor—loss of CDK2 further decreased growth after palbociclib treatment (Fig. 2g–j and Extended Data Fig. 5l, m). Similar to KP cells, in H838 and H1993 cells—which are relatively resistant to palbociclib—CDK2 loss created de novo sensitivity to CDK4/6 inhibition (Fig. 2h–j and Extended Data Fig. 5l, m). These data suggest that CDK2-specific inhibitors may be a potential additional therapeutic option that would synergize with existing CDK4/6-targeted therapies to more potently reactivate the RB pathway and suppress tumour growth.

Progression of lung adenocarcinoma is marked by a worsening of nuclear pleomorphism (grades 3 and 4) and the presence of desmoplasia and transition to a poorly differentiated phenotype (grade 5)11,12. At 14 weeks after tumour initiation, when more advanced tumours are likely to occur, KP carcinomas were primarily grade 3 and 4, whereas a significant fraction of KP;RbTR/TR carcinomas were already grade 5 lesions (Fig. 3a). Notably, around 40% of KP;RbTR/TR mice also had widespread metastases to local lymph nodes and/or the pleural cavity. No metastases were observed in KP mice at this time point (Fig. 3a and Extended Data Fig. 6a). Within human lung adenocarcinoma datasets1–22, RB pathway deficiency did not affect tumour stage, which is a measure of size and position (Fig. 3b). However, patients whose tumours had RB pathway alterations were significantly more likely to have metastases and higher grade tumours, indicating a preponderance of poorly differentiated disease (Fig. 3b, c). Before gaining metastatic ability, KP tumours silence the lineage-specifying transcription factors NKX2-1 and FOXA2, and derepress HMGA2, a metastasis-associated, chromatin-regulating factor the expression of which is normally restricted to embryonic cell types23–25 (Fig. 3d, e and Extended Data Fig. 6b). However, KP;RbTR/TR tumours and metastases frequently co-expressed HMGA2, NKX2-1 and FOXA2 (Fig. 3d, e and Extended Data Fig. 6b–d). Moreover, derepression of HMGA2 was frequent in grade 3 KP;RbTR/TR carcinomas that expressed NKX2-1 (Fig. 3d, f). Although no KP;RbTR/TR tumours expressed club cell or neuroendocrine markers, a subset lost the surfactant protein C (SPC) lineage marker despite maintaining NKX2-1 and a well-differentiated morphology; a pattern that was not observed in KP tumours (Fig. 3d, g). Therefore, RB deficiency subverts lineage fidelity and promotes alternative pathways to gain metastatic ability.

On the basis of the expression of NKX2-1 and HMGA2, KP cell lines can be classified into those that are derived from tumours that lacked a metastatic ability (NKX2-1–HMGA2–) and those that had metastatic potential (NKX2-1–HMGA2+). Because RB deficiency in KP;RbTR/TR tumours promotes metastasis, we determined whether the RB pathway is deregulated spontaneously in metastatic KP tumours. We found that NKX2-1–HMGA2– KP cell lines strongly express p16INK4A, but that NKX2-1–HMGA2+ KP cell lines did not (Extended Data Fig. 7a, b). Primary metastatic tumours and extra-pulmonary metastases that were sorted directly from KP lesions also had fewer p16INK4A-specific transcripts than non-metastatic tumours26 (Extended Data Fig. 7c, d). KP;RbTR/TR tumour-derived cell lines also co-expressed NKX2-1 and HMGA2 and therefore did not clearly fit into NKX2-1–HMGA2+ or NKX2-1–HMGA2– groups (Extended Data Fig. 7e). When injected subcutaneously in syngeneic mice, multiple KP and KP;RbTR/TR lines grew at similar rates at the injection site, but KP;RbTR/TR tumours seeded approximately fourfold more metastases in the lung as well as frequent metastases in the liver, diaphragm and pleura. KP;RbTR/TR tumours also formed more than threefold more liver metastases than NKX2-1–HMGA2– KP cell lines after intrasplenic injection (Extended Data Fig. 7f, g).

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**Fig. 3 | RB inactivation accelerates onset of metastasis and enables alternative pathways to gaining metastatic competency.** a. Left, grades for carcinomas 14 weeks after tumour initiation. KP, n = 258 tumours from 9 mice; KP;RbTR/TR, n = 580 tumours from 12 mice. Significance was calculated by $\chi^2$ test for trend. $P = 2.9 \times 10^{-2}$. Data are mean ± s.d. Right, number of metastases per mouse. KP, n = 9 mice; KP;RbTR/TR, n = 12 mice. Significance was calculated by two-tailed unpaired Student’s $t$-test. $P = 0.0201$. Data are percentage of all carcinomas that fall into each grade (left) or mean number of metastases (line) and all metastases (dots) in individual mice (right). b, Tumour stage, and metastasis to the lymph node and distant sites in patients with lung adenocarcinoma in which the RB pathway is lost or intact. Data are mean ± s.d. $P = 0.0066$ ($\chi^2$ test for trend) and two-sided Fisher’s exact test. c, Grade and size of tumours from patients with lung adenocarcinoma in which the RB pathway is lost or intact. Data are mean ± s.d. Significance of tumour grade was calculated by $\chi^2$ test for trend and significance for tumour size by two-tailed unpaired Student’s $t$-test. d, Number of samples n is shown. Data were obtained from a previously published study1, d, NKX2-1, HMGA2 and SPC immunohistochemistry from KP and KP;RbTR/TR tumours. A summary of the results is shown at the bottom. Red dashed lines separate lower and higher grade regions. Left (L) and right (R) sides of images are indicated where relevant. e, Percentage of HMGA2+ tumours that are also positive for NKX2-1. KP, n = 22 tumours from 4 mice; KP;RbTR/TR, n = 84 tumours from 4 mice). Significance was calculated by two-sided $\chi^2$ test. f, Percentage of grade 3 tumours that were positive for HMGA2. KP, n = 88 tumours from 4 mice. KP;RbTR/TR, n = 86 tumours from 4 mice. Significance was calculated by two-sided Fisher’s exact test. g, Percentage of SPC+ and SPC− areas by grade in NKX2-1–HMGA2+ tumours from individual KP;RbTR/TR mice. n = 28 tumours from 3 mice. Significance was calculated by $\chi^2$ test for trend.
RB is a pleiotropic tumour suppressor with canonical roles that respond to mitogenic cues and regulate cell cycle progression, as well as multiple less well understood non-canonical roles that regulate plasticity of cellular states. Our study uncovers RB suppression as a major effect of oncogenic MAPK signal transduction during early carcinoma progression, and highlights the role of RB in maintaining lineage commitment as a critical barrier to the development of metastatic disease (Extended Data Fig. 10a, b). We identified CDK2 as an effector of the MAPK signalling pathway and determinant of the success of cancer therapies that are aimed at reactivating the RB pathway, reinforcing the need for potent and selective CDK2 inhibitors (Extended Data Fig. 10c–f). Finally, our data reveal that therapies that aim to reactivate the RB pathway may have tumour-suppressive effects, not by suppressing cell proliferation, but instead by reverting cell state changes associated with advanced tumour progression.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-1172-9.

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METHODS

Animal studies and treatment. Animal studies were performed under strict compliance with Institutional Animal Care and Use Committee at University of Pennsylvania (804774). KrausSL-G12D mice (Jax stock number 001879), Trp53fl/fllox mice (Jax stock number 008462), RbRTR/Tr mice12, Trp53fl/flloxRbRTR/Tr mice13 (p130 is also known as Rbl2) mice and Rosa26p16+ mice have previously been described. Mice are mixed B6:129S4/Avi. Mice were transduced with either 2.5 × 10^6 plaque-forming units (PFUs) of Ad-CMV-Cre obtained from University of Iowa Viral Vector Core or Cre-expressing lentivirus (PKG-Cre) at 4 °C and then exposed to fluorescent secondary detection reagents (Jackson ImmunoResearch Laboratories, SK-4105) were prepared and used according to the product instructions. Immunofluorescence was performed on the paraffin-embedded sections following the same antigen-retrieval protocol. Sections were incubated in primary antibodies for 16–20 h at 4 °C and then exposed to fluorescent secondary detection reagents (Jackson ImmunoResearch Laboratories). Photomicrographs were captured on a Nikon Eclipse E800 microscope using Eclipse Ti-E Software (Nikon) with an iXon EMCCD camera, and analysis was performed using NIS-Elements software (Nikon). Histological quantification of human tissue microarrays was performed by quantifying the frequency of cells that were positive and then scoring high and low as follows: For Ki67 and p-RB S807/S811: <10% positive cells per tumour, low; ≥10% positive cells per tumour, high. For p-ERK and p-ERK-low tumours were defined as <30% positive cells per tumour and p-ERK-high tumours were defined as ≥70% positive cells per tumour. Penn Vet Comparative Pathology Core determined individual mouse tumour grades by using established tumour-grading schemes in experiments12-21. Histological quantification of human tissue microarrays was performed by quantifying the frequency of cells that were positive and the staining intensity for Rb, p16, p-ERK and p-RB S807/S811. Rb+ and p16+ tumours were both defined as those with greater than 20% of cells having medium or strong staining intensity, and the Rb pathway was designated as intact if the tumour was positive for both Rb and p16. p-RB and p-RB S807/S811-high tumours were defined as those containing greater than 10% positive cells. p-ERK staining was scored on a four-point scale with score 0 indicating no staining, score 1 indicating either low staining or less than 20% of cells with medium or high staining, score 2 indicating medium staining in greater than 20% of cells, and score 3 indicating high staining in greater than 20% of cells.

In vivo metastasis models. KP and KRbTR/TR mice were subcutaneously implanted in the lower right flank of B6129F1/J mice (Jax stock number 100492) at ≥5 × 10^5 cells per inoculum. Mice were euthanized 6 weeks after inoculation. Primary tumour, lung, liver and kidney tissues were collected and processed for histology. Mice were visually examined for macroscopic metastatic tumours and lung metastases were quantified from H&E-stained sections. For the intrasplenic injection model of metastasis, B6129F1/J mice were anaesthetized with isoflurane. The left subcostal area was shaved, then prepared with 70% ethanol and iodine. A small incision was made in line with the left ear through the peritoneum. Through the excision, the exposed spleen was divided by placing a microvascular clip down the centre. Tumour cells were injected at a concentration of 1 × 10^5 in a 50-μl volume of PBS. After 10 min, a microvascular clip was placed on the downstream vascularule at the injection site and then the spleen was removed. Mice were euthanized after 4 weeks and macroscopic liver metastases were counted. During the experimental time course, subcutaneous tumours never grew above 1.0 cm^3 and mice with liver tumours were never under duress (that is, with a body weight less than 2). Mouse cell lines were authenticated for genotype. Human lung cancer cell lines were tested for the expected, genotype-associated expression pattern of GAPDH and compared to standardized DNA ratios of RbR and RbTR alleles. Primers used: GFP forward 5′-GAGCTTAAAAACGGCCACAAGTT-3′ and reverse 5′-GAACTCCTAGGTTGACGGTTG-3′; Gapdh forward 5′-TGTCGCTGCTGGATCGAC-3′ and reverse 5′-CTGCTGTCACGCTTTGG-3′.

Cell lines. Mouse cell lines were generated from primary mouse tumours and propagated using standard techniques. Human lung adenocarcinoma cell lines were obtained from ATCC or NIH Cell line repository. HEK293FT cells that were used for lentivirus production were obtained from Invitrogen. GreenGo cells used for lentivirus titration were obtained from the laboratory of T. Jacks and are a derivative of NIH3T3 cells. Mouse cell lines were authenticated for genotype. Human and mouse lung cancer cell lines were tested for the expected, genotype-associated protein expression patterns by western blot. HEK293FT cells that were used for lentivirus production were validated by verifying that high titre virus production was possible. NIH3T3-Green Go cells were validated by measuring Cre-induced GFP expression. All human cell lines tested negative for mycoplasma as performed by the cell line repository or manufacturer. Mouse cell lines were not tested. Human lung adenocarcinoma cell lines H2009 and A549 were grown in F12K medium and high glucose DMEM (Invitrogen). Lung and tumour tissues for BrdU, Ki67, p-MEK, p-ERK and p-RB S807/S811 by immunohistochemistry. Lung and tumour tissues for BrdU, Ki67, p-MEK, p-ERK and p-RB S807/S811 by immunohistochemistry. Histological quantification of mouse tumours was performed by quantifying the frequency of cells that were positive and then scoring high and low as follows: For Ki67 and p-RB S807/S811: <10% positive cells per tumour, low; ≥10% positive cells per tumour, high.

Histological quantification. ImageJ software was used to determine the percentage of lung area occupied by the tumour on H&E-stained slides, and the frequency of cells that were positive for specified antigens as a fraction of total tumour cells.

Data points represent individual mice for tumour burden graphs and individual tumours for BrdU, Ki67, p-MEK, p-ERK and p-RB S807/S811 by immunohistochemistry. Histological quantification of mouse tumours was performed by quantifying the frequency of cells that were positive and then scoring high and low as follows: For Ki67 and p-RB S807/S811: <10% positive cells per tumour, low; ≥10% positive cells per tumour, high.

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and cultured in high-glucose DMEM supplemented with 10% fetal bovine serum, GlutaMAX and antibiotics at 37°C and 5% CO2 until cell line establishment. Proliferation of cell lines was determined by plating the indicated cell number and analysed by manual cell counts using a haemocytometer at the indicated time points. This was performed after manufacturer's instructions for the APC BrdU Flow Kit (BD Pharmingen). Clonogenic survival assays were performed by plating 500 cells per well in a six-well plate. After approximately 16h, cells were treated with DMSO or palbociclib (Selleck Chemicals) at the indicated doses every 3 days for 1.5 or 3.5 weeks. Cells were then fixed by incubating in ice-cold methanol for 10 min and then stained with crystal violet dye for 15 min. Plates were imaged and the relative area covered by cell colonies was determined with Image software.

**Immunoblot analysis.** KP and KP;RbTR/TR cell lines were lysed in RIPA buffer, resolved on NuPage 4–12% Bis-Tris protein gels (Thermo Fisher) and transferred to polyvinylidene fluoride (PVDF) membranes. Blocking, primary and secondary antibody incubations were performed in Tris-buffered saline (TBS) with 0.1% Tween-20. Total ERK1/2 (Cell Signaling, c4969), CDK2 (Abcam, ab32147), CDK4 (Abcam, ab199728), CDK6 (Abcam, ab151247), β-actin (Sigma Aldrich, A2066), HSP90 (BD Transduction Laboratories, 610418), p-RB S780 (Cell Signaling, 9307), p16 (Abcam, ab211542) and cyclin D1 (Abcam, ab16663) were assessed by western blotting. HSP90 and β-actin were used as loading control. All other antibodies are the same as indicated for immunohistochemistry.

**RNA sequencing data analysis.** Libraries for RNA sequencing, RNA was isolated from 4 KP and 4 KP;RbTR/TR lung tumour-derived cell lines using the RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. RNA concentrations were measured using the Qubit RNA HS Assay Kit (Invitrogen) and sample quality was determined using the RNA 6000 Nano Kit on a 2100 BioAnalyzer (Agilent). Sequencing libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) as per the manufacturer's instructions and subjected to 75 bp paired-end sequencing on the Illumina NextSeq 500 platform. Fastq files for each sample were aligned against the mouse genome, build GRCm38.p4, using the STAR aligner (v.2.5.2b)34. FeatureCounts (v.1.5.0-p1) was used to quantify alignments against the mouse genomic annotations from Gencode (v.M11)35. Differentially expressed genes were identified with DESeq2 (v1.14.0)36 and an ‘R signature’ gene signature was defined as genes with a false-discovery rate adjusted P value less than 0.05 and log2-normalized fold change in expression greater than ±0.8 (46 genes were downregulated and 63 genes were upregulated in KP;RbTR/TR cells). Single-sample gene set enrichment analysis (ssGSEA) was performed at https://genepattern.broadinstitute.org/gp/ using the ssGSEAproject module35. Provisional TCGA lung adenocarcinoma z-score mRNA-expression data extracted from CBioPortal was used as the input expression dataset and the Rb signature gene signature was used as the gene set38. RB signature samples were defined as those with an enrichment score greater than 1,000; RB signature-low samples were defined as those with an enrichment score less than 1,000. Kaplan–Meier analysis was performed to examine the differential survival of the RB signature high samples and RB signature low samples. MSK-IMPACT-NSCLC survival analysis was performed by Kaplan–Meier analysis using data from CBioPortal35. Rb pathway alterations were defined as mutations or copy number alterations in RBl, CDKN2A, CDK2, CDK4, CDK6, CCND1 or CCNE1. For ONCOMINE-based analyses of tumour stage, lymph node metastasis, distant metastasis, tumour grade and tumour size, 35. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose RNA-seq program for assigning sequence reads to genomic features. *Bioinformatics* **29**, 80–84 (2013).

**Statistics and reproducibility.** All analyses were performed in the Graphpad Prism (v.7.0a) software package. For tumour burden, BrdU, Ki67, p-ERK, p-MEK, p27, p27 S10 and p27 T187, ratio of p16:Arf reads and human tumour size, unpaired Student's t-tests were performed. For tumour grade distribution, human tumour stage distribution and p-ERK staining in relation to Rb pathway status and p-ERK clone staining, contingency analyses with a χ2 test for trend was performed and was used. Other contingency analyses used a standard χ2 test. A log-rank (Mantel–Cox) test was performed to determine significance in survival studies. Compusyn software (ComboSyn) was used to evaluate synergy between inhibition of CDK4/6 and CRISPR-mediated knockout of CDK2, whereby a Combination Index score less than 1 indicates synergy33. For Fig. 1c, d, experiments were repeated 5 times each for mice at 8 weeks and 9 and 12 times for KP and KP;RbTR/TR mice at 14 weeks, respectively, with similar results. For Fig. 2a, the experiment was repeated 3 times on separate mice, with similar results. For Fig. 3d, the experiment was repeated 4 times in KP and 6 times in KP;RbTR/TR mice for HMGAA2 and NKX2-1 staining, with similar results. The experiment was repeated 3 times each in KP and KP;RbTR/TR mice for SFCP staining, with similar results. For Fig. 4d, the experiment was repeated 3 or more times for KP and KP;RbTR/TR mice, with similar results each.

**Extended Data Fig. 2** c, e, f, g, i, 8a, d, 9b, c, e are available in the online version of the paper. Raw images from western blots are shown in Supplementary Fig. 1. In gels with multiple bands per lane or in which specific lanes were selected, the locations at which gels were cropped are also shown. For Extended Data Figs. 2b, c, 3k, 7e, controls were run on separate gels as sample-processing controls; for Extended Data Fig. 7a, loading controls for each gel are provided in the Supplementary Information.

**Data availability** All data generated or analysed during this study are included in this published Letter (and its Supplementary Information) with the exception of raw RNA sequencing data associated with Extended Data Fig. 2e, f, which have been deposited publicly in the Gene Expression Omnibus (GEO) under accession number GSE84447. Source data associated with Figs. 1e–i, 2b–e, g, i, j, 3a–c, e–g, 4c, e–h and Extended Data Figs. 2d–f, 3c, d, 4b, d–i, 5a–h, j, m, 6c, d, 7d, f, g, i, 8a, d, 9b, c, e are available in the online version of the paper. Raw images from western blots are shown in Supplementary Fig. 1. In gels with multiple bands per lane or in which specific lanes were selected, the locations at which gels were cropped are also shown. For Extended Data Figs. 2b, c, 3k, 7e, controls were run on separate gels as sample-processing controls; for Extended Data Fig. 7a, loading controls for each gel are provided in the Supplementary Information.
The RB pathway is frequently altered in human lung adenocarcinoma. 

**a**. Oncoprint from CBioPortal showing frequency and co-occurrence of mutations and copy number alterations in RB pathway components, \textit{KRAS} and \textit{TP53} in the provisional lung adenocarcinoma TCGA dataset. 

**b**. RB pathway components and their corresponding mutation frequencies in the provisional lung adenocarcinoma TCGA dataset. 

**c**. Kaplan–Meier survival analysis of patients with lung adenocarcinoma whose tumours do (\( n = 456 \) patients) or do not (\( n = 987 \) patients) contain alterations in RB pathway members. Patient data were obtained from the MSK-IMPACT clinical sequencing cohort. Significance was determined by two-sided log-rank test (\( P = 0.0015 \)). Source Data can be found at the cBioPortal. 

Extended Data Fig. 1
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | *RbXTR* allows Cre-dependent inactivation and FlpO-dependent reactivation of *Rb1*. a, Top, *RbXTR* (expressed): XTR gene trap cassette consists of a splice acceptor (SA), GFP complementary DNA ‘GeneTrap’, and the polyadenylation transcriptional terminator sequence (pA). Stable inversion is achieved by the use of two pairs of mutually incompatible mutant *loxP* sites (Lox2272 and Lox5171) arranged in the ‘double-floxed’ configuration. In the germline and in normal somatic cells, RB expression is normal in *RbXTR/XTR* mice10. Middle, *RbTR* (trapped): inhalation of Cre-expressing adenoviral or lentiviral vectors induces the permanent conversion of the *RbXTR* allele to the *RbTR* allele that inactivates *Rb1* gene expression. Transcripts are spliced from the upstream exon to the GFP reporter gene and downstream transcription is terminated to functionally inactivate gene function. RB expression is inactivated only in the tumour cells. Bottom, *RbR* (restored): the Rosa26*FlpO-ERT2* allele enables tamoxifen-dependent conversion of trapped *RbTR* to its restored *RbR* allelic state via excision of the gene trap.

b, Western blot analysis of 3 KP and 3 KP;*RbTR/TR* tumour-derived cell lines. c, Western blot analysis of 2 KP;*RbTR/TR* tumour-derived cell lines treated with Adeno-Cre as a control, or Adeno-FlpO to restore RB expression. d, Quantitative RT–PCR analysis of 3 KP;*RbTR/TR* tumour-derived cell lines treated with Adeno-FlpO to restore RB expression. Data are normalized to Adeno-Cre-treated cells (control). The log2-normalized fold change in expression ± s.d. is shown. *n* = 3 technical replicates for each cell line. e, Volcano plot of differentially expressed genes from RNA sequencing data obtained from KP (*n* = 4) versus KP;*RbTR/TR* (*n* = 4) cell lines. The RB signature, defined by genes for which the change in expression is ≥2-fold and the *P* value adjusted for multiple testing is ≤ 0.05, is boxed. Statistical significance was determined by two-sided Wald’s test using Benjamini–Hochberg correction using DESeq2. f, Kaplan–Meier survival analysis of patients with lung adenocarcinoma whose tumours exhibit a high (*n* = 114 patients) or low (*n* = 189 patients) Rb signature. Significance was determined by two-sided log-rank test. *P* = 0.0175.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | RB deficiency is associated with low MAPK pathway signalling in mouse and human lung adenocarcinomas. a, Top, H&E staining images of tumours from KP and KP;Rb{Tm/Tm} mice 8 weeks after tumour initiation. Bottom, corresponding immunohistochemistry for p-ERK. b, Immunohistochemistry for Ki67 in tumours from KP and KP;Rb{Tm/Tm} mice 8 and 14 weeks after tumour initiation. c, Quantification of Ki67+ cells from b. Symbols represent individual tumours. KP 8 weeks, n = 9 tumours from 2 mice; KP;Rb{Tm/Tm} 8 weeks, n = 9 tumours from 2 mice; KP 14 week, n = 31 tumours from 3 mice; KP;Rb{Tm/Tm} 14 weeks, n = 31 tumours from 3 mice. Significance was determined by two-sided unpaired Student’s t-test with Welch’s correction for 8-week (P = 0.0034) and 14-week (P = 0.2686) analyses. Data are mean ± s.d. d, Plot showing the relationship of p-ERK+ fraction versus Ki67+ fraction in KP and KP;Rb{Tm/Tm} tumours at 14 weeks after tumour initiation. KP, n = 15 tumours from 2 mice; KP;Rb{Tm/Tm}, n = 16 tumours from 2 mice. Significance was determined by linear regression analysis and the line of best fit for each is shown (P = 0.0376). e, Immunohistochemistry for RB depicting an RB-negative core (left) and an RB-positive core (right). f, Immunohistochemistry for p16 depicting a p16-negative core (left), a core with low staining (middle) and a core with high staining (right). g, Immunohistochemistry for p-RB S807/S811 depicting two examples of cores with low expression (left) and high expression (right). h, Immunohistochemistry for p-ERK depicting a core with a p-ERK staining score of 0 (left), 1 (middle left), 2 (middle right) and 3 (right). Scale bars, 100 μm; inset images are magnified 5×.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | RB and p27 phosphorylation are enhanced by amplification of the MAPK signal and suppressed by inhibition of MEK1/2. a, Representative images of immunohistochemistry for p-ERK and p-RB S807/S811 in KP tumours treated with vehicle control or BRAF inhibitor PLX4720. b, Contingency analysis of p-ERK and p-RB S807/S811 from a. n = 40 tumours from 4 mice treated with vehicle control; n = 66 tumours from 5 mice treated with PLX4720. Significance was determined by two-sided χ² test for the vehicle-treated group (P = 6.4 × 10⁻⁵) and for the PLX4720-treated group (P = 0.0003). c, Representative images of immunohistochemistry for p-ERK, p27, p-p27 S10 and p-p27 T187 in KP tumours treated with vehicle control, BRAF inhibitor PLX4720 or MEK inhibitor PD0325901. Tumour sections were stained with anti-p-p27 S10 antibody as a measure of non-CDK2-dependent suppression of p27. Significant changes in phosphorylation at this site were not observed. d, Analysis of p27 levels in p-ERK low (n = 28) and p-ERK high (n = 68) tumours as determined by immunohistochemistry in KP and KP;RbTR/TR mice. n = 2 KP mice and n = 2 KP;RbTR/TR mice. Data are mean ± s.d. Significance was determined by two-tailed unpaired Student’s t-test. P = 0.0350. e, Analysis of p-p27 S10 levels in p-ERK low (n = 43) and p-ERK high (n = 70) tumours as determined by immunohistochemistry in KP and KP;RbTR/TR mice. n = 2 KP mice and n = 2 KP;RbTR/TR mice. Significance was determined by two-tailed unpaired Student’s t-test. P = 0.6937. f, Analysis of p-p27 T187 levels in p-ERK low (n = 40) and p-ERK high (n = 73) tumours as determined by immunohistochemistry in KP and KP;RbTR/TR mice. n = 2 KP mice and n = 2 KP;RbTR/TR mice. Significance was determined by two-tailed unpaired Student’s t-test. P = 0.0127. g, Analysis of p27 levels in KP (n = 85) and KP;RbTR/TR (n = 54) tumours as determined by immunohistochemistry. n = 2 KP mice and n = 2 KP;RbTR/TR mice. Significance was determined by two-tailed unpaired Student’s t-test. P = 0.0018. h, Analysis of p-p27 S10 levels in KP (n = 82) and KP;RbTR/TR (n = 74) tumours as determined by immunohistochemistry. n = 2 KP mice and n = 2 KP;RbTR/TR mice. Significance was determined by two-tailed unpaired Student’s t-test. P = 0.4372. i, Analysis of p-p27 T187 levels in KP (n = 86) and KP;RbTR/TR (n = 72) tumours as determined by immunohistochemistry. n = 2 KP mice and n = 2 KP;RbTR/TR mice. Data are mean ± s.d. Significance was determined by two-tailed unpaired Student’s t-test. P = 3.1 × 10⁻⁶. j, Representative images of immunohistochemistry for p-ERK and p-RB S807/S811 in KP tumours treated with vehicle control or MEK1/2 inhibitor PD0325901. k, Contingency analysis of p-ERK and p-RB S807/S811 from c. n = 42 tumours from 2 mice treated with vehicle control; n = 60 tumours from 3 mice treated with PD0325901. Significance was determined by two-sided χ² test for vehicle-treated group (P = 1.1 × 10⁻⁵) and for PD0325901-treated group (P = 2.7 × 10⁻⁵).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | CDK2 blockade enhances the effects of CDK4/6 inhibition in mouse KP and human lung adenocarcinoma cell lines. 

a, BrdU/7AAD double labelling of KP clones expressing GFP (n = 1 biological replicate) or Cdk2 (n = 2 biological replicates) targeting sgRNAs with 0, 0.1 or 1.0 μM palbociclib. Percentage of cells in G1, S or G2/M phase are shown (2 technical replicates for each sample). Data are mean ± s.d. where appropriate. 

b, Top, cell cycle analysis (BrdU/7AAD double labelling) of KP clones expressing GFP (n = 1 clone per cell line) or Cdk2 (n = 2 clones per cell line) targeting sgRNAs. Bottom, the percentage of cells in each stage of the cell cycle is shown. Data are mean ± s.d. Significance was determined by ANOVA with Dunnett’s multiple comparisons test (n = 4 for all). 

c, d, Cell proliferation assay performed in duplicate showing number of cells 3 days after initial plating treated with 0, 0.1 or 1.0 μM palbociclib for either KP54 (c) or KP62 (d) cells. Two independent Cdk2 knockout clones and control GFP cells are shown. Data are means. 

e, f, Cell proliferation assay performed in triplicate showing number of cells 24, 48 and 72 h after initial plating for KP54 (e) or KP62 (f) cells. Three independent Cdk2 KO clones and one control is shown. Data are mean ± s.d. Significance was determined by ANOVA with Dunnett’s multiple comparisons test (n = 3 for all). 

e, Treatment for 24 h with GFP shRNA compared to CDK2-2 (P = 0.0013), CDK2-3 (P = 0.0029) or CDK2-8 (P = 0.0302) shRNA. Treatment for 48 h with GFP shRNA compared to CDK2-2 (P < 0.0001), CDK2-3 (P < 0.0001) or CDK2-8 (P < 0.0001) shRNA. Treatment for 72 h with GFP shRNA compared to CDK2-2 (P < 0.0001), CDK2-3 (P < 0.0001) or CDK2-8 (P < 0.0001) shRNA. f, Treatment for 48 h with GFP shRNA compared to CDK2-2 shRNA (P = 0.0415). Treatment for 72 h with GFP shRNA compared to CDK2-2 (P < 0.0001) or CDK2-5 (P < 0.0001) shRNA. 

g, Proliferation of KP (left) and KP;RbTR/TR (right) cells in quadruplicate, 72 h after addition of roscovitine (red outlines) and/or palbociclib (increasing grey tones) at the indicated concentrations. Cell numbers normalized to the average of the vehicle (DMSO) controls (white). Data are mean ± s.d. Significance was determined by ANOVA with Dunnett’s multiple comparisons test (n = 4 for all). 

h, Proliferation of KP cells in quadruplicate, stably transduced with a tet-regulated dominant negative Cdk2 allele (CDK2DN) 72 h after addition doxycyclin (red) and/or palbociclib (increasing grey tones). Cell numbers normalized to the average of the vehicle (DMSO) controls (white). Data are mean ± s.d. Significance was determined by ANOVA with Dunnett’s multiple comparisons test (n = 4 for all). Control compared to 1 μM doxycycline to induce CDK2DN (P < 0.0001) or 1 μM palbociclib (P = 0.0194), 1 μM doxycycline compared to 1 μM doxycycline and 0.1 μM palbociclib (P = 0.4184) or 1 μM doxycycline and 1 μM palbociclib (P = 0.0020). 

i, Analysis of KP clones targeted with GFP-targeting or Cdk2-targeting sgRNAs. Western blot for Rb pathway component expression: RB, p-RB S807/S811, Cdk2, Cdk4, Cdk6 and p-RB 780. Actin was used as loading control. 

j, Effects of CDK4/6 inhibition and Cdk2 knockout on human lung adenocarcinoma cell lines. Data mined from the Sanger Center’s COSMIC database showing the relative sensitivity (IC50) of independent human lung adenocarcinoma cells lines to palbociclib. 

k, Western blot showing CDK2 loss following CRISPR-mediated knockout in indicated human lung adenocarcinoma cell lines. HSP90 was used as loading control. 

l, Representative images of clonogenic survival analysis of human lung adenocarcinoma cell lines performed in triplicate, treated every 3 days for 1.5 weeks with 0, 0.1 or 1.0 μM palbociclib. Cell lines were targeted with either an inert sgRNA or one targeting CDK2. 

m, Quantification of culture area covered by cells in l. Dark grey bars indicate inert sgRNA and red bars indicate CDK2 sgRNA. Data are mean ± s.d. Significance was determined by ANOVA with Sidak’s multiple comparisons test (n = 3 for all). 

A549 with inert sgRNA compared to CDK2 sgRNA in combination with 0 μM palbociclib (P > 0.9999), 0.1 μM palbociclib (P = 1.0 × 10⁻⁶) or 1 μM palbociclib (P = 0.0823). H1993 with inert sgRNA compared to CDK2 sgRNA in combination with 0 μM palbociclib (P > 0.9999), 0.1 μM palbociclib (P = 0.0331) or 1 μM palbociclib (P = 0.6557). EKVX with inert sgRNA compared to CDK2 sgRNA in combination with 0 μM palbociclib (P = 0.5412), 0.1 μM palbociclib (P = 0.0003) or 1 μM palbociclib (P = 0.5929).
Extended Data Fig. 6 | Loss of RB promotes alternative pathways towards gaining metastatic competency. a, H&E photomicrographs of metastases that formed from KP;RbTR/TR tumours. b, Immunofluorescence analysis of KP and KP;RbTR/TR tumours for co-expression of HMGA2 and NKX2-1. c, Immunohistochemistry staining of serial sections from KP and KP;RbTR/TR tumours for HMGA2 and FOXA2. Orange dotted lines outline mutually exclusive staining and red dotted lines outline co-expressing staining patterns. Quantification of staining pattern (right) showing percentages of HMGA2⁺ tumours from KP or KP;RbTR/TR mice that are FOXA2⁺ or FOXA2⁻. KP, n = 29 tumours from 3 mice; KP;RbTR/TR, n = 37 tumours from 3 mice. Significance was determined by two-sided \( \chi^2 \) test, \( P = 1.1 \times 10^{-5} \). d, Left, histological analysis of KP;RbTR/TR metastases. H&E staining and immunohistochemistry for NKX2-1, HMGA2 and FOXA2 of serial sections from representative metastases that are NKX2-1⁺ or NKX2-1⁻. Right, quantification. e, Immunohistochemistry staining of KP and KP;RbTR/TR tumours for club (CC10) and neuroendocrine (synaptophysin) cell markers. For control and comparison, a synaptophysin-positive small-cell lung cancer from a Trp53flox/flox;RbflkJlac;P130flox/flox mouse model is shown.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Loss of p16 or RB is associated with increased metastatic proclivity. 

**a**, Western blot analysis of KP;RbTR/TR and KP (NKX2-1−HMGA2−(T_Met) and NKX2-1+HMGA2−(T_tumor)) tumour-derived cell lines examining cyclin D1 and p16 expression. HSP90 was used as loading control. 

**b**, RNA sequencing reads at the Cdkn2a locus for NKX2-1+HMGA2−KP (n = 2) and NKX2-1−HMGA2−KP (n = 2) cell lines. Reads from exon 1α encoding p16 (left) and exon 1β encoding Arf (right) are shown. 

**c**, Representative Sashimi plots comparing the number of p16 and Arf exon-spanning reads from the Cdkn2a locus. Plots are shown for a representative NKX2-1+HMGA2−tumour (red), NKX2-1−HMGA2+ tumour (blue) and metastasis (green). The number of reads that span each exon–exon junction is displayed. The range of minimum to maximum read count for the given plot is displayed in the top left corner. 

**d**, Quantification of the ratio of p16 to Arf reads from the Cdkn2a locus. RNA sequencing results examining NKX2-1+HMGA2−tumours (n = 8), NKX2-1+HMGA2−tumours (n = 8) and extrapulmonary metastases (n = 19) were obtained from the GEO (accession GSE84447)25. Significance for each comparison was determined by two-tailed unpaired Student’s t-test. NKX2-1+HMGA2−versus NKX2-1−HMGA2+, P = 0.0682; NKX2-1−HMGA2+versus metastases, P = 0.0504; NKX2-1+HMGA2−versus metastases, P = 0.0006. Data are represented by box and whisker plots, with the line indicating the median and whiskers indicating the minimum and maximum values. 

**e**, Western blot analysis of KP and KP;RbTR/TR tumour-derived cell lines for NKX2-1, HMGA2 and RB. Actin was used as loading control. 

**f, g**, Analysis of subcutaneous tumour growth (f) and associated lung metastases (g) of KP and KP;RbTR/TR tumour-derived cell lines from e. Symbols represent individual mice injected with either one of two NKX2-1−HMGA2+ KP cell lines (n = 4 and 5 mice per cell line), two NKX2-1−HMGA2+ KP;RbTR/TR cell lines (n = 3 and 4 mice per cell line) or two NKX2-1+HMGA2+ KP;RbTR/TR cell lines (n = 4 mice per cell line). Significance was determined by unpaired two-tailed Student’s t-test with Welch’s correction. 

**h**, H&E photomicrographs of representative metastases from KP;RbTR/TR cell line allografts. Insets show magnification of boxed areas. Scale bars, 100 μm. 

**i**, Liver metastases after intrasplenic injection of KP and KP;RbTR/TR tumour-derived cell lines (top) from Extended Data Fig. 5i. Symbols represent individual mice injected with a NKX2-1+HMGA2− KP cell line (n = 2 mice), a NKX2-1−HMGA2+ KP cell line (n = 5 mice), two NKX2-1−HMGA2+ KP;RbTR/TR cell lines (n = 4 mice for each cell line) or two NKX2-1+HMGA2+ KP;RbTR/TR cell lines (n = 5 mice for each cell line). Significance was determined by two-tailed unpaired Student’s t-test with Welch’s correction. P = 0.0007. Data are mean ± s.d.
Extended Data Fig. 8 | Growth of lung adenocarcinomas after RB reactivation. **a,** Imaging of individual tumour growth by μCT. Images were taken weekly starting 11 weeks after tumour initiation. RB reactivation (tamoxifen treatment) initiated at week 12. Average fold change after week 12 is shown. KP;RbTR/TR, n = 6 mice; KP;RbR/R, n = 6 mice. Data are mean ± s.d. Significance at 14 weeks was determined by unpaired two-tailed Student’s t-test. *P* = 0.2617. **b,** Representative μCT images quantified in **a.** **c,** Low-magnification scans of sections through tumour lobes showing relative tumour burden 2 weeks after RB reactivation in KP;RbTR/TR and KP;RbR/R cohorts. **d,** Quantitative PCR detection of the GFP cDNA within the RbTR allele. DNA templates for PCR were isolated by laser capture microdissection of individual tumours. KP;RbTR/TR, n = 8 tumours from 2 mice; KP;RbR/R, n = 8 tumours from 2 mice. Significance was determined by two-tailed unpaired Student’s t-test. *P* = 0.0023. Data are mean ± s.d.
Extended Data Fig. 9 | Impact of RB reactivation on proliferation, MAPK signalling and RB phosphorylation. a, Ki67 immunohistochemistry of KP;RbTR/TR and KP;RbR/R tumours 3, 7 and 14 days after Rb1 restoration. b, Quantification of a. n = 10 tumours from 3 mice for 0-, 3- and 7-day time points; n = 15 tumours from 3 mice for the 14-day time point. Significance was determined by χ² test for trend. P = 0.3289. c, Quantification of the percentage of total tumour cells that are Ki67+ in KP;RbTR/TR and KP;RbR/R tumours 14 days after Rb1 restoration. KP;RbTR/TR, n = 14 tumours from 1 mouse; KP;RbR/R, n = 14 tumours from 1 mouse. Significance was determined by two-tailed unpaired Student’s t-test with Welch’s correction. P = 0.0110. Data are mean ± s.d. d, Immunohistochemistry for RB, p-RB S807/S811 and p-ERK in KP;RbTR/TR and KP;RbR/R tumours 3, 7 and 14 days after Rb1 restoration. Scale bars, 100 μm; insets are magnified 5×. e, Contingency test for NKX2-1+ HMGA2+ KP;RbTR/TR and KP;RbR/R tumours 2 weeks after Rb1 restoration. NKX2-1+ HMGA2+ KP;RbTR/TR, n = 23 tumours from 2 mice; KP;RbR/R, n = 12 tumours from 2 mice. Significance was determined by two-sided χ² test. P = 0.0019.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | RB controls multiple barriers to tumour progression and is repressed by multiple pathways that will require multiple pharmacological interventions to reverse. a, In the KP model, adenomas transit through an ‘early barrier’ that limits progression to the carcinoma state by amplifying the MAPK signalling cascade (red). The ‘late barrier’ limits the onset of metastatic ability and is characterized by the loss of lineage fidelity marked by lost expression of lineage-specific transcription factors, NKX2-1 (blue) and FOXA2 (purple), and the differentiation marker of alveolar type 2 cells, SPC (green). Loss of these lineage commitment factors precedes the derepression of the embryonic-restricted chromatin factor HMGA2 that functionally drives metastasis and marks the metastatic cell state (yellow). Downregulation of p16^{INK4a} expression is associated with the metastatic cell state (grey). b, The additional suppression of Rb1 in the KP;Rb^{TR/TR} model alters the molecular trajectory of these tumours by first abrogating the early barrier through the elimination of the requirement for amplification of the MAPK signal (lack of red) and then by facilitating loss of lineage fidelity to overcome and blur the late barrier. Carcinomatous KP;Rb^{TR/TR} tumours can rapidly derepress HMGA2 (yellow) and lose the lineage identity marker SPC (green); however, loss of lineage fidelity is unlinked from NKX2-1 and FOXA2, which normally enforce lung cell identity in these tumours. Notably, metastatic primary tumours and distant metastases can sometimes maintain expression of NKX2-1 (blue) and FOXA2 (purple). Expression of p16^{INK4a} is maintained in RB-deficient metastatic cell states (grey). c, In lung adenomas that maintain RB, RB tumour suppressor activity blocks progression to carcinomatous stages and the onset of metastatic cell states and enforces lineage fidelity. d, In lung adenocarcinomas that maintain RB expression, amplification of the MAPK signal activates CDK2-dependent hyperphosphorylation of RB to promote carcinoma progression. e, Inactivation of RB removes early barriers that limit carcinoma progression, removes constraints that reinforce lineage fidelity and disrupts late barriers that suppress metastatic competency. f, Reactivation of RB in tumours that lack Rb pathway function highlights a need for a multi-pronged approach to inhibit CDK4/6 as well as CDK2 and/or MAPK pathway signalling (for example, through MEK inhibition) to fully reactivate RB-mediated tumour suppression. These data emphasize the need for the development of selective CDK2 inhibitors.
**Statistical parameters**

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- **n/a**
- **Confirmed**
  - The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement
  - An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
  - A description of all covariates tested
  - A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
  - A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
  - For null hypothesis testing, the test statistic (e.g. \(F, t, r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted
  - Give \(P\) values as exact values whenever suitable.
  - For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
  - For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
  - Estimates of effect sizes (e.g. Cohen’s \(d\), Pearson’s \(r\)), indicating how they were calculated
  - Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

*Our web collection on statistics for biologists may be useful.*

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**Software and code**

Policy information about availability of computer code

**Data collection**

Micro-CT acquisition was performed on u-CT (Mi Labs) with Acquisition 7.45 software. Image reconstruction and visualization used performed with Mi Labs REC-7.09. Analysis was performed with Microview version 2.5. Quantification of IHC, clonogenic growth, was performed with FIJI (Imagej). Compusyn software (ComboSyn, Inc.) was used for determination of synergy. CBioportal and was used for enumerating mutation frequency in the indicated human genomic data sets. Oncomine was used for establishing genotype dependent associations with tumor stage, grade, frequency of metastasis, and size in the indicated human genomic data sets.

**Data analysis**

Statistical analyses were performed using Prism Software version 7. All statistical methods are indicated in the figure legends and in the methods. Reads from RNA-Seq were mapped using STAR aligner (v2.5.2b). FeatureCounts (v1.5.0-p1) was used to quantify alignments against the mouse genomic annotations from Gencode (vM11). Differentially expressed genes were identified with DESeq2 (v1.14.0). Single sample gene set enrichment analysis (ssGSEA) was performed at https://genepattern.broadinstitute.org/gp/ using the ssGSEAProjection module. BAM files were indexed using samtools (v.1.9) and quantified using Integrative Genomics Viewer (v.2.4.10). Sashimi plots were generated using Integrative Genomics Viewer (v.2.4.10).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files) with the exception of raw RNA-Seq data associated with Extended Data Fig. 2e,f which have been deposited publicly in the Gene Expression Omnibus under accession number XXXXX

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

No statistical methods were used to predetermine sample sizes. The size of each animal cohort was determined by estimating biologically relevant effect sizes between control and treated groups and then using the minimum number of animals that could reveal statistical significance using the indicated tests of significance.

**Data exclusions**

In analysis of Cdkn2a reads from RNA-Seq datasets, outliers were excluded based on pre-established criteria (greater than Q3 + 1.5x interquartile range or less than Q1 - 1.5x interquartile range). No other data were excluded from analyses.

**Replication**

Cell culture experiments described were repeated with at least 3 independent replicates and on at least two separate occasions with significant results in the same direction as those represented in the figures. Independent experiments (or experimental groups) were combined as indicated and when appropriate (e.g. when multiple cell lines with the same genotype had the same phenotype).

**Randomization**

All animal studies were randomized in ‘Control’ or ‘Treated’ groups. However, all animals housed within the same cage were generally placed within the same treatment group.

**Blinding**

For analysis of tumour grades sample identity and group identity were blinded from histopathological assessment.

Reporting for specific materials, systems and methods

**Materials & experimental systems**

|      | Involved in the study |
|------|----------------------|
| n/a  |                      |
| ☑    | Unique biological materials |
| ☑    | Antibodies |
| ☑    | Eukaryotic cell lines |
| ☑    | Palaeontology |
| ☑    | Animals and other organisms |
| ☑    | Human research participants |

**Methods**

|      | Involved in the study |
|------|----------------------|
| n/a  |                      |
| ☑    | ChIP-seq |
| ☑    | Flow cytometry |
| ☑    | MRI-based neuroimaging |

**Unique biological materials**

Policy information about availability of materials

Obtaining unique materials

A subset of the tumor tissue micro-arrays (TMA) are unique and not available commercially. Other TMAs were obtained from BioMax.
Antibodies

Antibodies used

Primary Antibodies used for Immunohistochemistry and Immunofluorescence:

- **Rb** [Abcam; ab181616], Lot GR3188052-5, 1:200, Commercially verified by Abcam, using Knockout Validation
- **GFP** [Cell Signaling; 2956], Lot: 4, 1:100 Commercially verified by Cell Signaling, using HCC827 cells, untransfected or GFP-transfected.
- **phosphorylated Mek 1/2** [Cell Signaling; cs2338], Lot: 9, 1:50 Commercially verified by Cell Signaling, using A375 cells, untreated or treated with Raf1 Kinase Inhibitor 1
- **phosphorylated Erk 1/2** [Cell Signaling; cs4370], Lot: 17 1:800 Commercially verified by Cell Signaling, using human lung carcinoma, untreated or λ phosphatase-treated
- **BrdU** [BD Transduction Laboratories; 347580], lot: 36576, 1:50, Commercially verified by BD
- **Ki67** [Vector Laboratories; VP-RM04], lot: X0302, 1:1000, Commercially verified by Vector Labs (product sheet says human but here’s one of 37 citations used in mouse Zheng et al. (2008) Nature https://www.nature.com/articles/nature07443
- **Nkx2-1** [Abcam; ab76013], Lot: GR76790-21, 1:250, Commercially verified by Abcam, using Human lung carcinoma and thyroid carcinoma tissue
- **Hmga2** [Biocheck; 59170AP], Lot: RN-31993, 1:300, Chiou et al. (2018) Scientific Reports, https://www.nature.com/articles/s41598-018-32159-x
- **Foxa2** [santa cruz sc-6554] lot 1715 1:250
- **Spc** [Millipore; AB3786], lot: 2965119, 1:200, Commercially validated by Millipore, using staining for localization of prosurfactant protein C in adult mouse lung.
- **CC10** [Santa Cruz; sc-9772], Lot: 1613, 1:50 García-Sanmartín, J. et al. 2015. Histology and histopathology.
- **phospho-Rb Ser807/811** [Cell Signaling; cs8516], Lot: 6 1:100 Commercially verified by Cell Signaling, using human colon carcinoma tissue
- **Cc1d2** [Abcam; ab32147], Lot: GR292523-18, 1:100, Commercially verified by Abcam, using Knockout Validation
- **Cc1d4** [Abcam; ab199728]. Lot: GR207212-2, 1:100 Commercially verified by Abcam, using Knockout Validation

Secondary Antibodies used for Immunofluorescence:

- **Invitrogen; Anti-rabbit-Alexa594** [A21207] Lot: 1890862, 1:200, Commercially verified by ThermoFisher
- **Anti-mouse-Alexa647** [A31571] Lot: 423849, 1:200, Commercially verified by ThermoFisher

Primary Antibodies used for Immunoblots:

- **Total Erk 1/2** [Cell Signaling; cs4696], Lot:22, 1:1000, Commercially verified by Cell Signaling using analysis of extracts from NIH/3T3, PC12 and COS cells
- **Cc1d1** [Gift from L. Busino/Michele Pagano], 1:4000
- **Cc1d4** [Abcam; ab199728], GR207212-2,1:1000, Commercially verified by Abcam, using Knockout Validation
- **Cc1d6** [Abcam; ab151247], no lot info: 1:1000, commercially verified by Abcam using 293T, A431, H1299, HeLa, HepG2, MOLT4 and Raji whole cell lysates; HeLa cells; Human SW480 xenograft tissue
- **Cyclin D1** [Abcam; Ab5285], Lot: 423670, 1:2000, Commercially verified by Abcam, using Knockout Validation
- **Cyclin E1** [Bioss Antibodies; bs-0573R], Lot: 9810X4, 1:3000 Cited by Lv, H., Ren, J., et al. 2012 PLoS ONE
- **E2f1** [Abcam; ab179445], Lot: GR155150-19, 1:1000, Commercially verified by Abcam using NIH 3T3, HeLa, HepG2, T47D, Human spleen and fetal muscle lysates; E2F1-DDDDK tag transfected 293T lysate; HepG2 cells

Authentication When possible, antibodies were validated beyond the manufacturers data sheet specification by staining slides (IHC) or blots (western) with known deficiencies for expression of the antigen.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Murine cell lines were generated from primary mouse tumors and propagated using standard techniques. Human lung adenocarcinoma cell lines were obtained from ATCC or NIH Cell line repository. HEK293FT cells used for lentivirus production were obtained from Invitrogen. GreenGo cells used for titering lentivirus were obtained from Tyler Jack’s Laboratory and are a derivative of NIH3T3 cells.

Authentication

Mouse cell lines were authenticated for genotype. Human and mouse lung cancer cell lines were tested for the expected, genotype-associated protein expression patterns by western blot. HEK293FT cells used for lentivirus production were validated by verifying high titer virus production was possible. NIH3T3-GreenGo cells were validated by measuring Cre-induced GFP expression.

Mycoplasma contamination

Human cells were tested negative by the cell line repository or the manufacturer. Mouse cell lines were not tested.

Commonly misidentified lines

We have not used any commonly misidentified cell lines in this study.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Species: Mus musculus
Laboratory animals

Strain and Sex: KrasLSL-G12D, p53flox, RbXTR, and Rosa26FipO-ER strains are maintained on a mixed 129sv/Jae, C57Bl6J background (Male and Female), tumors initiated by 20 weeks of age. Xenograft recipient mice: CrTac:NCr-Foxn1nu (Males); NOD Rag1-/-;Il2rg-/- (Males), Age = 6-12 weeks.

Wild animals

N/A

Field-collected samples

N/A

Flow Cytometry

Plots

Confirm that:

☑️ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑️ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑️ All plots are contour plots with outliers or pseudocolor plots.
☑️ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells were isolated from culture dishes using trypsin-EDTA, washed 3x with PBS and fixed according to the APC BrdU Flow Kit (BD Pharmingen).

Instrument

Attune acoustic focusing cytometer

Software

FlowJo v10

Cell population abundance

Greater than 20,000 total events/cells were acquired.

Gating strategy

All events were separated based on APC and 7-AAD staining and gated as shown. BrdU negative cells were used to establish thresholds for gates. Clear separation between G1 and G2/M populations were established by assuming that G2/M cells would be twice as bright for 7-AAD than G1 cells.

☐️ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.