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A Braf kinase-inactive mutant induces lung adenocarcinoma

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Abstract

The initiating oncogenic event in almost half of human lung adenocarcinomas is still unknown, a fact that complicates the development of selective targeted therapies. Yet these tumours harbour a number of alterations without obvious oncogenic function including BRAF-inactivating mutations. Inactivating BRAF mutants in lung predominate over the activating V600E mutant that is frequently observed in other tumour types¹. Here we demonstrate that the expression of an endogenous Braf(D631A) kinase-inactive isoform in mice (corresponding to the human BRAF(D594A) mutation) triggers lung adenocarcinoma *in vivo*, indicating that BRAF-inactivating mutations are initiating events in lung oncogenesis. Moreover, inactivating BRAF mutations have also been identified in a subset of KRAS-driven human lung tumours. Co-expression of Kras(G12V) and Braf(D631A) in mouse lung cells markedly enhances tumour

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Author Contributions D.S. and M.B. designed experiments and research aims, analysed data and wrote the manuscript with help from co-authors. P.N. performed experiments and analysed the data with help from C.A., L.E. and M.T.B. R.C. carried out critical interpretation of the tumour phenotype. R.M. provided the *Braf^{+/LSLD631A}* strain. Z.Y., N.R., R.M. and R.C. contributed critical information and helpful discussions. D.G.P. and G.G.-L. performed the bioinformatic analysis of human datasets.

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initiation, a phenomenon mediated by Craf kinase activity^{2,3}, and effectively accelerates tumour progression when activated in advanced lung adenocarcinomas. We also report a key role for the wild-type Braf kinase in sustaining Kras(G12V)/Braf(D631A)-driven tumours. Ablation of the wild-type *Braf* allele prevents the development of lung adenocarcinoma by inducing a further increase in MAPK signalling that results in oncogenic toxicity; this effect can be abolished by pharmacological inhibition of Mek to restore tumour growth. However, the loss of wild-type Braf also induces transdifferentiation of club cells, which leads to the rapid development of lethal intrabronchiolar lesions. These observations indicate that the signal intensity of the MAPK pathway is a critical determinant not only in tumour development, but also in dictating the nature of the cancer-initiating cell and ultimately the resulting tumour phenotype.

The RAS–MAPK signalling cascade serves as a central node in transducing signals from membrane receptors to the nucleus. This pathway is aberrantly activated in a substantial fraction of human cancers⁴. Moreover, germline mutations resulting in limited activation of this signalling cascade cause developmental disorders known as RASopathies⁵. There is also abundant evidence that elevated RAS–MAPK signalling results in cellular toxicity that may serve as a natural barrier to cancer progression early in tumorigenesis⁶. Finally, genetic abrogation of this pathway in adult mice results in their rapid death⁷. These findings suggest that defined thresholds of RAS–MAPK activity are required for homeostasis as well as for malignant transformation, but compelling genetic evidence is missing.

In order to augment MAPK signalling in controlled increments we have taken advantage of the expression of an endogenous Braf(D631A) kinase-dead isoform (corresponding to the human BRAF(D594A) mutant) that is known to induce Erk phosphorylation in a Craf-dependent manner^{2,8}. This effect, known as the MAPK paradox, is due to enhanced heterodimerization and activation of the catalytically competent Craf protomer in Braf(D631A)–Craf complexes^{2,3}. In agreement with these observations, lack of wild-type Braf expression in *Kras*^{G12V} cell lines expressing Braf(D631A) increased the intensity and duration of MAPK signalling (Extended Data Fig. 1), probably as a result of the exclusive formation of Braf(D631A)–Craf heterodimers. Thus, to generate controlled thresholds of MAPK intensity *in vivo*, we combined wild-type *Braf*, conditional knockout *Braf*^{lox} and conditional knock-in *Braf*^{LSLD631A} with an inducible *Kras*^{LSLG12Vgeo} allele⁹ (where *LSL* indicates a *lox-STOP-lox* motif). The resulting *Kras*^{+/LSLG12Vgeo} (hereto designated as K), *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (designated as KB) and *Kras*^{+/LSLG12Vgeo}; *Braf*^{lox/LSLD631A} (designated as KBL) strains were intratracheally infected with adenovirus expressing Cre recombinase (Ad-Cre). Cre-mediated recombination of these alleles results in the induction of distinct levels of Ras–MAPK signalling, with *Braf*^{+/+} driving lower activity, *Braf*^{+/D631A} intermediate intensity and *Braf*^{–/D631A} maximal activation. This strategy allowed us to investigate the effect of various MAPK activity thresholds on cell transformation, adenocarcinoma development and cellular toxicity *in vivo*.

Lung cells expressing these conditional alleles were identified by co-expression of the Kras(G12V) oncoprotein with β-geo, a chimaeric bacterial protein with β-galactosidase activity⁹ (Extended Data Fig. 2). As illustrated in Fig. 1a, small X-gal⁺ hyperplasias could

be readily detected in the lungs of K mice 1 month after Ad-Cre infection. By contrast, Ad-Cre-infected KB mice displayed abundant hyperplastic areas together with adenomas, a lesion that is extremely infrequent in K mice at this early stage¹⁰. Remarkably, X-gal⁺ alveolar hyperplasias and adenomas were nearly absent in Ad-Cre-infected KBL animals. These mice, however, displayed hypertrophic bronchi as their most prominent feature (Fig. 1a). The lack of *Kras*^{G12V}-driven alveolar lesions in KBL mice is unlikely to be a consequence of the absence of an active Braf, since this kinase is dispensable for the development of *Kras*^{G12V}-driven lung adenocarcinoma^{7,11}. Thus, we explored the possibility that the absence of alveolar lesions was due to some sort of cellular toxicity such as that caused by the induction of senescence or DNA damage, two phenomena known to act as barriers to tumour development^{6,12,13}. Indeed, examination of lung lysates and sections from KBL mice detected p19^{Arf} and p53 tumour suppressors, active caspase-3 and γ -H2AX, a marker for DNA damage, as early as 1 week following Ad-Cre infection (Fig. 1b, c). We did not observe differences in senescence-associated β -gal staining (data not shown). The concomitant increase in the phosphorylation of Erk1/2 and p90Rsk suggested that elevated MAPK signalling triggered a stress response that impaired tumour cell proliferation. Yet it has been recently described that mutant *Kras*-driven lung adenocarcinomas display a tonic activation of the DNA damage response to prevent the induction of genotoxic stress¹⁴. We hypothesize that the exclusive formation of Braf(D631A)–Craf complexes in KBL mice exceeded such a toxic signalling threshold and induced a stress response incompatible with tumour development. Thus, our results indicate that Ras-oncogenic toxicity is quantitatively dictated by MAPK function. In support of this hypothesis, whereas Mek inhibition prevented tumour growth in Ad-Cre-infected KB mice, it rescued the toxic phenotype in KBL animals in a dose-dependent manner and restored tumour progression at intermediate drug concentrations, most likely by curtailing MAPK activity to levels compatible with cell proliferation (Fig. 1d). This is in agreement with the observation that RAS-induced senescence in primary cells is bypassed by inhibition of MEK and ERK kinases¹⁵. These observations suggest that *Kras*^{G12V}-driven lung tumour cells can only proliferate within a limited range of MAPK activity. Whereas excess MAPK activity induced DNA damage-mediated cellular toxicity, insufficient MAPK signalling cannot sustain tumour growth.

Next, cohorts of Ad-Cre infected K, KB and KBL mice were followed over time. In agreement with the earlier onset and the more rapid progression of the alveolar lesions, the KB animals displayed significantly shortened survival (Fig. 2a). Histopathological analysis of their lungs at 6 months post Ad-Cre infection revealed a 7.5-fold increase in tumour burden compared to K controls (Fig. 2b). Moreover, KB mice displayed advanced adenocarcinomas, a tumour stage that is extremely infrequent at this time in tumours driven by *Kras*^{G12V} alone (Fig. 2c). Tumours present in KB mice displayed SPC⁺CC10⁺ immunostaining, which suggests an alveolar type II (AT2) origin as previously described for adenocarcinomas driven by oncogenic *Kras* alone^{10,16} (Fig. 2d). Altogether, these observations suggest that MAPK hyperactivation by coexisting *Kras*(G12V) and Braf(D631A) mutations resulted in increased transformation of AT2 cells and accelerated tumour progression. The MAPK paradoxical activation model postulates that the observed tumour phenotype is mediated by Craf kinase activity^{2,8,17}. To genetically validate this hypothesis in the lung tumours studied here, we added conditional knock-in *Craf*(also

known as *Raf1*) kinase-dead alleles (*Craf^{L5LD468A}*) to KB mice. The resulting strain, *Kras^{+/LSLG12Vgeo};Braf^{+/LSLD631A};Craf^{L5LD468A/LSLD468A}* (designated as KBC^{KD}) was used to determine whether genetic inhibition of the *Craf* kinase reverted the increased tumorigenic phenotype displayed by KB mice. Expression of the *Craf*(D468A) kinase-dead isoform led to a substantial decrease in the levels of phosphorylated (p-)Erk1/2 and overall tumour burden (Fig. 2e, f and Extended Data Fig. 3), significantly extending the survival of Ad-Cre-infected KBC^{KD} mice compared to KB animals. Similarly, *Craf* ablation (KBC^L mice) also resulted in prolonged survival (Fig. 2g). Altogether, these results demonstrate that the increased tumour burden and faster adenocarcinoma progression in KB animals is due to *Craf*-mediated hyper-activation of MAPK signalling.

As indicated above, the tumour burden of the KBL cohort was significantly decreased compared to KB mice, suggesting that excessive MAPK activity in the absence of wild-type *Braf* expression may be detrimental for lung adenocarcinoma development. Yet, in spite of the reduced tumour burden, KBL animals reached humane end point at the same time as KB mice (Fig. 2a, b). Detailed examination of the lungs of KBL mice revealed the presence of intrabronchiolar carcinomas (112 of 250 bronchi, 45%), a rare lesion in K (0 of 162) or KB (20 of 145, 14%) cohorts (Fig. 3a). Immunostaining with a collection of markers including *Ttf1* and *SPC*, characteristic of lung adenocarcinoma and *Sox2*, *p63* and *CK5*, diagnostic of squamous cell carcinoma, did not clarify the origin of these intrabronchiolar lesions since the resulting expression pattern was not in accordance with the expected profile of either of these tumour types¹⁸ (Fig. 3b). Analysis of the initial tumour stages of these lesions revealed protruding papillary structures accompanied by loss of *CC10* expression, a marker characteristic of the bronchiolar epithelium. Most of these lesions (95%) acquired expression of the AT2 marker *SPC*, thus suggesting a transdifferentiation process (Fig. 3c and Extended Data Fig. 4).

To further clarify the origin of these intrabronchiolar lesions we performed *in vivo* tracing experiments by infecting K, KB and KBL mice with lineage-specific Ad-*CC10*-Cre or Ad-*SPC*-Cre viruses that restrict Cre-mediated recombination to club or AT2 cells respectively¹⁹. Analysis of lungs of K and KB mice after Ad-*SPC*-Cre infection revealed the presence of adenomas that, as expected, were larger and more abundant in KB animals. Likewise, KBL mice predominantly developed alveolar hyperplasias that failed to progress to advanced stages. Importantly, infection with Ad-*CC10*-Cre did not induce tumour growth in K or KB mice but caused abundant *SPC*⁺ intrabronchiolar carcinomas in KBL animals (Fig. 3d, e). These results indicate that the threshold of MAPK activity toxic for AT2 cells resulted in the efficient transformation of cells present in bronchial epithelium that are usually refractory to *Kras^{G12V}*-driven oncogenesis^{10,20,21}. Altogether, these results suggest a quantitative impact of MAPK activity controlling various aspects of *Kras* oncogene-driven lung carcinogenesis. As such, oncogenic transformation may critically depend on cell- or tissue-specific programs that are hijacked to adjust MAPK activity and avoid senescence or other tumour-suppressive stress responses. This quantitative model is not exclusive to MAPK signalling as a similar threshold response level has been proposed for Wnt/ β -catenin-driven²² or PI3K/AKT-driven tumorigenesis²³.

Mutational analysis of different human cancers has recently uncovered that among the BRAF hot spots in lung adenocarcinoma, those resulting in inactivating mutations predominate over the V600E activating substitution¹. However, the contribution of BRAF-inactive mutants to lung cancer progression is unclear. Interestingly, a percentage of BRAF-inactivating mutations (11%) coexist with upstream *RAS* alterations (Table 1). Thus, we decided to investigate whether expression of the *Braf*(D631A) kinase-inactive mutant in pre-existing *Kras*^{G12V}-driven tumours may enhance tumour progression. To this end, we added the *Braf*^{LSLD631A} allele to a lung tumour model driven by the Flp recombinase. The resulting strain, *Kras*^{+/-FSFG12V}; *Tg.hUb-cre-ERT2*^{+/-T}; *Braf*^{+/-LSLD631A} (designated as K^{FB}) allows the temporal separation of tumour initiation (Flp-mediated *Kras*(G12V) expression) from genetic events induced during tumour progression (Cre-mediated *Braf*(D631A) expression). Induction of the *Braf*(D631A) kinase-inactive isoform in pre-existing *Kras*^{G12V}-driven tumours resulted in reduced survival owing to the accelerated progression of these lesions (Fig. 4a and Extended Data Fig. 5). Thus, these results provide an experimental explanation for the concurrence of oncogenic *KRAS* and *BRAF*-inactivating mutations in certain human lung tumours. These observations also reinforce the current clinical indication that class I RAF inhibitors (vemurafenib and derivatives targeting the activated form of the BRAF kinase) should not be used in patients with *KRAS*-mutant lung adenocarcinoma.

Yet, the majority of BRAF-inactivating mutations present in human lung cancer (89%) do not coexist with *RAS* mutations (Table 1). To assess whether *Braf* inactive mutants could induce lung adenocarcinoma formation in the absence of *Kras* mutations, we infected *Braf*^{+/-LSLD631A} mice intratracheally with Ad-Cre. Analysis of their lungs 12 months after infection revealed the presence of tumours in 9 of 22 mice (41% incidence) compared to 14 of 18 (78%) in *Kras*^{+/-LSLG12Vgeo} animals. All tumours studied displayed histology characteristic of lung adenocarcinoma with SPC⁺CC10⁻ immunostaining (Fig. 4b and Extended Data Fig. 6a). Notably, p-Erk1/2 levels in *Braf*^{D631A}-driven lung adenocarcinomas were higher than those observed in *Braf*^{V637E} (equivalent to human *BRAF*^{V600E}), *Kras*^{G12V} or *Kras*^{G12V}; *Trp53*^{-/-} tumours (Fig. 4c). As control, we confirmed that the *Braf*^{LSLD631A} allele was efficiently recombined in tumour tissue. Moreover, we determined that there were no *Ras* mutations in these tumours (data not shown).

These results suggest that *Braf*-inactivating mutations initiate lung adenocarcinoma (Extended Data Fig. 6b). Our results are in good agreement with data from the Rosen laboratory indicating that increased levels of wild-type Ras-GTP complexes can cooperate with *Braf* hypoactive mutants to trigger tumour development in epithelial cells²⁴. Similarly, increasing the pool of Ras-GTP by a dominant active Sos is sufficient to induce MAPK hyperactivation and the formation of epithelial tumours in response to Raf inhibition²⁵. Likewise, MAPK activation in primary keratinocytes carrying the *Braf*^{LSLD631A} allele depended both on *Craf* and RTK signalling. Of note, elimination of the *Braf* wild-type allele induced a further increase in p-Erk1/2 (Extended Data Fig. 6c). In lung, the high levels of endogenous Ras-GTP present in adult AT2 cells²⁶ may explain the oncogenicity of the *Braf*(D631A) inactive mutant in the absence of *Kras*(G12V). By contrast, hypoactive BRAF mutants require the presence of *RAS* or *NF1* mutations to trigger melanoma development, both in experimental GEM models² as well as in human tumours (Table 1).

In summary, we provide the first genetic evidence demonstrating that a kinase-inactivating *Braf* mutation induces lung adenocarcinoma development. Importantly, in lung adenocarcinoma patients kinase-inactivating BRAF mutations are more prevalent than the activating V600E allele¹. Furthermore, a recent retrospective study identified BRAF(D594G) (affecting the same residue as the mouse mutation used in this study) as the most frequent BRAF alteration in these patients²⁷. Our analysis of human lung adenocarcinomas with hypoactive BRAF revealed co-occurring alterations such as mutations in RTK signalling antagonists that might cooperate in sustaining MAPK activity (Extended Data Fig. 7). The accompanying manuscript by Yao *et al.*²⁴ suggests that RTK profiling of tumours driven by kinase-impaired BRAF mutants can identify the dominant receptor for the design of specific treatment for individual patients. Alternatively, our results suggest that these patients could benefit from therapies based on selective CRAF inhibitors. The mouse model described here will be useful to evaluate effective drug combinations.

METHODS

Mice

Kras^{LSLG12V_{geo}} (ref. 9), *Braf*^{L_{SLD}631A} (ref. 2, described therein as *Braf*^{L_{SLD}594A}), *Braf*^{lox} (ref. 28), *Braf*^{L_{SLV}637E} (ref. 29, described therein as *Braf*^{CA}) and *Tip53*^{lox/lox} (ref. 30) strains have been previously described. The targeting vector for the *Craf*^{L_{SLD}468A} allele was generated by Gene Bridges GmbH. The D468A misscoding mutation (GAC (Asp) to GCC (Ala)) was engineered within the *Craf* locus present in the BAC RP23-37K24 by multi-site Red/ET ('triple recombination'). A *loxP*-cDNA-*STOP*-*PGK*-*Neo*-*loxP* cassette containing a partial *Craf* cDNA sequence encompassing exons 13 to 17 was inserted 150 bp upstream of the 5' end of the mutated exon13 by Red/ET recombination. The resulting targeting vector was linearized with NotI and SalI restriction enzymes and electroporated into B6129SF1/J ES cells. Clones having undergone proper homologous recombination were identified by Southern blot analysis. Two independent recombinant ES cell clones were microinjected into FVB donor blastocysts and implanted into pseudo-pregnant females. Chimaeric mice were backcrossed to C57BL/6J mice and germline transmission of the *Craf*^{L_{SLD}468A} allele was confirmed by Southern blot analysis. The targeting vector used to obtain the *Kras*^{FSFG12V} allele was generated by Taconic Artemis. In brief, the homology arms including the first exon containing the oncogenic G12V mutation were amplified by PCR using as a template a targeting vector previously developed to generate a *Kras*^{LSLG12V_{geo}} allele⁹. A *PGK*-*Neo*-*STOP* cassette was generated by PCR amplification of the 1,377 bp *STOP* cassette derived from the *Kras*^{LSLG12V_{geo}} targeting vector with primers that incorporated NdeI restriction sites. The *STOP* cassette was subsequently cloned into the NdeI restriction site of pBASIC10 (Taconic Artemis) flanked by FRT sequences. The resulting targeting vector was linearized with NotI and electroporated into B6129SF1/J ES cells. Two independent recombinant ES cell clones were microinjected into C57BL/6J blastocysts and transplanted into pseudo-pregnant females. Chimaeric mice were backcrossed to C57BL/6J mice and germ line transmission of the targeted allele was confirmed by Southern blot analysis. All animal experiments were approved by the Ethical Committee of CNIO and performed in accordance with the guidelines stated in the International Guiding Principles for Biomedical

Research Involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS). All strains were genotyped by Transnetyx.

Tumour induction and drug treatments

Tumours were induced in 8- to 12-week-old mice (both male and female animals were used) by single intratracheal infection with 10^6 adenoviral particles (unless stated otherwise) after anaesthesia (i.p. injection of ketamine 75 mg kg^{-1} , xylazine 12 mg kg^{-1}) as previously reported⁷. Ad-Cre, Ad-CC10-Cre, Ad-SPC-Cre and Ad-Flp were purchased from University of Iowa Vector Core Facility. The Mek inhibitor PD-0325901 (Tocris Bioscience) was dissolved in 0.5% hydroxypropyl methyl cellulose, 0.2% Tween-80 water solution and administered by daily gavage. Following the ethical committee guidelines all mice were euthanized when showing respiratory problems.

Histopathology and immunohistochemistry

For routine histological analysis, all lung lobes from each mouse were fixed in 10% buffered formalin (Sigma), embedded in paraffin and evaluated in serial sections by conventional haematoxylin and eosin (H&E) staining according to previously published criteria³¹. All whole mount X-gal-stained sections were counterstained with Nuclear Fast Red. Antibodies used for immunostaining included those raised against: SPC (Millipore, AB3786); CC10 (Santa Cruz Biotechnology, SC-9772); Ttf1 (Epitomics, 2044-1); Sox2 (Cell Signaling Technology, 3728); p63 (Thermo Scientific, MS-1081-P); Ck5 (Covance, PRB-160P) and p-Erk1/2 (Cell Signaling Technology; 9101).

Cell culture

Kras^{+/G12V};Araf^{-/-};Braf^{lox/lox};Craf^{lox/lox};Tip53^{-/-};Tg.hUb-cre-ERT2^{+/T} lung adenocarcinoma cell lines were generated from primary tumours. Cells were infected with lentiviruses expressing Braf, Braf(D631A) and Craf (pLVXpuro) using routine procedures and exposed to 50 MOI (multiplicity of infection) of Ad-Cre. 4-hydroxytamoxifen (4-OHT) (Sigma) was used at 600 nM. Cell lines were confirmed mycoplasma free at every freeze/thaw cycle. Primary keratinocytes were obtained from adult tail skin. The antibodies used for western blotting included those raised against: Araf (Cell Signaling Technology, 4432); Braf (Santa Cruz Biotechnology, SC-5284); Craf (BD Biosciences, 610151); p-Erk1/2 (Cell Signaling Technology, 9101); Erk1 (BD Biosciences, 554100); Erk2 (BD Biosciences, 610103); γ -H2AX (Millipore, 05-636); p-p90Rsk (Cell Signaling Technology, 9341); p90Rsk (Santa Cruz Biotechnology, SC-231); p53 (Cell Signaling Technology, 2524); p19^{ARF} (Abcam, ab80); cleaved caspase-3 (Cell Signaling Technology, 9661) and Gapdh (Sigma, G8795).

Computed tomography

Mice were anaesthetized with 1% to 3% flow of isoflurane/oxygen, and the chest area was visualized with the GE eXplore Locus micro PET-CT scanner (GE Healthcare). The resulting raw data were reconstructed to a final image volume of $875 \times 875 \times 465$ slices at $93 \mu\text{m}^3$ voxel dimensions. Reconstructed slices were output in the manufacturer's raw format

and corrected equal to Hounsfield units and analysed with MicroView analysis software (GE Healthcare).

Digital image quantification

Digital images of immunostained slides were obtained using a whole slide scanner (Dotslide Olympus) with resolution 0.32 μm per pixel (20 $\times$ /NA 0.75). For automated image quantification the tumour areas on scanned H&E sections were manually delimited within the normal lung tissue according to histological criteria and quantified by Dotslide viewer software.

Statistical and data analysis

The product limit method of Kaplan and Meier was used for generating the survival curves, which were compared by using the log-rank (Mantel–Cox) test. A *P* value that was less than 0.05 was considered statistically significant for all datasets. All statistical analysis was performed using GraphPad Prism software. No statistical method was used to predetermine sample size in animal studies. Only mice carrying tumours >1 mm in diameter on CT were enrolled in longitudinal studies assessing tumour volume variation. For all animal experiments block randomization was used to ensure a balance in sample size across groups over time. The investigators were blinded during evaluation of tumour size variations following tamoxifen diet.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

Extended Data

Extended Data Figure 1. Braf(D631A)-dependent activation of MAPK signalling *in vitro*

a, Western blot analysis of the expression levels of the three Raf isoforms in lysates derived from *Kras^{+G12V};Araf^{+/+};Braf^{lox/lox};Craf^{lox/lox};Trp53^{-/-};Tg.hUb-cre-ERT2^{+fl}* lung adenocarcinoma cell lines. The endogenous *Araf* alleles were eliminated using CRISPR–Cas9 editing where indicated. *Braf^{lox}* and *Craf^{lox}* alleles were eliminated by addition of 4-hydroxytamoxifen (4-OHT) to induce their Cre-mediated recombination where indicated. Gapdh is shown as a loading control. **b**, *Kras^{+G12V};A-Raf^{-/-};Braf^{lox/lox};Craf^{lox/lox};Trp53^{-/-};Tg.hUb-cre-ERT2^{+fl}* lung adenocarcinoma cell lines were infected with lentiviral particles expressing combinations of wild-type Braf, kinase-inactive Braf(D631A) and wild-type Craf as indicated before the addition of 4-hydroxytamoxifen to induce elimination of the endogenous conditional *Braf* and *Craf* alleles. Cells were serum-starved for 24 h and re-stimulated with serum for the indicated times. The activation of MAPK signalling was assessed by western blot analysis of p-Erk1/2. Expression levels of the exogenous *Braf* and *Craf* alleles as well as endogenous Erk1/2 and Gapdh are shown as loading controls. For gel source data, see Supplementary Fig. 1.

Extended Data Figure 2. Mouse alleles and strains used in the study

a, Expression of the endogenous *Kras*^{G12V} oncogene is achieved by Cre-mediated excision of the transcriptional *STOP* cassette preceding the *Kras*^{LSLG12Vgeo} allele. Cells expressing the oncogene can be identified by X-gal staining owing to the co-expression of a bicistronic β -galactosidase (β -geo) reporter. Likewise, Cre-mediated excision of the *lox-STOP-lox* cassette (containing the indicated wild-type exons) induces the expression of the kinase-dead Brafl(D631A) and Crafl(D468A) mutants instead of the wild-type isoforms. Finally, Cre-mediated excision of the conditional *Brafl*^{lox} allele results in the elimination of the wild-type Brafl protein. **b**, Schematic representation of the proteins expressed in lung cells following intratracheal infection with Ad-Cre virus in the compound strains used in the study.

Extended Data Figure 3. Activation of p-Erk1/2 in lung adenocarcinomas driven by concomitant expression of Kras(G12V) and Braf(D631A) depends on Crf kinase activity

Representative images of p-Erk1/2 immunostaining of paraffin-embedded lung sections ($n = 3$ per genotype) from Ad-Cre-infected *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB), *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A}; *Craf*^{LSLD468A/LSLD468A} (KBC^{KD}) and *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A}; *Craf*^{lox/lox} (KBC^L). Tumour samples were collected 6 months after Ad-Cre intratracheal infection coincident with the humane end point of the KB strain. Scale bar, 1 mm.

Extended Data Figure 4. MAPK hyperactivation induces transdifferentiation of the bronchiolar epithelium leading to papillary carcinoma

Immunostaining of paraffin-embedded sections showing transdifferentiation of the bronchiolar epithelium at early stages (1 week) after Ad-Cre infection of *Kras*^{+/LSLG12V_{geo}, *Braf*^{lox/SLD631A} (KBL). Images display staining using antibodies against CC10 (club cell marker, left panels) and SPC (AT2 marker, right panels). Protruding bronchiolar papillary growth is invariably associated with a transdifferentiation process illustrated by the acquisition of SPC⁺ staining (red arrowheads). Scale bar, 100 μ m (top panels), 50 μ m (bottom panels).}

Extended Data Figure 5. Expression of an endogenous Braf(D631A) inactive mutant allele in mice harbouring *Kras*^{G12V}-driven lung adenocarcinomas detectable by CT increases tumour growth and reduces survival

a, Survival of *Kras*^{+/FSFG12V}; *Braf*^{+/LSLD631A}; *Tg.hUb-cre-ERT2*^{+/T} (K^FB, solid circles $n = 14$) and control *Kras*^{+/FSFG12V}; *Tg.hUb-cre-ERT2*^{+/T} (K^F, empty circles $n = 13$) mice bearing lung adenocarcinomas detectable by CT. Upon tumour detection mice were maintained on a tamoxifen-containing diet to activate expression of the Braf(D631A) kinase-dead isoform. $P < 0.0058$, obtained using the log-rank test (Mantel–Cox). **b**, Waterfall plot representation of the tumour volume increase (measured as fold change) when re-evaluated by CT after 8 weeks of continuous tamoxifen-containing diet in *Kras*^{+/FSFG12V}; *Braf*^{+/LSLD631A}; *Tg.hUb-cre-ERT2*^{+/T} (K^FB, $n = 29$ tumours from 14 mice) and control *Kras*^{+/FSFG12V}; *Tg.hUb-cre-ERT2*^{+/T} (K^F, $n = 38$ tumours from 15 mice) cohorts. The same dataset is represented in Fig. 4a.

Extended Data Figure 6. Histology of Braf(D631A)-driven lung adenocarcinoma and evaluation of Braf(D631A)-dependent activation of MAPK signalling *in vitro*.

a, Haematoxylin and eosin staining of paraffin-embedded lung sections from Ad-Cre-infected *Braf*^{+/L^{SLD}631A mice killed at humane end point. Sections display moderately to poorly circumscribed tumours occupying nearly an entire lung lobe, with compressed adjacent lung parenchyma and peripherally infiltrated by mononuclear inflammatory cells, mainly macrophages. Tumour masses are composed by papillary outgrowths or less-differentiated areas with increased cellular pleomorphism. Images correspond to three independent mice. Scale bar, 100 μ m. **b**, Schematic representation of BRAF mutations detected in human tumours with high (top) or low/absent (bottom) kinase activity when compared to the wild-type isoform (see also the accompanying manuscript by Yao *et al.*²⁴). **c**, Primary keratinocytes derived from *Braf*^{+/L^{SLD}631A (B), *Braf*^{lox/L^{SLD}631A (BL) and *Braf*^{+/L^{SLD}631A; *Craf*^{lox/lox} (BC) mice were infected with Ad-Cre (5 MOI) and treated with recombinant EGF (150 ng ml⁻¹ for 1 h) where indicated. The activation of MAPK signalling was assessed by western blot analysis of p-Erk1/2, demonstrating that the activation of MAPK signalling by Braf(D631A) in epithelial cells requires upstream RTK activation and is enhanced by elimination of the wild-type Braf allele. Efficient recombination of the Craf conditional alleles in Ad-Cre-infected BC cells is also shown, demonstrating that MAPK activation by Braf(D631A) is Craf-dependent. Expression levels of Erk1/2 and Gapdh are shown as loading controls. For gel source data, see Supplementary Fig. 1.}}}}

Extended Data Figure 7. Co-occurring alterations in lung adenocarcinoma patients with BRAF-inactivating mutations

Whole-genome sequencing data available from the TCGA LUAD study at cBioportal (<http://www.cbioportal.org>) was used to generate a gene network for the analysis of genetic alterations coincident with BRAF hypoactive mutations. PIK3CA mutations (Q296E, E542K and E545K) were the most frequent co-occurring event. In addition, mutations or gene deletions in known RTK signalling antagonists (DOK2, SPRY2), MAPK scaffolds (KSR1), members of the RAS subfamily (RAP1A, RIT1) or the guanine nucleotide exchange factor TRIO might potentially cooperate with inactive BRAF to sustain MAPK activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Combinations of *Kras*^{G12V}, *Braf*^{D631A} and wild-type *Braf* alleles establish a MAPK activity window that determines cell transformation and oncogene toxicity

a, Whole-mount X-gal staining of representative lung sections ($n = 5$ per genotype) from *Kras*^{+/LSLG12Vgeo} (K), *Kras*^{+/LSLG12Vgeo}*Braf*^{+/LSLD631A} (KB) and *Kras*^{+/LSLG12Vgeo}*Braf*^{lox/LSLD631A} (KBL) mice 1 month after Ad-Cre infection. X-gal staining identifies β-galactosidase expression as a surrogate marker for *Kras*^{G12V}-expressing cells. Scale bar, 1mm. Insets show high-magnification images. Scale bar, 100 μm. **b**, Western blot analysis of lung lysates from *Kras*^{+/LSLG12Vgeo} (K), *Kras*^{+/LSLG12Vgeo}*Braf*^{+/LSLD631A} (KB) and *Kras*^{+/LSLG12Vgeo}*Braf*^{lox/LSLD631A} (KBL) mice 1 week after Ad-Cre infection. Migration of p19^{ARF}, p53, γ-H2AX, cleaved caspase-3 (C3A), p-Erk1/2, Erk1/2, p-p90Rsk and p90Rsk is indicated by arrowheads. Gapdh was used as loading control. Lysates from two independent animals per genotype are shown. **c**, Representative immunostaining of paraffin-embedded lung sections ($n = 5$ per genotype) from *Kras*^{+/LSLG12Vgeo} (K), *Kras*^{+/LSLG12Vgeo}*Braf*^{+/LSLD631A} (KB) and *Kras*^{+/LSLG12Vgeo}*Braf*^{lox/LSLD631A} (KBL) mice 1 week after Ad-Cre infection using the indicated antibodies. Scale bar, 50 μm. **d**, Whole-mount X-gal staining of representative lung sections ($n = 3$ per genotype) from *Kras*^{+/LSLG12Vgeo} (K), *Kras*^{+/LSLG12Vgeo}*Braf*^{+/LSLD631A} (KB) and *Kras*^{+/LSLG12Vgeo}*Braf*^{lox/LSLD631A} (KBL) mice 1 month after infection with 10⁸ Ad-Cre particles. During this period, mice were treated with the indicated doses of the MeK inhibitor PD-0325901. The percentage of *Kras*^{G12V}-expressing cells (X-gal-positive area) per lung section is indicated in each panel (top left, shown as mean ± s.d.). Scale bar, 200 μm. Insets

in each panel show high-magnification images (top right; scale bar, 50 μm) or representative images of p-Erk1/2 immunostaining (bottom right; scale bar, 25 μm).

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Figure 2. Mice concomitantly expressing Kras(G12V) and Braf(D631A) show reduced survival and increased tumour development mediated by Craf kinase activity

a, Survival of *Kras*^{+/LSLG12Vgeo} (K, open circles, *n*=14), *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB, solid circles, *n*=22) and *Kras*^{+/LSLG12Vgeo}; *Braf*^{lox/LSLD631A} (KBL, open triangles, *n*=17) mice after Ad-Cre intratracheal infection. *P* < 0.0001, obtained using the log-rank test (Mantel–Cox). **b**, Quantification of average tumour (adenoma and adenocarcinoma) burden (per cent of total lung area) in lung sections from *Kras*^{+/LSLG12Vgeo} (K, *n*=5), *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB, *n*=7) and *Kras*^{+/LSLG12Vgeo}; *Braf*^{lox/LSLD631A} (KBL, *n*=7) mice 6 months after Ad-Cre intratracheal infection. **P < 0.01, *P < 0.05. **c**, Representative haematoxylin and eosin staining of paraffin-embedded lung sections obtained from *Kras*^{+/LSLG12Vgeo} (K, *n*=5), *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB, *n*=7) and *Kras*^{+/LSLG12Vgeo}; *Braf*^{lox/LSLD631A} (KBL, *n*=7) mice 6 months after Ad-Cre infection. Scale bar, 5 mm. **d**, Representative immunostaining of paraffin-embedded sections showing tumours from Ad-Cre-infected *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB, *n*=7) mice killed at humane end point using antibodies against SPC and CC10. Scale bar, 1 mm. **e**, Quantification of average tumour (adenoma and adenocarcinoma) burden (per cent of total lung area) in lung sections from *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB, *n*=7) and *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A}; *Craf*^{+/LSLD468A/LSLD468A} (KBC^{KD}, *n*=5) mice 6 months after Ad-Cre intratracheal infection. **P < 0.01. **f**, Representative haematoxylin and eosin staining of paraffin-embedded lung sections (*n*=5 per genotype) from *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB), and *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A}; *Craf*^{+/LSLD468A/LSLD468A} (KBC^{KD}) mice 6 months after Ad-Cre infection. Scale bar, 5 mm. **g**, Survival of *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A} (KB, solid circles, *n*=22), *Kras*^{+/LSLG12Vgeo}; *Braf*^{+/LSLD631A}; *Craf*^{+/LSLD468A/LSLD468A} (KBC^{KD}, solid triangles, *n*=

12) and $Kras^{+/-LSLG12Vgeo}, Braf^{+/-SLD631A}, Craf^{lox/lox}$ (KBC^L, empty squares, $n = 7$) mice after Ad-Cre intratracheal infection. $P < 0.0001$, obtained using the log-rank test (Mantel–Cox).

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Figure 3. Hyperactive MAPK signalling triggers bronchiolar carcinoma by transdifferentiation of club cells

a, Haematoxylin and eosin staining of paraffin-embedded sections showing the presence of intrabronchiolar carcinoma in Ad-Cre-infected *Kras*^{+/LSLG12Vgeo}, *Braf*^{lox/LSLD631A} (KBL) mice killed at humane end point. Two different magnifications are shown. Scale bar, 200 μ m (top) and 50 μ m (bottom). **b**, Representative immunostaining of paraffin-embedded sections showing intrabronchiolar tumours from Ad-Cre-infected *Kras*^{+/LSLG12Vgeo}, *Braf*^{lox/LSLD631A} (KBL, *n* = 5) mice using antibodies against, SPC, CC10, Ttf1, Sox2, p63 and Ck5. Scale bar, 50 μ m. **c**, Immunostaining of paraffin-embedded consecutive sections showing early intrabronchiolar lesions (1 week after Ad-Cre infection) in *Kras*^{+/LSLG12Vgeo}, *Braf*^{lox/LSLD631A} (KBL) mice using antibodies against SPC and CC10. Arrowheads indicate protruding bronchiolar papillary growth undergoing transdifferentiation. Scale bar, 50 μ m. **d**, Representative haematoxylin and eosin staining of paraffin-embedded lung sections (*n* = 3 per genotype) from *Kras*^{+/LSLG12Vgeo} (K), *Kras*^{+/LSLG12Vgeo}, *Braf*^{+/LSLD631A} (KB) and *Kras*^{+/LSLG12Vgeo}, *Braf*^{lox/LSLD631A} (KBL) mice killed 2 months after infection with cell-type-restricted Ad-Cre viruses that selectively induce Cre-mediated recombination in AT2 (Ad-SPC-Cre) (top) and club cells (Ad-CC10-Cre) (bottom). Scale bar, 500 μ m. Insets show high-magnification images. Scale bar, 100 μ m. **e**, SPC and CC10 immunostaining of paraffin-embedded consecutive sections showing intrabronchiolar tumours from *Kras*^{+/LSLG12Vgeo}, *Braf*^{lox/LSLD631A} (KBL) mice infected with lineage-specific Ad-CC10-Cre. Scale bar, 100 μ m.

Figure 4. Activation of endogenous *Braf*^{D631A} results in lung adenocarcinoma development
a, *Kras*^{+/FSFG12V}; *Brac*^{+/LSLD631A}; *Tg.hUb-cre-ERT2*^{+/T} (K^{FB}) and control *Kras*^{+/FSFG12V}; *Tg.hUb-cre-ERT2*^{+/T} (K^F) mice were infected intratracheally with Ad-Flp and tumour formation was monitored by computed tomography (CT). Mice bearing tumours visible on CT (K^{FB}, *n* = 29 tumours from 14 mice and K^F, *n* = 38 tumours from 15 mice) were fed *ad libitum* a tamoxifen-containing diet resulting in expression of the *Brac*(D631A) kinase-dead isoform in K^{FB}. Tumour volume increase (measured as fold change) was re-evaluated by CT after 8 weeks in continuous diet. **P < 0.01. **b**, Haematoxylin and eosin (H&E) staining, as well as SPC and CC10 immunostaining, of paraffin-embedded lung sections from Ad-Cre-infected *Brac*^{+/LSLD631A} mice killed at humane end-point. Scale bar, 500 μm and 50 μm (top right). **c**, Representative images of p-Erk1/2 immunostaining of paraffin-embedded lung sections (*n* = 3 per genotype) from Ad-Cre-infected *Kras*^{+/LSLG12Vgeo}, *Kras*^{+/LSLG12Vgeo}; *Trp53*^{lox/lox}, *Braf*^{+/LSLV637E} (equivalent to human *BRAF*^{V600E}) or *Brac*^{+/LSLD631A} mice. Scale bar, 100 μm.

Table 1

BRAF hypoactive mutants with NF1 and RAS mutations

Human skin cutaneous melanoma					
Sample	BRAF	NF1	KRAS	NRAS	HRAS
TCGA-EE-A29N-06	D594N	Y80*	K117N	-	-
TCGA-EE-A2GJ-06	G466E	-	-	G12D	-
TCGA-EE-A2GC-06	G466E	Q2239*	-	-	-
TCGA-EE-A19F-06	G466E	X69_splice, L274F	-	-	-
TCGA-EE-A1ZZ-06	N581S	Q684*, S1599F	-	P34L	-
TCGA-EE-A181-06	S467L	Q519*, R440*, L2604F	-	-	-
TCGA-EE-A3JD-06	S467L	P1851S, R1276Q	-	-	-
Human lung adenocarcinoma					
Sample	BRAF	NF1	KRAS	NRAS	HRAS
TCGA-05-5428-01	D594N	-	-	-	-
TCGA-50-5044-01	D594H	-	-	-	-
TCGA-05-4410-01	G466V	-	-	-	-
TCGA-78-7537-01	G466V	-	-	-	-
TCGA-78-7153-01	G466A	-	-	-	-
TCGA-44-6744-01	N581S	-	-	-	-
TCGA-44-2662-01	N581S	-	-	-	-
TCGA-05-4249-01	A762E	-	G12C	-	-
TCGA-55-6979-01	P367R	-	-	-	-

The co-occurrence of BRAF hypoactive mutants with NF1 and RAS mutations in human cancer datasets. Data from skin cutaneous melanoma and lung adenocarcinoma patients obtained from The Cancer Genome Atlas cutaneous melanoma and lung adenocarcinoma studies, respectively. Sample ID as well as the precise mutations identified in these patients are indicated. Asterisks indicate that the mutation creates a *STOP* codon, and therefore a truncated protein. X69_splice represents a mutation affecting the splice site adjacent to amino acid 69.