Ras Effector Mutant Expression Suggest a Negative Regulator Inhibits Lung Tumor Formation

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Abstract

Lung cancer is currently the most deadly malignancy in industrialized countries and accounts for 18% of all cancer-related deaths worldwide. Over 70% of patients with non-small cell lung cancer (NSCLC) are diagnosed at a late stage, with a 5-year survival below 10%. KRAS and the EGFR are frequently mutated in NSCLC and while targeted therapies for patients with EGFR mutations exist, oncogenic KRAS is thus far not druggable. KRAS activates multiple signalling pathways, including the PI3K/Akt pathway, the Raf-Mek-Erk pathway and the RaIGDS/Ral pathway. Lung-specific expression of BrafV600E, the most prevalent BRAF mutation found in human tumors, results in Raf-Mek-Erk pathway activation and in the formation of benign adenomas that undergo widespread senescence in a Cre-activated Braf mouse model (BrafCA). However, oncogenic KRAS expression in mice induces adenocarcinomas, suggesting additional KRAS-activated pathways cooperate with sustained RAF-MEK-ERK signalling to bypass the oncogene-induced senescence proliferation arrest. To determine which KRAS effectors were responsible for tumor progression, we created four effector domain mutants (S35, G37, E38 and C40) in G12V-activated KRAS and expressed these alone or with BrafV600E in mouse lungs... The S35 and E38 mutants bind to Raf proteins but not PI3K or RaIGDS; the G37 mutant binds to RaIGDS and not Raf or PI3K and the C40 mutant is specific to PI3K. We designed lentiviral vectors to code for Cre recombinase along with KRAS mutants (V12, V12/S35, V12/G37, V12/E38 or V12/C40) or EGFP as a negative control. These lentiviruses were used to infect BrafCA and wild-type mice. Surprisingly there was a significant decrease in tumor number and penetrance with each KRAS effector domain mutant relative to controls, suggesting that KRAS directly activates effectors with tumor suppressive functions.

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide [1] and can be categorized into two main histological subtypes: small cell lung cancer and non-small cell lung cancer (NSCLC). The latter can be further divided into three subtypes: large cell carcinoma, squamous cell carcinoma and adenocarcinoma. In never smokers, 62% of lung cancers diagnosed are of the adenocarcinoma subtype, which makes it the most frequent subtype among that group, while it accounts for 19% of smoking-adenocarcinoma subtype, which makes it the most frequent

unfortunately, not only are NSCLC patients with KRAS mutations not responsive to targeted therapies against EGFR or ALK, KRAS mutations are also predictive of a reduced survival under conventional chemotherapy when compared to patients with EGFR mutations [14–16].

The RAS family of genes is composed of three members with high sequence homology: HRAS, NRAS and KRAS, of which there are two alternative splicing isoforms, KRAS 4A and 4B. In lung adenocarcinoma, KRAS is the predominant RAS gene found mutated (20%), whereas HRAS and NRAS are found mutated in other types of epithelial cancers [17]. The protein products of KRAS (KRAS 4A and KRAS 4B) are small GTPases of 21 kDa that serve as molecular switches for signal transduction from the cellular membrane, by alternating between an active, GTP-bound state and an inactive GDP-bound conformation [18,19]. Oncogenic KRAS mutations in humans almost exclusively alter codons 12 (85% of RAS mutations), 13 (14%) or 61 (1.5%) [7] and disrupt the catalytic activity of KRAS, leaving RAS in a permanent active, GTP-bound “active” state [20,21]. GTP-bound RAS proteins adopt a conformation that exposes two regions named switch I (residues 32–40, also known as the effector domain), and the switch II region (residues 60–76) [22]. The switch I and II regions allow Ras to activate downstream signalling by recruiting Ras effector proteins to the plasma membrane. These Ras effector
proteins interact with Ras through one of three types of Ras binding domains (RBD). The first type is found on the phosphatidylinositol 3-kinase (PI3K) p110 subunits (α, β, γ or δ) [23,24]. The second interaction domain is a Raf-type RBD that is found in Raf proteins and also in Tiam1 [25]. The third type of RBD is the RA domain (RasGDS/RAF-6 or Ras-association domain), found in RasGDS family (Ral-guanine nucleotide dissociation stimulator) also known as RasGEFs, Ral-guanine exchange factors) [26,27]. It is widely held that this interaction with effector proteins and/or membrane proximity can alter catalytic activity and/or substrate partner availability thereby facilitating signal transduction. There is a series of Ras效应or domain mutants (RASED mutatants), which allow for the differential activation of downstream signalling pathways [24,28]. For instance, expression of point activated HRASV12 bearing an S35 or E38 mutation preferentially interacts with and activates Raf proteins with minimal recruitment of PI3K and RasGDS members [24,28]. In a similar fashion C40 RASED mutants preferentially activate the PI3K pathway and G37 RASED mutants induce predominantly activate Ras-GDS. The KRAS effector domain mutants have an abrogated binding to certain effectors (S35 and E38 bind to Raf proteins but not RasGDS or PI3K; G37 binds to RasGDS but not Raf or PI3K; C40 binds to PI3K but not Raf and RasGDS) [28–30]. That stated, there are a number of other proteins that interact with RAS’s effector domain in a GTP-dependent manner, which are thus potential Ras effector proteins [23]. The binding of these proteins to different RASED mutants has been assessed allowing the use of RASED mutants to probe RAS signalling further [23].

A number of mouse models exist to explore the role Ras plays in tumor initiation and progression [31,32]. Two genetically engineered mouse (GEM) models have been developed to express physiological levels of KRAsG12D mutants following Cre-mediated recombination: KRaS12L/L mice, where recombination leads to KRasG12D expression [33] and KRasG12D/IK?-IRES-βGal mice, which express KRasG12D following Cre expression [34]. Lung-specific [33] or systemic activation [34] of these alleles leads to the formation of atypical adenomatous hyperplasia and epithelial hyperplasia of the bronchioles as early as 2 weeks post-infection. These lesions progress to adenomas and eventually to adenosarcomas [33,34]. To determine whether Ras-Raf-Mek-Erk signalling was sufficient to initiate this cancer phenotype, the Braf locus was engineered to express constitutively active BrafV600E protein at physiological levels following Cre-mediated recombination in BrafV600E mice [35]. Lung-specific BrafV600E expression causes the formation of atypical adenomatous hyperplasia and multiple tumors with an adenomatous morphology. At the earlier stages, these tumors are phenotypically similar to those observed with the KRasG12D mice [33,35]. Tumors that develop in both models stain negative for the Clara cell marker, CC10. While many of the KRasG12D-driven lung tumors remain adenomas due to a stable proliferative arrest (i.e.: oncogene-induced senescence) [36], adenosarcomas appear as early as 16 weeks post-induction. Strikingly BrafV600E lung adenoma progression to adenosarcomas is a very rare event and has not been observed before 40 weeks post- BrafV600E expression. Much like Kras-induced adenomas [36], these BrafV600E lung adenomas appear to be senescent [35]. Together this suggests that while sustained RAF-MEK-ERK MAPK pathway plays a role as an initiator of disease [35,37,38], additional Ras effector proteins mediate a signal required for lung adenoma progression.

We sought to use genetic complementation in mouse lungs to determine whether an additional Kras effector protein(s) could cooperate with constitutive MAPK pathway activation to bypass oncogene-induced senescence (OIS) and permit tumor progression. Specifically we expressed different G12V-activated KRAS effector domain mutants (KRASV12+, KRASV12/S35, KRASV12/ C37, KRASV12/E38 or KRASV12/C40) alone or in conjunction with induction of BrafV600E expression in BrafCA mice and assessed tumor formation and pathology. To do this we designed a Gateway-compatible lentiviral vector (gLEX-iCL) to express cDNA (here either EGFP or KRAS mutants) along with bicistronic expression of the Cre recombinase (here to permit BrafV600E expression). These lentiviruses were used to infect the lungs of wild-type and BrafCA mice to assess how each effector domain mutant of KRAS cooperates with sustained MAPK pathway activation. Tumor size was significantly elevated in BrafV600E lesions expressing either KRASV12 or KRASV12/C40 suggesting co-activation of the PI3K pathway cooperate to increase tumor growth. Surprisingly we found that, expression of each activated KRASED lead to a substantial decrease in BrafV600E induced tumors relative to the EGFP control suggesting the existence of a KRAS activated negative regulator of tumorigenesis.

Results

Development and titration of lentiviral expression vectors

To efficiently co-express activated KRAS, KRASED and EGFP along with Cre recombinase we engineered a bicistronic lentiviral vector, gLEX-iCL (Figure 1). This second-generation lentiviral vector contains a Gateway selection cassette to facilitate the cloning of cDNAs upstream of an internal ribosome entry sequence (ires), which allows for the translation of the downstream Cre recombinase and luciferase. The latter proteins are encoded as a open reading frame and are separated by a Threase ascina virus-derived 2A peptide allowing for “translational cleavage” [39,40] between the Cre and luciferase proteins [41]. Lentiviral expression vectors containing the KRAS effector domain mutants or EGFP as a control were produced via Gateway recombination. The selective activity of the KRAS effector domain mutants and the Cre recombinase activity were confirmed in vitro (supplemental data, Figure S1, S2, S3).

Lentiviruses, to be used as vectors, are often tiered using clonogenic assays for drug resistance markers, flow cytometric analysis for fluorescent protein markers or with immune-based assays for viral or encoded proteins engineered into the virus. The LEX-iCL viruses encoding KRAS derivatives lack a selectable marker to determine their titre directly, thus an alternate method of lentivirus titration was needed, which would be applicable to all lentiviruses used for mouse infections. As such, we used reverse transcription quantitative PCR (RT-qPCR) to measure the amount of lentiviral vector genomic RNA in different virus productions. To this end we initially focused our efforts on LEX-EGFP-iCL, where we could correlate the number of RNA molecules with the number of infectious units as determined by flow cytometric analysis for EGFP in this vector. The primers used for the RT-qPCR analysis are specific for the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) region. This element is commonly used in lentiviral vectors to enhance expression, thus rendering the RT-qPCR titration method applicable to any lentivirus containing this WPRE region [42,43]. Unconcentrated lentiviral supernatants were divided into 2 aliquots. RNA was extracted from one viral aliquot and the number of virion RNA molecules was determined by RT-qPCR. The titre of lentivirus from the other aliquot was determined by infecting HEK 293T cells and subsequently determining the number of EGFP-positive cells using fluorescence-activated cell
sorting (FACS). The number of virion RNA molecules was strongly correlated ($r^2 = 0.972$) to the percentage of EGFP-positive cells for the five different undiluted lentiviruses of varying sizes (Figure 2A). These findings are consistent with others [42,43] and demonstrate that RT-qPCR of RNA isolated from lentiviral supernatant can be used as a surrogate to measure viral titre.

We additionally determined the feasibility of this approach to quantify concentrated virus. Specifically we generated seven independent lentiviruses preparations of LEX-EGFP-iCL (EGFP-iCL) and using serial dilutions of these concentrated viruses we determined titre with FACS relative to number of RNA molecules following the same qRT-PCR approach. Despite ultracentrifugation and concentration of the virus by over 200-fold we obtained similar results (supplemental data, Figure S4). Hence, the titre of all lentiviruses produced in the same manner can be determined by measuring the number of RNA molecules through RT-qPCR. We noted that the absolute slope of this line was affected by alterations to the protocol used for the production and concentration of lentiviruses. Specifically during the optimization of large-scale virus production and concentration we noted that multiple factors may modify the ratio of infectious to defective virion particles (e.g. virus collection time, pH of media)(data not shown). As such, all large-scale lentiviral production followed the specific protocol described in materials and methods.

**Lentiviral transduction to initiate BrafV600E expression**

To determine whether lentiviruses containing Cre recombinase could be administered efficiently to the lungs of mice, we used intranasal administration of LEX-EGFP-iCL, a virus that encodes both EGFP and Cre recombinase. BrafCAd/+ or their wild type littermates were infected with $10^8$ infectious units (IU) of LEX-EGFP-iCL. The mice were pretreated with sodium caprate prior to the intranasal instillation of the virus to increase infection efficiency as this has been shown to increase the viral transduction through disruption of the tight junctions [44]. Using this method and $10^8$ IUs of LEX-EGFP-iCL virus, we observed tumors formed in each mouse with an average of 33 tumors/per mouse when assessed at 16 weeks post-infection (Table 1). The tumors initiated with lentiviral Cre in BrafCA mice had a papillary adenomatous phenotype indistinguishable from those formed with Adenovirus Cre [35]. These adenomas stain positive for the type II pneumocyte marker surfactant protein C (SP-C) and negative for the Clara cell antigen 10 ([35] and supplemental data, Figure S5). Moreover, we did not detect tumors in any of the wild-type mice infected with LEX-EGFP-iCL (n = 8) demonstrating that lentiviral integration and Cre recombinase activity do not themselves initiate tumor formation.

Using the same viral dose we then infected BrafCAd/+ and wild type littermate mice with lentiviruses additionally encoding KRASV12 or the activated effector domain mutant KRASG12V, KRASG12D, KRASG12E by intranasal instillation. Surprisingly, while each mouse infected with LEX-iCL virus expressing KRASV12 developed tumors by 16 weeks postinfection, there was a 15-fold reduction in tumor number relative to BrafCAd activation alone (Table 1). Additionally, a large variance in tumor formation in LEX-EGFP-iCL mice was apparent with tumor induction...
number ranging from 1 to 79 tumors per animal. We believe that this variation, in part, is due to the intranasal administration of sodium caprate. Caprate at these concentrations is viscous and this pretreatment appeared to render breathing while under sedation more difficult. This irregular breathing made the subsequent administration of the virus variable. Moreover, the large variation and relatively low number of tumors formed with intranasal lentivirus administration led us to explore other means of administering virus. We then compared intranasal versus direct intratracheal administration of dye and found that a much higher proportion of dye enters the lungs when infection intratracheally (not shown). We thus chose to conduct subsequent experiments with intratracheal virus administration.

Intratracheal administration of 10^8 IUs of LEX-EGFP-iCL was sufficient to initiate over 1000 tumors in BrafCA mice as early at the 8- and 16-week time points. In this instance caprate pretreatment caused a transient reduction in breathing rate, which returned to normal prior to lentivirus administration (see methods). Additionally, there was a decreased variability in tumor number by this method. Again the lung sections of BrafCA/+ mice receiving EGFP-

Table 1. Tumor penetrance, expressed in average number of tumors observed per lung ± SEM, using the intranasal instillation technique of BrafCA/+ mice.

| Virus      | 8weeks       | 16weeks      |
|------------|--------------|--------------|
|            | Mice with tumors | Avg. tumors/lung | Mice with tumors | Avg. tumors/lung |
| EGFP       | 3/3          | 1.0          | 5/5            | 33.0±13.2        |
| KRA512     | 5/5          | 1.2±0.2      | 5/5            | 2.2±0.6          |
| KRA512/s53 | 1/3          | 1.0          | 3/4            | 2.7±1.2          |
| KRA512/G37 | 1/3          | 1.0          | 2/3            | 1.0              |
| KRA512/E38 | 1/3          | 1.0          | 3/4            | 2.0±1.0          |
| KRA512/C40 | 2/5          | 1.0          | 3/6            | 1.0              |

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iCL virus contained many well-differentiated adenomas per section (Figure 3). Using EGFP-iCL or similar vectors we reproducibly obtained 1000 lung adenomas in each mouse (Table 2 and not shown).

Expression of KRAS ED inhibits Braf V600E-driven tumor initiation

Having determined that we could routinely infect BrafCA/+ mice and obtain a large number of tumors, we infected BrafCA/+ mice

**Table 2.** Tumor penetrance, expressed in average number of tumors observed per lung ± SEM, in BrafCA/+ mice infected by tracheal intubation with 1–2×10^8 infectious units of indicated lentivirus.

| Virus     | 8weeks | 16weeks |
|-----------|--------|---------|
|           | Mice with tumors | Avg. tumors/lung | Mice with tumors | Avg. tumors/lung |
| EGFP      | 2/2 | >1000 | 4/4 | >1000 |
| KRAS V12  | 1/2 | 2.0 | 1/2 | 1.0 |
| KRAS V12/S35 | 1/2 | 2.0 | 2/2 | 8.0±7.0 |
| KRAS V12/G37 | 0/2 | 0 | 2/3 | 3.5±1.5 |
| KRAS V12/E38 | 2/3 | 2.5±0.5 | 3/3 | 17.0±5.8 |
| KRAS V12/C40 | 1/2 | 2.0 | 2/3 | 2.0 |

Figure 3. KRas expression inhibits tumor formation in after Cre-mediated expression of Braf V600E in Braf CA/+ mice. Sections of lungs from BrafCA/+ mice infected with LEX-iCL lentiviruses expressing EGFP, KRAS V12 or the indicated KRAS V12/ED mutants. Slides were stained with haematoxylin and eosin and are shown at low (upper panels) and high (lower panels) magnification. The box in the upper panels depicts region shown at higher power. All images are representative for each lentiviral construct. Bars, 3 mm.

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with LEX-KRAS\textsuperscript{V12} -iCL. KRAS\textsuperscript{V12} coexpression resulted in a large decrease in tumor number relative to the control EGFP-iCL virus both at high (10\textsuperscript{9} IU, Table 1) and lower viral titres (10\textsuperscript{7} IU, supplementary Table S1). Histological analysis of the tumors (n = 24) induced in each of these conditions revealed that all KRAS\textsuperscript{V12} -induced tumors possessed a papillary adenoma phenotype (Figure 3). Surprisingly, there was a lack of adenocarcinoma in Braf\textsuperscript{V600E} mice induced with lentiviruses expressing KRAS\textsuperscript{V12} and Cre. Presumably the lack of adenocarcinoma is reflective of a low tumor number. Indeed, in Kras\textsuperscript{LSL} mice expressing KRAS\textsuperscript{G12D} only a fraction of tumors progress to adenocarcinoma [45]. To determine the effects of KRAS\textsuperscript{V12} on its own, we infected wild type littermate control mice with lentiviruses at high titre (10\textsuperscript{9} IU) using the tracheal intubation method. We observed a reduced penetrance (2 of 5 mice developed tumors) and these mice developed only 2 and 4 tumors per mouse.

To determine if any of the effector mutants genetically cooperate with Braf\textsuperscript{V600E} to induce adenocarcinoma formation, we used intratracheal administration of lentiviruses to express each of the KRAS\textsuperscript{ED} mutants. In each case KRAS\textsuperscript{ED} expression inhibited tumor formation from concomitant activation of the Braf\textsuperscript{V600E} allele relative to the LEX-EGFP-iCL control virus. Figure 3 shows the difference between lung sections of Braf\textsuperscript{V600E} mice infected with either LEX-EGFP-iCL or LEX-KRAS\textsuperscript{ED} -iCL at 16 weeks post-infection. At 16 weeks post-infection, the lowest number of tumors was observed with KRAS\textsuperscript{V12} (1 tumor in 1 lung out of 2 mice), whereas the KRAS\textsuperscript{S35} and KRAS\textsuperscript{V12/C40} conditions had an average of 8 and 17 tumors per lung, respectively (Table 1). These last two mutants both activate the BRAF-MEK-ERK pathway but not the RalGDS or PI3K/Akt pathways.

We sought to determine if other tumor parameters were altered by the expression of different Ras effector mutants. We assessed the median size of the tumors and the distribution of tumor sizes were compared using a two-tailed Mann-Whitney U test between the LEX-EGFP-iCL negative control and the five conditions where KRAS is present (Figure 4). We found that tumors expressing Braf\textsuperscript{V600E} and either KRAS\textsuperscript{V12} or KRAS\textsuperscript{V12/C40} were significantly larger than those expressing Braf\textsuperscript{V600E} alone or Braf\textsuperscript{V600E} and the other KRAS\textsuperscript{ED} mutants. We additionally assessed proliferation rates of these tumors by immunohistochemical staining for Ki67 (supplemental figure S6). The size and proliferative status of each adenoma were plotted and are shown in Figure 5. A general trend was observed where smaller tumors had a high proliferation rate corresponding to the tumor initiation stage, whereas larger tumors had a lower proliferation rate. As expected, the presence of KRAS\textsuperscript{V12} expression resulted in larger tumors relative to the mice receiving LEX-EGFP-iCL (p = 0.0008). The tumors arising from the KRAS\textsuperscript{S35} -iCL virus were bigger than in the LEX-EGFP-iCL condition (p = 0.0245), yet the median tumor size for KRAS\textsuperscript{S35} tumors was less than two times that of those obtained with control LEX-EGFP-iCL virus, indicating there wasn’t a strong effect on proliferation induced by that mutant. The KRAS\textsuperscript{S35} mutant did not lead to a statistically significant increase in tumor size compared to those seen with EGFP-iCL. Surprisingly, expression of KRAS\textsuperscript{S35}, which selectively activates the MAPK pathway, in the Braf\textsuperscript{V600E} mice led to smaller tumors than with Braf\textsuperscript{V600E} expression alone (i.e., with LEX-EGFP-iCL infection). This is in contrast with the KRAS\textsuperscript{S35} mutant, which is also activates the MAPK pathway, suggesting an excess of MAPK signalling can lead to either more proliferation or a more abrupt OIS response. The difference in phenotype between the KRAS\textsuperscript{S35} and KRAS\textsuperscript{V12} mutants could be caused by other effectors that they selectively bind to, which could shift the balance either way. Expression of Braf\textsuperscript{V600E} along with KRAS\textsuperscript{C40}, which selectively activates the PI3K/AKT pathway, mimicked the expression of KRAS\textsuperscript{V12}. Here the median tumor size caused by the LEXKRAS\textsuperscript{C40} -iCL virus was more than 3 times larger than with LEX-EGFP-iCL (p < 0.0001), although there were fewer tumors (Figure 4).

**Discussion**

Here we describe a genetic strategy to concomitantly activate the MAPK pathway through Cre-dependent expression of Braf\textsuperscript{V600E} along with subgroups of KRAS effectors by simultaneously expressing KRAS\textsuperscript{V12} effector domain mutants. Lentiviruses encoding EGFP and Cre recombinase efficiently induced lung adenomas similar to those observed with adenovirus activation of the Braf\textsuperscript{C4} allele. Co-expression of Braf\textsuperscript{V600E} and activated KRAS significantly reduced tumor formation, with this very low tumor burden precluding our ability to observe progression to the adenocarcinoma stage as would be predicted. It is possible that the elevated expression of KRAS\textsuperscript{V12} coupled with Braf\textsuperscript{V600E} induces senescence at an early stage in tumor development precluding detection.

Sustained elevated Ras-Raf-MEK signalling in human fibroblasts leads to a senescence response with the induction p16INK4A and p21 expression [46–49]. There is ample evidence to support the notion of oncogene and stress induced senescence both in mouse models and in human tumors [50]. For example, in a Kras\textsuperscript{V12}-driven model of lung cancer, the majority of tumors that form are adenomas that express senescent markers along with a low proliferative index. In these mice when adenocarcinomas are observed, they lack these markers [36,49]. Constitutively active Braf\textsuperscript{V600E} expression in melanocytes gives rise to nevi (more commonly known as moles), which are benign lesions that typically display hallmarks of senescence. In humans these lesions often remain dormant for decades but can progress to malignant melanoma [51–53]. This ability of Raf proteins to induce a growth arrest in vitro and in vivo is dependent on their expression levels, where lower expression levels can induce proliferation and high levels leads to rapid cell cycle arrest [46,54] [54]. The correlation between OIS and expression level can additionally be inferred

**Figure 4. Median tumor size.** Tumor size was measured by section area at 16 weeks post-infection along with Cre-mediated activation of the Braf\textsuperscript{C4} allele. Distributions of tumor size for all the KRAS mutants were compared with the LEX-EGFP-iCL negative control with the Mann-Whitney U test (**p < 0.005; ***p < 0.001; ****p < 0.0001). Note median tumor size was significantly larger in KRAS\textsuperscript{V12} and KRAS\textsuperscript{V12/C40} expressors.

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from studying human Spitz nevi, melanocytic lesions, which possess amplification of an activated HRAS allele (most commonly a G12V allele). These lesions have elevated HRAS yet rarely progress to full malignancy [55]. Taken together these data demonstrate that elevated levels of Ras/Raf signalling are linked to senescence induction.

Recent studies have demonstrated an approximately 10-fold higher tumor burden with BrafV600E expression in mouse lung compared to KrasG12D, while adenocarcinoma formation was exclusively observed with KrasG12D but not BrafV600E expression [56,57]. This suggests one or more of the following may be true: that BrafV600E is less efficient at inducing senescence than is KrasG12D, that BrafV600E is a more efficient tumor initiator, or that there are fewer cells infected by adenovirus Cre that express Kras when compared to those expressing Braf. Here we have forced KRASV12 expression thus it is likely that the reduced tumor number observed is a consequence of increased engagement of senescence.

**KRAS effector domain mutants**

Of the four KRAS effector domain mutants, KRASV12/C40 produced the largest increase in tumor size in BrafCA/+ mice, relative to BrafV600E expression alone. The C40 mutant is the only mutant capable of interacting with the p110x subunit of PI3K [23]. It additionally interacts with Tiam1, RASSF4 and RIN3, as do the S35, G37 and E38 mutants [23,25]. As the PI3K pathway activation is specific to the C40 mutant, it suggests that this pathway cooperates with constitutive MAPK signalling to increase tumor growth. Of the PI3K genes, only p110a and p110c are activated by Kras, whereas p110b is activated by Kras and TC21. The p110b isoform activation occurs in cells infected with adenovirus Cre that express Kras when compared to those expressing Braf. Here we have forced KRASV12 expression thus it is likely that the reduced tumor number observed is a consequence of increased engagement of senescence.

The PIK3CA gene, coding for the p110a subunit of PI3K, is mutated in 2% of lung adenocarcinomas [7], which suggests that this isoform might be a key interactor of KRASV12/C40 that promoted tumor growth. It will be of interest to formally test the cooperation between activated alleles of PIK3CA and Braf using a newly engineered PIK3CA GEM [58].

Both the S35 and E38 KRASED mutants activate MAPK signalling while failing to interact with RalGDS or PI3K yet differences in the phenotypes were observed. Expression of the KRASV12/S35 or KRASV12/E38 mutants permitted the formation of more tumors than the KRASV12, KRASV12/G37 and KRASV12/C40 mutants when coexpressed with BrafV600E, yet far fewer than with BrafV600E expression alone. The combined expression of BrafV600E and KRASV12/E38 resulted in smaller tumors than BrafV600E expression alone. In fibroblasts, the levels of activity of Raf-1 determine whether cells enter a proliferative state or a growth state [46]. Moreover the different Raf proteins (Araf, Braf, and Raf-1) display different kinase activities and the levels of induction of a growth arrest correlates with these levels [46]. It is not known if KRASV12/S35 or KRASV12/E38 display different affinities for the three Raf proteins. If so, it can be hypothesized that this differential affinity could result in a premature or delayed senescence response. Another possibility comes from previous protein pull-down experiments, in which the E38 mutant seems to have a greater affinity for RIN and RASSF1 compared to the S35 mutant [23]. RASSF1 is a tumor suppressor that is often silenced in NSCLC [59]. Also, two studies have shown that the S35 mutant, and not E38, is able to bind weakly to RalGDS and RGL, another Raf GEF, although it is still unclear if this is sufficient to cause a physiologically relevant increase in Raf signalling [23,24].
All of the above suggests that, even though S35 and E38 mutants of KRAS have been used in multiple studies as specific activators of MAPK signalling, they may not have the same functional properties and thus should not be considered as genetic equivalents.

Our data makes it difficult to conclude if RalGDS signalling has a positive effect on NSCLC progression. KRASV12/E38 interacts with more RA domain-containing proteins (including RIN, RIN2, Nore1, RASSF1, RGL, RGL2) than any other effector mutant. Also, as the RASSF family of tumor suppressor proteins all possess an RA domain, it is possible that the G37 mutant, by having a higher affinity for the RA domain than the S35, E38 and C40 mutants, activated a RASSF-dependent tumor suppressive response that counteracted oncogenic Ras signalling [23,60].

Oncogene-induced senescence blocking tumor initiation

Our results indicate that lentiviral driven KRASV12 expression concomitant with BrafV600E expression reduces tumor number by over 100-fold compared to BrafV600E expression alone. The most straightforward explanation would be that increased signalling output from KRASV12 lead cells into an oncogene-induced senescence (OIS) response. This notion becomes difficult to reconcile with the observations that decreased tumor formation occurred when each of the KRASED mutants were coexpressed along with the BrafV600E. There is the possibility that the activation of single pathways downstream of KRAS with each effector domain mutant expressed is sufficient to induce OIS. However, this model would fail to explain why the KRASV12/S35 or KRASV12/E38 mutants, which activate the RAF-MEK-ERK MAPK pathway, fail to produce tumors when expressed alone in wild-type mice but do give rise to tumors when expressed concomitantly with an activated BrafV600E allele. This would be predicted to further increase the MAPK oncogenic signalling output and to elicit a stronger OIS response. None of the KRAS effector domain mutants were capable of causing tumor formation on their own, and KRASV12 expression led to tumor formation in only two wild-type mice out of eight. It is thus improbable that increased downstream oncogenic signalling is the only cause of the abrogation of tumor formation and suggests an additional mechanism to explain this tumor suppression exists.

Direct activation of a negative regulator by KRAS

The RASSF family of proteins are direct Ras effectors that act as tumor suppressors and can antagonize the antiapoptotic and pro-proliferative effects of Ras. They are frequently found transcriptionally silenced early in the development of various cancers [60]. There are 10 members of the RASSF family proteins in mammals. RASSF1–6 possess a C-terminal Ras association (RA) domain, whereas RASSF7–10 possess an N-terminal RA domain [60,61]. RASSF1–6 proteins also share a Sav-RASSF-Hpo (SARAH) domain, which interacts with the proapoptotic MST1/2 kinases [60]. There are several lines of evidence that points to a role of RASSF proteins mediating negative signal downstream of Ras.

Drosophila possesses a single RASSF ortholog, dRASSF, which shares a C-terminal RA domain and a SARAH domain like its mammalian RASSF1–6 counterparts. Through the SARAH domain Drosophila dRASSF physically interacts with Hippo (Hpo), which is homologous to mammalian MST1/2. Hpo is a central protein kinase of the Salvador/Warts/Hippo pathway controlling organ size in animals through regulation of cell proliferation and apoptosis [62]. Hpo and MST1/2 kinases are negative regulators of the Drosophila Yorkie or mammalian YAP/TAZ proteins respectively, which function to activate transcription of anti-apoptotic and pro-proliferative genes. Hpo loss in Drosophila results in tissue overgrowth [62], which phenocopies elevated Yorkie expression [62,63]. Similarly, mouse Mst1/2 deficiency in the liver results in the loss of inhibition of Yap1, massive liver overgrowth and hepatocellular carcinoma formation [64]. Although biochemical evidence in Drosophila points to an inactivation of the Hpo kinase by dRASSF, concomitant loss of Hpo and dRASSF enhances the tissue overgrowth phenotype observed with Hpo loss alone, suggesting a tumor suppressor function for dRASSF that is independent of its interaction with Hpo [65]. Moreover loss of Ras1 in Drosophila reduces cell growth and increases apoptosis [65,66], while ommatidia-specific loss of function dRASSF alleles rescue the growth defects observed with mutant Ras1 alone by increasing proliferation and decreasing apoptosis [65]. Together, this suggests that dRASSF is a genetic antagonist of Ras signalling in Drosophila.

In humans, RASSF1, RASSF3, RASSF4 and RASSF5 each are implicated in mediating a negative signal to repress proliferation and/or survival. Of these, RASSF1A has received the most attention. Its promoter is found methylated and inactivated in a variety of cancers and in particular in 33% of NSCLC. This is associated with poor overall relapse-free survival. Moreover, RASSF1A has the properties of a tumor suppressor gene [59,67], RASSF1A-specific knockout mice, while fully viable and fertile, display increased spontaneous- and carcinogen- induced tumor susceptibilities, particularly in the lung [68,69]. Moreover, Raf1 inhibits MST2 activity thus preventing apoptosis [70]. Expression of RASSF1A can disrupt MST2-Raf1 association thereby relieving this Mst2 inhibition and inducing apoptosis [71]. Additionally RASSF4 is located in a region with frequent LOHs observed in prostate cancer [72] and it is mutated in lung and breast cancers [73]. When tethered to the plasma membrane via a CAAX membrane localization sequence, RASSF4 induces apoptosis [73]. RASSF3 is a Ras-dependent proapoptotic protein [74] that is frequently found transcriptionally silenced in many cancers including in 24% of NSCLC [67,75,76] and has been identified in a screen for genes that suppress MMTV-Neu-driven breast cancer [77]. Finally, RASSF3 can bind MST1/2 via its SARAH domain [74], which is also used by other RASSF proteins (RASSF1A, RASSF2, RASSF5) to induce apoptosis [78,79]. Taken together these data suggest that at least four RASSF family members can function as negative regulators of tumorigenesis.

While the methylation and mutational status the other RASSF family members is as yet unknown it is interesting to note that RASSF7, RASSF8 and RASSF10 genes are in close proximity with the HRAS, KRAS and RRAS loci respectively, suggesting a potential common evolution with Ras GTPases [61]. It should be noted that while binding to KRASED mutants has not been evaluated for all the RASSSF members, RASSF4 binds all the KRASED Mutants (S35, G37, E38, C40) [23]. If other RASSF proteins display similar binding this would provide an explanation for the decreased tumor reduction phenotype observed in each condition where KRASV12 alleles is present. Taken with the above data, one can speculate that the decrease tumor formation we see with each KRASED mutant could be due to recruitment of one or more RASSF-members to the activated KRAS alleles. Perhaps this was a consequence of the levels of KRASED mutant expression obtained from lentiviral expression.

Further studies can be done to test this notion. A cleverly conceived transgenic mouse (called RasE Multi-Hit) has been recently developed to study which pathways may cooperate to induce HRAS-driven cancer [80]. This allele codes for three effector domain mutants (HRASV12/S35, HRASV12/G37, HRASV12/C40) each of which may be expressed at physiological
levels upon Cre mediated recombination in a stochastic manner [90]. This approach allows for the expression of one, two or three HRas<sup>V12/13D</sup> mutants. The majority of lung tumors formed after Cre activation expressed all three alleles thus simultaneously activating the MAPK, PI3K and RalGDS pathways. Interestingly, no tumors were found coexpressing the HRas<sup>V12/G37</sup> and HRas<sup>V12/C40</sup> in the absence of HRas<sup>V12/S35</sup>, even though individually the G37 and C40 alleles were found in 0.6% and 6% of the tumors formed respectively. This is another indication supporting the hypothesis that the sum of the signalling pathways activated by Ras leads to a direct increase in both pro-proliferative and tumor suppressive functions.

**Materials and Methods**

**Ethics Statement**

Administration of lentiviral vectors and subsequent euthanasia was performed under 2,2,2 Tribromoethanol anaesthesia. All efforts were made to minimize suffering throughout the course of these studies. All mouse experiments were carried out in strict accordance with the recommendations in the Canadian Council on Animal Care (CCAC) “Guide to the Care and Use of Experimental Animals” and under the conditions and procedures approved by the Animal Care Committee of McGill University (AUP number: 5819).

**Cloning and mutagenesis**

Human KRAS 4B cDNA was PCR amplified from a cDNA provided by Pablo Rodriguez-Viciana (University College of London) using forward primer 5’ CACCCGGATCCCATGGACTGTAATATAA-CTTTGG and reverse primer 5’ ACATTAGATCCCTATTTGTTGAATC-TCCTCTATTG with the Phusion polymerase (Thermo scientific), as per the manufacturer’s instructions. The PCR product was cloned into pENTR-d-TOPO (Invitrogen) following manufacturers instructions.

Mutagenesis of the KRAS cDNA was performed using overlapping forward and reverse primers that included the desired mutation, using the Phusion DNA polymerase (Thermo scientific). The following primers pairs were used for mutagenesis to create the G12V (GV0004 fwd, GGTAGTTGGAGCCGCTTGCAAGACCTAAACAT, GV0005 rev, CTGGCTCTGCTGCTGCAAGATCTCAATTCACATTTTG-TCTTGGT) using the Phusion polymerase (Thermo scientific), as per the manufacturer’s instructions. The PCR product was cloned into pENTR-d-TOPO (Invitrogen) following manufacturers instructions.

**Lentivirus Production and Purification**

Lentivirus was produced in HEK 293T cells by co-transfection using a Polyelectrolycine (P.E.L.) solution at a 2:65:1 ratio (P.E.L. mass:DNA mass). Specifically ten 175 cm<sup>2</sup> tissue culture-treated flasks were seeded with 8x10<sup>6</sup> HEK 293T cells and were transfected the following day with psPAX2 packaging plasmid (11.7 µg), pMD2.G envelope plasmid containing VSV-G (6.3 µg), the recombinant lentiviral plasmid (18 µg) and 95.9 µg P.E.L. per plate as described [82]. 16 hours post-transfection the media was changed to 20 ml DMEM containing 10% FBS and 50 mM HEPES pH 7.3, 24 hr later the supernatant was collected, filtered through 0.22 µm filter and concentrated by ultracentrifugation through a 20% sucrose cushion for 2 hr at 4°C using a Beckman Coulter SW32 Ti rotor at 29,500 RPM [82,000 x g] [83]. Lentiviral pellets were suspended in 600 ul 1 x PBS at 4°C for two hours with occasional gentle vortexing. Concentrated virus was aliquoted and immediately stored at −80°C.

**Lentivirus Titration - Flow Cytometry**

Flow cytometry was used to titre the EGFP-iCL virus and the infectious titre obtained was correlated with the RT-qPCR data obtained from the same viral preparations. 6-well dishes were seeded with 1x10<sup>5</sup> 293T cells per well. The following day, cells were infected with 10 or 100 µl of 1:10 and 1:100 dilutions of the virus in individual wells in the presence 4 µg/ml of polybrene. The following day, the medium was changed and the proportion of EGFP-positive cells was determined by standard flow cytometry analysis 72 hours post-infection using a Becton Dickinson FACScan with the 530/ 30 filter. The infectious titre of each virus dilution could be calculated as (titre = %EGFP+ cells x number of infected cells x dilution factor)/volume of virus tested.

**Lentivirus Titration - RT-qPCR**

RNA was extracted from 1:10 and 1:100 dilutions of viruses using an RNaseasy kit (QIAGEN) according to the manufacturer’s instructions. Potential DNA carryover was removed with a 15 minute treatment with DNase1 (Invitrogen). cDNA was generated using the GoScript Reverse Transcription kit (Promega) according to the manufacturer’s
instructions, except for the use of a specific oligonucleotide (5’-GCAGAGTCCAGTGGCACA) to prime cDNA synthesis to replace poly d(T) or random primers.

cDNA concentration was determined through quantitative PCR (qPCR). A standard curve was produced each time using 10 fold dilutions spanning 10 ng/μl to 10 fg/μl of pLEX-EGFP-iCL plasmid DNA as an internal control. The primers and probe used were 5’-CCCTTTCCGGGACCTTGGCTTT (Gy0022 fwd), 5’-GCCAGATCCAGTGGCAACA (Gy0022 rev) and 5’-6-FAM/ACTTCATGCCGGCATTGGGC/IABkFQ/-3’ (probe), as described previously [42].

Lentiviral infections of mouse lungs

Strains and genotyping. The BrafCA mouse strain, described previously [35], was backcrossed 7 times in a C57BL/6 background. Matings were set up to obtain both BrafCA+/+ and wild-type mice in a 1:1 ratio for the different experiments. Genomic DNA for PCR was extracted as described [84] and the BrafCA allele was identified by PCR as described [35].

Anaesthetic preparation. A 1 g/ml solution of Avertin (2,2,2-tribromoethanol, Sigma) was made in 2-methyl-2-butanol. This solution was then diluted to 25 mg/ml with a sterile solution of 1 mM Tris-HCl pH 7.4, 250 mM EDTA and 137 mM NaCl and subsequently 0.22 μm filtered prior to use. While use of isoflurane would be preferred, we were unable to obtain sufficient anaesthesia to routinely administer lentiviruses.

Mouse lung infections. The mice used for the lung infections were all female FVB/Nj mice. Mice were anesthetized by intraperitoneal injection of Avertin at a dose corresponding to 0.5 mg of Avertin per gram of mouse weight. 25 μl of 40 mM sodium caprate (Sigma) in 1 PBS was administered either by intranasal instillation or by using an I.V. catheter (Becton Dickinson, cat. #381223), followed by 62.5 μl of lentivirus (2×10−9−108 IU [intranasal]; 106 IU [tracheal instillation]) ten minutes later. During the procedure and up until recovery, the mice were kept on a 37°C pad to prevent hypothermia. Mice were euthanized at 18 to 19 weeks post-infection to harvest the lungs for analysis.

Histology analysis

Tissue processing. Lungs were perfused with 1×PBS, fixed at 4°C in zinc formalin (Sigma) for 24 hours and subsequently embedded in paraffin. The paraffin blocks were cut in 5 μm sections by step-sectioning at 200 μm intervals.

Histopathological analysis. Lung sections were stained using a standard haematoxylin & cosin (H&E) staining protocol. For the Ki67 immunohistochemistry, antigen retrieval step was initiated with either adenovirus or lentivirus stain negative for SPC.

For the KI67 immunohistochemistry, antigen retrieval step was performed using a standard haematoxylin & eosin (H&E) staining protocol. Endogenous peroxidises were blocked with a 20 minute incubation in 3% H2O2. Slides were washed once with distilled H2O2, twice in 1×PBS for and then blocked with 2% BSA/1×PBS for 1 hr. Sections were incubated for 45 minutes at room temperature with a biotin-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch #711-065-152) diluted to 1:500 in the 2% BSA/1×PBS solution. The slides were then washed in distilled H2O2 and then twice with TBST prior to the application of the biotin-streptavidin ABC complex (Vectorstain #PK-4000) according to the manufacturer’s instructions. The slides were washed three times in PBS and the staining was revealed using DAB (Invitrogen #750118). The slides were thereafter counterstained with haematoxylin as described [35].

Slides stained with H&E and Ki67 were scanned using an Aperio Scanscope AT. Individual slides were analyzed using the Aperio ImageScope software, in which each tumor was circumscribed to obtain the section area (μm2) and the percentage of Ki67-positive cells was obtained using the IHC Nuclear Algorithm.

Supporting information

Figure S1 Infection of 293T L9.2 Cre reporter cells that express dsRed upon Cre expression. In each condition, 2.5×105 cells were infected with 6.9×106 IU the same viruses that were used to infect mice. (TIF)

Figure S2 Focus formation in 3T3 cells stably expressing the different KRAS effector domain mutants. 1×104 cells of each stable cell line were seeded with 2.5×106 of the parental 3T3 C5 cell line and left at confluency for 14 days. In the KRASV12 condition, 1×103 cells were seeded instead of 1×104 cells to be able to count single foci and the number was normalized thereafter. Error bars: standard error of the means (SEM). (TIF)

Figure S3 Colony formation in soft agar of 3T3 cell lines expressing the KRAS effector domain mutants. In each well, 1×103 cells were seeded in DMEM with 0.35% low melting point agarose. After 14 days, the number of colonies was stained with MITT and counted. Error bars: SEM. (TIF)

Figure S4 Determining titre of concentrate lentivirus. A Schematic representation of pLEX-EGFP-ires Cre(2a)Luc (pLEX-EGFP-iCL), a gLEXiCL-derived that expresses eGFP transcriptonally upstream of theCre(2a)Luc fusion. Indicated is the location of the PCR primers used to quantify lentiviral RNA-derived molecules. B Seven independent LEX-EGFP-iCL lentiviral preparations were generated and were concentrated, with a small aliquot of preps 1−5 being frozen directly. These viral preps were split, diluted and used to infect 293T cells or to isolate RNA for RT PCR analysis. The graph represents correlation between infectious units, as judged by GFP positivity with FACS analysis, and RNA molecules, by RT PCR. Asterisks indicate the values obtained for undiluted virus and are color-coded with the corresponding prep the figure legend. Preps 6 and 7 were not tested as undiluted viruses. The dotted line indicates the line of best fit the correlation coefficient of r = 0.93. (TIF)

Figure S5 Lentiviral and Adenoviral activation of BrafCA produce similar tumors. A Braf+/− or B) BrafCA/− mice were infected with 106 IU of pLEX-EGFP-iCL lentivirus and lung tissue was obtained 16 weeks postinfection. The tissue of was analysed by H&E and Ki67 stained lung tissues at low (upper) and high (lower) magnification. Aperio
software was used to quantify the percentage of Ki67 positive nuclei. Analysis was focused on individual tumours, which were manually circled (6 tumors are shown circled in green as an example).

(TIF)

Methods S1

Table S1

References

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