Reduced HRASG12V-Driven Tumorigenesis of Cell Lines Expressing KRASC118S

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Abstract

In many different human cancers, one of the HRAS, NRAS, or KRAS genes in the RAS family of small GTPases acquires an oncogenic mutation that renders the encoded protein constitutively GTP-bound and thereby active, which is well established to promote tumorigenesis. In addition to oncogenic mutations, accumulating evidence suggests that the wild-type isoforms may also be activated and contribute to oncogenic RAS-driven tumorigenesis. In this regard, redox-dependent reactions with cysteine 118 (C118) have been found to promote activation of wild-type HRAS and NRAS. We sought to determine if this residue is also important for the activation of wild-type KRAS and promotion of tumorigenesis. Thus, we mutated C118 to serine (C118S) in wild-type KRAS to block redox-dependent reactions at this site. We now report that this mutation reduced the level of GTP-bound KRAS and impaired RAS signaling stimulated by the growth factor EGF. With regards to tumorigenesis, we also report that oncogenic HRAS-transformed human cells in which endogenous KRAS was knocked down and replaced with KRASC118S exhibited reduced xenograft tumor growth, as did oncogenic HRAS-transformed KrasC118S/C118S murine cells in which the C118S mutation was knocked into the endogenous Kras gene. Taken together, these data suggest a role for redox-dependent activation of wild-type KRAS through C118 in oncogenic HRAS-driven tumorigenesis.

Introduction

The RAS family of small GTPases is comprised of three genes in humans, HRAS, NRAS and KRAS that encode the proteins HRAS, NRAS, KRAS4A and KRAS4B. Activation of growth factor receptors as well as other receptors recruits Guanine nucleotide Exchange Factors (GEFs), which stimulate the exchange of GDP on RAS for GTP, rendering the protein active. In this active state, RAS recruits and activates RAF kinases, PI3 kinases (PI3K), RalGEFs, and other proteins that are well known to mediate a host of cellular phenotypes, including cell proliferation and survival. RAS is, in turn, returned to the inactive GDP-bound state through association with GTPase Activating Proteins (GAPs) [1,2].
In up to a third of all human cancers, one of the three RAS genes harbor a mutation, typically at G12, G13 or Q61, that leads to chronic GTP binding and, correspondingly, oncogenic activation of the protein [3]. A wealth of studies demonstrate that expression of any of the RAS proteins containing an oncogenic mutation can impart transformed and tumorigenic phenotypes to cells, and when oncogenic mutations are engineered into endogenous Ras genes in mice, such changes can induce tumorigenesis [4].

In addition to the oncogenic RAS protein, accumulating evidence supports both tumor-suppressive and tumor-promoting roles for the remaining wild-type RAS proteins in cancer. With regards to tumor-suppressive roles, loss of one or both of any of the wild-type Ras alleles increases the sensitivity of mice to the carcinogen urethane, which induces lung adenomas with an oncogenic mutation in Kras [5,6]. With regards to tumor-promoting roles, loss of Hras or Nras renders mice more resistant to DMBA and TPA treatment, which induces skin papillomas with an oncogenic mutation in Hras [5]. In cultured cells, wild-type HRAS, NRAS or KRAS have been shown to promote proliferation of oncogenic RAS-driven cancer cell lines by mediating EGF signaling [7]. Knocking down Hras also sensitizes oncogenic Kras-transformed murine cells to DNA damaging chemotherapeutics [8]. Finally, wild-type KRAS has been shown to inhibit apoptosis induced by oncogenic KRAS [9].

We previously found that oncogenic KRAS stimulates the PI3K-AKT pathway [4], and activated AKT can phosphorylate S1177 of endothelial nitric oxide synthase (eNOS), activating the enzyme to produce nitric oxide (NO) [10–12]. NO as well as other free radical oxidants have been shown to facilitate S-nitrosylation or S-glutathiolation of wild-type HRAS in a manner dependent upon the thiol residue of C118, and further, such alterations activate HRAS [13–15]. Redox-dependent reactions on C118 of wild-type HRAS or NRAS can promote oncogenic KRAS-driven tumorigenesis. In more detail, substitution of C118 for serine (C118S), a very minor modification in which the thiol residue of this cysteine is replaced with a hydroxyl group, renders HRAS completely insensitive to activation by free radical oxidants, with no measurable effect on the protein structure, GTPase activity, intrinsic and GEF-mediated guanine nucleotide dissociation rate, or the ability to bind an effector [13,15–22]. Taking advantage of this very specific separation-of-function mutation to specifically block redox-dependent reactions on C118 of RAS, we previously demonstrated that knocking down wild-type HRAS or NRAS by shRNA reduced oncogenic KRAS-driven tumorigenesis in some cell lines, and that this effect was rescued by re-expressing wild-type HRAS or NRAS, but not the C118S mutant variants [23].

While blocking redox-dependent reactions on C118 of wild-type HRAS and NRAS can inhibit oncogenic KRAS-driven tumorigenesis, it was not known if the C118S mutation had the same effect on wild-type KRAS. This cysteine is conserved between HRAS, NRAS and KRAS and resides in a rather well conserved region (80% homology between amino acids 101–140 in the three RAS proteins). As such, it stands to reason that it may similarly function in an analogous fashion in KRAS. Thus, we tested whether introducing the C118S mutation into wild-type KRAS reduced the amount of GTP-bound KRAS and inhibited oncogenic HRAS-driven tumorigenesis.

Materials and Methods

Plasmids

pBabePuro, pBabeNeo-SV40-T/t-Ag (encoding the early region of SV40), pBabePuro-Flag-HRAS<sub>G12V</sub>, pBabeBleo-p110-CAAX, pBabeBleo-eNOS<sub>S1177D</sub>-HA and pBabeBleo-eNOS<sub>S1177A</sub>-HA were previously described [23,24]. pSuperRetroGFP/Neo-KRAS-shRNA was designed with the targeting sequence GTTGGAGCTGGTGGCGTAG. pSuperRetroGFP/Neo-scramble...
shRNA was designed with the sequence GATTTGGGAATCTTATAAGTTCCCTATCAGTGATAGAGATGGTCAGCGCACTCTTGCCTTTTTA. pBabe Hygro-Flag-KRAS\(^{C118S}\), KRAS\(^{C118S}\) and KRAS\(^{G12V,C118S}\) were created by introducing C118S and/or G12V mutations by site-directed mutagenesis into the previously described pBabeHygro-Flag-KRAS [23] and KRAS\(^{+}\) [25] vectors, which encode shRNA-resistant and N-terminal Flag epitope-tagged human KRAS4B cDNA, either comprised of the wild-type sequence (KRAS) or one in which a number of rare codons were converted to common codons to increase protein expression (KRAS\(^{+}\)).

**Cell lines**

HEK-TtH cells (primary human embryonic kidney cells transduced with vectors encoding the early region of SV40 and hTERT) were previously described [26]. Mouse embryonic fibroblasts (MEFs) were prepared using standard approaches [27] from embryos isolated from Kras\(^{C118S/+}\) females bred with Kras\(^{C118S/+}\) males. Each primary MEF line was immortalized by stably infection [24] with a retrovirus derived from pBabeNeo-SV40-T/t-Ag. Genotypes of resultant MEF cell lines were determined by PCR with the primer pair P7+P8 (see S1 Table) that distinguishes the wild-type and C118S Kras alleles by amplification of a 621 bp versus a 517 bp product, respectively, as previously described [28]. HEK-TtH cells or SV40-immortalized Kras\(^{+/+}\) or Kras\(^{C118S/C118S}\) MEFs were stably infected [24] with retroviruses derived from vectors encoding no transgene as a control or encoding the indicated transgenes or shRNAs.

**Immunoblot analysis**

The indicated cells were lysed with RIPA buffer (1% NP-40, 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol and 2mM EDTA) and protein concentrations determined by Bradford assay (Bio-Rad). Equal amount of protein lysates (50 μg) were resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with primary antibodies anti-Kras F234 (Santa Cruz sc-30, diluted 1:200), anti-Flag M2 (Sigma F1804, diluted 1:1000), anti-HA (Covance, diluted 1:1000), anti-β-actin (Sigma A2228, diluted 1:10000), anti-β-tubulin (Sigma T5201, diluted 1:2000), anti-Erk1/2 (Santa Cruz sc-94, diluted 1:2000), anti-P(Thr 202/Tyr 204)-ERK1/2 (Santa Cruz sc-7383, diluted 1:500), anti-AKT (Cell Signaling, diluted 1:1000) or anti-P(Thr 308)-AKT (Cell Signaling, diluted 1:200), followed by incubation with either goat anti-rabbit (Santa Cruz sc-2004, diluted 1:5000) or anti-mouse (Invitrogen G21040, diluted 1:10000) IgG-HRP (Horseradish peroxidase) conjugated secondary antibodies and detected by ECL (GE healthcare). Bands of immunoblot were quantified using Image J software. Full length blots are shown in S1 Fig.

**Ras-GTP analysis**

Cell lysates were prepared and protein concentrations determined as above from the indicated cells cultured over night in medium supplemented with 0.5% fetal bovine serum. Equal amount (0.5–2 mg) of lysates were incubated with recombinant glutathione S-transferase protein fused with the Ras-binding domain of Raf (GST-RBD) bound to glutathione agarose beads (GE healthcare) and rotated for 45 minutes at 4°C as previously described [29]. The beads were washed with 0.5 ml of RIPA buffer three times for 10 minutes each at 4°C, boiled in sample buffer, resolved using SDS-PAGE and immunoblotted with anti-Kras or anti-Flag antibodies, as described above. Full length blots are shown in S1 Fig.
RT-PCR analysis

RNA was extracted from cells with the RNA-BEE reagent according to the manufacturer’s protocol (Fisher Scientific). 0.5–2.0 μg of RNA was reverse transcribed using Omniscript RT kit (QIAGEN) with an Oligo dT (QIAGEN) primer. Resultant cDNA was used as a template to amplify targets of interest using the primers (P1-P6) listed in S1 Table. Full length gels are shown in S1 Fig.

Tumor xenograft analysis

All animal experiments were approved by an Institutional Animal Care and Use Committee at Duke University. 5x10⁶ of the indicated HEK-TtH-derived cell lines or 1x10⁶ of the indicated SV40-immortalized MEF-derived cell lines were mixed with 500 μl Matrigel (BD Biosiences) and injected subcutaneously into each flank of five, 8-week-old female immunocompromised SCID-Bg mice (Charles River). Seven days later, the length (L) and width (W) of tumors were measured with a caliper three times a week. Tumor volumes were calculated by 0.5L²W². When the average tumor volume in one mouse in a group reached 1.5 cm³, all mice were euthanized and the tumors removed, photographed and weighed. For survival studies, mice were injected and monitored as above, except that each individual mouse was euthanized when tumors reached 1.5 cm³ or if a moribundity endpoint was reached, and time to reach endpoint was recorded. Moribundity was defined by changes in the hair coat, changes in the activity level lasting more than one week, changes in posture of ambulation lasting more than one week, changes in facial expression or weight loss >15%. Euthanasia was performed by carbon dioxide asphyxiation followed by thoracotomy in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Kaplan-Meier survival curves were generated with Graphpad Prism 5 software.

Soft agar growth analysis

The indicated SV40-immortalized Kras*+/+ and KrasC118S/C118S MEFs stably infected with a retrovirus encoding no transgene (vector) or HAsG12V were plated in triplicates in 6-well plates in soft agar, as previously described [30]. Four weeks later each well was imaged, which was used to quantitate the number of colonies using Image J software.

Statistical analysis

Data were presented as mean values ± standard error of the mean (SEM). Statistical analyses were performed with Graphpad Prism 5 software. The two-tailed unpaired student’s t test was used to compare two groups. The one-way ANOVA plus post-hoc Bonferroni’s multiple comparison tests were used to compare three or more groups. The long-rank test was performed to compare the survival between two groups. P values < 0.05 were considered significant.

Results

A C118S mutation introduced into wild-type KRAS impairs oncogenic HRASG12V-driven tumorigenesis

To evaluate the effect of blocking redox-dependent reactions with C118 in wild-type KRAS on oncogenic HRASG12V-driven tumorigenesis, we monitored tumor growth of oncogenic HRASG12V-transformed cells upon replacing the endogenous wild-type KRAS protein with the C118S mutant version. To create the cell lines for this experiment, oncogenic HRASG12V-transformed human HEK-TtH cells [23] were stably infected with a retrovirus encoding...
CAAX, a constitutive active PI3K, to activate AKT and promote S1177 phosphorylation and activation of eNOS [10–12]. Appropriate expression of p110-CAAX was validated by RT-PCR (Fig 1A). To then parse out the contribution of wild-type KRAS protein to the tumorigenic growth of these cells, this cell line was stably infected with a retrovirus encoding a scramble or KRAS-specific shRNA. Appropriate knockdown of endogenous wild-type KRAS was validated by immunoblot (Fig 1B). Resultant cells in which the expression of wild-type KRAS was knocked down were stably infected with a retrovirus encoding N-terminal, Flag epitope-tagged wild-type or C118S-mutant KRAS4B (termed KRAS hereafter for ease of discussion) that was engineered to be resistant to the KRAS-specific shRNA. Re-expression of wild-type and C118S Flag-KRAS was confirmed by RT-PCR (Fig 1A).

To next evaluate the effect of replacing endogenous wild-type KRAS with the C118S mutant form on tumorigenesis, all four of the above cell lines were each injected into the flanks of five immunocompromised mice, after which tumor growth was monitored over time. Consistent with the finding that knocking down wild-type HRAS or NRAS can reduce tumor growth of some KRAS mutation-positive cancer cells [23], knocking down wild-type KRAS also reduced tumor growth over time (Fig 1C), resulting in a statistically significant 91% reduction in tumor weight at endpoint compared to scramble shRNA control cells (Fig 1D and 1E). This effect was fully reversed by re-expressing shRNA-resistant wild-type Flag-KRAS, as evident from the similar tumor growth and tumor weight at endpoint between the KRAS-knockdown cells re-expressing wild-type Flag-KRAS and the scramble shRNA control cells (Fig 1C–1E). However, this ability of Flag-KRAS to rescue the poor tumor growth of KRAS-knockdown cells was lost if the C118S mutation was introduced into KRAS. Specifically, there was no statistical difference between KRAS-knockdown cells re-expressing Flag-KRAS<sup>C118S</sup> and KRAS-knockdown cells with regards to tumor growth or tumor weight at endpoint (Fig 1C–1E). Thus, wild-type KRAS promotes xenograft tumor growth of oncogenic HRAS<sup>G12V</sup>-transformed cells expressing constitutively active PI3K in a manner dependent upon C118.

The C118S mutation impairs activation of wild-type KRAS

We previously demonstrated that the C118S mutation reduced the ability of wild-type HRAS to be activated in an eNOS-dependent fashion and to promote oncogenic KRAS-driven tumor growth [23]. As the same mutation in wild-type KRAS also reduced oncogenic HRAS<sup>G12V</sup>, driven tumorigenic growth (Fig 1), we reasoned that this may similarly be due to the C118S mutation blocking activation of wild-type KRAS. To test this possibility, we measured the amount of active (GTP-bound) KRAS in the absence or presence of the C118S mutation in cells expressing eNOS. To create the cell lines for this experiment, HEK-TtH cells were stably infected with retroviruses encoding a C-terminal, HA epitope-tagged S1177D constitutively active [11] or S1177A inactive [11] mutant form of eNOS in conjunction with the wild-type or C118S mutant form of Flag-KRAS<sup>*</sup>, a N-terminal, Flag epitope-tagged version of KRAS optimized for expression [25]. Ectopic expression of HA-eNOS and Flag-KRAS was validated by immunoblot analysis (Fig 2A).

To next assess whether the C118S mutation inhibited the activation of Flag-KRAS<sup>*</sup> downstream of eNOS, GTP-bound RAS proteins were captured from all four of these cell lines using the RAS-binding domain of RAF1 [29], followed by immunoblot with an anti-Flag antibody to specifically detect ectopic Flag-KRAS<sup>*</sup>. Consistent with the findings that HRAS and NRAS are activated downstream of eNOS [23], the level of GTP-bound Flag-KRAS<sup>*</sup> was higher in cells expressing HA-eNOS<sup>S1177D</sup> compared to those expressing HA-eNOS<sup>S1177A</sup>, even though the latter cells had higher expression of both Flag-KRAS<sup>*</sup> and HA-eNOS<sup>S1177A</sup>. Importantly, this
Fig 1. Introducing a C118S mutation into wild-type KRAS impairs HRAS$^{G12V}$-driven tumor growth. (A) RT-PCR amplification of Flag-KRAS, Flag-HRAS, p110CAAX and GAPDH mRNA isolated from oncogenic Flag-HRAS$^{G12V}$-transformed HEK-TtH cells infected with retroviruses encoding p110-CAAX and either scramble (scram) control shRNA or KRAS shRNA in the absence (-) or presence of shRNA-resistant wild-type (WT) or C118S mutant Flag-tagged KRAS. One of two replicate experiments. (B) Immunoblot detection of endogenous KRAS, Flag-tagged HRAS$^{G12V}$ and tubulin in oncogenic Flag-HRAS$^{G12V}$-transformed HEK-TtH cells infected with retroviruses encoding p110-CAAX and either KRAS shRNA or a scramble control (scram) shRNA. One of three
activation could be ascribed to C118, as the level of GTP-bound Flag-KRAS/C118S remained low, regardless of whether cells express HA-eNOS1177D or HA-eNOS1177A (Fig 2A).

To determine whether the effect of C118S on the level of GTP-bound KRAS was reproducible, we repeated the same experiment in another cell line. Specifically, SV40-immortalized mouse embryonic fibroblasts (MEFs) were stably infected with retroviruses encoding either Flag-KRAS/C3 or Flag-KRAS/C3C118S in conjunction with either HA-eNOS1177D or HA-eNOS1177A. Appropriate expression of these transgenes was verified by immunoblot (Fig 2B). As above, cells expressing Flag-KRAS/C3 and HA-eNOS1177D exhibited higher levels of GTP-bound ectopic Flag-KRAS than cells expressing Flag-KRAS/C3 and HA-eNOS1177A or cells expressing Flag-KRAS/C3C118S and HA-eNOS1177D or HA-eNOS1177A (Fig 2B). Thus, the C118S mutation impairs eNOS-dependent activation of wild-type KRAS in two independent types of cells.

The C118S mutation impairs signaling by wild-type KRAS

GTP-bound RAS proteins undergo a conformational change that results in binding effector proteins like Rafs and PI3K, leading to activation of the MAPK and PI3K pathways, respectively [31,32]. Since introducing the C118S mutation into KRAS reduced the level of GTP-bound KRAS detected in cells expressing activated eNOS (Fig 2A and 2B), we tested whether this mutation also impeded the ability of KRAS to activate effector pathways. To this end, the aforementioned oncogenic HRASG12V-transformed HEK-TtH cells expressing p110-CAAX and either scramble control or KRAS shRNA in the absence or presence of shRNA-resistant Flag-tagged wild-type (light green triangles, KRASi + KRAS) or C118S-mutant (dark green reverse triangles, KRASi + KRASC118S) KRAS. ns: non-significant, *, P<0.05, **: P<0.01 and ***: P<0.001, as determined by one-way ANOVA plus post-hoc Bonferroni’s multiple comparison test using GraphPad Prism 5 Software. Full-length immunoblots and gels are shown in S1A and S1B Fig.
Fig 2. Introducing a C118S mutation into wild-type KRAS impairs EGF stimulation of AKT and ERK1/2 phosphorylation. Immunoblot detection of input and GTP-bound Flag-KRAS captured by the Ras-binding domain of Raf1, as well as HA-eNOS and tubulin, in (A) HEK-TtH cells or (B) SV40-immortalized MEFs stably infected with retroviruses encoding either wild-type (WT) or C118S-mutant Flag-tagged KRAS in conjunction with either the HA-tagged S1177D constitutively-active or the S1177A inactive mutant versions of eNOS. High molecular weigh bands were variably detected using this assay, and because they are above the size of Ras, they were considered to be non-specific. Relative mean ± SEM of GTP-bound KRAS (normalized

**Reduced HRAS Oncogenesis in Cell Lines Expressing KRAS C118S**
An activating mutation in wild-type KRAS overcomes the reduction of tumor growth imparted by the C118S mutation

The findings that the C118S mutation inhibited the activation and signaling as well as tumorigenesis mediated by wild-type KRAS argues that these effects are related. To genetically test whether the defect inflicted by the C118S mutation could be overcome by activating KRAS<sup>C118S</sup>, the above oncogenic HRAS<sup>G12V</sup>-transformed HEK-TtH cells expressing p110-CAAX and KRAS shRNA were engineered to express no other transgene or shRNA-resistant Flag-tagged KRAS, KRAS<sup>C118S</sup> or KRAS<sup>C118S</sup> with an activating (G12V) mutation. Re-expression of the three versions of Flag-KRAS was confirmed by RT-PCR (Fig 3A). The four resultant cell lines were then each injected into the flanks of five immunocompromised mice, after which tumor growth was monitored over time. As already observed (Fig 1), the reduced tumor growth of the KRAS-knockdown cells was reversed by re-expressing shRNA-resistant wild-type Flag-KRAS, as these cells formed significantly larger (7.3 fold) tumors than the KRAS-knockdown cells. Again, re-expressing the C118S mutant version did not rescue this phenotype, as evidenced by similar tumor growth and tumor size and weight at endpoint in tumors derived from the KRAS-knockdown cells and the KRAS-knockdown cells expressing Flag-KRAS<sup>C118S</sup> (Fig 3B–3D). Importantly, the inability of Flag-KRAS<sup>C118S</sup> to restore the reduced tumor growth upon knocking down endogenous wild-type KRAS was rescued if Flag-KRAS<sup>C118S</sup> was engineered to contain an activating (G12V) mutation, as evident from the similar growth kinetics (Fig 3B) and tumor size and weight at endpoint between the KRAS-knockdown cells expressing wild-type Flag-KRAS and those expressing Flag-KRAS<sup>C118S,G12V</sup> (Fig 3B–3D). These results support the contention that the C118S mutation blocks activation of wild-type KRAS and the ability of this protein to support tumor growth.

The C118S mutation introduced into the endogenous murine wild-type Kras gene impairs oncogenic HRAS<sup>G12V</sup>-driven transformation

Admittedly, one caveat to the above experiments was that KRAS was restored in KRAS-knockdown cells by ectopic expression of various versions of KRAS protein. Thus, to assess the effect on tumorigenesis when the C118S mutation was introduced into the endogenous Kras gene, MEFs were isolated from three Kras<sup>C118S/C118S</sup> mouse embryos in which the C118S mutation was knocked into both alleles of the endogenous Kras gene [28], as well as three control Kras<sup>+/+</sup> embryos. These six cultures were immortalized by stable infection with a retrovirus encoding the early region of SV40, and the Kras genotype confirmed by PCR in the resultant immortalized MEF cell lines (Fig 4A). Immunoblot analysis revealed varying levels of Kras protein regardless of the genotype (Fig 4B). Given this, Kras<sup>+/+</sup> cell line 3 and Kras<sup>C118S/C118S</sup> cell line 5 were chosen for analysis, owing to their similar levels of Kras protein expression. These two cell lines were stably infected with a retrovirus encoding no transgene (vector) or oncogenic HRAS<sup>G12V</sup>. Appropriate expression of oncogenic HRAS<sup>G12V</sup> was validated by immunoblot (Fig 4B). The four resultant cell lines were then assayed in triplicate for the transformed phenotype of growth in soft agar. As expected, vector control cells did not grow in soft agar, but...
introduction of oncogenic HRAS\textsuperscript{G12V} permitted this growth. However, there was a statistically significant, 70% reduction in the mean number of colonies seeded by oncogenic HRAS\textsuperscript{G12V}-transformed \textit{Kras\textsuperscript{C118S/C118S}} MEFs compared to \textit{Kras\textsuperscript{+/+}} MEFs (Fig 4C and 4D). Thus, introduction of the C118S mutation into the endogenous \textit{Kras} gene inhibits oncogenic HRAS\textsuperscript{G12V}-mediated transformation.

**The C118S mutation introduced into the endogenous murine wild-type \textit{Kras} gene impairs oncogenic HRAS\textsuperscript{G12V}-driven tumorigenesis**

To assess whether the reduction in growth in soft agar observed with the oncogenic HRAS\textsuperscript{G12V}-transformed \textit{Kras\textsuperscript{C118S/C118S}} MEFs reflected a defect in the more relevant \textit{in vivo} phenotype of tumor growth, these cells and the control oncogenic HRAS\textsuperscript{G12V}-transformed \textit{Kras\textsuperscript{+/+}} MEFs were injected into the flanks of five immuno-compromised mice each, after...
Reduced HRAS Oncogenesis in Cell Lines Expressing KRAS<sup>C118S</sup>

**Panel A**

| Kras:       | +/+   | C118S/C118S |
|-------------|-------|-------------|
| MEF line:   | 1, 2  | 3, 4, 5, 6  |
|             | 621 bp| 517 bp      |

**Panel B**

| Kras:       | +/+  | C118S/C118S |
|-------------|------|-------------|
| MEF line:   | 1, 2| 3, 4, 5, 6  |
| Kras        | 1.17 | 1.48        |
| Hras        | 1.26 | 0.78        |
| Tubulin     | 1.08 | 0.93        |

**Panel C**

- Vector
- HRAS<sup>12V</sup>
- Kras<sup>+/+</sup>
- Kras<sup>C118S/C118S</sup>

**Panel D**

Number of colonies

**Panel E**

Tumor volume (cm<sup>3</sup>)

**Panel F**

Kras<sup>+/+</sup>
- Kras<sup>C118S/C118S</sup>

**Panel G**

Tumor weight at end point (g)

**Panel H**

Tumor volume (cm<sup>3</sup>)

**Panel I**

Percent survival (%)
Fig 4. Introducing a C118S mutation into the endogenous wild-type Kras gene impairs HRASG12V-driven tumor growth. (A) PCR amplification of genomic DNA yielding a 621 bp or 517 bp fragment indicative of the wild-type or C118S mutant Kras alleles in three SV40-immortalized Kras+/+ or KrasC118S/C118S MEF cell lines, respectively. (B) Immunoblot detection of endogenous Kras, HRAS and tubulin in the indicated SV40-immortalized Kras+/+ or KrasC118S/C118S MEF cell lines transformed with HRASG12V. Relative Kras or HRAS levels normalized to tubulin are shown beneath the immunoblot. (C) Representative images and (D) the mean ± SEM number of colonies growing in soft agar by SV40-immortalized Kras+/+ versus KrasC118S/C118S MEF cell lines stably infected with a retrovirus encoding no transgene (vector) or HRASG12V, seeded in triplicate. (E) Mean ± SEM tumor volume over time. (F) Photographs of excised tumors at end point and (G) mean ± SEM tumor weight at end point of tumors developing in immunocompromised mice (n = 5) injected with SV40-immortalized Kras+/+ (pink boxes) versus KrasC118S/C118S (orange squares) MEF cell lines transformed with HRASG12V. (H) Mean ± SEM tumor volume over time of tumors developing in immunocompromised mice (n = 5) injected with SV40-immortalized and HRASG12V-transformed Kras+/+ MEFs (pink circles), KrasC118S/C118S MEFs (orange squares) or KrasC118S/C118S MEFs stably infected with a retrovirus encoding KRAS* (light green triangles) or KRAS* (dark green reverse triangles). (I) Kaplan-Meier survival curves based on the time to reach end point of immunocompromised mice (n = 5) each injected with one of the three SV40-immortalized Kras+/+ (pink line) versus KrasC118S/C118S (orange line) MEF cell lines transformed with HRASG12V. *: non-significant, **: P<0.01, ***: P<0.001 and ****: P<0.0001, as determined by one-way ANOVA plus post-hoc Bonferroni’s multiple comparison test (D, H), two-tailed unpaired t test (E, G) or long-rank test (I) using GraphPad Prism 5 Software. Full-length immunoblots and gels are shown in S1F and S1G Fig.

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which tumor growth was monitored over time. This analysis revealed that tumors derived from oncogenic HRASG12V-transformed KrasC118S/C118S MEFs grew more slowly than the control Kras+/+ counterparts (Fig 4E), which was manifested at endpoint as smaller tumors (Fig 4F) that weighed 78% significantly less (Fig 4G). As a control, we demonstrate that re-expression of ectopic wild-type KRAS* was more effective than KRAS* to restoring tumor growth of oncogenic HRASG12V-transformed KrasC118S/C118S MEFs (Fig 4H). Finally, to assess the impact of the C118S mutation on the clinically relevant endpoint of survival, the above three Kras+/+ and three KrasC118S/C118S immortalized MEFs were also transformed with oncogenic HRASG12V and the resultant six cell lines were each injected into the flank of five immunocompromised mice. Mouse were then euthanized if they reached a maximum tumor volume or moribundity. A plot of the percent of mice surviving over time revealed that mice injected with oncogenic HRASG12V-transformed KrasC118S/C118S MEFs exhibited a 62% greater, significant survival advantage over mice injected with the oncogenic HRASG12V-transformed Kras+/+ MEFs (Fig 4I). Thus, introducing the C118S mutation into the endogenous Kras gene inhibits oncogenic HRASG12V-mediated xenograft tumor growth.

Discussion

We report here that xenograft tumor growth of an oncogenic HRASG12V-transformed human cell line engineered to express activated PI3K is diminished upon knockdown of endogenous wild-type Kras, and that this effect was rescued by re-expressing the wild-type, but not the C118S-mutant KRAS. Oncogenic HRASG12V-transformed KrasC118S/C118S MEFs also exhibited reduced anchorage-independent and tumorigenic growth compared to similarly transformed Kras+/+ MEFs. Again, there was a trend towards these cells being more tumorigenic upon re-expression wild-type KRAS compared to C118S-mutant KRAS. Thus, introducing the C118S mutation into wild-type KRAS inhibits oncogenic HRASG12V-mediated tumorigenesis in two different experimental settings. The same C118S mutation engineered into HRAS and NRAS was previously shown to reduce the tumor growth of the KRAS mutation-positive human pancreatic cancer cell line CFPac-1 [23]. As such, mutating C118 in the different wild-type RAS proteins may reduce tumorigenesis driven by different oncogenic RAS isoforms.

We also demonstrate that the level of GTP-bound wild-type KRAS in the presence of functionally-active eNOS was reduced when the C118S mutation was introduced into the KRAS transgene. Moreover, EGF stimulation of the MAPK and PI3K pathways in HRASG12V-transformed human cells was blunted when endogenous KRAS was replaced with the C118S mutant version. It was previously demonstrated that knocking down endogenous eNOS reduced the level of S-nitrosylated and GTP-bound HRAS and NRAS in cells expressing p110-CAAX to activate eNOS [23]. There is also accumulating evidence that wild-type RAS proteins are
activated in cells harboring an oncogenic RAS mutation [7,23,37]. In this regard, we genetically demonstrate that the inability of KRAS<sup>C118S</sup> to restore tumor growth of HRAS<sup>G12V</sup>-transformed human cells upon knocking down endogenous KRAS could be restored, in large part, by introducing an activating mutation into the KRAS<sup>C118S</sup> transgene. As the C118S mutation does not alter other known activities of RAS aside from activation by redox-dependent reactions [13,15–22], these results collectively support the contention that activation of eNOS through the PI3K/AKT signaling arm of oncogenic RAS leads to S-nitrosylation or other redox-dependent reactions with C118 in the remaining wild-type RAS proteins, leading to their activation and promotion of tumorigenesis.

The effect of the C118S mutation on the ability of wild-type KRAS to promote HRAS oncogenesis may nevertheless be dependent upon the stage of tumorigenesis or types of cancer. Specifically, we previously demonstrated that Kras<sup>C118S/C118S</sup> mice are more resistant to urethane-induced lung tumorigenesis that their Kras<sup>+/+</sup> counterparts, this effect was linked to the oncogenic, rather than the remaining non-mutated Kras<sup>C118S</sup> allele [28]. These results suggest that either later stages of tumorigenesis, such as those modeled in cancer cell lines, or specific types of cancer are sensitive to loss of redox-dependent reactions with C118 of wild-type KRAS. In agreement, knocking out wild-type Ras genes in mice can be either tumor promoting or suppressing depending upon the carcinogen, cancer type or the Ras isoform acquiring the oncogenic mutation [5]. Nevertheless, in the settings studied, we propose that redox-dependent reactions with C118 of wild-type KRAS activate the protein to stimulate xenograft tumor growth of oncogenic HRAS<sup>G12V</sup>-driven cell lines. These results, coupled with previous findings of a similar role for wild-type HRAS and NRAS in oncogenic KRAS-driven tumorigenesis [8,23,37], suggest that activation of wild-type RAS isoforms can promote oncogenic RAS-driven tumorigenesis in certain settings.

Supporting Information

S1 Fig. Full-length gels and blots for figures. Full-length gels or blots for (A) Fig 1A and Fig 3A, (B) Fig 1B, (C) Fig 2A, (D) Fig 2B, (E) Fig 2C, (F) Fig 4A, and (G) Fig 4B.

(S1 Table. PCR primers.)

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Author Contributions

Conceived and designed the experiments: LH CMC. Performed the experiments: LH. Analyzed the data: LH CMC. Wrote the paper: LH CMC.

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