c-RAF Ablation Induces Regression of Advanced *Kras/Trp53* Mutant Lung Adenocarcinomas by a Mechanism Independent of MAPK Signaling

Hannah Goddard and Ella Colistro

Introduction

- Mutations in KRAS proteins have been identified in ¼ of all human cancers.
  - Most prominent in lung, pancreatic, and colorectal cancers.
- This identification was discovered over 30 years ago, yet there are still no selective therapies against KRAS.
- This is due to KRAS targeting being very difficult, so most efforts have focused on targeting its downstream kinases.
- Main pathway: MAPK

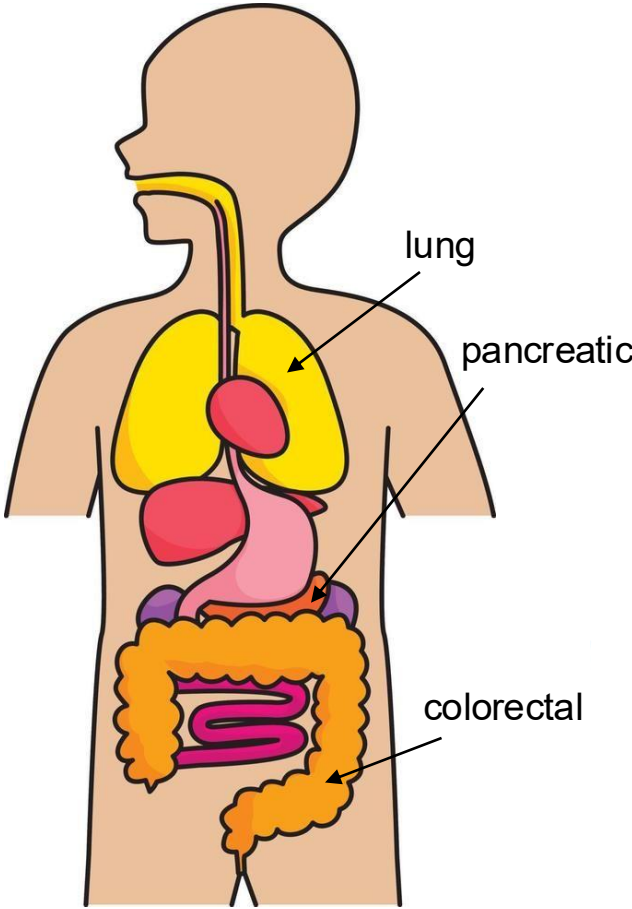


Figure 1. Diagram of human body showing location of lung, pancreatic, and colorectal cancer.

Introduction

The role of KRAS in the MAPK pathway:

- KRAS phosphorylates and activates a cascade of protein kinases that amplify and regulate mitogenic signals.
- RAS → RAF → MEK → ERK

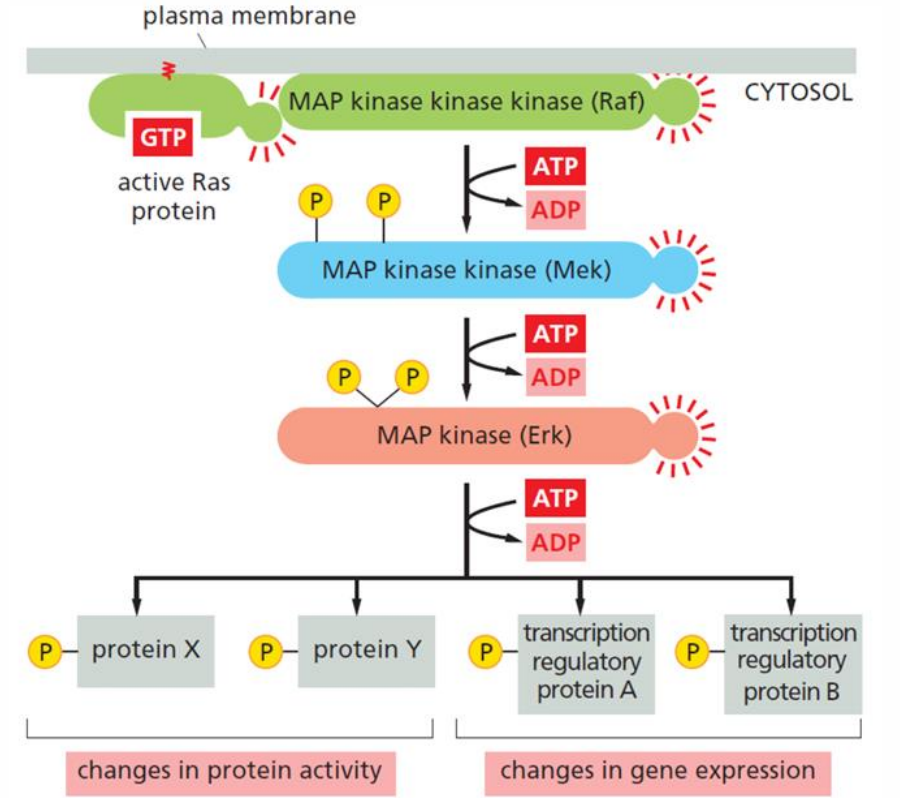


Figure 2. MAPK reaction scheme.

Introduction

Previous Studies:

- In mice, the inactivation of MEK1/2 effectively prevented KRAS-driven tumor development.
- Similar results were obtained when inactivating ERK1/2.
- Ablation of each of these kinases resulted in high levels of toxicity in mice, leading to rapid death.

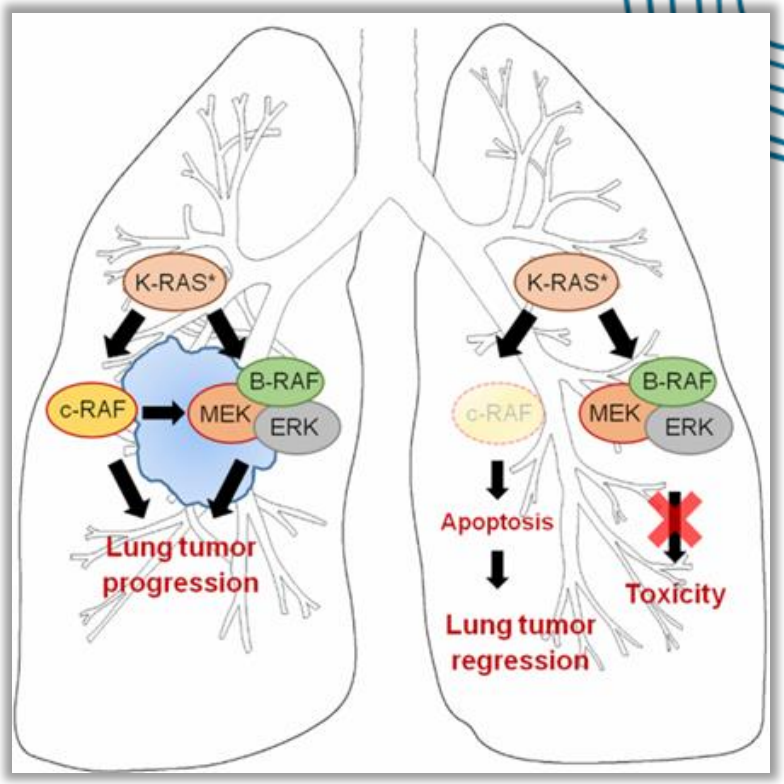


Figure 3. Schematic demonstrating the effects of ablation of various protein genes.

Introduction

Previous Studies:

- Targeting c-RAF but not B-RAF revealed equally effective inhibition of tumor development without inducing significant toxicities.
- Suggests these kinase isoforms likely play different roles in mediating KRAS oncogenic signals.

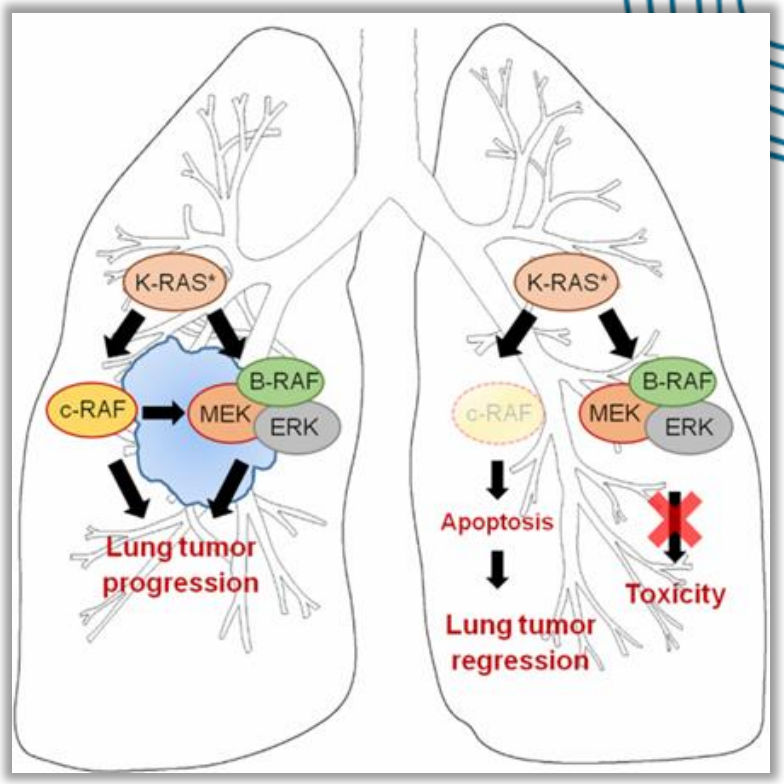


Figure 3. Schematic demonstrating the effects of ablation of various protein genes.

Introduction

Goal of the Study:

To determine the role of c-RAF in lung tumor progression of already established tumors and to assess the effects of its inhibition in adult mice.

Methods I

Developing a genetically engineered mouse model:

- *Kras* gene locus targeting using a Neo-resistant STOP cassette flanked by frt (F) sequences within the first intron.
- Mutated first exon encoding a Gly to Val substitution.

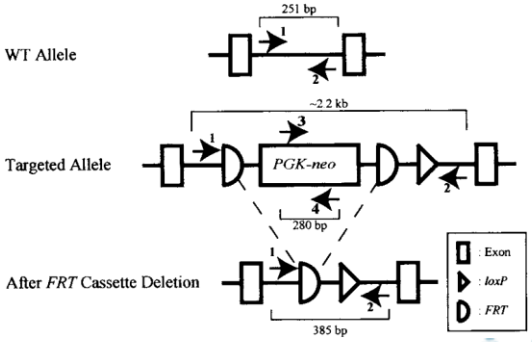


Figure 4. Demonstration of a Neo-resistant STOP cassette flanked by frt (F) into an intron.

- **Neo-resistant**: cassette is linked to a gene that provides resistance to the drug neomycin.
- **STOP cassette**: sequence of DNA that prevents expression of a gene by stopping its transcription.
- **frt (F) sequence**: DNA sequence that can be recognized by Flp recombinase, that can remove the STOP cassette.

Methods I

c-Raf ablation reduces tumor growth:

- The genetically engineered mice (with the mutant *Kras* gene) were crossed with mice carrying conditional floxed c-Raf and B-Raf alleles, along with an inducible CreERT2 recombinase.
- CreERT2 recombinase is activated in response to a drug (tamoxifen).
- It cleaves portions of the c-Raf and B-Raf alleles, inhibiting their expression.

Control
$c\text{-}Raf^{+/+};Braf^{+/+}$

c-Raf inhibition
$c\text{-}Raf^{L/L};Braf^{+/+}$

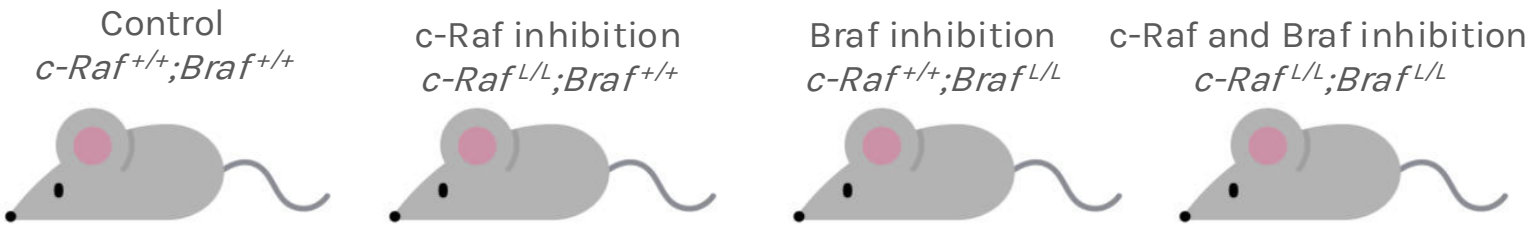
Braf inhibition
$c\text{-}Raf^{+/+};Braf^{L/L}$

c-Raf and Braf inhibition
$c\text{-}Raf^{L/L};Braf^{L/L}$

Methods I

c-Raf ablation reduces tumor growth:

- The mice were infected with Flp recombinase via intratracheal instillation to allow *Kras* expression in lung tissue.
- This induced the formation of a small number of tumors in their lungs, which were monitored using CT scans.
- Once at least 1 tumor reached 1 mm in diameter, the mice were fed a TMX-containing (tamoxifen) diet for 4 months to inactivate the c-Raf and/or B-Raf alleles.



Methods I

c-Raf ablation reduces tumor growth:

- Tumor growth was monitored throughout the 4 months of TMX exposure.
- Histological analyses of the tissues were conducted to assess any potential toxicities.
- The survival of the mice and the development of tumors were observed, and potential toxic effects of the ablation of c-RAF and/or B-Raf were evaluated.

Control
c-Raf$^{+/+}$;Braf$^{+/+}$

c-Raf inhibition
c-Raf$^{L/L}$;Braf$^{+/+}$

Braf inhibition
c-Raf$^{+/+}$;Braf$^{L/L}$

c-Raf and Braf inhibition
c-Raf$^{L/L}$;Braf$^{L/L}$

Results I

- Western blot analysis of c-Raf and B-RAF expression from five independent tumors.
- Demonstrates that the treatments inhibiting the production of each kinase were successful.

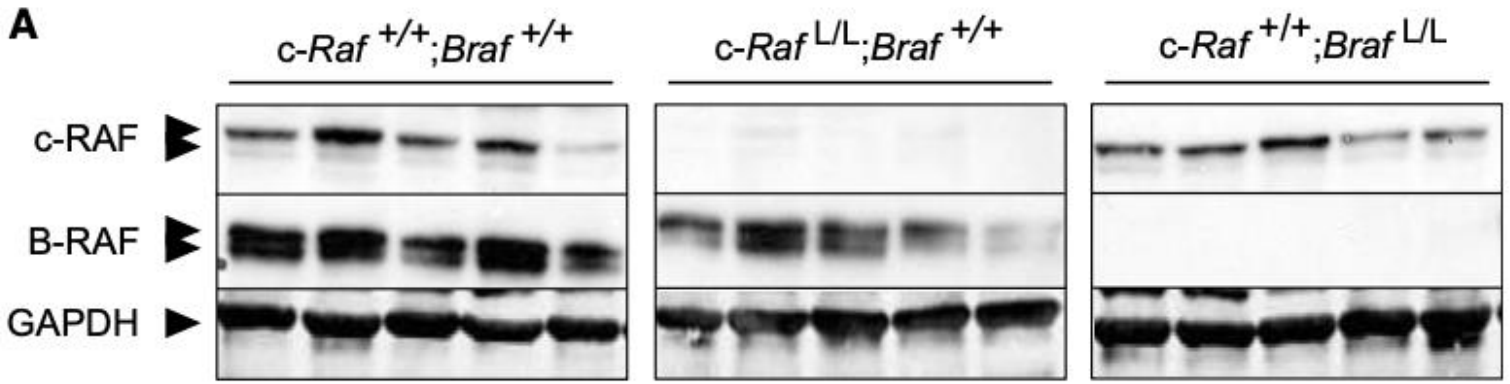


Figure 5. Western blot analysis of c-Raf and B-Raf expression in lysates derived from five independent tumors obtained from mice exposed for 4 months to a TMX diet. GAPDH used as the loading control.

Results I

Control and B-Raf inhibition:

- All tumors grew significantly, with more tumors appearing throughout time.
- Indicates B-Raf doesn't play a relevant role in oncogenic signaling.

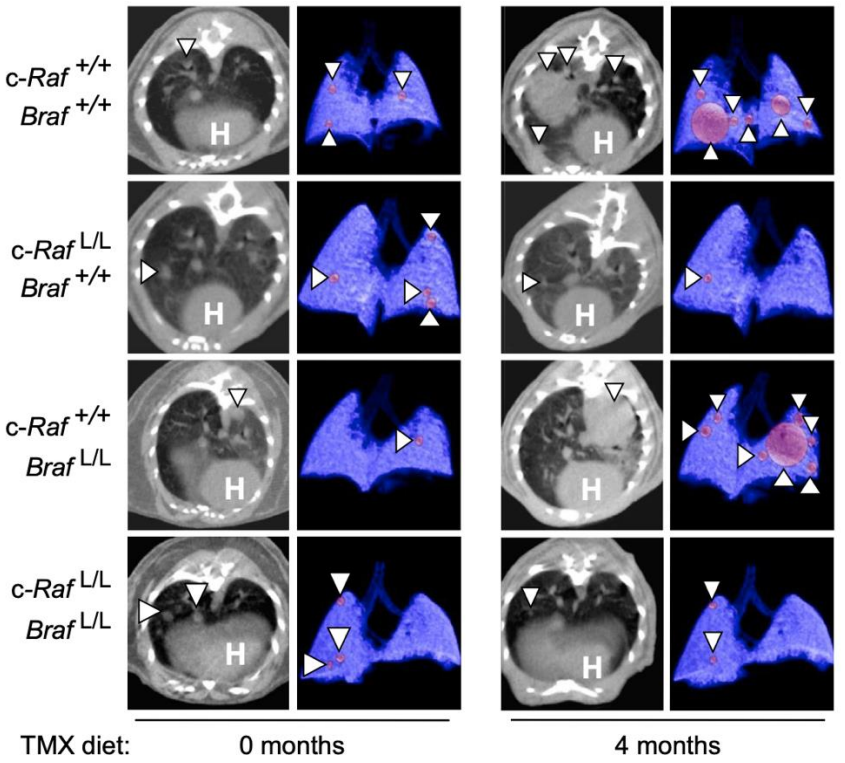


Figure 6. CT axial projection images and 3D reconstructions of lungs of representative mice carrying the indicated c-Raf and B-Raf alleles before and after 4 months of TMX exposure.

Results I

c-Raf and B-Raf inhibition:

- Led to the greatest reduction in tumor growth.
- However, ablation of both kinases was not well tolerated, so the mice were sacrificed within the first 4 months of TMX exposure.

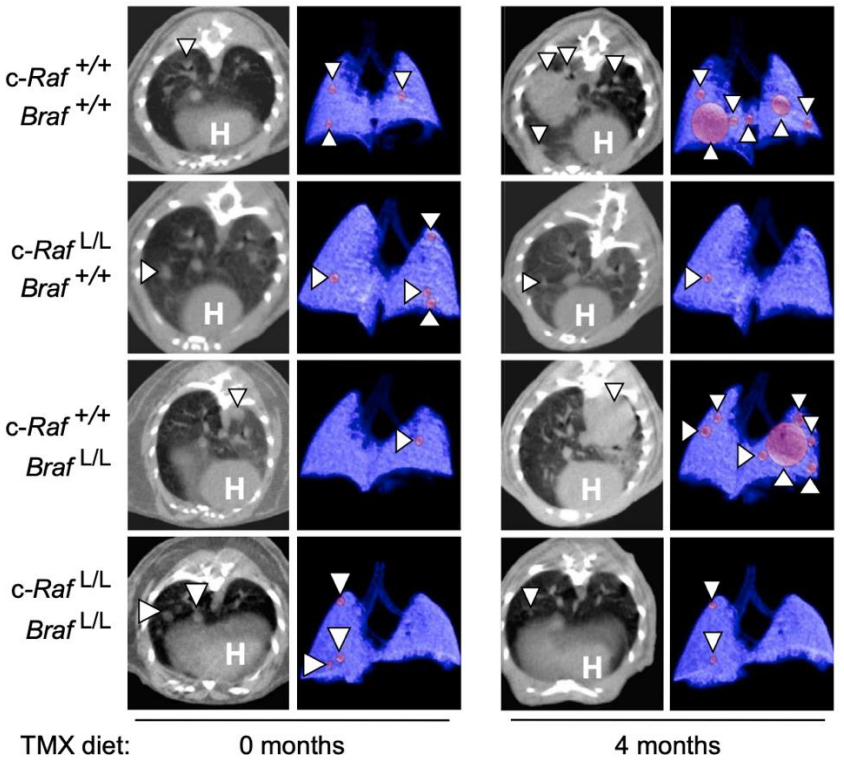


Figure 6. CT axial projection images and 3D reconstructions of lungs of representative mice carrying the indicated c-Raf and B-Raf alleles before and after 4 months of TMX exposure.

Results I

c-Raf inhibition:

- Caused the tumors to shrink and some to disappear entirely.
- All tumors were benign, so non-cancerous.
- Analysis of tissues didn't reveal any toxicities.
- These mice survived up to 9 months, and there was no development of resistance mechanisms during this time.

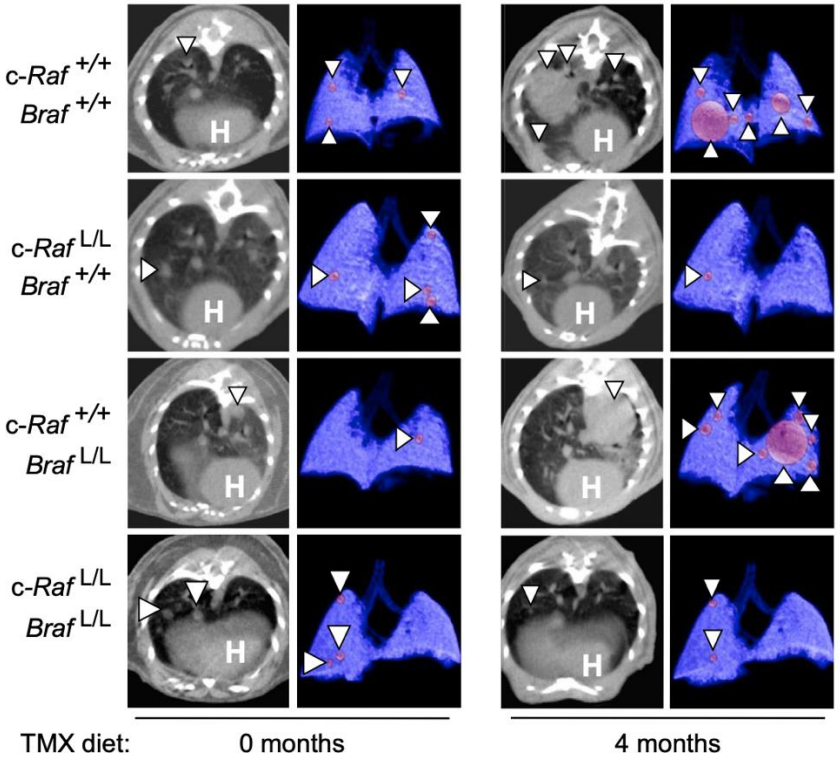


Figure 6. CT axial projection images and 3D reconstructions of lungs of representative mice carrying the indicated c-Raf and B-Raf alleles before and after 4 months of TMX exposure.

Results I

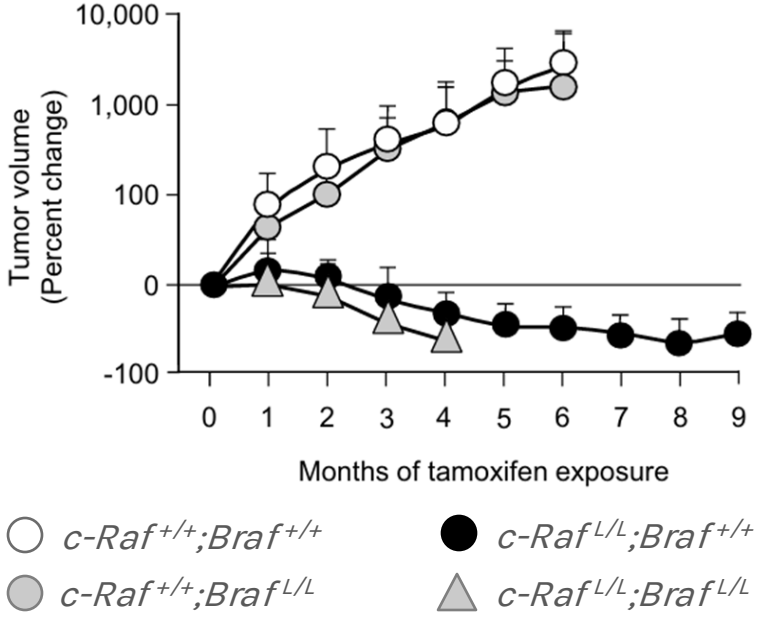


Figure 7. Average tumor volume variation during TMX exposure.

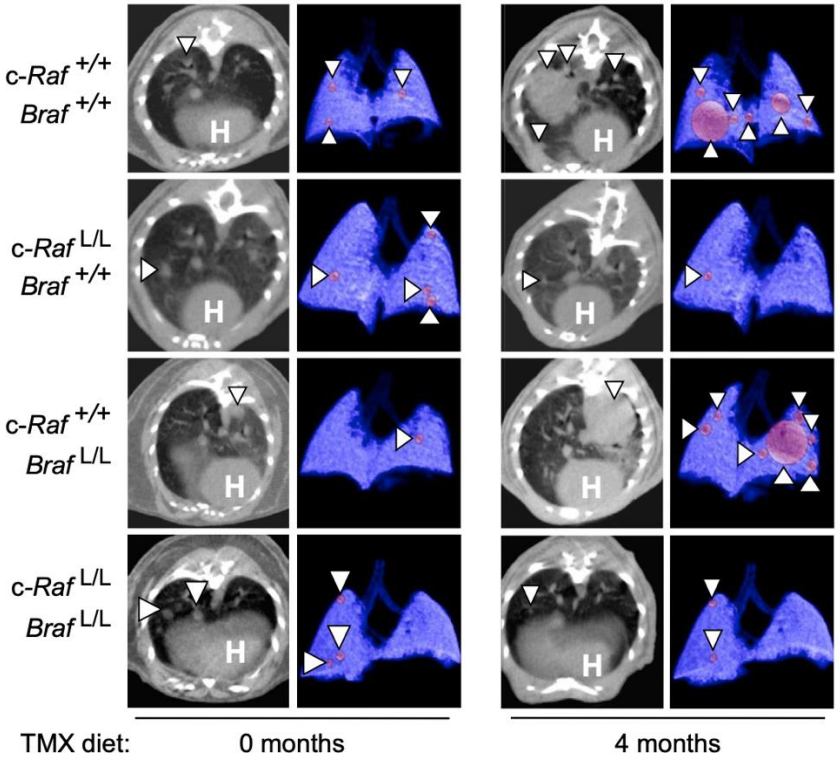


Figure 6. CT axial projection images and 3D reconstructions of lungs of representative mice carrying the indicated c-Raf and B-Raf alleles before and after 4 months of TMX exposure.

Methods II

c-RAF expression is essential for proliferation of patient-derived xenograft models:

1. Lung tumor lines were extracted from viable patients to xenograft tumor models.
    i. Tumor lines carried a mutation in the KRAS gene.
2. Cells were infected with a lentivirus containing shRNA to target the c-Raf gene.
    i. Deplete c-Raf expression.
    ii. Scrambled shRNA (-ve control).
3. After 2 weeks, injected cells into lungs of immune compromised mice.

- **xenograft**: cell transplantation of tumor cells from a patient to a model.
- **lentivirus**: genus of virus that delivers chronic diseases in mammals.
- **shRNA**: short hairpin RNA – type of RNA used to knock down and silence certain genes.

Results II – Infected Cells

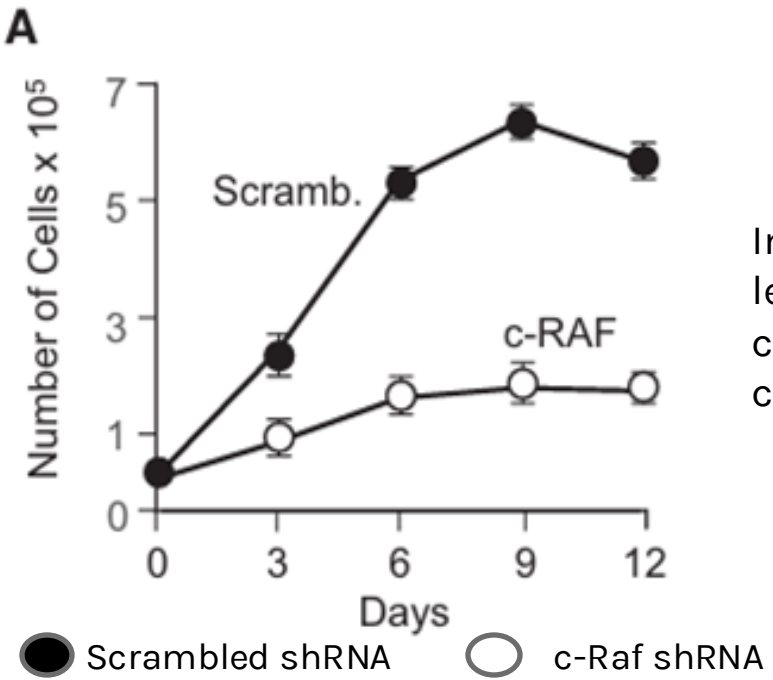


Figure 7. Cell proliferation assay expressing scrambled shRNA (.solid circles) and shRNA against c-Raf (open circles)

Infection of cells with shRNA-containing lentivirus resulted in partial inhibition of cell proliferation within the c-Raf shRNA cells.

Results II – Infected Mice

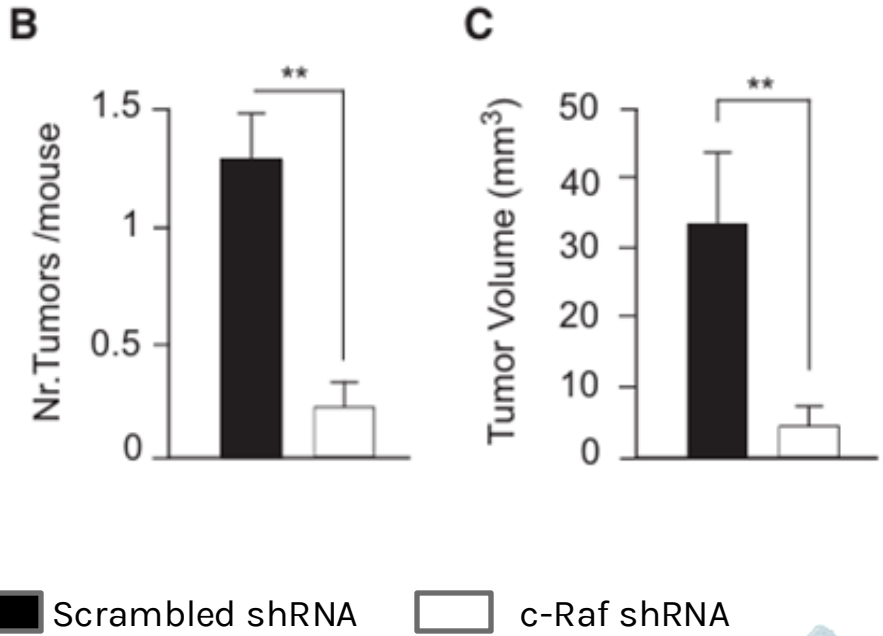


Figure 8. Quantification of tumor size and number per implantation (scrambled shRNA – solid bar; shRNA against c-Raf – open bar). n=10 mice

B) Cells exposed to c-Raf shRNA formed significantly fewer tumors than the cells containing scrambled shRNA.

C) Tumors in the c-Raf shRNA cells were significantly smaller in size volume.

Discussion – Previous Research

- Research has investigated the roles of KRAS effector proteins within the MAPK signaling pathway:
    - No effect on lung tumor growth (A-Raf and B-Raf).
    - Prevent tumor growth but cause unacceptable toxicities (MEK and ERK).
    - Prevent tumor growth and cause no significant toxicities (c-Raf).

Discussion – This Research

Mouse model findings

c-Raf ablation:

- Significant reduction in tumor growth after 4 months of TMX exposure.
- Suggests c-Raf plays a role in tumor growth.

B-Raf ablation:

- Had no effect on tumor progression, as tumors grew at a similar rate to the control.
- Indicates B-Raf is not critical for KRAS oncogenic signaling.

Toxicity:

- Systemic ablation of c-Raf in adult mice showed no toxic effects.

Discussion – This Research

Human tumor cell line findings

c-Raf ablation:

- Lentiviral infection with c-Raf shRNA led to significant tumor growth inhibition.

Tumor formation:

- Cells with c-Raf shRNA formed fewer and smaller tumors in immune-compromised mice.
- Confirms the role of c-Raf in tumor growth.

Future Work

- c-Raf ablation itself is not sufficient for complete regression of most tumors – not sufficient for optimal therapeutic benefits to lung cancer patients. May have to be combined with other targeted therapies.

- c-Raf cannot be completely ablated long-term as it has other effects within the body contributing to the MAPK pathway.

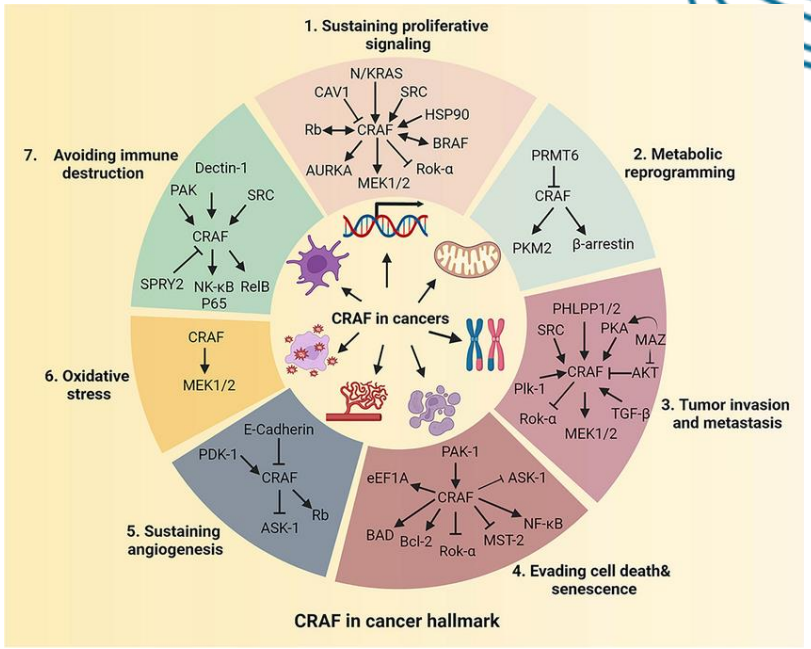
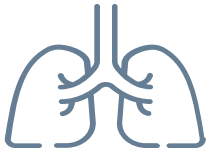


Figure 9. c-Raf promotion of 7 features in tumors.

Conclusion



c-Raf is a key mediator of KRAS driven lung tumor progression.

c-Raf ablation requires selectivity due to toxicity induced by B-Raf ablation.

c-Raf inhibition is unlikely to completely regress tumors in a clinical setting.

c-Raf is still important for other aspects of the MAPK pathway, so we can't eliminate it completely.

References

Blasco, R.B., Francoz, S., Santamaria, D., Canamero, M., Dubus, P., Charron, J., Baccarini, M., and Barbacid, M. (2011). c-Raf, but not B-Raf, is essential for development of K-Ras oncogene-driven non-small cell lung carcinoma. Cancer Cell 19, 652–663.

Castellano, E., Sheridan, C., Thin, M.Z., Nye, E., Spencer-Dene, B., Diefenbacher, M.E., Moore, C., Kumar, M.S., Murillo, M.M., Gro¨nroos, E., et al. (2013). Requirement for interaction of PI3-kinase p110alpha with RAS in lung tumor maintenance. Cancer Cell 24, 617–630.

Wang P;Laster K;Jia X;Dong Z;Liu K; “Targeting CRAF Kinase in Anti-Cancer Therapy: Progress and Opportunities.” Molecular Cancer, U.S. National Library of Medicine, pubmed.ncbi.nlm.nih.gov/38111008/. Accessed 25 Mar. 2025.