Peer Review

Mai, Benjamin J. 2025. "Selective KRAS Dependency in Cancer: Therapeutic Implications for KRAS-Mutant Tumors." *Journal of High School Science* 9 (2): 375–90.

1.Figure 4, T3M4 cell line, KRAS KO increases growth. Explain and discuss in the manuscript.
2.The manuscript lacks the sourcing of various reagents/chemicals as well as methodology detail. Please ensure that all information is provided which will enable your work to be duplicated in other labs. For example, "……proteins were separated by capillary electrophoresis….." needs description of equipment/apparatus, vendor, model, and operating conditions, including sources of consumables and or kits. Similarly "…..Immunodetection was carried out by incubating proteins with primary antibodies specific to the target protein (diluted 1:50 to 1:200), followed by HRP-conjugated secondary antibodies. After washing with Jess wash buffer, chemiluminescent detection was performed using a substrate for HRP. The resulting signals were captured and quantified using the ProteinSimple Compass software……." needs sourcing and cat# of antibodies (primary and secondary), substrate for HRP, chemiluminescent detection apparatus/instrument and its settings, ProteinSimple Compass software settings, version #, manufacturer…… etc. If manufacturer's or vendor's recommended procedure was used, then state as such in the manuscript. If a different procedure was used, it must be described in detail so as to enable others to replicate your work.
3."……was measured using PherastarFSX….." need conditions of measurement, operating procedure.
4.Figure 4B, KRAS KO has only been confirmed in 4 cell lines. What about the remaining three? PANC-1 KRAS does not appear to be completely KO. Explain and discuss in the manuscript.
5.You state "……Immediately after electroporation, cells were transferred into pre-warmed complete medium and allowed to grow for 5 days for cell growth assay and protein detection….." How did you select those cells or colonies which were gene-edited? Please explain the procedure in detail.
6.Did you perform the work shown in the Kaplan-Meier plot? I am assuming not. Please therefore clearly mention that work related to Figure 5 was not performed in your study but you are using those results from other studies to emphasize your point.
7.Provide evidence that KRAS KO does not simultaneously result in PLK1 KO, and vice' versa. Detailed explanation and discussion is required especially in the light of this published artcle: https://doi.org/10.1016/j.cell.2009.05.006
8.References must be in consistent format throughout and must include 6 authors followed by an et al., where the number of authors is > 6. When the number of authors is <=6, all authors must be listed. A live link must be provided for each reference. Please see the formatting requirements for the Journal for more details or use one of the published papers at the website as a template.

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1. Figure 4, T3M4 cell line, KRAS KO increases growth. Explain and discuss in the manuscript.
   Response:
   Thank you for your comment. I have now included the discussion in the manuscript.
   "In the T3M4 cell line, a slight increase in cell number was observed in sgKRAS-treated cells compared to sgNC controls (118% vs. 100%) (Fig. 4A). This apparent increase in growth may be attributed to experimental variation or potential off-target effects, particularly since no KRAS protein depletion was detected in these cells (Fig. 4B). The lack of effective KRAS knockout suggests that the observed difference is unlikely to reflect a true biological effect of KRAS loss."

2. The manuscript lacks the sourcing of various reagents/chemicals as well as methodology detail. Please ensure that all information is provided which will enable your work to be duplicated in other labs. For example, "……proteins were separated by capillary electrophoresis….." needs

description of equipment/apparatus, vendor, model, and operating conditions, including sources of consumables and or kits. Similarly "…..Immunodetection was carried out by incubating proteins with primary antibodies specific to the target protein (diluted 1:50 to 1:200), followed by HRP-conjugated secondary antibodies. After washing with Jess wash buffer, chemiluminescent detection was performed using a substrate for HRP. The resulting signals were captured and quantified using the ProteinSimple Compass software……." needs sourcing and cat# of antibodies (primary and secondary), substrate for HRP, chemiluminescent detection apparatus/instrument and its settings, ProteinSimple Compass software settings, version #, manufacturer…… etc. If manufacturer's or vendor's recommended procedure was used, then state as such in the manuscript. If a different procedure was used, it must be described in detail so as to enable others to replicate your work.

Response:

Thank you for your detailed and constructive feedback. In response, I have thoroughly revised the Materials and Methods section to include all relevant details (as labeled in red). These additions have been made to ensure that the methods are transparent and fully replicable by other researchers.

"Cell culture

AsPC1 (CRL-1682, ATCC, Manassas, VA, USA), T3M4 (Cat#CSC-C6425J, Creative Bioscience, Salt Lake City, UT, USA), and BxPC3 (CRL-1687, ATCC) cells used in this study were cultured in RPMI-1640 medium (Cat# 11875093, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Cat# A5670701, Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (Cat# 10378016, Gibco, Thermo Fisher Scientific). Panc 04.03 cells (CRL-2555, ATCC) were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 10 μg/ml insulin (Cat# 12585014, Gibco, Thermo Fisher Scientific). Capan-1 cells (HTB-79, ATCC) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Cat# 12440053, Gibco, Thermo Fisher Scientific) supplemented with 20% FBS and 1% penicillin-streptomycin. PANC-1 cells (CRL-1469, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cat# 11965118, Gibco, Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin-streptomycin. MIA PaCa-2 cells (Cat#85062806, Sigma Aldrich, St. Louis, MO, USA) were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 2.5% horse serum (Cat#16050130, Gibco, Thermo Fisher Scientific). The cultures were maintained at 37°C in a humidified incubator with a 5% CO_2 atmosphere. The medium was replaced every 2-3 days, and the cells were sub-cultured when they reached approximately 70-80% confluency. For passage, cells were detached using 0.25% trypsin-EDTA (Cat# 25200056, Gibco, Thermo Fisher Scientific) after washing with Dulbecco's phosphate-buffered saline (DPBS) (Cat# 14190144, Gibco, Thermo Fisher Scientific) and reseeded at appropriate densities (9)."

"Protein detection

Protein detection was performed using the Jess system (Cat# 004-650, ProteinSimple, San Jose, CA, USA) following the manufacturer's guidelines (11). Cell lysates were prepared using RIPA buffer (Cat# 89900, Thermo Fisher Scientific) supplemented with protease inhibitors (Cat# 78429, Thermo Fisher Scientific), with cells lysed on ice for 30 minutes and centrifuged at 14,000 × g for 10 minutes at 4°C. Samples were diluted in Jess sample buffer and denatured by heating at 95°C for 5 minutes, with final concentrations adjusted to 0.5–1.0 μg/μL. The Jess system was loaded with 5 μL of each sample, and proteins were separated by capillary electrophoresis, followed by immobilization within the capillaries. Immunodetection was carried out by incubating proteins with primary antibodies specific to the target proteins, PLK1 (Cat# 4535, Cell Signaling Technology, Danvers, MA, USA), KRAS (Cat#

415700, Invitrogen, Carlsbad, CA, USA), GAPDH (Cat# 9545, Sigma Aldrich), followed by HRP-conjugated secondary antibodies, Anti-Mouse Secondary HRP (Cat# 042-205, ProteinSimple) and Anti-Rabbit Secondary HRP (Cat# 042-206, ProteinSimple). After washing with Jess wash buffer, chemiluminescent detection was performed using a substrate for HRP. The resulting signals were captured and quantified using the ProteinSimple Compass software following the user manual (Compass for SW Version 7.0)."

"Cell viability assay

Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's protocol. Briefly, cells were seeded in white-walled 96-well plates (Cat# 3903, Corning, Corning, NY, USA) at a density of 1250 cells per well and allowed to grow for 5 days. 100 µL of CellTiter-Glo reagent (Cat# G9241, Promega, Madison, WI, USA) was added to each well, followed by gentle shaking for 2 minutes to induce cell lysis. The plate was incubated at room temperature for 10 minutes to stabilize the luminescent signal. Luminescence, which is proportional to the amount of ATP present and thus an indicator of metabolically active cells, was measured using PherastarFSX multi-mode plate reader (BMG Labtech, Cary, NC, USA) equipped with Luminescence Detection Module, using the following settings: integration time of 1.0 second, gain set to 3000, and top-read mode following the manufactural setting. All measurements were performed at room temperature in triplicate. Luminescence data were recorded as relative luminescence units (RLUs) and normalized to control sgRNA- or DMSO-treated samples, as previously described (12). Data were analyzed using GraphPad Prism (version 10.0), and results are presented as mean ± standard deviation."

"Gene editing by CRISPR

CRISPR-mediated gene editing was performed using the ribonucleoprotein (RNP) approach with gRNAs and Cas9 (SpCas9 2NLS Nuclease, Synthego, Redwood City, CA, USA) from Synthego. Synthetic single guide RNAs (sgRNAs) targeting KRAS, PLK1 or control sgRNAs (Gene Knockout Kit, Synthego) were designed using Synthego's CRISPR design tool and purchased pre-assembled. The sgRNAs were complexed with recombinant Cas9 protein at a ratio of 3:1 to form RNP complexes. The cells were electroporated with the RNP complexes using the Neon Transfection System (Cat# MPK5000, Thermo Fisher Scientific). Specifically, 100,000 cells were resuspended in Neon R Buffer (Cat# MPK1025, Thermo Fisher Scientific) and mixed with the RNP complex. The mixture was electroporated (program 1750/20/1) according to the manufacturer's protoco1 (13). Immediately following electroporation, the entire cell population was transferred into pre-warmed complete medium and cultured for 5 days prior to performing the cell growth assay and protein detection. No cell selection was carried out after RNP electroporation."

3. "……was measured using PherastarFSX….." need conditions of measurement, operating procedure.

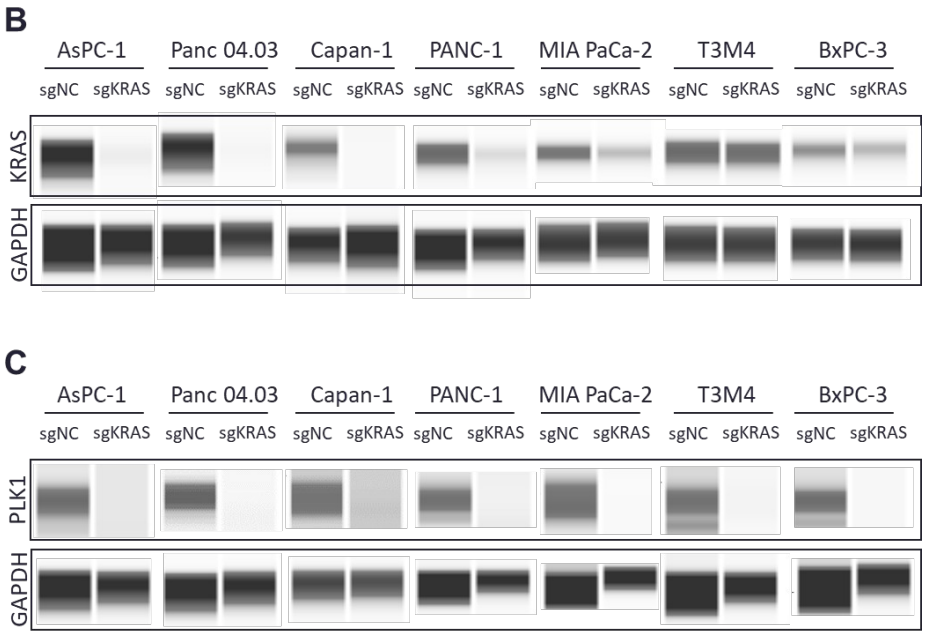
   Response:

   Thank you for your comment. I have now included the measurement conditions in the manuscript.

"The plate was incubated at room temperature for 10 minutes to stabilize the luminescent signal. Luminescence, which is proportional to the amount of ATP present and thus an indicator of metabolically active cells, was measured using PherastarFSX multi-mode plate reader (BMG Labtech, Cary, NC, USA) equipped with Luminescence Detection Module, using the following settings: integration time of 1.0 second, gain set to 3000, and top-read mode following the manufactural setting.

All measurements were performed at room temperature in triplicate. Luminescence data were recorded as relative luminescence units (RLUs) and normalized to control sgRNA- or DMSO-treated samples, as previously described (12). Data were analyzed using GraphPad Prism (version 10.0), and results are presented as mean ± standard deviation."

4. Figure 4B, KRAS KO has only been confirmed in 4 cell lines. What about the remaining three? PANC-1 KRAS does not appear to be completely KO. Explain and discuss in the manuscript.
   Response:
   Thank you for your comment. I have now included KRAS knockout data for all 7 cell lines in the revised manuscript. As noted, KRAS knockout in PANC-1 and MIA PaCa-2 cells was partial, and minimal knockout efficiency was observed in T3M4 and BxPC-3. This variable knockout efficiency likely contributes to the reduced impact on cell growth observed in these cell lines.
   I have added this explanation and discussion to the Results section of the manuscript to clarify the relationship between knockout efficiency and phenotypic outcomes.
   "Upon KRAS knockout, I observed a substantial reduction in cell viability in AsPC-1, Panc 04.03, and Capan-1 cells (Fig. 4A), underscoring the critical role of KRAS in promoting the growth and survival of these pancreatic cancer cells."
   "As noted, KRAS knockout was partial in PANC-1 and MIA PaCa-2 cells, and minimal in T3M4 and BxPC-3. This variability in knockout efficiency likely accounts for the attenuated impact on cell growth observed in these cell lines."



5. You state "……Immediately after electroporation, cells were transferred into pre-warmed complete medium and allowed to grow for 5 days for cell growth assay and protein detection….." How did you select those cells or colonies which were gene-edited? Please explain the procedure in detail.
   Response:
   Thank you for your comment. I would like to clarify that no selection of cells was performed following RNP electroporation. After electroporation, the entire cell population was directly used for subsequent analyses, including the cell growth assay and protein detection. This

approach allows me to assess the overall impact of RNP editing on a heterogeneous cell population without introducing selection bias.
I have now added this clarification to the Material and Methods section of the revised manuscript for transparency and completeness.
"Immediately following electroporation, the entire cell population was transferred into pre-warmed complete medium and cultured for 5 days prior to performing the cell growth assay and protein detection. No cell selection was carried out after RNP electroporation."

6. Did you perform the work shown in the Kaplan-Meier plot? I am assuming not. Please therefore clearly mention that work related to Figure 5 was not performed in your study but you are using those results from other studies to emphasize your point.
Response:
Thank you for your observation. You are correct that the Kaplan-Meier plot shown in Figure 5 was not generated from my own experimental data. It was obtained from The Human Protein Atlas, a publicly available resource. I have now clearly stated this in the revised manuscript, indicating that the data were derived from an external source and included to support and emphasize my findings.
"Figure 5B displays a Kaplan-Meier survival analysis (plot from The Human Protein Atlas)"
7. Provide evidence that KRAS KO does not simultaneously result in PLK1 KO, and vice' versa. Detailed explanation and discussion is required especially in the light of this published article: https://doi.org/10.1016/j.cell.2009.05.006
Response:
Thank you for your thoughtful comment and for sharing the article. The referenced publication suggests that cancer cells harboring mutant Ras may experience elevated mitotic stress and could be more dependent on mitotic proteins such as PLK1. In my experiments, complete knockout of PLK1 in 6 KRAS-mutant pancreatic cancer cell lines and one KRAS wild-type cell line (BxPC-3) consistently resulted in significant inhibition of cell growth, supporting PLK1's essential role regardless of KRAS mutation status.
In my study, PLK1 was included as a positive control to benchmark the effects of KRAS knockout on cell viability, given its well-established role as an essential mitotic regulator in various cancer types. While I did not assess PLK1 protein levels in sgKRAS-treated samples or KRAS protein levels in sgPLK1-treated samples (since this was beyond the scope of my current experimental design), I am not aware of any published evidence suggesting that KRAS knockout directly affects PLK1 protein expression, or vice versa.
To clarify this in the manuscript, I have now included the following statement in the Discussion section:
"Literature suggests that cancer cells with mutant Ras experience elevated mitotic stress and are more dependent on key mitotic regulators such as PLK1. However, in my study, the complete knockout of PLK1 in 6 KRAS-mutant pancreatic cancer cell lines and one KRAS wild-type line (BxPC-3) resulted in significant cell growth inhibition. These findings indicate that the impact of PLK1 loss is robust across genotypes and supports its role as a broadly essential gene. I did not assess reciprocal protein depletion between KRAS and PLK1 knockouts, and to my knowledge, no direct regulatory relationship between these proteins has been established."

8. References must be in consistent format throughout and must include 6 authors followed by an et al., where the number of authors is > 6. When the number of authors is <=6, all authors must be listed. A live link must be provided for each reference. Please see the formatting

requirements for the Journal for more details or use one of the published papers at the website as a template.

Response:

Thank you for pointing this out. I have carefully revised the reference list to ensure consistency with the journal's formatting guidelines. Specifically, I list all authors when the number of authors is <=6, and use "et al." when the number exceeds 6. Additionally, I have added live links to all references as required.

Thank you for addressing my comments. Accepted.