Master thesis

Title: Screening for inhibitors of different KRAS mutants, a key oncogene in nonsmall-cell lung Cancer

Student: Madina Turmagambetova

Program: Master in Molecular Medicine

PI: Dmitriy Gushchin, Assistant Professor

Co-PI: Nikolai Barlev

ORCID ID: 0009-0001-9300-1525

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ABSTRACT

Nonsmall-cell lung cancer is the most prevalent type of lung cancer accounting for 85% of all lung cancer cases. Ki-ras2 Kirsten rat sarcoma viral oncogene homolog(KRAS) belongs to the RAS family. KRAS is a common oncogenic signal transducer protein predominantly mutated in cancers at positions G12, G13, and Q61. In this study, we successfully knocked out the coding region(Exon 2) of KRAS in the H1299 cell line by CRISPR/Cas9 system. Overall 8 positive clones were detected from 128 individual colonies. The clonal subpopulations have shown various growth rates. Some clones showed higher growth rates, while others grew more slowly, due to the off-targeted effects of the CRISPR/Cas9 system. The deletion efficiency was proved by the Sanger sequencing analysis.

INTRODUCTION

1. Nonsmall-cell lung cancer: classification, risk factors, and statistics

Nonsmall-cell lung cancer is the most prevalent type of lung cancer accounting for 85% of all lung cancer cases *(Sher, T et al., 2008)*. There are two types of lung cancer cells, small-cell lung carcinoma(SCLC) and nonsmall-cell lung carcinoma(NSCLC). SCLC is more aggressive and treatment methods include chemotherapy and radiotherapy depending on the level of malignancy*(Mustafa, et al., 2016)*. In addition, its frequency is lower than NSCLC, and the incidence rate has gradually decreased since 2006 in the United States(*https://www.cancer.net/cancer-types/lung-cancer-small-cell/statistics*). At the same time, common treatments for NCSLC are surgery, immunotherapy, and stereotactic body radiation therapy depending on the level of malignancy(Oda et al., 2004). At present, combination therapy of immune checkpoint inhibitors(ICI) such as Atezolizumab and nivolumab in complex with other treatment tools has increased the median survival rate twice. They are considered the most effective treatment at any stage of NSCLC *(Reck, M et al., 2022)*.

Most of the genetic alteration in NSCLC is detected in EGFR, the tyrosine kinase growth factor receptor, and the RAS proto-oncogene family. KRAS is most likely to have point mutations at codon 12 while in-frame deletions or single missense mutations overexpress in

EGFR. In addition, somatic mutations in the EGFR gene were detected in people who never smoked (*Raso & Wistuba, 2007*). The prevalence of lung cancer depends on the risk factor, ethnicity, sex, etc. Mostly, NSCLC is categorized into 3 main groups, adenocarcinoma, squamous cell carcinoma, and large cell carcinomas. Other undefined subtypes exist.

The most common risk factor for lung cancer is smoking. Smoking cigarettes causes various genetic alterations such as the formation of covalent DNA adducts which later cause the altered protein-coding with activation of oncogene drivers or inactivation of tumor suppressor genes *(Hecht S. S.2012)*. Although smoking is the major cause of lung cancer, asbestos, radon, and polycyclic aromatic hydrocarbons are also considered a risk factor. Exposure to any of these carcinogens can cause NSCLC explaining that former smokers and non-smokers can develop NSCLC too. There are also genes with an increased risk of cancer such as TP53. Cigarette smokers and at the same time carriers of this gene are 3 times more likely to develop lung cancer than other people (*Zappa & Mousa, 2016)*.

2. KRAS molecular structure and function

Ki-ras2 Kirsten rat sarcoma viral oncogene homolog(KRAS) belongs to the RAS family. The HRAS, NRAS, and KRAS(KRAS4A, KRAS4B) proteins are the well-known isoforms of RAS genes. KRAS is a common oncogenic signal transducer protein predominantly mutated in cancers at positions G12, G13, and Q61 *(Pantsar, 2020)*. All RAS isoforms share the identical G domain and hypervariable region which is different in each isoform*(Kim et al., 2021)*. The G domain comprises 6 beta strands and 5 alpha helices, P-loop, switch-I, and switch-II. Beta strands and alpha helices form the protein core; the P-loop is where the most common mutations occur (G12D/V/C)(Figure 1). The C-terminal domain also called the hypervariable domain, helps attach to the cell membrane (Pantsar, 2020).

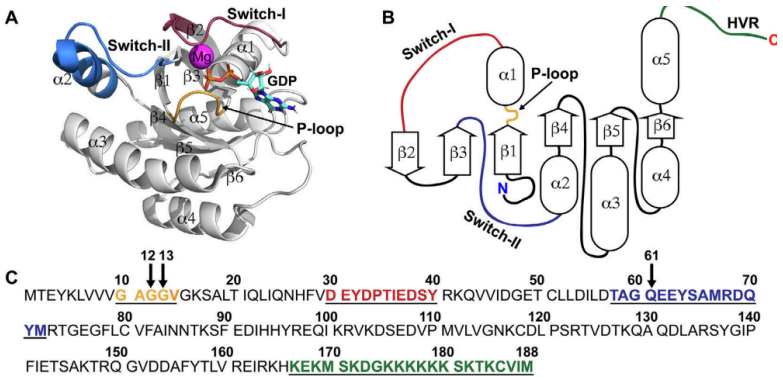


Figure1. Structure and sequence of KRAS4b. (Pantsar, T. (2020)) (A) Molecular structure of the wild type(WT) KRAS in GDP bound state. (B) Secondary structure of KRAS protein. (C) Sequence of KRAS4b. The arrows indicate the most common mutation sites.

Usually, RAS is overactivated in cancer cells and if mutated does not need upstream receptor activation to promote downstream signaling *(Kattan & Hancock, 2020)*. Normally, the RAS family links upstream effectors such as epidermal growth factor receptors(EGFR) and downstream signaling molecules, eventually leading to the activation of Ras-like signaling pathway(RAL), mitogen-activated protein kinases(MAPK), and phosphoinositide -3-kinase(PI3K). The activation of these cascades leads to cell growth, differentiation, and division.

KRAS has two regulatory proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which switch to active and inactive forms. GAPs maintain KRAS inactive conformation. When the upstream cell surface receptor such as EGFR is activated, GDP is replaced by GTP in the presence of GEFs*(Kim et al., 2021)*. This switch helps KRAS bind to its downstream kinases. Generally, KRAS works as a molecular switcher. Downstream pathways that KRAS activates include mitogen-activated protein kinases(MAPK).

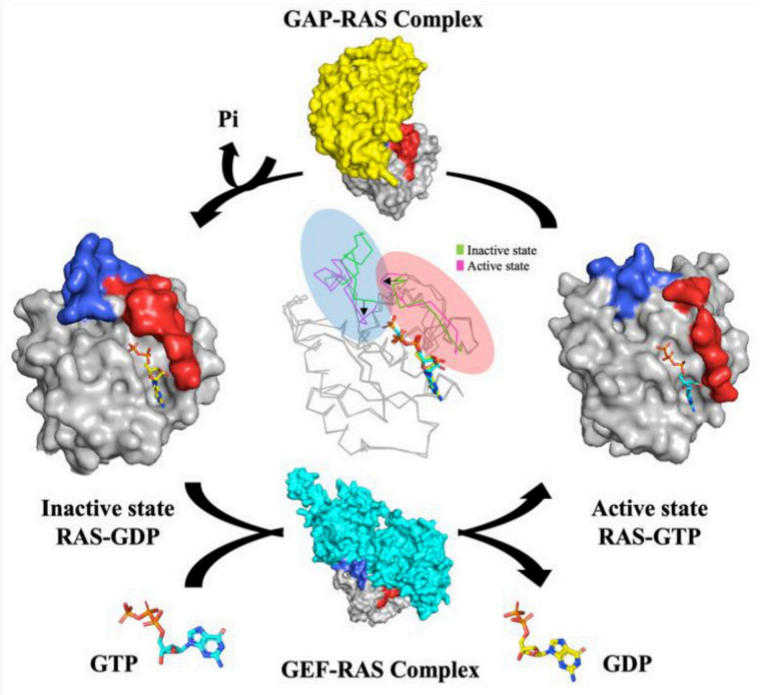


Figure 2. 3D depiction of activation and inactivation conformation of RAS by regulatory proteins(Cancers (Basel). 2021 Nov). The regulatory proteins of RAS are GEFs(guanine nucleotide exchange factors) and GAPs(GTPase-activating proteins). GEFs maintain RAS-GTP binding conformation while GAPs hydrolyze the GTP to GDP maintaining inactive conformation.

3. KRAS and downstream signaling pathways

Activation of RAF-MEK-ERK pathway

The receptor tyrosine kinases are the upstream signaling molecules of the mitogen-activated protein kinase(MAPK). The EGFR is a well-explained and major receptor tyrosine kinase for cell proliferation and growth. It is overexpressed in most of the tumorogenesis(Colorectal cancer (27-77%), pancreatic cancer (30-50%), lung cancer (40-80%), and non-small cell lung cancer (14-91%)). However high expression of EGFR is not well correlated with its overactivation *(Kriegs, 2019)*.

The EGFR is activated when its ligands bind to its extracellular receptor binding domain. Activated EGFR autophosphorylates its tyrosine residues and causes the recruitment of downstream molecules. For instance, EGFR is phosphorylated when EGF binds to it and causes heterodimerization of the receptor. It leads to the autophosphorylation and recruitment of the

GRB2-SOS(Son of Sevenless) complex. Activated GRB2-SOS complex undergoes a conformational change and works as GEF for RAS activation *(Roberts & Der, 2007)*. The RAF is phosphorylated after RAS is switched on. Then, hetero-dimerization of cytosolic serine/threonine kinases(BRAF, CRAF, and ARAF) happens. Hetero-dimerization usually takes place when BRAF activates CRAF. The mutated form of BRAF works in a RAS-independent manner. There are also other mechanisms that BRAF uses to activate CRAF, such as CRAF transphosphorylation, heat-shock protein90(hs90), and 14-3-3 mediated hetero-oligomerization*(Garnett et al., 2005)*. So, RAF activates MEK1 and MEK2(MAPKK) dual specificity protein kinases which phosphorylate ERK1 and ERK2(MAPK)*(Roberts & Der, 2007)*. The RAF-MEK-ERK signaling cascade eventually activates the genes for cell proliferation, migration, and differentiation *(Huang et al., 2021)*.

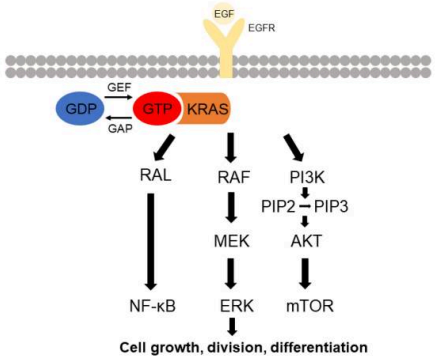


Figure 3. KRAS and downstream signaling pathways(Cancers (Basel). 2021 Nov) Activation of RTKs leads to the KRAS activation. The regulatory proteins of RAS are GEFs(guanine nucleotide exchange factors) and GAPs(GTPase-activating proteins). GEFs maintain RAS-GTP binding conformation while GAPs hydrolyze the GTP to GDP maintaining inactive conformation. The main effector pathways of the RAS are RAF-MEK-ERK, PI3K-AKT, and RAL pathways. All of them are important in cellular neoplasia, apoptosis, and division.

PI3K activation by RAS and their interaction

Phosphatidylinositol 3-kinases(PI3K) are lipid kinases important in cell proliferation and apoptosis signaling pathways and it is another effector pathway of RAS. There are three well-known variations of PI3K: class I, class II, and class III (Shuttleworth et al., 2009). The hyperactivation of PI3K is established in most human cancers, especially in the colon, gastric,

lung, and brain(Figure 4)*(Samuels, Yardena, et al. 2004)*. In this study, scientists sequenced tumor cells of patients to evaluate the prevalence of PI3K mutations. They analyzed that from 234 colon cancer cells, 74 tumors represented mutations in the PIK3CA gene; out of 15 brain cancer cells 4 PIK3CA gene mutations were detected; for gastric tumorigenesis 3 out of 12 tumor cells mutated in the PIK3CA gene; one mutation in breast cancer cells out of 12 and one in lung cancer cells out of 24 tumor cells represented mutations in PIK3CA gene.

PI3K can be activated by at least three mechanisms (Figure 5). The direct activation happens through the regulatory subunit of PI3K by RTKs, also this activation can happen by GRB2 molecule, and the last activation happens by active RAS through its effector site*(Rodríguez-Viciana et al., 1994)*. The RAS activates directly the catalytic subunit(p110) of PI3K. The activation of PI3K causes the formation of PIP3(phosphatidylinositol 3,4,5-triphosphate) from PIP2(phosphatidylinositol 4,5-triphosphate). This signaling is highly detected in tumor progression*(Castellano, E., & Downward, J. (2011)*. PIP3 phosphorylates PDK1 in the PH domain and recruits into the cell membrane. Subsequently, PDK1 activates AKT by phosphorylation of threonine residue 308*(Castellano, E., & Downward, J. 2011.)*

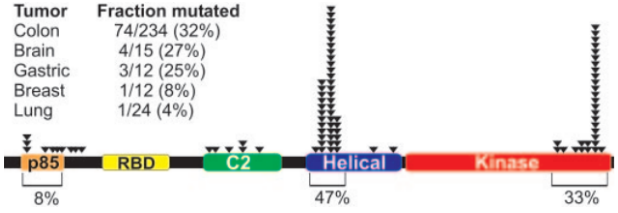


Figure 4. Mutations in PIK3CA(Samuels, Yardena et al.) Arrowheads indicate the location of missense mutations, and boxes represent functional domains (the p85 binding domain, Ras binding domain, C2 domain, helical domain, and kinase domain).

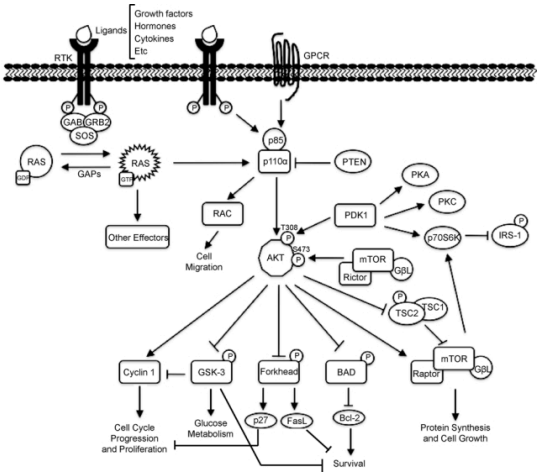


Figure 5. PI3K activation pathway. The regulatory domain of PI3K is directly activated by RTKs. The GTP-binding RAS can activate the catalytic subunit of PI3K p110. The PTEN(phosphatase and tensing homolog) is the regulator of PI3K and dephosphorylates PIP3 to PIP2. AKT is activated when the PH domain of PDK1 is phosphorylated by PIP3. This phosphorylation recruits the PDK1 and AKT to the cellular membrane, and then PDK1 activates AKT by phosphorylation at threonine residue.

There are four main hot spot regions for the interaction of RAS RBD(receptor binding domain) and p110a domain*(Pacold et al., 2000)*. Two of them were selected to express mutant PI3K in mice that lack RAS binding ability. They are threonine 208 and lysine 227 in mammalian pl10α. The analysis showed inhibition of RAS-p110a domain interaction leads to defects in lymphatic development and inhibits in vitro transformation of fibroblasts, however, these mice models are resistant to RAS oncogene-induced tumorigenesis*(Gupta et al., 2007)*.

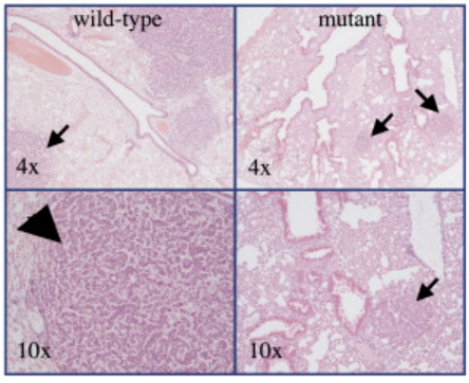


Figure 6. H&E staining of oncogenic K-Ras mouse lungs. The arrows indicate small adenomatous hyperproliferations seen in the lungs of Pi3kca mutant mice. The large arrowhead indicates a typical adenocarcinoma in a mouse with wild-type Pi3kca.

Ral-NF-kB pathway

The interaction between KRAS oncogene and Nuclear factor kappa B(NF-kb) pathway is still not well understood. However, some studies illustrated that glycogen synthase kinase-3 (GSK-3) which is downstream of KRAS implicates the canonical activation pathway of NF-kB. The GSK-3 activation leads to the expression of TGFbeta activated kinase 1(TAK1) which leads to the degradation of IκBα and activation of NF-kB. TAK1 phosphorylates the IKKβ which in turn phosphorylates inhibitors of kB alpha *(Pak, C., & Miyamoto, S. 2013)*. In addition, these authors identified that GSK3 can activate the noncanonical activation pathway of NF-kB that happens through IKKα. When they depleted the GSK3 in KRAS-positive Pancreatic ductal adenocarcinoma(PDAC cells), the levels of p100 and p52 subunits also decreased meaning that the noncanonical activation pathway was inhibited.

4. Common RAS oncogene-inducing mutations detected in NSCLC

G12C and its inhibitors.

Even though Kras mutations were first discovered in 1980, it is still problematic to target them. One of the main reasons is the picomolar affinity of GDP and GTP *(Mao et al., 2022)*. Several common somatic mutations occur in codon 12 that eventually lead to impaired GTP hydrolysis activity. As a result, more GTP binds to the effector site of the KRAS protein and keeps it in an active state*(Qunaj et al., 2023)*. When glycine is replaced by cysteine it gives the protein a unique nucleophilicity property. Cysteine thiols can be a target for allosteric inhibitors and help monitor their binding affinity to cysteine residue(G12C)*(Ostrem et al., 2013*). The Food and Drug Administration approved Sotorasib and Adagrasib for clinical use in the treatment of NSCLC. The Adagrasib has a longer half-life(24 hours) than Sotorasib(5.5±1.8 hours). Acquired resistance to these therapies is the main problem because cancer etiology is specific for each patient. Co-occurring mutations in genes such as TP53, KEAP1, and STK11 and secondary mutations in KRAS decrease the efficacy of therapy and increase the rate of acquired resistance*(Oya & Mitsudomi, 2023)*.

G12D and its inhibitor

The other well-known KRAS point mutation is G12D. It impacts more than 50% of pancreatic ductal adenocarcinoma cases, lung cancer, and some other cancer types. Scientists testified a compound that binds aspartic acid of G12D that forms salt bridge interaction and induces allosteric pocket-switch-II. These inhibitors could bind the GTP-bound and GDP-bound state of KRAS G12D, while G12C inhibitors bind only the GDP-bound state. As a result, those inhibitors decreased MAPK signaling leading to tumor regression *(Mao et al., 2022)*.

5. Crispr Cas9-mediated gene knock-out

Clustered Regularly Interspaced Short Palindromic Repeats(CRISPR cas9) system helps to generate knock-out cancer cell lines by guide RNAs (gRNA). Gene knock-out helps us to study the role of specific regions. The manipulated DNA can be repaired by non-homologous end joining(NHEJ) or homology-directed gene repair(HDR). The NHEJ causes small insertions or deletions(INDELs) in the gene leading to premature termination codons(PTCs)*(Tang et al., 2018)*. Generally, the sgRNAs guide Cas9 to the target site, and Cas9 makes double-stranded breaks.

Materials and methods

1. Extraction of plasmids: KRAS(wt), G12V, G12C, G12D

    a. Making media LB and LB+agar

The LB broth and LB agar were provided by the university. The formulation per liter was 10g Peptone, 5g yeast extract, and 5g sodium chloride, for LB agar 12g of Agar was used. All the ingredients were mixed properly in Milli-Q water after autoclaved for further work.

    b. Plasmid extraction from filter paper

Plasmids were ordered and received from the Addgene company. Half the filter paper is cut with sterilized scissors and placed into 50ul of milli-Q. Gently mix with vortex and incubate at room temperature for 5-10 minutes. Plasmids are ready for further usage.

    c. Cellular transformation into DH5α competent cells

Before the transformation prepare Petri dishes with LB+agar and ampicillin. Turn on the water bath at +42 degrees. 200ul of DH5α cells were thawed on ice for 10 minutes. For 50ul of competent cells 5 ul of extracted plasmids were placed, gently mixed with tips, and incubated on ice for 30 minutes. Heat shock cells for 45-60 seconds and return to ice for 3-5 minutes. Added 500ul of SOC media and incubated at +37 degrees for 1 hour. After incubation centrifuge cells for 5 minutes and delete 400ul of media. Resuspended pellet in 100ul of media and placed gently on LB+agar with ampicillin 100mg/ml. Leave petri dishes overnight at +37 degrees.

The next day, few colonies were selected for each plasmid and placed into 5ml of LB with ampicillin for mini-prep plasmid extraction.

1.1 Mini-prep plasmid extraction

Centrifuged 5ml of bacterial culture for 5 minutes, 1700g. Deleted the supernatant and added 250ul of Resuspend solution. Gently resuspended the cells. The same quantity of the lysis solution was added to break the cell membrane. Incubated at room temperature for 1 minute and added 350ul of neutralization solution. Incubated at room temperature for 1 minute and centrifuged for 10 minutes at high speeds(7000g). Prepare the new 1.5ml Eppendorf tube with the spin column. Place the spin column into the tube and add 20ul of endotoxin removal solution. After centrifugation collect the supernatant gently and put it into the spin column. centrifuged for 30 seconds at high speeds. At this step, we expect the plasmid to stay in the column. To provide efficient binding of target DNA with the column, we added 200ul of endotoxin removal solution again and centrifuged for 30 seconds. The column was washed with 700ul of washing buffer, centrifuged and the supernatant deleted. After centrifuging the empty column for 1 minute to fully delete the washing buffer. Then the column should be dried for 5 minutes to evaporate the ethanol. Too much drying time can cause the DNA degradation. Finally, the DNA was eluted with 50ul of Elution solution. Extracted plasmids are kept in the freezer at -20 degrees.

1.2 Restriction digestion reaction for extracted plasmids

a. 1% Agarose gel preparation

Firstly, the 50x TAE buffer was prepared and diluted to 1x. To make 1L 50x TAE buffer 0.5M EDTA(pH8.0), 2M TRIS, and 57.1 ml acetic acid were mixed. 20ml of 50x TAE was

diluted in 1000ml of milli-Q water to make 1x buffer. Agarose was provided by the university. For 1% agarose gel, 1g of agarose melted in 100 ml of 1x TAE buffer.

b. Set up restriction endonuclease digestion solution

For each plasmid, 3 solutions were prepared. All the plasmids were cut with HindIII, HindIII&Xbal, HindIII&EcoRI.

Restriction Enzyme	10 units are sufficient, generally, 1µl is used 1. HindIII 2. XBal 3. EcoRI
DNA	1 µg 1. Kras 2. G12V 3. G12C 4. G12D
10X Cutsmart	2.5µl (1X)
Total Reaction Volume	20 µl
Incubation Time	1 hour*

Incubation Temperature	37°C

1.3 Midi-prep plasmid extraction

The overnight 300ml of bacterial culture was transferred to 500ml centrifuge tubes. Centrifuges at high speeds for 15 mins at +4 degrees and the supernatant was discarded. The pellet was resuspended in 5 ml of resuspension solution with RNAse. Mixed and vortexed gently to make the suspension homogenous. The same quantity of the lysis solution was added and mixed by shaking. The suspension should be viscous. Incubated lysed cells for 4-5 minutes at room temperature and added 5ml of neutralization solution. Should be gently mixed by inverting the tube. Then, the cells for 20 minutes, 3900g at +4 degrees to collect the supernatant. The new tubes with spin columns were prepared and 1 ml of endotoxin removal solution was added to the column. Then purified bacterial lysate was transferred to the column. The tube with the spin column was centrifuged for 3 minutes 1000g at room temperature and the supernatant was discarded. The 1 ml of endotoxin removal solution was added and the column was centrifuged for 1 minute 3900g at room temperature. The spin column was washed with 15 ml of washing buffer 2 times for 1 minute, 3900g at room temperature. The supernatant was removed. The empty spin column was centrifuged to fully remove the solution. For elution, deionized water was pre-heated at +50 degrees in the water bath. The spin column was placed into the new tubes and 500ul of pre-heated deionized water was added and centrifuged to collect the DNA. The filter was eluted with 500ul of deionized water. To clarify the DNA the eluted solutions were centrifuged for 5 minutes at the highest speed, and the supernatant was transferred to the new 1,5 ml Eppendorf tubes. Extracted DNAs kept at -20 degrees.

2. Build a sgRNA expressing vector

2.1 Annealing

The target sgRNA oligonucleotides and reverse oligonucleotides were ordered from ANL facility. First, these forward and reverse oligonucleotides should anneal. They compose 5’CACC(g-19bp-sgRNA)3’ and 3’(c-19bp-sgRNA) CAAA5’ sticky ends. They mixed in 1x

NEB2 buffer in 1:1 proportion and annealed using a T-100 PCR machine. Protocol for PCR machine:

+95C 3 min

+95C to +85C in -2C/sec increment

+85C to +25 in-0.1 C/sec

Hold at +4C(total time is 15 minutes)

2.2 Set ligation reaction for each sgRNA

The vector plasmid pRG2 was provided by the supervisor and it was already cut with restriction endonuclease Bsa1(double cut). The ligation reaction mix was composed of 5.5il of milli-Q, 2il 5x of ligation buffer, 1ul of T4 ligase, 0.5ul pRG2(-GG), and 1 ul of annealed gRNA double-stranded nucleotide. The mixture is incubated at room temperature for 30 minutes and transformed into competent cells DH5α.

2.3 Transformation into competent cells

For 50ul of competent cells 5 ul of ligation mixture was added. Gently mixed with tips, and incubated on ice for 30 minutes. Heat shock cells for 45-60 seconds and return to ice for 3-5 minutes. Added 500ul of SOC media and incubated at +37 degrees for 1 hour. After incubation centrifuge cells for 5 minutes and delete 400ul of media. Resuspended pellet in 100ul of media and placed gently on LB+agar with ampicillin 100mg/ml. Leave petri dishes overnight at +37 degrees.

3. Cell culture

3.1 Cell thawing

Before the cell thawing complete DMEM media was prepared. The complete DMEM(Gibco) is composed of 10% of fetal bovine serum(Capricorn DMEM), and 1% of penicillin-streptomycin antibiotics. The supervisor provided the frozen vial of H1299 lung adenocarcinoma cells and the DMEM. The cells were plated into a +37 degree water bath for 5 minutes to thaw them and resuspended in 5 ml of complete DMEM media. The cell suspension

was centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and the pellet was resuspended in 5 ml of complete DMEM media. The resuspended cells were transferred into a T75 culture flask with 20 ml of complete DMEM media, and stored in an +37 degrees incubator for further cell passaging.

3.2 Cell culturing and passaging

After a day of incubation, the cell confluency was observed under the light microscope. When the confluency reaches 90-95% cells can be used for further analysis. The complete DMEM media was aspirated and 3 ml of TrypLE(Gibco) was added, and incubated for 10 minutes in a +37degree incubator. When most of the cells are detached from the flask, transfer the cells with TrypLE into a 15 ml Falcon tube. Centrifuge falcon tube for 5 minutes at 1000 rpm. The cell pellet was resuspended in 4 ml of fresh DMEM media. Into two T75 flasks were placed 20ml of fresh DMEM media. Resuspended cells were transferred into the flasks, and stored in an +37 degrees incubator with a supply of 5% CO2 for further cell passaging.

3.3 Cell freezing

When the cell confluency reached 90-95%, the DMEM media aspirated. Then 3 ml of TrypLE was added and incubated for 10 minutes at a +37 degree incubator. Poured all TrypLE and cells into a 15 ml falcon tube and centrifuge at 1000rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 5 ml of freezing media. Freezing media preparation: 40% DMEM, 50% FBS, 10% DMSO. Transfer 1 ml of cells with freezing media into a 1.5 ml tube.

3.4 Cell counting

When the confluency of the cells was 80-90% we counted the concentration of viable cells. The H1299 cells incubated with TrypLE and the cell pellet were resuspended in 3 ml of complete DMEM media as mentioned above. From that suspension 10ul of aliquot was placed into the hemocytometer.

The final cell concentration was calculated using the formula:

x = an average number of cells/4x10^4

Average number of cells=viable cells/4

10^4= factor to convert the square area of the hemocytometer in 1 ml cell volume.

3.5 Passaging cells into a 6-well plate

From the last step, we had an overall 3 ml of cell suspension for 6 well-plate. The required well number for transfection is 7. To each plate, 3ml of complete DMEM media was poured. After, 5x10^5 cells were plated to each well for further analysis.

4. Cellular transformation

4.1 Transfection

The cell confluency has to be reached 70-90% in 6-well plates for the transfection reaction. These 6-well plates hold 6 combinations of 4 sgRNA extracts. For instance: 1-2, 1-3, 1-4, 2-3, 2-4, 3-4. One more well was used for GFP control. The 0.5 – 0.75 ug each of the 2 target sgRNAs and 5ug Cas9 plasmid were diluted in 0.5 ml Opti-MEM medium and mixed with 0.5 ml Opti-MEM medium and 15 ul of Lipofectamine 2000. The 1 ml of transfection solution was mixed by vortex and incubated for 10 minutes. The complete DMEM media was aspirated from a 6-well plate and replaced by 1 ml of transfection media. Then, cells were incubated in the incubator for 4 hours at +37°C. After 4 hours the transfection solution was replaced by DMEM media without antibiotics(penicillin/streptomycin). The next day the media was replaced by complete DMEM media. After 3 days transfection efficiency was detected.

4.2 Deletion detection

The 50 ul of TrypLE was used to collect the ¼ of the cells and DNA extracts have been prepared. The rest of the cells were kept for further analysis. The PCR machine was set up for one cycle of 5 min at 95C, 20 min at 60C, 5 min at 95C, and cool down to 16C. When cellular suspensions are at 60C we have added 1/10 volume of 1mg/ml Proteinase K (NEB #P8107S). 1/10 volume of the DNA extracts was used to conduct the PCR reaction. The deletions have been detected in 2% Agarose gel electrophoresis.

4.3 Cell cloning

The remaining cells were collected by 250 ul of TrypLE, spun down, and resuspended in 200 ul of complete DMEM media. Then 10 ul of aliquot of cells were diluted in 90 ul of Trypan Blue solution. To make limited dilution cloning the exact concentration of cells was counted. The cells were diluted three times by 10x. The final purpose was to get 1 cell per well.

Prepared 3 plates for each ocombination of knockouts; 2 with 1 cell/well and 1 with 2 cells/well. 200 μL of complete DMEM media was added for each well.

The calculation for dilution:

Number of cells —x

10^5—2 ml

X gives us the number of cells for Dilution 1.

Dilution 1 (D1)	2 ml complete DMEM+X number of cells
Dilution 2 (D2)	2 ml complete DMEM+X number of D1
Dilution 3 (D3)	2 ml complete DMEM+X number of D2
Dilution 4 (D4)	20 or 10 ml complete DMEM+X number of D3

4.4 Screening of the clones

Colonies after cell cloning can be visible after 5-7 days under the microscope. The important step is to identify where the single colonies are rising. At this step, wells with two or

more colonies should be eliminated. The media from side wells evaporates very fast, therefore always check plates. Otherwise, cells can die.

Colonies from single clones were raised for 3 weeks. When the confluency of the cells reached 80-100% they passaged into 24-well plates and to confirm the deletion rate 10 ul of the cells went to the PCR analysis. The cells were trypsinized with 50 ul of TrypLE and incubated for 2-3 minutes. 40 ul of the cells were transferred into 24-well plates with 1 ml of complete DMEM media and 10 ul were transferred to PCR tubes.

Preparation of DNA extracts

PCR setup:

95°C-5 min

60°C-20min

95°C-5 min

16°C-∞

When the cellular suspension is at 60°C we used 1/10 volume of Proteinase K(NEB#P8107S).

PCR reaction for screening of the clones

PCR clone analysis is performed to identify clones with 100% deletion. PCR set up:

95°C-3 min

95°C-30 sec

52°C-1 minute

72°C-40 sec

40 cycles

72°C-5 min

The total volume of the PCR reaction was 20 ul.

5. Western Blot

Cell lysate preparation

The plate with positive clones was prepared for protein sample extraction. When the confluency of the cells reached 90-100% cells were washed with 500 ul of ice-cold PBS twice.

Then, cells were lysed in 100 ul of RIPA buffer with 1mM phenylmethylsulfonyl fluoride(PMSF). RIPA buffer consists of 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 5 mM EDTA, and 1% Triton-100. PMSF is degraded during the day and added before the sample collection. After adding RIPA buffer, using a cell scraper cells were collected and transferred into a 1.5 ml Eppendorf tube. The protein samples were kept on ice during all steps.

Sample preparation

First, we determined the concentrations of each protein sample and calculated how much we needed for 4x Laemmli buffer. We determined that 10 ul of Laemmli buffer can be used for our 30 ul of lysates. Samples in Laemmli buffer were denatured at 95°C for 5 minutes. Samples are ready for SDS-Page gel electrophoresis.

SDS-Page gel electrophoresis

The expected protein size for our lysates was 21kDa, so 12% Gel was prepared. The protein samples were loaded into wells to run the gel, and the electrophoresis apparatus was filled with 1x running buffer. Gels run for 1,5 hours at 100 V.

Separating gel recipe:

Bis-acrylamide - 4 ml

Tris-HCl pH 8.8 - 2.5 ml

H2O - 3.3 ml

SDS 10% - 100 ul

APS 10% - 80 ul

Temed - 10 ul

Stacking gel recipe:

Bis-acrylamide - 500 ul

Tris-HCl pH 6.8 - 1 ml

H2O - 2.4 ml

SDS 10% - 40 ul

APS 10% - 50 ul

Temed - 7 ul

4x Laemmli buffer recipe:

Tris 0.5M pH6.8 - 2 ml

Glycerol - 1.6 ml

SDS 10% - 3.2 ml

2-mercaptoethanol - 0.8 ul

Bromophenol blue1%-0.8 ml

10x running buffer

30.3 g Tris-base

144.0 g glycine

Protein transfer

To assemble the transfer sandwich we used the wet transfer method. The 10X Transfer buffer, membrane, filter paper, transfer cassette, and sponges were prepared before the experiment.

The membrane was first wet in 96% ethanol and then soaked into a transfer buffer. The gel, filter papers, and sponges are also pre-soaked in 1X Transfer buffer for 10 minutes. Then the transfer sandwich was collected and placed into the transfer cassette. The transfer setup runs overnight at 20V. The next day we expected that the membrane contained target proteins.

1X Transfer buffer recipe:

25 mM Tris base

192 mM glycine

20 % methanol

Immunoblotting

At first, the membrane was incubated in 1X TBST buffer for 10 minutes and then incubated in blocking buffer for 1 hour at room temperature. Second, the membrane was incubated overnight at +4°C in a primary antibody solution. We have used RAS antibodies, which contain monoclonal antibodies against KRAS, NRAS, and HRAS. The next day, the membrane was washed with 1X TBST for 30 minutes and incubated with a secondary

antibody(Antirabbit IgG) solution for 2 hours. After this step, the membrane was washed and went to the detection step.

1X TBST buffer

20 mM Tris base
150 mM NaCl
0.1% Tween-20

Blocking buffer

1X TBST
5% non-fat dry milk OR 5% BSA

Primary antibody solution

Diluted antibody in 1X TBST with 1% milk

Secondary antibody solution

Diluted antibody in 1X TBST with 1% milk

Detection

ECL substrates were prepared before the experiment in 1:1 proportion. The membrane was incubated in ECL for 2-5 minutes and proteins were detected with the chemiluminescent imaging system.

4. Sanger sequencing

PCR

To obtain the nucleotide sequence of the target DNA fragment we designed Sanger sequencing. The PCR products of positive clones were used to run the PCR with Big Dye solution. According to the size of the PCR product, the exact quantity of primer and Big dye solution was calculated.

Mixture ratio:

Reverse primer - 2 ul (10pmol/ul)

Big dye - 2 ul

Plasmid - 4 ul

H20- 2 ul

Cycler program:

95°C -1 min

95°C -10 sec

52°C -5 sec

60°C -4 min

25 X

4°C - ∞

DNA precipitation

The sodium acetate solution that contains 37.5 ul 3M CH3COONa ph 5.2, 782.5 ul of ethanol(96%), and 180 ul of dH2O have been prepared. The 100 ul of this solution was added to the sequence reaction and incubated at room temperature for 30-40 minutes. The mixture was centrifuged at 13200g for 30 minutes at +4°C. The precipitant was washed with 75% ethanol and centrifuged at 13200g for 30 minutes. The pellet then dried at room temperature for at least 30 minutes. Then the pellet was resuspended in Hi-Di formamide. The sequence was denatured at 95°C for 2 minutes and went to the sequencing machine.

Results

1. Extraction of plasmids: KRAS(wt), G12V, G12C, G12D

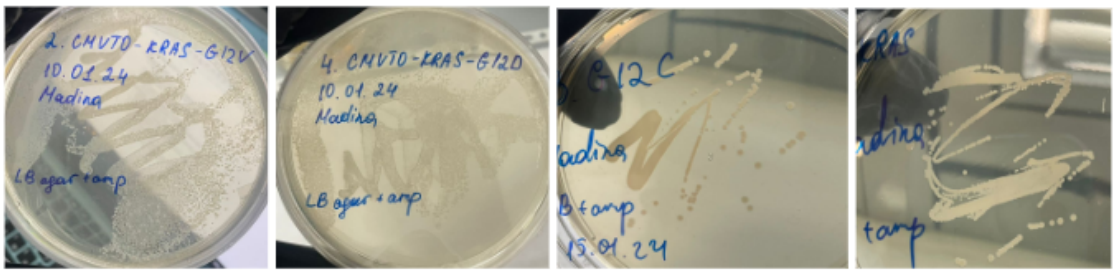


Figure 1. Plates with bacterial colonies containing amp-resistant plasmids.

Clones after transformation were detected at 1 day of incubation at 37°C. The individual clones were selected for inoculation of enriched bacterial culture.

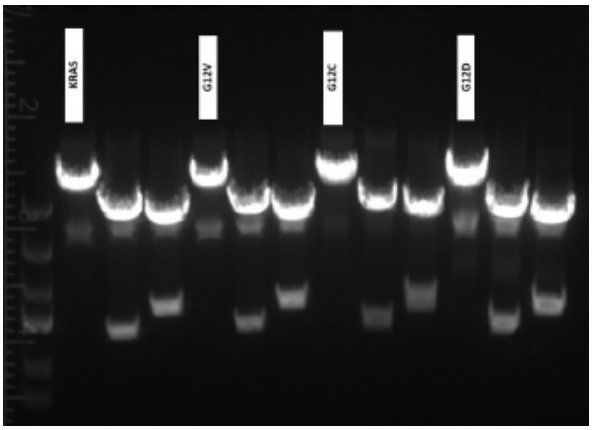


Figure 2. Picture of gel with plasmids restriction analysis.

Mini-prep plasmids were used to make restriction maps for extracted plasmids. The expected lengths were 5.7 kb for HindIII. 4.5 kb and 1.2 kb for HindIII+XbaI, and 4.2 kb and 1.5 kb for HindIII+EcoRI digestions. All plasmids show the identical positive results.

2. Build a sgRNA expressing vector

All the annealed oligonucleotides were inserted into vector pRG2 and ligated for 30 minutes. Followed by transformation into DH5α.

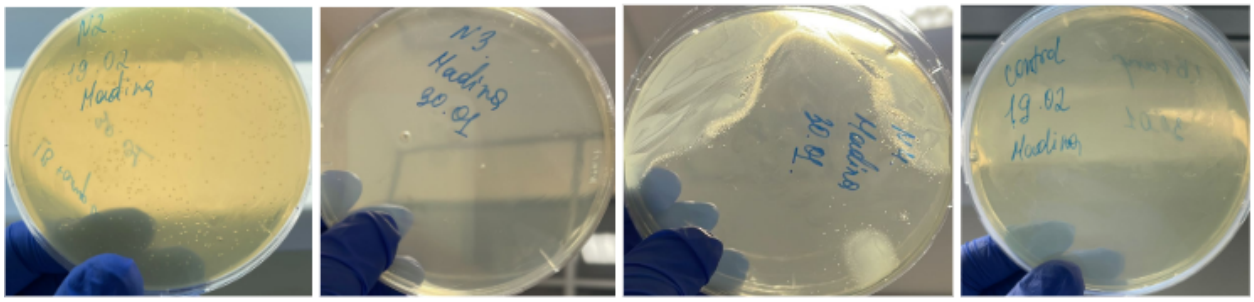


Figure 3. Clones after transformation.

The clones were growing differently. Plate 2 has shown a high number of clones. Plates 3 and 1 grew similarly and only 2-3 clones can be visible. In plate 4 we can detect huge colonies. Individual clones were selected for enriched bacterial growth to check the ligated sgRNAs.

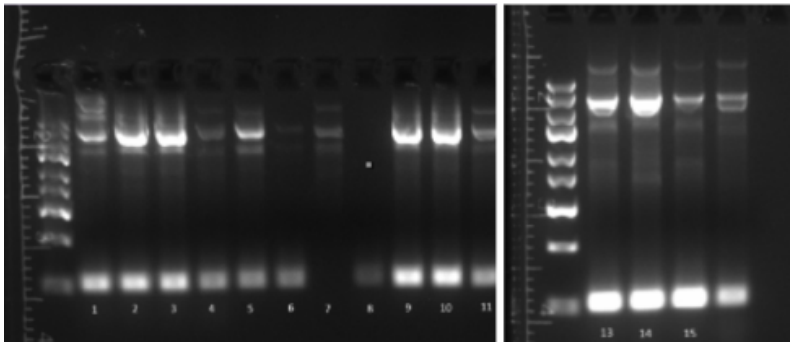


Figure 4. PCR analysis of mini-prep clones.

U6 primer and reverse primers for each clone were used to conduct the PCR reaction. The expected length for sgRNAs was 100bp. №1,2,3 are the clones from plate 1(sgRNA 1) the №4 is control for sgRNA1. For control, the plasmid from other sgRNA was used. As we can see control also gives a band for sgRNA. This can be a pipetting problem or primers find the complementary sequence from the other sgRNA plasmid. The only 2 clones were selected for sgRNA2(№5,6,7). The №7 was the control. This is a clear depiction of how control must look like. The pipetting problem happened for sgRNA 3 and 4 as well.

3. Cellular transfections

3.1 Transfection

The alive and growing cells were detected in all plates. To show that transfection happened successfully GFP proteins were checked under a microscope.

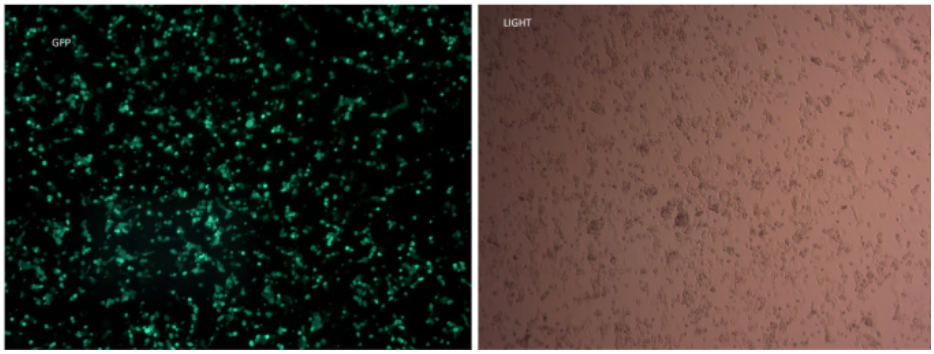


Figure 5. After three days of transfection, GFP control plasmid.

Most of the cells were alive after transfection and were growing well. It was impossible to calculate the exact transfection efficiency until the cell cloning. However, the GFP control reaction has shown that transfection was done successfully.

3.2 Deletion detection

To prove that gene deletion happened in H1299 cell lines, 1/10 of cells from the 6-well plate were collected by TrypLE. The PCR reaction has been performed with their DNA extraction.

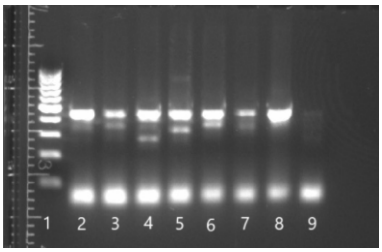


Figure 6. PCR analysis of deletion detection

Deletions happened with all sgRNA combinations. Parental KRAS is present in all samples(440bp). Lower bands from parental KRAS indicate the presence of a deletion in the gene. 1- Ladder; 2- GFP control; 3-combination of sgRNA1,2; 4-combination of sgRNA 1,3; 5-combination of sgRNA 1,4; 6-combination of sgRNA 2,3; 7-combination of sgRNA 2,4; 8-combination of sgRNA 3,4; 9-water. For further cell cloning, we selected combinations of sgRNA 1,3 and 1,4

3.3 Cell cloning

Cell counting before limited dilution cloning

	sgRNA1+sgRNA3	sgRNA1+sgRNA4
Number of cells	35x10^5	55x10^5

Limited dilution cloning

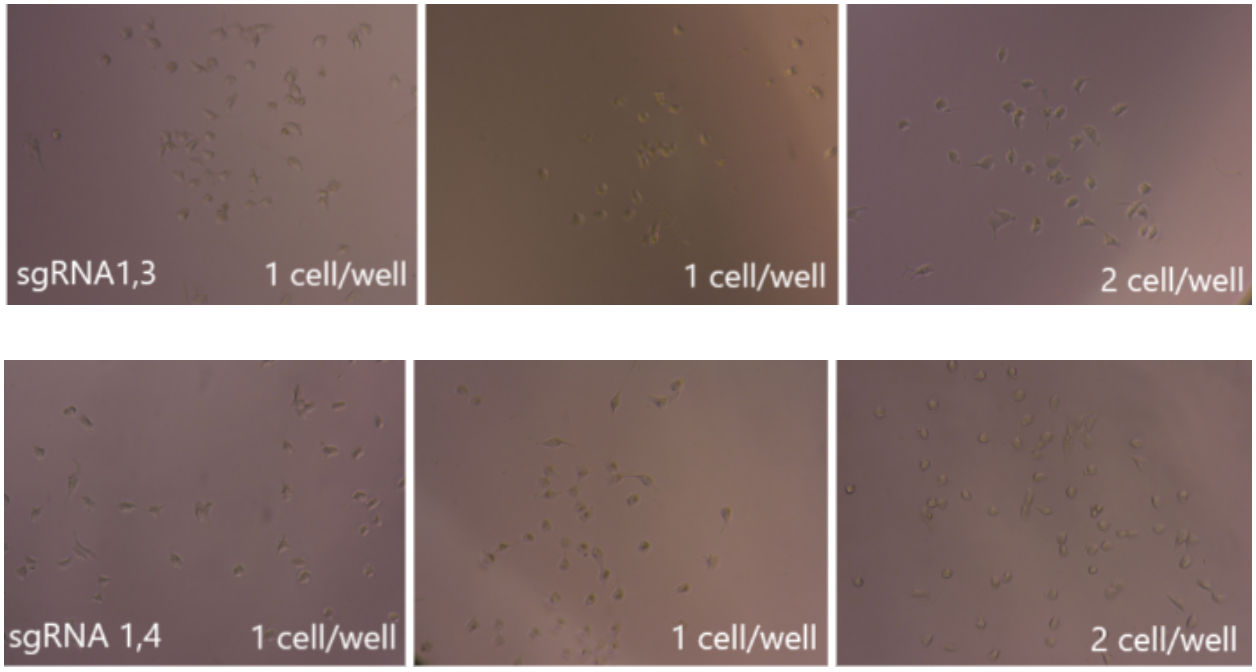


Figure 7. Clones from the 96-well plate after a week of incubation.

Single colonies of clonal subpopulations were a priority because the aim was to identify the cells with deletion in both chromosomes. Individual colonies and two or three colonies of cells per well were identified in the 96-well plate.

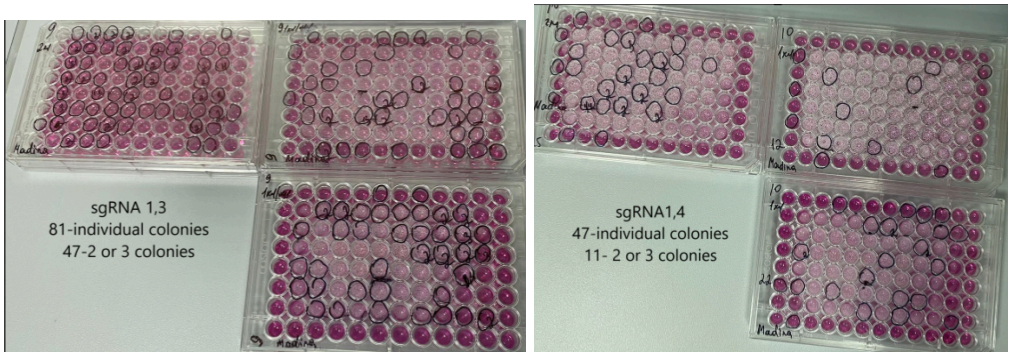


Figure 8. 96-well plates. The depiction of clone counting after 2 weeks of incubation.

3.4 Screening of the clones

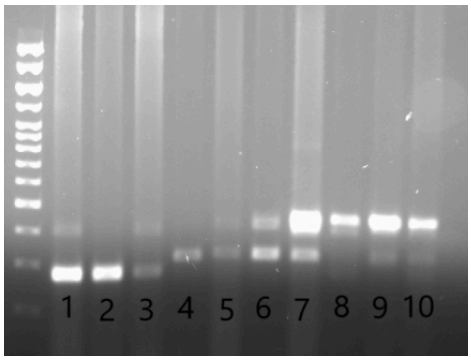


Figure 9. Screening of clones from 24-well plates.

After 3 weeks of incubation, it was detected that half of the colonies died, and others passaged into a 24-well plate. Figure 9 represents clones with full deletion and clones where deletion happened only in one chromosome. The numbers 2 and 4 are the positive clones for sgRNA combinations 1&3 and 1&4. The numbers 1,3,5,6,7, 9, and 10 contain full-length KRAS, 440 base pairs.

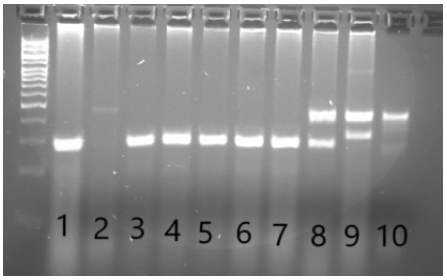
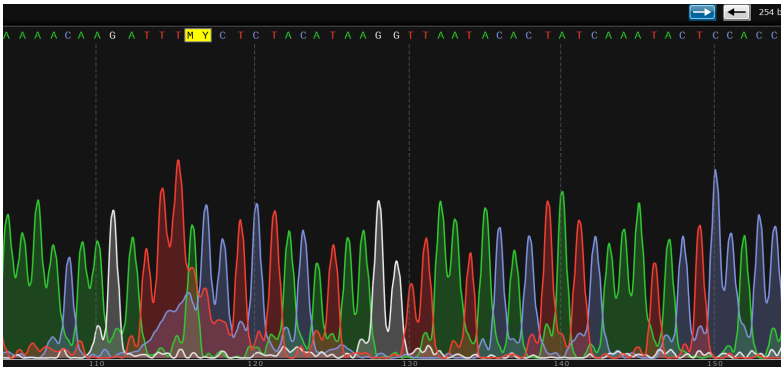


Figure 10. Final PCR analysis for positive clones.

This figure represents the final PCR analysis for positive clones. Overall there were 8 positive clones. The number 2 is a mistakenly selected clone. The numbers 8 and 9 are controls from pool cells for sgRNA 1&3 and 1&4. There is no clone for combination 1&4 in this gel because that clone had a very low growth rate. It was impossible to do PCR analysis with a small number of cells.

Sanger sequencing

All six positive clones were tested with Sanger sequencing. Even if they cut with the same sgRNAs the sequencing results were specific for each clone. Only clone 4 has shown a clear chromatogram and 100% match with wild-type KRAS.



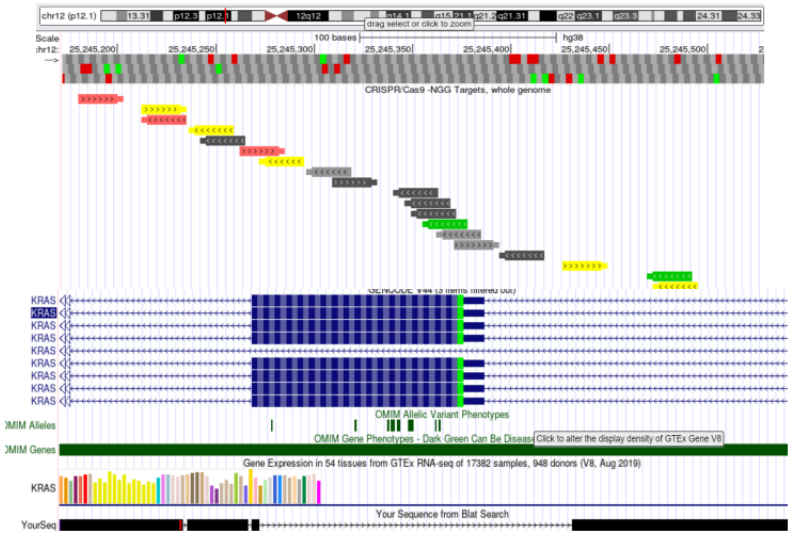


Figure 11. Representation of clone 4 sequence compared to full-length kRAS in the genome browser. The lowest black bar represents the sequence from clone 4.

The upper red, yellow, and black boxes are sgRNA sequences that can be used. Below represented wild-type KRAS. the blue color represents the exon 2. The lowest black line shows the presence of deletion for our clone 4. The combination of sgRNA 1&3 has deleted whole exon 2.

Discussion

1. Different growth rates of clones

In the pool of cells, there were several different perturbations may happen. Our main target was to select clones with the kRAS KO through the deletion of exon 2. The off-target effects of the CRISPR/Cas9 systems may happen. That is why the growth rates of our positive clones may be different. We speculated that the deletion of KRAS coding exon leads to the inhibition of downstream signaling cascades, such as MAPK, PI3K, and RAL-NfKB. Eventually, this leads to decreased cell proliferation and differentiation. However, we analyzed all clones despite the growth rate. Clone 4 grew more slowly than some other clones. The clones that grow very fast have shown the deletion at the correct site of the coding region, but sequence analysis has not shown the presence of wild-type KRAS. In a study by Panda, A., et al. scientists studied the CRISPR/cas9 off-target effects in independent 3 experiments at different universities. Results were similar for all three experiments, and they detected each clone represented a unique

mutation and altered copy number. In our study, we were confined to Sanger sequencing analysis because of the time limitation. In addition, the more clones there are, the higher the probability of getting the right genetically engineered clones.

2. Optimization of bacterial cellular transformation conditions.

There were several problems extracting mutant plasmids. For about a month, we have not been able to get a successful transformation into competent cells. The zero colonies were detected on agar plates after a night incubation at 37°C. To solve this problem, we replaced the antibiotic with a new one; also, tried to express mutants in other competent cells(XL1); and tried to optimize the transformation protocol. Despite all these attempts, we have not been able to get a large number of colonies on agar plates. Spreading cells by a triangle-shaped spreader gave a much higher number of colonies. To get concentrated transformed cells, we centrifuged 350 ul of cells in SOC media(after incubation), and isolated the pellet of cells. From the supernatant, 300 ul of media was discarded and the pellet was resuspended in 50 ul of SOC media. All 50 ul of cells spread on the agar plate. During colony inoculation in 5 ml of liquid bacterial culture, the number of plasmids grew at a low level, and it also took two or three days to obtain a large number of cells.

3. PCR reaction optimization after cell cloning.

To detect the positive clones after passaging them into 24-well plates, we had to conduct a PCR reaction with DNA extracts of clones. It was impossible to see the PCR results from 10 ul of DNA samples extracted from 96-well plates. After that, it was decided to grow clones in 24-well plates. Even if the number of DNA extracts was high, we could not detect the bands on gels. It was assumed that the PCR reaction did not assemble well. We have tried to decrease and increase the annealing temperature; change the initial denaturation time; and increase the extension time or the number of PCR rounds. We also changed the PCR buffer and checked our primers. Eventually, the PCR reaction worked when we increased the annealing time to 1 minute. Theoretically, an increase in the annealing time would have given us additional bands, but this did not happen.

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Supplemental materials and data

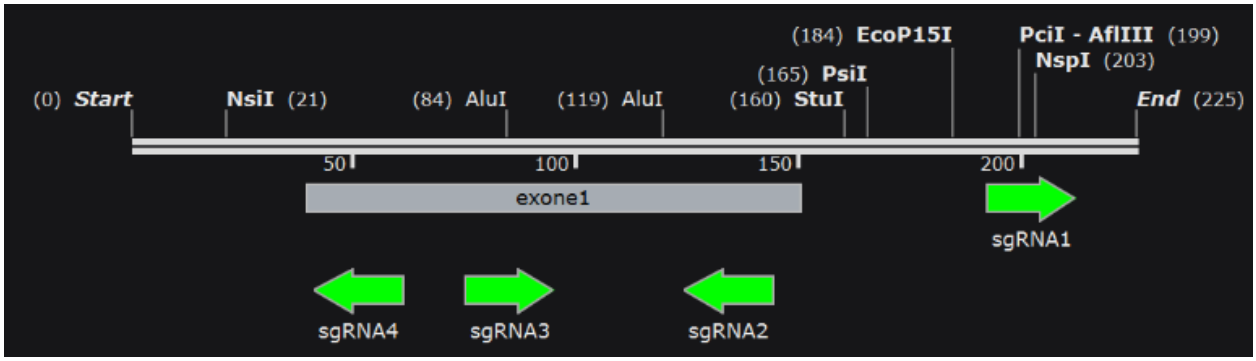

Figure 1. The sites where sgRNA binds

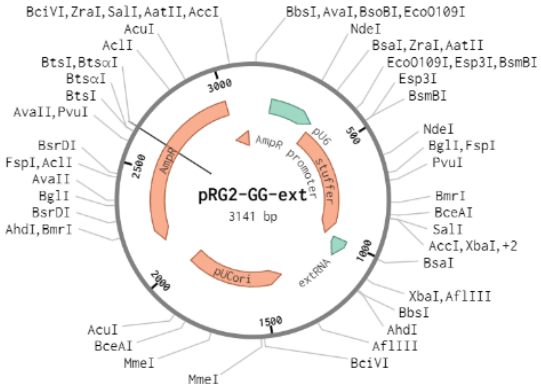

Figure 2. The vector plasmid map for sgRNA expression

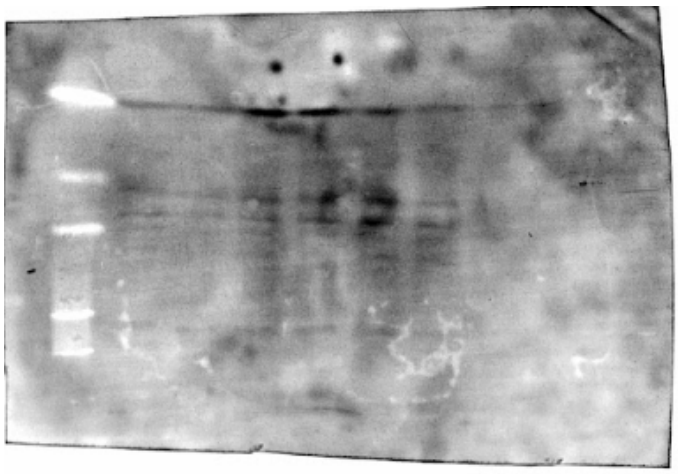

Figure 3. Western blot analysis with RAS antibody.

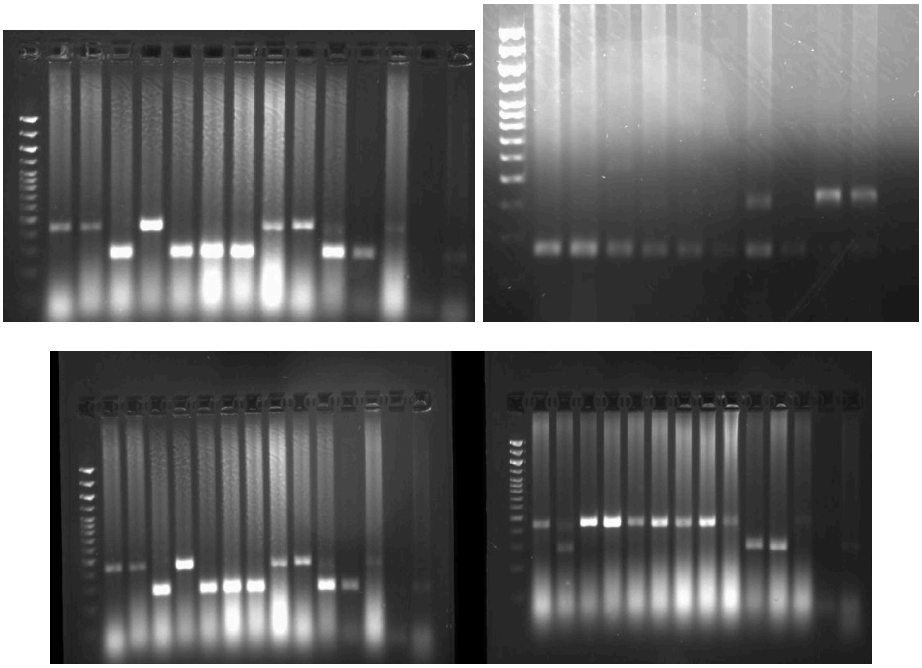

Figure 4. Screening of positive clones

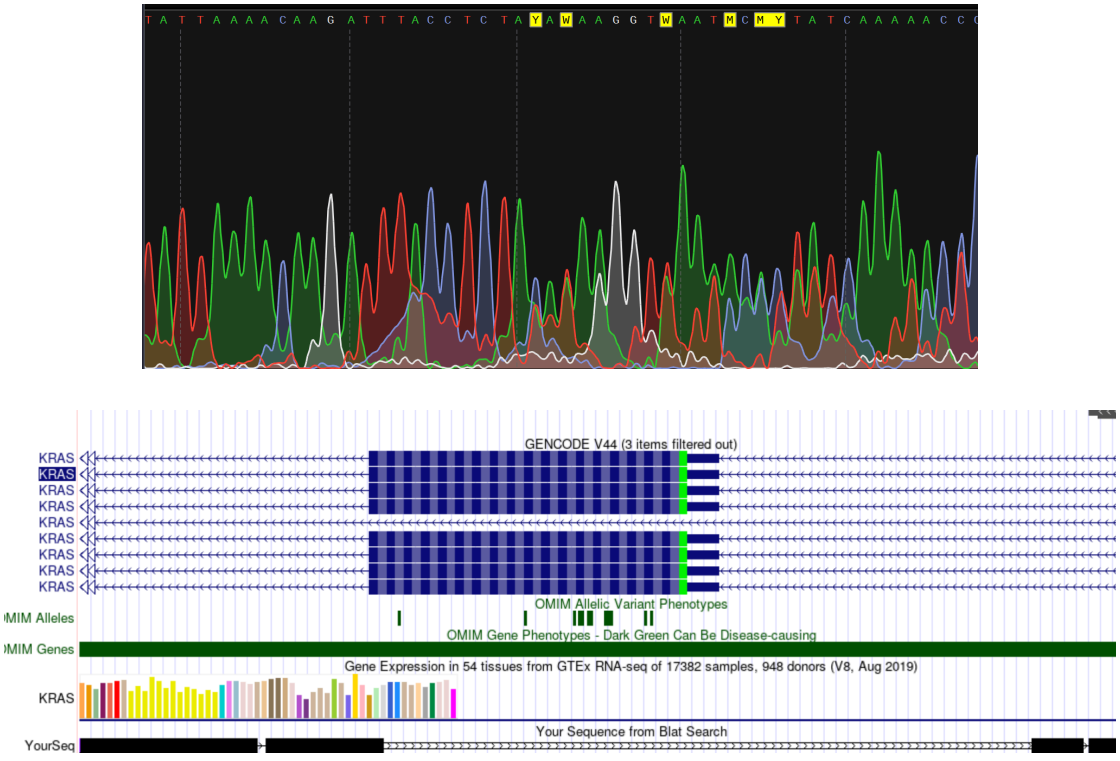

Figure 5. Sanger sequencing result for fast-growing clone.