Identification of the Cyclin Responsible for the Activation of Cancer Dependency CDK11

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Abstract

According to the National Cancer Institute, over 600,000 people in the United States die from cancer every year. One promising area of research to address this crisis is the identification of cancer dependencies as drug targets. CDK11, a cyclin-dependent kinase responsible for cell cycle progression through phosphorylation of key target proteins, was recently discovered to be a cancer dependency. CDK11 must first be activated by a cyclin protein in order to phosphorylate, but currently, which cyclin is responsible for this cancer dependency's activation is unknown. 11 recombinant plasmids were designed and harvested expressing GFP, ampicillin resistance, and a guide RNA targeting either cyclin L1, cyclin L2, or both. These were used to create lentivirus to infect three different Cas9-expressing cancer cell lines, A375 (melanoma), HCT116 (colon cancer), and MDA-MB-231 (breast cancer), using CRISPR/Cas9 to induce a double-stranded break on the genes responsible for coding certain cyclins. These samples were subjected to dropout assays, a type of competition assay measuring relative percent GFP+ to GFP-, to test their viability. If the cyclin targeted is responsible for activating CDK11, those cells will die, since it has already been proven that cancer cells need CDK11 in order to survive. The intended "knock-out" of targeted cyclins was then confirmed using Western blotting and genomic DNA sequencing. The identification of the activating cyclin of CDK11 can help researchers understand the true function of CDK11, especially as a cancer dependency, characterize the structure of CDK11 itself, synthesize novel therapeutics targeting CDK11, and identify a new potential drug target for cancer therapies.

Introduction

According to the National Cancer Institute, over 600,000 people in just the United States die due to cancer every year (National Cancer Institute, n. d.). Facing a severe public health crisis, significant research efforts are dedicated to finding new and improved treatments for those suffering from cancer. One promising area of research is the identification of cancer dependencies as drug targets. Also called cancer addictions, these dependencies are required for cancer cell proliferation, and without them, the cancer would not be able to survive (Luo, Solimini, & Elledge, 2009). A vast majority of cancer cells exhibit aneuploidy, which is the condition in which cells contain an abnormal number of chromosomes. This aneuploidy rewrites the genetic framework of the cancer cell, which is thought to be the cause of newly arising dependencies that are present specifically in cancer and not found in the non-cancer cells of the types. The discovery of these cancer dependencies provides an avenue to novel therapeutics, such as small molecule inhibitors that can impede cancer cell proliferation by targeting a protein the cells have become addicted to (Lin et al., 2019). Dependencies become "drug targets" due to their cruciality to cancer cells; if drugs are synthesized that effectively inhibit a cancer dependency, it may theoretically effectively discriminate between cancer and noncancer cells, the inability of which is a consistent fault of common cancer treatments.

One recently discovered cancer dependency in multiple types of cancers is CDK11, a protein belonging to the family of cyclin-dependent kinases, which are responsible for cell cycle progression through the phosphorylation of key proteins. This was discovered after an effective small molecule inhibitor named OTS964 was found to have a mischaracterized target, and its true mechanism of action was discovered to be the inhibition of CDK11 (Lin et al., 2019). Cyclin-dependent kinases are extremely important proteins responsible for activating other key proteins through phosphorylation, but they themselves must first be activated by a complementary protein, called a cyclin. Cyclins both activate their target CDK and direct it to necessary target proteins during the appropriate cell cycle period, thus functioning as critical cell cycle progressors (Alberts, 2002).

In non-cancer cells, CDK11 has been observed to have a role in transcriptional initiation and elongation as well as pre-mRNA splicing, and seem to interact with cyclins L1, L2, and D3 (Loyer, 2008). However, CDK11 has been described as "poorly-characterized" (Lin et al., 2019), as there is little to no consensus on its activating cyclin(s), function in cell cycle progression, and most importantly, role as a cancer dependency. Despite observed interactions with specific cyclins that hint towards regulatory partners to CDK11, there has been no definitive evidence linking any cyclin(s) to the activation of this cyclin-dependent kinase.

In order to identify the cyclin(s) responsible for the activation of CDK11, a powerful biological tool called CRISPR/Cas9 system was used. CRISPR, which is short for Clustered Regularly Interspaced Short Palindromic Repeats, is used to permanently alter DNA, and this specific variant allows researchers to "knock-out" a gene by cutting the targeted gene at a specified sequence. By knocking out a gene, the development of the protein it codes for is inhibited, meaning that this tool can be used to develop cell line variants without a certain target protein. This tool consists of three important components: a Cas9 protein, a guide RNA, and a PAM sequence, short for Protospacer Adjacent Motif sequence. The Cas9 protein acts as a biological scissor to cut DNA and is naturally found in *Streptococcus pyogenes* bacteria. The Cas9 protein, however, cannot function without binding to a guide RNA, frequently abbreviated as gRNA, that directs the proteins as to where to cut in a cell's genome sequence. Even with both of these components, the system cannot function without a PAM sequence, which is a three base pair sequence immediately downstream of the site targeted by the gRNA. Without this sequence, CRISPR/Cas9 will not be able to bind or cut target DNA (CRISPR Guide, n.d.).

In this project, CRISPR/Cas9 will be used to induce changes in three different cell lines: A375, a melanoma cell line, MDA-MB-231, a breast cancer cell lines, and HCT116, a colon cancer cell line. In order to test whether a cyclin is responsible for activating CDK11, the effect of knocking out that cyclin on cell viability can be observed. Since it has been shown that CDK11 is a cancer dependency (Lin et al., 2019), and therefore without its effective action cancer cells will die, if a cyclin that is responsible for CDK11's activation is not present in the cell, CDK11 is never activated, and therefore, cancer cells should die. Following this logic, in this project, guide RNAs were specifically designed to cut the genes coding for cyclins that may activate CDK11 to test the proliferation of these cells. The inability of cells shown to be lacking a specific cyclin to survive is evidence to support the argument that that cyclin activates CDK11.

The identification of the activating cyclin is extremely important for a number of reasons. The identification of a cyclin regulatory partner can help researchers understand the true function of CDK11, both in non-cancer cells and as a cancer dependency, by adding another piece to the puzzle of this protein. Even though research has shown some potential function of CDK11 in transcriptional initiation and elongation as well as pre-mRNA splicing, due to the proliferative and aggressive nature of cancer cells, the role of CDK11 as a cancer dependency, one that cancer has become overly reliant on, is unkown. Additionally, since cyclins function by binding to their partner CDKs, the identification of a specific cyclin, and subsequent characterization of its structure, can help researchers elucidate the exact structure of CDK11 itself, thereby aiding in the effort to synthesize novel therapeutics to treat cancers by targeting CDK11. Finally, since a CDK is functionally inactive without activation by its partner cyclin, the

identification of cyclin(s) responsible for activating CDK11 may also produce a new drug target for cancer therapies as an alternative way to interrupt the pathway of a crucial cancer dependency. The newly developed drugs, most likely to be small molecule inhibitors, can serve as more effective treatments for cancer than current options due to the discriminatory nature of targeting cancer dependencies. Cancer cells are proven to be unable to survive without these dependencies, but non-cancer cells may be able to compensate for their loss and therefore survive the small molecule inhibition that kills cancer.

Methodology

New gRNA Recombinant Plasmid Formation

Four new CRISPR gRNAs, two targeting cyclin L1 and two targeting cyclin L2, were cloned into a GFP-expressing backbone (Figure 1). First, a GFP-expressing backbone plasmid was linearized with the BsmB1 restriction enzyme and digested at 55° C for 3 hours in NE buffer 3.1 to produce a vector suitable for ligation. After digestion, Calf Intestinal Phosphatase (CIP) was added to dephosphorylate the ends of linearized vectors in order to prevent re-ligation of the desired backbone and filler region. Gel electrophoresis with a 1% agarose gel was then conducted on samples to separate the 2kb filler region and the 11kb backbone, which was cut out of the gel and purified. To purify, 11kb bands were cut out of gel and vortexed after adding NTI buffer and incubated in a heat block at 50° C for 10 minutes at 500 RPM. The samples were then loaded into NucleoSpin Gel and PCR Clean-up Columns, centrifuged to discard flow-through, washed with NT3 buffer, and eluted in NE buffer.

Forward and reverse strands of gRNA primers were annealed together and the 5' end was phosphorylated using T4 ligase buffer and T4 PNK enzyme to form a double-stranded vector suitable for ligation. The samples were put in a thermocycler to ensure accurate annealing and subsequently diluted 1:200. The digested backbone and the annealed gRNA primers were then ligated using T4 ligase buffer and T4 ligase enzyme to produce complete transformable plasmids.

Following ligation, newly created recombinant plasmids were transformed into Stbl3 *E. coli* cells on plates containing Lysogeny broth (LB) and ampicillin by adding the ligation reaction to culture plates. The backbone used for guide cloning also expressed ampicillin-resistance, so only cells successfully transformed with recombinant plasmids formed colonies. After 16 hours, these colonies were picked and

inoculated into liquid LB + ampicillin to be grown for another 16 hours in a shaker. Following inoculation, transformed Stbl3 cells were stored at -80° C in a 50% glycerol solution.

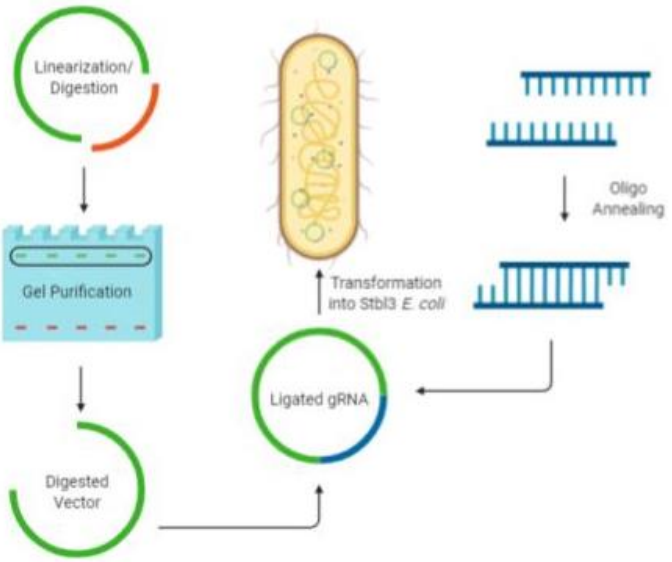

Figure 1. Guide RNA Recombinant Plasmid Formation workflow

Digested backbone vector was ligated with annealed oligos customized with desired guide RNA sequence and transformed into Stbl3 *E. coli*. (Created by Student Researcher)

CRISPR/Cas9 gRNA Sequence Validation

A total of 15 gRNA recombinant plasmids were sent to MCLAB for DNA sequencing and checked using the Nucleotide BLAST database in order to verify that the gRNA sequence cloned into the backbone vector was accurate before using the plasmids for lentiviral generation (Figure 2). This included the four new plasmids with gRNAs targeting either cyclin L1 or cyclin L2, eight already made plasmids with gRNAs targeting either cyclin L1 or cyclin L2, and three already made "double guide" plasmids with gRNAs targeting both cyclin L1 and cyclin L2.

Stbl3 *E. coli* glycerol stocks were inoculated into liquid LB + ampicillin to be grown for 16 hours in a shaker. Following inoculation, plasmid DNA was extracted and purified using the QIAGEN QIAprep Spin Miniprep Kit. Stbl3 cells were pelleted by centrifuge and resuspended in resuspension buffer P1 after aspirating the LB + ampicillin solution. Lysis buffer P2 was then added to each sample, which were subsequently mixed by inverting. Next, neutralization buffer N3 was added to each sample, which were again mixed by inverting. Samples were then centrifuged to separated lysed cells from their

internal content, including the desired plasmid DNA. QIAprep Miniprep columns were then attached to a vacuum manifold, and the internal contents of cells from each sample were passed through the column. Then, binding buffer PB followed by wash buffer PE was passed through each column. Columns were then placed into collection tubes and centrifuged to extract any remaining buffer liquid. Next, DNA bound to the columns was eluted using deionized (DI) water and centrifuged again to collect plasmid DNA. The concentration of plasmid DNA in each sample was determined using a NanoDrop Spectrophotometer.

A portion of each sample was diluted to a DNA concentration of 100 ng/µL in order to be suitable for sequencing, and 10 µL of each sample was packaged and sent for sequencing. The rest of the plasmid DNA was frozen at -20° C and saved to use in following experimentation. After sequencing was complete, the sequence of each plasmid was downloaded from the MCLAB website and viewed in SnapGene to verify the specific desired gRNA sequence in each plasmid.

Next, this verified sequence was checked against the Nucleotide BLAST database to ensure the guide is accurate. This is a further test to ensure the gRNA sequence is present in the genomic sequence of the targeted gene, which is required for CRISPR to function properly.

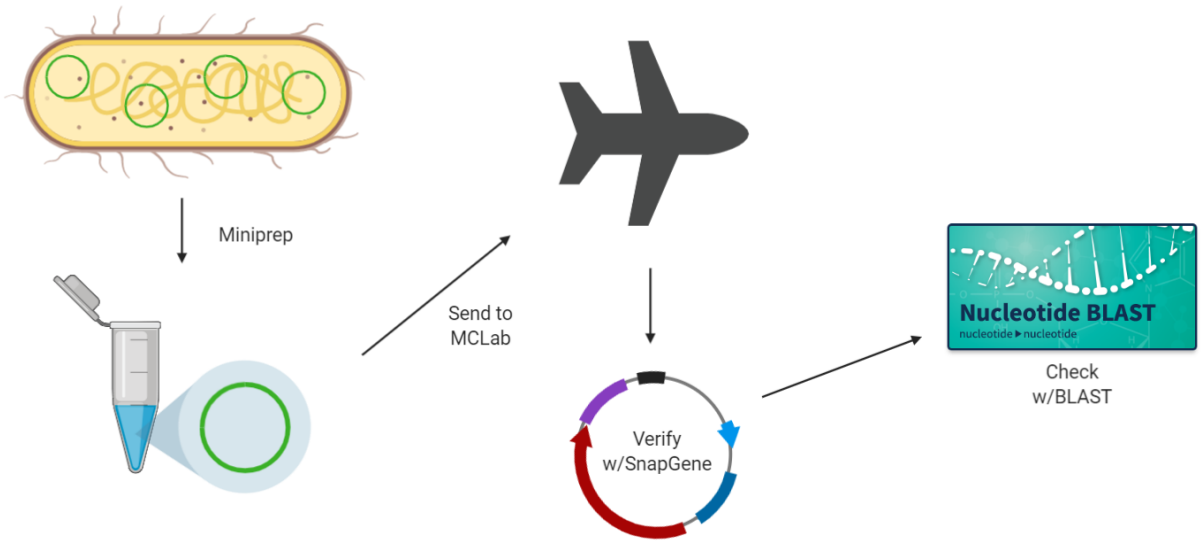

Figure 2. Sequence Validation workflow

Plasmid DNA was harvested from Stbl3 *E. coli* cells and sent to MCLAB for sequencing. Sequencing was verified using SnapGene and BLAST. (Created by Student Researcher)

Lentiviral Generation

Calcium-phosphate transfections of gRNA plasmids into HEKFT cells from ThermoFisher Scientific, a human embryonic kidney cell line, were used to produce lentivirus capable of incorporating the desired gRNA sequence into the genomic DNA of cancer cell lines through transduction (Figure 3). A solution consisting of plasmid DNA, plasmid DNA expressing CMVGagPol, plasmid DNA expressing the envelope protein pVSVG, H_2O, and $CaCl_2$ was mixed in drop wise to HBS buffer while using a pipet aid to bubble the mixture. During a 15-minute incubation of the solution at room temperature, HEKFT cells were plated with chloroquine in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and 1% glutamine. After drop wise addition of the transfection solution, plates were incubated at 37° C for 8 hours, after which media was changed and plates were incubated again. 24, 48, and 72 hours later, lentivirus was collected using a syringe and 0.45 µM filter and stored at -80° C (Smale, 2010).

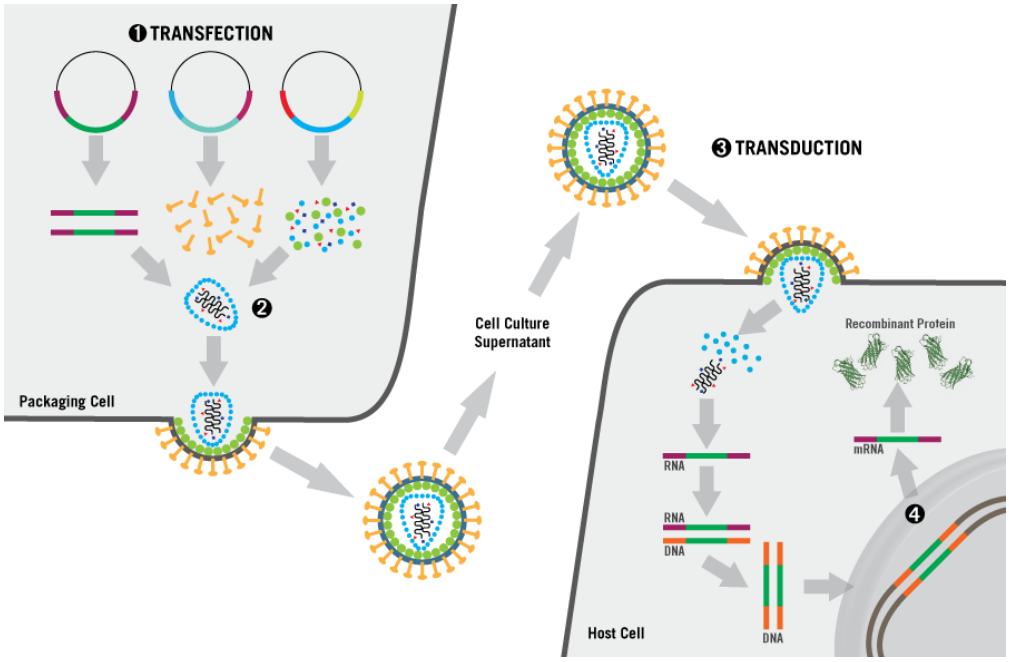

Figure 3. Visualization of Lentiviral Generation and Infection

Calcium phosphate transfections (**1**) were used to generate lentivirus, packaged in HEKFT cells (**2**). After collection, lentivirus was transduced into HEKFT cells to determine titer (**3**). (Mirus Bio LLC)

Test Lentiviral Infections

On the first day of collection, generated lentivirus was transduced into HEKFT cells to determine titer (Figure 3). 10,000 HEKFT cells in 100 µL of media with 0.1 µL polybrene were plated onto each well in a 96-well plate. 100 µL of virus was added to each well, excluding an un-transduced control group. After incubating at 37° C for 24 hours, each well was replaced with 100 µL of fresh media. After incubating at 37° C for another 24 hours, samples were run through the MacsQuant Analyzer, a flow cytometer used to count the percentage of the cell population in each sample expressing GFP to quantify the efficiency of lentivirus.

Dropout Assays

Cancer cell lines A375 (melanoma), MDA-MB-231 (breast), and HCT116 (colon), all from ATCC, already transduced to express Cas9, were thawed from liquid nitrogen storage to be used in dropout assays. Cancer cells were plated onto a 12-well plate, 50,000 cells per well in 1 mL media, for transduction. The next day, new media with 1 µL polybrene was added to the wells, along with the appropriate amount of virus based on the titers determined through test transductions. Media was changed after 48 hours. Cells were passaged and time points were taken on the MacsQuant Analyzer every 3-5 days post-transduction up until five passages to measure the percent of cells expressing GFP throughout the dropout assay (Lin et al., 2017).

Flow Cytometry

Cas9-expressing A375, MDA-MB-231, and HCT116 cells transduced with gRNA lentivirus were sorted using flow cytometry to purify the cell population to only GFP-expressing cells in order to isolate cells that have been successfully transduced (Figure 4). After expanding, cells were then either set aside for Western Blotting or frozen using freezing media with DMSO and stored in liquid nitrogen.

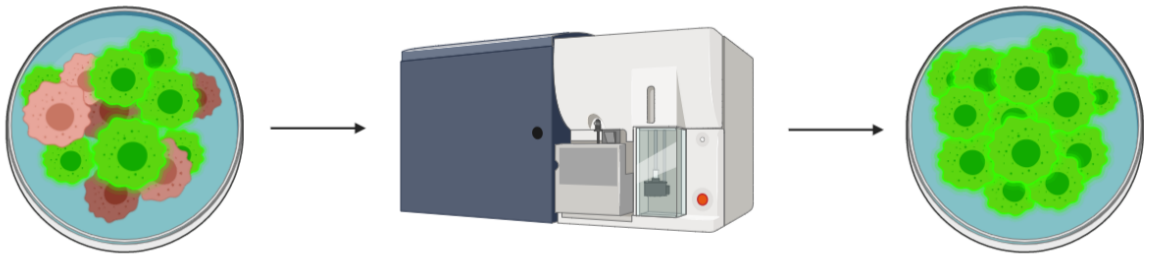

Figure 4. Flow Cytometry workflow

Transduced populations were sorted for GFP florescence to produce a pure GFP+ population. (Created by Student Researcher)

Western Blot Validation

Protein lysate was harvested from sorted GFP+ cell samples to for use in Western blotting to validate the presence or lack thereof of the targeted cyclin(s) (Figure 5). Samples were trypsinized, quenched, and centrifuged for 5 minutes at 1000 rpm, followed by a wash step where they were resuspended in 500 µL of PBS, centrifuged again, and the supernatant was aspirated. Samples were then resuspended in 100 µL of RIPA buffer modified with Protease inhibitor and Phosphatase inhibitor to preserve protein lysate. Samples were then placed in a shaker in the cold room for 30-60 minutes followed by centrifuging at max speed for 20-60 minutes. Supernatant was then transferred to a new tube and stored at -20° C.

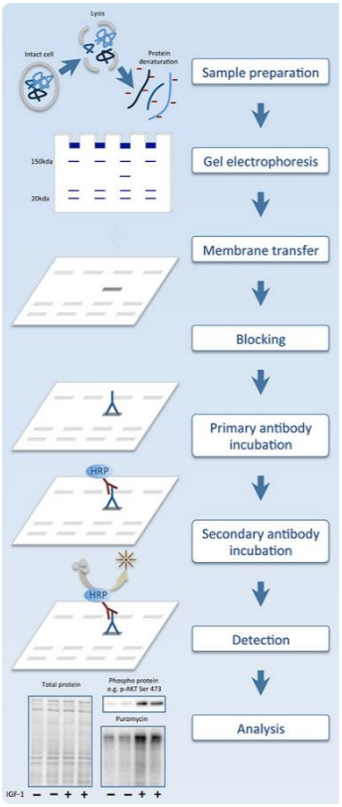

Figure 5. Western Blot workflow

Western blotting was used to validate knock-out of targeted protein. (Precision Biosystems)

The resolving and stacking gels for Western blotting were then made using Acrylamide, TRIS, SDS, APS, and TEMED, and methanol was used to smooth out bubbles. After polymerization, gels were loaded and locked into the cassette.

Protein lysate samples were then quantified in order to ensure effective Western blotting. 4 µL RIPA plus 1 µL of each protein sample was added in triplicates to 96 well quantification plates. In a separate tube, 1 mL of DC Reagent A was combined with 20 µL of DC Reagent S, after which 25 µL of the combined solution was added to each sample and the standards. 200 µL of DC Reagent B was then added to each sample and standard and samples were incubated at room temperature for 15 minutes. Using a plate reader, protein samples were quantified and concentrations were used to calculate the volume of each sample needed to test a consistent amount of protein for each sample.

Samples were prepared with the calculated volume plus RIPA buffer to reach 25 µL per samples, to which 8 µL of loading dye was added. To prepare samples for the gel, a heating block was turned on to high and samples were loaded in when it reached ~90° C. Samples were boiled for 10 minutes and spun down using a tabletop centrifuge. The western blotting cassette was filled with running buffer, and protein ladder and samples were added to the gel. Samples were run for about 2 hours at 120 V.

In order to transfer the blotting from the gel onto a membrane, two filter pads and one membrane were prepared. The filter pads were soaked in 1X transfer buffer, while the membrane was soaked in methanol, after which one pad and one membrane were transferred to 1X transfer buffer and laid on the transfer cassette. The stacking gel was cut out from the previous cassette and laid on top of the membrane, on top of which another filter pad was laid. The cassette was locked and placed into the transfer machine, which was run at a constant current of 1.3A for 30-45 minutes.

After transfer, the membrane was soaked in 100 mL TBST with 5 g milk for 1 hour for blocking. Primary antibody was then added in a 5% milk solution to the membrane, covered with tin foil, and placed in a 4° C shaker overnight. The next day, the membrane was washed three times with TBST for 10 minutes each. Then, secondary antibody was added in a 5% milk solution for 1 hour at room temperature. Membranes were then washed again with TBST three times for 10 minutes each. Next, black and white solution with 500 µL of each was added for developing. Membranes were then developed in the dark room using the developing machine.

After developing, blots were put in Ponceau and shaken for 20 minutes. Then, the 5% milk solution was added, and blots were put in the shaker for 1 hour for blocking. Primary antibody was then added in milk and shaken overnight at 4° C.

Results

As a first step in this research, gRNA plasmids with a GFP marker were successfully created with the ligation reaction detailed previously, and the sequences of these plasmids were verified. Before moving on with the procedure, all plasmids were found to contain accurate sequences, matching both the gRNA sequence desired and a portion of the targeted gene according to BLAST. These plasmids were then used to create lentivirus suitable for transduction, particularly for the dropout assays. The lentiviral generation successfully yielded effective virus, determined through test lentiviral infections. After

obtaining the titer of each virus from HEKFT cells, the virus was able to be transduced into A375, HCT116, and MDA-MB-231.

The first A375 dropout assay tested only single guide RNAs targeting either CCNL1 or CCNL2 (Figure 6). There were 16 experimental samples, consisting of four CCNL1 guides, four CCNL2 guides, two Rosa26 guides, two AAVS1 guides, two RPA3 guides, and two PCNA guides. Rosa26 and AAVS1 functioned as negative controls with no dropout expected, while RPA3 and PCNA functioned as positive controls with high dropout expected. There seems to be noticeable dropout in all guides targeting either cyclin L1 and L2, but none are to a magnitude even remotely close to the positive controls. Also, the cyclin L1-targeted populations seem to exhibit slightly higher dropout than their cyclin L2 counterparts.

A

A375 Dropout #1					
Guide	% GFP				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	28.66%	22.56%	18.34%	15.46%	13.49%
CCNL1 g2	44.60%	37.18%	30.88%	27.17%	24.59%
CCNL1 g3	23.16%	16.18%	12.52%	10.28%	8.90%
CCNL1 g4	35.02%	29.02%	25.33%	21.86%	19.31%
CCNL2 g1	41.30%	36.48%	31.40%	30.26%	28.60%
CCNL2 g2	62.71%	57.51%	51.79%	49.32%	46.34%
CCNL2 g3	32.13%	26.19%	21.61%	19.77%	18.20%
CCNL2 g4	47.02%	43.69%	39.30%	37.46%	36.58%
Rosa26 g1	33.38%	26.80%	22.48%	22.15%	21.07%
Rosa26 g2	80.03%	92.87%	93.45%	92.57%	93.21%
AAVS1 g1	75.53%	93.41%	91.89%	91.65%	91.12%
AAVS1 g2	81.89%	94.32%	94.84%	94.68%	95.20%
RPA3 g1	78.83%	22.40%	8.21%	7.05%	6.80%
RPA3 g2	39.65%	10.52%	3.06%	2.21%	2.10%
PCNA g1	13.74%	1.56%	0.52%	0.45%	0.18%
PCNA g2	81.09%	72.34%	38.41%	22.69%	17.40%
No Guide	0.02%	0.10%	0.00%	0.08%	0.00%

B

A375 Dropout #1					
Guide	Fold Dropout				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	1	1.27	1.56	1.85	2.12
CCNL1 g2	1	1.20	1.44	1.64	1.81
CCNL1 g3	1	1.43	1.85	2.25	2.60
CCNL1 g4	1	1.21	1.38	1.60	1.81
CCNL2 g1	1	1.13	1.32	1.36	1.44
CCNL2 g2	1	1.09	1.21	1.27	1.35
CCNL2 g3	1	1.23	1.49	1.63	1.77
CCNL2 g4	1	1.08	1.20	1.26	1.29
Rosa26 g1	1	1.25	1.48	1.51	1.58
Rosa26 g2	1	0.86	0.86	0.86	0.86
AAVS1 g1	1	0.81	0.82	0.82	0.83
AAVS1 g2	1	0.87	0.86	0.86	0.86
RPA3 g1	1	3.52	9.60	11.18	11.59
RPA3 g2	1	3.77	12.96	17.94	18.88
PCNA g1	1	8.81	26.42	30.53	76.33
PCNA g2	1	1.12	2.11	3.57	4.66

C

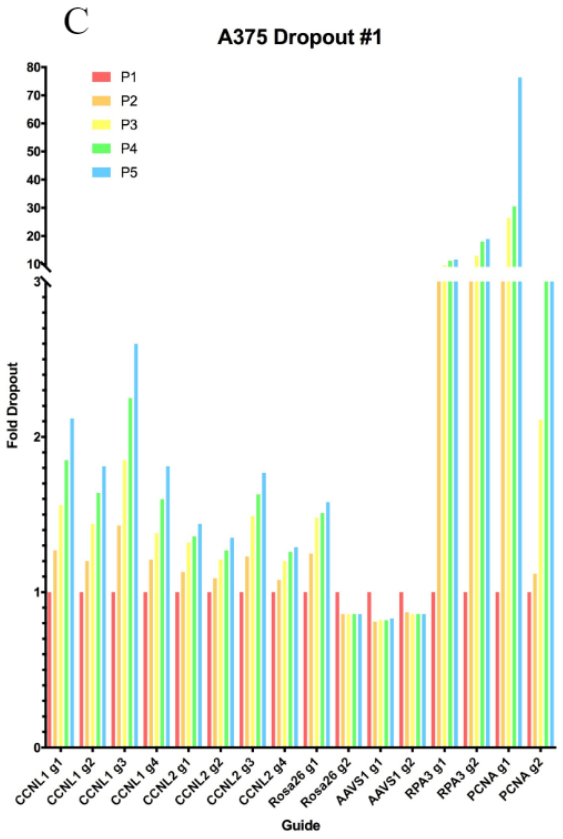

Figure 6. A375 Dropout Assay #1

A375 cells were transduced with four guide RNAs targeting CCNL1, the gene that codes for cyclin L1, and four guide RNAs targeting CCNL2, the gene that codes for cyclin L2. Guide RNAs targeting Rosa26 and AAVS1 were transduced as negative controls for dropout while guide RNAs targeting RPA3 and PCNA were transduced as positive controls. (**A**) The raw percentages of GFP+ cells are displayed at each passage for 16 samples transduced with guide RNAs as well as an untransduced control. (**B**) The fold dropout, which is the fold change in the percentage of GFP+ cells relative to the initial percentage of GFP+ cells from passage 1, for each of the transduced samples in (**A**) is displayed. (**C**) A graph illustrating the dropout of each sample at each passage from (**B**) is displayed. (Created by Student Researcher)

The second A375 dropout assay tested the same guide RNAs with the addition of three "double" guide RNAs targeting both CCNL1 and CCNL2 (Figure 7). Just as in the first dropout assay, cyclin L1 seems to be experiencing slightly higher dropout than cyclin L2, but interestingly, the double guides also experience slightly higher dropout in a more consistent manner. However, in this trial, there was a problem with RPA3 g2, a positive control expected to experience a much higher fold dropout.

A

A375 Dropout #2					
Guide	% GFP				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	25.24%	24.62%	19.85%	17.72%	15.36%
CCNL1 g2	26.94%	25.53%	20.52%	18.16%	15.66%
CCNL1 g3	14.47%	12.00%	8.59%	7.29%	6.17%
CCNL1 g4	30.98%	28.62%	23.82%	22.08%	19.81%
CCNL2 g1	31.01%	30.22%	24.94%	22.90%	21.30%
CCNL2 g2	45.08%	46.67%	40.61%	38.53%	35.44%
CCNL2 g3	29.45%	25.78%	19.09%	17.36%	15.52%
CCNL2 g4	39.98%	38.99%	31.79%	31.35%	29.26%
Double g1	69.12%	69.57%	63.39	61.72%	58.32%
Double g2	66.89%	61.59%	43.16%	36.73%	29.24%
Double g3	53.54%	46.44%	29.05%	26.84%	22.62%
Rosa26 g1	9.64%	6.60%	5.74%	5.78%	5.14%
Rosa26 g2	30.31%	32.70%	32.42%	33.12%	32.77%
AAVS1 g1	28.26%	30.04%	22.50%	22.77%	20.95%
AAVS1 g2	90.48%	90.33%	87.42%	90.63%	88.91%
RPA3 g1	83.20%	47.80%	8.21%	5.85%	4.60%
RPA3 g2	96.81%	85.80%	70.06%	71.75%	63.93%
PCNA g1	38.92%	17.02%	2.53%	1.90%	1.37%
PCNA g2	33.72%	26.52%	5.96%	5.34%	3.20%
No Guide	0.15%	0.00%	0.00%	0.00%	0.00%

B

A375 Dropout #2					
Guide	Fold Dropout				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	1	1.03	1.27	1.42	1.64
CCNL1 g2	1	1.06	1.31	1.48	1.72
CCNL1 g3	1	1.21	1.68	1.98	2.35
CCNL1 g4	1	1.08	1.30	1.40	1.56
CCNL2 g1	1	1.03	1.24	1.35	1.46
CCNL2 g2	1	0.97	1.11	1.17	1.27
CCNL2 g3	1	1.14	1.54	1.70	1.90
CCNL2 g4	1	1.03	1.26	1.28	1.37
Double g1	1	0.99	0.01	1.12	1.19
Double g2	1	1.09	1.55	1.82	2.29
Double g3	1	1.15	1.84	1.99	2.37
Rosa26 g1	1	1.46	1.68	1.67	1.88
Rosa26 g2	1	0.93	0.93	0.92	0.92
AAVS1 g1	1	0.94	1.26	1.24	1.35
AAVS1 g2	1	1.00	1.04	1.00	1.02
RPA3 g1	1	1.74	10.13	14.22	18.09
RPA3 g2	1	1.13	1.38	1.35	1.51
PCNA g1	1	2.29	15.38	20.48	28.41
PCNA g2	1	1.27	5.66	6.31	10.54

C

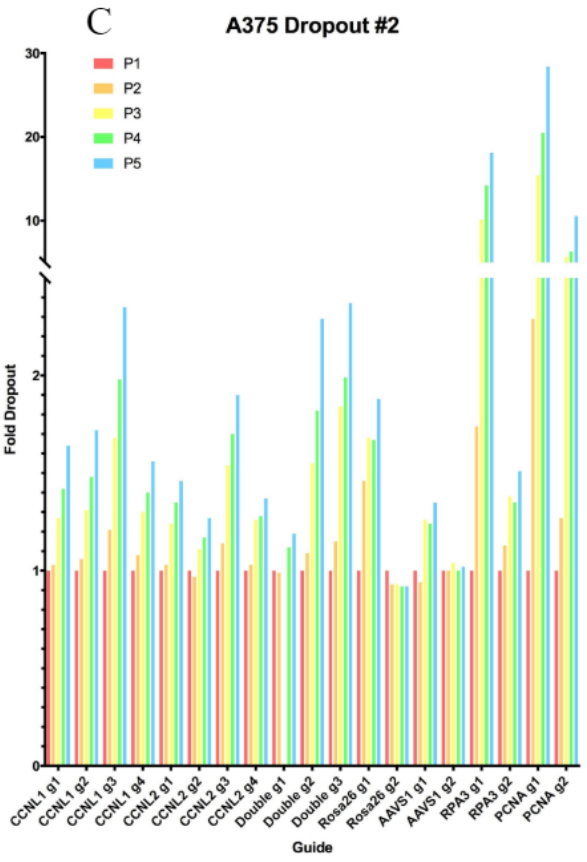

Figure 7. A375 Dropout Assay #2

A375 cells were transduced with four guide RNAs targeting CCNL1, the gene that codes for cyclin L1, four guide RNAs targeting CCNL2, the gene that codes for cyclin L2, and three "double" guide RNAs targeting both CCNL1 and CCNL2. Guide RNAs targeting Rosa26 and AAVS1 were transduced as negative controls for dropout while guide RNAs targeting RPA3 and PCNA were transduced as positive controls. (**A**) The raw percentages of GFP+ cells are displayed at each passage for 19 samples transduced with guide RNAs as well as an untransduced control. (**B**) The fold dropout, which is the fold change in the percentage of GFP+ cells relative to the initial percentage of GFP+ cells from passage 1, for each of the transduced samples in (**A**) is displayed. (**C**) A graph illustrating the dropout of each sample at each passage from (**B**) is displayed. (Created by Student Researcher)

Next, the HCT116 dropout assay tested the same guide RNAs as the second A375 dropout assay, with 19 experimental samples (Figure 8). The relative dropout levels are consistent even in HCT116: the cyclin L1 populations and the double targeted populations experience slightly higher dropout than the cyclin L2 populations, but none are as substantial as the included positive controls. In HCT116, however, the positive controls did not dropout nearly as much as in A375, especially in the first trial, making the relatively low dropout of the experimental samples more meaningful.

A

HCT116 Dropout					
Guide	% GFP				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	25.69%	23.58%	18.72%	15.18%	29.93%
CCNL1 g2	52.34%	43.43%	37.29%	32.80%	30.06%
CCNL1 g3	27.03%	19.51%	16.48%	13.26%	11.68%
CCNL1 g4	30.58%	27.45%	23.07%	20.14%	17.47%
CCNL2 g1	41.30%	43.37%	43.00%	40.82%	40.00%
CCNL2 g2	63.36%	62.12%	61.56%	59.08%	57.48%
CCNL2 g3	40.30%	39.16%	36.93%	34.17%	32.21%
CCNL2 g4	46.49%	45.60%	44.29%	42.68%	40.73%
Double g1	74.90%	69.47%	67.31%	63.00%	61.47%
Double g2	72.58%	52.83%	37.81%	29.34%	23.97%
Double g3	61.13%	50.78%	43.73%	38.80%	35.36%
Rosa26 g1	33.28%	34.00%	31.38%	29.45%	29.01%
Rosa26 g2	30.97%	34.95%	35.19%	35.01%	35.61%
AAVS1 g1	82.28%	82.15%	81.61%	79.99%	79.89%
AAVS1 g2	80.01%	79.40%	79.08%	77.06%	78.59%
RPA3 g1	74.52%	30.00%	15.20%	10.70%	10.21%
RPA3 g2	10.77%	3.92%	1.59%	0.91%	0.67%
PCNA g1	42.77%	10.21%	6.06%	4.33%	3.68%
PCNA g2	42.09%	26.76%	20.24%	15.27%	12.69%
No Guide	0.00%	0.00%	0.00%	0.00%	0.01%

B

HCT116 Dropout					
Guide	Fold Dropout				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	1	1.09	1.37	1.89	0.86
CCNL1 g2	1	1.21	1.4	1.6	1.74
CCNL1 g3	1	1.39	1.64	2.04	2.31
CCNL1 g4	1	1.11	1.32	1.52	1.75
CCNL2 g1	1	0.95	0.96	1.01	1.03
CCNL2 g2	1	1.02	1.03	1.07	1.1
CCNL2 g3	1	1.03	1.09	1.18	1.25
CCNL2 g4	1	1.02	1.05	1.09	1.14
Double g1	1	1.08	1.11	1.19	1.22
Double g2	1	1.38	1.92	2.47	3.03
Double g3	1	1.2	1.4	1.58	1.73
Rosa26 g1	1	0.98	1.06	1.13	1.15
Rosa26 g2	1	0.89	0.88	0.88	0.87
AAVS1 g1	1	1	1.01	1.03	1.03
AAVS1 g2	1	1.01	1.01	1.04	1.02
RPA3 g1	1	2.48	4.90	6.96	7.30
RPA3 g2	1	2.75	6.77	11.84	16.07
PCNA g1	1	4.19	7.08	9.88	11.62
PCNA g2	1	1.57	2.08	2.76	3.32

C

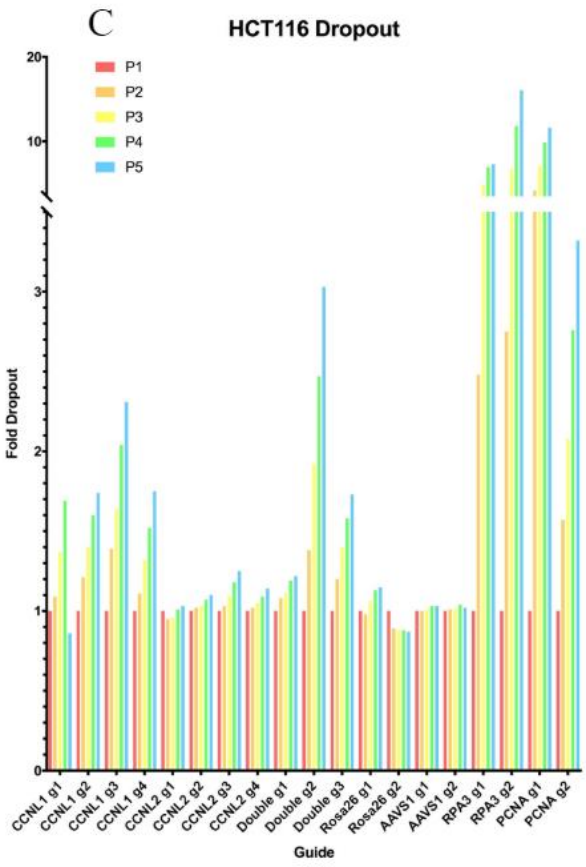

Figure 8. HCT116 Dropout Assay

HCT116 cells were transduced with four guide RNAs targeting CCNL1, the gene that codes for cyclin L1, four guide RNAs targeting CCNL2, the gene that codes for cyclin L2, and three "double" guide RNAs targeting both CCNL1 and CCNL2. Guide RNAs targeting Rosa26 and AAVS1 were transduced as negative controls for dropout while guide RNAs targeting RPA3 and PCNA were transduced as positive controls. (**A**) The raw percentage of GFP+ cells is displayed at each passage for 19 sampes transduced with guide RNAs as well as an untransduced control. (**B**) The fold dropout, which is the fold change in the percentage of GFP+ cells relative to the initial percentage of GFP+ cells from passage 1, for each of the transduced samples in (**A**) is displayed. (**C**) A graph illustrating the dropout of each sample at each passage from (**B**) is displayed. (Created by Student Researcher)

Finally, the dropout assay was conducted on MDA-MB-231 with the same 19 guide RNAs (Figure 9). Interestingly, the cyclin L1 and cyclin L2 guides both seem to experience relatively similar, but still low, dropout, and cyclin L1 does not have an obviously higher magnitude of dropout as observed with other cell lines. However, the double guide RNAs did experience noticeably higher dropout, indicating both cyclin L1 and L2 must be knocked out to hamper the functionality of CDK11.

A

MDA-MB-231 Dropout					
Guide	% GFP				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	12.29%	12.35%	9.75%	8.54%	8.21%
CCNL1 g2	25.76%	24.88%	20.64%	20.01%	18.34%
CCNL1 g3	10.08%	9.99%	7.34%	7.33%	6.51%
CCNL1 g4	18.57%	17.31%	14.05%	13.96%	12.82%
CCNL2 g1	22.46%	21.97%	18.56%	18.39%	17.14%
CCNL2 g2	33.22%	35.28%	28.96%	28.11%	27.47%
CCNL2 g3	18.03%	19.64%	16.15%	15.07%	14.22%
CCNL2 g4	21.22%	22.95%	18.28%	18.05%	17.65%
Double g1	48.65%	47.20%	37.06%	32.92%	28.79%
Double g2	40.72%	32.11%	19.69%	15.46%	11.41%
Double g3	30.42%	25.14%	16.37%	13.86%	12.25%
Rosa26 g1	18.26%	19.34%	15.79%	13.57%	13.91%
Rosa26 g2	4.08%	5.40%	4.60%	5.16%	5.52%
AAVS1 g1	64.29%	69.17%	61.21%	57.22%	58.13%
AAVS1 g2	16.40%	20.14%	15.78%	15.11%	17.16%
RPA3 g1	61.59%	41.19%	3.29%	1.38%	1.42%
RPA3 g2	6.21%	4.55%	0.70%	0.36%	0.32%
PCNA g1	6.71%	3.80%	0.44%	0.23%	1.06%
PCNA g2	22.94%	16.07%	3.39%	1.98%	2.51%
No Guide	0.05%	0.00%	0.10%	0.03%	0.01%

B

MDA-MB-231 Dropout					
Guide	Fold Dropout				
	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
CCNL1 g1	1	1	1.26	1.44	1.5
CCNL1 g2	1	1.04	1.25	1.29	1.4
CCNL1 g3	1	1.01	1.37	1.38	1.55
CCNL1 g4	1	1.07	1.32	1.33	1.45
CCNL2 g1	1	1.02	1.21	1.22	1.31
CCNL2 g2	1	0.94	1.15	1.18	1.21
CCNL2 g3	1	0.92	1.12	1.2	1.27
CCNL2 g4	1	0.92	1.16	1.18	1.2
Double g1	1	1.03	1.31	1.48	1.69
Double g2	1	1.27	2.07	2.63	3.57
Double g3	1	1.21	1.86	2.19	2.48
Rosa26 g1	1	0.94	1.16	1.35	1.31
Rosa26 g2	1	0.76	0.89	0.79	0.74
AAVS1 g1	1	0.93	1.05	1.12	1.11
AAVS1 g2	1	0.81	1.04	1.09	0.96
RPA3 g1	1	1.5	18.72	44.63	43.37
RPA3 g2	1	1.38	8.87	17.25	19.41
PCNA g1	1	1.77	15.25	29.17	6.33
PCNA g2	1	1.43	6.77	11.59	9.14

C

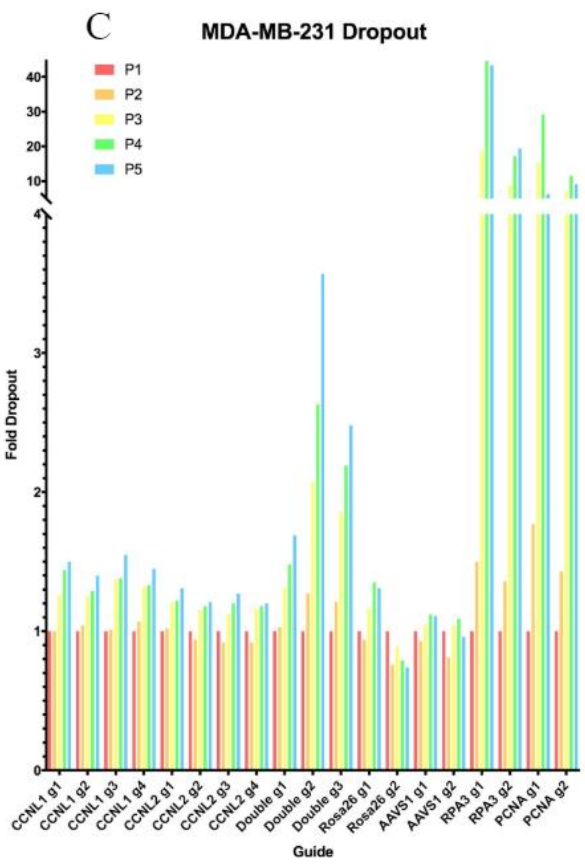

Figure 9. MDA-MB-231 Dropout Assay

MDA-MB-231 cells were transduced with four guide RNAs targeting CCNL1, the gene that codes for cyclin L1, four guide RNAs targeting CCNL2, the gene that codes for cyclin L2, and three "double" guide RNAs targeting both CCNL1 and CCNL2. Guide RNAs targeting Rosa26 and AAVS1 were transduced as negative controls for dropout while guide RNAs targeting RPA3 and PCNA were transduced as positive controls. (**A**) The raw percentage of GFP+ cells is displayed at each passage for 19 sampes transduced with guide RNAs as well as an untransduced control. (**B**) The fold dropout, which is the fold change in the percentage of GFP+ cells relative to the initial percentage of GFP+ cells from passage 1, for each of the transduced samples in (**A**) is displayed. (**C**) A graph illustrating the dropout of each sample at each passage from (**B**) is displayed. (Created by Student Researcher)

Transduced cells from the dropout assay were sorted using flow cytometry to produce a pure GFP+ population. Bulk sorts for tested A375, HCT116, and MDA-MB-231 cells were all successful and produced viable GFP+ populations. These cells were then expanded, with some being froze down for future experimentation, some used to harvest protein lysate, and some used to harvest genomic DNA for PCR amplification and sequence verification.

Harvested protein lysate samples were used in Western blots to verify knockout of the targeted cyclins. These Westerns are currently in progress, with multiple completed Westerns and some preliminary results.

Discussion

The dropout assays all demonstrated similar results, with relatively low, but noticeable fold dropout in the experimental samples. Although there was no dropout close to the positive controls, dropout was still for the most part higher than the included negative controls. Cyclin L1 experienced a maximum 2.6 fold dropout, cyclin L2 experienced a maximum 1.9 fold dropout, and the double guides experienced a maximum 3.57 fold dropout. Given that the negative controls experienced at most 1.88 fold dropout, there does seem to be significant dropout of experimental samples. This is consistent with previous observations of interaction between CDK11 and cyclin L1 and L2 (Loyer, 2008), as knocking these cyclins may have hampered the functionality of CDK11 without preventing its activation altogether.

However, the dropout is still too low to conclusively establish either cyclin L1 or cyclin L2, or both working in conjunction, as responsible for the activation of CDK11. There are multiple possible explanations for noticeable, but not extremely high, dropout of the cyclin guide RNAs. First, it is possible the dropout occurred independent of CRISPR mutagenesis and is solely due to fitness defects from experimental procedure. In that case, regardless of whether CRISPR successfully knocked out the target protein, the population would dropout out due to problems with cell care or induced stress from experimentation. However, given that tested guides dropped out more than the negative controls, this is unlikely, and there is probably a biological mechanism behind the observed decreased cell fitness.

Another possibility may have been a problem with the CRISPR/Cas9 system used to modify the genomic DNA of cancer cells. Sequence validation of plasmids rules out the possibility of nonfunctional guide RNAs, but off-target effects are still possible. Additionally, the non-homologous end joining used

by cells to repair the double stranded break induced by CRISPR may have introduced a silent mutation with no observable effect on the targeted protein.

Due to fold dropout essentially presenting data in relative terms, a calculation of statistical significance is not the norm in the scientific community for dropout assays (Lin et al., 2019). Rather, an arbitrary threshold value for fold dropout is usually set, which in the case of Lin et al., 2019 was 2.5-fold dropout, and results are compared to this threshold. Therefore, statistical significance was not calculated for these dropouts, but the magnitude of fold dropout is instead analyzed.

At the current stage, the completed Western blots do not clearly show a knock-out or a lack of it, due to a potential problem with the primary antibodies used to target cyclin L1 and L2. Due to successful detection of the GAPDH control, which is being used in place of Tubulin due to the similarity in molecular weight of Tubulin (55.0 kDa) and the targeted cyclins (L1 - 60.0 kDa; L2 - 58.0 kDa), the problem seems to lie in the antibody, rather than the Western itself, but either is possible.

Conclusions

Due to the ambiguity of dropout, there are a variety of potential interpretations from this project's results so far. It cannot be concluded that cyclin L1 nor cyclin L2 have no role in the activation of CDK11, as some dropout was observed, and it was of a magnitude higher than negative controls. However, it also cannot be concluded that either cyclin L1 or cyclin L2 are responsible for the activation of CDK11, as the magnitude of dropout was relatively low and not obviously higher than negative controls in all cases. Also, without conclusive Western blotting results, it is unknown whether the targeting cyclins were even knocked-out from samples as intended.

However, it is worth noting that double guide transduced populations tended to dropout more than their single guide counterparts, which could potentially implicate both cyclin L1 and L2 in the activation of CDK11. It is also important to note that this is relatively new project that is still in progress. Additional trials of dropouts, as well as Western blotting and PCR amplification for sequencing, are currently underway. Furthermore, this project may potentially incorporate new guide RNAs targeting cyclin L1 and/or cyclin L2 for dropout assays, and may possibly be expanded to include an investigation into cyclin D3, involving a repeat of this entire methodology, due to its observed interaction with CDK11 in non-cancer cells (Loyer, 2008).

Even without a conclusive identification, this project serves as an important first step in the identification of the cyclin responsible for the activation of cancer dependency CDK11. What dropout exists warrants further investigation into both cyclin L1 and L2, possibly concurrently, as activating cyclins, and supports a continuation and possible expansion of this project to include the previously mentioned adjustments.

Future Work

The next steps in this project include primarily the Western blotting and PCR amplification for sequence verification detailed in the methodology and currently in progress, which will determine whether or not the transduced gRNAs from the dropout assays successfully produced a knock-out of the targeted cyclin(s).

Next, even if further experimentation provides more conclusive results, other experiments should be conducted to verify a particular cyclin as the activator for CDK11. These can include biochemical investigations into the pathways of these cyclins, as well as the elucidation of the structures of these cyclins and CDK11 to look for complementary structures. Other more complicated approaches could include the development of a small molecule inhibitor targeting the activating cyclin to investigate whether the same effect is seen, or using homology directed repair (Pyhtila, 2016) to modify an activating cyclin to confer some biological marker or conformational change onto CDK11, and then testing for that marker or change.

This project itself can also be expanded in the future to increase comprehensibility. This includes developing gRNAs targeting cyclin D3 as well as combinations of this cyclin with cyclins L1 and/or L2. The dropout assays can also be repeated on an assortment of additional cancer cell lines to enhance the reliability of any results and conclusions drawn from them, or can likewise incorporate more guide RNAs targeting all of the aforementioned cyclins.

Once an activating cyclin is successfully identified, researchers can in the future study the function and biological pathway of this cyclin in depth to better understand both the cyclin itself and CDK11, both in non-cancer cells and as a cancer dependency. Similarly, an understanding of the activating cyclin can be used to aid efforts to elucidate CDK11's exact structure that are underway right now. By extension, this will also aid the effort to synthesize drugs targeting CDK11, which can potentially save lives as a novel cancer therapy. Finally, since cancer cells cannot survive without

CDK11, if the activating cyclin is inhibited and CDK11 is never activated, the same effect would occur, producing a new drug target. Small molecule inhibitors (or other drugs) targeting CDK11's activating cyclin would theoretically also kill cancer cells, creating another avenue with which to save lives.

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