CRISPR/SPCAS9 MEDIATED SCREENING OF P53-RELATED MIRNA GENE TARGETS

by

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Dedicated to my parents, John and Mojgan Withers

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by

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THESIS

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The p53 network plays a vital role in regulating cell growth and protecting against DNA damage. Recent research has uncovered that many microRNAs (miRNAs) are activated by p53 and assist in its regulatory functions. These miRNAs act by targeting sequences on messenger RNA (mRNA) transcripts that bare partial or complete complementarity to the seed region of the miRNA. Thus, miRNAs can affect apoptosis, cell-cycle arrest, mesenchymal-epithelial transition, and stemness in cells depending on their targets. Using CRISPR/SpCas9, the targets of p53-related miRNAs were studied, as well as their relation to cell viability by building a sgRNA library. The sequences that miRNAs interact with were edited in the 3'untranslated region (3'UTR) of mRNAs and negatively select against the cells by triggering cell death with miRNA transfection. This library provides a novel approach to miRNA network analysis by allowing for high throughput analysis via edge removal.

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CHAPTER 1

INTRODUCTION

1.1 MicroRNA

First discovered in 1993 in *C. elegans,*[1] microribonucleic acids (miRNAs) are a class of small, non-coding RNAs implicated in post-transcriptional gene regulation. Since their discovery, miRNAs have been found in a wide variety of organisms, from plants [2] to humans and even in their mitochondria.[3] miRNAs are exported to the cytoplasm complex with a member of the Argonaute protein family to form the RNA induced silencing complex (RISC),[4] which uses the miRNA to target mRNAs for either translation inhibition or trigger mRNA degradation.[5] Due to their role in regulating gene expression, miRNAs are a rich topic of study for scientists seeking to understand cellular processes, fetal development, and disease pathologies.

miRNAs are first transcribed as hairpin shaped transcripts called pri-miRNAs.[6] These transcripts are often several kilobases long. A RNAse type III protein called Drosha binds to pri-miRNAs to cleave the stem of the hairpin, resulting in the release of a 65 nucleotide hairpin called a pre-miRNA.[7] After processing in the nucleus, the pre-miRNA is exported to the cytoplasm via exportin-5.[8] Once in the cytoplasm, the small hairpin is processed by Dicer.[9] The pre-miRNA is cleaved near its terminal loop and released as a duplex of approximately 22 nucleotides. The two strands of the duplex are referred to as the guide strand (miRNA) and the passenger strand (miRNA*). The guide strand is loaded onto the RISC complex, and the passenger strand is released, resulting in the active miRISC complex.[10]

mRNAs are targeted by miRNAs based on the complementarity of target sequences in their 3'Untranslated Region (3'UTR) with the seed region of the miRNA, nucleotides two through eight.[11,12] In animals, perfect complementarity between the target sequence and seed region is rare. Partial complementarity is more common and usually results in preventing the translation of the mRNA, not in its degradation.[13] The disruption of translation is believed to be due to similarities between the Ago2 protein of the RISC and eIF4E, resulting in competition between the two proteins to bind to the mRNA.[14] It has been observed that a single miRNA can target many genes, with some genes being targeted by more than one miRNA.[15] Furthermore, some genes are predicted to have multiple target sequences for a given miRNA.[16]

The ability of miRNAs to regulate a wide variety of genes confers them multiple functions in cells. The miRNA let-7 is temporally controlled during larval development in *C. elegans* to regulate the timing of gene expression.[18] miRNAs can also curb protein expression levels or introduce adaptation to copy number variability.[19] Some miRNAs have been found to be tissue-specific, such as miR-1 in the heart, miR-122 in the liver, and miR-124 in the brain,[17] lending credence to the hypothesis that miRNAs play a role in tissue differentiation in mammals during development. Other miRNAs are differentially expressed in various hematopoietic tissues, implicating them in affecting the differentiation of hematopoietic cells. Chenz et al. supported this hypothesis by ectopically expressing miRNAs of interest in hematopoietic progenitor cells and observing the resulting altered lineage differentiation.[20]

As of June 2014, the Human microRNA Disease Database (HMDD) had tallied 378 human diseases influenced by miRNAs from 3,511 papers.[21] The diseases detailed range across a variety of body systems, from Crohn's Disease [22] to Parkinson's Disease [23] to many different types of

cancer.[24] The ability to quantify and respond to endogenous miRNA levels therefore provides potential diagnostic and therapeutic applications in responding to diseases.[25,26,27] miRNAs related to the tumor suppressor protein p53 are of particular interest for studying the development of cancer. These miRNAs are intricately involved in the functions of p53, such as regulation of the cell cycle and induction of apoptosis.[28,29]

1.2 CRISPR/Cas9

Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) were first discovered in *E. coli* genomes in 1984.[30] The functional role of CRISPRs was elucidated in 2007 when new CRISPR spacer sequences were found in bacterial DNA following phage infection, and the bacteria with these new sequences gained resistance to the particular virus.[31] This experiment supported the hypothesis that CRISPRs, along with CRISPR associated proteins (Cas) were a form of bacterial defense against viruses. Various Cas proteins have nuclease and helicase domains,[32] which allow them to use CRISPR-RNA (crRNA) to target viral DNA.[33] There are multiple modes of action for CRISPR systems.[34]

Type II systems use a proto-spacer-adjacent motif (PAM) on a crRNA structure that lacks a hairpin loop, which is used to load the crRNA into the Cas protein in other systems.[35] They use trans-encoded small RNA (tracrRNA) to associate with pre-crRNA, leading to cleavage by RNase III.[36] This cleavage results in mature crRNA, which directs the Cas9 protein that has complexed to a targeted DNA sequence, and the Cas9 protein then cleaves the DNA.[37]

The development of a combined tracr/crRNA molecule, called a short-guideRNA (sgRNA) opened up the possibility of using Cas9 protein from *Streptococcus pyogenes* to make a simple two structure CRISPR/SpCas9 system for intentional targeting of DNA.[38,39] As understanding of

the CRISPR/Cas9 system advanced, researchers pursued using the system to make targeted edits in the human genome.[40] Since its first use to make specific alterations to eukaryotic cells, the various purposes for genome editing with CRISPR/Cas9 have expanded. It has been used to make transgenic animal models,[41] to fluorescently label DNA for imaging in live cells,[42] and in library screening models.[43] The PAM constraint can be used for single nucleotide targeting,[44] while a self-cleaving mechanism allows for controllable gene delivery.[45]

Wang et al., used CRISPR/Cas9 mediated gene knockout as a negative screen for genes involved with cell viability in cancer cells and as a positive screen for genes that contribute to vemurafenib resistance in melanoma cells. By constructing a sgRNA library, they were able to build a lentivirus library with their lentiCRISPR system. Using the lentiCRISPR system, they grew lentiviruses that contained their sgRNA vectors. They were then able to integrate these vectors into mammalian cells by infecting them with the viruses. This system allowed for integration of a single sgRNA into each cell that survived selection. That sgRNA acted with SpCas9 to introduce insertions and deletions (indels) into targeted genes through non-homologous end joining (NHEJ), disabling, or knocking out, the gene. These indels resulted in the disability of the targeted gene. For their first screen, cells that received an edit that was detrimental to cell survival died, and they detected these changes by using Next Generation Sequencing (NGS) to detect the sgRNAs in surviving cells. NGS allows for the collection of large amounts of reads of short DNA strands, making it ideal for sgRNA libraries. They compared the starting sgRNA profile with the final sgRNA profile to determine which edits were important.

CRISPR libraries have been used in a variety of applications. However, they have not yet been used to screen for target sequences for miRNAs. In the p53 network, p53-regulated miRNAs can

target mRNA substrates that are essential in cell cycle progression and apoptosis. Thus, expanding the knowledge of miRNA-mRNA functional interactions can provide insight into the efficacy of miRNA therapy.

CHAPTER 2

LITERATURE REVIEW

2.1 MicroRNAs and the p53 Pathway

As a tumor suppressor protein, p53 responds to many sources of stress in cells.[46] As such, it is often mutated in cancers. miRNAs also respond to sources of stress, such as oxidative stress.[47] Because miRNAs are good at modulating gene levels, many are involved in the p53 pathway, being directly and indirectly activated by p53.[48,49] By acting on genes in the p53 pathway, in response to stress, miRNAs can induce cell cycle arrest, senescence, or apoptosis.[50] Among the many miRNAs that can be activated by p53 are miR-34a, miR-145, miR-192, miR-194, and miR-215. These miRNAs are notable because they affect multiple p53 related processes in the cell.

2.2 miR-34a

miR-34a is one of the most studied miRNAs of the p53 pathway. Treatment of cells with miR-34a has been found to lead to apoptosis, and ectopic expression of miR-34a has been demonstrated to lead to G1-cell cycle arrest.[51] Though a large part of miR-34a's induction of apoptosis is p53-dependent, by using a double p53 knockout cell model, Chang et al. found that miR-34a has some p53-independent role in inducing apoptosis.[52] miR-34a's p53 dependent apoptotic response is because miR-34a can regulate p53 indirectly through a positive feedback loop, repressing the p53 repressor, SIRT1.[53] Part of miR-34a's relationship with cell cycle arrest is from the repression of cell cycle regulator, E2F3.[54]

Additionally, miR-34a is implicated in mesenchymal-epithelial translation (MET). When miR-34a is downregulated in cancer cells, it can no longer repress the transcription factor SNAI1, which induces epithelial-mesenchymal transition (EMT), a process that contributes to the

metastatic nature of cancers.[55] The transcription of the miR-34a gene can be repressed by SNAI1, constituting a double negative feedback loop, which confers bistability on cells, making them less likely to undergo MET or EMT.[56] Another way that miR-34a reduces metastasis in cancer cells is by repressing the receptor tyrosine kinase MET, an observation found by Hwang et al. by transfecting ovarian neoplastic cells with pri-miR-34a and measuring MET expression.[57] With miR-34a's various roles in cancer repression, it should come as no surprise that it is often deactivated epigenetically in cancer cells through CpG methylation.[58]

2.3 miR-145

miR-145 contributes to cell cycle arrest by repressing the expression of a myriad of genes. Among the mRNAs targeted by miR-145 are the mRNAs for MYC, Cyclin D1, and eIF4E.[59] CDK4, and CDK6 are also regulated by miR-145.[60] Cyclin D1, eIF4E, CDK4 and CDK6 are G1 regulators, and when they are repressed, the cell enters G1 arrest. miR-145 is found to be deregulated in epithelial cancers, resulting in a loss of regulation of MDM2, which inhibits p53 activation. Reintroducing miR-145 into these cancers was thus observed to cause p53 dependent apoptosis.[61] miR-145 provides another check against cancer by regulating OCT4, KLF4, and SOX2. By repressing these factors, miR-145 limits the ability of cancer to develop into stem cells.[62] miR-145 has been observed to be silenced in prostate cancers through CpG methylation.[63]

2.4 miR-192/194/215

miR-192, miR-194, and miR-215 all belong to the same family of miRNAs. miR-192 and miR-215 inhibit many cell cycle regulators, and as such, it has been demonstrated that induction with these miRNAs can trigger G1 arrest and G2-M arrest.[64] They also help stabilize p53 by regulating DTL, a protein that interacts with the DDB1-CUL4 complex to destabilize p53.[65] miR-192, miR-

194, and miR-215 play an indirect role in modulating apoptosis by repressing IGF1 expression thereby reducing the signaling threshold necessary to induce apoptosis.[66] These miRNAs also contribute to apoptosis in a similar manner to miR-145, by inhibiting MDM2.[67] The interactions of miR-34a, miR-145, miR-192, miR-194, and miR-215 in the p53 pathway are multitudinous, and new explorations of these interactions continue to unfold.

CHAPTER 3

CRISPR/SPCAS9 MEDIATED SCREENING OF P53-RELATED MIRNA GENE TARGETS

3.1 Objective

p53 and miRNAs involved in the p53 pathway are known to regulate important cellular events, such as apoptosis, cell cycle checkpoints, and epithelial-to-mesenchymal transition. These events are often implicated in cancers, whether it be the disruption of apoptosis and cell cycle regulation or the stimulation of EMT. As such, the relationships of miRNAs and their gene targets have been researched extensively. However, the relationships of miRNAs and the specific target sequences on which they act have not been explored deeply. Recent advances in library screening techniques due to the discovery of the CRISPR-cas9 as a eukaryotic DNA editing device allow for the possibility of screening for specific miRNA target sequences and their impact on cell survival.

To screen for miRNA target sequences involved in cancer cell survival, a short-guideRNA (sgRNA) vector library of sgRNAs that resulted in the formation of insertions or deletions (indels) that disrupted the Watson-Crick complementarity interaction of miRNA with mRNA was built. This library and the LentiCRISPRv2 system were used to build a viral library. This viral library was to affect changes on colon cancer cells. It was hypothesized that the continued transfection of super-physiological concentrations of miRNA mimic, which well exceeded endogenous miRNA levels, will cause cells to die, resulting in the survival of only cells that received a sgRNA that affected an important miRNA target sequence. Additionally, vectors were constructed to express the miRNAs of interest and tested the efficacy of these miRNA vectors with reporter plasmids.

Finally, vectors were built to express various miRNAs and fluorescent proteins to be packed into lentivirus for future experiments.

3.2 Experimental Design

As a screen for miRNA gene targets, a sgRNA library to disrupt miRNA gene targets was built. Five candidate miRNAs that are regulated by p53 were identified: miR-34a, miR-145, miR-192, miR-194, and miR-215. Genes regulated by these miRNAs were researched and the most likely miRNA targets in the 3'UTRs of these mRNAs were found (Figure 3.1a). sgRNAs were designed to allow the CRISPR-cas9 system to introduce indels into the miRNA targets and oligonucleotide primers were designed to form these sgRNAs. The annealed primers were integrated into a lentiviral vector, which was used to grow lentiviruses. With this lentivirus pool, cells were infected and grown under puromycin selection so only the cells that had received lentivirus and stably integrated the CRISPR cassette could survive and grow (Figure 3.1b). Once cells had grown to confluency, cells were collected, genomic DNA was extracted, and PCR was performed to amplify sgRNA sequences. These sequences were sent out to be sequenced by Sanger sequencing to determine that the sgRNA profile had been maintained through all these steps. The cell library was exposed to miR-34a mimic and a miRNA control (ce-miR67) for six days, passing and transfecting every three days. Cells were collected at passage zero, one, and two. Genomic DNA was collected from each sample, PCR was performed to amplify sgRNA sequences (Figure 3.1c), and these sequences were sent to be sequenced by Next Generation Sequencing (NGS). The sgRNA profiles of miRNA treated with miR-34a from day six were compared with untreated cell profiles from day zero (Figure3.1d). Cells from the first passage were tested for cell-cycle arrest using a propidium iodide assay by Chance Nowak.

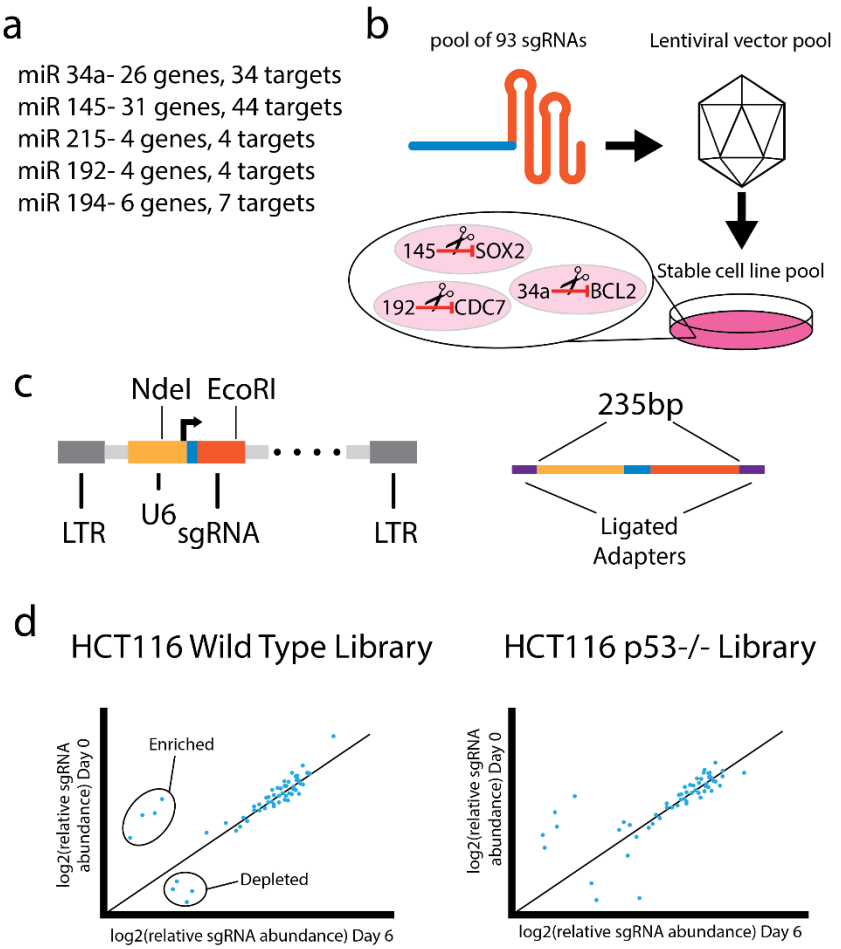


Figure 3.1. An overview of experimental design. a) Selection of miRNAs and targets b) Construction of sgRNA, lentiviral, and cell libraries c) extraction of sgRNAs for NGS d) mock data of a comparison of sgRNA profiles

Plasmids to express miR-34a, miR-145, miR-192, miR-194, and miR-215 were designed and cloned using a library of plasmids from the Bleris lab. The miR-192 vector was tested using a pre-existing reporter plasmid that expresses cyan fluorescent protein (CFP) with three perfectly complementary seed sequences for miR-192 in the CFP's 3'UTR. Plasmids to express miR-34a, miR-125b, miR-146a, and miR-155 along with yellow fluorescent protein (YFP) were designed and integrated into an AAVS plasmid. Plasmids to express mKate fluorescent protein with three seed sequences in the mKate's 3'UTR that are perfectly complementary to miR-34a, miR-125b,

miR-146a, and miR-155 were designed and integrated into an AAVS plasmid. These plasmids were sent for Sanger sequencing and will be used in a future project in the lab.

CHAPTER 4

MATERIALS AND METHODS

4.1 Cell Lines

HCT116 and HCT116 $p53^{-/-}$ cell lines, obtained from the Michael White lab at UT Southwestern Medical Center were used in this experiment. These cells are derived from human colorectal carcinoma and were chosen due to the pairing of a wild type and a p53 knockout cell line. The cells were grown in an incubator at 37℃, 100% humidity, and 5% CO_2. The medium used was Dulbecco's modified Eagle's medium (DMEM, Gibco, Cat-No# 11965-092) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Cat-No# S11550), 0.1 mM non-essential amino acids (Gibco, Cat-No# 11140-050), 0.045 µg/mL of Penicillin, 0.045 µg/mL of Streptomycin (Pen-Strep, Gibco, Cat-No# 15140-122), and 1 mmol of additional glutamine (Gibco, Cat-No# 25030-081). The medium was filter sterilized (Corning, Cat-No# 430758). Cells were grown to 60-90% confluency then passed to a new plate. To pass cells, cells were washed with phosphate buffer saline (PBS, Mediatech, Inc., Cat-No# 21-030-CM), trypsinized with 0.25% Trypsin-EDTA (Gibco, Cat-No# 25200-056), and diluted with fresh medium. Cells were maintained in 10 cm Cell Culture Dishes (Greiner Bio-One, Cat-No# 664160).

AAV HEK293 cell line obtained from Agilent was used to grow lentivirus. These cells are derived from human embryonic kidney cells. Cells were grown in an incubator at 37℃, 100% humidity, and 5% CO_2. The medium used was Dulbecco's modified Eagle's medium (DMEM, Gibco, Cat-No# 11965-092) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Cat-No# S11550), 0.1 mM non-essential amino acids (Gibco, Cat-No# 11140-050), 0.045 µg/mL of Penicillin, and 0.045 µg/mL of Streptomycin (Pen-Strep, Gibco, Cat-No# 15140-

122). The medium was filter sterilized (Corning, Cat-No# 430758). Cells were grown to 60-90% confluency then passed by the same method described above.

4.2 Recombinant DNA Cloning

For PCR, cloning, and ligation, 2X Q5 Master Mix, most restriction enzymes, Taq polymerase, 10X T4 ligase, and buffers were obtained from NEB. Restriction enzyme BsmBI and the Tango buffer were obtained from Thermo Scientific. Plasmid isolation kits, gel extraction kits, genomic DNA isolation kits, and PCR purification kits were obtained from QIAGEN, and these procedures were performed per manufacturer's instructions. NEB 5-alpha Competent *E. coli* (Cat-No# C2987H) and XL-10 Gold Ultracompetent cells (Stratagene, Cat-No# 200314) and used for transformation. Bacterial culture media (Difco), agar (Difco), and antibiotics (Mediatech, Inc., Teknova) were prepared as per manufacturer's instructions. Primers were designed using A Plasmid Editor (ApE v2.0.50) and ordered from Sigma. Primers were diluted to 100 µM. Sanger sequencing was performed by Genewiz.

4.3 Constructs

Pre-existing vectors from the Bleris lab were used to build the constructs. Vector sequences are supplied in the Supplement section.

4.3.1 *Vector Library Constructs*

Genes were selected that had been validated with a Reporter Assay, Western Blot, and qPCR, which were found to be reported on MirtarBase. miRNA target sites within the 3'-UTRs were identified by Targetscan. In total, 93 such targets sites from 71 target genes were identified. sgRNAs were designed to direct the spCas9 protein to miRNA target sites with a NGG PAM located close to the sites. Sense and antisense oligos were designed to form sgRNAs with

overhangs complementary to BsmBI cleavage sites and ordered mixed on a 96-well plate from Sigma Aldrich (Supplement Table 7.1). The oligos were diluted to 100 µM and annealed in an annealing PCR reaction (Supplement Protocol 7.1.1). One microliter of each annealed oligo was combined with 9,904 µL of dH_2O to result in a 1:100 mixed dilution. Vector D15 (Supplement 7.3.1) was digested with BsmBI at 37°C for 1 hour, and the 12 kb band was gel purified. One microliter of the mixed annealed oligo solution was ligated with the BsmBI treated D15 12 kb segment at 16°C overnight. The solution was treated with ClaI for one hour at 37°C to ensure only vector ligated with oligos remained. Five microliters of this solution were transformed into 50 µL of gold ultracompetent cells, which were grown in 450 µL of outgrowth media, and the full volume was plated onto an antibiotic plate for 16 hours at 37°C. All colonies grown, of which there were over 300, were scraped from the plate and plasmids were harvested. Plasmids were sequenced by Sanger sequencing using Primer 1 to ensure they matched the planned construct (Figure 4.1).

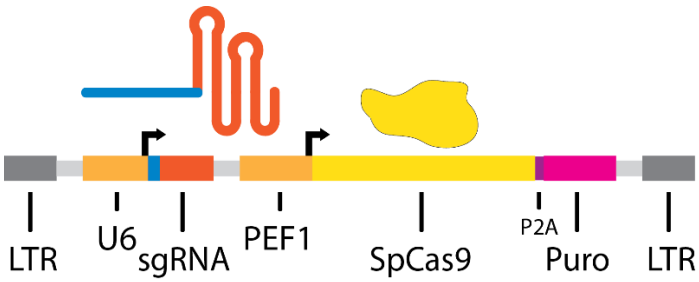


Figure 4.1. Construct of library plasmid

4.3.2 *MicroRNA Vector Constructs*

MiRNA vectors were designed using vector C1 (Supplement 7.3.2). Oligos to produce a miR-30 hairpin with miR-34a, miR-145, miR-215, miR-192, and miR-194 seed sequences were designed and annealed with PCR (Supplement Protocol 7.1.1) (Primers 2-11). Annealed oligos were buffer exchanged and diluted at a 1:10 dilution. 600 ng of vector C1 was digested for three

hours at 37℃ with XhoI and EcoRI. Digested vector was run through a 1% agarose gel at 150 V for 45 minutes. The digested band was excised and purified. Annealed oligos were ligated with the purified C1 digest at 16℃ overnight. Ligation solutions were used to transform NEB 5α cells, and cells were allowed to grow on agar plates with carbenicillin for 16 hours at 37℃. Three colonies from each plate were collected, grown in broth, and had their plasmids extracted. Plasmids were sent for Sanger sequencing using Primer 12, and one successful sample for each miRNA was retained. A successful sample was considered to match the design of Figure 4.2.

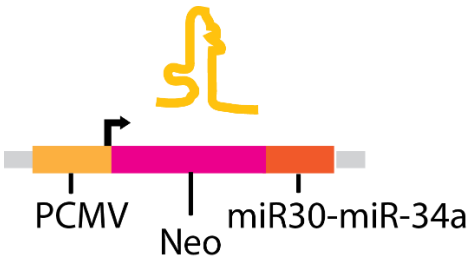


Figure 4.2. Construct of miRNA vector plasmid. miR-34a structure is replaced with miR-215, miR-192, or miR-194 in other vectors from this construct.

4.3.3 *MicroRNA with YFP Constructs*

PCMV and YFP sequences were amplified from vector D30 (Supplement 7.3.3) using PCR (Supplement Protocol 7.1.2) using Primers 11 and 12. PCR amplicons were buffer exchanged. PCR products and vector D34 were digested with AgeI and NotI for one hour at 37℃ and gel purified. The insert was ligated with the digested vector in a ratio of 3:1 at 16℃ overnight. Ligation product was used in transformation of NEB 5α cells, and four colonies were selected for expansion. Plasmids were extracted and digested with AgeI and NotI to test for successful ligation. A successful sample was retained for further use.

Oligos were designed to produce a miR-30 backbone with miR-34a, miR-125b, miR-146a, and miR-155 seed sequences (Primers 2-3,15-20). Oligos were also designed to have XhoI and EcoRI

cut sites at the 5' and 3' ends. Oligos were annealed using PCR (Supplement Protocol 7.1.1), buffer exchanged, and diluted at a 1:10 dilution. The vector from the previous step was digested with XhoI and EcoRI and gel purified. The vector and oligos were ligated overnight and transformed. Three colonies for each miRNA were selected and expanded. Their plasmids were sent for sequencing with Primer 23. One successful sample was retained for each miRNA.

The PCMV, YFP, and miRNA encoding sequences were amplified using Primers 21 and 22, buffer exchanged, and digested with SbfI and AscI. Vector D11 (Supplement 7.3.4) was digested with SbfI and AscI as well. Digested products were gel purified and ligated overnight. Ligated products were used to transform NEB 5α cells, and three successful colonies for each sample were expanded. Plasmids were extracted and sequenced with Primer 23 to ensure they matched Figure 4.3.

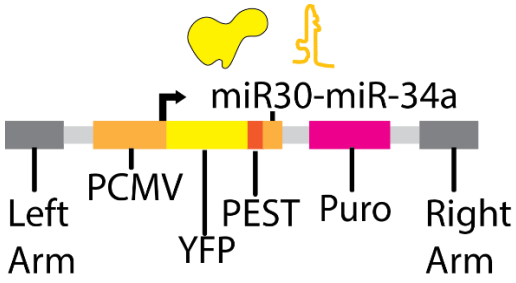


Figure 4.3. Construct of miRNA donor vector with YFP. miR-34a structure is replaced with miR-125b, miR-146a, and miR-155 in other vectors from this construct.

4.3.4 *mKate with MicroRNA Target Constructs*

Using vector CPF_CCR1.1.8 (Supplement 7.3.5), the sequences for UBC and mKate were amplified with Primer 24 and the reverse primers, Primers 25, 26, 27, and 28 (Supplement Protocol 7.1.2). The reverse primers were designed to adjust the miRNA targets on the 3'UTR of the mKate mRNA encoded by these amplicons. PCR products were buffer exchanged and digested with NheI

and XhoI. CPF_CCR1.1.8 was also digested with NheI and XhoI. Digested products were gel purified, ligated, and used to transform NEB 5α cells. Successful colonies were picked, expanded, and had their plasmids extracted. These plasmids were sent for sequencing using Primer 29. One successful sample for each miRNA was retained.

Using Primers 30 and 31, the UBC, mKate, miR target sequences were amplified (Supplement Protocol 7.1.2). PCR products were buffer exchanged and digested with ClaI and AscI. Vector D11 was digested with ClaI and AscI as well. Digested products were gel purified and ligated. Ligation products were used to transform NEB 5α cells. Successful colonies were picked, expanded, and had their plasmids extracted. Plasmids were verified by digestion with ClaI and AscI and gel electrophoresis. One successful sample for each miRNA was retained.

A vector with the sequences for PCMV and CFP flanked by AscI sites and each of the successful samples described above were digested with AscI and gel purified. The PCMV CFP fragment was ligated into the digested destination plasmids. Ligation products were used to transform NEB 5α cells. Successful colonies were picked, expanded, and had their plasmids extracted. Plasmids were verified by sequencing using Primer 29 to ensure they matched Figure 4.4.

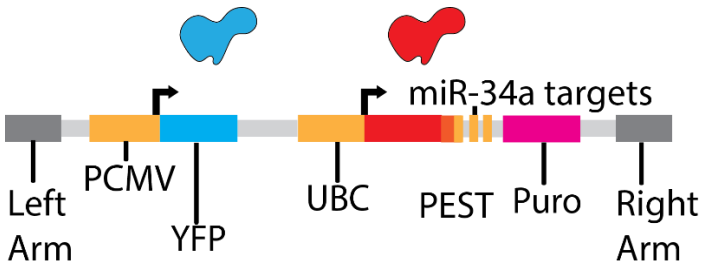


Figure 4.4. Construct of miRNA receiver with mKate and CFP. miR-34a targets are replaced with miR-125b, miR-146a, and miR-155 targets in other vectors from this construct.

4.4 Preparation of the pLentiCRISPRv2 Library

Protocol for pLentiCRISPRv2 library preparation was developed by Dr. Zhang Feng. Three point three micrograms of plasmid library, three point three micrograms of D16 (Supplement 7.3.6), and three point three micrograms D17 (Supplement 7.3.7) were transfected into AAV-293 cells. Five milliliters of medium containing the viral vectors were harvested at days two, three, and four for a final volume of 15 mL. The viral titer was determined by qPCR Lentivirus titration (Titer) Kit (ABMGOOD, Cat No# LV900). The viral titer was found to be $2.07*10^7$ IU/mL. This viral titer was used to determine the volume of viral medium to be used to infect cells for a desired multiplicity of infection (MOI) of 0.3.

4.5 Preparation of the Cell Libraries

HCT116 and HCT116 $p53^{-/-}$ cells were seeded into five wells on two six-well plates each, at concentrations of $2.5*10^5$ cells per well. Cells were exposed to one-half, one, two, three, and four µg/mL puromycin. Cell death was observed to determine the appropriate concentration of puromycin for drug selection. 0.5 µg/mL puromycin was determined to be appropriate.

HCT116 and HCT116 $p53^{-/-}$ cells were seeded onto two collagen coated cell culture dishes at concentrations of $9*10^6$ and $7.5*10^6$ cells per plate respectively. Cells were grown overnight and exposed to 250 µL and 211 µL of viral media to result in a MOI of 0.3. This low MOI ensured that each cell only received one copy of sgRNA. Cells were grown for 48 hours and split 1:3. Cells were then selected by 0.5 µg/mL puromycin for two weeks. Stable cells were combined back into one plate. Cells were passed onto a new plate in puromycin as they grew to 60-90% and several samples were stored in liquid nitrogen after the first passage. Genomic DNA samples were collected from the HCT116 sgRNA library cells, and the sgRNA library was amplified using PCR

(Supplement Protocol 7.1.3) (Primers 1 and 32). PCR products were sent for sequencing using Primer 32 to ensure the sgRNA profile had been maintained.

4.6 Transfection of Cell Libraries with miRNA Mimics

Reverse transfection was performed using RNAiMax transfection reagent (Invitrogen, Cat-No#13778-030) to achieve a final concentration of 25 μM of miRNA mimic. miRNA mimic 34a (QIAGEN, Cat-No# MSY0000255) and miRNA mimic Cel-miR-67 (Dharmacon, Cat-No# CN-001000-01-05) were used as experimental and control treatments respectively. Cells were seeded into six-well plates (Greiner Bio-One, Cat-No# 657160) at concentrations of $5*10^5$ cells per well. Cells were collected, re-transfected, and passed every three days for six days total. Cells were collected at passage zero, one, and two of the experiment. Cells from the first passage were also collected for cell cycle analysis performed by Chance Nowak using a propidium iodide kit (abcam, Cat-No# ab139418) and flow cytometry. Genomic DNA was purified from each set of cell samples, and the sgRNA libraries were amplified using PCR (Supplement Protocol 7.1.3) using Primers 1 and 32. PCR products were sent for Next Generation Sequencing (UT Health Science Center at San Antonio) using primers 33 and 34. The sgRNA library of the HCT116 cells from passage zero was sent for Sanger sequencing using primer 32 to ensure the sgRNA profile had been maintained since the cells were first selected.

4.7 Transfection of Cells Using miRNA Vectors

HCT116 cells were seeded into seven wells of a twelve-well plate at $1*10^5$ cells per well and grown for 24 hours. Three wells were treated with 100 ng of reporter plasmid and 500 ng of miR-192 vector, and three wells were treated with 100 ng of reporter plasmid and 500 ng of C1 control

vector using JetPrime (PolyPlus, Cat-No# 114-15). One well was left untreated. Cells were incubated for 48 hours before analysis with fluorescence microscopy and flow cytometry.

4.8 Data Collection with Fluorescence Microscopy and Flow Cytometry

Microscopy images were collected using the Olympus motorized inverted research microscope (IX81). The microscope is equipped with an environmental chamber (Weather station) held at 37℃. Images were collected using a Hamamatsu camera at 10X objective. The filter sets for CFP are 430/25x (excitation) and 470/30m (emission). The software used to image photographs was Slidebook Version 5.0.

Flow cytometry data were collected using a BD LSRFortessa cell analyzer. Cells were prepared for FACS analysis by trypsinizing each well with 100 µL 0.25% trypsin-EDTA, quenching with 900 µL cell medium, and centrifuging the cell suspension at 500 rpm for five minutes. Medium was removed, and the cell pellets were re-suspended by vortexing in 0.5 mL PBS. CFP was measured with a 200nm laser and a 450/50 emission filter. The data were analyzed using FlowJo, which allows for sample gating and obtaining of sample statistics. Sample statistics were further analyzed using R Version 3.3.1 and the EZR Plugin.

CHAPTER 5

RESULTS

5.1 Sequencing of Cloned Vectors

Vectors from section 4.3 were confirmed to be accurately produced by Sanger sequencing. The 20 nucleotide stretch of sgRNAs in the sgRNA library appears to be correct (Figure 5.1)

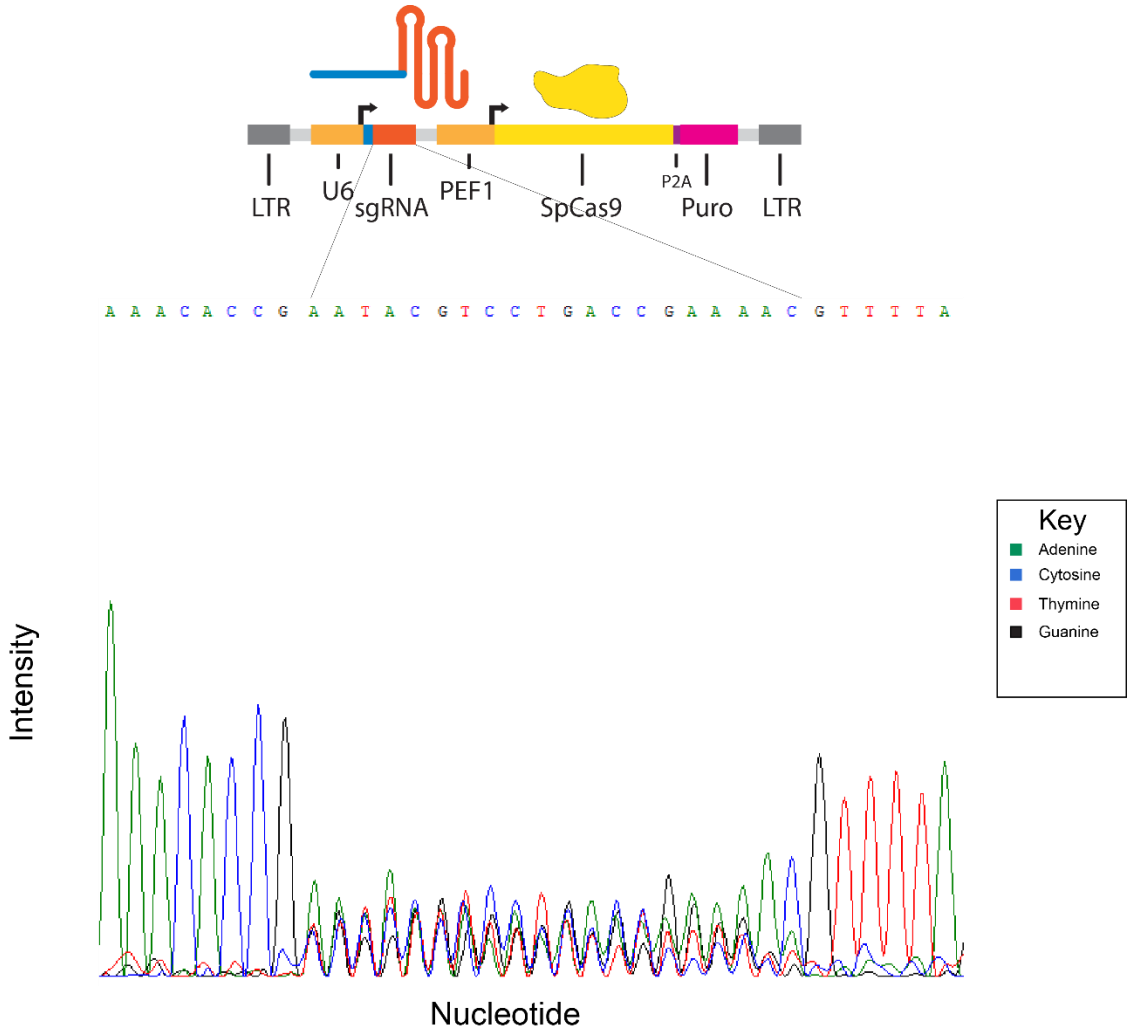


Figure 5.1. The sequence trace file from sequencing of the sgRNA vector library. Overlapping nucleotides indicate that annealed oligos were randomly ligated into the vectors.

5.2 miR Vector Function

Live cells were gated on FlowJo using untransfected cells. Mean CFP of untransfected cells was subtracted from the mean CFP levels of the three control replicates and three experimental replicates, and adjusted means were extracted to R, a statistical analysis software. A one-sided two-variances F-test was performed, and the variances were found not to differ significantly (α=0.05, p-value=0.114). A one-sided two-sample t-test with equal variances was performed, and it was found that there was a significant difference in mean CFP expression between the control and the experimental treatments (α=0.05, p-value=0.0308). The miR vector expressing miR-192 decreased the expression of CFP from the reporter plasmid (Figure 5.2).

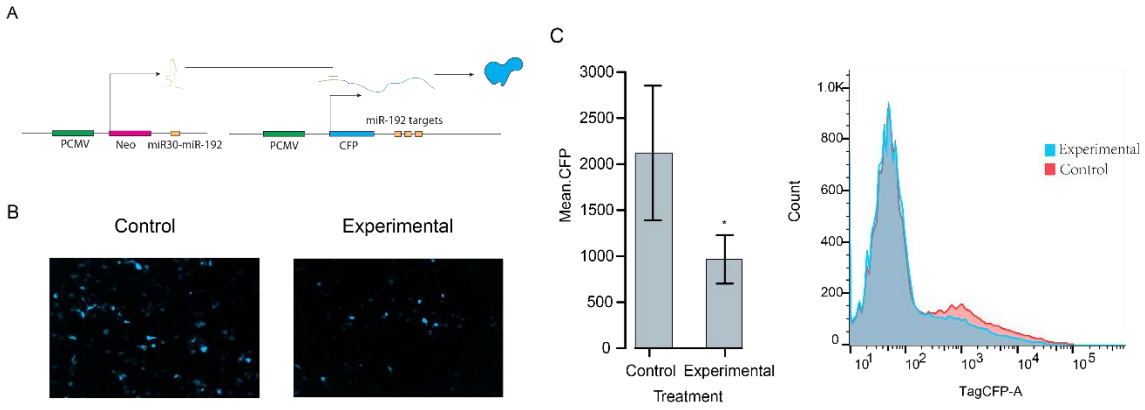


Figure 5.2. Repression of CFP via miR-192 expressing vector. A) Scheme of interaction between the products of the miR-192 vector and the reporter plasmid. B) Fluorescence microscopy images of HCT116 cells transfected with miR reporter and control and miR reporter and miR-192 vector. C) Bar graph of mean CFP expression of treatments, and histogram of CFP expression. Significance level is 0.05.

5.3 Maintenance of sgRNA Profile in sgRNA Library Cell Lines

HCT116 library cells were collected after drug selection to ensure that the sgRNA profile was maintained after viral selection (Figure 5.3a). The sgRNA profile appears to have been maintained. Because silencing is a concern with DNA inserted into mammalian genomes, cells were collected

just before the final experiment was started. The sgRNA profile of these cells was compared with the sgRNAs of the cells collected from their first passage after drug selection (Figure 5.3b). The sgRNA profile at this point still appeared to be maintained.

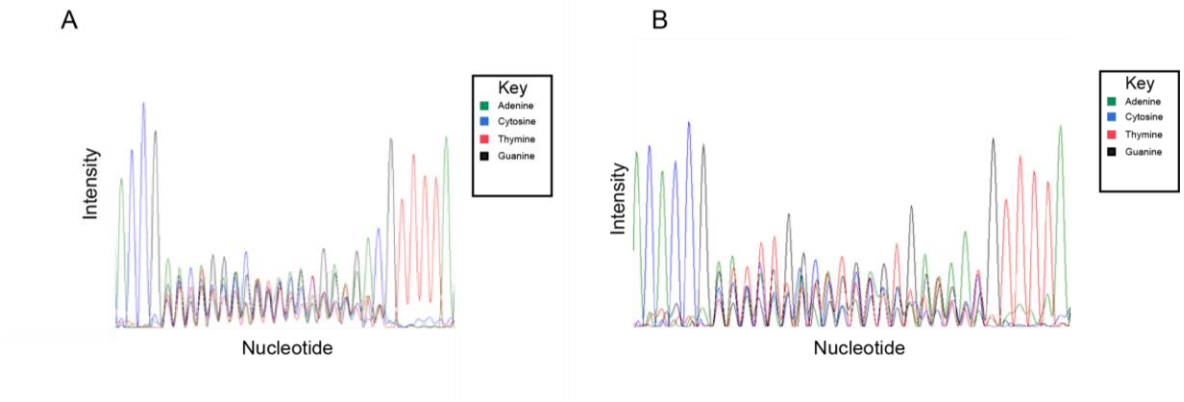


Figure 5.3. The sequence trace files from the HCT116 sgRNA library. A) Cells collected from the first passage after drug selection. B) Cells collected from the zeroth passage of miR transfection

5.4 Cell Cycle Arrest Following miR Treatment

A propidium iodide assay for cell cycle analysis was performed after the first transfection with miR-34a mimic. The assay results in propidium iodide binding to DNA, which results in a fluorescence proportional to the amount of DNA present in the cell. Flow cytometry was performed, and DNA content for each cell was analyzed using FlowJo (Figure 5.4).

Among the HCT116 library cells, the miR-34a treatment had less cells in the S and G2 phases than in the negative control treatment, indicating that G1 arrest had occurred. There were also more cells undergoing apoptosis in the miR-34a treatment. Among the HCT116 $p53^{-/-}$ library cells, there was not a noted difference in cells in the G2 or S phase, indicating that G1 arrest did not occur.

There was an increase in cells undergoing apoptosis, which is consistent with previous findings that miR-34a has some p53 independent function in inducing apoptosis.[51]

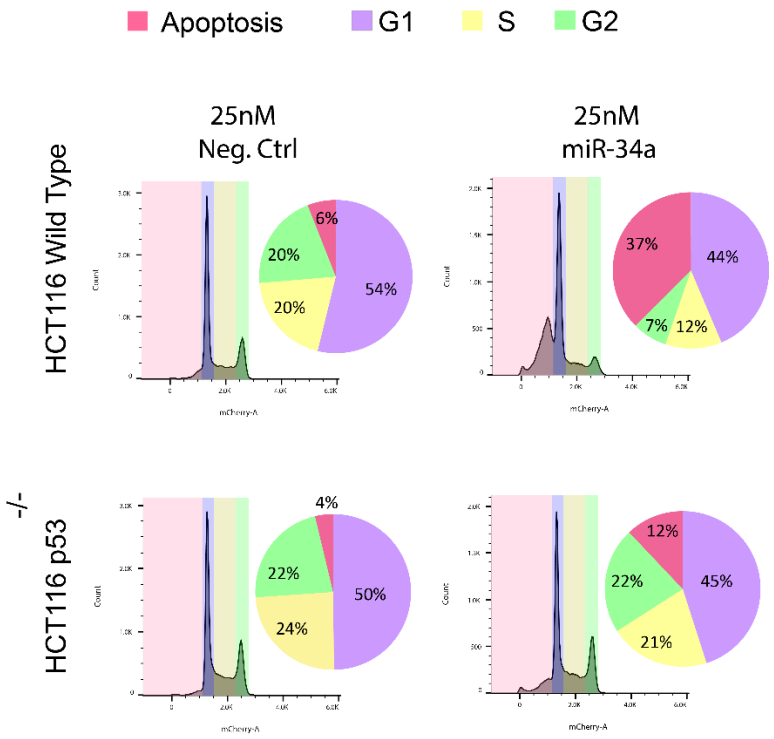


Figure 5.4. Graphical representation of cell cycle analysis.

5.5 Continued Transfection of sgRNA Library Cell Lines with miR-34a

Results were processed by counting each sgRNA read and dividing by the total number of reads. The relative sgRNA abundance was multiplied by 1,000,000 and had one added to each value [42]. Each value was $\log_2$ transformed and plotted on a scatterplot for which sgRNA abundance from day six of the experiment was represented on the y-axis, and sgRNA abundance from day zero was represented on the x-axis (Figure 5.5). Important sgRNAs were chosen based on if they were enriched in the HCT116 wild type cells but not in the HCT116 $p53^{-/-}$ cells, if they were diminished in the HCT116 wild type cells but not in the HCT116 $p53^{-/-}$ cells, and vice versa for both cases. Only sgRNAs for miR-34a were chosen. sgRNAs for FOSL1, DLL1, Notch1, MYCN,

MAP2K1, YY1, and SRC were depleted in the wild type library. sgRNAs for FOSL1, BCL2, and DLL1 were enriched in the $p53^{-/-}$ library. The sgRNA for PDGFRA was depleted in the $p53^{-/-}$ library.

Figure 5.5. Relative abundance of sgRNAs. Important sgRNAs were chosen that were differentially expressed across cell lines.

CHAPTER 6

DISCUSSION AND CONCLUSION

From the library experiment, several notable miR-34a targets were identified; DLL1, Notch1, YY1, SRC, FOSL1, MYCN, and MAP2K1 in the wild type library and FOSL1, BCL2, DLL1, and PDGFRA in the $p53^{-/-}$ library. Interestingly, two DLL1 targets were identified in the wild type library and one other DLL1 target was identified in the $p53^{-/-}$ library. The roll for these miR-34a targets should be explored in future experiments. Of special interest are the targets that were enriched in the $p53^{-/-}$ library and diminished in the wild type library, such as FOSL1 and DLL1.

The team of Couts et al. explored the effects of miR-34a on FOSL1 regulation and the role of FOSL1 in contributing to cancer metastasis.[68] By using combinatorial treatments of miRNAs, they found that miR-34a repression is strong enough that repression by other miRNAs gets overshadowed.

DLL1 is a ligand of the Notch1 receptor protein. Both are regulated by miR-34a, and this loss of regulation for both was found to diminish the populations of cells that received those edits in the cell line. Their regulation by miR-34a has been reported to result in a diminishment of cell proliferation.[69] The interaction of these genes with other miRNAs in the p53 pathway could be explored to determine the mechanism responsible for reducing cell proliferation in the wild type cell line, which has intact p53.

MYCN is a gene found to be amplified in 20-30% of neuroblastoma tumors.[70] Its regulation by miR-34a has been found to result in apoptosis.[71, 72, 73] The observation that removing miR-34a regulation results in a loss in cell viability is notably new. By learning more about the relationship between miR-34a and MYCN, miRNA therapy approaches can be refined. miRNA therapy is

currently a promising route to take in treating MYCN positive neuroblastoma tumors, which have a survival rate less than 30% in patients over a year old.[70]

MAP2K1 contributes to the proliferation of cells as a response to extracellular signals.[74] It is repressed by miR-34a, but this relationship has not been investigated as thoroughly as many other miR-34a targets. The novel finding that the loss of MAP2K1's regulation by miR-34a can somehow result in a loss of cell viability encourages further study on the subject. SRC is a gene that leads to the activation of oncomir miR-21.[75] It was also found to be a part of the same extracellular response kinase (ERK) pathways as MAP2K1.[76] Its proliferative qualities in cancer have been found to be downregulated in response to miR-34a.

In future work, the specific sgRNAs will be explored further by creating monoclonal cell lines with just those sgRNAs. Other studies that can be performed using the cell library include testing the other miRNAs (Figure 3.1a). Additionally, new cell libraries with multiple edits can be constructed by increasing the MOI at the infection step. This library screening offers the novel opportunity to identify miRNA interactions and properties via network edge removal.

CHAPTER 7

SUPPLEMENTARY MATERIALS

7.1 Protocols

7.1.1 PCR for Annealing Oligos

Reaction mix-

a. 1 µL Forward Primer (100 µM)
b. 1 µL Reverse Primer (100 µM)
c. 2 µL 10X T4 ligase buffer
d. 16 µL dH_2O

Reaction conditions-

a. 95℃ for 4 mins
b. Cool on benchtop at room temperature for 1 hour

7.1.2 PCR (Taq Polymerase)

Reaction mix-

a. DNA (10 ng)
b. 1 µL Forward Primer (10 µM)
c. 1 µL Reverse Primer (10 µM)
d. 10 µL 2X Q5 Master Mix
e. dH_2O to a final volume of 20 µL

Reaction conditions-

95℃ for 30 sec

40 cycles:

I. 95℃ for 10 sec

II. 58℃ for 30 sec

III. 72℃ for 1 min/Kb

72℃ for 5 min

Hold: 4℃

7.1.3 Genomic PCR

Reaction mix-

a. Genomic DNA (100 ng)

b. 1 µL Forward Primer (10 µM)

c. 1 µL Reverse Primer (10 µM)

d. 10 µL 2X Q5 Master Mix

e. dH_2O to a final volume of 20 µL

Reaction conditions-

98℃ for 30 sec

40 cycles:

I. 98℃ for 10 sec

II. 60℃ for 30 sec

III. 72℃ for 1 min/Kb

72℃ for 2 min

Hold: 4℃

7.2 Primer Tables

Table 7.1. Oligos used for constructing plasmid library.

Name	Forward Oligo	Reverse Oligo
A1	caccgtccaagctaagcactgccac	aaacgtggcagtgcttagcttggac
A2	caccgaatcagctatttactgccaa	aaacttggcagtaaatagctgattc
A3	caccgagaaaactaaaactagtaca	aaactgtactagttttagttttctc
A4	caccgtttgccaacaaggcagtgtg	aaaccacactgccttgttggcaaac
A5	caccgtgcaacggcgacgtcacgga	aaactccgtgacgtcgccgttgcac
A6	caccgagaatccaaaatatacactc	aaacgagtgtatattttggattctc
A7	caccgcgtatcaaaaaggacaataa	aaacttattgtccttttgatacgc
A8	caccgaaagcagctagaaagtattc	aaacgaatactttctagctgctttc
A9	caccgtcacctgctgctgctggcag	aaacctgccagcagcagcaggtgac
A10	caccggtcacttaaattacacaata	aaactattgtgtaatttaagtgacc
A11	caccgtctttttgaagcactgccac	aaacgtggcagtgcttcaaaaagac
A12	caccgcataaagagactttcataca	aaactgtatgaaagtctctttatgc
A13	caccgagagggcaggcagtgtttct	aaacagaaacactgcctgccctctc
A14	caccggaaggtgaaggtgaaggcag	aaacctgccttcaccttcaccttcc
A15	caccgaaaaataacttttttttcaaa	aaactttgaaaaaaagttatttttc
A16	caccgcctcagactctacctagcgg	aaacccgctaggtagagtctgaggc
A17	caccgttgatttcctcacttaaggc	aaacgccttaagtgaggaaatcaac
A18	caccgaaaaatactgaattctcttc	aaacgaagagaattcagtatttttc
A19	caccgtggtgaagccccagctatca	aaactgatagctggggcttcaccac
A20	caccgtcgatggccttttaaaggtc	aaacgacctttaaaaggccatcgac
A21	caccgttcagttcagctgcaggtat	aaacatacctgcagctgaactgaac
A22	caccgaacagtacatataaataaaa	aaacttttatttatatgtactgttc
A23	caccgagatcagtaaaaagtttgaa	aaacttcaaacttttttactgatctc
A24	caccgacagcttgtcataaatgttt	aaacaaacatttatgacaagctgtc
A25	caccgagttaaaaaaatataaacga	aaactcgtttatatttttttaactc
A26	caccgttacactgaacatcagcatc	aaacgatgctgatgttcagtgtaac
A27	caccgagtaaccttaacattcccta	aaactagggaatgttaaggttactc
A28	caccgaaaaatcaaaatgtgcaaag	aaacctttgcacattttgatttttc
A29	caccgagcgtcatactaaagtatac	aaacgtatactttagtatgacgctc
A30	caccggagatgtcactgctaggtct	aaacagacctagcagtgacatctcc
A31	caccgccacatccttctcactgcca	aaactggcagtgagaaggatgtggc
A32	caccgcagctgggctgagcgggcag	aaacctgcccgctcagcccagctgc
A33	caccgacacagccagtcctggtgca	aaactgcaccaggactggctgtgtc
A34	caccgctggctggctcaagaacccc	aaacggggttcttgagccagccagc

A35	caccggttttggcagccatgataat	aaacattatcatggctgccaaaacc
A36	caccgagcagaaggtaaataatgtc	aaacgacattatttaccttctgctc
A37	caccgatacaaatcagttagttttt	aaacaaaaactaactgatttgtatc
A38	caccggtgcaactctttcttatgat	aaacatcataagaaagagttgcacc
A39	caccgctgtaaacaagaggtaacag	aaacctgttacctcttgtttacagc
A40	caccgaagtgcctagactgttactt	aaacaagtaacagtctaggcacttc
A41	caccgaagttgtaacagttcagaaa	aaactttctgaactgttacaacttc
A42	caccgatcatgtcttgcactgtaac	aaacgttacagtgcaagacatgatc
A43	caccgtatatttataaaaatacttt	aaacaaagtatttttataaatatac
A44	caccgtctacaagcagcgtgtaaca	aaactgttacacgctgcttgtagac
A45	caccgatttgtaattaggtaacagc	aaacgctgttacctaattacaaatc
A46	caccggttttggcagccatgataat	aaacattatcatggctgccaaaacc
A47	caccgtatactcgcacaacttaaat	aaacatttaagttgtgcgagtatac
A48	caccgtcagttagtttttaggtcaa	aaacttgacctaaaaactaactgac
A49	caccggtgcaactctttcttatgat	aaacatcataagaaagagttgcacc
A50	caccgcatttatttgtgatgacaac	aaacgttgtcatcacaaataaatgc
A51	caccggattgggggatgtagcaaac	aaacgtttgctacatcccccaatcc
A52	caccggcaaactggactttaagaac	aaacgttcttaaagtccagtttgcc
A53	caccgttctagatctcactaactac	aaacgtagttagtgagatctagaac
A54	caccgaaaatgcagctgttctgaac	aaacgttcagaacagctgcattttc
A55	caccgaccaataatcctttggaaac	aaacgtttccaaaggattattggtc
A56	caccgaaagctctgttccatgcaac	aaacgttgcatggaacagagctttc
A57	caccgttatctcaataaaagcccac	aaacgtgggcttttattgagataac
A58	caccgtatatatatatatagctgac	aaacgtcagctatatatatatatac
A59	caccggctttgtgttaagtgcctac	aaacgtaggcacttaacacaaagcc
A60	caccggtggtgggtggtttatacac	aaacgtgtataaaccacccaccacc
A61	caccgccttgaagggaagacaaaac	aaacgttttgtcttcccttcaaggc
A62	caccgcaaattcagtggatggcaac	aaacgttgccatccactgaatttgc
A63	caccgcagtgaagttttcacccaac	aaacgttgggtgaaaacttcactgc
A64	caccgagccccttgcctttcaaac	aaacgtttgaaaggcaaggggctc
A65	caccgccctgggcgtgtagtgtaac	aaacgttacactacacgcccagggc
A66	caccgtcctttcaccctagcctgac	aaacgtcaggctagggtgaaaggac
A67	caccgccatgatagtagcttcaaac	aaacgtttgaagctactatcatggc
A68	caccgtacctaccggtttccacaac	aaacgttgtggaaaccggtaggtac
A69	caccgtcaaactaccgatttaaaac	aaacgttttaaatcggtagtttgac
A70	caccgcacttttaaacaaaattaac	aaacgttaattttgtttaaaagtgc
A71	caccgcaaaatgccaaggggtgac	aaacgtcacccccttggcattttgc
A72	caccgaagaaaaccacaactaaaac	aaacgttttagttgtggttttcttc
A73	caccgatggcacagtaccatataac	aaacgttatatggtactgtgccatc

A74	caccgaggtggcatagtggcttaac	aaacgttaagccactatgccacctc
A75	caccgtaacggttactaaggaaaac	aaacgttttccttagtaaccgttac
A76	caccggatgtttaaaaaataaaaac	aaacgtttttatttttaaacatcc
A77	caccgggcagcggccctcccatcac	aaacgtgatgggagggccgctgccc
A78	caccggggtacccatagccaataac	aaacgttattggctatgggtacccc
A79	caccgaacccttcctggaagaaaac	aaacgttttcttccaggaagggttc
A80	caccggccttcatctgggacaaaac	aaacgttttgtcccagatgaaggcc
A81	caccgctagtctaaattatttcaac	aaacgttgaaataatttagactagc
A82	caccggaaacttgcctcatgtaaac	aaacgtttacatgaggcaagtttcc
A83	caccgtgtttttgttgaaaacaaac	aaacgtttgttttcaacaaaaacac
A84	caccgtcttcacattgtgtgaatac	aaacgtattcacacaatgtgaagac
A85	caccgcttagttttgtgcttataac	aaacgttataagcacaaaactaagc
A86	caccgttgtaatttttttaaataac	aaacgttatttaaaaaaattacaac
A87	caccggtcagatccataaagcaaac	aaacgtttgctttatggatctgacc
A88	caccgttatttacatgagaggaaac	aaacgtttcctctcatgtaaataac
A89	caccgtctgggttatcagcataaac	aaacgtttatgctgataacccagac
A90	caccgcagacctggtcggggcccac	aaacgtgggccccgaccaggtctgc
A91	caccggcatgtttttaatggtaaac	aaacgtttaccattaaaaacatgcc
A92	caccgtgaagaactggagtctgact	aaacagtcagactccagttcttcac
A93	caccgctgtgtctactttaaaaaac	aaacgtttttttaaagtagacacagc

Table 7.2. Primers used for constructs other than vector library and for sequencing

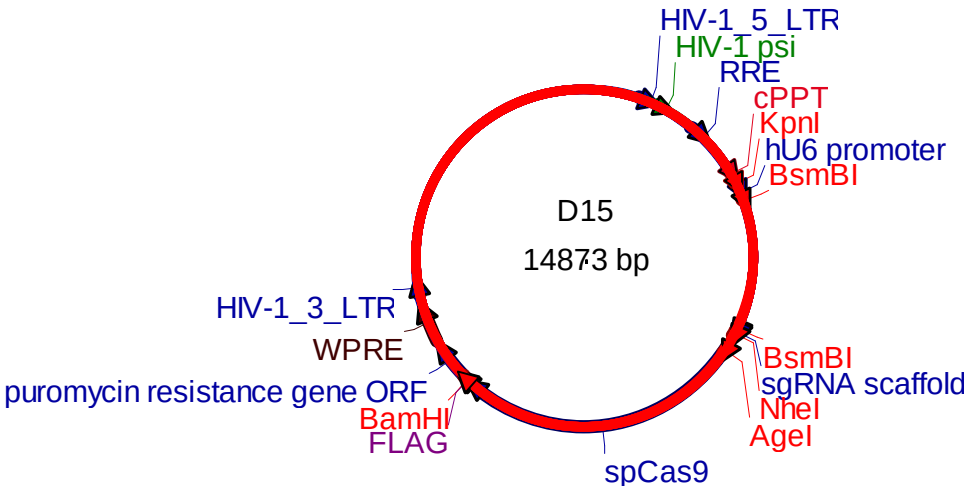
Name	Sequence
1	gggcctatttcccatgattccttca
2	tcgagtgctgttgacagtgagcgtggcagtgtcttagctggttgttagtgaagc cacagatgtaacaaccagctaagacactgccatgcctactgcctcggag
3	aattctccgaggcagtaggcatggcagtgtcttagctggttgttacatctgtggc ttcactaacaaccagctaagacactgccacgctcactgtcaacagcac
4	tcgagtgctgttgacagtgagcgtctgtcatttctttaggccaatatagtgaagc cacagatgtatattggcctaaagaaatgacagatgcctactgcctcggag
5	tattctccgaggcagtaggcatctgtcatttctttaggccaatatacatctgtggct tcactatattggcctaaagaaatgacagacgctcactgtcaacagcac
6	tcgagtgctgttgacagtgagcgctgacctatgaattgacagcctagtgaagc cacagatgtaggctgtcaattcataggtcagtgcctactgcctcggag
7	aattctccgaggcagtaggcactgacctatgaattgacagcctacatctgtggc ttcactaggctgtcaattcataggtcagcgctcactgtcaacagcac
8	tcgagtgctgttgacagtgagcgtgtaacagcaactccatgtggatagtgaag ccacagatgtatccacatggagttgctgttacatgcctactgcctcggag

9	aattctccgaggcagtaggcatgtaacagcaactccatgtggatacatctgtgg cttcactatccacatggagttgctgttacacgctcactgtcaacagcac
10	tcgagtgctgttgacagtgagcggtccagttttcccaggaatcccttagtgaag ccacagatgtaagggattcctgggaaaactggactgcctactgcctcggag
11	aattctccgaggcagtaggcagtccagttttcccaggaatcccttacatctgtgg cttcactaagggattcctgggaaaactggaccgctcactgtcaacagcac
12	cgagttcttctgagcggccgcaagc
13	cagtacaccggtcgccaccatggttagcaaaggcgaggagct
14	cagtacgcggccgcttagacgttgatcctggcgctggcgcaagca
15	tgctgttgacagtgagcgtccctgagaccctaacttgtgatagtgaagccacag atgtatcacaagttagggtctcagggatgcctactgcctcggag
16	tccgaggcagtaggcatccctgagaccctaacttgtgatacatctgtggcttca ctatcacaagttagggtctcagggacgctcactgtcaacagcac
17	tcgagtgctgttgacagtgagcgtgagaactgaattccatgggtttagtgaagc cacagatgtaaacccatggaattcagttctcatgcctactgcctcggag
18	aattctccgaggcagtaggcatgagaactgaattccatgggtttacatctgtgg cttcactaaacccatggaattcagttctcacgctcactgtcaacagcac
19	tcgagtgctgttgacagtgagcgttaatgctaatcgtgatagggttagtgaag ccacagatgtaacccctatcacgattagcattaatgcctactgcctcggag
20	aattctccgaggcagtaggcattaatgctaatcgtgatagggttacatctgtgg cttcactaacccctatcacgattagcattaacgctcactgtcaacagcac
21	cagtaccctgcaggtagttattaatagtaatcaattacggggt
22	cagtacggcgcgcccgcttacaatttacgcgttaagatacattgat
23	gcccgcgaccacatggtgctcctgg
24	cagtacgctagcatgtttatgcacagggtggaacaagat
25	cagtacctcgagacaaccagctaagacactgccaacaaccagctaagacact gccaacaaccagctaagacactgccaggatccttagacgttgatcctggcgct ggcgcaagc
26	cagtacctcgagtcacaagttagggtctcagggatcacaagttagggtctcag ggatcacaagttagggtctcagggaggatccttagacgttgatcctggcgctg gcgcaagc
27	cagtacctcgagaacccatggaattcagttctcaaacccatggaattcagttctc aaacccatggaattcagttctcaggatccttagacgttgatcctggcgctggcg caagc
28	cagtacctcgagacccctatcacgattagcattaaacccctatcacgattagcat taaacccctatcacgattagcattaaggatccttagacgttgatcctggcgctgg cgcaagc
29	aaagagacctacgtcgagcagcacg
30	cagtacatcgatggccagatatacgcgccggcctccgcgc
31	cagtacggcgcgcccttaatgcgccgctacagggcgcgtggg
32	gaattcaaaaaagcaccgactcggtg
33	tcgtcggcagcgtcagatgtgtataagagacagcatatgcttaccgtaacttga aagt

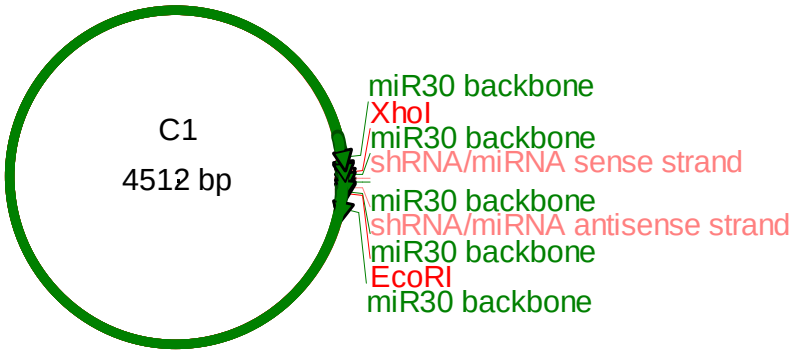
34	gtctcgtgggctcggagatgtgtataagagacaggaattcaaaaaagcacc gactcggtg

7.3 DNA Plasmids

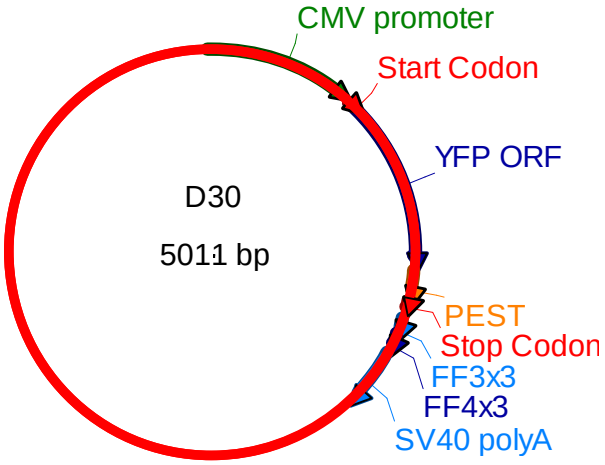
7.3.1 D15



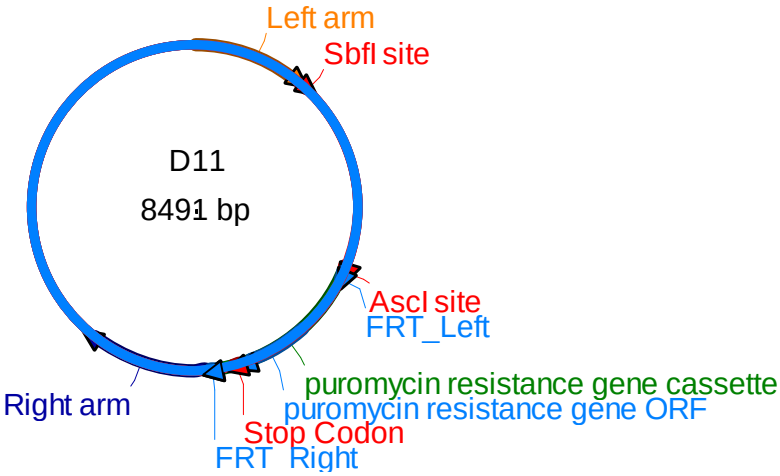
7.3.2 C1



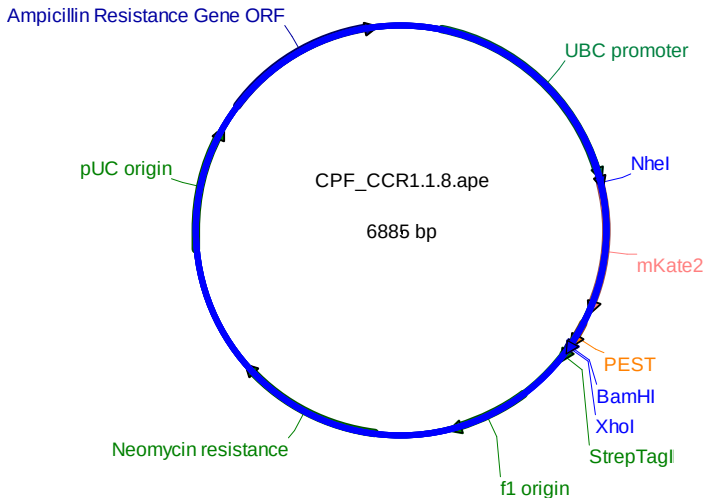
7.3.3 D30



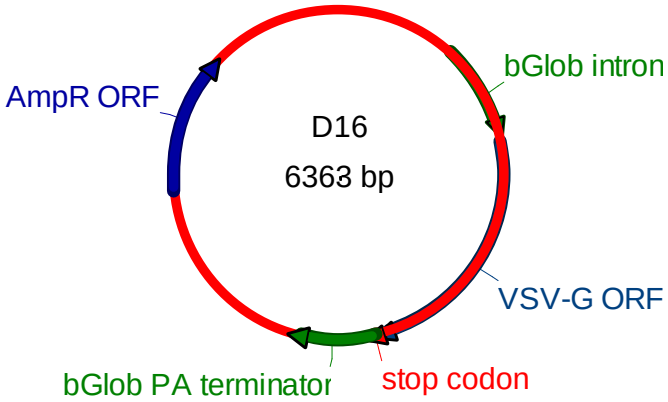
7.3.4 D11



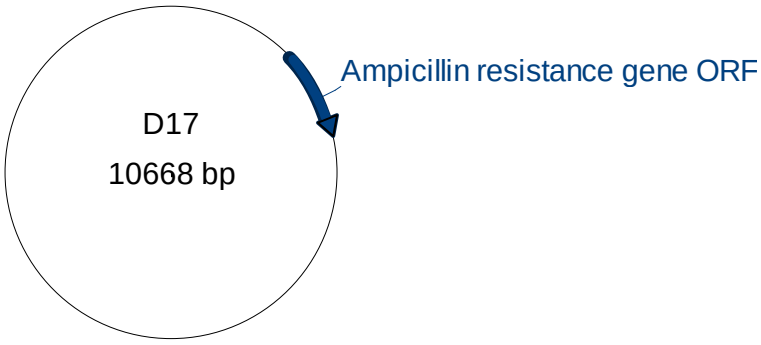
7.3.5 CPF_CCR1.1.8



7.3.6 D16



7.3.7 D17



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BIOGRAPHICAL SKETCH

Daniel Pashang Withers was born December 1, 1992 in Dallas, Texas. He is the son of John H. Withers, Jr. and Mojgan Z. Withers and the brother of Robert D. Withers and Michael C. Withers. After completing high school in Dallas in 2011, he entered college at Southwestern University in Georgetown, Texas. He majored in Biology and Spanish and minored in Chemistry, graduating in 2015 with a Bachelor of Science. He began his studies at The University of Texas at Dallas in the fall of 2015 and joined Dr. Bleris' Systems and Synthetic Biology Lab in October 2015. He graduated as a Master of Science from the Department of Bioengineering in May 2017.

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Daniel Withers

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Bachelor of Science in Biology and Spanish

University of Texas at Dallas 2017
Master of Science in Biomedical Engineering

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Research Assistant in Bleris Lab at University of Texas at Dallas 2015-2017

Research Assistant in Rawji Lab at Southwestern University 2014

Technical Skills

Flow Cytometry, Plasmid Ligation, Bacterial Transformation, Mammalian Cell Culture, Plasmid Transfection, Gel Electrophoresis, Poly-Acrylamide Gel Electrophoresis, Western Blot, Fluorescence Microscopy, Fluorescence Spectroscopy

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Eagle Scout, Boy Scouts of America 2008

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