Oklahoma State University- Ruhl Laboratory

Deciphering the Role of the INI-1 Protein within the SWI/SNF Remodeling Complex

Honors Thesis

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Abstract

INI-1 is a core subunit of the SWI/SNF chromatin remodeling complex, which stands for SWItch/Sucrose Non-Fermentable, and is a known tumor suppressor gene. INI-1 is a 47kDa long protein with 385 amino acids present in its sequence. It's expressed ubiquitously in all cell types including developmental cells (Fagerberg). It is primarily found in the nucleus, but can be found in small concentrations in the cytoplasm. The mechanism behind the SWI/SNF complex work is still being discovered and not much is known about the role of INI-1 in the complex. 20% of cancer cells contain a mutation in the SWI/SNF complex and a specific type of cancer, Atypical Teratoid Rhadboid Tumors are classified by their lack of INI-1 in the cell (Kim). This report documents how CRISPR-Cas9 was utilized to create INI-1 knockout cells along with developing mononucleosomes to measure how INI-1 affects nucleosome movement. In future studies, the INI-1 knockout cells will be used to compare the cell proliferation and protein expression between the presence and absence of INI-1 in cancer cells. The mononucleosomes will be used to identify the role that INI-1 plays on nucleosomes movement.

Acknowledgements

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Introduction

INI-1 Structure

INI-1 is roughly 47kDa long with 385 amino acids present in its sequence. Within the sequence of INI-1, there are several different binding domains present. From residue 1 to residue 113, this region is responsible for binding to DNA (Allen). The region spanning from residue 183 to residue 243 is responsible for HIV-1 integrase-binding. In almost the same area, from residue 186 to residue 245, the MYC-binding element is present. The last known interacting region for INI-1 occurs from residue 304 to residue 318. This section of the sequence interacts with PPP1R15A. The sequence for INI-1 also contains tandem repeats. The first repeat occurs from residue 186 to 245. The second repeat occurs from residue 259 to residue 319 (Opentarget).

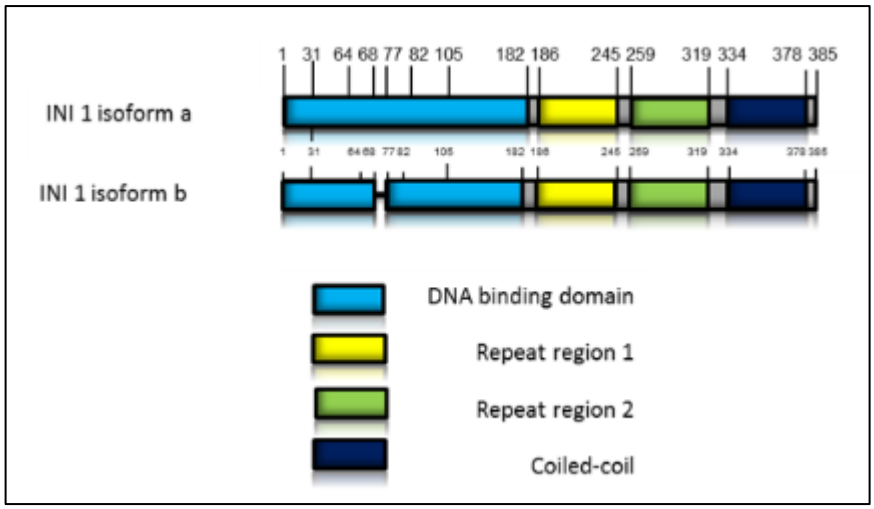

Figure 1- The structure of INI-1 with the DNA Binding domain, repeats, and coiled-coil labelled (Steele).

This protein has a total of 16 different alpha helices spanning across the entire length of the protein sequence. The first alpha helix begins at residue 29 and the last helix ends at residue 380. INI-1 has a maximum of 385 residues which means that there are alpha helices placed all throughout the protein. The largest helix is 22 residues long and is from residue 358 to 380 (Uniprot). Alpha helixes aren't the only structure found in this protein because there are 14 beta strands that also occur. These beta strands tend to be small in nature only occurring for two to

four residues except for a few strands that are at a nine-residue maximum (Uniprot). The final structures found in this sequence are two beta turns. The first turn is two residues long at residue 98 to 100. The second turn is six residues long at residue 343 to 349.

INI-1 is a self-associating protein in which case the arrangement of the protein depends on the concentration of the protein in a solution. At low concentrations, INI-1 creates a dimer. When INI-1 is in a high concentration solution, the protein has been seen in tetramer and octamer arrangements (Das). Repeats within the sequence are necessary for the multimerization along with different binding regions and these repeats are highly conserved throughout eukaryotes and some plants.

INI-1 undergoes very few post translational modifications. At residue 129, there is a phosphoserine which helps control the cellular processes for protein kinases (Matsuoka). INI-1 also contains 4 cross-link modifications. At residue 106, a Glycyl lysine isopeptide is formed with SUMO2. Another Glycyl lysine isopeptide cross-link is formed at residue 108 again with the SUMO2. This same Glycyl lysine isopeptide cross-link is formed again at 124 and 161 with SUMO2. These 5 post translational modifications are the only modifications made to INI-1 and most of the modifications pertain to the interaction of INI-1 with the SUMO2 gene (Open targets).

INI-1 Function

INI-1 plays an integral role in the formation of the SWI/SNF complex, which stands for SWItch/Sucrose Non-Fermentable and. When INI-1 is not expressed in a cell environment, there is a decrease in the amount of peptides corresponding to the SWI/SNF complex. On the flip side, when INI-1 is reintroduced, there are markedly increased levels of the SWI/SNF complex

(Wang). This evidence explains that INI-1 helps stabilize the formation of the SWI/SNF complex and that is one part of INI-1's overall function.

Monitoring the gene expression within cells before and after they begin expressing INI-1 allows for a change in the type of gene expressed and the enhancers that the SWI/SNF complex was able to bind to. INI-1 is largely responsible for activating differentiation-related regular enhancers. However, INI-1 has no effect on super enhancers. When INI-1 was not present in the cell, genes enhanced by regular enhancers were relatively undetectable, but were expressed as normal when INI-1 was present in the cell. Genes controlled by super enhancers had no change in expression regardless of the presence of INI-1 (Wang). This concluded that INI-1 also plays a role in gene expression and regulation in a cellular environment.

INI-1 is responsible for binding to the nucleosome acidic patch in order to effectively unwind DNA (Valencia). The ability of INI-1 to bind to the nucleosome acidic patch is attributed to the C-terminal domain. In this domain, there is a cluster of basic, positively charged amino acids within an alpha helical structure. This structure is required for the ability to bind to the nucleosome acidic patch. When given a mutation within the C-terminal alpha-helical domain, INI-1 can still exhibit normal targeting to chromatin. The mutation does render INI-1 defective for generating DNA accessibility and activating critical genes (Valencia). This means that there is a difference between the functionality of SWI/SNF targeting and DNA accessibility within INI-1 since the outcomes don't mirror one another in experiments. These experiments help determine the complex role of INI-1 in the SWI/SNF complex, because the mutations don't result in a complete loss of the INI-1 subunit, but it can affect the remodeling function.

SWI/SNF Chromatin Remodeling Complex

SWI/SNF is a relatively large complex at about 2MDa which is composed of about 15 different subunits (de la Serna). There are several core members present in most SWI/SNF complexes, INI-1, SMARCC1, SMARCC2 and SMARCA4 or SMARCA2, one of two mutually exclusive ATPase Subunits (Alver). The exact mechanism for the SWI/SNF complex is still being discovered. However, there are some ideas about what each of the core units are responsible for. INI-1 is thought to be responsible for binding to the nucleosome acidic patch (Valencia). SMARCA4 and SMARCA2 mobilize nucleosomes using energy obtained from ATP hydrolysis (Alver). SMARCC1 and SMARCC2 help stabilize the subunit and can negatively regulate transcription (Li). There are two different forms of the SWI/SNF complex, the BAF and the PBAF forms (Figure 2). The difference between these two different forms is their subunit composition and what accessory subunits are utilized (Kalimuthu).

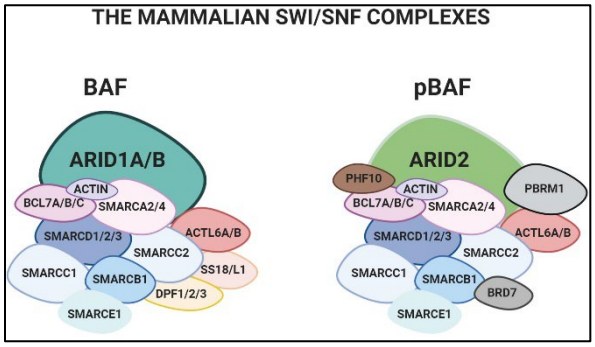

Figure 2- The two different subunits, BAF and pBAF, and their most commonly associated subunits (Pagliaroli).

INI-1 Regulation

INI-1 interacts with eight different proteins in terms of known regulatory interactions. INI-1 is inactivated by CHFR which is an E3 ubiquitin-protein ligase. INI-1 is responsible for inactivating CCNA1, E2F1, CDC6, and CCNA2. CCNA1 and CCNA2 function as regulators of CDK kinases. E2F1 is a member of a family of transcription factors that are in control of the cell cycle and the action of tumor suppressor proteins. CDC6 is a regulator of DNA replication in the early stages. On the flip side, INI-1 is activated by CSF1, SMARCA2, and SMARCA4. CSF1 is a cytokine that controls macrophages. SMARCA2 and SMARCA4 are members of the SWI/SNF

complex with INI-1 (Alver). If the nucleosome interface is modified or disrupted, there would be inhibition of the INI-1 protein.

INI-1 Expression

INI-1 is ubiquitous in expression across all tissue types in the human body (Fagerberg). INI-1 has also been measured during fetal development from 10 weeks to 20 weeks (Fagerberg). During this period, the protein is still expressed ubiquitously throughout the major organs. This explains that the protein maintains a constant expression across the human body from fetal development into adulthood. INI-1 is primarily found within the nucleus of a cell. It can occasionally be found in the cytoplasm; however the highest concentrations of this protein are found within the nucleus (NCBI).

Mutations in INI-1

Eleven different mutations within the INI-1 gene have been correlated with Coffin-Siris syndrome (Medline Plus). Current research is still being done to determine how the amino acid changes within INI-1 affect the SWI/SNF complex as a whole. Most of the diseases associated with INI-1 are different forms of cancer and tumor production. There are more than 50 germline variants of INI-1 that cause rhabdoid tumor predisposition syndrome. This syndrome is marked by the increased risk for developing malignant growths called rhabdoid tumors (Medline Plus). Schwannomatosis is also common with variants in INI-1 with the hallmark symptom of multiple non-cancerous growths around nerves. However, it has been noted that variants in INI-1 aren't enough to cause the development of a schwannomas (Medline Plus). The last main disease caused by INI-1 is the development of Atypical Teratoid Rhabdoid Tumors. This form of cancer is specifically determined by the absence of INI-1 in tumor cells. Overall, INI-1 has a strong

association with different cancers and the development of tumors due to its role as a tumor suppressing gene.

Mononucleosome

Chromatin remodeling is an important process during transcription and DNA repair and is the movement of a histone octamer. This histone octamer typically has DNA wrapped around it and the unit containing both DNA and the histone octamer is known as a nucleosome (Figure 3). Due to the sheer size of DNA and the need to be compact within the cell, each nucleosome wraps approximately 147 nucleotides in order to make chromatin. This process allows for a large amount of DNA to be contained in a small amount of space. However, DNA transcription can't occur while the DNA is wrapped around the nucleosome. SWI/SNF is responsible for unwinding the DNA on the nucleosome to make localized regions of DNA accessible.

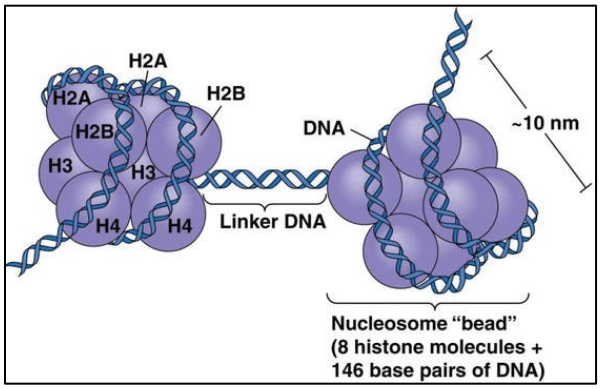

Figure 3- This is the depiction of a nucleosome. The histone octamer is visible with the eight spheres along with how the DNA is wrapped around the histones to create a condensed form (Caputi).

CRISPR-Cas9

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. These repeats were first identified in bacteria cells, and they were separated by non-repeating DNA sequences. The CRISPR associated genes, Cas, were relatively well conserved in bacteria and archaea species (Adli). The CRISPR Cas system was identified as a bacterial immune system.

CRISPR-Cas9 is able to make a precise double strand DNA cut and then allow DNA repair processes to occur. The plasmid used in transfection, for our laboratory, contained guide sequences for the knockout of INI-1, a puromycin resistance gene, and the Cas9 enzyme. By

transfecting the cells with the guide RNA sequences, researchers are able to control exactly where the genome will be cut. CRISPR-Cas9 gives researchers the ability to either add in a specific gene or mutation, or to knock-out a specific gene. In Dr. Ruhl's laboratory, we focused on being able to knock out the INI-1 gene. The gene knock outs are a result of the double strand cut that CRISPR employs. In an effort to connect the DNA strands back together, non-homologous end joining occurs which produces an insertion-deletion in the exons of the desired protein. The insertion-deletion creates a premature termination codon which causes the knockout protein to not be fully expressed or expressed at all. The knockout function of CRISPR-Cas9 helps study the function of proteins by documenting the changes in cells depending on their expression of the protein.

Project Goals

The first goal of this project is to develop a CRISPR-Cas9 INI-1 knockout cell line which will undergo further testing for protein concentrations and proliferation in future laboratory experiments. The second goal of this project is to utilize an in vitro mononucleosome in remodeling assays to understand the impact INI-1 has, if any, on how DNA is remodeled.

Materials and Methods

Cell Media Preparation

The MCF-7 cells were grown in Minimum Essential Medium Eagle (Sigma) media. To make the media, a 1 L jar of Minimum Essential Medium Eagle was mixed with 2.22 g $NaHCO_3$ and 880 mL of nanopure H_2O. This was mixed until homogenous then a pH meter was used to ensure the mixture was at a pH of 7.2. Under a sterile fume hood, 100 mL of 10% Fetal Bovine Serum (Atlanta Biologicals), 10 mL of penicillin (Sigma), 10 mL of streptomycin (Fisher), and 20 mL of HEPES (Thermofisher) are added. After filtration, the media is ready for use.

The HeLa cells were grown in Dulbecco's Modified Eagle's Medium (Sigma). To make the media, a 1L jar of Dulbecco's Modified Eagle's Medium (Sigma), 1.2 g $NaHCO_3$, and 900 mL of nanopure water. This was mixed until homogenous. A pH meter was utilized to ensure that the media was at a pH of 7.2. Under a sterile fume hood, 100 mL of 10% Fetal Bovine Serum, and 10mL of Pen/strep were added to the media. After filtration, the media was ready for use.

Cell Maintenance

The media must be warmed up in a 37℃-water bath until no longer cold to the touch. Using a vacuum pump, the old media was vacuumed out. Using a sterile pipette, 10 mL of media was dispensed back onto the culture plate. The plate was then returned to the incubator. The incubator was set to 37℃ with 5% CO_2. The MCF-7 cells underwent a media change approximately every four days while in 10cm plates and once they hit about 90% confluency, they were transferred to new plates. The HeLa cells underwent a media change approximately every 2 days while in 10cm plates and were transferred to new plates once they hit 100% confluency. All cells were grown in 10 cm cell culture dishes (Cell Star).

To transfer cells to new plates, all confluent plates were removed from the incubator and all media was discarded. The plates were rinsed with 10mL of 1X PBS (8g NaCl, 0.2g KCl, 0.92g $Na_2HPO_4 \cdot 7H_2O$, 1 L Nanopure H_2O). 1 mL of trypsin (Sigma) was added to the plate then watched under a microscope. The MCF-7 cells sat in trypsin for approximately 15 minutes before all cells were detached from the plate. HeLa cells sat in trypsin for approximately 20 minutes before all cells were detached from the plate. After all cells were detached from the plate, 10 mL of their respective media was added back to the plate. Depending on how many new plates were being made, the 10mL will be divided among the new plates. Each plate needed

a total amount of 10 mL of media before being placed back into the incubator. After 24 hours, the media was changed.

Stocks of MCF-7 and HeLa cells were created by taking five 100% confluent 10cm plates. The media was removed, and the plate was rinsed with PBS. 1 mL of trypsin was added to each plate and allowed to remain on plate until all cells were detached from the plate. 2 mL of media was added to the plate to stop the trypsin. A pipette was used to move the media/trypsin containing the cells into a 15 mL conical. All 5 plates were combined into a single 15 mL conical. Using a centrifuge, the cells were pelleted, and the supernatant was discarded. The cells were resuspended in 5 mL of media and set on ice for 10 minutes. 5 mL of ice cold 2X Freezing media (3 mL regular cell media, 5 mL 10% Fetal Bovine Serum, and 2 mL DMSO) was added and mixed together. 1 mL of the mixture was aliquoted into freezing vials to create 10 stock vials. They were placed in a cell freezing container and stored in a -80℃ freezer.

Cell Counting

After reaching confluency on 10cm plates, each plate underwent cell counting. Trypsin was used to detach all the cells on the plate. 10mL of media were added to the plate and transferred to a 15mL conical. 10 µL of cells were dispensed on to the hemacytometer. The average number of cells got multiplied by 10,000 to determine the cells per mL of media. For MCF-7 cells, about 700,000 cells were wanted for each well in a 6 well plate for transfection. HeLa cells needed about 700,000 cells for each well in a 6 well plate.

Guide Sequence Determination

The guide sequences for the CRISPR-Cas9 experiments were designed using the online software, CRISPOR. sgRNA sequences that were selected had a value of 80 or higher for the MIT score, and a 50 or higher for the Doench score. The selected sequences were also chosen

based on minimizing the off-target sites. CRISPOR was ran using the sequence for INI-1 that was obtained from the National Center for Biotechnology Information database (NCBI, SMARCB1: **NM_001007468.3**). There were three sgRNA sequences that were used for CRISPR-Cas9 as can be seen in figure 4.

		Sequence
sgRNA #1	120 Forward	5'- AACTACCTCCGTATGTTCCGAGG -3'
sgRNA #1	120 Reverse	5'- AAACCGGAACATACGGAGGTAGTTCCA -3'
sgRNA #2	327 Forward	5'- CACCATGGAGCTACCTCCGTATGTTCCG -3'
sgRNA #2	327 Reverse	5'- AAACCGGAACATACGGAGGTAGTTCCAT -3'
sgRNA #3	1196 Forward	5'- AAACTCCGGAACACGGGCGATGCGCCAT -3'
sgRNA #3	1196 Reverse	5'- CACCATGGCGCATCGCCCGTGTTCCGGA -3'

Figure 4-The sgRNA sequenced that were cloned and transfected into the HeLa and MCF-7 Cells.

Guide Sequence Cloning

For each of the sgRNA sequences, the forward and reverse oligos were annealed into a pX459 plasmid, which contains a puromycin selection marker. This process began with a mixture of 11.5 µL Nanopure H_2O, 1 µL pX459 Plasmid (100 ng), 1.5 µL 10X Restriction Buffer, & 1 µL BbsI Enzyme to digest the plasmid. This mixture was placed in a 37°C-water bath for 60 minutes, then a 65°C-water bath for 20 minutes, before placing mixture at room temperature.

To anneal the primers, 7.0 µL Nanopure H_2O, 1.0 µL 10X T4 Ligation Buffer, 1.0 µL Forward sgRNA primer, and 1.0 µL Reverse sgRNA Primer were mixed together. This mixture was created for each of the three sgRNA sequences. The mixtures were placed in a 95°C-water bath for five minutes. The water bath was then turned off and allowed to cool to room temperature overnight. The annealed primers were diluted in a 1:200 ratio in nanopure H_2O.

The annealed primers were ligated into the digested plasmid in a mixture of 8.0 µL Nanopure H_2O, 7.0 µL digested plasmid, 2.0 µL 10X T4 Ligation Buffer (New England Biolabs), 2.0 µL annealed primers, and 1.0 µL T4 DNA ligase (New England Biolabs). This mixture was incubated for five minutes in a 37℃-water bath then stored in the fridge.

The sgRNA plasmids were transformed into DH5α competent Escherichia *coli* cells and grown onto ampicillin agar plates. 5 µL of the ligated plasmid and 20 µL of competent cells were combined and set on ice for thirty minutes. The cells quickly underwent a temperature change by transferring them to a 42℃-water bath for forty seconds before returning back to ice for another ten minutes. 175 µL of Super Optimal Catabolite and rocked in a 37℃ incubator for one hour. This process was repeated for a positive control of the uncut pX459 plasmid. Under an aseptic area, the mixture was spread onto ampicillin agar plates and left to incubate over night at 37℃. The isolated colonies were selected and transferred to culture tubes containing 5 mL of LB broth. The culture tubes were allowed to incubate overnight in a 37℃-incubator shaking at 200 rpm.

The sgRNA plasmids were isolated and quantified. To isolate the sgRNA plasmids, 1.5 mL of the culture was centrifuged for four minutes at 4000 rpm. The supernatant was discarded. 100 µL of Tris, EDTA; pH 8.0 with RNase A was added to the pellet and resuspended. 200 µL of SDS in NaOH was added and inverted to display a uniform sample. 150 µL of 3M Potassium Acetate, pH 5.0 was added and immediately inverted. The sample was centrifuged for 10 minutes at 13,000 rpm. The supernatant containing the plasmid DNA was saved and the tube containing the pellet was discarded. 600 µL of isopropanol was added to the supernatant and then set on ice for 10 minutes. The sample was centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and discarded. 500 µL of 70% ethanol was added then centrifuged for

five minutes at 13,000 rpm. The ethanol was removed from the sample and discarded. The pellet was resuspended in 50 µL of nanopure H_2O.

Guide Sequence Confirmation

To ensure the plasmid had the desired sgRNA sequence, a restriction digestion was performed. 1 µL Cutsmart Buffer (New England Biolabs), 0.5 µL NcoI (New England Biolabs), 4.5 µL of nanopure H_2O, and 4 µL of the isolated DNA was combined and incubated overnight at 37℃. The restriction digest was run on a 1.5% agarose gel made with 1X TBE buffer. Each well contained 4 µL of orange G dye (Sigma) and 10 µL of the restriction digest. The agarose gel was run at 100 volts for 40 minutes. It was imaged using the BioRad ChemiDoc XRS+ with Image Lab Software.

To confirm the sequence of each isolated plasmid, a 0.75% agarose gel was run with the isolated plasmid DNA against a high mass ladder (Invitrogen). The isolated plasmid DNA was measured using the nanodrop then diluted to 125 ng/µL. Each well of the 0.75% agarose gel contained 4 µL of orange G dye (Sigma), 2 uL of diluted DNA, and 4 µL of sterile H_2O. The gel was run at 100 volts for 35 minutes then imaged using BioRad ChemiDoc XRS+ with Image Lab Software. After using the gel to determine the actual concentration of the isolated plasmid DNA sample, it was sent off for sequencing.

Transfection

Once cells were 70-90% confluent in a 6 well plate, transfection for CRISPR-Cas9 occurred. The transfection protocol was run with both high and low concentrations of lipofectamine 3000 (Invitrogen). For each well ran with the high concentration of Lipofectamine 3000, 125 µL of opti- MEM medium (Gibco) and 7.5 µL of lipofectamine 3000 were combined and mixed. For each well ran with a low concentration of Lipofectamine 3000, 125 µL of opti-

MEM medium and 3.75 µL of lipofectamine 3000 were combined and mixed. In a separate tube, 125 µL of opti-MEM medium, 2.5 µg of each sgRNA, 5 µL of P3000 (Invitrogen) reagent were mixed together. In a 1:1 ratio, the diluted lipofectamine 3000 and the diluted DNA were mixed together and incubated at room temperature for 15 minutes. 250 µL of the incubated mixture was added to each well.

A media change occurred after leaving the transfection medium on each well for 24-48 hours. The same media as regular cell maintenance was used, however 1 µg/mL puromycin (MP Biomedicals) was added. The cells underwent daily media changes while using the puromycin media. Puromycin media was halted once a control plate that underwent no transfection being treated with puromycin displayed complete cell death. Regular maintenance media was continued throughout the rest of cell culture with puromycin media only being used every few weeks to ensure that the cells were retaining the mutations. Serial dilutions were utilized to create single cell colonies for future research.

Polymerase Chain Reactions for Mononucleosome Assembly

The desired DNA product was created through PCR. Each tube for the PCR reaction made 100 µL of product. It consisted of 53.5 µL of dH_2O, 10 µL 10X ThermPol Buffer, 20 µL betaine (Sigma), 1 µL p601 ERE, 6.25 µL 5 µM forward primer, 6.25 µL 5 µM reverse primer, 2 µL 10 mM dNTPs, and 1 µL XL TAQ. They were placed in a thermocycler for a 2-hour run. The thermocycler ran as follows: 94℃ for 5 minutes, 37 cycles of 94℃ for 30 seconds, then 50℃ for 40 seconds, then 72℃ for 1 minute, and 72℃ for 7 minutes. The products were removed and stored in the fridge.

To ensure the proper PCR product was created, 1.5% agarose gels using 1X TBE were ran. Each well contained 4 µL of orange G dye (Sigma), and 8 µL of PCR product. It was run against a 1 Kb DNA ladder (Invitrogen). The standard contained 4 µL of 1 Kb DNA Ladder, 4 µL of H_2O, and 4 µL of orange G dye (Sigma). The agarose gel was run at 100V for 35 minutes and then imaged on a BioRad ChemiDoc XRS+ with Image Lab Software.

For mononucleosome assembly, the DNA products were at a concentration of 0.5 µg/ µL. To quantify the concentration of the PCR product, the samples were nanodropped then diluted to 125 ng/µL. The diluted samples were ran on a 0.75% agarose gel. Each well of the 0.75% agarose gel contained 4 µL of orange G dye (Sigma), 2 uL of diluted DNA, and 4 µL of sterile H_2O. The standard was a high mass ladder that was 4 uL ladder, 4 uL orange G dye (Sigma), and 4 uL H_2O The gel was ran at 100 volts for 35 minutes then imaged using the BioRad ChemiDoc XRS+ with Image Lab Software. The gel was analyzed, and the concentration of our DNA products was determined.

Mononucleosome Assembly

In a siliconized microfuge tube, the DNA PCR product, histones, and 2X Assembly Buffer (1.0 mL 5M NaCl, 40 uL 2M Tris at 7.5 pH, 8 uL 500mM EDTA at pH 8.0, 80 uL 500mM DTT, 40 uL 10 mg/mL BSA, and 232 uL ddH_2O) was mixed. The histones were previously purified from HeLa cells in the laboratory. The ratio of DNA to histone was empirically determined with a desired 1:1 molar ratio. Titrations were used to determine the exact amounts utilized. This mixture was placed into a modified siliconized microfuge tube cap and covered with Hominal MWCO 3,500 dialysis membrane (Fisherbrand). The lid mechanism was snapped shut on top of the dialysis membrane (figure 5).

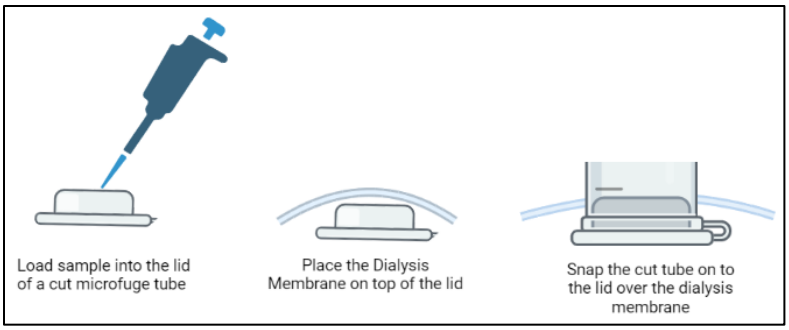

Figure 5-The lid mechanism that was utilized in the mononucleosome assembly. Created with BioRender.com

Using a biorad Econo pump, The input tube was placed into a chilled low salt buffer (70mL 5M NaCl, 7 mL 2M Tris at pH 7.5, 2.8 mL 500 mM EDTA at pH 8, 4.3 mL of 65g/L Sodium Azide, and 1316 mL of nanopure H_2O). The output tube was placed into a 400mL beaker of a chilled high salt buffer (160mL 5M NaCl, 2 mL 2M Tris at pH 7.5, 0.8 mL 500 mM EDTA at pH 8, 1.23 mL of 65g/L Sodium Azide, and 236 mL of nanopure H_2O). The pump was set to 0.8 diameter, with a speed of 0.65 mL/min for 18 to 22 hours to create a gradient salt dialysis. The modified siliconized tube floated dialysis membrane down in the high salt buffer. The high salt buffer sat on a stir plate and had slow movement of the buffer to ensure the low salt buffer was mixed into the beaker (Figure 6). After 18-22 hours, pump speed was increased to 1.27 mL/min for 4 hours. The modified siliconized tube was transferred to a chilled no salt buffer (2 mL 2M Tris at pH 7.5, 0.8 mL 500 mM EDTA at pH 8, 1.23 mL of 65g/L Sodium Azide, and 396 mL of nanopure H_2O) overnight with constant movement of the buffer. The mononucleosome assembly occurred in a cold room for the duration of experiment and then mononucleosomes were stored in a fridge.

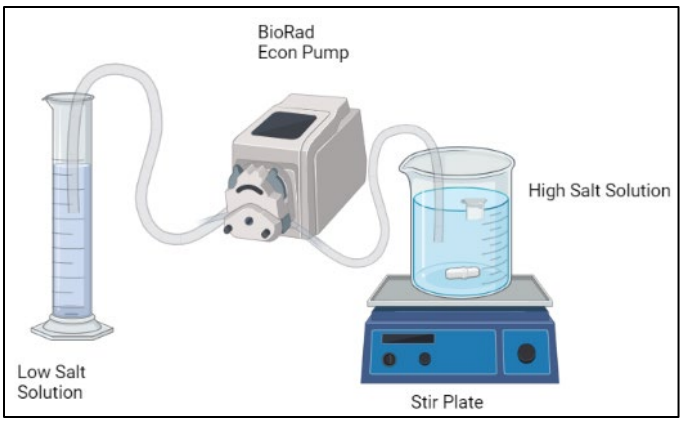

Figure 6-The set up associated with the mononucleosome assembly. Created with BioRender.com

Native Page Gel Electrophoresis

The 4% native PAGE gel (4.06 mL of ddH_2O, 0.8 mL of 50% Glycerol, 2 mL of 1X TBE, 1.06 mL of acrylamide, 0.08 mL of Ammonium persulfate, and 0.006 mL of TEMED) is used to ensure the mononucleosome was assembled. It was gently mixed so as to not incorporate oxygen. The solution was poured quickly into a PAGE set up with a 1.5mm glass plate and a 15 well comb and hardened in about 15 to 20 minutes.

Each well in the gel includes 5 uL of mononucleosomes, 4 uL of orange G dye (Sigma), and 5 uL of ddH_2O. The gel is ran against a well with 3 uL of 1Kb DNA ladder, 4 uL of orange G dye (Sigma), and 5 uL of ddH_2O for a DNA size standard. The PCR product utilized in the mononucleosome assembly will also serve as a standard. It is run in a well with 5 uL of PCR product, 4 uL of orange G dye (Sigma), and 5 uL of ddH_2O. After the gel is loaded, the chambers were filled with chilled 0.25X TBE buffer and placed in a bucket of ice. The gel was ran at 140 Volts for an hour and 20 minutes. The orange G dye (Sigma) will be ran off the end of the gel. The completed gel was stained in a water solution that contains 10 uL of Ethidium Bromide for five minutes with gentle shaking. The gel was rinsed with water and imaged using the BioRad ChemiDoc XRS+ with Image Lab Software.

Nucleosome Mapping

A digestion with HhaI (NEB) on the mononucleosome will be performed to analyze the positioning of the nucleosome (Figure 7 & 8). A total of four samples will be created. The first sample will contain 1 uL of the assembled mononucleosomes, 1 uL of 10X NEB Buffer #4, and 8 uL of ddH_2O. The second sample will contain 1 uL of assembled mononucleosome, 1 uL of HhaI, 1 uL of 10X NEB Buffer #4, and 7 uL of dH_2O. The third sample will consist of 1 uL naked DNA (0.25 mg/mL), 1 uL 10X NEB Buffer #4, and 8 uL ddH_2O. The fourth sample will

contain 1 uL of naked DNA (0.25 mg/mL), 1 uL of HbaI (NEB), 1 uL of 10X NEB Buffer #4, and 7 uL of ddH_2O. Each sample is in incubated in a water bath at 37℃ for 30 minutes. 2 uL of 50% glycerol was added to each sample.

GATAACA ATTTCACACA GGAAACAGCT ATGACCATGA TTACGCCAAG CTATTTAGGT

GACACTATAG AATACTCAAG CTTCAGGTCA CAGTGACCTG CCCTATACGA TATCGCGGCC

GCCCTGGAGA ATCCCGGTGC CGAGGCCGCT CAATTGGTCG TAGACAGCTC TAGCACCGCT

TAAACGCACG TACGCGCTGT CCCCCGCGTT TTAACCGCCA AGGGGATTAC TCCCTAGTCT

CCAGGCACGT GTCAGATATA TACATCCTGT GCATGTATTG AACAGCGACC TTGCCGGTGC

CAGTCGGATA GTGTTCCGAG CTCCCACTCT AGAGGATCCC CGGGTACCGA GCTCGAATTC

GCCCTATAGT GAGTCGTATT ACAATTCACT GGCCGTCGTT TTACAACGTC GTGACTGGGA

AAACCCTGGC GTTACCCAAC TTAATCGCCT TGCAGCACAT CCCCCTTTCG CCAGCTGGCG

TAATAGCGAA GAGGCCCGCA CCGATCGCCC TTCCCAACAG TTGCGCAGCC TGAATGGCGA

ATGGCGCCTG ATGCGGTATT TTCTCCTTAC GCATCTGTGC GGTATTTCAC ACCGCATATG

GTGCACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCAGCCCC GACACCCGCC

AACACCCGCT GACGCGCCCT GACGGGCTTG TCTGCTCCCG GCATCCGCTT ACAGACAAGC

TGTGACCGTC TCCGGGAGCT GCATGTGTCA

Figure 7 -The 120 Forward / 480 Reverse Primers created this DNA product. The primers are highlighted in blue. The Nucleosome positioning element is underlined. The HhaI cut sites are highlighted in yellow.

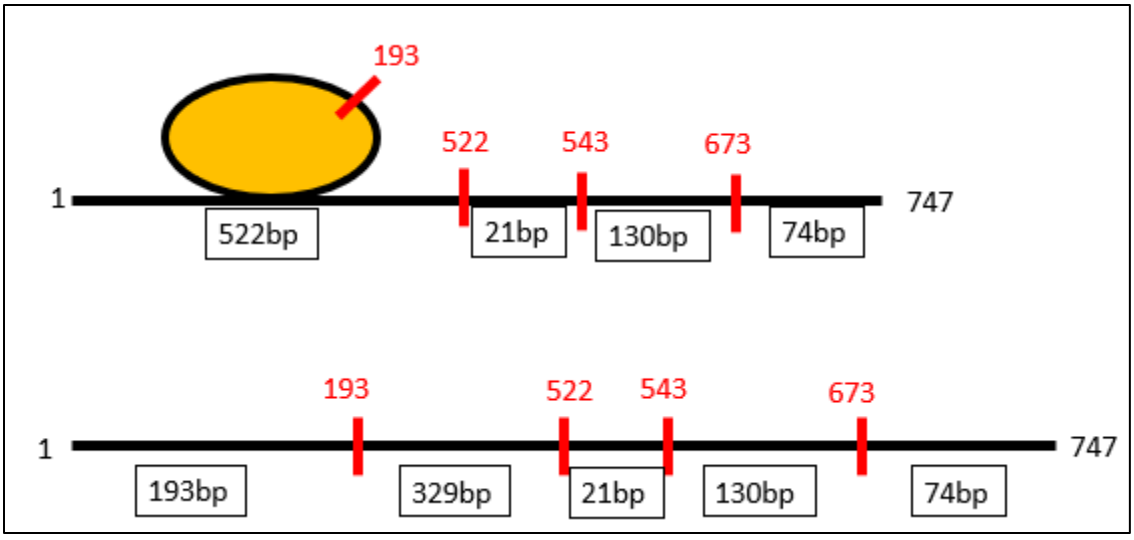

Figure 8-Each red mark indicates a cut site for HhaI. The yellow circle represents the nucleosome. When a nucleosome is binded to the DNA only 4 DNA bands are present at 522bp, 21bp, 130bp, and 74bp. When a nucleosome is not present, there are 5 DNA bands at 193bp, 329bp, 21bp, 130bp, and 74bp.

The entirety of each sample was loaded into a well. On an outside well, place a loading dye with bromophenol blue to measure the rate that the samples are moving through the gel. Fill the chambers with chilled 0.25X TBE buffer and place the chamber in a bucket of ice. Run the gel at 140 Volts for at least an hour and 20 minutes. The blue dye will be ran off the end of the gel. The completed gel is stained in a water solution that contains 10 uL of Ethidium Bromide for five minutes with gentle shaking. It was rinsed with water then imaged using the BioRad ChemiDoc XRS+ with Image Lab Software.

The same four samples from above will be created as stated above, however, after incubation 1 µL pk will be added to each sample and incubated for 20 minute at 37 ℃ in a water bath. The samples will be run on a 1.5% agarose gel for 35 minutes at 105 volts then imaged using BioRad ChemiDoc XRS+ with Image Lab Software.

Results

Selection and Cloning of 3 sgRNAs for CRISPR-Cas9

There were four sgRNA clones that we attempted, 92, 120, 327, and 1196. The sgRNA's targeted the coding region of the INI-1 gene. The numbers are created by CRISPOR.com for each sgRNA primers set. 327 and 1196 were previously identified by the undergraduate biochemistry laboratory course. 92 and 120 were selected by the criteria outlined in Figure 9. These primers had the highest MIT, and doench scores with the lowest amount of off target effects (figure 9). They were selected from a pool of six options that matched all four isoforms of the INI-1 gene.

#guideId	targetSeq	Mit Spec Score	Cfd Spec Score	Off target Count	Doench '16-Score	Moreno-Mateos-Score	Out-of-Frame-Score	Lindel-Score
92forw	GTTCTACATGATCGGCTCCGAGG	99	98	17	70	60	61	83
120forw	AACTACCTCCGTATGTTCCGAGG	98	98	15	66	28	65	77

Figure 9-The scores obtained from CRISPOR.com for the two sgRNA sequences that were designed.

Three clones from the 120 primer sequences were able to be identified as seen in figure 10. No clones from the 92 primer sequences were able to be identified. The proper clones can be seen as double bands around the 500 bp mark on the ladder. The 120-primer clone was confirmed by sequencing. For transfection, the plasmid concentration was needed for the 327, 1196, and 120 were needed. Using the high mass ladder, it was determined that the samples had concentrations of 17.54 µg/µL, 15.04 µg/µL, and 30.30 µg/µL respectively.

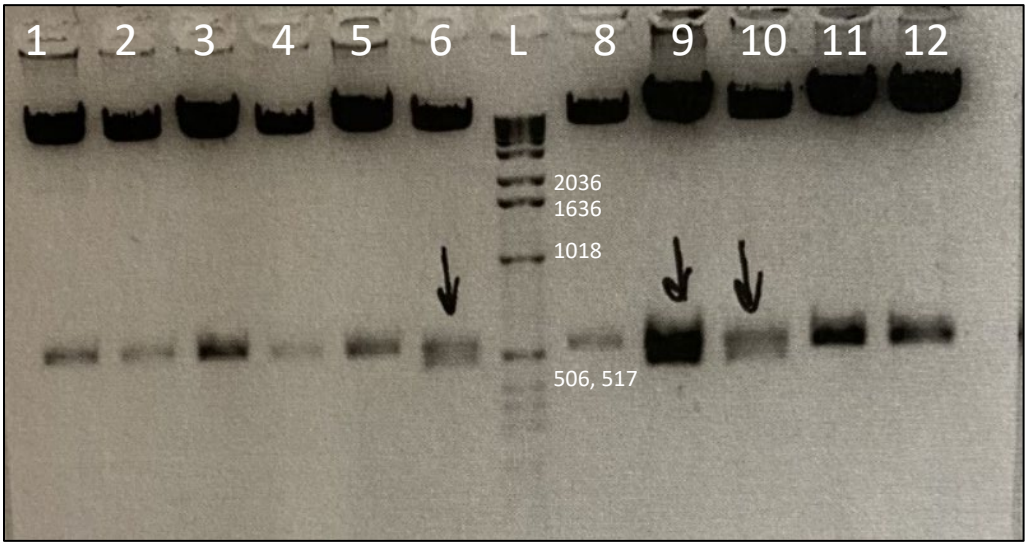

Figure 10-A 1.5% agarose gel. Lane L was ran with 4 µL of orange DNA dye, 5 µL of H2O, and 5 µL of the 1Kb DNA Ladder. Lanes 1 through 10 were the 120 primer set that contained 4 µL of Orange G dye and 10 µL of the restriction digest. Lanes 12-18 were the 92 primer set and contained 4 µL of Orange G DNA dye and 10 µL of the restriction digest. Lanes 13- 18 are not shown but had no double bands. Lanes 6,9 and 10 contained the properly cloned plasmid as can be seen by the double bands around the 1000bp mark.

Human-derived cells lines respond differently to INI-1 sgRNA

To establish the mechanistic connection between the loss of INI-1 and cancer, an approach to genetically knockout INI-1 using CRISPR-Cas9 from a human cell line was employed. The 3 sgRNA's were transfected into two human-derived cancer cell lines, MCF-7 and HeLa. After counting the MCF-7 cells on the hemocytometer, there was an average of 176.75 cells per 0.1 uL of media. After scaling, there was an average of 1,767,500 cells per mL of media. To seed each 6 well plate with about 700,000 cells, each well got 396 uL of media which was approximately 699,930 cells per well. HeLa cells underwent the same protocol and cell counting.

All of the cells were transfected with lipofectamine 3000. The cells were treated with puromycin media one day after transfection to kill off any cells that were not properly transfected. The MCF-7 cells reacted poorly to the puromycin media and never survived past 72 hours with puromycin media. MCF-7 cells were able to produce puromycin resistance since the transfected cells were able to survive longer than the control plate with no transfection. The control plate didn't have living cells past 24 hours with puromycin, which meant that the MCF-7 cells were able to be transfected and produce resistance. There was no apparent difference in the efficiency of transfection between the wells treated with high or low lipofectamine. In future transfection experiments, low lipofectamine 3000 can be used to achieve the same results. The HeLa cells had a much higher rate of efficiency for transfection with multiples cells from each well surviving after the addition of puromycin media.

The HeLa cells are being used to continue the INI-1 knockout cell line. They have been able to grow at levels equal to the original cell line and follow the same media protocol as before transfection. The cells are not able to grow as quickly in puromycin media, so the cells are

usually kept in regular media with a puromycin media change every few weeks. This helps ensure that all the cells in the culture have the INI-1 knockout. The cells are being frozen down as they are grown in order to use the cellular protein extracts in western blots to confirm the lack of protein expression for INI-1.

Production of DNA through 601B primers

PCR was utilized to create a DNA strand that contained a nucleosome positioning element that would allow the development of mononucleosomes in future assays. While there was some difficulty attaining the PCR products for the 120F/480R primers and the 300F/300R primers, we were able to produce the 601B primer products. The products were measured around 600bp and were in between the markers for 1000 bp and 500 bp (Figure 11). When we quantified the DNA concentration for each sample, there was an average of 50.66 ng/µL which when modified to be at a concentration of 0.5 µg/µL equals 56 µL.

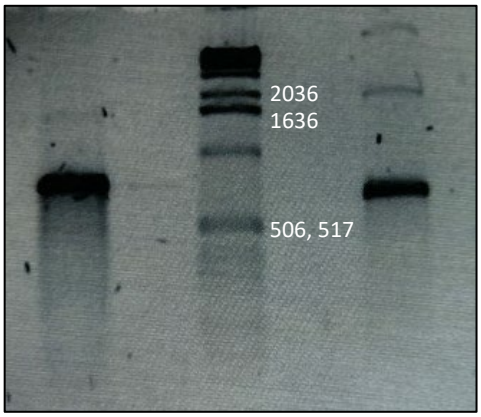

Figure 11 -Lane 1 contains a control sample of 0.5 ng/ µL of the desired PCR product. Lane 1 was ran with 8 µL of control DNA sample, 4 µL of Orange G dye. Lane 2 was the 1 Kb DNA Ladder. It was ran with 4 µL of Orange G Dye, 4 µL of H20, and 4 µL of 1 Kb DNA Ladd Ladder. Lane 3 was the PCR product that was created. Lane 3 contained 4 µL of Orange G Dye, and 8 µL of the PCR product. Lanes 1 and 3 display the desired PCR product.

Utilization of salt gradient to create mononucleosomes

After imaging the 4% Native PAGE gel, there was a clear difference in size between the PCR product and the mononucleosome assembly product (Figure 12). The mononucleosome assembly product was measuring about 2000 bp, while the PCR product was around 600bp. The difference in the sizing is due to the histones binding to the DNA and the mononucleosome product taking longer to move through the gel. This 4% Native gel proves that with 1 µL of histones and 45 µL of PCR product that the histones can bind to the DNA and make nucleosomes.

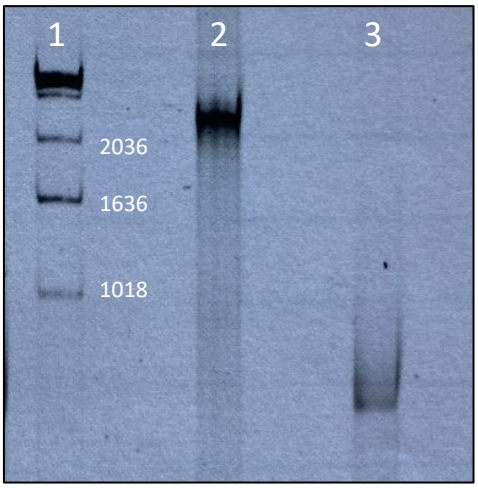

Figure 12-Lane 1 contains 3 µL of 1Kb+ Ladder, 6 µL of H2O, and 4µL of Orange G dye. Lane 2 contains 5 µL of Mononucleosome assembly sample, 4µL of Orange G Dye, and 5 µL of H2O. Lane 3 Contains 1 µL of PCR DNA, 4 µL of Orange G Dye, and 7 µL of H2O. Lane 2's band didn't travel as far down the gel as the band in Lane 3. This is indicative of the presence of a mononucleosome in lane MN.

The 4% Native PAGE gel was ran after using HhaI, however the results displayed that a mononucleosome was not created (Figure 13). There was a slight shift in size between lane 1 and 2 which is evidence that HhaI made a cut on the DNA, however, the shift in size is not large enough for a mononucleosomes assembly. Instead, it appears that multiple histones binded to the DNA which blocked off more than one HhaI cut site. There are no visible bands in lane 3 for the HhaI cut PCR product. There should be 4 bands, however the gel was ran too long and we believe the cut DNA was ran off the gel since the smaller bands for the ladder are also not visible.

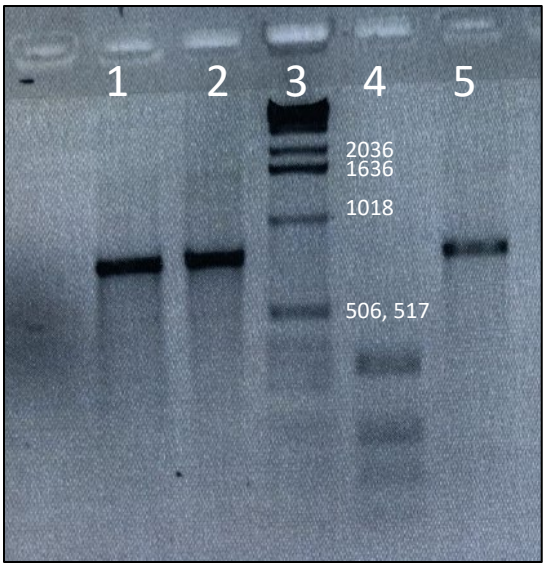

Figure 13- 1.5% Agarose gel. Lane 1 contains 5 µL of Mononucleosome, 1 µL of HhaI buffer, 0.5 HhaI, 3.5 µL of H2O, and 4 µL of Orange G Dye. Lane 2 contains 5 µL of Mononucleosome, 1 µL of HhaI buffer, 4 µL of H2O, and 4 µL of Orange G Dye. Lane 3 contains 3 µL of 1Kb+ Ladder, 6 µL of H2O, and 4µL of Orange G dye. Lane 4 contains 1.5 µL of PCR Product, 1 µL of HhaI buffer, 0.5 HhaI, 3.5 µL of H2O, and 4 µL of Orange G Dye. Lane 5 contains 1.5 µL of PCR Product, 1 µL of HhaI buffer, 4 µL of H2O, and 4 µL of Orange G Dye. There is a slight shift between lane 1 and 2 indicating that HhaI cut one site. Lane 4 displays no bands indicating that the gel was ran too long.

The agarose gel confirmed the findings from the 4% native PAGE gel (figure 14). There is a small difference in size between lane 1 and 2 as seen in the native gel. The agarose gel clearly displayed the cut DNA in lane 3 to prove that the HhaI enzyme was functioning properly. It is also evident that the pk enzyme also functioned properly in removing the histones since the bands in lane 1,2, and 4 are roughly the same size. Our mononucleosomes assembly did not create a mononucleosomes and instead binded too many histones to the DNA.

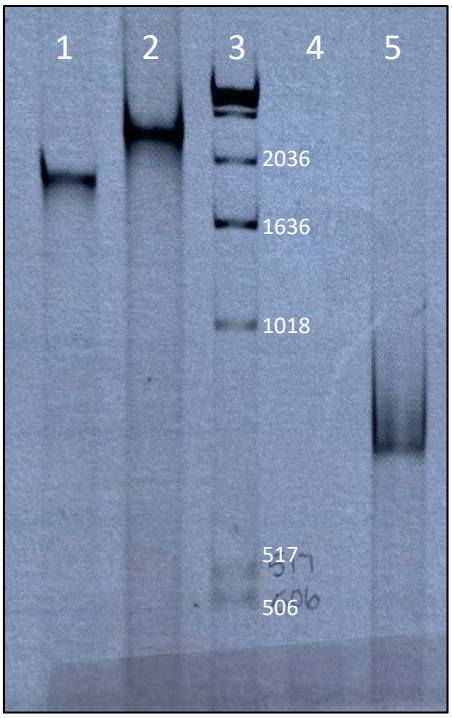

Figure 14-1.5% agarose gel. Lane 1 contains 5 µL of Mononucleosome, 1 µL of HhaI buffer, 0.5 HhaI, 3.5 µL of H2O,1 µL pk and 4 µL of Orange G Dye. Lane 2 contains 5 µL of Mononucleosome, 1 µL of HhaI buffer, 4 µL of H2O, 1 µL pk and 4 µL of Orange G Dye. Lane 3 contains 3 µL of 1Kb+ Ladder, 6 µL of H2O, and 4µL of Orange G dye. Lane 4 contains 1.5 µL of PCR Product, 1 µL of HhaI buffer, 0.5 HhaI, 3.5 µL of H2O, 1 µL pk and 4 µL of Orange G Dye. Lane 5 contains 1.5 µL of PCR Product, 1 µL of HhaI buffer, 4 µL of H2O, 1 µL pk and 4 µL of Orange G Dye.

Discussion

PCR Optimization to increase DNA concentration

Due to low concentrations in the PCR reactions, different optimization protocols were used to increase the concentration of DNA. A Betaine and $MgCl_2$ Titration was used to determine if our bands could be tightened or darkened on the agarose gel. The best result came from only using betaine with no $MgCl_2$ present in the solution. There were new stocks made of SOC, betaine, and primers. dNTP's, xL TAQ, and TAQ buffer were all compared against the Biochemistry Department Teaching Lab's reagents. The Biochemistry Teaching Lab had been running PCR reactions and knew that their reagents were working. Our reagents matched the same DNA concentration after the PCR protocol with the teaching lab reagents which determined that our reagents weren't the reason for a low DNA concentration. Different thermocyclers were used with the same protocol settings, and we noticed a two-fold difference in the amount of PCR product that was obtained.

The second change to our PCR protocol that elicited a difference in the DNA concentration of our products was changing the location of the primers (Figure 15). We tried three different primers and noticed that the p601B resulted in the highest concentration of DNA. The 300Forward/300Reverse resulted in the second highest DNA concentration while the 120 Forward/480 Reverse primers were the least high DNA concentration and frequently didn't make a PCR product at all. The 120 Forward/480 Reverse primers had previously worked in the laboratory, so they were reordered to check if the primers had degraded in the freezer. However, the new set of 120 Forward/480 Reverse primers still had the same problems. It was attempted to create 300Forward/480 Reverse and 120Forward/300 Reverse products to check if a single

primer was degraded. These PCR products were minimal and matched the concentration of the 120 Forward/480 Reverse.

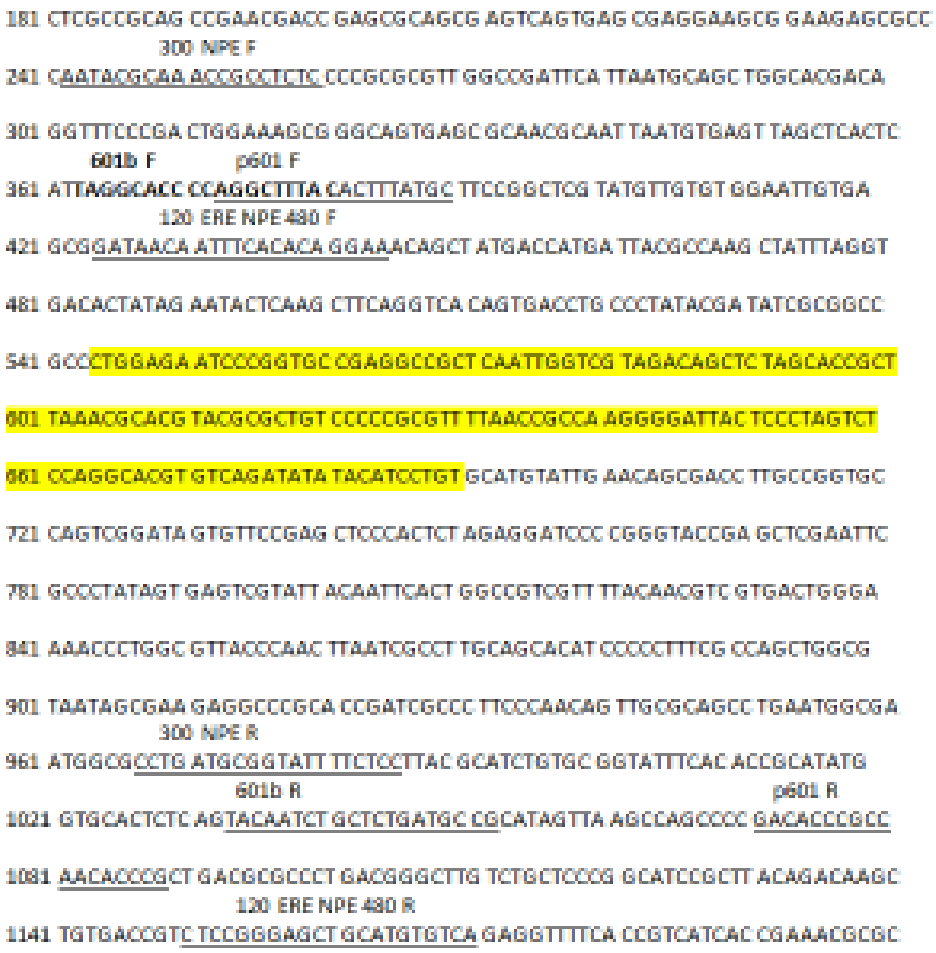

Figure 15-A portion of the plasmid sequence is displayed ranging from nucleotide 181 to 1200. In the yellow highlight is the Nucleosome positioning element. The four different primer sequences are labelled and underlined. 601bF is bolded due to overlapping with p601F.

Mononucleosome Assembly Optimization

The 4% Native PAGE gel is an important aspect of testing the mononucleosomes, because this is the only percentage that properly allows the mononucleosomes to be visible. A 6% Native PAGE gel was attempted in order to have a more solid gel to work with. The 6% Native PAGE didn't allow the mononucleosomes to move into the gel. Instead, the mononucleosomes stayed clustered together inside the well. Once the gel percentage was moved down to 4%, the mononucleosomes were able to move into and through the gel.

The ratio of DNA to histones is important in order to both create a mononucleosome and be able to visualize your samples in a 4% native PAGE. When there are too many histones in the assembly, the histones stick together and make it impossible for the DNA to move into the gel. At a lower DNA to histone ratio, the DNA will be able to move into the gel and allow a visible change to be seen. However, there can still be too many histones attached to the DNA strand even if the nucleosomes are visible on the native PAGE. This is why titration is important to determine the closest 1:1 ratio of DNA to Histones. Utilizing HhaI is also helpful in this endeavor to determine if the mononucleosomes is actually attached to the nucleosome positioning element or how many mononucleosomes are attached to the DNA.

Future Direction

The single cells that were created from the CRISPR-cas9 will undergo future testing with a Western Blot to ensure that no INI-1 activity is present in the cells. They will then be able to be used in experiments that can measure the rate of proliferation between cell lines that contain INI-1 and have no INI-1. These cells can also be utilized to measure if giving supplemental INI-1 can reverse any of the changes that appeared due to the knockout. These cells will set the stage for future studies in our laboratory to be conducted on the comparison of cancer cells with or

without INI-1. This could potentially set the stage for therapeutic avenues to cure cancers that contain no INI-1.

While the samples that were prepared are believed to contain multiple nucleosomes, instead of a single nucleosome, the optimization and proof of concept was completed. In future assemblies, a titration containing less histones will be completed. This titration will allow for the laboratory to create a DNA strand that has a single nucleosome present. The creation of the mononucleosomes will allow for future studies to monitor the movement of the nucleosome based on the addition of INI-1. This will help uncover more about the role of INI-1 within the SWI/SNF complex and help scientists understand how nucleosomes are able to move.

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