Characterization of the histone binding properties of the epigenetic reader protein UHRF2 to H3 in PHD domain

Marissa Gilliam

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Abstract
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Degree Type
Open Access Senior Honors Thesis

Department
Chemistry

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Keywords
UHRF2, epigenetics, protein binding

Subject Categories
Chemistry
CHARACTERIZATION OF THE HISTONE BINDING PROPERTIES OF THE EPIGENETIC READER PROTEIN UHRF2 TO H3 IN PHD DOMAIN

By

Marisa Gilliam

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Chemistry

Approved at Ypsilanti, Michigan, on this date 5/13/2019

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Abstract:

Every cell contains the same genetic code, to have cell differentiation this genetic code needs to be regulated on what is expressed. The regulation of gene expression without altering the genetic code itself is known as epigenetics. An example of epigenetic regulation can be seen between the binding of UHRF2 and H3 histones. The purpose of this project is to determine if D363 in UHRF2 is important for histone H3 binding. To do so, D363 was mutated to alanine, lysine or asparagine. The mutant protein was expressed, purified, and its ability to binding to H3 was tested by fluorescence polarization. Compared to wildtype UHRF2 PHD which bound histone H3, the D363A, D363K and D363N mutants showed no binding. This suggests D363 is critical for the UHRF2: H3 interaction.

Introduction:

Cancer is a growing problem throughout the world, it is estimated that nearly one out of three Americans will be diagnosed with cancer throughout their lifetime. This means that in 2018 an estimated 1,735,350 newly cases of cancer will be diagnosed, and 609,640 Americans will die of cancer\(^1\). While cancer is not the leading cause of death in the United States, it is second, coming in behind heart disease\(^2\). Common treatments for cancer include chemotherapy, surgery, radiation therapy, and immunotherapy, with a large push for preventative action also playing a role in fighting cancer\(^1\). However, most of these treatments are harsh on the body and remission is not guaranteed. As such new approaches to cancer treatments are constantly researched and new drugs developed to try and better every patients’ odds.

One such approach is by using the field of epigenetics. Epigenetics is the alteration to gene and/or protein expression without altering the DNA sequencing itself\(^3\). By controlling what
genes are being expressed, epigenetics can be used to fight cancer by slowing cancer growth and thus allow for less treatment needed to become cancer free. Eukaryotic DNA is organized into higher order structures called nucleosomes. Nucleosomes are composed of approximately 150 DNA base pairs coiled around an octamer of histone proteins, with each octamer containing 2 sets of the 4 histone proteins H2A, H2B, H3, and H4 and packaged as chromatin Modifications on the tails of histone proteins are epigenetic markers that function to regulate gene expression. This can happen through the addition of small molecules to proteins/DNA, such as the addition of a methyl, acetyl, or phosphoryl group, or by the binding of proteins known as reader proteins to the histones. Within cancer cells, certain proteins are up regulated to cause gene expression that encodes for cell growth. One such protein that does this and is often overexpressed in cancer is Ubiquitin-like containing plant Homeodomain and Ring Finger 1 or UHRF1. UHRF1 has been extensively studied and the crystalized protein complex of UHRF1 bound to the H3 histone has already been determined.

However, a genetically similar reader protein to UHRF1 is Ubiquitin-like containing plant Homeodomain and Ring Finger 2 called UHRF2 for short. Unlike UHRF1, UHRF2 has not been extensively studied and while a crystalized structure of just UHRF2 is available, a crystalized structure of UHRF2 in complex with H3 is not available. While UHRF1 is commonly overexpressed in cancers, UHRF2 is both overexpressed as well as repressed in cancers. This means UHRF2 is capable of being both an oncprotein and a tumor suppressor protein. An oncprotein causes a cell to be transformed into a cancerous one; while a tumor suppressor protein prevents this transformation or suppresses the proliferation of cancerous cells if transformation does occur. One function of UHRF2 is that it recruits transcription proteins for expressing genes that turn off growth genes. Within both UHRF1 and UHRF2 are the same two
histone binding domains that have 40-60% similarity in genetic sequencing, these domains are called Tandem-Tudor Domain (TTD) and a Plant Homeo Domain (PHD), as shown in figure 1.8,9 UHRF1 has been shown to interact with multiple recognition sites on histone H3 through the TTD and PHD domains. The similarities between UHRF2 and UHRF1 suggest that similar binding mechanisms exist in UHRF210. Using an overlay of H3 bound UHRF1 crystal with unbound UHRF2 (figure 1), sites of UHRF2:H3 binding within the TTD and PHD domain can be predicted and tested.

As shown in figure 1, UHRF1 and UHRF2 TTD domains overlay very well, while the PHD domains do not. This is likely being caused by the flexibility of the linker domain between the two proteins and not due to the domains not being similar. This can further be shown to be true by figure 2 which shows the overlay of the two PHD domains.
The binding between H3 and UHRF1 PHD domain is known to be done by two charge interactions between Arginine residue in the H3 and Aspartic Acid residue in the PHD domain. By comparing the overlay of UHRF1 in complex with H3 with UHRF2, the Aspartic Acid in position 363 was found to be close to one of the Arginine residues in H3. As binding between these two residues was already shown in UHRF1, it is hypothesized that D363 in UHRF2 was a point of binding between the UHRF2 PHD domain and the H3.

The focus of this specific research project is to interrogate the H3 binding interactions within the single UHRF2 domain PHD. This is tested by removing the Aspartic Acid and replacing it with three other residues. By characterizing the D363 binding site of UHRF2 to histone H3, an anticancer drug can eventually be developed to inhibit UHRF2 by disrupting histone interactions.

**Experimental Procedure:**

**Plasmid:** The mutations were performed on the pGEX-KG UHRF2 PHD plasmid. This small, double stranded, circular piece of DNA contains multiple segments that are used throughout the
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The experimental process. The first is an origin of replication where the plasmid begins the process of DNA replication. Next is a lac repressor gene that codes for a protein that prevents the expression of the proteins encoded within the plasmid. Then, there is a tac promoter region that drives high expression of downstream genes. After this is a gene that codes for glutathione s-transferase (GST-enzyme tag). Downstream is the gene that codes for the UHRF2 PHD domains. In the final protein product, the GST is at the N terminal end of and covalently attached to the expressed UHRF2 PHD. The GST tag is useful for its purification and also plays a helpful role in the expression, folding, and solubility of the protein. Finally, the last gene is one that codes for the enzyme β-lactamase. This enzyme provides cells that take up this plasmid with resistance to the antibiotic ampicillin.

**Primers:** The mutation in the pGEX-KG UHRF2 PHD plasmid was added using forward and reverse primers. These short pieces of single stranded DNA each introduce the mutation into one of the strands of the double stranded plasmid. They serve as a starting point for DNA replication by DNA polymerase with the mutation already incorporated. The primers were designed and ordered for creation by Dr. Albaugh.

**Cite Directed Mutagenesis Using Inverse PCR:** An inverse polymerase chain reaction (PCR) was performed to create and then amplify the mutated version of UHRF2 PHD. To prevent contamination from foreign DNA from being amplified in the sample, reagents were assembled in biosafety hood. 10 ng of pGEX-KG UHRF2 PHD template DNA, 200 nM of forward and reverse primers, 25 µL Clone Amp Hifi PCR Premix, and 22 µL nuclease free water were combined in a PCR tube. The PCR premix contains buffer, salts, metals, water, dNTPs (deoxynucleotide triphosphates, or bases), and DNA polymerase. The tube was then placed in a thermocycler with two other empty PCR tubes to prevent melting of the tube of interest. The
thermocycler first heats the sample to 98°C for 10 seconds to break the hydrogen bonds between the strands of the pGEX-KG UHRF2 PHD plasmid DNA, this separates the strands. The thermocycler then switches to 55°C for 10 seconds which allows the primers to bind to the complementary sequenced bases on the separated strands. The temperature is then raised to 72°C for 30 seconds in which DNA polymerase synthesizes DNA using the plasmid as a template. The three temperature stages are then cycled to amplify mutant DNA. Inverse PCR was performed in which the primers anneal and amplify DNA in opposite directions to amplify the entire template rather than standard PCR in which the primers face one another and amplify only a portion of the template. The resultant mutant DNA is linear which allows for only the circular DNA template to be degraded during DPNI Digest.

**DPNl Digest:** 40 uL of nuclease free water, 10 uL of CutSmart buffer, 48 uL of PCR product, and 2 uL of the DPNl enzyme were combined in a microfuge tube and centrifuged at 13000 rpm for one minute. The tube was then placed in a 37°C water bath for 1 hour to allow the enzyme to cleave the methylated DNA at its optimal temperature. Following this, the tubes were heated at 80°C for 20 minutes to denature the enzyme and stop its function.

**Plasmid Transformation into Rosetta DE3 cells:** 20µl of Rosetta DE3 competent cells were added to pre-chilled microfuge tubes on ice followed by either 1 µl of DNA or no DNA (negative control tube), gently mixed the tubes and incubated on ice for 15 minutes. The cell/DNA mixtures were heat shocked in a 42°C water bath for 30 seconds and then returned to ice for 2 minutes. 250 µl of Super Optimal Broth (SOB) was to each tube and placed in 37°C rotating incubator for 40 minutes. The whole sample was spread on the prewarmed LB-Agar-Ampicillin-Choramphenicol plates and placed back in a 37 incubator for overnight growth.
DNA Purification: Colonies from the overnight transformation plates is grown in SOB overnight. Colonies selected were uniform in appearance and were not touching other colonies. These cells are pelleted at top speed for 1 minute in two 1.5ml portions, the supernatant was decanted each time. Pellets were resuspended in 250 µl of Cell resuspension solution and 250 µl of cell lysis solution was added. The solution was gently inverted three times and 10 µl of Alkaline Protease solution was added and incubated for 5 minutes. 350 µl of neutralization solution was added and the tubes were centrifuged at top speed for 10 minutes. The resulting supernatant was then added to spin columns attached to a vacuum adaptor and pulled through with a vacuum. 750 µl of wash solution was vacuumed through followed by a second wash of 250 µl. After the washes the samples were left on the vacuum running for 10 minutes. The columns were transferred into new microfuge tubes and centrifuged at top speeds for 2 minutes. To free the DNA from the column, 100 µl of nuclease free water was added to the columns and spun again at top speed for 1 minute.

Nanodrop Spectrophotometric Analysis of DNA Samples: Nanodrop spectrophotometry was performed to determine the purity and concentration of DNA samples. DNA absorbs at a wavelength around 260 nm, protein at 280 nm, and salts and buffers at 230 nm. The concentration value is given, and purity can be determined by the 260/280 and 260/230 values. The 260/280 value describes how much protein is in the sample with a value above 1.8 corresponding to low protein levels. The 260/230 value describes how much salt and buffer contamination is contained in the sample with a value around 1.8-2 being ideal.

DNA Gel Run: 50ml of Agarose DNA gel was made by mixing together 50ml of 0.5X TBE and 0.35g agarose to a 250ml flask and left to settle for at least two minutes. The flask opening was covered with plastic wrap and a small whole pierced in the plastic wrap for ventilation. The flask was microwaved until bubbles begin to appear in the solution (about 1 minute). The flask was
removed and stirred before returning to microwave for an additional minute. The solution was cooled for 2-4 minutes and then 1 µl of Gel Red Staining Reagent added. The gel casting tray was set up and made sure that it is properly sealed and the correctly sized comb for the number of wells needed added before pouring in the gel. The gel to cooled for at least 30 minutes.

For the DNA samples, a total of 12 µl volumes was prepared: 2 µl of the DNA, ladder, or water for the negative controls, 2 µl of purple loading dye, and 8 µl of nuclease free water. To each well, only 10 µl were added. The gel was then ran at 120V for up to a total of 45 minutes and checked every 15 minutes to insure the samples did not run off the gel.

**Protein Expression:** One colony from a transformation plate was inoculated into 50ml LB growth media containing 50 µl of 1000X Ampicillin and 50µl of 1000X Chloramphenicol in a sterile environment and left to incubate in the rotating incubator at 37°C overnight. 10ml of this overnight solution was added to a 1L container of LB growth media along with 1 ml of 1000X Ampicillin and 1000X Chloramphenicol and incubated in rotating incubator for two hours at 37°C. After 2 hours the absorbance was measured. As a rule of thumb for this bacterium, cell growth doubles every 30 minutes. The absorbance was checked every 30 minutes and removed from incubator when OD600 was between 0.4-0.5. 1ml IPTG was to the 1L sample and the temperature lowered to 21°C and shaken in the incubator overnight. The centrifuge, a Sorvall RC 5B Plus with a Sorvall SLA-3000 Super-Lite fixed angle rotor, was used to pellet the cells for 10 minutes at 5000rpm and supernatant decanted. The pellet was then frozen in a 50ml conical and placed in the -20°C freezer.

**Protein Purification:** Frozen pelleted cells from protein expression were placed on ice to thaw. 3.5 µl BME was added to 50ml of Triton Lysis Buffer II (0.5% Triton, 10% glycerol, 150 mM
NaCl, and 50 mM Tris, 1 mM 2-Mercaptoethanol (BME)). From this solution, 10ml of 10mg/ml Lysozyme in Triton Lysis Buffer II was made. 27ml of Triton Lysis Buffer II and 3ml of Lysozyme in Triton Lysis Buffer II was added to the thawed cell pellet. PMSF at a stock concentration of 100 mM, leupeptin at a stock concentration of 10 mg/mL, and aprotinin at a stock concentration of 5 mg/mL were added to a final concentration of 1x to the thawed cell pellet and mixed until the mixture is homogenous. If the solution did not become homogenous after 10 minutes, up to an additional 3ml of the Lysozyme in Triton Lysis Buffer II solution was added in 1 ml increments with 3-5 minutes of stirring to see if homogenous before another 1ml was added. 30 µl of DNAse1 was added to the tube and stirred on ice for 10-15 minutes. This should leave the solution as homogenous and with the consistency of water, if it is not, more DNAse1 was added and stirred at room temperature for 10-15 minutes. 100 µl of sample was collected and stored in test tube labeled all of cell for SDS gel run. The sample was then centrifuged for 30 minutes at 13000 rpm. A portion of the pellet and the supernatant was collected and placed into labeled tubes for the SDS gel run. The supernatant was ran on a GST-tagged column in the cold room through tubing with gravity pulling the supernatant through at a rate around 0.5ml per minute, the liquid removed from the column is collected and known as the flow through. Between the flow through and elution the column is washed with around 100ml of Triton Lysis Buffer II to remove all unbound proteins from the column. The eluent was collected at a rate around 0.25ml per minute to allow the resin beads to release the bound UHRF2 protein in favor for the elute buffer of 10 mL of 50 mM Tris, pH 8.0, 10 mM reduced glutathione. The eluent was collected and placed in Thermo Fisher Scientific 10,000 MWCO (molecular weight cut off) snakeskin dialysis tubing and dialyzed overnight in 1 L of 10% glycerol, 50 mM Tris, 150 mM NaCl, 1 mM BME dialysis buffer at a pH of 7.5 with stirring. The next day, the solution
was centrifuged in Corning Spin-X UF 30,000 MWCO concentrator tubes in an Allegra 25R centrifuge in a Beckman Coulter TA-10-250 fixed-angle aluminum rotor at 6000 rcf and 4 C until concentrated to around 1mL. The 1mL was then aliquoted as 100 µl into 1.7mL microfuge tubes. To prevent protein degradation, the microfuge tubes were snap frozen in liquid nitrogen and stored in -80 C freezer.

**DC Assay:**

From 25mg/ml BSA, 2.5mg/ml BSA was made with water. Six tubes were set up with the following concentrations.

<table>
<thead>
<tr>
<th>µg BSA</th>
<th>µl 2.5 BSA</th>
<th>µl water</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>5</td>
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<tr>
<td>10</td>
<td>4</td>
<td>21</td>
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<td>15</td>
<td>6</td>
<td>19</td>
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<tr>
<td>20</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>25</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

The protein was diluted to 1/5 by adding 20µl of protein to 80µl of water. Three test tubes were prepared with varying concentrations of protein 1/5.

<table>
<thead>
<tr>
<th>µl of 1/5 protein</th>
<th>µl water</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>
First, 2ml reagent A and to 40 µl reagent S was prepared in 15ml tube and mixed to vortex. 150µl of this solution was added to each of the nine test tubes prepared. 1ml of reagent B was added to each of the nine test tubes, vortexed and incubated for 15 minutes at room temperature. Nine cuvettes were labeled and 1ml of each sample was added to the matching cuvettes. The Spectrophotometer was used to read samples at 750nm. A Beckman Coulter DU 800 UV/Vis Spectrophotometer was used to read samples at 750nm. The BSA results were graphed and a standard curve (Fig. 3) was created. As Beer’s Law (Fig. 4) shows, there is a direct relationship between absorbance and concentration. Thus, the BSA standard curve can be used to calculate the protein concentration from the absorbance readings of the 1/5 protein concentration samples using the linear trendline, by plugging the absorbance in for x and solving for y.

![Standard Curve](image)

$y = 75.406x - 0.7124$

$A = ebc$

Figure 3: Example graph of BSA µg protein against Absorbance with a trendline fit to it showing the linear relationship

Figure 4: Beer’s Law equation

Fluorescence Polarization:
A Fluorescence Polarization Buffer was prepared by combining 50ml of 50mM Tris with a pH of 7.5 with 0.05g of BSA. This solution was then filter sterilized using a sterile syringe filter with a 0.2µm Polyethersulfone Membrane and a 60ml sterile syringe. To the sterilized Fluorescence Polarization Buffer 50µl of 1000x stock protease inhibitors were added: Aprotinin, Leupeptin, and PMSF. When the buffer was not in use it was kept on ice. H3 peptides had covalently attached to the C terminus a 5-carboxyfluorescein (5-FAM) fluorophore molecule that is capable of fluorescing. When light of a wavelength of 485 nm is shone on the fluorescein, it excites the electrons within it from the ground energy state, or unexcited state, to an excited electron energy state. This light is then emitted at a different wavelength, 528 nm, as the electron returns to ground state after a set amount of time. Preparation of the Histone H3 peptides differed due to concentrations when unmodified or modified H3 was used. Thus, using nuclease free water, the peptide concentrations were made to be 100nmol per 1000ml. This was done to a total volume of 1ml in a 1.7ml microcentrifuge tube and kept on ice. To protect the fluorescing peptide, the amount of exposure to light should be kept to a minimum as well and was done so by wrapping H3 peptide containers in aluminum foil. To perform the FP assay, four replicates of 11 binding reactions were performed containing the Fluorescence Polarization Buffer with PMSF, Leupeptin, and aprotinin protease inhibitors added to it, 10 nM of the H3K9me3-fluorescein peptide, and varying concentrations of the mutant UHRF2 PHD. To do so, a black, opaque, 96 well plate was prepared. The 96 well plate had a serial dilution done in row A starting with 100% protein solution in column 12 and then being diluted by mixing with the Fluorescence Polarization Buffer from columns 11 through 3 and no protein is added to column 2. In column 1 the 100nM of H3 peptide is added to B, C, D, and E. From row A the column dilutions of protein are pulled...
down in 10µl amounts, and the H3 peptide in column 1 is pulled across the columns in 10µl amounts as shown in table 3 which has the assumed protein concentration of 100µM.

Table 3: Table showing example FP Assay 96 well plate provided by Zeineb ElMohri.

<table>
<thead>
<tr>
<th>Row/Column</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>100 µM Protein and 10 nM H3 peptide</td>
<td>0.0153 µM Protein and 10 nM H3 peptide</td>
<td>0.0906 µM Protein and 10 nM H3 peptide</td>
<td>0.7813 µM Protein and 10 nM H3 peptide</td>
<td>1.325 µM Protein and 10 nM H3 peptide</td>
<td>3.35 µM Protein and 10 nM H3 peptide</td>
<td>6.5 µM Protein and 10 nM H3 peptide</td>
<td>12.5 µM Protein and 10 nM H3 peptide</td>
<td>25 µM Protein and 10 nM H3 peptide</td>
<td>50 µM Protein and 10 nM H3 peptide</td>
<td>120 µM Protein and 10 nM H3 peptide</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>100 µM Protein and 10 nM H3 peptide</td>
<td>0.0153 µM Protein and 10 nM H3 peptide</td>
<td>0.0906 µM Protein and 10 nM H3 peptide</td>
<td>0.7813 µM Protein and 10 nM H3 peptide</td>
<td>1.325 µM Protein and 10 nM H3 peptide</td>
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<td>6.5 µM Protein and 10 nM H3 peptide</td>
<td>12.5 µM Protein and 10 nM H3 peptide</td>
<td>25 µM Protein and 10 nM H3 peptide</td>
<td>50 µM Protein and 10 nM H3 peptide</td>
<td>120 µM Protein and 10 nM H3 peptide</td>
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<tr>
<td>D</td>
<td>0</td>
<td>100 µM Protein and 10 nM H3 peptide</td>
<td>0.0153 µM Protein and 10 nM H3 peptide</td>
<td>0.0906 µM Protein and 10 nM H3 peptide</td>
<td>0.7813 µM Protein and 10 nM H3 peptide</td>
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<td>25 µM Protein and 10 nM H3 peptide</td>
<td>50 µM Protein and 10 nM H3 peptide</td>
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<tr>
<td>E</td>
<td>0</td>
<td>100 µM Protein and 10 nM H3 peptide</td>
<td>0.0153 µM Protein and 10 nM H3 peptide</td>
<td>0.0906 µM Protein and 10 nM H3 peptide</td>
<td>0.7813 µM Protein and 10 nM H3 peptide</td>
<td>1.325 µM Protein and 10 nM H3 peptide</td>
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<td>6.5 µM Protein and 10 nM H3 peptide</td>
<td>12.5 µM Protein and 10 nM H3 peptide</td>
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<td>50 µM Protein and 10 nM H3 peptide</td>
<td>120 µM Protein and 10 nM H3 peptide</td>
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</table>

Graph Creation and Kd Calculation:

Using the concentrations of protein along with the intensities of parallel to perpendicular light from the Fluorescence Polarization Assays, a graph was created. This graphed data was then normalized and set to the equation seen in figure 3 using the computer program Prism to calculate a Kd value.

\[
\text{Percent Bound} = \frac{[\text{protein}]}{K_d + [\text{protein}]} \times 100
\]

Figure 5: Prism equation used to create a best fit curve for Fluorescence Polarization Assay Data.

**Results and Discussion:**

Inverse PCR was performed on 50 µl samples to create and amplify mutant pGEX-KG UHRF2 PHD DNA. Once finished, a 2 µl sample of each reaction was run, along with negative controls
where nuclease free water was used, on a 0.7% agarose gel at 120 V for 45 minutes alongside a 1 KB ladder. The resultant gel was imaged, and the bands were compared to the 1KB DNA ladder (Fig. 6) to determine success of the PCR reaction.

The gel shows a single dark band in the lanes that had DNA and this signifies that the replication of DNA was successful; the smaller and lighter bands at the bottom of the gel are likely primers that bound to each other and should have no effect. With no bands of significance in the negative control lanes, contamination can be ruled out and the mutant DNA inverse PCR was successful.

After DNA gel electrophoresis, the mutants underwent a DPNI digest to remove the methylated template DNA from the samples. The samples were then purified using PCR column purification and transformed into Stellar Competent E. coli cells. The Stellar Competent E. coli cells mass produced the plasmid while dividing and colony forming units were selected for plasmid
purification. Afterwards, Nanodrop spectrophotometry was used to determine sample concentration and purity. The values collected are seen in Table 4.

Table 4: Nanodrop results showing concentration and purification of DNA mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Concentration</th>
<th>260/280 Purity</th>
<th>260/230 Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D363A</td>
<td>1.) 97.1 ng/ul</td>
<td>1.89</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td>2.) 76.9 ng/ul</td>
<td>1.90</td>
<td>3.11</td>
</tr>
<tr>
<td></td>
<td>3.) 37.4 ng/ul</td>
<td>1.83</td>
<td>-34.13</td>
</tr>
<tr>
<td></td>
<td>4.) 102.3 ng/ul</td>
<td>1.94</td>
<td>2.64</td>
</tr>
<tr>
<td></td>
<td>5.) 58.1 ng/ul</td>
<td>1.91</td>
<td>7.08</td>
</tr>
<tr>
<td></td>
<td>6.) 140.7</td>
<td>1.89</td>
<td>3.03</td>
</tr>
<tr>
<td>D363N</td>
<td>1.) 54.2 ng/ul</td>
<td>1.78</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>2.) 96.4 ng/ul</td>
<td>1.66</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>3.) 98.1 ng/ul</td>
<td>1.91</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>4.) 98.9 ng/ul</td>
<td>1.92</td>
<td>1.76</td>
</tr>
<tr>
<td>D363K</td>
<td>1.) 161.7 ng/ul</td>
<td>1.95</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>2.) 191.5 ng/ul</td>
<td>1.78</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>3.) 95.7 ng/ul</td>
<td>1.92</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>4.) 161.3 ng/ul</td>
<td>1.85</td>
<td>1.37</td>
</tr>
</tbody>
</table>

The samples were sent to the University of Michigan to be sequenced by Sanger Sequencing and for D363A samples 2, 4, and 6 were correct; for D363N 1, 2, and 3 were correct and D363K had 1, 2, and 4 with correct sequencing.
The mutant DNA was then transformed into Rosetta E. coli cells and left to grow overnight. The overnight E. Coli culture was then used to inoculate a 1-liter LB culture and protein induction was performed using IPTG once the cell density absorbance value reached approximately 0.4. The protein was then extracted from the cells and purified by GST affinity chromatography. Once a purified sample was obtained, SDS-PAGE was conducted to determine protein purity and if the protein purification process was successful. This process was done for each mutant protein produced as well as the Wildtype protein. The results for these four purifications are seen in figures 7 through 10.

![Protein Purification](image)

*Figure 7: Protein Purification of UHRF2 WT checked by SDS-Gel for successful purification.*
Figure 8: Protein Purification of D363A checked by SDS-Gel for success.

Figure 9: Protein Purification of D363K checked by SDS-Gel for success.
The samples were ran on the SDS-PAGE gels to check if protein purification was successful. The amount of other protein in the purified protein column is negligible compared to the amount of UHRF2 protein present. The method can be improved as seen by the presence of UHRF2 protein in the Flow through and Resin columns meaning that desired protein was lost. To lessen this, the amount of resin used should be increased and the speed of the Flow through dripping through the column should go at a slower speed. The protein lost on the resin could also be prevented by having the elution go at a slower pace. Lastly, figures 9 and 10 are missing the pellet that sample was not collected.

The purified protein samples were then run through a DC Assay using BSA of known concentrations to make the standard curve. Using the trendline produced (Table 5) from the
graphs and the absorbances of the purified proteins, the concentrations were solved as seen in the example calculation in figure 11, which is from D363A Trial 1 using the absorbance of the 10µl of protein at a protein concentration of 1/5 total protein concentration. As multiple volume amounts were tested, the calculated concentrations in table 6 are the average of the tests that fit within the standard curve.

Table 5: The trendline equations created for each DC Assay done.

<table>
<thead>
<tr>
<th>Mutant/Trial</th>
<th>Standard Curve Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D363A Trial 1</td>
<td>( y = 75.406x - 0.7124 )</td>
</tr>
<tr>
<td>D363K and D363N Trial 1</td>
<td>( y = 74.512x - 0.3533 )</td>
</tr>
<tr>
<td>D363A Trial 2 and WT Trial 1</td>
<td>( y = 101.48x - 0.5625 )</td>
</tr>
<tr>
<td>D363K, D363N and WT Trial 2</td>
<td>( y = 90.363x - 0.5333 )</td>
</tr>
</tbody>
</table>

\[ y = 75.406x - 0.7124 \]

\[ y = 75.406(0.2921) - 0.7124 \]

\[ y = 21.31\mu g \]

\[
\left( \frac{21.31 \mu g}{10ul} \times 5 \times \frac{1 \text{ mol}}{27010 \text{ g}} \right) \times \frac{1000000 \mu \text{ mol}}{1 \text{ mol}} = 394.4 \mu \text{M}
\]

*Figure 11: Sample calculation for finding the protein concentration of purified proteins.*
Table 6: Final calculated concentrations from DC Assay

<table>
<thead>
<tr>
<th>Mutant/Trial</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>D363A Trial 1</td>
<td>389.8221 µM</td>
</tr>
<tr>
<td>D363K Trial 1</td>
<td>329.2249 µM</td>
</tr>
<tr>
<td>D363N Trial 1</td>
<td>169.4104 µM</td>
</tr>
<tr>
<td>D363A Trail 2</td>
<td>505.0471 µM</td>
</tr>
<tr>
<td>WT Trial 1</td>
<td>614.5047 µM</td>
</tr>
<tr>
<td>D363K Trial 2</td>
<td>34.9763 µM</td>
</tr>
<tr>
<td>D363N Trial 2</td>
<td>80.9957 µM</td>
</tr>
<tr>
<td>WT Trial 2</td>
<td>194.9308 µM</td>
</tr>
</tbody>
</table>

The concentrations are all are of usable amounts, the low level of protein expression in the D363N Trial 2 and D363K Trial 2 are believed to have been caused by loss of competency in the E. coli cells used.

To determine the binding affinity between the mutants and the modified H3 with three methyl groups on the Lysine in the ninth position (H3K9me3) peptide, FP assays were run. The nonlinear curve of UHRF2 Wildtype PHD binding to the H3K9me3 shown in figure 12 is used to calculate the dissociation constant (Kd). In the case of the UHRF2 WT PHD binding to H3K9me3 the Kd measured is 0.44µM. The Kds of the mutant UHRF2 PHD proteins are then shown in figures 12 through 15.
Figure 12: The Fluorescence Polarization Assays done on UHRF2 PHD Wildtype, a Kd of 0.44 µM.

Figure 13: The Fluorescence Polarization Assays done on UHRF2 PHD D363A, no Kd can be calculated as no binding occurs.
Figure 14: The Fluorescence Polarization Assays done on UHRF2 PHD D363K, no Kd can be calculated as no binding occurs.

Figure 15: The Fluorescence Polarization Assays done on UHRF2 PHD D363N, no Kd can be calculated as no binding occurs.

The calculated Kd of the wildtype UHRF2 from using the Fluorescence Polarization Assay matches the results found in other studies of what the expected Kd should be in the single PHD domain (Fig. 12). As no binding occurs with the changing of the 363 Aspartic Acid to an Alanine (fig. 13), it can be concluded that this is an important H3 binding site in the PHD domain. The lack of binding between the H3 and the D363K mutation (fig. 14) is expected, as
the predicted interaction between the PHD domain and H3 is that of a charge-charge interaction. This mutation switches the negatively charged Aspartic Acid, to a positively charged Lysine and the predicted repulsion between the Lysine on the PHD and the Arginine on the H3 is seen with the lack of binding. The importance of the negative charge on the PHD domain for binding to occur is seen in figure 15, for the mutation from an Aspartic Acid to an Asparagine is a small change in charge while the overall shape of the two are very similar. As no binding occurs it can be concluded that both shape and charge are highly specified for binding to occur.
References:


