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# Facilitated Binding of High Mobility Group Box 1 and p53 in Transcriptional Regulation

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# **Facilitated Binding of High Mobility Group Box 1 and p53 in Transcriptional Regulation**

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**Abstract:**

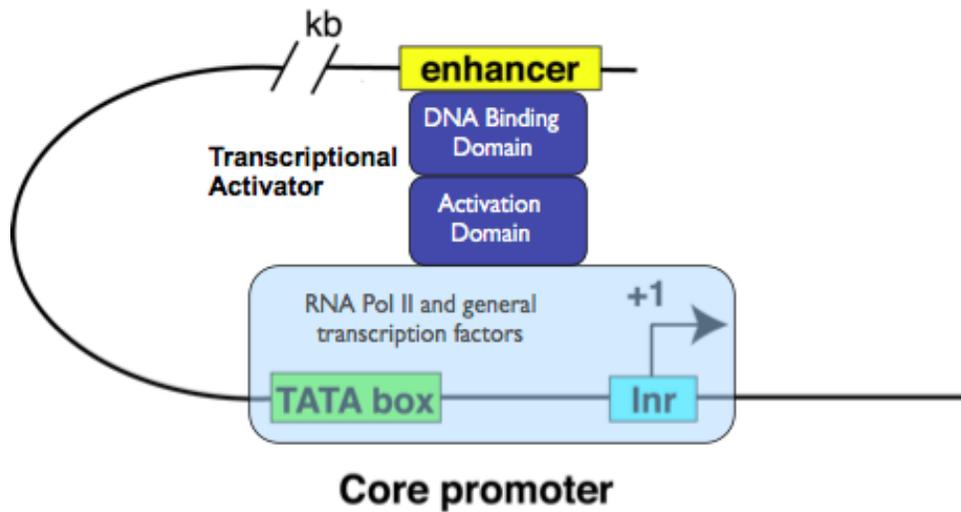
Transcription of mRNA is a very important and highly regulated processes necessary for the growth and viability of cells. Human p53 is a transcriptional activator that can act as a tumor suppressor by inducing cell cycle arrest and apoptosis. HMGB1 is an architectural protein that can bind and bend DNA to aid transcriptional regulation. This project investigated the potential for HMGB1 to facilitate DNA binding by p53. Various gel shift assays were performed to see if the addition of HMGB1 to reactions containing p53 and DNA would increase the amount of p53/DNA binding observed. On wild-type DNA containing two p53 response element half sites, the addition of HMGB1 did not result in an increase in binding. Similarly, on a shorter piece of DNA containing only one p53 response element half site, no additional p53 binding was observed in the presence of HMGB1. In a gel shift assay with DNA that was the same length as the wild type DNA but contained only one p53 response element half site, some facilitated binding of DNA by p53 was observed with HMGB1. In addition to looking at wild type tetrameric p53, two p53 mutants were created, one that forms only monomers, and one that forms only dimers. The monomeric mutant did not bind DNA. The dimeric p53 mutant bound DNA but at 10-fold the concentration at which wild type p53 bound. When HMGB1 was added to the dimeric mutant and DNA containing only one p53 response element half site, increased binding of a dimer to a single half site was observed. It is possible that HMGB1 increases specific binding of p53 to its consensus sequences. Further elucidation of the mechanism of p53 and HMGB1 binding DNA is important, as these complexes can play a key role in determining cell fate.

## **Introduction:**

### *Transcription by RNA polymerase II*

Transcription is one of the most highly regulated steps in gene expression. Protein coding DNA sequences are transcribed into mRNA molecules by the enzyme RNA polymerase II (Pol II). The mRNAs—after modification—are translated to create proteins, the essential building blocks of cellular processes. There are many steps that go into regulating transcription.

Transcriptional initiation is regulated in part by the DNA sequences in core promoters, for example, elements such as the TATA box and Inr sequence help guide the transcriptional machinery to the start site. This machinery includes the general transcription factors that are required at all genes (TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, TFIIF). These factors must associate with the DNA to recruit Pol II and all the needed co-factors for the process to begin. Additional regulatory factors include mediator regulatory non-coding RNAs, and protein complexes important for the remodeling of chromatin. An important class of transcription factors are transcriptional activators. These are proteins that bind to DNA elements upstream or downstream of the core promoter region and communicate with the general transcription machinery in order to increase transcription of a specific gene. Activators bind to specific consensus sequences using their DNA binding domains and interact with other transcription machinery in order to stimulate transcription (Figure 1). The order and mechanisms by which these factors are recruited and assembled is an essential field of today's research.



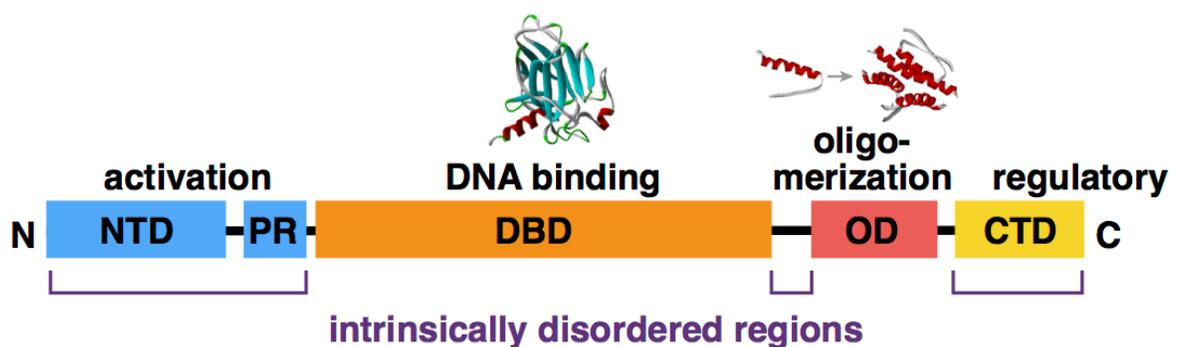
**Figure 1:** Transcriptional activators communicate with general transcription factors in order to achieve high levels of transcription.

### *P53's Role in Transcription*

Gene expression is highly regulated by transcription, and transcriptional malfunctions can lead to toxic or malignant cells. Human p53 is an important transcriptional activator that binds DNA recognition elements in the promoters of genes to facilitate transcriptional activation (Vaughan et al., 2014). By nature of the genes it controls, p53 acts as a tumor suppressor, responsible for maintaining genome integrity and preventing malignant transformation and the proliferation of cancer cells (van Dieck et al., 2009; Okorokov and Orlova, 2009). p53 is activated in response to genotoxic stress and can then initiate cell cycle arrest or apoptosis through transactivation of target genes (Okorokov and Orlova, 2009). Mutations in the gene encoding p53 are observed in about 50% of all known human cancers, making it an essential part of cellular processes (Joerger and Fersht, 2008).

There are four key domains of p53, the N-terminal activation domains, the DNA binding domain, the tetramerization domain, and the C-terminal domain (Figure 2). In cells, p53 can

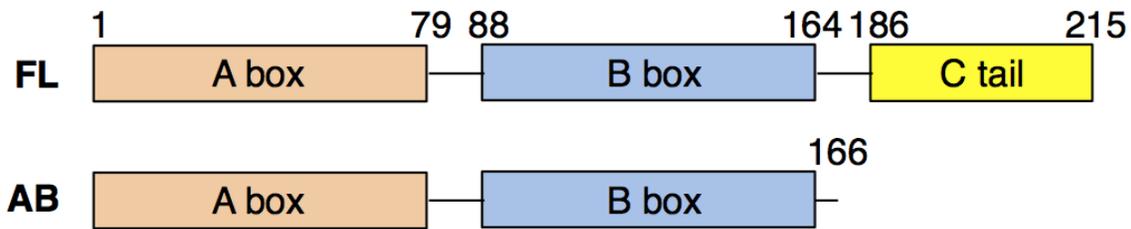
oligomerize into a tetrameric state, the predominate form of p53. The contribution of the four DNA binding domains allows p53 to bind DNA in the major groove to p53 DNA response elements (Chen et al., 2010). These response elements are composed of two decameric half sites that can be separated by 0-13 base pairs, with each decameric site being occupied by one p53 dimer (Kitayner et al., 2006). The oligomerization of p53 is a dynamic process influenced by cellular p53 protein levels and is likely regulated (Fischer et al., 2016). It is unclear whether in cells dimers can bind DNA and activate transcription, or if the complex must form a complete tetramer first. In vitro, dimer/DNA and tetramer/DNA complexes have been observed. (Fischer et al., 2016)The tetramerization domain of p53 is stabilized by hydrophobic interactions, with a Leucine at position 344 playing a role in the oligomerization process. (van Dieck et al., 2009; Joerger and Fersht, 2008) Mutation of this residue results in different conformations of p53: one forming only dimers when the leucine is mutated to an alanine, and one that forms only monomers when the leucine is mutated to a proline. (Fischer et al., 2016) These mutations lead to a reduction or loss of transcriptional regulatory activity at some classes of p53 target genes, highlighting the importance of oligomerization in p53 function (Fischer et al., 2016).



**Figure 2:** Domain Schematic of p53

### *High Mobility Group Box 1 Protein*

High Mobility Group Box 1 (HMGB1) is an architectural protein important for many nuclear processes, including transcription. HMGB1 contains two HMG-boxes (A box and B box) that can bind directly to DNA and are thought to help organize chromatin structure and facilitate DNA binding by some transcription factors (Ranzato et al., 2015). When HMGB1 binds DNA, it can also bend the DNA, which could help transcriptional protein complexes access the DNA as well (Livesey et al., 2012; McKinney and Prives, 2002). In addition to the two DNA binding domains, HMGB1 has a 30 residue acidic tail on the C-terminus (Figure 3). The A and B boxes bind DNA but can be negatively regulated by this C-terminal acidic tail, as interactions between the tail and the A and B boxes can disrupt HMGB1's ability to bind and bend DNA (Lee and Thomas, 2000; Tros et al.). HMGB1 binds non-specifically to DNA and this is important in its proposed role of facilitating sequence-specific DNA binding by transcriptional activators (Tros et al.; Watson et al., 2007). Some literature suggests HMGB1 can facilitate DNA binding by p53, potentially regulating levels of autophagy and apoptosis (Banerjee and Kundu, 2003; Jayaraman et al., 1998; Livesey et al., 2012). In gel shift assays, the addition of HMGB1 increased the amount of p53 bound to DNA (Banerjee and Kundu, 2003). In p53-null cells, co-transfection of p53 with HMGB1 increased transcription of a p53 reporter gene (Jayaraman et al., 1998). However, how and under what conditions HMGB1 is facilitating p53 binding to DNA is unclear.



**Figure 3:** Domain Schematic of HMGB1. The AB protein is a construct we made that lacks the acidic tail (Blair et al., 2013).

### Methods:

#### *HMGB1 and p53 Gel Shift Assays*

Four different DNAs containing either two half sites or one half site from the p53 response element in the GADD45 promoter were used in various gel shift assays. DNA-1 was 32 base pairs with the forward strand reading: 5' TAG AGC **GAA CAT GTC TAA GCA TGC TGG** CGT CG 3'. The reverse strand was the reverse complement of this sequence. The red characters indicate the two decameric p53 half sites. Three other DNA sequences were used as well. DNA-2 was only 22 base pairs long, and included one of the p53 half sites, however, the second half site was removed, leaving one half site and a short flanking region (forward strand): 5' TAG AGC **GAA CAT GTC** TGC GTC G 3'. DNA-3 included both p53 half sites but was flanked on both sides by additional bases, totaling to 60 base pairs (forward strand): 5' TGA TAT CGA ATT CTC GAG CAG **AAC ATG TCT AAG CAT GCT** GGG CTC GAG AAT TCC TGC AGC 3'. A final DNA used in these assays (DNA-4) was similar to DNA-1, but the second half site was scrambled, resulting in a 32 base DNA that only included one half site (forward strand): 5' TAG AGC **GAA CATG TCT** TTC AAAG GCG GCG TCG 3'. To create the double stranded DNAs, the forward and reverse strands were annealed by heating and slow cooling.

For all HMGB1 gel shifts, a native 7% polyacrylamide gel in 0.33x TBE was poured and pre-run for 45 minutes at 125V. HMGB1 (previously expressed and purified by two graduate students in the lab: Elina Ly and Abigail Horn) was diluted with a 1x reaction buffer and mixed with a 4x reaction buffer and water to obtain 20 $\mu$ L reactions with final concentrations varying with experiment. The final buffer component concentrations in the 20 $\mu$ L reaction were as follows: 25mM Hepes pH 7.9, 50mM KCl, 10% glycerol, 5mM MgCl<sub>2</sub>, 0.1% NP-40, 1mM DTT, and 0.05mg/mL BSA. Radioactive <sup>32</sup>P gamma-ATP was used to label all four of the DNAs used to perform these gel shifts. <sup>32</sup>P DNA was added to the diluted protein-buffer mixture to obtain a 0.1nM DNA concentration and the reactions incubated at room temperature for 45 minutes before being loaded on the gel. Once loaded, the gel ran for 45 minutes in 0.33x TBE buffer. The gel was dried for one hour and cooled in a vacuum before exposing to a phosphor screen and being imaged.

For gel shift assays containing wild-type p53, a combination of p53 and HMGB1, and p53 mutants, the same four sequences of DNA were used. For these assays, a native 4% polyacrylamide gel in 0.5x TBE was poured, and pre-run at 150V. Wild-type p53 was expressed in insect cells and purified by Elina Ly. The proteins were diluted in a similar 1x reaction buffer (this one containing a final concentration of 1mM EDTA in the 20 $\mu$ L reaction) and mixed with 4x reaction buffer and water as described above. After addition of the DNA, the reactions were incubated at room temperature for 30 minutes before being loaded on the gel. In assays with HMGB1 and p53, the reactions incubated at room temperature for 10 minutes before the DNA probe was added, then for another 30 minutes after the addition of the DNA. The native gels were dried and imaged as described above.

### *Cloning, Expression, and Purification of Mutant p53*

To create the monomeric and dimeric p53 mutants, two primers were designed to create single base pair changes in the oligomerization domain. A mutation from CTG to CCT replaced leucine 344 with proline (L344P) to create the monomeric mutant. Sequences of the primers were as follows. L344P forward: 5' TTC GAG ATG TTC CGA GAG **CCT** AAT GAG GCC TTG GAA CTC 3'. L344P reverse: 5' GAG TTC CAA GGC CTC ATT **AGG** CTC TCG GAA CAT CTC G 3'. A mutation from CTG to GCC replaced leucine 344 with alanine (L344A) to create the dimeric mutant. Primer sequences were as follows. L344A forward: 5' TTC GAG ATG TTC CGA GAG **GCC** AAT GAG GCC TTG GAA CTC 3'. L344A reverse: 5' GAG TTC CAA GGC CTC ATT **GGC** CTC TCG GAA CAT CTC G 3'. After multiple failed attempts to create a new double stranded DNA sequence via quick change mutagenesis, the forward and reverse primers were used in separate PCR reactions to generate overlapping DNA fragments containing the mutation using a primer sequence for the 5' end of p53 and a primer sequence for the 3' end of the C-terminals SNAP-tag in our p53 construct. The primer sequences were as follows. P53 forward: 5' GAT TCA GAA TTC ATG CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC CCA ACG ACC GAA AAC CTG TAT TTT CAG GGC ATG GAG GAG CCG CAG TCA GAT C 3'. SNAP reverse: GAT TCA TCT AGA CTA ACC CAG CCC AGG CTT GCC CAG 3'. The 5' end and 3' end fragments were then annealed together and used as a template in a final PCR step using Vent polymerase and the p53 forward and SNAP reverse primers.

After the p53 gene was created with point mutations, restriction enzymes EcoRI<sup>+</sup> and XbaI<sup>+</sup> from New England Bioscience (NEB) were used to digest the gene and a pACEBac1 plasmid containing a gene for gentamicin resistance. The p53 fragment and cut vector were

ligated together overnight at 16 °C using T4 DNA ligase from NEB. The plasmid was transformed into XL1 Blue E. coli cells. Half of the 25 µL ligation reaction was added to 100µL of cells, heat shocked at 42 °C for 45 seconds, rested on ice for 2 mins and then mixed with 100µL of Luria Broth (LB). It was then incubated at 37 °C (shaking) for an hour and a half. 100µL was then plated onto LB agar plates containing the antibiotic gentamicin. Single colonies were selected after overnight growth on the plate and allowed to grow overnight again in 4mL of LB. The overnight colonies were mini-prepped using a EZNA DNA extraction kit, isolating the plasmid containing the mutant p53 gene. The purified plasmid was again digested with the same restriction enzymes to confirm that the isolated plasmid contained an insert corresponding to the size of the mutated p53 gene. The DNA was sequenced by Quintara Bio to confirm the presence of the mutations. The plasmids were then individually transformed into DH10 EmBacY E. coli cells for 8 hours and plated on blue-white screening plates and grown for 48 hours. Half of one large white colony was selected for overnight culture and the other half was re-streaked on a plate to confirm a white phenotype. Bacmids were isolated from the culture and sent to a protein expression facility in Denver to be expressed in insect cells.

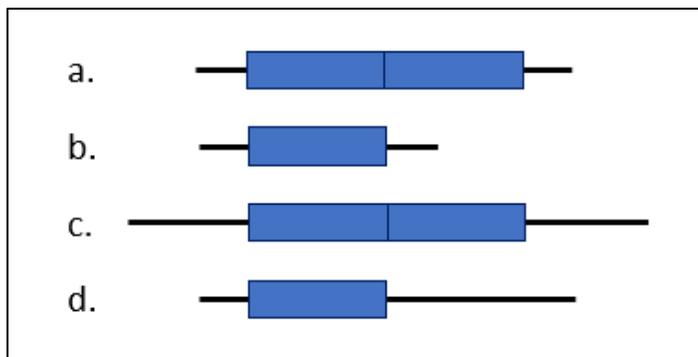
Upon receiving the pelleted insect cells from the expression facility, western blots were performed to confirm the presence of p53, a SNAP tag, and a his tag. The primary antibodies used were: p53 DO-1 SC126 mouse antibody, SNAP-tag rabbit antibody, and his-tag mouse antibody. The secondary antibodies were rabbit-anti-mouse and goat-anti-rabbit. After confirming the presence of these three features, both the L344P and L344A mutants were purified. The cells were lysed using a lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 10% glycerol, 0.5% NP-40, 2mM, DTT, 1mM PMSF, 1x Phosphatase Inhibitor, and 1x Protease Inhibitor). A Nickle-NTA column in a buffer similar to the lysis buffer—but containing 50mM

imidazole—was used to purify the protein. The protein was then eluted using 250mM imidazole. Purification was monitored using SDS-PAGE.

## Results:

### *HMGB1 Does Not Facilitate Wild Type p53 DNA Binding*

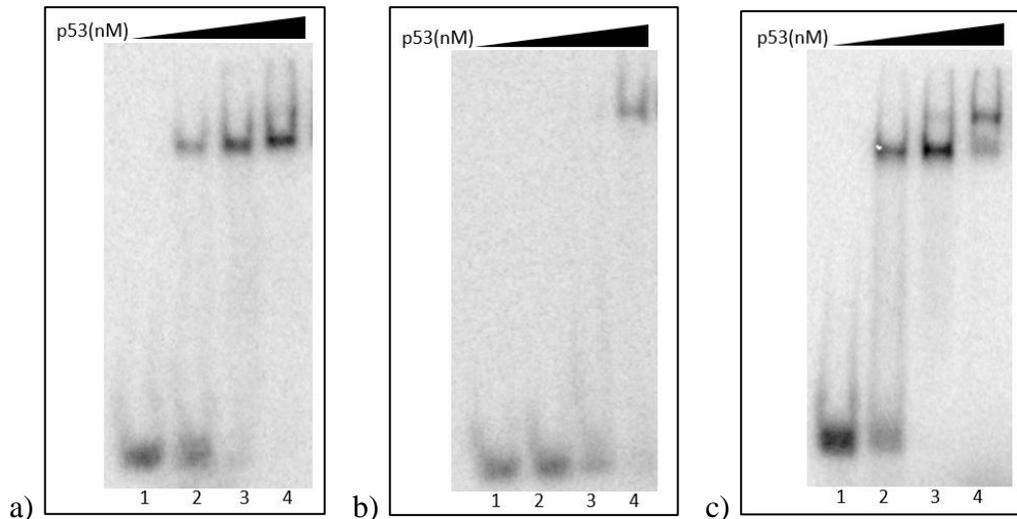
To examine how p53 and HMGB1 interact with DNA together, it was necessary to first perform gel shift assays to determine how each protein binds individually to DNA. A p53 dimer binds a specific 10 base pair half site sequence with the consensus: 5' G/A G/A G/A C [A T] OR [T A] G C/T C/T C/T C/T 3'. For these experiments, I used four different DNAs to study the protein binding, DNA-1, DNA-2, DNA-3, and DNA-4 (Figure 4, sequences listed in methods).



**Figure 4:** Representation of DNAs with each p53 half site shown as a blue rectangle.  
a) DNA-1, 32 bp b) DNA-2, 22 bp c) DNA-3, 60 bp d) DNA-4, 32 bp

DNA-1 is 32 base pairs and is the p53 response element found in the GADD45 promoter. This is a consensus sequence that has been extensively studied and is known to be bound by p53 (Chin et al., 1997). DNA-2 is shorter by 10 bases, as it only contains only one of the half-sites in the response element. I intended to see if the lack of one half site would change the DNA binding pattern of p53, with or without HMGB1. DNA-3 contains both half sites, but they are in the middle of a longer sequence, which was previously used to observe HMGB1-facilitated DNA

binding by p53 (McKinney and Prives, 2002). The first set of gel shift assays I completed looked at p53 binding on each of the DNAs. (Figure 5).

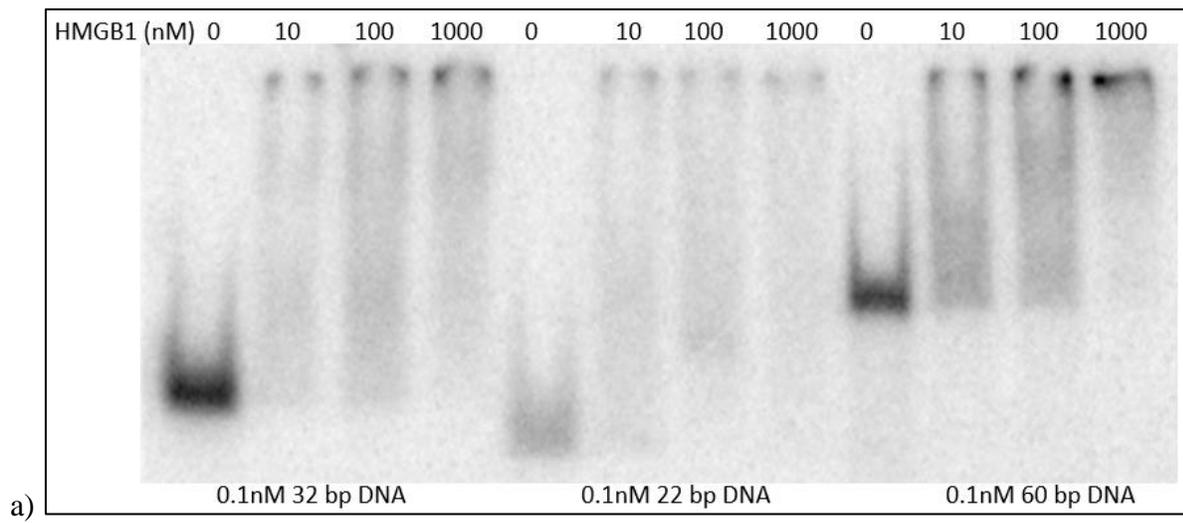


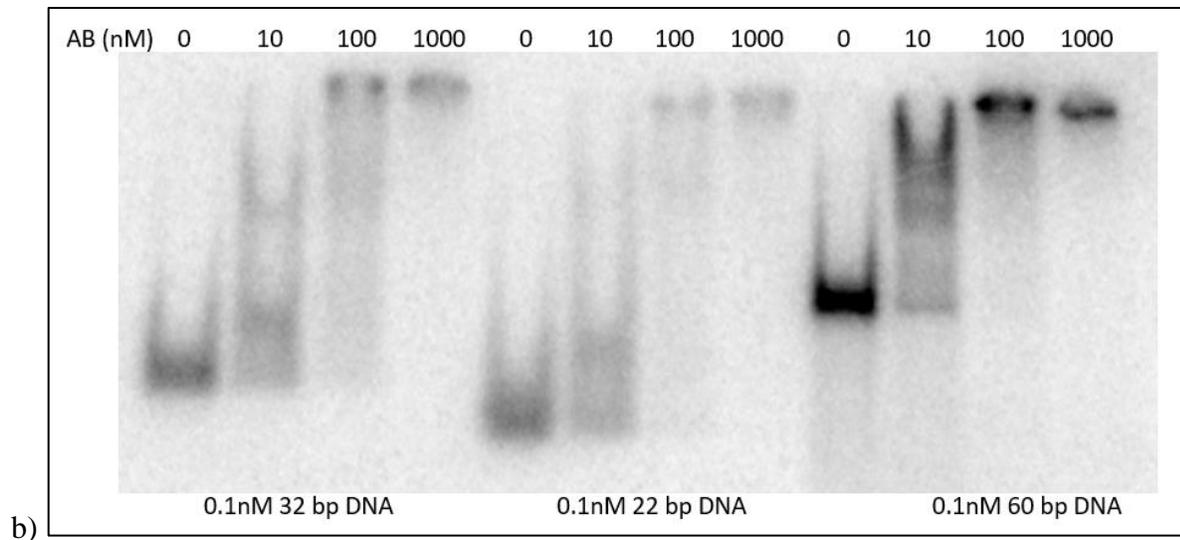
**Figure 5:** p53 bound DNAs 1-3 with various affinity. In all panels the DNA concentration was 0.1nM, no protein was added to lane 1, and the protein concentrations were 0.1nM, 1nM, and 10nM in lanes 2, 3, and 4 respectively. a) P53 on DNA-1, b) P53 on DNA-2, c) P53 on DNA-3. The gels were run at different times so the distance between free and bound DNA cannot be compared between the panels.

p53 bound DNAs 1-3 with different affinities. The first lane in each gel shift assay contained no DNA, and as p53 was titrated in, more protein bound the DNA and the DNA clearly shifted up in the gel. p53 binds to DNA-1 with high affinity, as a shifted band can be seen at the lowest titration point of p53. The apparent  $K_D$  is estimated to be 0.1nM. DNA-2 eventually is bound by p53 at the highest concentration (10nM) but the signal is less intense compared to the other DNAs, with an estimated  $K_D$  of 6nM. This shows that in vitro p53 can bind to DNA containing only one half site. p53 bound more tightly to DNA-3, and binding was observed at the lowest titration point. An estimated  $K_D$  for DNA-3 is 0.1nM, similar to DNA-1. In the last two lanes of the gel shift assays with DNA-3, there are two different bands running in separate places. Some studies suggest that two p53 tetramers can bind DNA simultaneously (Gaglia et al., 2013). This

higher running band might be evidence of an octameric complex that can form when p53 is saturated on a significantly longer piece of DNA.

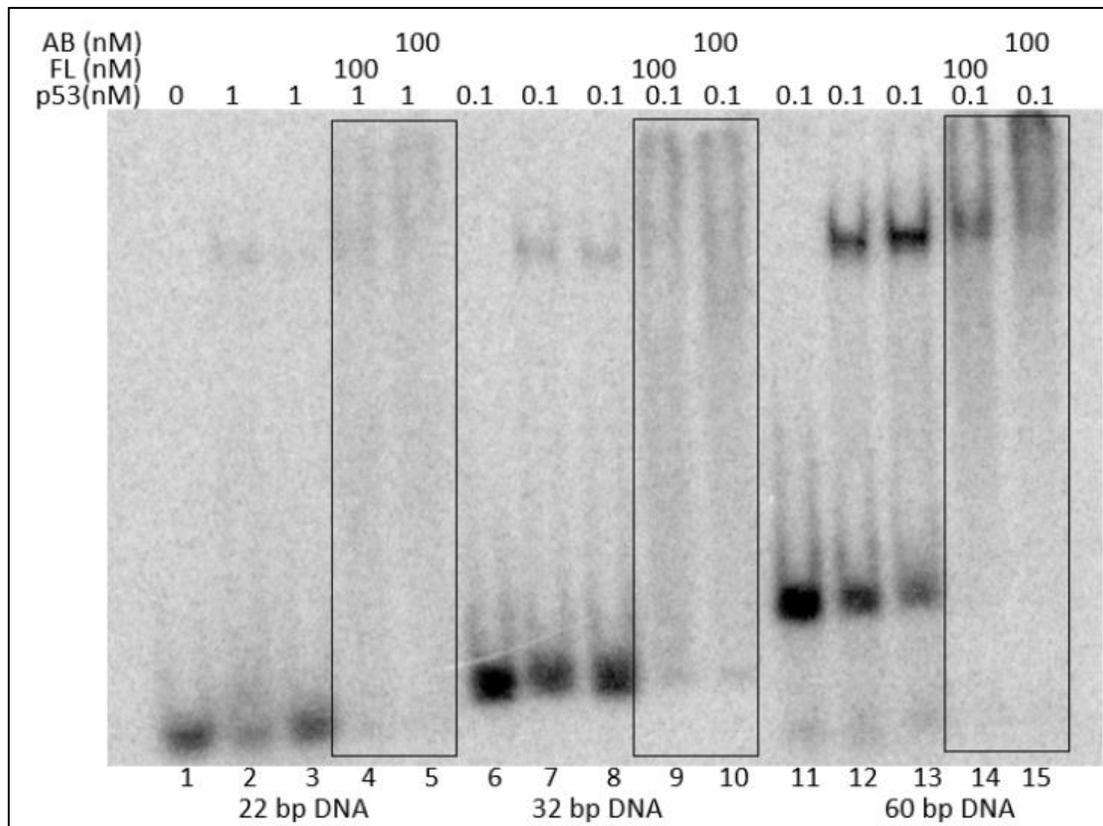
The next step in this process was to look at how HMGB1 binds these same DNAs. Two different forms of this nuclear protein were used, one was a full length HMGB1 protein that was 26kDa, and another was a construct of this protein that was 20kDa because the C-terminal tail had been deleted, leaving only the A box and the B box (AB protein). It has been shown that the acidic tail of HMGB1 can interact with the HMG boxes to interfere with HMGB1 binding and bending DNA (Blair et al., 2013; Lee and Thomas, 2000). I performed two more gel shift assays to show the binding of both full length HMGB1 and the AB construct to DNAs 1-3. One unfortunate characteristic of HMGB1 is that it smears in gel shift assays, resulting in a blurry path down the gel that makes it difficult to distinguish clear protein bound bands. However, the assays still enabled me to conclude the HMGB1 proteins were active for binding to DNA. As shown in Figure 6, while it is difficult to locate a distinct bound band, the lanes with no protein look significantly different than when HMGB1 was added. These assays also show the relative sizes of the different DNAs used, as all three DNAs were run on the same gel.





**Figure 6:** HMGB1 and AB binding to DNA-1, DNA-2, and DNA-3. DNA concentration was 0.1nM and protein concentrations were 10, 100, and 1000nM. HMGB1 smears in gel shifts, resulting in large smudges rather than clear bands. a) Full length HMGB1 with 1nM 32, 22, and 60 base pair DNA (DNA-1, DNA-2, and DNA-3) b) AB construct of HMGB1 with 1nM 32, 22, and 60 base pair DNA (DNA-1, DNA-2, and DNA-3).

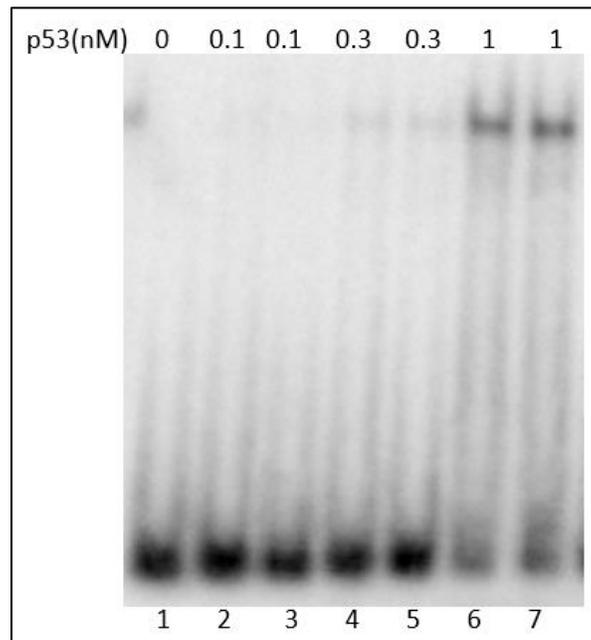
After looking at each protein individually on the three different DNAs, I performed multiple gel shifts looking at the interaction of p53 with these DNAs in the presence HMGB1. To determine if the presence of HMGB1 could increase p53 binding to DNA, I chose concentrations of p53 where very partial binding was observed. Based on my previous gel shifts, I chose to hold the p53 concentration constant at 1nM on DNA-2, and 0.1nM on DNA-1 and DNA-3.



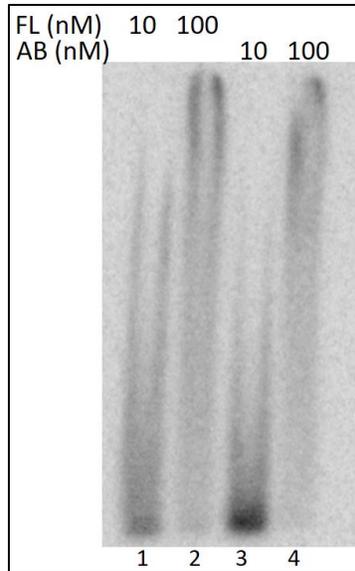
**Figure 7:** HMGB1 does not facilitate p53 binding. In this gel shift with p53, HMGB1, and AB construct on DNA-1, DNA-2, and DNA-3, DNA was held constant at 0.1nM. 1nM p53 was added to lanes 1-5 on DNA-2, and 0.1nM p53 was added to lanes 6-15. 100nM HMGB1 or AB was added to the final lanes to test for facilitated binding.

At low concentrations of p53, where partial binding is observed, the addition of HMGB1 had very little impact on p53 binding (Figure 7). The lanes where HMGB1 was added are highlighted by boxes. In these lanes, the lack of free DNA at the bottom of the gel indicates that the addition of HMGB1 leads to increased DNA binding, but this is likely due to HMGB1 binding the free DNA and smearing, rather than facilitating specific p53 binding. This observation was also seen in control gel shift assays that included HMGB1 alone. Upon closer inspection, it appears that the specific p53/DNA bound band was actually reduced when HMGB1 was added, suggesting perhaps HMGB1 is directly competing with p53 for DNA binding.

After looking at the DNA binding patterns of these two proteins on DNA, a fourth DNA sequence was developed and used (DNA-4, Figure 4d). This sequence was also 32 base pairs, but the second half site sequence was scrambled so that it no longer followed the p53 binding consensus sequences. This new DNA that was thus the same length as the wild type DNA but contained only one half-site. To see if HMGB1 would facilitate p53 binding on DNA-4, it was necessary to perform gel shifts with each protein alone first. Especially with p53, I needed to see the concentration range that p53 would bind to this DNA, to choose the right concentration at which to titrate in HMGB1 to test for potential facilitated binding. Figure 8 shows that p53 bound DNA-4 with an estimated  $K_D$  of 1nM, again indicating that p53 could bind one half site in vitro. Figure 8 shows the binding of HMGB1 on DNA-4, again greatly smearing the DNA up the lanes.

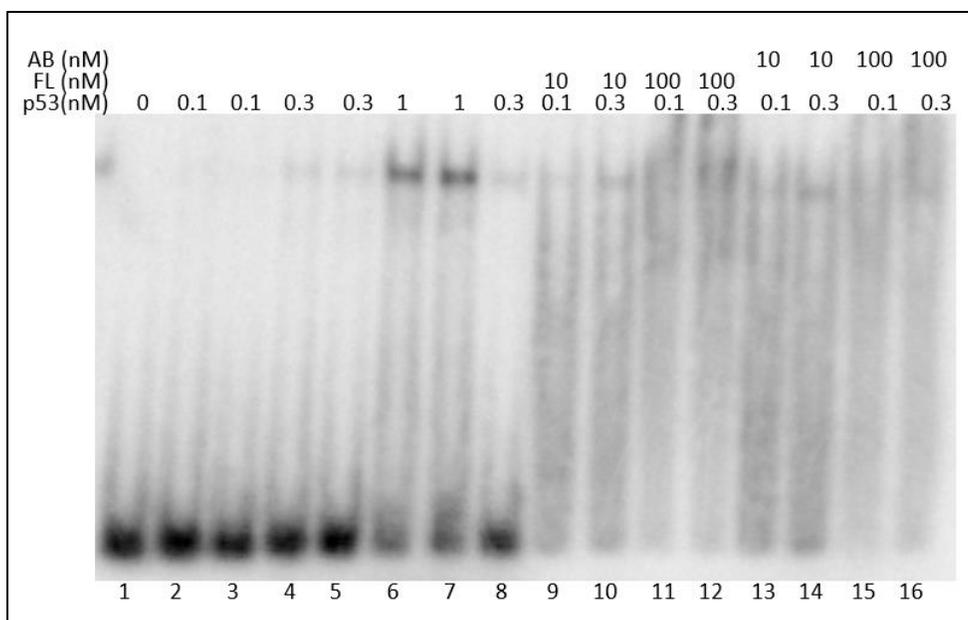


**Figure 8:** WT p53 could bind a DNA with only one half site (DNA-4). DNA concentration was held constant at 0.1nM and p53 was titrated in at concentrations labeled on the gel.



**Figure 9:** HMGB1 and AB can also bind to DNA-4. This gel shift was done at a DNA concentration of 0.1nM

After looking at just p53 binding to DNA-4 I chose two p53 concentrations to use in experiments in which HMGB1 was added: 0.1nM and 0.3 nM, since little to no binding was observed at these concentrations. Much like the gel shifts with DNAs 1-3, the addition of HMGB1 smeared the DNA signal (Figure 10), however, HMGB1 and the AB construct also slightly enhanced the binding of p53. For example, at 0.1nM of p53, there is essentially no binding observed (lanes 2 and 3). After the addition of HMGB1 or AB, there are p53/DNA bands in lanes 9 and 13. This effect is also observed at 0.3 nM p53. Moreover, it appears to be specific to 10 nM HMGB1. At 100 nM HMGB1 most of the DNA is smeared up the gel and specific p53/DNA complexes cannot be observed.



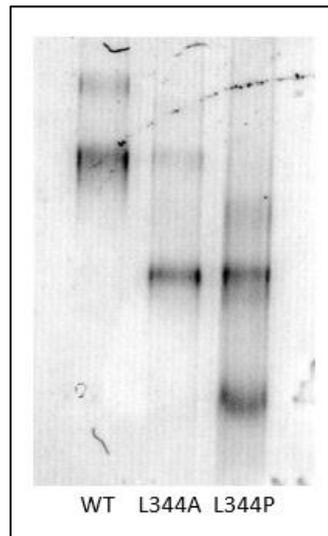
**Figure 10:** HMGB1 facilitates p53 binding on DNA-4. Lanes 9, 10, 13, and 14 show that at low concentrations of p53—0.1 and 0.3nM—HMGB1 can increase p53/DNA binding. DNA was held constant at 0.1nM.

### *Activity of Mutant p53*

To further investigate whether HMGB1 could aid p53 binding, I created two mutant variants of p53. A leucine amino acid located at position 344 is essential to the oligomerization of p53. By mutating that amino acid, it is possible to disrupt the protein from forming higher oligomeric states. Two mutants were created for this experiment; one of them prevented tetramerization and thus formed only dimers, and the other prevented dimerization and formed only monomers (Fischer et al., 2016). The dimeric mutant contained an alanine in place of leucine 344 (L344A) and the monomeric mutant contained a proline in that place (L344P). The mutated p53 gene was cloned into a plasmid containing a His tag, allowing me to use a nickel column to purify the mutant protein.

After purifying the mutant p53's, it was important to run a native gel to confirm their native oligomeric states. The native gel showed that the monomer and dimer mutants and wild

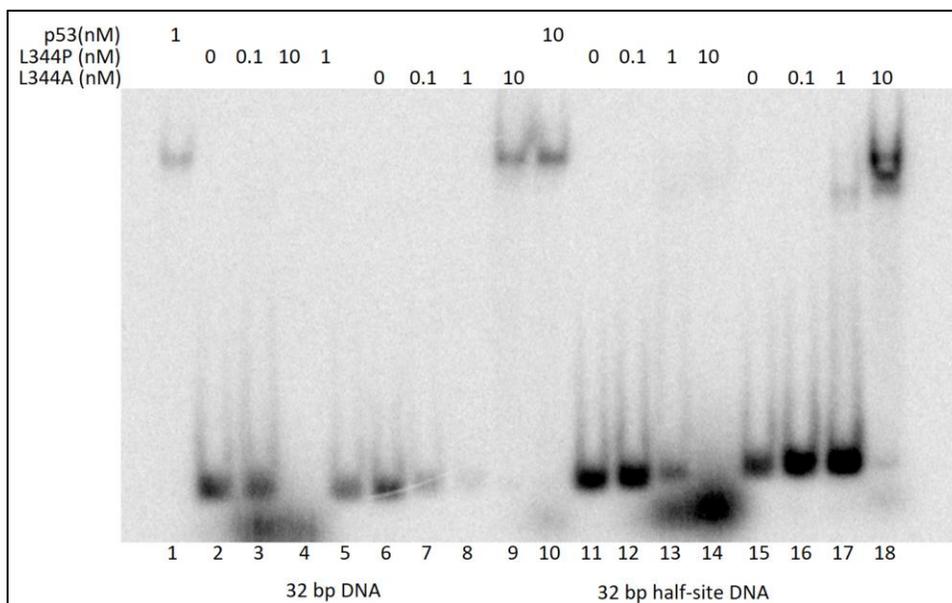
type tetramer all run at distinct locations on the gel. (Figure 11). In each lane, a band running at the next highest oligomeric state appears. The monomeric p53 also has a population of dimers. This dimer population is unlikely to form at the lower concentrations of proteins used in gel shift assays.



**Figure 11:** Wild type and mutant p53 form different oligomeric states. Wild type p53 is primarily tetrameric, L344A p53 forms mainly dimers, and L344P p53 forms mainly monomers, but also shows a population of dimers at this concentration.

After confirming the oligomeric states of the mutants I created, I performed additional gel shift assays to determine their DNA binding patterns. Two different DNAs were used in these assays, DNA-1 with both natural p53 half sites, and DNA-4 with only one half site but the same length as DNA-1. I wanted to examine how the dimeric mutant would interact with DNA containing only one-half site. While some research shows that the dimer can still actively bind DNA, the monomer is believed to be inactive for DNA binding (Fischer et al., 2016). This was confirmed (Figure 12) as the monomeric mutant failed to bind DNA at any concentration (lanes 3-5 and 12-14). There was phosphatase contamination in the L344P p53 preparation, so as the protein was titrated higher, the  $^{32}\text{P}$  label on the DNA was cleaved and lost. The dimeric mutant did bind DNA. On DNA-1 (lanes 1-10), both wild type (lane 1) and L344A shifted DNA to a

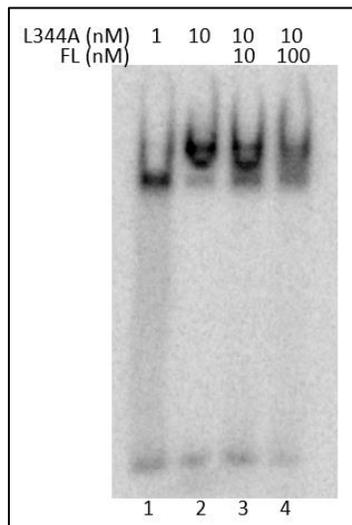
similar position, but 10-fold more L344A was needed to observed binding compared to wild type. For DNA-4, the DNA/L344A band ran lower than the band corresponding to wild type p53/DNA (lane 17 vs. lane 10). However, at a higher concentration (10nM), the DNA/L344A band shifted higher, more similar to that of wild type p53 bound to DNA-4, indicating two different sized populations of DNA/L344A complexes. This suggests that with L344A there is a population of dimer/DNA complexes that are smaller than the wild type tetramer on the DNA. In addition, on DNA-1 L344A bound to DNA runs at the same size as wild-type p53 bound to DNA, which suggests that wild type likely binds as a single tetramer and L344A binds as two individual dimers, one to each half-site.



**Figure 12:** Monomeric mutant is inactive while dimer can still bind DNA. The monomeric mutant fails to bind either DNA-1 or DNA-4 at any concentration. The dimeric mutant binds both DNAs at 10nM, and some binding is observed on DNA-1 at 1nM. Both DNA concentrations were held constant at 0.1nM.

A final gel shift was performed using the dimeric mutant in order to see if HMGB1 could facilitate binding of this mutant p53. HMGB1 was added to the dimeric mutant on DNA-4 to see

if there was increased binding, and what size the DNA/L344A complex was. Binding of a single dimer was observed at 1nM of L344A and potentially binding of two dimers was observed at 10nM. (Figure 13, lanes 1 and 2). Addition of full length HMGB1 appeared to slightly shift the binding pattern back down to favor a single dimer binding to the DNA (Figure 13, lanes 3 and 4). Perhaps HMGB1 enhances the specificity of L344A binding to the proper half site sequence by directly competing against p53 dimers for binding to the non-specific DNA sequence.



**Figure 13:** HMGB1 slightly affects DNA binding by the dimeric p53 mutant on DNA-4. DNA-4 concentration was held constant at 0.1nM.

## Discussion:

Because of p53's role as a tumor suppressor, its binding cooperativity to DNA is important. Mutations in p53, as observed in this project, can impair DNA binding and lead to cancer susceptibility as p53 loses its function to regulate transcription. (Schlereth et al., 2010). This project sought to examine how HMGB1 facilitated the binding of p53 to various sequences of DNA. To test this, I performed gel shift assays using p53, full length HMGB1, an HMGB1-AB construct and multiple different DNAs. Both proteins showed binding to all of the DNAs individually. When HMGB1 and AB were titrated in to low concentrations of p53 where binding was previously unobserved, I was able to conclude that the presence of HMGB1 can enhance p53 binding only on certain DNAs. On DNA-1, DNA-2, and DNA-3, regardless of the number of half sites, the addition of HMGB1 did not facilitate p53 binding. While it was hard to determine estimated  $K_D$  values after HMGB1 was added due to increased smearing of the  $^{32}P$  signal, there were less distinct p53/DNA bands and they seemed no more intense than the p53/DNA bands before addition of HMGB1 or AB. On DNA-4 however, there was increased p53 binding to DNA upon addition of HMGB1. 0.1 and 0.3 nM concentrations of p53 did not show much binding to DNA-4, but in the presence of HMGB1 and AB, distinct p53/DNA bands were observed at those concentrations. This suggests that HMGB1 may be increasing sequence specific binding of p53 to its specific half site. This stipulation may be supported by the fact that HMGB1 seemed to enhance binding of the p53 L344A dimeric mutant on DNA-4 (Figure 13). The addition of 100nM HMGB1 appeared to favor the dimer of p53 binding a single half site.

These results differed from previous publications that showed HMGB1 can facilitate DNA binding by p53. Other groups have seen a significant fold increase in p53 DNA-binding activity upon addition of HMGB1 (Jayaraman et al., 1998; Livesey et al., 2012; McKinney and

Prives, 2002; Tros et al., 2014). The reason(s) their results were not reproducible are not clear, however, there were a few differences in my experiment compared to previous published experiments. First of all, the DNAs I used were slightly different than those previously used. I would be surprised if this was the full reason for different results, but it may have been a contributing factor. Furthermore, in the EMSA gel shift assays performed by other groups, DNA concentration was held constant at 0.15nM and p53 concentration was held constant at 8.5nM. Under these conditions, very little DNA binding by p53 was observed (McKinney and Prives, 2002). This is very unlike my experimental conditions, where DNA binding was observed at p53 conditions as low as 0.1nM. These very low nM  $K_{DS}$  observed in my experiment agree with traditional measurements of p53 binding affinity. Perhaps HMGB1 can activate p53 that isn't very active to begin with, but has less of an effect on active p53. Finally, previous experiments showed that the ability of HMGB1 to facilitate p53 binding changed 10-fold to 50-fold depending on the purification method of HMGB1, showing that variation in protein preparation impacted HMGB1 facilitated p53 DNA binding.

This project leaves a lot of room for future work. More experiments with DNA-4 and both wild-type p53 and L344A could be performed to better characterize the conditions for binding enhancement by HMGB1. Other p53 mutants could be created to determine if removal of the C-terminal tail, for example, also alters p53-DNA binding activity with and without HMGB1 present. It would also be interesting to better characterize how the L344A dimeric p53 binds to different DNA constructs. Furthermore, HMGB1 and p53 could be fluorescently labeled and examined using single molecule microscopy to measure how binding kinetics of wild type, dimeric and monomeric mutant p53 to DNA differ, and the potential role of HMGB1 in each situation.

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