Determination of An Interaction between Nipped B-Like Protein and MLL

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LOYOLA UNIVERSITY CHICAGO

DETERMINATION OF AN INTERACTION
BETWEEN NIPPED B-LIKE PROTEIN AND MLL

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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BY
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To my family
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ABSTRACT

The Mixed Lineage Leukemia (MLL) protein is a large, multi-domain protein that serves as a positive regulator of gene expression during hematopoietic and embryonic development. The MLL gene is associated with chromosomal translocations, which produce MLL fusion proteins that can lead to the onset of myeloid, lymphoid, and mixed-lineage leukemias. Each fusion protein retains the MLL amino-(N-)terminus; within this portion of MLL is the repression domain. A recent yeast two-hybrid screening experiment initiated by our laboratory, which used the repression domain of MLL as bait, yielded nine positive clones of the Nipped B-like (NIPBL) protein.

NIPBL is a crucial member of the cohesin-loading complex. The cohesin complex functions in the segregation of sister chromatids during mitosis and meiosis. However, more recent evidence suggests the cohesin complex machinery can function as a transcriptional regulator through enhancer-promoter looping of genes. An interaction between the MLL repression domain and NIPBL could contribute additional insight on MLL-dependent gene regulation in cells with wild type MLL and in MLL leukemias.

In the present study, we want to confirm this interaction using an independent system. To validate the interaction of NIPBL with the MLL repression domain, GST pull-down assays will be used. Because the NIPBL protein is quite large and is difficult to express, subclones expressing smaller regions were created. NIPBL was divided into
seven distinct regions based on sequence and previous functional information. All seven regions were amplified using PCR and subcloned into an expression vector. Expression of the subcloned domains and full length NIPBL in HEK 293T cells was shown at the protein level through western blotting analysis. To confirm the interaction of NIPBL with the repression domain of MLL, GST-tagged MLL repression domain protein was expressed to perform GST pull-downs with each of the NIPBL subclones. The results of this project have shown and confirmed that NIPBL interacts, through amino acids 450-637, with the repression domain of MLL.
CHAPTER ONE
LITERATURE REVIEW

Mixed Lineage Leukemia

The Mixed Lineage Leukemia (MLL) gene is located on chromosome 11, band q23 and was first identified due to its involvement in chromosomal translocations that can lead to acute myeloid leukemia (AML), acute lymphoid leukemia (ALL) or those with a mixed-lineage phenotype. The chromosomal rearrangements generated cause an in-frame fusion between MLL and over 60 different fusion partners (Figure 1). In all translocations, the amino-terminus of MLL is fused in frame to a partner gene. This results in the formation of a chimeric protein. The leukemias that are caused by the translocations of the MLL gene account for 5-10% of all human acute leukemias. They are the predominant type of leukemia in infants as well as in patients who develop leukemia after treatment with drugs targeted to DNA topoisomerase II. In comparison with other leukemias, the MLL leukemias are often more aggressive and have a worse prognosis.

Wild type MLL belongs to the Trithorax (trx) group of proteins based on functional properties. These proteins are crucially involved in the positive regulation of the homeobox (HOX) genes during embryonic and hematopoietic development. Several
key domains are conserved between MLL and the *Drosophila* trx protein. MLL is a large, multi-domain protein (432 kD) that is present within the nucleus\(^7\). The wild type MLL protein contains several functional domains within the carboxyl terminus of the protein. Included in this region are the plant homeodomain (PHD) fingers and the SET domain, both of which are conserved with trx. The PHD fingers function in protein-protein interactions, including with post-translationally modified histone 3 (H3) tail\(^7\). The SET domain has enzymatic histone methyltransferase activity, which is responsible for trimethylation of H3K4\(^8,9\). However, when the chromosomal rearrangements cause the generation of MLL fusion proteins, only the N-terminal regions of MLL remain intact. Within the N-terminus are the AT-hooks, which bind to AT-rich, nucleosomal, and bent DNA. Also within this region is the repression domain (RD)\(^10\).

The repression domain, initially coined for its ability to repress transcription of certain genes, is now associated with transcription activation and repression as well as protein-protein interactions\(^10\). The repression domain has been shown to interact with histone deacetylases (HDAC1) and other co-repressor proteins, including CtBP and polycomb proteins HPC2 and BMI1\(^11\). Within the repression domain is a CXXC domain that binds to non-methylated CpG DNA sequences. The domain is named for the conserved cysteine residues within the region; eight cysteine residues coordinate two zinc atoms. The CXXC domain structure determines specific non-methyl-CpG DNA binding activity through electrostatic and hydrogen bonding interactions\(^12\). The CXXC domain and its DNA-binding ability have been shown to be essential for MLL translocations to generate acute leukemias\(^12,13\). The repression domain is present in both wild type and
Wild type MLL and an MLL fusion protein produced after chromosomal translocation. Wild type MLL contains various regions including: AT hooks (DNA binding), nuclear localization sequences (transport), repression domain (DNA and protein interactions), PHD fingers (protein-protein interactions), and SET domain (enzymatic histone methyltransferase). The MLL fusion protein retains some critical functional regions, including the repression domain.
MLL fusion proteins (Figure 1), thus understanding the function of this domain would be relevant to both normal and aberrant MLL proteins.

The CXXC domain of MLL (MLL1) is homologous to the CXXC domains of both MLL2 (MLL4) and DNMT1. The MLL2 protein, much like MLL, contains AT-hooks, PHD fingers, and CXXC and SET domains\textsuperscript{14}. The presence of these similar functional domains and amino acid sequence of these two proteins would suggest they have similar enzymatic and regulatory functions within the cell. However, through domain swapping of the MLL2 CXXC domain within the MLL CXXC of a MLL fusion protein, the protein is unable to immortalize bone marrow progenitor cell\textsuperscript{15,16}. DNMT1 CXXC domain, though, is able to confer transforming ability when swapped into a MLL fusion protein\textsuperscript{17}.

These studies have illustrated that the CXXC domain and the larger repression domain of MLL are important regions in the normal and leukemia-causing functions of MLL. There may be additional critical functional protein-protein interactions with MLL that have yet to be determined. The repression domain is essential to in the function of wild type and MLL fusion proteins and should be studied further\textsuperscript{11}.

**Yeast Two-Hybrid Screening for Protein-Protein Interactions**

The interactions between proteins are vital to a cell. Cellular processes including cell adhesion, transcription and translation, and antigen recognition are dependent on protein-protein interactions. The yeast two-hybrid screen is one molecular and biochemical technique that has been used to study these protein-protein interactions\textsuperscript{18}. The basis behind the screen is the functions of transcription factors of yeast. Transcription factor DNA binding and activation domains can be expressed separately,
and when they are brought together in the yeast cell through protein-protein interaction, can generate a signal through activated gene expression. This idea has been modified into an unbiased technique to identify novel protein-protein interactions. However, yeast two-hybrid screens have yet to differentiate between a direct or indirect interaction between the two resulting proteins.

While several variations of the yeast two-hybrid screen exist, all are designed to allow for the successful identifications of protein interactions. The simplest of examples utilizes the yeast Gal4 transcriptional activator. Gal4 contains both a DNA-binding domain and a transcriptional activation domain. Both domains can exist as separate proteins, but must still be in proximity to allow for expression of the genes necessary for survival of the yeast cells. This model allows for the generation of chimeric proteins bound to either of the two domains within yeast cells that are starved of nutrients. If the two proteins of interest interact with one another, then the Gal4 domains will be in close enough proximity to allow for transcription of target genes to allow for survival under specific experimental conditions. The expression of the required genes acts as the reporting mechanism for the yeast two-hybrid screen since only the cells with an interaction will survive.

This experimental system has been modified over thirty years to improve specificity. LexA and Tet-R modifications allow for stricter selection of positive interactions. cDNA libraries have been created to scan through large numbers of proteins in a single screening. The yeast two-hybrid is quite a powerful tool, but caveats of the technique must be taken into consideration. The screen identifies novel interactions between two proteins within a yeast cell in an impartial selection process. However, to
properly identify the interaction as *bona fide*, it must be re-confirmed in a different system through another independent method.

Our laboratory recently initiated a yeast two-hybrid screen that used the repression domain of MLL as bait. The screen yielded positive-interacting results for two different proteins: NIPBL (nine clones) and the ribosomal protein S18 (RPS18) (one clone).

**Cohesin Complex**

The cohesin complex is an integral group of proteins that allow for proper cell functioning and survival\(^{22}\). Cohesin interacts with chromosomal and naked DNA. Four proteins make up the cohesin machinery—the structural maintenance of chromosomes (SMC) proteins 1 and 3 (Smc1 and Smc3), Rad21, and Stag2\(^{23}\). The basis of the cohesin complex functioning is the ATPase activity of Smc1 and Smc3. The ATPase regions of Smc1 and Smc3 are located in the head domains of each protein. The SMC proteins form a ring-like structure around DNA through a bridged interaction with Rad21. Stag2 interacts with the carboxyl-terminus of Rad21 within the protein complex (Figure 2; center)\(^{24,25}\).

In addition to the four core proteins, two additional proteins are crucial for function of the complex. Nipped B-like (NIPBL) and Mau2 form a separate heterodimer that interact with both DNA and the SMC complex (Figure 2; center). Nipped B-like is the ortholog of Drosophila *Nipped-B*. *Nipped-B* facilitates the activation of Drosophila *cut* and *Ultrabithorax* homeobox genes through the interactions of distant enhancer regions\(^{35}\). Mau2 is a homolog of the *C. elegans* *mau-2*, which has been shown to have a role in mitotic segregation\(^{31}\). This dimer of NIPBL and Mau2 are known as the cohesin-
loading complex; they function to load the cohesion ring structure around DNA\textsuperscript{26}. Coupled together, the activated ATPase domains of the Smc proteins interact with the NIPBL/Mau2-loading complex to allow for proper functioning of the complex\textsuperscript{27}.

For many years it has been known that the cohesin complex functions in three main processes within the cell (Figure 2; left panel). After DNA replication, sister chromatids are attached to each other prior to their separation during mitosis and meiosis. The cohesin complex allows for the cohesion of the sister chromatids. The complex also facilitates the spindle attachment to the chromosomes as well as the segregation and condensation of the chromosomes. Lastly, cohesin plays a role in DNA repair through the recombination mechanism\textsuperscript{22,28}.
The cohesin complex: Smc proteins 1 and 3, Rad21, and SA/Stag2. Cohesin executes several canonical functions and has more recently been shown to be involved with additional gene regulatory processes with the aid of its loading complex (NIPBL and Mau2). Modified from Dorsett 2011.
Cornelia de Lange syndrome (CdLS) is a rare heterogeneous developmental disorder. This multisystem congenital anomaly is characterized by patients that have a variety of defects, including upper limb reduction, craniofacial dysmorphia, cardiac and gastrointestinal abnormalities, mental retardation, and pre- and post-natal growth delay. The prevalence of CdLS is estimated between 1/10,000 and 1/50,000 births; however, due to the variable types of abnormalities, this could be an underestimate. In 2004, the genetic basis of CdLS was discovered. Mutations in three genes have been reported to cause CdLS in humans. Mutations in Smc1a and Smc3 genes account for 5% of the patients with CdLS. These two genes code for subunits of the cohesin complex. However, approximately 60% of CdLS patients have mutations in the NIPBL gene. The phenotype of the syndrome is often dependent on which gene is mutated—patients with Smc1a/3 mutations have mild structural problems with severe mental retardation, while patients NIPBL mutations have severe organ and limb dysmorphia with mild cognitive effects.

Since the genetic foundation of CdLS was uncovered, NIPBL has been the focus of many studies. The Nipped B-like (NIPBL) gene codes for the NIPBL protein, which is an ortholog of the Drosophila Nipped B, identified as a transcription factor, and yeast Scc2, a component of the cohesin complex. Much like in yeast, human NIPBL protein was shown to be an active member of the cohesin complex that regulates processes involving sister chromatids. The NIPBL protein is one half of the cohesin-loading complex along with Mau2 (Figure 2; center). NIPBL interacts with Mau2 and Rad21.
Full-length Nipped B-like protein is 2804 residues and contains many domains. The glutamine-rich region (418-462) as well as the domain (450-637) that was shown through yeast two-hybrid screen to interact with MLL are located in the N-terminus. Seven, NIPBL-specific PETPK amino acid repeats are located in residues 597-759. A caldesmon domain was shown through NIPBL residues 489-1025. Also contained within the protein are nuclear localization and export signals as well as PEST sequences, which are important in proteosomal degradation. Located in the C-terminal portion of the protein are five, HEAT domain repeats, which are important in protein-protein interactions. Modified from Wierzba et al/2006.
within the cohesin complex to allow for the Smc proteins to encircle chromosomal DNA\textsuperscript{25,36}.

The NIPBL protein is approximately 2800 amino acids in length. The three-dimensional structure has yet to be solved; however, several specific sequences and domains have been identified (Figure 3)\textsuperscript{29}. Beginning with the amino-terminus, NIPBL contains a glutamine (Gln)-rich region (aa 418-462), which has fourteen Gln residues in that stretch. Glutamine repeats have been shown to be integral in protein-protein interactions between co-activators. Also within the N-terminus is the domain (residues 450-637) that was identified through the yeast two-hybrid screen to interact with the repression domain of MLL. A previously shown caldesmon domain, which is important in binding interactions with calmodulin and actin proteins, is located in residues 489-1025. Residues 597-759 include seven NIPBL-specific amino acid repeats with the sequence PETPK. No function has yet been assigned to these repeats. NIPBL also includes bipartite nuclear localization and export signals. In addition, the protein contains five HEAT-domain repeats near the carboxyl-terminus of the protein\textsuperscript{37}. These HEAT domains also function in protein-protein interactions\textsuperscript{38}. Two PEST sequences, which are important in proteosomal degradation of proteins, are found in at residues 1149-1170 and 2627-2646. Through the analyses of CdLS patients, mutations have also been identified that span most of the NIPBL protein\textsuperscript{29}.

CdLS is caused by heterozygous loss-of-function mutations of NIPBL. The mutations reduce expression of NIPBL by approximately 30%, and a 15% decrease in expression is sufficient to cause CdLS\textsuperscript{37}. The diminished amount of NIPBL does not cause any effects on the cohesin complex or chromosomal segregation during mitosis and
meiosis\textsuperscript{38}. However, the gene expression profiles of heterozygous mutant Drosophila, yeast, mouse, and humans all show changes. The lack of effects on mitotic functions of the cohesin complex with the NIPBL mutations argues that CdLS is caused by a different mechanism—perhaps changes in gene expression\textsuperscript{25}. 
Figure 4: Gene Regulation Mechanisms of Cohesin and NIPBL

A) Cohesin complex interacts with CCCTC-binding factor (CTCF) leading to looping and sequestering of enhancer region from promoter, which causes inhibition of the target gene expression. B) Cohesin and NIPBL loop the enhancer towards the transcriptional start site where they can interact with RNA polymerase and the Mediator (a large, multi-subunit transcriptional activator) complex to lead to increased gene expression. Modified from Dorsett 2011.
Recent experiments support a model whereby NIPBL and cohesin act to regulate gene expression through the looping of DNA (Figure 2; right panel). Two hypothetical modes have been proposed for the effect of NIPBL and cohesin on gene regulation because experiments have shown both increased and decreased gene expression due to these proteins. The inhibitory mechanism has been shown to occur at several genes (Figure 4A). Through gene expression analysis and the technique Chromosome Conformation Capture (3C), the NIPBL-dependent cohesin complex has been shown to mediate DNA looping at the homeobox gene A locus and at the β-globin locus. At both loci, NIPBL and cohesin interact with the CCCTC-binding factor (CTCF; Figure 4A) at insulating elements to cause the inhibition of expression.

As for the activating mechanism, in a recent study, cohesin, Nipped B-like, and the mediator complex were shown to mediate DNA looping at genes with positive changes in expression. Again, 3C was used to determine looping between enhancer and promoter regions of Nanog, Phc1, Oct4, and Lefty1 genes. These genes are expressed in embryonic stem (ES) cells, but not in mouse embryonic fibroblasts (MEFs). It was shown that there is an increased frequency of looping between enhancer and promoter regions in ES cells over MEFs. Also, ChIP-seq was used to show that cohesin, mediator and Nipped B-like occupy the enhancer and promoter regions as well. Other such studies have shown positive regulation of other genes (c-Myc) by a Nipped B-like-dependent cohesin complex. Put together, these studies form the basis of a hypothetical mechanism through which the cohesin complex acts with the Mediator complex, a large, multi-subunit protein transcriptional regulator, and RNA polymerase to regulate gene expression through a DNA looping mechanism (Figure 4B).
CHAPTER TWO
METHODS

Yeast Two-Hybrid Screen

The repression domain of MLL (amino acids 1101-1433; sequence from NCBI L04284) was sent as bait for a yeast two-hybrid screening project to identify possible interactions with other proteins. The yeast two-hybrid screen was performed by ProteinLinks, Inc. (Pasadena, CA; www.proteinlinks.com). The screening was run against a human fetal brain library using the TetR yeast two-hybrid approach. A total of $2.6 \times 10^6$ cDNA clones were screened with the bait MLL resulting in 10 verified interacting clones.

Verification of full length Nipped B-Like

From the results of the yeast two-hybrid screen, the Nipped B-like protein (NIPBL) was a viable target to pursue. pFLAG-N3 NIPBL and pEGFP-N3 NIPBL plasmids were obtained from Frank J. Kaiser, PhD (University of Lubeck, Lubeck, Germany). The Kaiser lab had assembled full length NIPBL cDNA sequence and cloned it into these two different plasmids, but had not attempted western blot to determine protein expression (Kaiser, unpublished data). The plasmid DNA from each
construct was electroporated into DH5α electrocompetent cells using 1 µL (125 ng) of the plasmid DNA and 20 µL bacterial cells using the following electroporator settings: 4 kΩ resistance, 330 µF capacitance (Cell-Porator; Life Technologies, Inc.). The transformants were miniprepped using the GeneJET Plasmid Miniprep Kit (Fermentas, Catalog #K0503). The miniprepped plasmid DNA from each construct was digested with the restriction enzyme BamHI (New England BioLabs, Catalog #R0136) to linearize the plasmid DNA. The reactions included 5 µL ddH₂O, 1 µL (200 units) BamHI, 1 µL BamHI buffer, and 3 µL (375 ng) plasmid DNA and were incubated at 37°C for 2 hours. The resulting digested DNA was electrophoresed on a 1% agarose gel and compared with uncut control plasmid DNA to confirm the linearization of each plasmid.

Cloning of Nipped B-like Subdomains

In order to generate clones of the subdomains (Table 1) of Nipped B-like (NM_015384.4), the following PCR reaction was set up for each subclone: 31 µL ddH₂O, 10 µL 5X HF buffer, 5 µL dNTPs, 1 µL (125 ng) pFLAG-N3 NIPBL plasmid DNA, 1.25 µL (2 ng) Forward Primer (Table 2; Integrated DNA Technologies), 1.25 µL (2 ng) Reverse Primer (Table 2; Integrated DNA Technologies), 0.5 µL (2 units) Phusion DNA Polymerase (New England BioLabs, Catalog #M0530). Additional non-NIPBL sequence was added to the PCR primers to facilitate in-frame cloning in the vector (Table 2). The PCR reactions were run in the PCR Express Thermal Cycler (Hybaid) according to the following settings: one cycle of 95°C for 2 minutes; 40 cycles of 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 2.5 minutes; and 1 cycle of 72°C for 10 minutes with a holding temperature of 4°C.
Table 1: Nipped B-like subcloned coding regions and specifications

<table>
<thead>
<tr>
<th>Subclone Region (amino acid #)</th>
<th>Nucleotide Sequence (nucleotide #)</th>
<th>Restriction Enzyme Sites</th>
<th>Total Amino Acid</th>
<th>Estimated Size (kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-450</td>
<td>500-1850</td>
<td>SalI, BamHI</td>
<td>450</td>
<td>49</td>
</tr>
<tr>
<td>418-462</td>
<td>1754-1886</td>
<td>EcoRI, BamHI</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>450-637</td>
<td>1850-2411</td>
<td>EcoRI, BamHI</td>
<td>187</td>
<td>20</td>
</tr>
<tr>
<td>590-800</td>
<td>2270-2900</td>
<td>EcoRI, BamHI</td>
<td>210</td>
<td>24</td>
</tr>
<tr>
<td>800-1700</td>
<td>2900-5600</td>
<td>EcoRI, BamHI</td>
<td>900</td>
<td>101</td>
</tr>
<tr>
<td>1700-2400</td>
<td>5600-7700</td>
<td>EcoRV, BamHI</td>
<td>700</td>
<td>80</td>
</tr>
<tr>
<td>2400-2597</td>
<td>7700-8291</td>
<td>EcoRI, BamHI</td>
<td>197</td>
<td>22</td>
</tr>
</tbody>
</table>

The amplified DNA was cleaned using the QIAEX II PCR Clean-Up Kit following the manufacturer’s suggested protocol (Qiagen, Catalog #200051). The insert fragment DNA was digested with a specific set of two restriction enzymes (Table 1; SalI, 60 units, New England BioLabs, Catalog #R0138; EcoRI, 60 units, New England Biolabs, Catalog #R0101; EcoRV, 45 units, New England BioLabs, Catalog #R0195). The reactions involving EcoRI/BamHI and EcoRV/BamHI restriction enzymes were simultaneously incubated at 37°C for 2 hours. The SalI/BamHI combination was sequentially digested—SalI digestion occurred the night before. The following morning an additional 20 units of SalI was added with BamHI to the reaction mixture. Each insert fragment was then electrophoresed and subsequently cut from a 1% agarose gel. The DNA was purified using the QIAEX II Gel Extraction Kit (Qiagen, Catalog #20051), according to the manufacturer’s protocol. The insert DNA was stored at -20°C until further use.

The expression vector that the subcloned regions would be inserted into is pFLAG-CMV2 to generate FLAG-tagged proteins. To allow for the subcloned
constructs to be properly inserted into the vector, pFLAG-CMV2, was digested with the identical combinations of restriction enzymes as the matching inserts. The resulting digested vector was gel extracted using the QIAEX II Gel Extraction Kit.

The gel extracted inserts and vector were then ligated together under the following protocol: 5 µL (1.5 µg) DNA, 1 µL (0.5 µg) vector, 1.5 µL 10X T4 Buffer, 1 µL (400 units) T4 DNA Ligase (New England Biolabs, Catalog #M0202L), and 7.5 µL ddH2O. The reactions were left in a 16°C water bath overnight. The ligations were electroporated into DH5α electrocompetent cells using 1 µL DNA and 20 µL bacteria cells. The transformants were plated and selected on LB/ampicillin plates. The selected colonies were miniprepped using the GeneJET Plasmid Miniprep Kit. The plasmid DNA from each subclone was sent for sequencing at ACGT, Inc (Wheeling, IL). The resulting sequences were verified using NCBI Blast. Once verified, the correct clones were midiprepped (Qiagen Plasmid Midi Kit, Catalog #12145) and the resulting DNA was saved for future use.

Table 2: Primers for amplifying NIPBL subdomain regions for cloning

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primer Sets</th>
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<tbody>
<tr>
<td>NIPBL 1-450</td>
<td>Forward: NIP1SalIF 5’aaacgATGAGTATGGGGATATG3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: NIP450BamR 5’ccGATCTCTCACAGAAGTCTGG3’</td>
</tr>
<tr>
<td>NIPBL 418-462</td>
<td>Forward: NIP418RIF 5’aaGAATTCTGAATTTTATGCAGC3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: NIP462BamR 5’ccGATCTCTACTGTGTGATATCG3’</td>
</tr>
<tr>
<td>NIPBL 450-637</td>
<td>Forward: NIP452RIF 5’aaGAATTCTGTGGTACAGAATCAAC3’</td>
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<tr>
<td></td>
<td>Reverse: NIP637BamR 5’ccGATCTCTACAGAAGTCTGG3’</td>
</tr>
<tr>
<td>NIPBL 590-800</td>
<td>Forward: NIP590RIF 5’aaGAATTCTAAACACCCAGAAGAAAAC3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: NIP800BamR 5’ccGATCTCTAGCCTAGCTTCATCC3’</td>
</tr>
<tr>
<td>NIPBL 800-1700</td>
<td>Forward: NIP800RIF 5’aaGAATTCTAGCAATTTGAGAAG3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: NIP1700BamR 5’ccGATCTCTACAGAAGTCTCCAT3’</td>
</tr>
<tr>
<td>NIPBL 1700-2400</td>
<td>Forward: NIP1700RIF 5’aaGAATTCTGATGGAAACACCTC3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: NIP2400BamR 5’ccGATCTCTAGCCTAGCTTCAT3’</td>
</tr>
<tr>
<td>NIPBL 2400-2597</td>
<td>Forward: NIP2400RIF 5’aaGAATTCTGGAAACCCGCAAC3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: NIP2597BamR 5’ccGATCTCTACAGAAGTCTCCAT3’</td>
</tr>
</tbody>
</table>

In the above table, restriction enzyme sites are displayed as bolded text. The stop codons are highlighted in red, and the additional non-Nipped B-like bases of the oligos are lowercased. The plain, capitalized text is the coding sequence of Nipped B-like.
Cell Culture

All cell culture work was performed using the Human embryonic kidney cell line, HEK 293T. The HEK 293T cells were cultured in DMEM (HyClone), 10% FBS, 1% penicillin/streptomycin in a 37°C incubator with 5% carbon dioxide.

For the transfections of subclones, 2.4x10^6 HEK 293T cells were plated on 10cm plates with 10 mL of culture media. Each plate was transfected 24 hours later with 25 µg of plasmid DNA (pFLAG-CMV2 NIPBL, all seven subclones, pcDNA HDAC1 FLAG positive control, and untransfected HEK 293T negative control). The CalPhos Mammalian Transfection Kit (Clontech, Catalog #631312) was used for the transfections. Fresh media was added 24 hours post-transfection. After 48 hours post-transfection, the cells were counted and stored in 1.5mL microcentrifuge tubes at a concentration of 5x10^6 cells per tube. The cell pellets were snap-frozen and stored at -80°C.

Protein Extraction

The 5x10^6 cell aliquots were thawed on ice and combined with 500 µL cold IPH buffer (50 nM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1:100 Protease Inhibitor cocktail (Sigma, Catalog #P8340)) by gentling pipetting the mixture in a 1.5 mL microcentrifuge tube. The cells were incubated on ice for 10 minutes. The tubes were centrifuged at maximum speed (14,000 rpm) for 30 minutes at 4°C. The supernatant, which contained the expressed protein constructs, was removed and snap-frozen in 450 µL and 50 µL aliquots. The aliquots of FLAG-tagged proteins were stored at -80°C for future use.
Western Blot Analysis

SDS-polyacrylamide gels with 12%-resolving (4.4 mL ddH2O, 3.0 mL 40% acrylamide, 2.5 mL Tris pH 8.8, 0.1 mL 10% SDS, 50 µL 10% APS, 10 µL TEMED) and 4%-stacking (7.68 mL ddH2O, 0.975 mL 40% acrylamide, 1.25 mL Tris pH 6.8, 0.1 mL 10% SDS, 50 µL 10% APS, 10 µL TEMED) were poured with 10-well combs. The 50 µL protein samples were prepared by adding 10 µL of 6X SDS loading buffer (2.5 mL 1M Tris pH 6.8, 1 g SDS, 0.05 g Bromophenol Blue, 5 mL glycerol, 2.5 mL ddH2O, 500 mM DTT). The samples were boiled at 95°C for 5 minutes and then loaded into the SDS-PAGE gels. Precision Plus Protein Kaleidoscope Standard (10 µL; Bio-Rad, Catalog #161-0375) was used as the MW ladder. The gels were placed in 1X Running Buffer (15.1 g Tris base, 94 g Glycine, 50 mL 10% SDS, filled to 1 L with ddH2O) and electrophoresed at 150 V for 1 hour.

Immobilon PVDF Transfer Membranes (Millipore, Catalog #IPVH00010) were soaked in methanol for 10 seconds and then washed in ddH2O. The gels were transferred to the membranes in the BioRad Electro-Eluter at 70 V for 2.5 hours using 1X Transfer Buffer (2.42 g Tris base, 11.26 g Glycine, 100 mL methanol, fill to 1L with ddH2O).

Once transferred, the membranes were placed in 50 mL of blocking solution (PBS-0.05% Tween with 5% milk w/v; (Jewel Instant Nonfat Dry Milk)) overnight in the 4°C cold room while on the rocker (Boekel Orbitron Rotator II). The membranes were then incubated with primary antibody (Sigma-Aldrich Monoclonal anti-FLAG M2, Catalog #080M6034, in 8 mL of blocking solution; 1:3000 dilution) at room temperature for 2 hours in the glass rotator (AutoBlot, BellCo Glass, Inc.). Following the incubation, the membranes were washed three times for 5 minutes each with 50 mL of PBS-Tween
on the BellyDancer (Stovall Life Sciences, Inc.). The membranes were incubated with secondary antibody (Amersham ECL anti-mouse IgG HRP, Catalog #NA931, in 8 mL of blocking solution; 1:8000 dilution) for two hours at room temperature in the rotator. The membranes were washed three times with 150 mL of PBS-Tween on the BellyDancer.

To develop the membranes, Pierce ECL Western Blot Substrate (Catalog #32106) was added to the membranes. The solution was left on the membranes for 1 minute. The excess ECL solution was drained, and the membranes were wrapped in plastic wrap. The membranes were then placed in the x-ray film holder (Fisher Biotech Autoradiography Cassette). An x-ray film sheet (Hyblot CL Autoradiography Film, Catalog #E3012) was placed on the membrane in the dark room for 15 minutes. After that time duration, the film was placed in the Alpha Tek Developer (Model #AX390 SE) and allowed to develop. The film was marked for the prestained Kaleidoscope ladder following the development. The membranes were peeled from the plastic wrap, placed in PBS-Tween, and stored in the 4°C refrigerator for future blotting analyses.

**Immunoprecipitation Assay**

The anti-FLAG M2 beads (40 µL; Sigma-Aldrich anti-FLAG M2-agarose Catalog #045K6065) were pre-blocked with 200 µL of Z-Buffer (25 mM HEPES, pH 7.5, 12.5 mM MgCl₂, 150 mM KCl, 0.1% NP-40, 20% glycerol, final concentration 1 mg/mL BSA, 1:10 Protease Inhibitor cocktail) and left to incubate for 15 minutes on ice. The mixture was centrifuged at 1,500 rpm for 30 seconds. The supernatant was removed. The 450 µL aliquots of FLAG-tagged proteins were thawed and added to the pre-blocked anti-FLAG M2 beads. The mixture was placed on the circular rotator (Glas-Col) in the cold room overnight.
The following day, the mixture was centrifuged at 1,500 rpm for 30 seconds and the supernatant was removed. The beads were then washed four times, each with 1 mL of NETN Buffer (100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 20 mM Tris-HCl, pH 8.0, 1:10 Protease Inhibitor cocktail). After the final removal of supernatant, the beads were resuspended in 40 µL of 1X SDS loading buffer. The samples were boiled for 5 minutes at 95°C and centrifuged at 1,500 rpm for 1 minute. The supernatant from each sample was loaded into a 12%-resolving, 4%-stacking SDS-PAGE gel. The previously mentioned western blotting protocol was used thereafter (see above).

**Generation of GST fusion proteins**

GST-fusion MLL repression domain (pGEX-KT MLL RD; glycerol stock #55) and GST alone (pGEX-KT empty vector; glycerol stock #40) were streaked out on LB/ampicillin plates and incubated at 37°C overnight. From the plates, a single colony was picked and grown in 25 mL of LB media overnight. The cultures were then expanded into 1 L of LB media for 4 hours. To induce protein production, 1 mL of 100 mM IPTG was added, and the cultures were allowed to grow for 3 more hours. The cells were centrifuged at 5800 rpm for 15 minutes and then resuspended in 20 mL of Lysis Buffer (500 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, Protease Inhibitor cocktail 1:1000, 30 mg Lysozyme, 3 mL Triton).

The cultures were incubated on ice for 5 min with frequent inversion. Following the incubation, the cell lysates were sonicated twice for 20 seconds (setting 5). The lysates were centrifuged at 9000 rpm for 30 min at 4°C. GST beads (45 mg; Sigma-Aldrich, Catalog #G4510) were swelled the previous night in 10 mL ddH2O. The lysates were combined with GST beads and incubated on the rotator at 4°C overnight. The
following day, the beads were washed four times with 1 mL of Lysis Buffer. The beads were resuspended in a 50% bead/volume ratio and stored at 4°C for future use.

To determine expression of the GST-fusion proteins, Coomassie staining was used. The GST-fusion MLL repression domain (40 µL of 50% slurry) and GST-alone (10 µL of 50% slurry) beads were centrifuged at 1,500 rpm for 30 seconds. The supernatant was removed, and the beads were resuspended in 40 µL of 1X SDS loading buffer. The samples were boiled for 5 minutes at 95°C and centrifuged at 1,500 rpm for 1 minute. The supernatant from each sample was loaded into a 10%-resolving, 4%-stacking SDS-PAGE gel. The gel was placed in 1 L of 1X Running Buffer and electrophoresed at 150 V for 1 hour.

After the gel was run, it was placed in a Tupperware container filled with 200 mL of Coomassie Stain (0.25% Brilliant Blue, 45% methanol, 10% acetic acid, fill to 1 L with ddH₂O) and allowed to mix on the BellyDancer at room temperature for 2 hours. Following the staining, the gels were placed in 200 mL of Destain (40% methanol, 10% acetic acid, 50% ddH₂O) overnight. The gels were vacuum-dried on the Bio-Rad Gel Dryer (Model #583) to preserve the gel.

**GST Pull-down Assay**

The GST-fusion MLL repression domain (40 µL) and GST-alone beads (10 µL) were pre-blocked with 200 µL of cold Z-Buffer and left to incubate for 15 minutes on ice. The mixture was centrifuged at 1,500 rpm for 30 seconds. The supernatant was removed. The 450 µL aliquots of FLAG-tagged proteins were thawed and added to either the pre-blocked GST MLL repression domain or GST-alone beads. The mixtures were placed on the circular rotator in the cold room overnight.
The following day, the mixture was centrifuged at 1,500 rpm for 30 seconds and the supernatant was removed. The beads were then washed four times with 1 mL of cold NETN Buffer. After the final removal of supernatant, the beads were resuspended in 40 µL of 1X SDS loading buffer. The samples were boiled for 5 minutes at 95°C and centrifuged at 1,500 rpm for 1 minute. The supernatant from each sample was loaded into a 12%-resolving, 4%-stacking SDS-PAGE gel. The previously mentioned western blotting protocol was used thereafter (see above).
CHAPTER THREE

RESULTS

Aim 1: Clone subdomain regions of Nipped B-like protein into mammalian expression vector and verify expression of the full-length and subdomain proteins

In a recent yeast two-hybrid screen, the MLL repression domain (amino acids 1101-1433) was used as bait to determine interactions with other proteins. Through the screen, it was shown that the MLL repression domain interacts with a small region of Nipped B-like protein (nine positive clones) and human ribosomal protein S18 (one positive clone). Further studies to validate the interaction of MLL repression domain and the Nipped B-like protein were pursued because of the high number of clones obtained with the yeast two-hybrid system.

Nipped B-like, a homolog of Drosophila Nipped B, is part of the cohesin-loading complex, which has been shown to be integral to the cellular processes of mitosis and meiosis\(^\text{25}\). Recently, however, Nipped B-like and the cohesin complex have been shown to have a role in the process of regulating gene expression. It was not until the genetic basis for Cornelia de Lange syndrome (CdLS) was uncovered, that this non-canonical function of cohesin and Nipped B-like was realized\(^\text{30-33}\). Examples of cohesion complex proteins involved in the process of gene regulation have been shown in Drosophila, yeast,
and mouse models\textsuperscript{25}.

To confirm the interaction that was shown in the yeast two-hybrid screen, we must first express Nipped B-like protein, which has yet to be done. Nipped B-like is a very large protein (2804 amino acids), and is shown here to be expressed at a low level (Aim 1B and Aim 2). However, we would like to show a direct interaction between the full-length NIPBL protein and the repression domain of MLL. Thus, we decided to subclone it into smaller fragments to facilitate expression for interaction studies. The full-length protein was sub-divided into seven subclones for expression (Figure 5).
The full-length Nipped B-like (NIPBL) coding sequence as well as the seven subdomain coding regions, which were subcloned into mammalian expression vector, pFLAG CMV-2, in order to yield N-terminally tagged fusion proteins. The seven subdomain regions were created based on previous sequence comparisons and protein analyses. Amino acids (AA) 1-450 define the N-terminus of NIPBL. AA 418-462 contains the entire glutamine-rich region. AA 450-637 contains the NIPBL region identified to interact with the MLL repression domain by yeast two-hybrid screen. AA 590-800 contains the 7, NIPBL-specific PETPK repeats. AA 800-1700 contains both nuclear localization and export signals as well as a PEST sequence. AA 1700-2400 contains the HEAT domain repeats and AA 2400-2597 is the C-terminus of NIPBL, which contains a PEST sequence.
A study published in 2006 analyzed mutations in the *Nipped B-like* gene in Cornelia de Lange syndrome patients. The entire protein was also analyzed using web-based programs to identify functional domains and motifs. We therefore designed seven subclones, which span the entire protein, keeping previously identified or suggested domains intact (Figure 5)\(^{29}\). Table 1 (p. 17) depicts the specific nucleic acid bases and amino acids that the subclones encompass. We had received both pFLAG-N3 NIPBL and pEGFP-N3 NIPBL plasmid vectors from Frank J. Kaiser, Ph.D\(^{22}\). Dr. Kaiser had assembled and sequenced the full-length NIPBL in these vectors. However, he had not yet validated protein expression from these vectors. The NIPBL cDNA is approximately 8,300 base pairs. Upon enzymatic digestion of the two vectors with restriction enzyme BamHI to release the NIPBL cDNA insert, the proper sized insert band was confirmed on a 1% agarose gel (Figure 6).

DNA of full-length plasmid was then used as the template to PCR amplify the subdomain regions. Using pFLAG-N3 NIPBL as the template, primer sets were designed for each of the subclones (Table 2; p. 18). The subclones will be referred to based on their amino acid sequence: NIPBL 1-450 (amino-terminus), NIPBL 418-462 (glutamine-rich region), NIPBL 450-637 (region identified in yeast two-hybrid screen), NIPBL 590-800 (PETPK repeats), NIPBL 800-1700 (NLS/NES), NIPBL 1700-2400 (HEAT repeats), NIPBL 2400-2597 (carboxyl-terminus). As previously mentioned, the amino acid limits of the subclones were designed based on sequence and structural data, however, NIPBL 2400-2597 was shortened from the end of the full-length protein because of a splice variant that ends shortly thereafter.
Photo of the two different full-length NIPBL plasmid DNAs after undergoing restriction enzyme digestion. Undigested plasmid DNA (uncut) is supercoiled and does not migrate according to size. Restriction enzyme digestion (cut) linearized the plasmid DNA, which causes the DNA to migrate according to its size after electrophoresis on a 1% agarose gel. Both plasmids have an approximate length of 12kb. Approximate DNA sizes (base pairs) are indicated on the 1kb ladder.
Each of NIPBL subregions was PCR amplified as shown on a 1% agarose gel (Figure 7). The subcloned regions were inserted into the pFLAG-CMV2 expression vector. This would allow for the expression of FLAG-tagged proteins, so each subdomain protein could be detected with the same antibody. Once the inserts were ligated into the expression vector, the plasmid DNA was transformed into DH5α bacterial cells. After ampicillin selection of colonies, four individual colonies were selected per construct to miniprep. The minipreped supercoiled DNA from each subclone was electrophoresed on a 1% agarose gel, and compared against uncut pFLAG-CMV2 vector control. We analyzed the agarose gels by determining which clones had DNA which migrated more slowly in the gel. A slower migrating DNA would correspond to a larger plasmid containing an insert as opposed to the uncut, empty vector control. The clones with slower migration as compared to the control, were chosen to send for DNA sequencing using the pFLAG-CMV2 forward (CMV30) and reverse (CMV24) primers (Integrated DNA Technologies). The plasmid DNA from these subclones was sent for sequencing at ACGT, Inc. All of the seven clones’ sequences were verified using NCBI Blast to confirm the subclones did not have any mutations, started and ended at the correct sequences, and inserted properly into the expression vector (Figures A5-11).
Figure 7: PCR Amplified Nipped B-like subdomain coding regions

Photo of the PCR amplified NIPBL subdomain coding regions. NIPBL amplicons are shown on the 1% agarose gel and denoted by the amino acid sequence they encode. pFLAG-CMV2 expression vector DNAs are shown after being enzymatically digested by the indicated pairs of restriction enzymes (B-BamHI, EI-EcoRI, EV-EcoRV, S-Sall). Approximate DNA sizes (base pairs) are indicated.
Confirm expression of full length and subcloned NIPBL regions

In our work with Nipped B-like, we have generated the seven subclones and verified their DNA sequences. The subclones were inserted into the mammalian expression vector, pFLAG-CMV2. In order to confirm the interaction of MLL repression domain and Nipped B-like protein, we first had to determine whether protein was expressed from the full-length NIPBL plasmid and the seven subclone plasmids.

Plasmid DNA was transfected into HEK 293T cells using the calcium phosphate transfection technique. After 48 hours following the transfections, the cells were lysed and the whole cell lysate from each construct was prepared. To determine expression of each construct as well as the full-length protein, the whole cell lysates were electrophoresed on SDS-PAGE gel and transferred to PVDF membrane. The pFLAG-CMV2 vector allowed us to probe the blots with anti-FLAG M2 primary antibody to view the expression of the respective proteins. The full-length NIPBL protein (predicted 316 kD) was observed after a long exposure time (Figure 8A; left panel). The expression of the full-length, FLAG-tagged NIPBL was observed in one experiment.

Next, the seven subclones were expressed and western blot analysis was again used to view their expression in HEK 293T cells. Proteins from four of the seven subclones have only been observed in one experiment (Figure 8A). These are NIPBL 1-450, NIPBL 418-462, NIPBL 1700-2400, and NIPBL 2400-2597. Proteins from the two other subclones: NIPBL 450-637 and NIPBL 590-800 have been observed in multiple western blots (Figure 8B; right panel). NIPBL 450-637 has been shown to be expressed in over five separate experiments while NIPBL 590-800 was shown in four. As seen in Figure 8B, NIPBL 450-637 (region matching yeast two-hybrid screen) has multiple
bands. We believe the lowest band to be a degradation product within the cells, while the two upper bands likely represent the protein and a modified version of the protein, which would cause a super shift in the SDS-PAGE gel. Since this degradation product is observed on immunoblot, it must have the N-terminal FLAG tag within the protein. Thus, the lowest band of NIPBL 450-637 occurs towards the N-terminal portion of that region.

To further determine whether there was any protein expression from the remaining constructs, we chose to perform FLAG immunoprecipitations (IP). NIPBL 800-1700 was shown in two, separate FLAG-IP experiments to be expressed (Figure 8B; left panel). The four subclones NIPBL 1-450, NIPBL 418-462, NIPBL 1700-2400, and NIPBL 2400-2597 that were only shown to be expressed in one western blot have not been successfully immunoprecipitated using the anti-FLAG M2 agarose beads to this point. This could be due to multiple different reasons, including poor transfection efficiency. Repeats of the IP experiments will be performed in future.

From the protein expression analysis, we can conclude that we can express the full-length Nipped B-like protein as well as the seven subclones, however, optimized expression and detection of some subclones needs to be further pursued.
Immunoblots of NIPBL protein regions using anti-FLAG M2. A) Western blot analysis of full-length and subdomain regions of NIPBL. Control untransfected whole cell lysates (WCL) from either HEK 293T or Phoenix-Eco (ΦNX) cells were used for comparison. B) Left panel, FLAG-IP experiment of NIPBL 590-800 and NIPBL 800-1700. Proteins are boxed in red; IgG heavy and light chains are noted. Right panel, western blot of NIPBL 450-637 and NIPBL 590-800. Expressed proteins are boxed in red.
Aim 2: Determine whether the Nipped B-like protein interacts with the MLL repression domain

Since yeast two-hybrid screening is often confounded with false positive results, and it is performed in yeast cells, it was necessary to confirm the interaction between Nipped B-like and the MLL repression domain in another system. Here, we use HEK 293T cells for expression of Nipped B-like protein and subclones. Based on the information from the yeast two-hybrid screening, we predict Nipped B-like to interact with the repression domain of MLL. In this aim, our goal is to confirm this novel interaction through the biochemical technique, GST pull-down, and to determine which region(s) of NIPBL can mediate this interaction.

Confirm the interaction of NIPBL and the MLL repression domain

GST pull-down assays were used to test the hypothesis that the interaction between the repression domain of MLL and Nipped B-like protein was a \textit{bona fide} interaction \textit{in vitro}. The Nipped B-like proteins were expressed in HEK 293T cells and harvested as whole cell lysate. To perform the GST pull-down assay, we had to first express the MLL repression domain. The MLL repression domain (1100-1400) inserted into a GST expression vector was used to express a GST-tagged MLL RD, which could bind to glutathione agarose beads for GST pull-down assays. E.coli transfected with either pGEX-KT MLL RD (1100-1400) or pGEX-KT empty vector control were cultured and expression of the proteins was induced by IPTG. After induction, the cells were harvested and lysed. The GST proteins were enriched by incubating the cell lysates with glutathione agarose beads, followed by several washes.

The GST-tagged MLL repression domain and GST-alone proteins were first analyzed through Coomassie staining (Figure 9). The staining allowed for normalization
of the amount of protein/beads added to the GST pull-down reactions. The proteins were detected in four separate experiments.

We hypothesized that full-length Nipped B-like would interact with the repression domain to support the evidence of the yeast two-hybrid results. However, due to the size and low level of expression of the full-length Nipped B-like protein, we were unable to show the interaction with the full-length protein. The GST pull-down of the full-length NIPBL and GST MLL repression domain was tried once prior to continuing with the subdomain proteins. Thus, to confirm the interaction with MLL and to determine whether additional regions of the protein can contribute to binding, we will be looking at the individual subclones.
Figure 9: Coomassie staining of GST fusion proteins

Coomassie staining of the GST fusion proteins. The MLL repression domain (MLL RD) was generated as a GST fusion protein from pGEX-KT MLL RD (1100-1400). GST alone was generated from empty pGEX-KT vector. The proteins were bound to glutathione beads, washed, and then incubated with SDS loading buffer before electrophoresis on an SDS-PAGE gel. Coomassie stain was used to visualize the protein in the SDS-PAGE gel.
We hypothesized that the region identified in the yeast two-hybrid screen (NIPBL 450-637) would interact with the MLL repression domain. We also thought it was possible that other regions of NIPBL could interact with the repression domain of MLL.

All of the NIPBL peptides have been expressed and their expression detected through western blot or immunoprecipitation experiments. To determine the regions of NIPBL that interact with the MLL repression domain, GST pull-downs were used. The whole cell lysates containing the FLAG-tagged NIPBL proteins were mixed with GST-tagged MLL RD or GST alone and allowed to bind overnight. If an interaction between the repression domain of MLL and a subdomain of NIPBL occurred, the GST-tagged protein bound to beads would pull down any interacting proteins. The resulting mixture was thoroughly washed and electrophoresed on an SDS-PAGE gel, transferred to PVDF membrane, and probed with anti-FLAG primary antibody to allow for visualization of the FLAG-tagged NIPBL constructs. The GST-alone beads were used as a control in each experiment.

The GST pull-down assays showed that, in fact, the NIPBL 450-637 region does interact with the repression domain of MLL (Figure 10). As previously mentioned, NIPBL 450-637 shows multiple bands when run on a SDS-PAGE gel. The lowest band is believed to be a degradation product within the cells. As seen from the GST pull-down assays, the degraded product does not interact with the MLL repression domain, while the upper two bands do (Figure 10). The interaction was demonstrated in five, separate GST pull-down assays (Figures 10 and A1-4). Untransfected HEK 293T whole cell lysate was used as negative control. The negative control did not bind to the MLL
repression domain or to the GST-alone beads. To serve as a positive control, HDAC1, a histone deacetylase, which our lab has previously shown to interact with MLL via its repression domain, was used\textsuperscript{11}. As seen in Figure 10, we confirmed that HDAC1 is able to interact with the repression domain.

The NIPBL subdomain 590-800, which also expressed well, did not show an interaction with the MLL repression domain (Figure 11). This experiment was repeated twice. NIPBL 418-462, the glutamine-rich region, was not pulled-down with the repression domain of MLL in two separate experiments. GST pull-downs of the remaining four constructs were each performed once, but no interactions were seen. Once the expression of the remaining four constructs has been optimized, future GST pull-down assays will be performed.

From these experiments, we can conclude that the repression domain of MLL (amino acids 1100-1400) interacts with the Nipped B-like subclone 450-637 as proposed from the yeast two-hybrid screening results.
Figure 10: GST Pull-down Assay of MLL RD and NIPBL 450-637

GST pull-down assay. pFLAG-CMV2 NIPBL 450-637 HEK 293T cell lysate was incubated with GST MLL RD or GST alone. The mixtures were subjected to GST pull-down assay, then electrophoresed on SDS-PAGE (right side). Whole cell lysates of untransfected HEK 293T cells or of HEK 293T cells transfected with FLAG-tagged NIPBL or with FLAG-tagged HDAC1 expression plasmids showed input amounts of proteins (left side of figure). HDAC1, which is known to interact with MLL RD, was the positive control. Input lanes contained 2%, 5%, and 5%, respectively, of the amount of cell lysate used in the pull-down experiment. After transfer, the membrane was probed with anti-FLAG M2 antibody to detect the FLAG-tagged NIPBL and the FLAG-tagged HDAC1 proteins.
GST pull-down assay. pFLAG-CMV2 NIPBL 590-800 HEK 293T cell lysate was incubated with GST MLL RD or GST alone. The mixtures were subjected to GST pull-down assay, then electrophoresed on SDS-PAGE (right side). Whole cell lysates of untransfected HEK 293T cells or of HEK 293T cells transfected with FLAG-tagged NIPBL showed input amounts of proteins (left side of figure). Input lanes contained 5% for each construct of the amount of cell lysate used in the pull-down experiment. After transfer, the membrane was probed with anti-FLAG M2 antibody to detect the FLAG-tagged NIPBL.
CHAPTER FOUR

DISCUSSION

The Mixed Lineage Leukemia (MLL) gene was first identified due to its involvement in chromosomal translocations that can lead to acute myeloid and lymphoid leukemias\(^1\). These MLL leukemias account for nearly ten percent of human acute leukemias and often present more aggressively and with worse prognoses than other leukemias\(^3-6\).

MLL is a large, multi-domain protein\(^7\). The wild type MLL protein contains several functional domains within the carboxyl terminus of the protein. However, in all translocations, only the amino-terminus of MLL is fused in-frame to a partner gene. This results in the formation of a chimeric protein, with the N-terminus intact. Within the N-terminus are three AT-hooks as well as the repression domain\(^10\). The repression domain is present in both wild type and MLL fusion proteins (Figure 1), and functions in both protein-protein interactions and protein-DNA interactions via the included CXXC domain\(^11\). The repression domain has been shown to bind with histone deacetylases (HDAC1) and recruit polycomb group proteins, including BMI1 and HPC2. The repression domain has also been shown to interact with the co-repressor protein, CtBP. In addition, previous studies in our lab have illustrated that the CXXC/repression domain of MLL is important in the wild type and leukemic MLL proteins function\(^16,17\). There may still be
additional, functionally important protein-protein interactions with MLL that have yet to be determined.

In a recent yeast two-hybrid screen initiated by our lab, the MLL repression domain (residues 1101-1433) was used as bait to determine interactions with other proteins in an unbiased manner. This technique is used to identify novel protein-protein interactions within a yeast system. It was shown that the MLL repression domain interacts with Nipped B-like protein. With the large number of positive interacting clones, the interaction of MLL repression domain and Nipped B-like protein was chosen to validate in another system—GST pull-down from *in vitro* cultured human cell extracts.

The Nipped B-like protein is part of the cohesin-loading complex, which has been shown to be important in the cellular processes of mitosis, meiosis, and DNA repair. Recent studies have shown that Nipped B-like and the cohesin complex are implicated in regulating gene expression. Analyses have been performed on Nipped B-like protein function since the heterozygous loss-of-function mutation that causes Cornelia de Lange syndrome in humans was uncovered.

Our objective for this thesis was to experimentally confirm the novel interaction identified between the repression domain of MLL and the Nipped B-like protein. We hypothesized that the two proteins do interact as shown in the yeast two-hybrid screen. To validate the interaction, we used the biochemical technique, GST pull-down. This technique is often used to confirm interactions between two, independent proteins. In our experiments, we used GST fusion MLL repression domain to pull-down the FLAG-tagged Nipped B-like proteins. This combination allowed us to pull-down the proteins of interest and then blot for those same proteins using the same antibody for detection of
each NIPBL protein subdomain. Several steps had to first be completed before the interaction could be tested.

From our western blot and immunoprecipitation experiments, all seven subcloned regions were expressed—some better than others. NIPBL regions 450-637, 590-800, and 800-1700 were all expressed at an easily detected level with both techniques in multiple experiments. The remaining four constructs (NIPBL 1-450, 418-462, 1700-2400, and 2400-2597) are shown in one western blot with background, and thus, will need to be optimized in future experiments. None of these four constructs were detected after FLAG-immunoprecipitation, which would suggest transfection efficiency and protein stability as points of future technical optimization.

The full-length Nipped B-like protein, as previously mentioned, is quite large and was only observed in one western blot. When a GST pull-down assay was performed with the full length NIPBL, no interaction was seen. This was one reason for analysis of interaction with the subdomain regions. The subclones allowed for protein expression in higher amounts that could be used to perform multiple GST pull-down assays. In this thesis, NIPBL 450-637, NIPBL 418-462, and NIPBL 590-800 were the three subdomain constructs used in these pull-down experiments. In our experiments, NIPBL 418-462 and 590-800 failed to show an interaction in two independent experiments. However, the NIPBL subdomain region 450-637, which was the region identified to interact with the repression domain of MLL through the yeast two-hybrid screen, was shown to interact with the MLL repression domain in five, separate experiments.

In summary, we have successfully expressed full-length Nipped B-like protein. In addition, seven domains of Nipped B-like were also cloned and expressed. These
constructs were used to confirm an interaction between the repression domain of MLL and Nipped B-like protein as proposed from the results of the previous yeast two-hybrid screen. The interaction was shown using GST pull-down assays. The repression domain of MLL (amino acids 1100-1400) was shown to interact with Nipped B-like (450-637). Confirmation of this novel interaction is only a stepping-stone for future research on these two important proteins.

The results from this thesis present several possibilities for further investigations. Immediate experiments to perform hereafter include more experiments on the expression of the Nipped B-like protein and its subdomains. This thesis included expression of all seven subclones and the full-length NIPBL proteins; however, difficulties were sustained with transfection efficiency and antibody optimization. To address these problems, varying conditions of transient transfection, including amount of DNA with the calcium phosphate transfection method, or using different methods such as lipid-based lipofectamine may increase the amount of plasmid DNA taken into the cells. Altering the method used to solubilize and extract proteins from the cells, as well as conditions used for immunoprecipitation are possible. We have also obtained a new antibody, which were used in later experiments and gave less non-specific background on the blots. Using this better antibody along with optimized secondary antibody and washing conditions may improve the experiments as well.

It still remains to be determined if the full-length Nipped B-like interacts with the MLL repression domain, and how they interact. This can be established through additional protein work. To determine if the full-length Nipped B-like interacts with the repression domain of MLL, GST pull-down assays would be used again once the full-
length Nipped B-like was efficiently being expressed and solubilized in HEK 293T cells. Since we confirmed NIPBL 450-637 to interact with MLL repression domain, we would expect the full-length protein to interact as well. To deduce whether other regions of NIPBL can interact with the MLL repression domain, additional GST pull-down assays would be performed—again, with sufficient protein from new transfections. If other, separate regions of Nipped B-like appear to interact with the repression domain, we would have some insight as to how Nipped B-like is folding with regards to its interaction with MLL. However, if NIPBL 450-637 is the only region to be shown to interact with the MLL repression domain, the results of this thesis have already begun to determine Nipped B-like’s minimal interacting domain with the repression domain. As seen in Figure 10, the two slower migrating bands of NIPBL 450-637 interact with the MLL RD, while the lower, possibly degraded product does not. We state that the lower band must contain the N-terminal FLAG-tag as it is detected by anti-FLAG antibody immunoblot. Since it migrated farther in the SDS-PAGE gel, this band must be lower molecular weight than the two upper bands. We would predict that the MLL-interacting region of NIPBL is the carboxy-terminal region of this construct. From NIPBL 450-637, we could therefore make smaller subdomain regions and perform similar GST pull-down assays with MLL RD 1100-1400 to determine the minimal amino acid residues that are required for the interaction.

On the other side of the interaction, the MLL repression domain used in the yeast two-hybrid screen was amino acids 1101-1433, whereas the GST-tagged MLL repression domain used for pull-down experiments contained amino acids 1100-1400. We were therefore able to both confirm the interaction with MLL and determine that amino acids
1100-1400 were sufficient to mediate interaction with NIPBL. It is still not clear from the experiments performed which region(s) within the MLL repression domain are critical to mediate the NIPBL interaction. Within the repression domain is the important DNA-binding CXXC domain. To identify the minimal NIPBL-interacting domain of MLL repression domain, smaller constructs of the region could be cloned and expressed. Similar GST-tagged proteins would be created and bound to glutathione beads so that the pull-down experiments to determine the minimal MLL interaction domain could be pursued. It will be important to determine minimal interaction domains from both NIPBL and MLL in order for structural determination of the interaction by NMR spectroscopy to be pursued. That would be the next step toward identification of small molecules, which could inhibit the interaction and could possibly serve as novel therapeutics.

In addition, it should be noted that the results of the yeast two-hybrid screen and the GST pull-down assays with whole cell lysates, cannot confirm if this interaction is direct or indirect in nature. In both systems, additional proteins besides Nipped B-like and the MLL repression domain could help mediate the interaction. As previously stated, the MLL repression domain can interact with other proteins, and Nipped B-like could interact with other proteins through its HEAT repeats, Caldesmon domain, or glutamine-rich region. These subsequent proteins could be needed for proper folding and organization of Nipped B-like and MLL into a larger, multi-subunit protein complex. To confirm a direct interaction, purified proteins would have to be used to repeat the GST pull-down assay. To shed light on an indirect interaction through a larger complex, whole cell immunoprecipitations could be performed to determine whether other proteins
are pulled-down with either MLL or Nipped B-like. The identity of additional proteins could be determined by mass spectroscopy. These interaction studies would give the needed basis for future studies that look at the expanding role of Nipped B-like and cohesin as transcriptional regulators. As previously stated, MLL is a positive regulator of genes in embryonic and hematopoietic development. Cohesin and Nipped B-like have been linked to both positive and negative regulation of various gene loci\textsuperscript{39-41}. This thesis and future protein interaction studies would be the foundation of placing these two proteins at the same point within a cell.

To begin to elucidate functional roles of these two proteins within the cell, several experimental techniques could be used. First, ChIP and ChIP-seq could be used to determine if MLL and Nipped B-like are binding to the same genes, where they are binding to these genes, and if they are binding at different times. Both abovementioned techniques have been shown before for MLL and Nipped B-like separately, and using different cell types, but we would like to confirm that both MLL and Nipped B-like are located at the same genes simultaneously in the same cell type. If this experiment was performed, we would hope, based on the interaction results of this thesis, that both MLL and Nipped B-like would be positioned at the same genes as depicted in our model (Figure 12). Having the two proteins at the same genes would allow further analysis into the regulation and expression of the gene targets of MLL and Nipped B-like.

As noted previously, MLL fusion proteins cause both acute myeloid and lymphoid leukemias in humans whereas mutations in Nipped B-like lead to Cornelia de Lange syndrome (CdLS). Leukemias and CdLS show their effects in the early, developmental stages of life and, therefore, limit one’s years. Our ultimate focus of
future studies would be to gain information on how the two proteins, MLL and Nipped B-like, affect these two medical conditions in order to effectively treat future patients. Some of the known gene targets of MLL and MLL fusion protein function are the HOX genes, which are critical in development. It would be especially important to determine whether MLL and NIPBL co-regulated expression of HOX genes is crucial for both normal hematopoiesis as well as developmental pathways altered in CdLS.

In CdLS, we would like to ask if MLL is required to still see the same developmental abnormalities and effects of the syndrome. To do this, we would use MLL null (-/-) cells as the basis for ChIP studies. Any changes in gene regulation of genes related to CdLS in the null cells, as compared to wild type MLL, would be genes of interest.

For MLL leukemias, we could see if Nipped B-like heterozygosity (+/-), which causes CdLS, could be an effective therapeutic for inhibiting the transformational capacity of MLL fusion cells. In this experiment, we would transfect NIPBL +/- cells with a MLL fusion plasmid—to produce the MLL fusion protein within the CdLS environment. From here, we would determine if the transfected cells were able to transform (i.e. confer leukemia) or not. If the cells were unable to transform based on the NIPBL +/-, this could lead to insight of the fact that Nipped B-like and cohesin’s effects on gene regulation also have an effect on the MLL fusion’s ability to cause leukemia. Put together, if these results were seen, Nipped B-like could be a possible therapeutic for targeting MLL leukemias.

As seen in prior studies, chromosome conformation capture (3C) can be used to determine if there is DNA looping at genes. In our case, the previously mentioned
combinations of cell lines (MLL-/− and NIPBL +/−) would be used to determine looping at MLL- and Nipped B-like-specific genes. 3C would allow us to confirm DNA looping, such as between gene enhancers and promoters, and ChIP-seq would allow us to immunoprecipitate for proteins at the bases of the loops. From the thesis data, we would hypothesize to see both MLL and Nipped B-like at the bases of the looping regions (Figure 12) in wild type and MLL fusion cells leading to transcriptional upregulation. However, a decrease of recruitment would be noted in cells with decreased Nipped B-like (NIPBL +/−) levels, which mimic the effects of CdLS. Finally, the MLL −/− cells would show if MLL is required for Nipped B-like to be engaged at MLL-specific genes.

The combined data from this thesis and future research is what I hope to be used in accomplishing significant strides in the understanding how both the MLL leukemias and Cornelia de Lange Syndrome are caused by these proteins of interest, and potential points for therapeutic intervention.
Schematic model depicting the potential functional outcome of an interaction between Nipped B-like and the MLL repression domain. An interaction between NIPBL and MLL could lead to the possibility of positive regulation of MLL target genes within the cell. For this to occur, NIPBL, with MLL bound, would loop DNA and allow for recruitment of the Mediator complex and RNA polymerase to transcribe the genes.
APPENDIX A

SUPPLEMENTAL DATA
Figure A1: GST Pull-down Assay of MLL RD and Nipped B-like

GST pull-down assay. pFLAG-CMV2 NIPBL 450-637 HEK 293T cell lysate was incubated with GST MLL RD or GST alone. The mixtures were subjected to GST pull-down assay. Whole cell lysates were immunoblotted and probed with anti-FLAG M2 to detect the FLAG-tagged NIPBL protein. Untransfected HEK 293T whole cell lysate served as a negative control. Input lanes were 2% and 5%, respectively.
Figure A2: GST Pull-down Assay of MLL RD and Nipped B-like

GST pull-down assay. pFLAG-CMV2 NIPBL 450-637 HEK 293T cell lysate was incubated with GST MLL RD or GST alone. The mixtures were subjected to GST pull-down assay. Whole cell lysates were immunoblotted and probed with anti-FLAG M2 to detect the FLAG-tagged NIPBL protein. Untransfected HEK 293T whole cell lysate served as a negative control. Input lanes were 2% and 5%, respectively.
Figure A3: GST Pull-down Assay of MLL RD and Nipped B-like

GST pull-down assay. pFLAG-CMV2 NIPBL 450-637 HEK 293T cell lysate was incubated with GST MLL RD or GST alone. The mixtures were subjected to GST pull-down assay. Whole cell lysates were immunoblotted and probed with anti-FLAG M2 to detect the FLAG-tagged NIPBL protein. Untransfected HEK 293T whole cell lysate served as a negative control. Input lanes were 2% and 5%, respectively.
Figure A4: GST Pull-down Assay of MLL RD and Nipped B-like

GST pull-down assay. pFLAG-CMV2 NIPBL 450-637 HEK 293T cell lysate was incubated with GST MLL RD or GST alone. The mixtures were subjected to GST pull-down assay. Whole cell lysates were immunoblotted and probed with anti-FLAG M2 to detect the FLAG-tagged NIPBL protein. Untransfected HEK 293T whole cell lysate served as a negative control. Input lanes were 2% and 5%, respectively. NIPBL 450-637 is boxed in red.
In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
Figure A6: Sequencing Verification of NIPBL 418-462

> G5_CMV30 sequence exported from G5_CMV30.ab1
CAAAATTGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTG
AACGTCAGAATTGATCTACCATCGGACTCAAAGACGATGACGACAAGCTTGCGGCCGC
GTTTACAACAGAAACACTTCTGTTGTGCAAAAAAACCCAGACTTCTGTGTCAGAAT
CAACAAACAGATATCACAACAGTGAGGATCCGGGTGCGCATCCTGTGACCCCTCCCCAG
GCCCTCTCTGCGCCTCTGAAGTTGCGCACTCCAGTGCCACCAGCCTTGCTCTAAATTT
AAGTTGCACTATTGCTGACTAGTGTCCCTCTCATAAAATTATATTGGGTGAGGGGGGT
GGTACGGCACTGACCAAATATTATAGATAACCTCCAGGGTAGTCATCAATAACTCAAAGAAG
CATAGTACTAGTGACCTGTCCCTCATAATAGACTCAAAGCAACCCCCGATAAAGGGTAAG
CAACGGCCCAACCGTTTACGTTAAGCGGTATGTTCTCGCTTTCAAGATGTGTCAGACTTAT
TTCGATTTTGGTGAATTGTGGTATAATTAGCGCTGATCGCCCATATGTAAGTCAGCCC
ATGACTTCTAAAGCTGACTCGAAAGCCTCGTGACGGGCTTGTGTCAGTGCCTG

In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
Figure A7: Sequencing Verification of NIPBL 450-637

>F1_CMV30 sequence exported from F1_CMV30.ab1

ATGGGCGGTAGGCATTACGGGTGGGAAGGTATATGTAAGACAGCAGTCGTTTGTTGATTGAACCG
TCAGAAGTGATCTTACCATGGACTACAAAGACGATGACGACAAGCTTGCGGCCGC
GAAATTCTGTTGCTACAGATCAAACACAGATATCACAACAGGGACCTATATATGATGAAATT
GGATGCTATTGGTAAATTAGAGAGAGAAGTAGCTATGGAAAGGAGCGCTT
CTCAGAAAGGTCAAGATAAAGATAAGCCTTTGGAAAAAGAAAAAAAGATTTCTCACC
ACAGGGAGCTGGGGGTTGCTACAGAGGTAATAGACCCAGCTTCGGAGAAGCGGTTCTAC
GGGAAATGGTCAAGGCCAGCATTATGTTAGCATTGATCTTTTCATTCCAGACGGAAGGT
GAGCTCTAGGTCTTATATAACTCAGAGTcccTCAGACTCCATAAAAAGCCTGAAGAAATCA
ACAATGTAATGTCACCTGGGTTCGGTTGCTTCTTAGGGTTGAAAAATGCTGAGATATGGAG
GTCTCCAGAAAAACCATCTGAGACACCTAAAAAATCTCTGATCTTGACTTTCAAGAG
TGAAATGCAAAAAATCTGAAGTAACGTGAAAGATTTAGTACAGGATACCTAAAAATGAAAGATC
GGAGACAAAATCAACTGAATGAGATCCTCCGAGCTCCCTGCTGAGCCCTCCTCCCATCC
CTCTCTGAGCCCTGGAGATGCGCCACTCCAGTGGCCCACCGCCTTGTCTAATTAA
GTTGAGCTATTTTGATCTGACTAGGTGCTCTTCTATAATATTATGGGATTGGAAGGGGGGTG
GG

In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
Figure A9: Sequencing Verification of NIPBL 8000-1700

>C8_CMV30 sequence exported from C8_CMV30.ab1
AAGCGGAAAGGGACGCTGTCGAGGTTGAGCTATATATAAACGAGACTCGTCTTTTATTG
GAACCCGGTCAGAAATCCTTTTACCGTGTAAACGAGAACAGATTGCTAAGCTAGCTGA
CCCTGAAATATATGCTGAAGATAGATAGATAGATAGATAGATAGATGAAGCCTTTTGG
AATCTCTCAGGAAAACCATGGAAGATAGATAGATAGATAGATAGATGAAGCCTTTTGG
CCAGAAGACTCAGAAGATAGATAGATAGATAGATAGATGAAGCCTTTTGG

>C8_NIP-C-R sequence exported from C8_NIP-C-R.ab1
GTAGTATAGATACGCTCGTTAAAAGAATACCACCCTCTCTGGCAGAAATTTTAGT
CCACCTGCGACCTTTGGAGAGAAATGCAAACACGCAAGCTGACACTTTTAAATAG
AAAGCGAAGAAATAGCCTGAAATCTTGTATGTAGTGAAGTGAAGCTTTTGG
AATCTCTCAGGAAAACCATGGAAGATAGATAGATAGATAGATAGATGAAGCCTTTTGG
CCAGAAGACTCAGAAGATAGATAGATAGATAGATAGATGAAGCCTTTTGG

>C8_CMV24 sequence exported from C8_CMV24.ab1
CGGGCTAAATGTTCTACCACTACGATTAAAAGAATATGCTATTAACCGAGACTAGT
GACATGGTAGCATTAAAGTGGAGGAAACTACGATCTTACACGAGACTAGT
CCCTGAGTTTTCATCTGTACAACTTTTTTGTGGGAAATATTGAGTTAGT
TTGTTGTGCTAGTAAAAGGACAGAAAATATTGAGTTAGT
CCCTGAGTTTTCATCTGTACAACTTTTTTGTGGGAAATATTGAGTTAGT
TTGTTGTGCTAGTAAAAGGACAGAAAATATTGAGTTAGT

In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
In the above figure, restriction enzyme sites are displayed as bolded text. The stop codon is highlighted in red, and the pFLAG-CMV2 expression vector sequence is labeled in blue. The plain, capitalized text is the coding sequence of Nipped B-like.
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VITA

The author, Adam R. Marek, was born in Chicago, Illinois on October 27th, 1987 to Robert and Tracy Marek. He received his Bachelor of Science degree in Chemistry from the University of Illinois Urbana-Champaign in May 2010. As an undergraduate student, Adam excelled in both chemical and biological courses and laboratory sessions prompting him to continue his education in those fields.

Shortly after graduating, Adam entered into the Molecular and Cellular Biochemistry Program at Loyola University Chicago in August 2010. He later joined the laboratory of Nancy J. Zeleznik-Le, Ph.D., where he performed his research on the interaction of Mixed Lineage Leukemia (MLL) and a cohesin complex protein, Nipped B-like.

After completing his Master of Science degree, Adam will be joining the Integrated Biomedical Sciences PhD and Master of Business Administration Programs at Loyola University Chicago. With the PhD and MBA programs, Adam hopes to obtain vast knowledge in both fields during the remainder of his graduate career and bridge the gap between the two in a future profession.