Investigating The Role of MycN and p53 Binding in Pediatric Neuroblastoma

A Major Qualifying Project
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Abstract

Neuroblastoma is the most common extracranial solid tumor found in children and accounts for 13-15% of all pediatric cancer deaths. Amplification of MycN is one of the strongest indicators of poor prognosis, is present in 50% of all high-risk cases of neuroblastoma, and has been detected in 30% of all cancers; while its role in neuroblastoma has not been clearly elucidated, evidence suggests that it binds to the C-terminal domain of p53 and alters its response to genotoxic stress. The binding of p53 and MycN is a relatively novel discovery; more investigation into the role of p53-MycN binding can provide insight into neuroblastoma tumorigenesis and potentially other MycN-amplified cancers. This project sought to further investigate the role of MycN and p53 binding in neuroblastoma and to outline further research on the novel interaction.

Acknowledgments

I would like to express my gratitude for those who have supported me throughout the completion of this project. The success of this project would not have been possible without the tireless efforts of the many individuals who showed genuine interest in the realization and implementation of my project.

I would first like to give sincere thanks to my advisors, Dr. Manning and Dr. Shohet, who provided me with insight, support, and guidance throughout the entirety of the project. Their expertise helped me through every step of the way, and this project would not have been possible without their knowledge and their willingness to teach.

To the members of the Shohet lab, thank you for welcoming me into your group and for taking time out of your busy schedules to help me learn and to execute this project. Tingting, thank you for keeping me company during late nights in the lab. Kim, your humor and enthusiasm to help me learn has been a highlight of this experience. Finally, this project would not have been possible without the patient guidance of Esteban. You were always willing to drop what you were doing to help me and to teach me, and I cannot even begin to express how grateful I am to have had the opportunity to learn from you. I am sorry that my time working with you all has come to a more abrupt end than we had planned, but I am so grateful for the time spent learning with you.

I would like to thank Worcester Polytechnic Institute and the University of Massachusetts Medical School for the opportunity to learn through this project. The partnership of these institutions has laid the foundation for me and countless other students to learn and work alongside incredible biomedical researchers; I extend my heartfelt appreciation to each institution for making this project possible.

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1: Introduction

Neuroblastoma is a neural-crest derived cancer that comprises 8-10% of pediatric cancers and up to 15% of all pediatric cancer deaths. Neuroblastomas tend to be highly heterogeneous and as a result, some patients' tumors regress without chemotherapy while others undergo several aggressive therapy regimens. The most negative diagnostic factor in NB is the presence of MycN amplification, which is associated with high rates of progressive disease and low survival. MycN is a part of the Myc family of proteins, which also includes C-Myc and MycL, which is considered to be a family of proto-oncogenes found in many human cancers. Myc proteins form heterodimers with MAX to activate transcription; however, unbound Myc can act as a global amplifier of transcription. Typically, such a widespread increase in transcription and proliferation would trigger p53-mediated apoptosis; however, evidence suggests that cross-talk between p53 and MycN exists in neuroblastoma.

Agarwal et al (2018) suggested a model for neuroblastoma in which MycN, when found in the excesses seen in MycN-amplified neuroblastoma tumors, binds to the C-terminal domain of p53. The study also provides evidence that p53's transcriptional activity is altered in conditions wherein high levels of MycN are present as opposed to low-MycN conditions. Learning more about this novel interaction of p53 and MycN has the potential to reveal insight into the tumorigenesis of neuroblastoma and other MycN-amplified tumors. The goal of this project is to replicate and expand upon results discovered by the Shohet lab that describe the interaction of MycN and p53. Specifically, this project aims to examine the binding of p53 and MycN in various neuroblastoma models, to examine the C-terminal domain of p53 for post-translational modifications that may facilitate its binding to MycN, and to perform CUT&RUN chromatin profiling to confirm the results of Agarwal et al (2018) regarding p53 response element binding when bound to MycN.

Unfortunately, due to the closure of labs at both WPI and the University of Massachusetts Medical School in order to preserve personal protective equipment for clinicians, the final experiments were cut from the project plan and the last two objectives were unable to be met. However, the overarching goal of investigating the interaction of MycN and p53 in neuroblastoma was partially met prior to these closures, and robust interaction of the two proteins was observed in various models of neuroblastoma. It is the hope of the author that this work may be a foundation for future students to continue to research this novel interaction and its implications on neuroblastoma.

2: Background

In order to understand the roles of p53 and MycN in neuroblastoma, the development of neuroblastoma must be understood alongside the wild-type functions of p53 and MycN.

2.1: Pediatric Neuroblastoma is a Neural Crest Derived Tumor

Neuroblastoma arises as a result of malignancies in peripheral ganglia of the sympathetic nervous system. A majority (~60%) of tumors arise from the abdominal paraspinal ganglia, 30% from the adrenal medulla, and the remaining ~10% from ganglia in the chest, pelvis, head, or neck (Louis & Shohet, 2015). In addition to presenting in a variety of physical locations within the body, neuroblastoma tumors are highly heterogeneous and can exhibit a wide range of behavior; some patients' tumors regress spontaneously while others have highly aggressive tumors that metastasize and resist treatment (Louis & Shohet, 2015). The biologic heterogeneity of these tumors can be explained in part by the complexity of neural crest development during embryogenesis.

2.1.1: Neural Crest Development

Neural crest cells are embryonic cells that are transiently present during the processes of gastrulation and neurulation; they migrate throughout the developing embryo and can differentiate into a wide range of derivative cell types (Bae and Saint-Jennet, 2014). The process of neural crest development begins when cells at the edges of the neural plate, or neural plate border cells (NPBs), are induced to express a series of proteins including bone morphogenic protein (BMP), fibroblast growth factor (FGF), and Wingless/Int (Wnt); the expression of these proteins "primes" NPBs for differentiation into neural crest progenitors. Additionally, research has implicated all of these proteins in neuroblastoma tumorigenesis and differentiation (Tomolonis et al, 2017). These neural crest precursors are then committed to speciation into neural crest cells (NCCs) by two groups of transcription factors, early- and late-onset neural crest specifiers. Early-onset neural crest specifiers include c-Myc and its target Id3, proteins that help to control the cell cycle and are theorized to help NCCs to maintain their pluripotency (Tomolonis et al, 2017). Interestingly, c-Myc is closely related to MycN, which is widely viewed as the strongest negative prognostic indicator in neuroblastoma; both proteins are known to regulate a wide number of genes involved in pluripotency maintenance (Tomolonis et al, 2017). A large number of genes are considered to be late-onset neural crest specifiers (Sox9, Sox10, FoxD3, Snail2, and Twist1) that allow NCC's to delaminate from the neuroepithelium, beginning the epithelial-to-mesenchymal transition (EMT) (Tomolonis et al, 2017). During the EMT, a series of changes to the NCCs occurs that decrease their adherence to neighboring cells and increase their motility (Taneyhill and Padmanabhan, 2014). NCCs then migrate throughout the embryo to their target locations, where they differentiate into their final cell types based on their position and origin (Bae and Saint-Jennet, 2014). Most, if not all, of these pathways have been shown to be dysregulated in neuroblastoma, contributing to tumor pathogenesis and differentiation.

2.1.2: Neuroblastoma Tumorigenesis

Despite the origin of neuroblastomas from aberrant neural crest development, a single mutation responsible for all cases has not been identified. This in conjunction with the clinical, molecular, and pathologic heterogeneity of neuroblastoma tumors may signal that a range of molecular drivers are at play (Louis and Shohet, 2015). Stem cell-like populations within neuroblastomas have been shown to differentially express late-onset neural crest specifiers such as Sox10 and E-Cadherin (Hsu, Agarwal, et al, 2013), supporting the idea that cancer stem cells (CSCs) of neuroblastoma may correspond to different tumor phenotypes based on their precursors' developmental stage (Louis and Shohet, 2015). On the whole, Louis and Shohet (2015) suggest that tumor-initiating CSCs may yield less or more malignant tumors based on the stage of neural crest development from which the CSCs are derived, with more differentiated CSCs yielding less malignant tumors. MycN amplification has been implicated in aggressive isotypes of neuroblastoma; this is not surprising due to its function of maintaining pluripotency (Tomolonis et al, 2017).

2.1.3: Neuroblastoma Clinical Staging and Treatment

As explained above, neuroblastoma exhibits an extremely wide range of activity in clinic; as such, not all neuroblastoma patients can be treated in the same way. In order to categorize neuroblastoma patients for treatment, the International Neuroblastoma Staging System (INSS) was developed. Stages in the INSS were based upon the characteristics of tumors that were defined in surgery and can be found in Appendix A; however, staging according to this system was limited in scope. The currently-used International Neuroblastoma Risk Group Staging System (see Appendices B and C) provides staging categories and risk groups that consider a much wider range of variables in light of neuroblastoma's heterogeneity (Louis and Shohet, 2015). Ultimately, these categories are utilized to determine recommendations for treatment, the scope of which ranges widely from observation to chemotherapy, surgery, immunotherapy, and more. As stated above, MycN amplification is present in aggressive neuroblastoma tumors; patients with these tumors are frequently considered to have higher-stage disease. In this way, understanding the behavior of MycN can help to understand and, ultimately, treat those patients who are at the highest risk.

2.2: p53 Plays a Critical Role in Preventing Cancer

p53 is arguably one of the most well-known proteins in modern health science. The mutation of p53 is associated with over fifty percent of all cancers; of the tumors that do not carry a p53 mutation, most experience p53 inactivation through another mechanism. (Horn and Vousden, 2007). It plays an important role in cellular responses to stress by triggering cell cycle arrest, senescence, or apoptosis. When stressful conditions such as hypoxia or DNA damage occur, p53 binds to response elements in DNA and induces the downstream expression of genes such as p21, a CDK inhibitor that prevents the cell from progressing through the cell cycle (Mello and Attardi, 2018). Figure 1 describes some of the traditional pathways in which p53 activation is involved; however, recent research suggests that p53 has a wider reach within the body, affecting processes such as glycolysis, autophagy, and angiogenesis (Vousden and Lane, 2007).

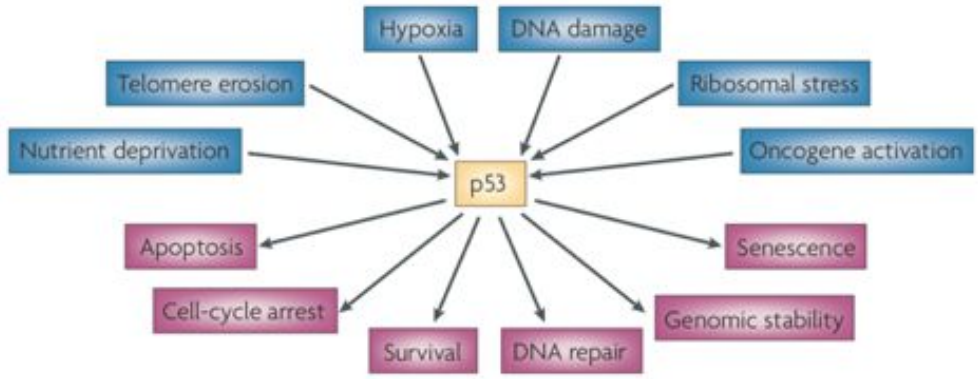

Figure 1: Activating stimuli and responses of p53 (Retrieved from Vousden and Lane, 2007)

2.2.1: Molecular Structure of p53

P53 is composed of 393 amino acids divided into five main domains: the transactivation domain, the proline-rich domain, the DNA binding domain, the tetramerization domain, and the C-terminal domain. Malignancy-causing mutations are most frequently found within the DNA binding domain; mutant p53 proteins with these mutations typically have both altered conformation and diminished DNA binding abilities (Dai and Gu, 2010). The transactivation domain is crucial for p53's binding to MDM2 and its human analog HDM2, which will be discussed later and plays an active role in suppressing p53's activity until the detection of cellular stress (Lee, Martinez-Yamout, Dyson, and Wright, 2010). The active form of p53 in complex with DNA is a tetramer; the tetramerization domain is responsible for the binding of dimerization of p53 monomers and the subsequent dimerization of these dimers (Aramayo et al., 2011). The function of the C-terminal domain has been subject to debate in recent years; however, research suggests that it facilitates site-specific binding to DNA response elements (Laptenko et al., 2015).

Figure 2: Structure of p53 and its domains (Adapted from Dai and Gu, 2010)

In order for p53 to complex with DNA, two molecules must first dimerize; the dimers then dimerize along with DNA to form the complex. However, the arrangement of this tetramer has been subject to debate and several Cryo-EM studies. Figure 3 depicts an accepted configuration for the p53 tetramer in complex with DNA in panel C, in which the C-terminal domains of two of the four monomers are at the core of the complex in order to stabilize the complex through additional interactions between the p53 tetramer and DNA. Additionally, the N-termini are located further from the core in order to allow for interactions with other proteins such as Mdm2, MdmX, and additional cofactors. (Aramayo et al., 2011).

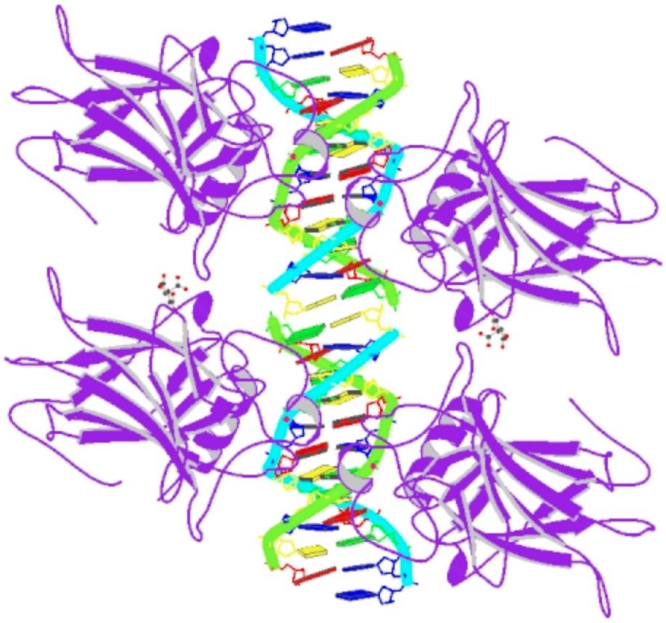

Figure 3: Structure of a p53 core bound to DNA (Retrieved from Malecka, Ho, and Marmorstein, 2009).

2.2.2: p53 Activation

In its inactive state, p53 is bound to DNA in its tetramer formation; however, its activity is repressed by the binding of Mdm2 and MdmX to the transactivation domains of the N termini. However, under stressful conditions within the cell, p53 undergoes a series of posttranslational modifications that prevent the binding of Mdm2 and MdmX, including acetylation of specific lysines in the DNA-binding domain and the phosphorylation of serines in the N- and C-termini. Specifically, the phosphorylation of serines 15 and 20 (S15/20) has been clearly implicated in the prevention of Mdm2 binding to p53 (Dai & Gu, 2010). When Mdm2 and MdmX are prevented from binding to the p53-DNA complex, several transcription factors, and cofactors are recruited alongside RNA polymerase II to begin the transcription of a variety of genes. Stress response pathways are activated according to the binding of the p53 complex to their specific response elements (Dai & Gu, 2010). Figure 4 provides a visual representation of the p53 activation pathway.

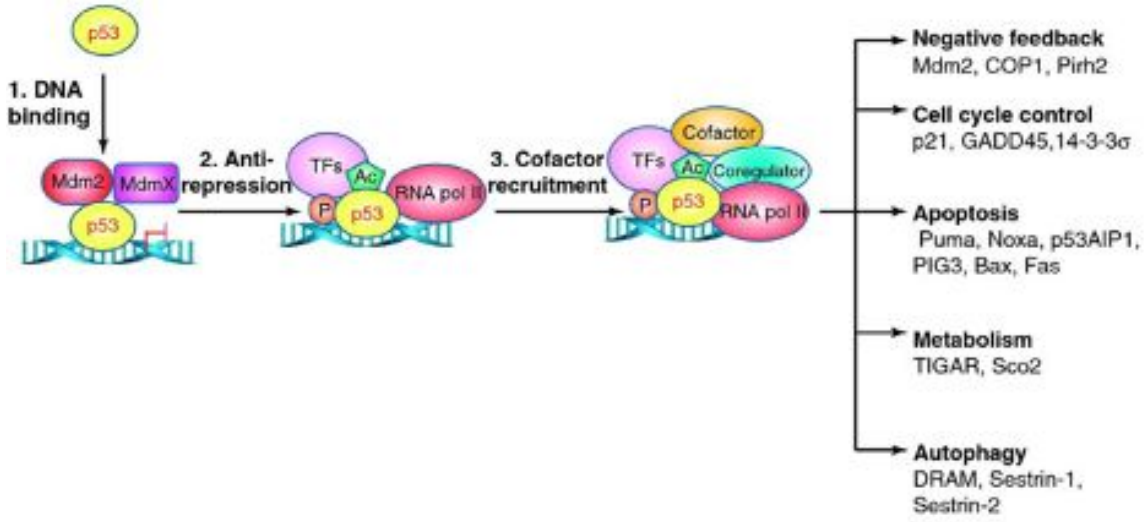

Figure 4: Activation of p53 transcriptional activity (Retrieved from Dai and Gu, 2010)

2.3: MycN Plays a Role in Neuroblastoma Tumorigenesis

The Myc family of proteins, including C-Myc and MycN, are global activators of transcription that are crucial for early embryonic development (Yoshida, 2018). It is a basic helix-loop-helix transcription factor that must form heterodimers with its binding partner, MAX, in order to bind E-box sequences (Agarwal et al, 2018). However, the amplification of MycN has been tied to nearly 30% of all cancers (Schaub et al, 2018). In neuroblastoma, it is present in 50% of high-risk cases, while patients whose tumors have low levels of MycN expression experience better responses to chemotherapy and higher survival rates (Agarwal et al, 2018).

2.3.1: A proposed mechanism for MycN's activity in neuroblastoma

The exact mechanism of MycN's activity within neuroblastoma tumors has not yet been defined. In MycN amplified tumors, MAX is not amplified; this may indicate that MycN functions independently of MAX in these tumors (Agarwal et al, 2018). Interestingly, p53 is typically wild-type at the time of diagnosis in most patients despite MycN functioning to drive proliferation and alter DNA damage responses; these functions would typically drive p53 to initiate apoptosis (Agarwal et al, 2018). As stated in 2.2.1, p53's binding to DNA response elements is facilitated by the C-terminal domain (CTD). Agarwal et al (2018) identified a direct interaction of MycN and the p53 CTD that affects the transcriptional responses of both MycN and p53. Additionally, Wang et al (2017, 2018) identified that post-translational modifications such as acetylation of the p53 CTD affect the binding of p53 to DNA and certain proteins. The following report seeks to replicate and expand upon the results obtained by Agarwal et al (2018); specifically, this report seeks to confirm and further explore the direct binding relationship between MycN and p53 and the potential effects of p53's post-translational modifications on the binding relationship.

3: Materials and Methods

The following section details the materials and methods utilized throughout the entirety of the project to obtain the results described in Section 4. A complete list of reagents can be found in Appendix D.

3.1: Cell Culture

The human neuroblastoma cell lines JF, MycN3 Tet-On, and NGP were maintained in RPMI medium containing 10% fetal bovine serum (FBS).

3.2: Western Blotting

Whole-cell lysates were collected and sonicated. The concentration of each lysate was determined via BCA assay (Thermo Fisher). Protein samples were boiled in Laemmli sample buffer, separated by SDS-PAGE using 14% tris-glycine gels, and transferred to a PVDF membrane. The membrane was blocked for 20 minutes, incubated with the primary antibody overnight at 4℃, washed with TBST for 20 minutes, incubated with the secondary antibody for one hour at room temperature, and imaged.

3.3: Co-Immunoprecipitation Assays

Crude protein extract from treated cells was prepared. Cells were lysed using 2X lysis buffer (See Appendix X for buffer formulation) and sonicated. Protein G Dynabeads (Invitrogen) were incubated for 1h at 4℃ with α-p53 mouse antibody (DO1, Santa Cruz). One to three milligrams of crude protein extract was incubated overnight with 40 µL of mAb-bound beads at 4℃. Following three washes with 1X lysis buffer 1, protein samples were eluted by boiling beads in Laemmli sample buffer, separated by SDS-PAGE, and analyzed via Western blot.

3.3: Mass Spectrometry and Post-Translational Modification Analysis

Crude protein extract from etoposide-treated (20 uM for 8 hours) NGP cells were prepared and immunoprecipitated for MycN according to the procedure listed in 3.3. The immunoprecipitated samples were eluted by boiling beads in Laemmli sample buffer and separated by SDS-PAGE. The band corresponding to p53 would have been cut out of the gel and sent to the WPI Mass Spectrometry Core, PI Scott Shaffer, for LC-MS/MS and post-translational modification analysis.

3.4: CUT&RUN Chromatin Profiling

The procedure described in Hainer et al (2019), omitted here due to its length, would have been used for CUT&RUN chromatin profiling using the α-p53 mouse antibody (DO1, Santa Cruz).

4: Goals and Objectives

The following report seeks to indicate that the binding of MycN and p53 plays a role in neuroblastoma while further exploring the interaction of the two molecules. This will be accomplished through examination of the procedures and findings outlined in Agarwal et al (2018) and can be summarized by the following main goal of this report:

> **Main Goal:** To replicate and expand upon the findings of Agarwal et al (2018) by further investigating the relationship between MycN and p53 and the impacts of this interaction in neuroblastoma.

In order to accomplish this main goal, three supporting objectives have been created and are listed and rationalized below.

> ***Objective 1:*** *To determine the extent of MycN and p53 binding in various models of neuroblastoma and to determine the conditions which yield the most robust indication of endogenous binding of MycN and p53.*

Before expanding upon the results obtained by Agarwal et al (2018), the preliminary procedures of this project should focus upon replicating the binding interaction of MycN and p53 in the first cell line utilized by Agarwal et al (2018) and at least two other cell lines. Additionally, in order to proceed to the remaining objectives, determining the conditions which consistently yield the most robust interaction of MycN and p53 is crucial.

> ***Objective 2:*** *To further examine the binding interaction of MycN and p53 by analyzing the post-translational modifications present in the C-terminal domain of MycN-bound p53.*

There is still much to be discovered about the relationship between MycN and p53. It is widely known that post-translational modifications of specific residues in p53 are vital to p53's functionality, and it has been suggested that the typical post-translational modifications of p53 are dysregulated in cancer tumorigenesis (Dai & Gu, 2010). Additionally, a wide number of post-translational modifications in p53 are localized to the C-terminus, which is also the location of MycN binding. By analyzing the post-translational modifications of MycN-bound p53, more can be learned about the interaction of the two molecules and whether or not a specific signature of post-translational modifications is required for the binding of MycN to p53's C-terminus.

Objective 3: *To investigate the chromatin binding profile of the MycN-p53 complex through the novel chromatin profiling technique CUT&RUN.*

Agarwal et al (2018) utilizes ChIP-seq to demonstrate the binding of the MycN-p53 complex at both p53 response elements and MycN E-boxes for a small number of genes. The use of CUT&RUN, a cheaper protocol that generates less background noise, can help to confirm and expand upon these results.

5: Results, Next Steps, and Discussion

The following section describes the results obtained by this project and discusses their importance in the context of neuroblastoma.

5.1: p53 and MycN Are Binding Partners

In order to validate the results of Agarwal et al (2018) and complete Objective #1, co-immunoprecipitations for p53 and MycN were performed under various conditions.

5.1.1: p53 and MycN binding in MycN3 cells

In order to most closely recreate the conditions used by Agarwal et al (2018), MycN3, a cell line with a tetracycline-controlled activator for MycN expression, was utilized. After a time-course assay using the dosage of doxycycline described in Agarwal et al (2018), it was determined that maximum expression of both p53 and MycN would occur between 6 and 10 hours of treatment. MycN3 cells were then treated under five conditions (untreated, doxycycline (1ug/mL), etoposide (20μM), doxycycline and etoposide for 6h, doxycycline and etoposide for 10h); p53 was then immunoprecipitated. As seen below, while the whole-cell lysate shows a large amount of MycN present, there is no MycN in the immunoprecipitated samples. The blot was re-blotted with larger amounts of primary antibody and exposed for a longer period of time, but there was still no signal indicating the presence of MycN in the blot (data not shown). At this time, it was determined that replicating the experiment in a cell line wherein MycN did not require doxycycline induction would yield better results and a more accurate depiction of the interactions of MycN and p53 in neuroblastoma tumors.

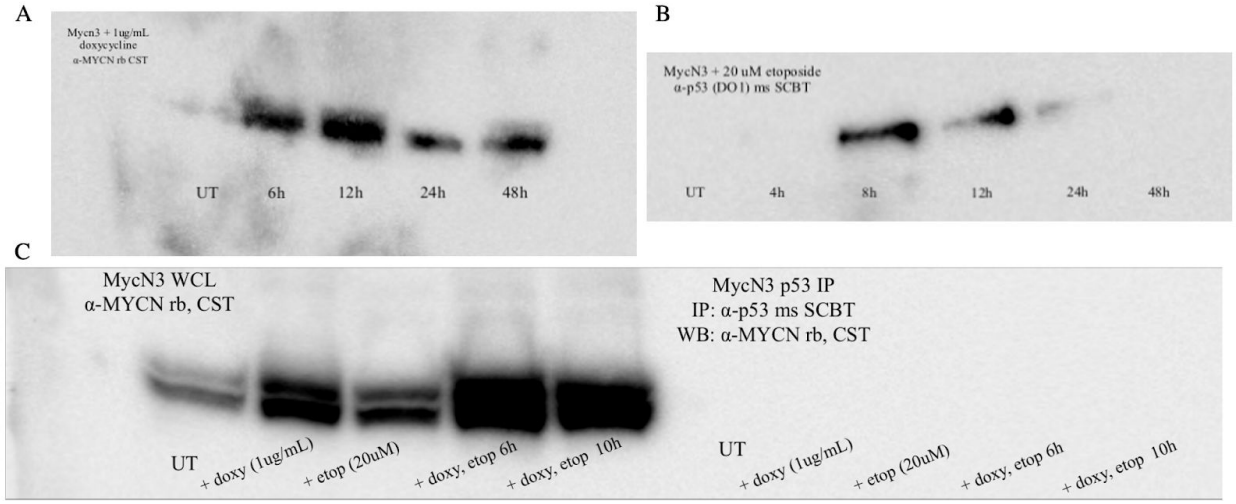

Figure 5: p53 and MycN binding in MycN3 cells (A) MycN3 cells were treated with 1 ug/mL doxycycline for varying amounts of time to determine the treatment condition leading to maximum MycN expression. **(B)** MycN3 cells were treated with 20 μM etoposide in order to determine the ideal treatment conditions for maximum p53

expression. **(C)** MycN3 cells were treated under various conditions to determine the best conditions for p53 and MycN co-immunoprecipitation.

5.1.2: p53 and MycN binding in JF cells

After a time-course and dose-response assay, it was determined that the proper dosage of etoposide treatment to maximize expression of both p53 and MycN was 20 μM for 6 hours (Figure 6A). Additionally, a series of ten lysis buffers was tested in order to determine the most effective lysis buffer to maximize protein concentration in the whole-cell lysate (See Appendix X for lysis buffer compositions). Bands in Figure XB are present, indicating weak interactions of p53 and MycN; however, the slight increase in band intensities in the lanes corresponding to lysis buffers 7, 9, and 10 provides a basis for further comparison of these lysis buffers for co-immunoprecipitation.

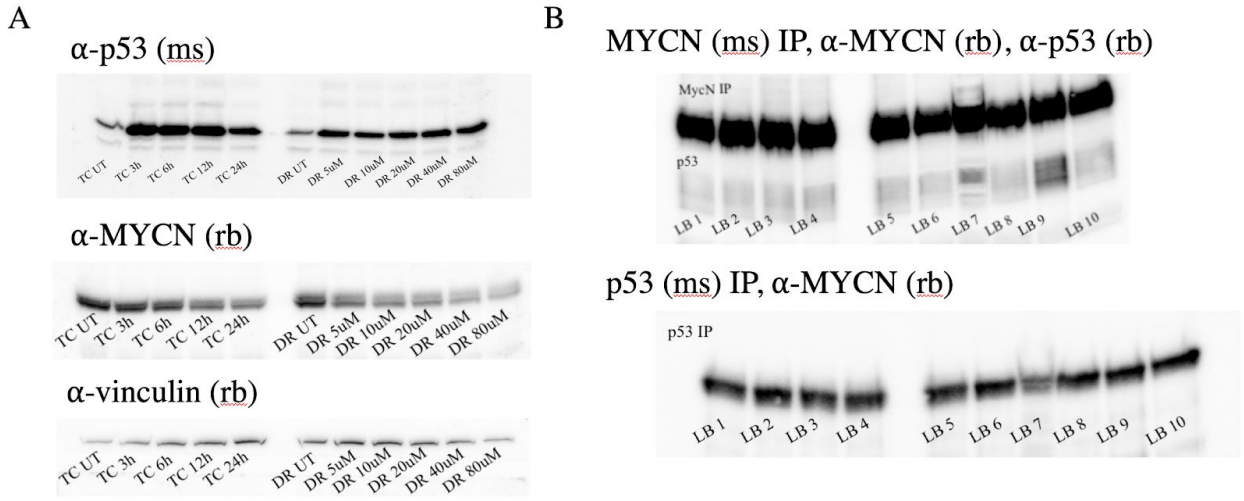

Figure 6: p53 and MycN binding in JF cells (A) JF cells were treated with either etoposide under varying treatment conditions to determine the ideal treatment condition for maximum MycN and p53 expression. **(B)** JF cells were lysed with various lysis buffers and immunoprecipitated in order to determine the lysis conditions to maximize co-immunoprecipitation protein concentrations.

5.1.3: p53 and MycN binding in NGP cells

In order to determine if the results seen in 5.1.2 were replicable in other NB cell lines, the binding of MycN and p53 was investigated in the neuroblastoma cell line NGP. Cells were treated with 20 μM etoposide for 8h; the initial NGP experiment compared the four lysis buffers discussed in section 5.1.2 (Buffers 1, 7, 9, 10). Immunoprecipitation was performed for all four conditions utilizing both p53 (ms) and MycN (ms) antibodies. As can be seen in Figure X, the immunoprecipitations yielded a small amount of protein corresponding to very faint bands; of the bands seen, lysis buffers 1 and 7 were chosen for additional analysis. In order to hopefully strengthen the bands seen in the immunoprecipitations, the following immunoprecipitations seen

in Figure 7B were performed using a MycN rabbit antibody. As can be seen, the α-MycN (rb) immunoprecipitation showed a much more robust interaction between p53 and MycN than had been seen in previous experiments. For this reason, the remaining experiments requiring co-immunoprecipitations utilized the MycN rabbit antibody.

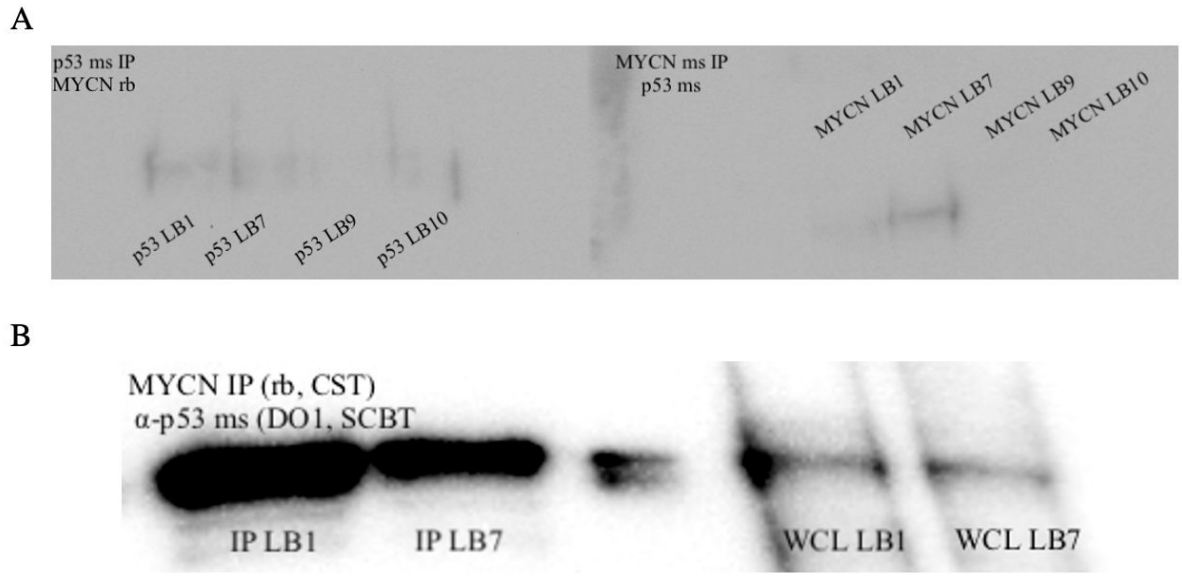

Figure 7: p53 and MycN binding in NGP cells (A) Immunoprecipitations for p53 (left) and MycN (right) utilizing the most successful lysis buffers from 4.1.2. **(B)** Immunoprecipitation for MycN utilizing a rabbit antibody as opposed to a mouse antibody.

5.1.4: p53 and MycN are binding partners, summarized

The data shown in Section 4.1 indicate that p53 and MycN do participate in a binding relationship in various models of neuroblastoma. These preliminary results indicate that it is possible that the interaction in the context of these cell lines is weaker than first described; however, further work should help to indicate the degree of this interaction.

5.2: Next Steps

Unfortunately, due to the onset of the SARS-CoV-2 outbreak, the labs at WPI and UMMS were closed to preserve personal protective equipment for clinicians. In light of these closures, the initially-planned mass spectrometry and CUT&RUN procedures were unable to be performed within the time frame of this project. This section will discuss future directions for this project should another student be interested in continuing to study the interaction between MycN and p53.

5.2.1: Mass Spectrometry for Analysis of Post-Translational Modifications

As discussed in Section 4: Goals and Objectives, Objective #2 centered around the investigation of MycN-bound p53's post-translational modifications. Due to the importance of

post-translational modifications for p53's function and the localization of several important post-translational modifications to the C-terminus of p53, it is possible that the post-translational modification profile of p53 helps to contribute to its interaction with MycN. The University of Massachusetts Medical School's Mass Spectrometry facility offers a workflow that utilizes LC-MS/MS to analyze the post-translational modifications of a protein. In order to determine MycN-bound p53's post-translational modifications, the following experimental setup should be utilized: Treat either NGP or JF cells with 20 μM etoposide for 8 hours and follow the co-immunoprecipitation protocol described in section 3.3; the immunoprecipitation should utilize the α-MycN rabbit antibody from Cell Signaling Technologies. The immunoprecipitate should then be separated via SDS-PAGE and stained with Coomassie blue. The band corresponding to p53 should then be cut out of the gel and transported to the University of Massachusetts Medical School's Mass Spectrometry facility for analysis.

5.2.2: CUT&RUN Chromatin Profiling

The findings demonstrated by Agarwal et al (2018) utilized ChIP-qPCR to investigate MycN and p53 binding at a select number of genes,including p21, CHEK1, SESN1, and CDC6. While ChIP has been the gold standard for chromatin profiling for a long time, the use of CUT&RUN yields better resolution results, is more efficient, and is much more cost-effective. The role of CUT&RUN in this project would have hopefully been to examine binding of the MycN-p53 complex at a wider variety of loci than the original publication due to its ability to examine more loci at a lower cost. For example, the paper mentions over 90 genes pulled from MycN-ChIP-seq and p53-ChIP-seq databases that contain either MycN E-boxes or p53 response elements. Examining the potential binding of the MycN-p53 complex at any number of these genes, including the original four genes, has the potential to confirm that Agarwal et al's (2018) conclusion that the MycN-p53 complex does indeed bind to both p53 RE's and MycN E-boxes, altering p53's binding activity.

6: Conclusion

The mechanisms underlying neuroblastoma, a neural-crest derived cancer accounting for up to 10% of pedric cancers, are not well defined. Previous research has suggested that a wide range of molecular players contribute to the tumorigenicity of neuroblastoma; one such molecular driver is MycN. In fact, MycN amplification is widely considered to be the most negative prognostic factor in neuroblastoma. Recent research has implicated a novel interaction of MycN with the tumor suppressor protein p53 in MycN-amplified models of neuroblastoma. This interaction is of great interest and has been proposed to be a potential explanation for MycN-amplified neuroblastoma's modified response to chemotherapy and genomic instability. This report sought to further investigate this interaction in order to learn more about the mechanisms underlying neuroblastoma.

The goal of further investigating the relationship between MycN and p53 remained the central focus throughout the duration of this project. However, with the transition to remote learning and the closure of academic labs due to SARS-CoV-2 pandemic, the methods of achieving this goal changed. Instead of performing the experimental work that would have led to the achievement of the final two objectives, the rationale and experimental outlines for these objectives were outlined above in section 5.2. This information can be utilized by students in the future who wish to continue to investigate the interaction of MycN and p53. However, the first objective of the project, determining the conditions and cell lines which demonstrate the most robust endogenous interaction of MycN and p53, was met. There is still some room to refine the co-immunoprecipitation protocol in order to maximize its efficiency and the amount of pulled-down proteins, however, the protocol was sufficient for the purposes of this project.

All in all, while this project may have been unfortunately cut short, the goal of further exploring the MycN-p53 interaction was met, even if to a smaller extent than originally planned. It is the author's hope that future students are able to utilize this foundational research to further investigate the complexities of this interaction and its further implications on neuroblastoma.

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Appendices

Appendix A: International Neuroblastoma Staging System

Stage	Surgical Characteristics
Stage I	Small, localized tumors; completely resected
Stage II	Small tumors; could not be completely resected; may or may not have had lymph node involvement
Stage III	Large tumors; crossed anatomic midline; could not be completely resected
Stage IV	Metastatic disease
Stage IVS	Metastatic disease in patients under one year; metastases confined to liver, skin, bone marrow

Adapted from Louis and Shohet, 2015

Appendix B: International Neuroblastoma Risk Group (INRG) Staging System

Stage	Definition
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

Retrieved from Louis and Shohet, 2015

Appendix C: International Neuroblastoma Risk Group (INRG) consensus pretreatment classification schema

INRG Stage	Age (mo)	Histologic category	Grade of tumor, differentiation	MycN	11q abberation	Ploidy	Pre-treatment risk group
L1/L2	Any	GN maturing or GNB intermixed	Any	Any	Any	Any	A Very low
L1	Any	Any, except GN maturing or GNB intermixed	Any	NA Amp	Any	Any	B Very low K High
L2	<18	Any, except GN maturing or GNB intermixed	Any	NA NA	No Yes	Any	D Low G Intermediate
L2	>18	GN nodular; Neuroblastoma	Differentiating Differentiating Poorly differentiated or undifferentiated	NA NA NA Amp	No Yes	Any	E Low H Intermediate H Intermediate N High
M	<18 <12 12 to <18 <18 ≥18	Any	Any	NA NA NA Amp	Any	Hyperdiploid Diploid Diploid	F Low J Intermediate J Intermediate O High P High
MS	<18	Any	Any	NA NA Amp	No Yes		C Very low Q High R High

Retrieved from Louis and Shohet, 2015

Appendix D: Lysis Buffer Compositions

	1	2	3	4	5	6	7	8	9	10
	original	testing conditions								
TrisHCl pH8	50mM	50mM	50mM	50mM	50mM	50mM	**50mM HEPES 7.5**	**50mM HEPES 7.9**	50mM	50mM
NaCl	150mM	150mM	150mM	**100**	**200**		150mM	150mM	150mM	150mM
NP-40	0.50%	**0.20%**	**1.00%**	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%	**TX-100 0,5%**
DTT	1mM	1mM	1mM	1mM	1mM	**0**	1mM	1mM	1mM	1mM
Prot-Phos Inh	1X	1X	1X	1X	1X	1X	1X	1X	1X	1X
EDTA	1mM	1mM	1mM	1mM	1mM	1mM	1mM	1mM	**0**	1mM