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Alternative regulation of MYC in lung cancer

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ALTERNATIVE REGULATION OF MYC IN LUNG CANCER

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Patrick N. Backman

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I dedicate this thesis to Rusty, the greatest dog who ever lived.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALK	anaplastic lymphoma kinase
EGFR	epidermal growth factor receptor
FL-MYC	full length, canonical MYC
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
lenti-pri-*let-7a-1*	a lentiviral plasmid that overexpresses pri-*let-7a-1*
mat-let-7a-1	mature let-7a-1
miR-34	microRNA-34
miRNA	microRNA
mRNA	messenger RNA
MYC	Myelocytomatosis oncogene cellular homolog
NSCLC	non-small cell lung cancer
PCR	polymerase chain reaction
pre-let-7a-1	precursor let-7a-1
pri-let-7a-1	primary let-7a-1
qPCR	real time polymerase chain reaction
RISC	RNA induced silencing complex

ABSTRACT

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Lung cancer is the leading cause of cancer deaths in the United States, accounting for 27% of all cancer induced deaths[1]. In an attempt to create a effective targeted therapy for the treatment of lung cancer, a strategy used to treat an activated *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ transgenic lung cancer mouse model was to deliver a known tumor suppressive microRNA (miRNA) to stop tumor growth. The tumor suppressive miRNA *let-7* was lentivirally delivered in the form of its primary transcript, pri-*let-7a-1*, and resulted in increased lung size and inflammation compared to lungs exposed to a control lentivirus. It was identified that *LIN28B* transcripts were elevated in this transgenic model[2] and a truncated MYC protein product, separate from canonical MYC, was overexpressed with activation of the transgenic lung cancer mouse model. LIN28B is a pluripotent factor and post-transcriptional inhibitor of *let-7* biogenesis[3–5]. Therefore, it was hypothesized that the LIN28B mediated accumulation of pri-*let-7a-1* transcripts promoted expression of the truncated MYC protein product, termed T-MYC. Through this work, it was determined that T-MYC expression is not dependent on the LIN28B mediated accumulation of pri-*let-7a-1* transcripts and that T-MYC is likely not a variant of canonical MYC.

CHAPTER 1: INTRODUCTION

Lung cancer is the largest contributor to the total number of cancer deaths worldwide with an estimated 158,040 deaths in the United States in 2015; 26.8% of all cancer associated deaths. Additionally, the number of new lung cancer diagnoses increases every year with an estimated 221,200 new cases in 2015 alone[1]. Despite efforts to create effective therapies for the treatment of lung cancer, the five-year survival rate for patients with this cancer type remains at a dismal 17%[1]. This poses a great challenge to both the medical and scientific communities to elucidate the underlying causes of lung cancer.

Patients who are diagnosed with lung cancer are typically recommended to undergo initial treatments that are more general in nature. This often includes measures such as the surgical removal of tumor tissue from the lungs in early stages of disease progression and chemotherapy or radiation therapy[6]. A major limitation in utilizing these techniques as a means of treating the disease is that lung cancer is typically not detected until later stages of progression, a point in time when metastatic disease is much more likely. This creates a situation in which traditional treatment measures, i.e. surgery, chemotherapy, and radiation therapy, are much less effective in halting lung cancer growth and progression[7]. In an effort to combat this problem, a great deal of research

effort has been expended on developing therapies that are specific and more effective in treating late-stage lung cancer, a more aggressive disease state.

Lung cancer, when diagnosed, is categorized into one of several subtypes depending on its clinical presentation. Non-small cell[8], small cell[9], and lung carcinoid tumors[10] are the subgroups that are used clinically to classify lung cancers. Non-small cell lung cancer (NSCLC) is the most prevalent type, comprising approximately 80% of diagnosed lung cancers. This lung cancer subtype encompasses several types of cancers, including squamous cell carcinoma, large cell carcinoma, and adenocarcinoma[11].

It is well known that the molecular landscape within a cancer cell is dysregulated when compared to its normal, healthy counterpart; that is, many normal cellular processes and molecular mechanisms are altered in cancer and much of this alteration has been established as being crucial for the development, maintenance, and growth of cancer. In cancer, this often results in the up- or down-regulation of factors that are important for development as well as maintaining a differentiated cellular state. Factors that are down regulated in cancer and are important for limiting cellular growth and promoting processes that result in cellular death are called tumor suppressors. Vice-versa, oncogenic factors are up regulated in cancer and allow cells to overcome processes that are important in controlling how cells grow and eventually die[12]. Efforts to create a targeted therapy for cancer are often focused on either the reintroduction of tumor-suppressive factors in a background where they are lost or blocking the action of oncogenic factors that are up regulated in a cancer type.

The major drugs currently on the market for the targeted treatment of late stage non-small cell lung cancer focus on blocking the action of such oncogenic factors.

Gefitinib and erlotinib are small molecule inhibitors that specifically target and block the action of the epidermal growth factor receptor, EGFR[13]. *EGFR* is a gene that has been found to be mutated and upregulated in approximately 43-89% of NSCLC tumors[14] and is responsible for activating signaling that promotes cell growth[14]. While it has been demonstrated that these drugs are effective in inhibiting the action of EGFR, their effectiveness is limited to lung cancers with *EGFR* upregulation. There are additional targeted therapies for the treatment of lung cancer, but they also rely on the upregulation of specific oncogenic factors that have implications in promoting lung cancer progression[15,16]. Inhibitors that block the anaplastic lymphoma kinase, ALK, are also used for the targeted treatment of lung cancer but are more limited as ALK rearrangements implicated in cancer formation are seen in approximately 2-7% of NSCLC tumors[15,17,18]. This poses a significant challenge to researchers to elucidate additional mechanisms that are widely activated in lung cancer that could serve as a basis of therapeutic intervention.

MYC is a family of proto-oncogenic transcription factors that have been established to be important in development and are also implicated in promoting cancer[19–21]. The increased expression of MYC, through varying mechanisms, is observed in approximately 30% of all cancers, signifying its importance and broad impact as an oncogene[22,23]. When expressed, MYC forms a dimer with a protein, MAX, that positively regulates the effects of MYC, binds to E-box sequences contained within the promoters of genes, and mediates transcriptional changes to its target genes[24,25]. Studies focused on understanding exactly what genes MYC regulates have revealed its ability to influence the expression of a large number of genes, especially ones that are already

being actively transcribed within a cell[26,27]. The response of cells to increased expression of MYC include, but are not limited to, changes in processes that promote cancer such as inducing cell cycle progression, influencing apoptosis, decreasing genomic stability, and enhancing cell growth[28]. The broad reaching effects of MYC on cells make it not only an important oncogene but therapeutically intervening on its action could prove to be an invaluable asset in the treatment of many different cancer types, including lung cancer.

Interestingly, there are several family members of MYC that have varying abilities of producing the pro-tumorigenic phenotype associated with MYC's oncogenic function. Of the family members, only N-MYC, c-MYC, and L-MYC have been demonstrated to have the potential to lead to neoplastic growth when expressed in cancer[29]. c-MYC is expressed in many different cancer types, L-MYC is expressed in small cell lung cancer[30], and N-MYC expression is seen in neuroblastoma[31]. Additionally, there are protein products that arise from alternative translation of *MYC* mRNA. These products are collectively called MYC-S and are truncated versions of c-MYC, lacking the N terminus of the canonical protein product. It has been demonstrated that these products have the ability to form a dimer with Max, the positive regulator of MYC, and inhibit transactivation of transcription of canonical MYC targets[32,33]. Together, this demonstrates one facet of the complexity surrounding the biology of MYC and its family members within the context of cancer.

In an effort to understand the regulatory mechanisms MYC's expression influences, it was identified that one subset of transcripts that MYC regulates is that of microRNAs (miRNAs)[34]. miRNAs are small, 18-24 nucleotide, non-coding RNAs that are transcribed in the nucleus as a longer primary miRNA transcript (pri-miRNA) and

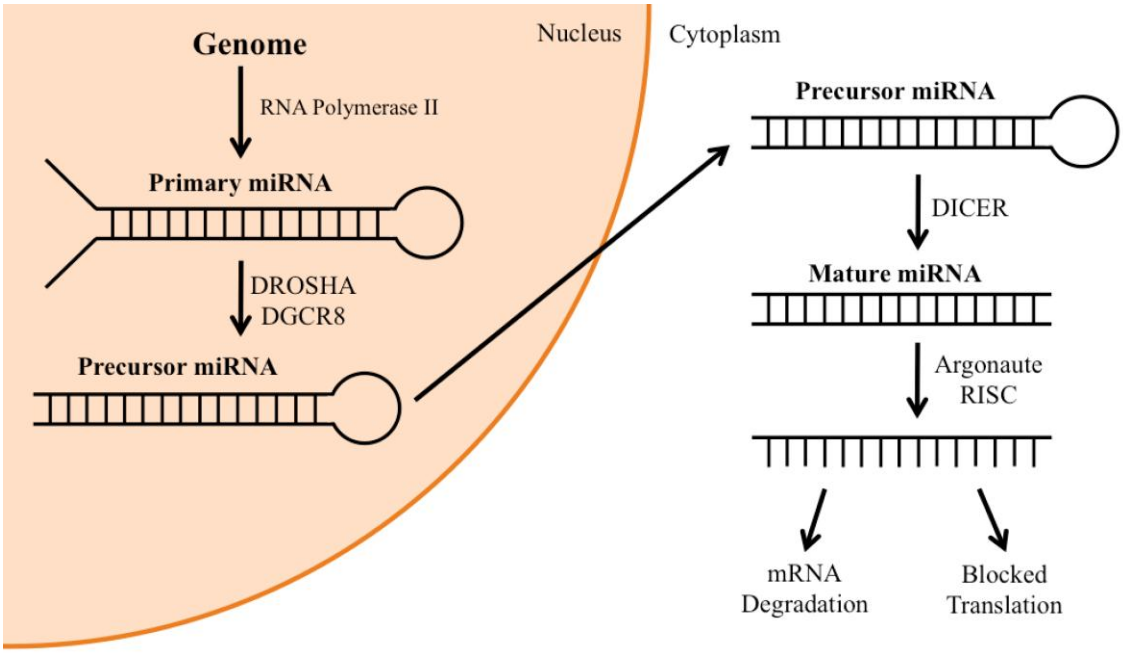

Figure 1: Canonical microRNA biogenesis pathway. The primary miRNA is transcribed by RNA Polymerase II and forms the characteristic hairpin-loop structure. The flanking regions of the transcript are cleaved by DROSHA and DGCR8 to form the precursor miRNA. The precursor miRNA is then exported into the cytoplasm where it is further cleaved by DICER. The guide strand is then loaded into Argonaute and further associates with the RNA Induced Silencing Complex to mediate mRNA degradation or inhibition of translation of mRNA targets.

form a hairpin loop structure, as seen in Figure 1[35]. The pri-miRNA is then processed into a shorter transcript, termed the precursor miRNA (pre-miRNA), by the RNAse processing enzyme DROSHA. The pre-miRNA is exported into the cytoplasm where it undergoes further cleavage into a double stranded RNA molecule by another RNase enzyme, DICER[36]. The guide strand of the miRNA duplex is then loaded into Argonaute, a component of the RNA Induced Silencing Complex (RISC). RISC is then poised for the post-transcriptional down-regulation of gene expression, and ultimately protein expression, through base complementarity of the mat-miRNA with its target messenger RNAs (mRNAs)[36,37]. miRNA mediated regulation of genes signifies an important regulatory mechanism that contributes to maintaining cellular states in development and differentiation and is a mechanism that is highly dysregulated in cancer.

Accordingly, MYC can both up- and down-regulate the expression of both tumor suppressive and oncogenic miRNAs, which can influence the fate of cells. For example, MYC has been shown to directly cause the expression of *miR-17-92*, an oncogenic miRNA cluster, which contributes to the tumorigenic phenotype associated with MYC by regulating genes that control chromatin modifications[38]. Alternatively, MYC also causes down-regulation of miRNAs important in controlling a proliferative phenotype, such as the tumor suppressive miRNA *let-7*. *let-7* was originally identified in *Caenorhabditis elegans* as an important developmental regulator and was later found to be conserved in humans. *let-7* expression is commonly down-regulated or lost in cancer, allowing for the uncontrolled expression of its target proto-oncogenes such as *KRAS, MYC,* and *LIN28B*[39–42]. As seen in Figure 2, MYC directly causes the expression of LIN28B, which

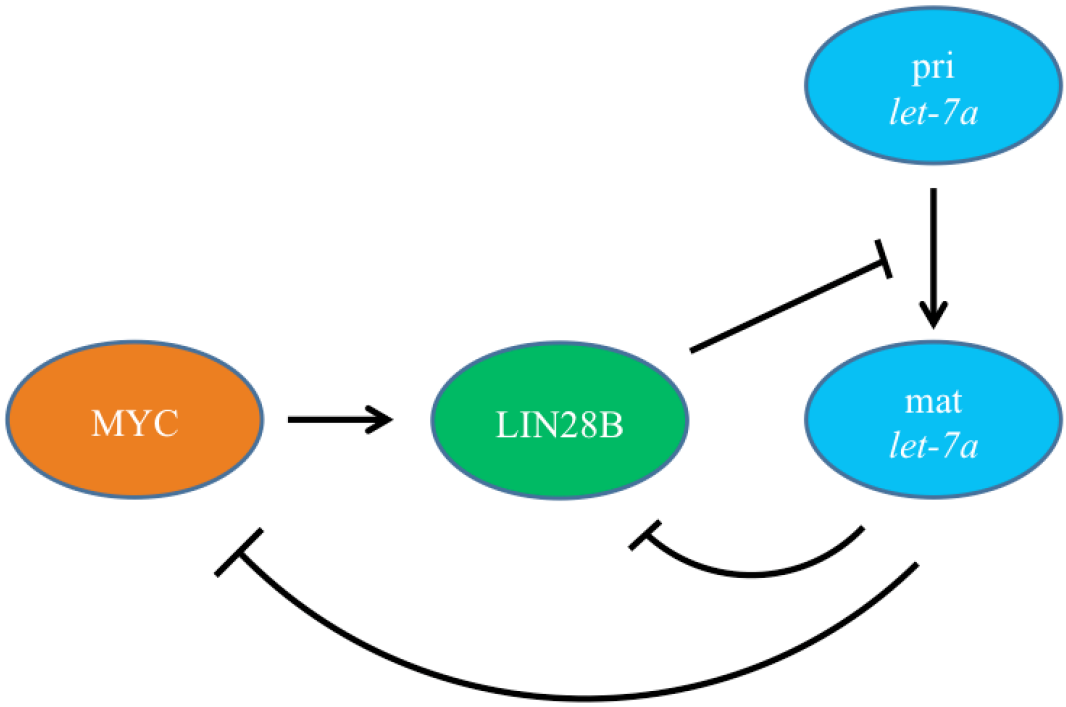

Figure 2: Negative feedback regulation of MYC, LIN28B, and *let-7*. In a differentiated cell, canonical *let-7* processing occurs allowing the negative regulation of MYC and LIN28B. In cancer, the expression of MYC promotes LIN28B expression, blocking the biogenesis of mat-*let-7a*, therefore losing the negative regulation of MYC and LIN28B.

in turn blocks the processing of *let-7* due to LIN28B being a post-transcriptional inhibitor of *let-7* biogenesis. This signifies an important negative feedback loop where in a differentiated cell, *let-7* targets and down-regulates both *MYC* and *LIN28B* but in a neoplastic state, such as cancer, the expression of these oncogenes is uncontrolled[3,39]. The overexpression of MYC and subsequently LIN28B then causes the decreased maturation of *let-7*[3]. MYC expression causes large changes in regulatory mechanisms that contribute to the tumorigenic state often associated with expression of this oncogene in cancers. Restoring regulatory mechanisms that are dysregulated by MYC not only contributes to understanding the role of MYC in cancer, but could also reveal potential therapeutic targets for the treatment of cancer.

Therapies have been developed to directly block the action of MYC within cancer which includes strategies to create small molecule inhibitors of MYC-Max dimerization[43] as well as targeting upstream mechanisms that influence MYC's expression and activity[44]. Creating effective targeted therapies against transcription factors has proven to be difficult due to the fact that they lack enzymatic activity, thus having less specific drug targets within their protein structure[45]. In the past few years, however, the therapeutic potential of using miRNAs as an intervention has shown promising results[46–48]. miRNAs as a whole can be classified as either being tumor suppressive or oncogenic. Tumor suppressive miRNAs usually down regulate processes that contribute to cancer and their expression is decreased or nonexistent in cancer. Oncogenic miRNAs are overexpressed in cancer and their expression results in a pro-tumorigenic phenotype[46,48]. Due to the fact that a single miRNA has many mRNA targets[37], the reintroduction of a single tumor suppressive miRNA or blocking the action of an oncogenic miRNA could mimic a drug

cocktail through reestablishing the canonical regulation of many genes at once. This has been demonstrated with the miRNA, *miR-34*, a tumor suppressive miRNA commonly down regulated in cancer[49]. A *miR-34* mimic is currently in clinical trials for the treatment of cancer due to its demonstrated ability to inhibit cellular processes that contribute to tumor formation and growth[50,51]. There is great potential in exploiting the use of miRNAs as a cancer therapy not only to reestablish tumor suppressive mechanisms but to also block oncogenic processes occurring within cancer. miRNA therapies could greatly contribute to the treatment of lung cancer, an aggressive cancer type with few effective therapeutic options.

To test the effectiveness in using microRNAs (miRNAs) as a therapeutic intervention, it was hypothesized that a tumor suppressive miRNA could be exogenously delivered and halt tumor progression. The lung cancer model being studied was a $Kras^{G12D/+};p53^{R172H/+}$ transgenic mouse model. The transgenes in this model are activated through genomic recombination of loxP sites with Cre Recombinase and results in production of mutant Kras and p53. *KRAS* mutation, and subsequent overexpression, has direct implications in lung cancer and is found in approximately 30% of lung adenocarcinomas[52]. *TP53* encodes the tumor suppressive protein p53 that is the most commonly perturbed gene in cancer, mutated in approximately 40% of all lung cancers[53]. To test the hypothesis that a tumor suppressive miRNA could halt tumor growth in this model, unprocessed, pri-*let-7a-1* was lentivirally delivered to the activated $Kras^{G12D/+};p53^{R172H/+}$ transgenic mouse model. Primary-*let-7a-1* (pri-*let-7a-1*) is the full length transcript of *let-7a* that requires processing through the canonical miRNA biogenesis pathway, Figure 1, to mediate the tumor suppressive function of *let-7* through

its mature transcript, mat-*let-7a*. The response seen with the lentiviral delivery of pri-*let-7a-1* was increased lung size and inflammation compared to lungs exposed to a lentiviral control, as seen in Figure 3. This result was in strict contrast to the hypothesis that pri-*let-7a-1* would be processed through the canonical miRNA biogenesis pathway, seen in Figure 2, and mat-*let-7a* and would subsequently downregulate its target oncogenes and ultimately halt tumor progression.

It was later determined in the $Kras^{G12D/+};p53^{R172H/+}$ transgenic model that *LIN28B* transcripts were overexpressed[2]. LIN28B is a post-transcriptional regulator of *let-7* that blocks maturation of *let-7* sequences, negating the tumor suppressive capabilities of *let-7*[4,5,54,55]. It was hypothesized that *let-7* targets would be dysregulated in this model due to the LIN28 mediated inhibition of *let-7* biogenesis. To determine if *let-7a* targets were dysregulated in the $Kras^{G12D/+};p53^{R172H/+}$ activated transgenic mouse model, lung tissue was assessed for the presence of MYC, a known oncogene that is targeted and down-regulated by mat-*let-7a*[39,56]. In tissues of a non-activated (normal) lung compared to lungs harvested from the $Kras^{G12D/+};p53^{R172H/+}$ activated model of lung tumor formation, the expression of canonical MYC was not affected. Interestingly, however, a truncated form of MYC was detected as being overexpressed in the activated tumor model compared to the normal lung tissue, seen in Figure 4. This led to the overall hypothesis of this work that the LIN28B mediated accumulation of pri-*let-7a-1* promoted the expression of a truncated form of MYC. To test this hypothesis, this work is divided into two main areas. First, since it was hypothesized that the truncated MYC protein product was indeed a variant of MYC, this protein was characterized. Secondly, the ability of pri-*let-7a-1* to induce the expression of this truncated MYC protein was evaluated.

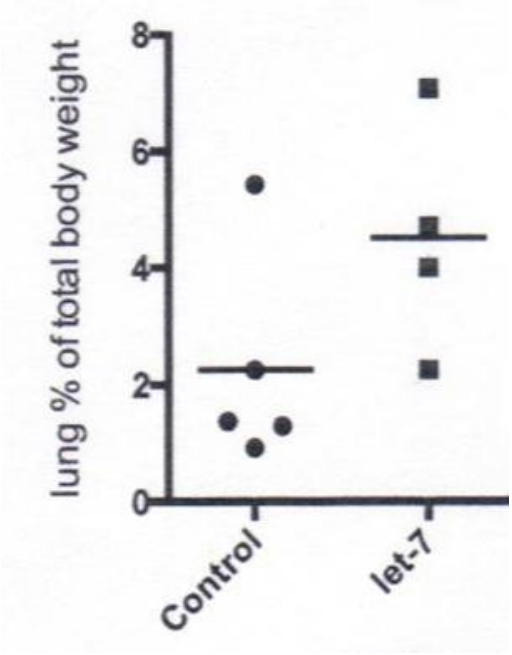

Figure 3: Lentivirally delivered pri-*let-7a-1* increases lung size. Pri-*let-7a-1* was lentivirally delivered to a $Kras^{G12D/+};p53^{R172/+}$ lung cancer mouse model, lungs were weighed and normalized to the total weight of the animal compared to lungs exposed to a control lentivirus.

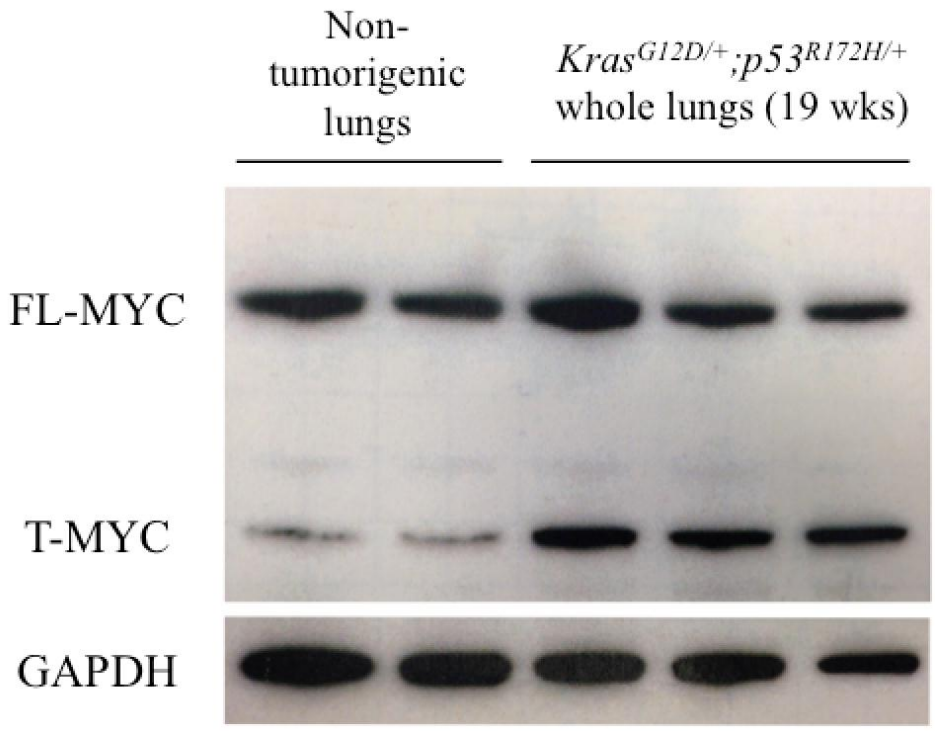

Figure 4: A truncated MYC protein product is induced in the $Kras^{G12D/+};p53^{R172/+}$ lung cancer mouse model. Transgenic mice were administered adenovirus-cre and tumor formation progressed for 19 weeks. Protein was extracted and assessed for the expression of MYC compared to non-tumorigenic lungs by SDS-PAGE and immunoblotting.

CHAPTER 2: CHARACTERIZATION OF THE TRUNCATED MYC PROTEIN PRODUCT, T-MYC

Activation of the *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ transgenic mouse model causes expression of mutated Kras and p53 which leads to tumor formation[2]. Molecularly, it was identified that a truncated variant of canonical MYC, termed T-MYC, was also overexpressed upon activation of this lung cancer model. As a means of determining if T-MYC was indeed a truncated variant of canonical MYC, several strategies were implemented to characterize this novel protein product.

The expression of T-MYC was first identified *in vivo* and was hypothesized to be induced by the accumulation of pri-*let-7a-1* through LIN28B. To test that the expression of the truncated form of MYC, termed T-MYC, could be replicated *in vitro*, the lentiviral plasmid that expresses the pri-*let-7a-1* sequence was transfected into A549 and Calu6 lung adenocarcinoma cell lines, both which have detectable levels of LIN28B expression. After transfection of lenti-pri-*let-7a-1* as well as a control lentiviral plasmid, cell lysates were assessed for the presence of T-MYC through SDS-PAGE and immunoblotting. As seen in Figure 5, T-MYC expression was induced in both A549 and Calu6 cell lines when the lenti-pri-*let-7a-1* plasmid was transfected compared to lysates obtained from cells transfected with the control lentiviral plasmid. This finding confirmed that T-MYC

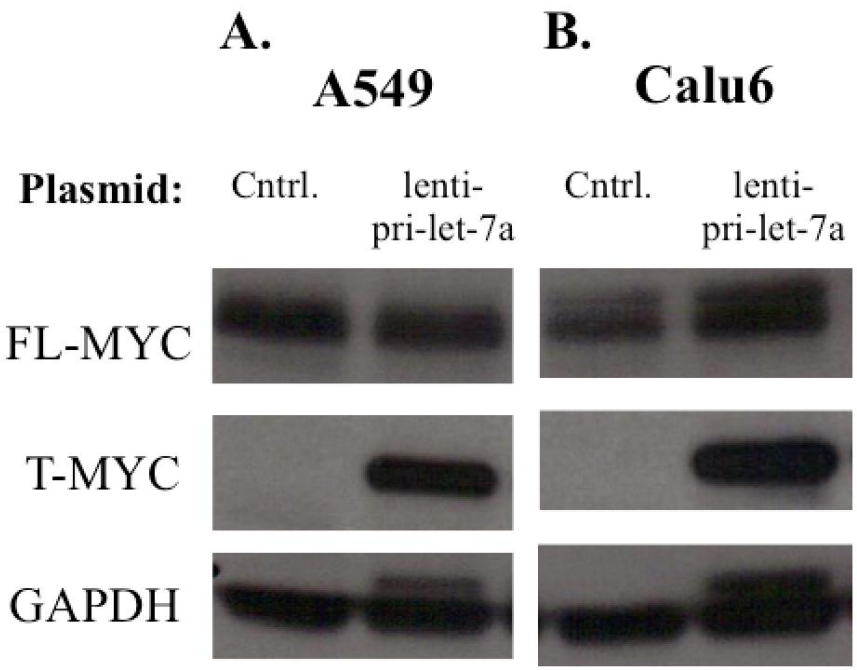

Figure 5: T-MYC expression is induced in cell culture. A control lentiviral plasmid or lenti-pri-*let-7a-1* was transfected in A549, A., and Calu6, B., lung adenocarcinoma cells. 48hrs post-transfection cells were lysed and protein was analyzed for the expression of T-MYC through SDS-PAGE and immunoblotting.

expression could be duplicated *in vitro* and signified its potential as a contributor to the pro-tumorigenic phenotype seen when lenti-pri-*let-7a-1* was transduced *in vivo*.

In an effort to further validate T-MYC as an alternate form of canonical MYC, several strategies were utilized. The first strategy was to simply determine if T-MYC could be detected through immunoblotting with several different primary antibodies generated to bind MYC. To do so, T-MYC expression was induced by transfection of the lenti-pri-*let-7a-1* plasmid in A549 cells and compared to cells transfected with a control lentiviral plasmid. Cellular lysates obtained post-transfection were assessed through SDS-PAGE and immunoblotting for the presence of T-MYC expression using several different primary antibodies that detect human MYC. Three antibodies were used to detect MYC, one that was generated to bind an epitope closer to the N-terminus of MYC made by Cell Signaling and two that were generated to recognize and bind closer to the C-terminus of MYC made by Sigma Aldrich and Abcam. Interestingly, T-MYC was detected only using antibodies generated to bind epitopes on the C-terminus of MYC, as seen in Figure 6. This finding suggested that T-MYC was a protein variant of canonical MYC and that it was lacking most of the N-terminus but retained the C-terminus of canonical MYC within its protein structure.

Small-interfering RNA (siRNA) mediated knock-down of T-MYC was a final strategy employed to understand if T-MYC was in fact a protein variant of MYC. It was hypothesized that if T-MYC is a truncated form of canonical MYC, then it would be knocked down with an siRNA that also knocks down canonical, full-length MYC. To test this hypothesis, A549 cells were co-transfected with either a control or lentiviral-pri-

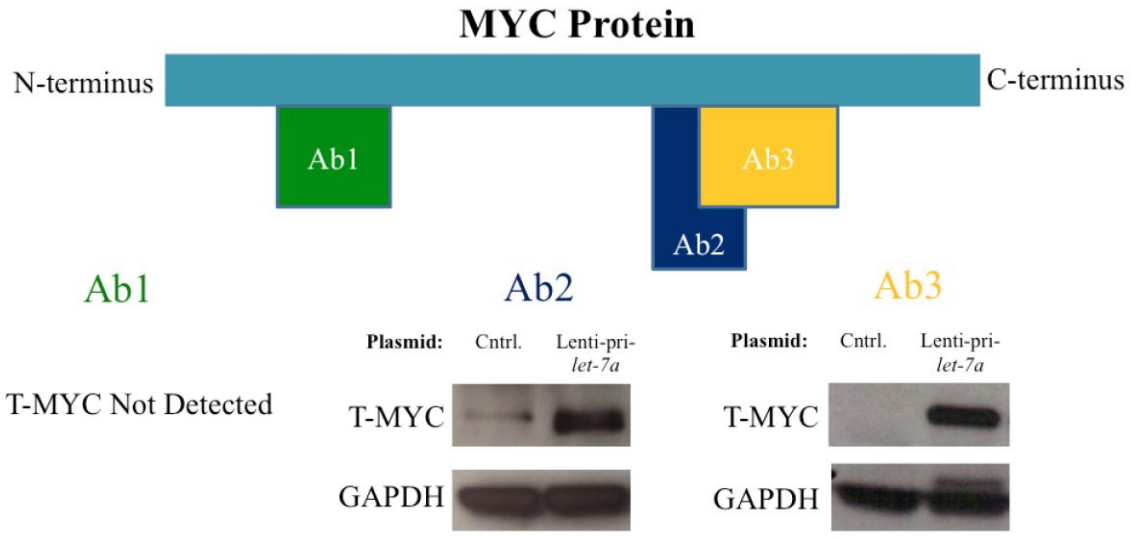

Figure 6: T-MYC is detected with two antibodies generated to detect the C-terminus of MYC. A549 lung adenocarcinoma cells were transfected with a control lentiviral plasmid or lenti-pri-*let-7a-1*. 48hrs post transfection, cells were lysed and protein extracted was assessed for the presence of T-MYC through SDS-PAGE and immunoblotting. Antibody 1 (Ab1) was produced by Cell Signaling, Antibody 2 (Ab2) by Sigma Aldrich and Antibody 3 (Ab3) by Abcam.

let-7a-1 plasmid along with a control, scrambled, siRNA or one of two siRNAs designed to target and downregulate MYC. After transfection, cell lysates were obtained and assessed for both canonical MYC and T-MYC expression through SDS-PAGE and immunoblotting. As seen in Figure 7, canonical MYC expression was only downregulated when the siRNAs targeting c-MYC was co-transfected, as expected. On the other hand, T-MYC was induced when the lenti-pri-*let-7a-1* plasmid was transfected into the cells, but its expression was not affected by co-transfection of the siRNA targeting the *5'* end of the *MYC* transcript. However, T-MYC was knocked down with co-transfection of the siRNA targeting the 3' end of the *MYC* transcript. This finding indicated that a transcript variant of *MYC* induced by pri-*let-7a-1* expression was possibly responsible for the expression of T-MYC.

To further investigate the possibility that a *MYC* transcript variant was responsible for the induction of T-MYC, it was hypothesized that the expression of total *MYC* transcripts would increase with the transfection of lenti-pri-*let-7a-1*. To test this hypothesis, protein and RNA were extracted after co-transfection of lenti-pri-*let-7a-1* with a scrambled, control siRNA or a siRNA targeting the *5'* end of *MYC* transcripts and compared to transfection with a control lentiviral plasmid in A549 lung adenocarcinoma cells. Protein was extracted from cell lysates and assessed for the expression of canonical MYC and T-MYC through SDS-PAGE and immunoblotting. As seen in Figure 8A, the expression of canonical MYC was decreased with co-transfection of the siRNA targeting *MYC*, as expected. The expression of T-MYC was induced by transfection of lenti-pri-*let-7a-1* and was unchanged by co-transfection of the siRNA targeting the *5'* end of *MYC*, as seen previously. Once it was confirmed that the expression of T-MYC was seen with

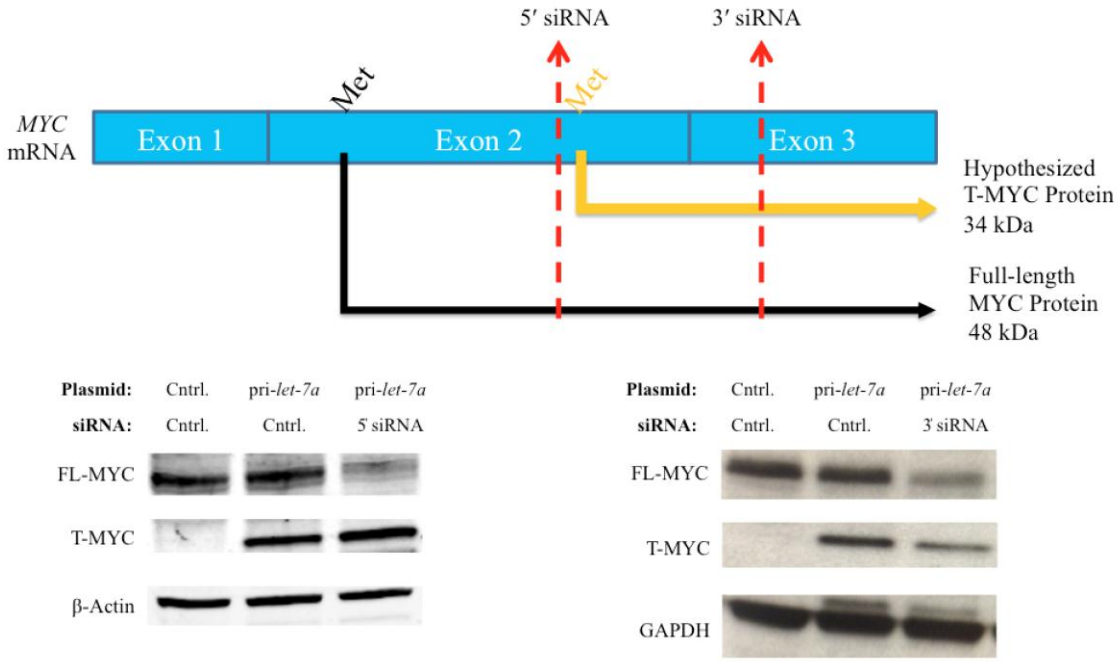

Figure 7: T-MYC is possibly expressed from a transcript variant of the canonical *MYC* transcript. A549 lung adenocarcinoma cells were cotrasfected with lenti-pri-l*et-7a-1* and either a control, scrambled siRNA or a siRNA targeting *MYC.* Lysates were assessed for the expression of canonical MYC and T-MYC through SDS-PAGE and immunoblotting.

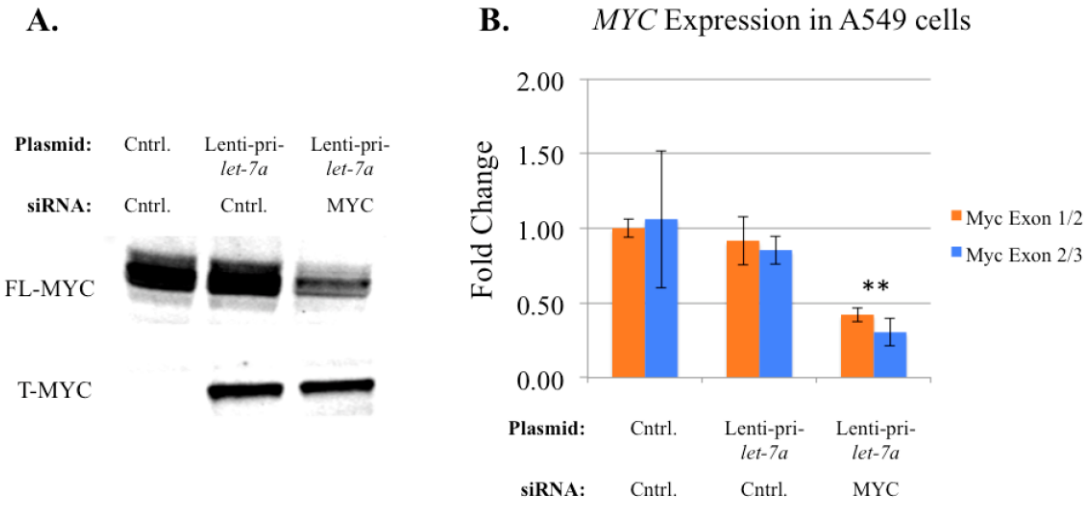

Figure 8: T-MYC is not a transcript or protein variant of canonical MYC. A549 lung adenocarcinoma cells were co-transfected with a control lentiviral plasmid or lenti-pri-*let-7a-1* and a control siRNA or a siRNA targeting Myc. 48hrs post-transfection protein and RNA were extracted. A. Protein was assessed for the expression of T-Myc through SDS-PAGE and immunoblotting. B. RNA was reverse transcribed and used in qPCR with primers spanning the two exon junctions in *MYC*, MYC Exon 1/2 and MYC Exon 2/3. *B-Actin* was used as the normalizer for gene expression and the control plasmid/control siRNA transfection served as the internal control. Error bars were generated by calculating the standard deviation of expression values obtained by utilizing the comparative Ct method with the control plasmid, control siRNA transfection serving as the internal control. Significance was determined through subjecting expression values to one-way ANOVA in GraphPad Prism. ** $p < .01$.

the transfection of lenti-pri-*let-7a-1*, the RNA also extracted from the cells after transfection underwent reverse transcription and was utilized in qPCR to further understand if a *MYC* transcript variant was induced by lenti-pri-*let-7a-1*. Two primer sets were used in qPCR, one that spanned the junction between exons 1 and 2 in *MYC*, MYC Exon1/2, and one primer pair that spanned the junction between exons 2 and 3 in *MYC*, MYC Exon2/3. As seen in Figure 8B, the expression of *MYC* was unchanged when lenti-pri-*let-7a-1* was transfected compared to the lentiviral control transfection. When lenti-pri-*let-7a-1* was co-transfected with the siRNA targeting *MYC*, however, the expression of *MYC* decreased. Since T-MYC expression was observed with transfection of lenti-pri-*let-7a-1* but there was no change in the expression of *MYC*, the hypothesis that a transcript variant was being expressed was false. Additionally, the co-transfection of lenti-pri-*let-7a-1* with the siRNA targeting *MYC* resulted in the decreased expression of the MYC protein product as well as *MYC* transcripts but the expression of T-MYC was unchanged. This indicated that T-MYC is likely not a protein or transcript variant of canonical MYC.

Although the expression of T-MYC was clearly induced by the transfection of lenti-pri-*let-7a-1* and detected with multiple antibodies that bind MYC, it was determined that T-MYC was not a protein or transcript variant of canonical MYC. The detection of T-MYC may be explained simply by conservation of the region that both the antibodies recognized. The c-terminus of MYC contains a basic helix-loop-helix (bHLH) domain, which is part of diverse family of transcription factors that also contain this domain[57–59]. It is possible that the transfection of lenti-pri-*let-7a-1* causes the induction of another bHLH containing protein that is detected by MYC antibodies that bind this domain.

Regardless, while T-MYC is likely not a variant of canonical MYC the implications of its expression in lung cancer has yet to be elucidated in regards to its expression seen *in vivo*.

CHAPTER 3: EVALUATING THE ABILITY OF PRI-LET-7A-1 TO INDUCE EXPRESSION OF T-MYC

The expression of T-MYC was first seen *in vivo* upon activation of a *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ mouse model and later when a lentiviral plasmid expressing primary-*let-7a-1* (pri-*let-7a-1*) was transfected in cell culture. The induction of T-MYC expression was consistently seen in backgrounds where LIN28B was also expressed which led to the hypothesis that the accumulation of pri-*let-7a-1* through LIN28B promoted the expression of T-MYC. Several strategies were used to understand if T-MYC expression was dependent upon pri-*let-7a-1* and LIN28B.

Since T-MYC expression was induced with the transfection of the lentiviral plasmid that expresses pri-*let-7a-1*, it was initially hypothesized that pri-*let-7a-1* induced expression of T-MYC. Several plasmids were created based on the lenti-pri-*let-7a-1* plasmid to test if T-MYC expression was dependent on pri-*let-7a-1*. First, pri-*let-7a-1* was excised from the lentiviral plasmid, lenti-pri-*let-7a-1*, and named lenti-Δpri-*let-7a-1*. An additional plasmid was created in which pri-*let-7a-1* was cloned from lenti-pri-*let-7a-1* into a non-lentiviral expression backbone and named pPNB-1. These constructs, along with a lentiviral plasmid that expresses pri-*let-7b*, were transfected into H441 and A549 lung adenocarcinoma cells. It was hypothesized that only the plasmids that expressed pri-*let-7a-1* would induce the expression of T-MYC in A549 cells and not H441 since A549 cells have detectable levels of LIN28B expression while H441 cells do not. As

seen in Figure 9, T-MYC expression was induced only by the lenti-pri-*let-7a-1* and lenti-Δpri-*let-7a-1* in both A549 and H441 lung adenocarcinoma cells. This result indicated that T-MYC expression is not dependent upon pri-*let-7a-1* expression or to other *let-7* family members, but is inherent to the lentiviral backbone. Additionally, since T-MYC expression was seen in both A549 and H441 cells, its expression is therefore not dependent upon pri-*let-7a-1* or LIN28B expression.

The preliminary data generated *in vivo* shows that T-MYC expression was induced when the $Kras^{G12D/+}$;$p53^{R172/+}$ model was activated through transduction with adenovirus Cre Recombinase, as seen in Figure 2. This in combination with the finding that the lentiviral backbone was responsible for inducing expression of T-MYC, Figure 6, led to the hypothesis that T-MYC expression may be conserved and inherent to transduction with viral particles. To test this, A549 cells were transduced with lentivirus and adenovirus and their cell lysates were probed for T-MYC through SDS-PAGE and immunoblotting. As seen in Figure 10, T-MYC was induced with transfection of the positive control, lenti-pri-*let-7a-1*, but not when transduced with lentivirus or adenovirus. These findings indicate that T-MYC expression is not induced by transduction of cells with lentivirus and adenovirus. The induced expression of T-MYC is inherent to the original lentiviral backbone that was used to overexpress genes of interest.

One caveat of this study that was identified was the fact that T-MYC expression is only induced in cell culture by transfection of lenti-pri-*let-7a-1* and that transfection of the lentiviral control plasmid did not promote expression of T-MYC. In the original study determining the effectiveness of the lentiviral delivery of pri-*let-7a-1*, the original lentiviral plasmids used for studying this effect were an older system created by System

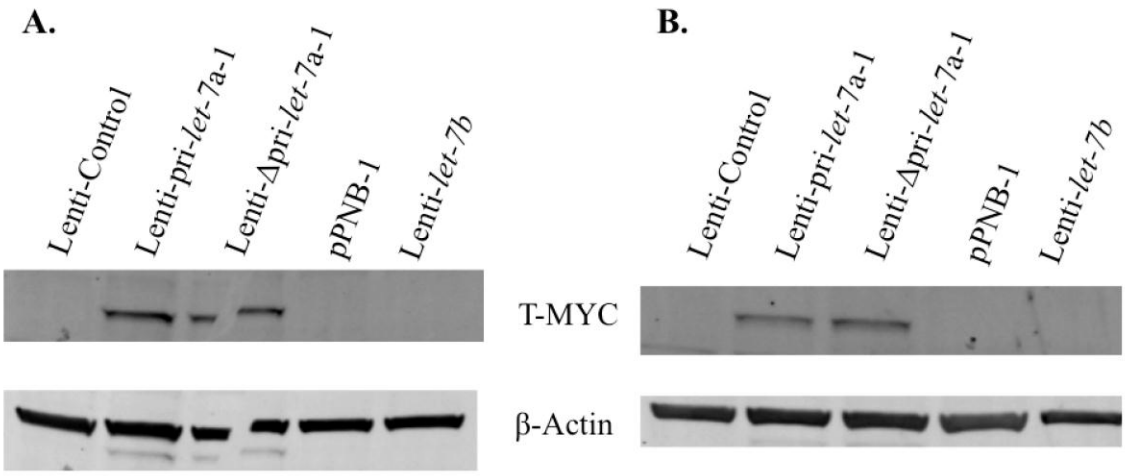

Figure 9: T-MYC expression is independent of pri-let-7a-1. H441, A., and A549, B., lung adenocarcinoma cells were transfected with plasmids that expressed (lenti-pri-*let-7a-1*, pPNB-1) and did not express pri-*let-7a-1* (Lenti-control, lenti-Δpri-*let-7a-1* and lenti-*let-7b*). 48hrs post transfection, cells were lysed and protein was assessed for the expression of T-MYC through SDS-PAGE and immunoblotting.

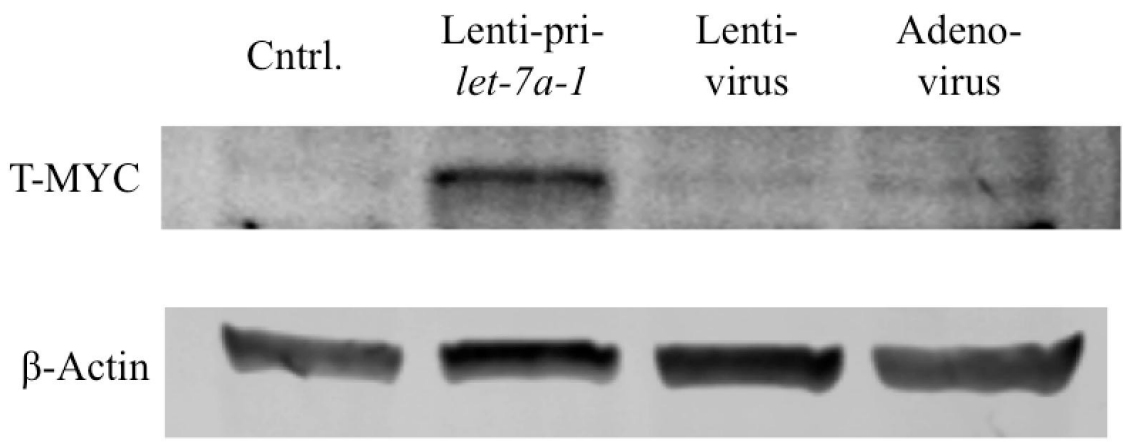

Figure 10: T-MYC expression is not induced by viral transduction in cell culture. H441 lung adenocarcinoma cells were transfected with a control lentiviral plasmid or lenti-pri-*let-7a-1*, or were transduced with lentivirus or adenovirus. 48hrs post transfection cells were lysed and protein was extracted and assessed for T-MYC and β-Actin expression through SDS-PAGE and immunoblotting.

Biosciences. When trying to propagate and transfect the lentiviral control, the plasmid was difficult to prepare at working concentrations and failed when used to try to create lentivirus for transduction *in vivo*. Thus, an updated lentiviral control was obtained from System Biosciences while pri-*let-7a-1* was still used in the older plasmid system. This indicates that T-MYC expression is somehow only inherent to the older lentiviral plasmid system by System Biosciences and T-MYC expression is not induced by the updated lentiviral system.

It was hypothesized that the accumulation of pri-*let-7a-1* through LIN28B expression induced the expression of T-MYC. However, it was determined that T-MYC expression is not dependent upon the expression pri-*let-7a-1* or LIN28B. The expression of T-MYC is promoted simply by the transfection of the lentiviral plasmid void of pri-*let-7a-1*, indicating that T-MYC expression inherent to a component of the older lentiviral backbone created by System Biosciences.

CHAPTER 4: CONCLUSION

The treatment of lung cancer is currently an area that represents a great deal of research effort both in academia and industry due to the fact that lung cancer is currently responsible for more cancer deaths per year than any other cancer type. In the United States in 2015, it was estimated that there would be 158,040 lung cancer deaths with an additional 221,200 new diagnoses[1]. Those who are diagnosed with lung cancer are faced with a 17% 5-year survival rate mainly due to most lung cancer cases not being detected until later stages of cancer progression[1]. A later diagnosis indicates a point in time when metastasis is much more likely, meaning a less treatable and much more aggressive time in disease progression. Creating effective targeted therapies for the treatment of lung could greatly increase the 5-year survival rate of lung cancer patients and decrease the number of lung cancer associated deaths in the United States and globally.

One strategy for the targeted treatment of lung cancer that has shown promising results is the use of microRNAs (miRNAs) as a therapeutic intervention. miRNAs are transcribed from the genome into a long, primary microRNA (pri-miRNA) and undergo further processing in the nucleus and cytoplasm, respectively, to form a shorter 18-24 nucleotide mature microRNA (mat-miRNA) duplex. The guide strand of the duplex then associates with the RNA induced silencing complex (RISC) and through base

complementarity with messenger RNAs (mRNAs), mediates the post transcriptional down-regulation of protein expression through mRNA degradation or blocking translation of mRNA targets[37]. Tumor suppressive miRNAs typically have decreased expression in cancer compared to normal, healthy tissue and function by targeting and regulating the expression of factors that promote tumorigenic processes. Oncogenic miRNAs have increased expression in cancer compared to normal tissues and target factors that are important in promoting anti-tumorigenic effects[46,48]. Therefore, the reintroduction of a tumor suppressive miRNA into a background where its expression is lost or blocking the action of an oncogenic miRNA could restore the regulation of many genes at one since one miRNA has many mRNA targets[37].

let-7 is a tumor suppressive miRNA that has been observed to have decreased expression in many cancer types[60,61]. The ability of a *let-7* mimic to decrease the tumorigenic phenotype seen in cancer has also been demonstrated *in vivo*[61]. These findings led to the hypothesis that the exogenous delivery of *let-7* would be advantageous in treating an aggressive lung cancer mouse model. The model being studied was a $Kras^{G12D/+};p53^{R172H/+}$ transgenic mouse model that is activated by the recombination of loxP sites with exposure to adenovirus-cre recombinase. Activation of this model results in the constitutive expression of a mutated form of Kras, a known oncogene[52,62], and mutation of the tumor suppressive factor p53[53]. To test the hypothesis that *let-7* could be exogenously delivered to this model and stop tumor growth and progression, primary-*let-7a-1* (pri-*let-7a-1*) was lentivirally delivered to the activated $Kras^{G12D/+};p53^{R172H/+}$ transgenic mouse model. Primary-*let-7a-1* (pri-*let-7a-1*) is the unprocessed transcript of *let-7* that requires processing by cellular machinery to form its mature transcript, mat-*let-*

7a, and mediate its proven tumor suppressive capabilities[37]. The effect seen with the lentiviral transduction of pri-*let-7a-1* was increased lung size and inflammation compared to lungs exposed to a control lentivirus. This result went against the hypothesis that pri-*let-7a-1* would be processed into mat-*let-7a*, target oncogenes and mediate a decrease in tumor growth within the *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ mouse model.

It was determined that within the *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ activated mouse model, that *LIN28B* transcripts were elevated[2]. LIN28B is a pluripotent factor that is overexpressed in some cancers and mediates part of its pro-tumorigenic capabilities through the post-transcriptional inhibition of *let-7* biogenesis[3–5]. Since LIN28B inhibits the tumor suppressive capabilities of *let-7*, it was hypothesized that *let-7* targets would be dysregulated within the activated *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ mouse model. One target of *let-7* is that of MYC, a well known oncogene that is overexpressed in approximately 30% of all cancers[22,23]. The expression of MYC was assessed within lung tissues of the *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ activated model compared to lungs harvested from non-tumorigenic mice. The expression of canonical MYC was not affected by activation of the transgenic model but a truncated MYC protein product was overexpressed in the *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ activated model compared to non-tumorigenic lung tissue, seen in Figure 4. This led to the hypothesis that increased LIN28B expression causes the accumulation of pri-*let-7a-1* transcripts, which promotes the expression of the truncated MYC protein product, termed T-MYC.

To understand if T-MYC was indeed a truncated form of canonical MYC, several strategies were employed. The induction of T-MYC was proven to be reproducible *in vitro*, seen in Figure 5 by transfection of the lenti-pri-*let-7a-1* plasmid also used in

transduction of the activated *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ model. This finding confirmed that T-MYC expression could be replicated both *in vitro* and *in vivo*. T-MYC was also only detected with multiple primary antibodies generated to detect the C-terminus of MYC in SDS-PAGE and immunoblotting, seen in Figure 6, but not with an antibody that binds to an epitope on the N-terminus of MYC. This result led to the hypothesis that T-MYC is a truncated form of canonical MYC that retains only the C-terminus of MYC but does not contain the N-terminus of MYC within its protein structure. In an attempt to further characterize T-MYC, small-interfering RNA (siRNA) mediated knock-down was employed with the hypothesis that if T-MYC is a protein variant of MYC, it would be knocked down when co-transfected with a siRNA targeting *MYC* in A549 cells. As seen in Figure 7, canonical MYC was knocked down with siRNAs targeting both the 5' and 3' end of the *MYC* transcript, determined through SDS-PAGE and immunoblotting. However, T-MYC was only knocked down with the siRNA designed to target the 3' end of *MYC*. This led to the hypothesis that a transcript variant separate from the canonical *MYC* transcript was being expressed with the transfection of the plasmid that promoted the expression of T-MYC, lenti-pri-*let-7a-1*. To understand if a *T-MYC* transcript variant was induced upon expression of T-MYC, A549 cells were co-transfected with lenti-pri-*let-7a-1* and a control scrambled siRNA sequence or a siRNA targeting the 5' end of *MYC*. As seen in Figure 8, the expression of canonical MYC was unchanged by the transfection of lenti-pri-*let-7a-1* and a control siRNA but decreased when lenti-pri-*let-7a-1* was co-transfected with the siRNA targeting *MYC*, as expected. Through using qPCR, it was found that the expression of *MYC* transcripts was unchanged when T-MYC was induced and *MYC* expression decreased when the siRNA targeting *MYC* was co-transfected while

T-MYC expression remained unchanged. Since the total expression of *MYC* did not change when T-MYC was induced, it was concluded that T-MYC was not expressed from a transcript variant of canonical *MYC*. Additionally, since MYC expression decreased both at the transcript and protein levels when cells were transfected with the siRNA targeting *MYC*, it was concluded that T-MYC is also likely not a protein variant of MYC.

The induced expression of T-MYC was first observed upon activation of the $Kras^{G12D/+};p53^{R172H/+}$ transgenic mouse model that has increased *LIN28B* transcript levels[2]. T-MYC expression was also seen through transfection of the lentiviral plasmid that overexpresses pri-*let-7a-1*, lenti-pri-*let-7a-1*, in cells that had detectable levels of LIN28B expression, A549 and Calu6. Since T-MYC expression was observed in backgrounds with detectable LIN28B expression, it was hypothesized that the LIN28B mediated accumulation of pri-*let-7a-1* transcripts induced the expression of T-MYC. To test this hypothesis, several plasmids based off of lenti-pri-*let-7a-1* were created. The pri-*let-7a-1* sequence was removed from the lenti-pri-*let-7a-1* plasmid, termed lenti-Δpri-*let-7a-1*, and was also cloned into a non-lentiviral backbone, termed pPNB-1. These constructs, along with a lentiviral plasmid that overexpressed pri-*let-7b*, were transfected in H441 and A549 lung adenocarcinoma cell lines. As seen in Figure 9, only the plasmids that were based on the original lentiviral construct, lenti-pri-*let-7a-1* and lenti-Δpri-*let-7a-1*, promoted the expression of T-MYC. This finding indicated that neither pri-*let-7a-1* nor other *let-7* family members, pri-*let-7b*, induce the expression of T-MYC. Additionally, since T-MYC expression was seen in both cells that have LIN28B

expression, A549, and cells that do not express LIN28B, H441, it was concluded that T-MYC expression is not dependent on pri-*let-7a-1* or LIN28B expression.

Finally, since expression of T-MYC was first observed simply by activation of the *Kras*$^{G12D/+}$;*p53*$^{R172H/+}$ transgenic mouse model by exposing mice to adenovirus-cre recombinase, it was hypothesized that T-MYC expression is conserved when cells are transduced with virus. To test this hypothesis, H441 lung adenocarcinoma cells were transduced with both lentivirus and adenovirus-cre recombinase and their cell lysates were assessed for the expression of T-MYC through SDS-PAGE and immunoblotting. As seen in Figure 10, T-MYC expression was not induced by either transduction with lentivirus or adenovirus. This finding indicates that T-MYC expression is not a conserved effect seen by the transduction of cells with lentivirus or adenovirus.

It was originally hypothesized that the accumulation of unprocessed, primary-*let-7a-1* (pri-*let-7a-1*) transcripts by LIN28B promoted the expression of a truncated form of MYC. Through this work, it was determined that pri-*let-7a-1* does not promote the expression of T-MYC and that T-MYC is likely not a protein variant of canonical MYC. While this work does not explain why the lentiviral delivery of pri-*let-7a-1* increases the pro-tumorigenc phenotype *in vivo*, the elucidation of this effect could reveal additional, unknown functions of the microRNA *let-7*. These findings could create a paradigm shift in the understanding of the functions microRNAs and lead to the creation of effective targeted therapies for the treatment of lung cancer.

CHAPTER 5: MATERIALS AND METHODS

Cell Culture and Transfection

A549 and H441 lung adenocarcinoma cell lines were grown in 1X Gibco® RPMI 1640 supplemented with fetal bovine serum (FBS) and penicillin/streptoMYCin in a cell culture incubator maintained at 37°C with 5% CO_2. Passaging and maintenance of cells took place in a biological safety cabinet under sterile conditions. For transfection, A549 cells were counted by using a hemocytometer and seeded at a volume of 2 mL at 2 x 10^5 cells/mL in sterile six well plates on Day 0. Transfection occurred on Day 1 by combining 3 μL of Lipofectamine® 2000 transfection reagent with 1 μg of plasmid and if co-transfected, with 25 nM of siRNA in serum and antibiotic free 1X Gibco® RPMI 1640. Four hours post transfection, serum free media was aspirated and replaced with 1X Gibco® RPMI 1640 supplemented with FBS and penicillin/streptoMYCin.

Viral Transduction in Cell Culture

H441 lung adenocarcinoma cells were counted by using a hemocytometer and seeded at a volume of 2 mL at 4 x 10^5 cells/mL in sterile six well plates on Day 0. Tranduction occurred on Day 1 at a multiplicity of infection (MOI) of 2, therefore a total concentration of 2 x 10^6 pfu were transduced. 2 μL of 1 x 10^9 pfu/mL feline immunodeficiency virus (lentivirus) and 2 μL of 4 μg/μL polybrene stock were combined

with 496 µL of serum and antibiotic free 1X Gibco® RPMI 1640, incubated at room temperature for 5 minutes and added to cells that had 1.5 mL serum and antibiotic free 1X Gibco® RPMI 1640 media. 500 µL of 4 x 10^6 pfu/mL adenovirus-cre diluted in serum/antibiotic free 1X Gibco® RPMI 1640 was combined with 2.4 µL 2M $CaCl_2$, incubated for 20 minutes at room temperature, and added to cells that had 1.5 mL serum and antibiotic free 1X Gibco® RPMI 1640 media. One day post transduction, Day 2, the media was aspirated and changed to 1X Gibco® RPMI 1640 media supplemented with FBS and penicillin/streptoMYCin.

SDS-PAGE and Immunoblotting

48 hours post transfection or Day 3 (protocol seen above), cells were lysed with 1X radioimmunoprecipitation assay (RIPA) buffer supplemented with 1X protease inhibitor cocktail with shaking for 10 minutes on ice. Remaining cellular debris was scraped from plates and cell lysates were centrifuged at 13,200 rpm for 10 minutes. The supernatant was moved to a new microcentrifuge tube and lysates were kept at -20°C until needed. A Pierce BCA Protein Assay was then used to determine the concentration of protein in each lysate fraction using a bovine serum albumin (BSA) standard curve. 50 µg of protein, with 1X Laemli buffer supplemented with 2-mercaptoethanol, was then boiled at 95°C for 5 minutes. The protein containing samples were then loaded onto Criterion™ TGX™ 12%, 12+2 comb stacking SDS polyacrylamide gels and run in an electrophoretic chamber with 1X Tris-SDS-Glycine running buffer at 150 volts for 1 hour. Protein was then transferred to polyvinylidene fluoride (PVDF) membranes activated for 5 minutes in methanol. The transfer occurred in a transfer chamber with 1X Glycine-SDS-Methanol transfer buffer at 4°C under 100 volts for 50 minutes.

The PVDF membranes were then blocked in Odyssey® Blocking Buffer (PBS) with gentle shaking for 1 hour at room temperature. Primary MYC antibodies, at a concentration of 1:500, were incubated on the PVDF membrane overnight at 4°C with gentle shaking. Membranes were washed three times with phosphate buffer solution supplemented with 0.01% Tween-20 (PBST) for 5 minutes with vigorous shaking. Secondary antibodies, LI-COR IRDye® 680RD/800RD Goat anti-Mouse or Goat anti-Rabbit, were diluted in Odyssey® Blocking Buffer (PBS) at a concentration of 1:15,000 and incubated on the PVDF membranes for 1 hour at room temperature. Membranes were then washed two times with phosphate buffer solution supplemented with 0.01% Tween-20 (PBST) for 5 minutes with vigorous shaking followed by one wash with phosphate buffer solution (PBS) for 10 minutes with vigorous shaking. Membranes were then imaged on a LI-COR Odyssey® CLx infrared imager. Primary β-Actin and GAPDH antibodies were incubated on PVDF membranes at a concentration of 1:10,000 for one hour at room temperature, followed by the same protocol above for secondary antibodies and imaging.

RNA Extraction and real-time PCR

RNA was extracted and purified from cell lysates post-transfection by direct lysis of cells in six well plates with 700 μL of QIAzol Lysis Reagent. Lysis and extraction of RNA was then performed using the Qiagen® miRNeasy Mini Kit following the manufacturer's protocol of "Purification of Total RNA from Animal Cells". The protocol was modified with a DNase digestion, protocol in Appendix B of the miRNeasy Mini Handbook, using a Qiagen® RNase-Free DNase Set.

Purified RNA was then quantified using a Thermo Scientific NanoDrop™ 2000 UV-Vis Spectrophotometer. 1 μg of total RNA was then reverse transcribed using a miScript® HiFlex buffer in the Qiagen® miScript® II RT kit for 1 hour at 37°C followed by 5 minutes at 95°C. The 20 μL reverse transcription reaction was then diluted to a total volume of 200 μL with nuclease free water and used in real-time polymerase chain reaction (qPCR). qPCR was performed using the Qiagen® miScript® SYBR® Green PCR Kit, using 10 μL reaction sizes in 384 well plates. Primer sets used are as follows: MYC Exon 1/2 primer pair was generated using the NCBI Primer-BLAST program and the sequences are as follows: MYC Exon 1/2 Forward: 5'-GGGAGGCTATTCTGCCCATT-3' and MYC Exon 1/2 Reverse: 5'-GAGGCTGCTGGTTTTCCACT-3'. The MYC Exon 2/3 and Actin primer pairs were commercially generated Qiagen® Quantitect Primer Assays. qPCR was performed on an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR system using the following thermal cycling conditions; Initial Hold Stage: 95°C for 15 minutes, PCR Stage: 40 cycles of denature at 95°C for 15 seconds, anneal at 55°C for 30 seconds, and extension with fluorescence data capture at 70°C for 30 seconds, and Melt Curve Stage: 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds with fluorescence data capture. Ct values obtained from qPCR were analyzed using the comparative Ct method using Actin as a gene for normalization of gene expression and the control transfection as the internal control for gene expression comparison.

Cloning

Lenti-pri-*let-7a-1* plasmids were propagated from pre-existing glycerol stocks in the lab kept at -80°C. Cells were grown in 4 mL overnight cultures in liquid Luria Broth

(LB) supplemented with 100 μg/mL of ampicillin with vigorous shaking at 37°C. Plasmids were extracted from bacterial cells using a Qiagen® QIAprep® Spin Miniprep Kit and quantified using a Thermo Scientific NanoDrop™ 2000 UV-Vis Spectrophotometer.

Lenti-Δpri-*let-7a-1* was created by excising the pri-*let-7a-1* sequence from the lenti-pri-*let-7a-1* plasmid. This was done by performing a double restriction endonuclease digestion of 1 μg of lenti-pri-*let-7a-1* with the restriction endonucleases NheI and PacI from New England Biolabs® (NEB®). A 20 μL reaction volume was used along with 0.5 μL of each NheI and PacI supplemented with 1X NEB® CutSmart® Buffer for 1 hour at 37°C and heat inactivated at 65°C for 20 minutes. 5' overhangs were filled in and 3' overhangs were removed by using NEB® T4 DNA Polymerase. The reaction was carried out by adding 1 unit of T4 DNA polymerase per μg of DNA, supplemented with 100 μM dNTPs and incubated on a thermal cycler for 15 minutes at 12°C. The reaction was then run on a 1% agarose gel with a Promega 1kb DNA ladder at 100 volts for 1 hour. The gel was visualized on a UVP Benchtop 2 Transilluminator and the digested band was extracted from the gel. The DNA was then extracted from the agarose gel using a Qiagen® QIAquick® gel extraction kit. 100 ng of plasmid in a 10 μL volume was then ligated with 1 μL of NEB® T3 DNA ligase and 10 μL of 2X T3 DNA ligase buffer and occurred at room temperature overnight. Ligated plasmids were then transformed in NEB® 5-alpha *Escherichia coli* competent cells. NEB® 5-alpha cells were thawed on ice for 30 minutes, 100 ng of ligated plasmid was added to 50 μL of competent cells and incubated on ice for 30 minutes. The cells were heat-shocked by placing the tube in a 42°C water bath for 45 seconds followed immediately by incubation

on ice for 2 minutes. 250 µL of SOC media was added to the cells in a microcentrifuge tube and was then incubated at 37°C for 45 minutes with vigorous shaking. The total volume was then spread onto LB agar plates supplemented with 100 µg/mL of ampicillin and grown overnight at 37°C. Multiple colonies were picked and grown in 4 mL overnight cultures in liquid LB supplemented with 100 µg/mL of ampicillin with vigorous shaking at 37°C. Plasmids were finally extracted from bacterial cells using a Qiagen® QIAprep® Spin Miniprep Kit followed by confirmation restriction endonuclease digestion and sequencing of the plasmid at the Purdue low throughput sequencing lab.

pPNB-1 was created by polymerase chain reaction (PCR) amplification of pri-*let-7a-1* from lenti-*pri-let-7a-1* and cloning into pTet-4a, a non-lentiviral plasmid maintained in the lab. pri-*let-7a-1* was PCR amplified from lenti-pri-*let-7a-1* using the following primer pairs generated using NCBI Primer-BLAST: let-7a-1_clon_F: 5'-GTACGTACGGCCGCCTCCCCGCCTTAATTA-3' and let-7a-1_clon_R: 5'-GAGATTTAAATACCTTGCTAGCGGCGCGCCAT-3'. PCR was carried out by using NEB® Q5® High Fidelity DNA Polymerase using the following conditions for each reaction: 5 µL 5X Q5 Reaction Buffer, 0.5 µL of 100 µM dNTPs, 1.25 µL of 10 µM forward and reverse primer, 10 ng of lenti-pri-*let-7a-1* plasmid DNA, 0.25 µL Q5® DNA Polymerase and 15.75 µL nuclease free water. The PCR thermal cycling conditions used are as follows: initial denaturation at 98°C for 30 seconds, 30 cycles of: denature at 98°C for 10 seconds, anneal at 60°C for 30 seconds and extension at 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes and hold at 4°C. Reactions were then run on a 1% agarose gel with the Promega 1kb DNA ladder at 100 volts for 1 hour.

Bands were extracted from the gel and DNA was extracted from the agarose gel using a Qiagen® QIAquick® gel extraction kit. The PCR amplified product and pTet-4a were then digested to create compatible ends for ligation. 1 μg of each were first digested with SwaI supplemented with with NEB® Buffer 3.1. The reaction components for digestion of the PCR product are as follows: 1 μL SwaI, 30 μL PCR product, 5 μL NEBuffer 3.1 and 14 μL of deionized water. The reaction components for digestion of the pTet-4a are as follows: 4.5 μL DNA, 2 μL NEBuffer 3.1, 0.5 μL SwaI and 15.25 μL of deionized water. Both reactions were incubated at 25°C for 1 hour followed by inactivation at 65°C for 20 minutes. 1 μL of BsiWi was then added to each reaction and incubated at 55°C for one hour. Digested pTet-4a was run on a 1% agarose gel with the Promega 1kb ladder and its DNA was extracted from the agarose gel using the Qiagen® QIAquick® gel extraction kit. The digested PCR insert was purified using the Qiagen® QIAquick® Purification kit. The digested pTet-4a vector was ligated to the digested pri-*let-7a-1* insert at a molar ratio of 1:3 by using NEB® T3 DNA ligase. The reaction was performed as follows: 1 μL digested pTet-4a, 6 μL digested pri-*let-7a-1* DNA insert, 10 μL NEB® T3 DNA ligase buffer and 1 μL NEB® T3 DNA ligase. The reaction was left at room temperature overnight. Ligated plasmids were then transformed in NEB® 5-alpha *Escherichia coli* competent cells. NEB® 5-alpha cells were thawed on ice for 30 minutes, 100 ng of ligated plasmid was added to 50 μL of competent cells and incubated on ice for 30 minutes. The cells were heat-shocked by placing the tube in a 42°C water bath for 45 seconds followed immediately by incubation on ice for 2 minutes. 250 μL of SOC media was added to the cells in a microcentrifuge tube and was then incubated at 37°C for 45 minutes with vigorous shaking. The total volume was then spread onto LB

agar plates supplemented with 100 μg/mL of ampicillin and grown overnight at 37°C. Multiple colonies were picked and grown in 4 mL overnight cultures in liquid LB supplemented with 100 μg/mL of ampicillin with vigorous shaking at 37°C. Plasmids were finally extracted from bacterial cells using a Qiagen® QIAprep® Spin Miniprep Kit followed by confirmation restriction endonuclease digestion and sequencing of the plasmid at the Purdue low throughput sequencing lab.

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