CHARACTERIZING C-MYC DEPENDENT NON-SMALL CELL LUNG CANCERS

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Dedicated to

My parents Steve and Nora Dospoy
My brothers Chris, Kevin and Sean
My late grandfather, Edwin Riner

CHARACTERIZING C-MYC DEPENDENT NON-SMALL CELL LUNG CANCERS

by

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DISSERTATION

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CHARACTERIZING C-MYC DEPENDENT NON-SMALL CELL LUNG CANCERS

Publication No._____

Patrick Dospoy, Ph.D.

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Supervising Professor: John D. Minna, M.D.

MYC is one of the most commonly deregulated oncogenes in human cancer, including breast, colorectal and lung. While mutations in *myc* are rare, MYC is overexpressed and in some cases amplified in these (and other) cancers. Recent reports demonstrate the utility of various drugs in selectively targeting MYC-driven cancers. However, given the lack of consistency across tissue types, particularly lung cancer, a multimodal approach to delineate MYC-dependent lung cancers is required. My goal is to characterize MYC deregulation in lung cancer, investigate the degree of differential dependence on MYC in lung cancer, and to elucidate the mechanism for resistance to MYC inhibition. A large panel of clinically and

molecularly annotated NSCLC lines was investigated for MYC mRNA, protein expression, and DNA copy number. In addition, publically available databases were interrogated to characterize the degree of MYC deregulation in lung cancer. Functional tests were performed on a large panel of NSCLC cell lines (n = 83) using four drugs that were recently shown to selectively target MYC-driven cancers. Further, we utilized the dominant negative mini-protein OMOMYC for functional classification. In all cases, drug effects were monitored by colony forming efficiency (CFE) assays. OMOMYC results were confirmed via xenograft experiments. Each of the four MYC inhibitors tested elicited a variable response in a subset of the 83 NSCLC cell lines, though the sensitive subset was not similar between any two drugs (highest correlation coefficient of 0.24). In order to determine which, if any, of the drugs targeted MYC-driven lung cancers, we stably expressed OMOMYC in a subset of the NSCLC cell line panel and performed functional assays. Most of the cell lines were sensitive to OMOMYC (with up to 100 fold reduction in CFE), compared to 3/8 that were totally resistant. The variability in the presence of OMOMYC showed a significant correlation with one of the four MYC inhibitors tested. These results support the idea that this sensitive subset represents a truly MYC-dependent class of lung cancers. Surprisingly, there was no correlation between MYC dependence and either MYC mRNA, protein expression or DNA copy number. OMOMYC levels were normalized in all cell lines tested and quantified using qRT-PCR. Additionally, in all cases, exogenous OMOMYC expression led to down regulation of MYC target genes as measured by both qRT-PCR and microarray. These data could be interpreted to suggest that the observed phenotype was the result of decreased MYC activity. Last, the NSCLC probed with OMOMYC showed a variable

response in the Wnt pathway, with some cell lines showing a dramatic activation of the Wnt pathway upon OMOMYC induction. This activation proved to be functionally important, as dual inhibition of β-catenin and MYC proved more effective than either approach alone. To investigate the clinical significance of this approach, a subset of the original panel of NSCLC (n = 15) was screened with the MYC inhibitor 10058-F4, Wnt inhibitor Wnt-C59, or a combination of both drugs. Here, 8/15 cell lines displayed a statistically significant increase in sensitivity to MYC inhibition when Wnt pathway inhibition was added. We conclude: there is a subset of NSCLCs that demonstrates dramatic growth inhibition by a single MYC-inhibitor, and these data are phenocopied by the more specific MYC-dominant negative protein, OMOMYC. We further conclude that activation of the Wnt pathway serves as a compensatory response in some cell lines that confers resistance to MYC inhibition. In conclusion, simultaneously targeting MYC and the Wnt pathway elicits superior sensitivity in a subset of NSCLCs, and thus provides rationale for a combinatorial approach in a subset of lung cancer patients.

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LIST OF ABBREVIATIONS

AC – adenocarcinoma

ALDH – aldehyde dehydrogenase

bHLH – basic helix loop helix

DMSO – dimethyl sulfoxide

EGFR – epidermal growth factor receptor

FACS – fluorescence activated cell sorting

RFP – red fluorescent protein

HBEC – human bronchial epithelial cell

IHC – immunohistochemistry

KRAS – v-Ki-Ras2 Kirsten rat sarcoma viral oncogene homolog

miR – microRNA

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NOD/SCID – non-obsese diabetic severe combined immunodeficiency mouse

NSCLC – non-small cell lung cancer

P53 – tumor protein 53

PI – propidium iodide

qRT-PCR – quantitative real-time polymerase chain reaction

RPMI – Roswell Park Memorial Institute medium

SCC – squamous cell carcinoma

SCLC – small cell lung cancer

shRNA – short-hairpin ribonucleic acid

siRNA – short-interfering ribonucleic acid

SNP – single nucleotide polymorphism

TMA – tissue microarray

NPC – Neural progenitor cell

TBST – Tris-Buffered Saline with Tween-20

CHAPTER ONE

AN OVERVIEW OF THE MYC ONCOGENE

1.1 c-Myc discovery and historical perspective

The *MYC* gene was discovered in 1979 as the oncogene responsible for inducing myelocytomatosis in birds (Sheiness and Bishop 1979). Specifically, the avian carcinoma virus, MC29, was found to contain a sequence of approximately 1,500 nucleotides, and Sheiness and Bishop presented evidence to suggest this sequence was host-obtained. They identified a homologous sequence in a variety of other vertebrate species, though the complementarity decreased in a manner proportional to the evolutionary divergence from the original host species (chickens) (Sheiness and Bishop 1979). This suggested the sequence was evolutionarily conserved. In 1982, Vennstrom et al isolated DNA from chickens that was found to be homologous to the viral oncogene of MC29 (Vennstrom, Sheiness et al. 1982). Thus, the cellular homolog to the *MYC* gene, *c-myc*, was identified.

The *c-myc* gene has been shown to be highly conserved in vertebrates, including >90% amino acid conservation between human and mouse genes (Bernard, Cory et al. 1983). The *c-myc* gene was initially found to have two exons that were both conserved across the avian, mouse and human genes as well as homologous to the viral sequences. However, later studies identified an untranslated first exon that lacked traditional start codons (Battey, Moulding et al. 1983). Battey et al showed that the mRNA of the normal c-myc gene is transcribed from two distinct transcriptional start sites (~160 base pairs apart), and that each

promoter initiates transcription of the untranslated exon 1. Whereas exons 2 and 3 demonstrated >90% homology between mouse and human, the untranslated first exon exhibits only 70% homology (Bernard, Cory et al. 1983).

Early *in situ* hybridization experiments showed that the *c-myc* gene is located on chromosome 8 band q24 (Taub, Kirsch et al. 1982). Later that same year, a separate group used Southern blotting to also show that the *c-myc* gene is located on chromosome 8, thus confirming the location of the gene (Dalla-Favera, Bregni et al. 1982).

1.2 c-Myc protein structure and function

Human *c-myc* encodes two different isoforms of the MYC protein. Depending on which transcriptional start site is used, either a 454 (Myc-1) or the much more abundant 439 (Myc-2) amino acid protein is produced (Hann, King et al. 1988). Myc-1 and Myc-2 are both nuclear phosphoproteins with the ability to initiate transcription, though their transcriptional activity appears to differ (Hann, Dixit et al. 1994). In both cases, the MYC protein contains several highly conserved regions, all of which are functionally important. The transactivation domain (TAD) of MYC was identified using a Gal4-based assay, which mapped transcriptional activation to the segment spanning residue 1 through 143 (Kato, Barrett et al. 1990). Of note, four evolutionarily conserved domains called MYC boxes exist in all *myc* family members. The amino-terminus of the MYC protein is largely unstructured, and contains the first two MYC boxes (MBI and MBII) (Conacci-Sorrell, McFerrin et al. 2014). MBI spans residues 45 – 65, and MBII spans residues 128 – 144 (Flinn, Busch et al.

1998). MBI contains several phosphorylation sites critical for the regulation of c-Myc protein, including Thr-58 and Ser-62 (Flinn, Busch et al. 1998). Additionally, deletion of MBI was shown to have a negative effect on the transforming ability of c-Myc (Sarid, Halazonetis et al. 1987). MBII is also important for the transforming ability of MYC, as well as the ability to repress transcription of certain targets (Li, Nerlov et al. 1994). The middle segment of the protein contains a conserved region rich in proline, glutamate, serine and threonine (PEST), as well as the second two MYC boxes (MBIII and MBIV), followed by a nuclear localization signal. MBIII spans residues 188 – 270 and is essential for transformation of Rat-1A fibroblasts (Stone, de Lange et al. 1987). MBIV spans residues 304 – 324 and overlaps the primary nuclear localization signal (NLS), which spans residues 320 – 328 (Meyer and Penn 2008). The basic region spans residues 355 – 369 and is essential for the interaction with DNA (Meyer and Penn 2008). The carboxy-terminal domain contains the helix-loop-helix leucine zipper (HLH-LZ) domain, which is essential for transformation due to its role in protein-protein interactions (Meyer and Penn 2008, Conacci-Sorrell, McFerrin et al. 2014). The topology of the MYC protein is depicted in Figure 1.1.

Like many other proteins of the bHLH-LZ family, dimerization of c-Myc is mediated through the HLH interface, which results in a four-helix structure when c-Myc binds to another HLH protein (Conacci-Sorrell, McFerrin et al. 2014). Nearly all known c-Myc functions require heterodimerization with another bHLH-LZ protein, Max (Blackwood and Eisenman 1991, Amati, Dalton et al. 1992). Max was identified as a binding partner of c-Myc via a cDNA library screen, wherein it was specifically found to bind to c-Myc in a manner dependent on an intact c-Myc HLH-LZ domain (Blackwood and Eisenman 1991). It

was further discovered that this complex bound DNA in a sequence-specific manner, while neither c-Myc nor Max alone could bind identical sequences (Blackwood and Eisenman 1991). Using the selected and amplified binding-sequence imprinting method, Blackwell et al identified 5'-CACGTG-3' as the sequence preferentially bound by MYC-MAX, which was mediated by the basic region in the carboxy-terminus of the c-Myc protein (Blackwell, Kretzner et al. 1990). basic regions become alpha-helical upon dimerization, allowing the helices to straddle the DNA (Ferre-D'Amare, Prendergast et al. 1993, Ferre-D'Amare, Pognonec et al. 1994). The preferential binding sequence falls within the family of E-box binding sequences, which take the form of 5'-CANNTG-3'. While MYC has been shown to bind to all E-box sequences, the affinity with which it binds is dependent on the sequence. Recent work has quantified this phenomenon, and found two important points. First, high-affinity E-boxes are found primarily in promoters, while low-affinity E-boxes are more often found in enhancers (Lin, Loven et al. 2012). Second, some combination of G/C at the third and fourth residues results in the highest affinity sequence, whereas A/T combinations result in the lowest affinity sequences (Lin, Loven et al. 2012). In addition to the role E-box sequences play, the flanking sequences (preference for GC residues adjacent to the E-box) and methylation patterns at CpG islands within the E-boxes can also affect MYC binding (Halazonetis and Kandil 1991, Prendergast and Ziff 1991). Last, the Structural studies have shown that MYC-MAX dimers have the ability to oligomerize to form tetramers, though the functional relevance of this structure has not yet been fully elucidated (Conacci-Sorrell, McFerrin et al. 2014). However, cooperative binding of MYC-Max heterodimers has been reported in genes that contain multiple binding sites in close proximity (Walhout, Gubbels et al. 1997).

The temporal expression pattern of Max does not mimic that of MYC, which led to the search for new Max binding proteins using a variety of approaches, including two-hybrid screening (Grandori, Cowley et al. 2000). These efforts resulted in the discovery of the Mad protein family, which consists of Mad1, Mxi1, Mad3 and Mad4 (Ayer, Kretzner et al. 1993, Zervos, Gyuris et al. 1993, Hurlin, Queva et al. 1995). Each protein in this family readily heterodimerizes with Max. In contrast to c-Myc, Mad-Max heterodimers repress transcription at the same binding sites (Grandori, Cowley et al. 2000). Max has also been shown to bind two other bHLH-LZ proteins, including Mnt, which has been shown to repress transcription in certain cell types, and Mga, which has been shown to activate transcription at T-box binding sites upon Max dimerization (Hurlin, Queva et al. 1997, Hurlin, Steingrimsson et al. 1999). Since c-Myc requires Max to activate transcription of target genes, any other proteins that dimerize with Max also serve as potential antagonists of c-Myc target transcriptional activity.

Binding of the MYC-MAX complex to E-boxes can facilitate either the initiation or elongation of transcription (Cole and Cowling 2008). The most common model for transcriptional initiation suggests that MYC-MAX binding at promoters results in an increase in histone acetylation, thus allowing acetyl-histone binding proteins to bind, resulting in increased transcription (Cowling and Cole 2006). Specifically, MYC (typically through a MBII-dependent interaction) binds to histone acetyl-transferase complexes including transformation/transcription-domain associated protein (TRRAP), as well as either general control of amino-acid-synthesis protein-5 (GCN5) or TIP60 (McMahon, Wood et al. 2000,

Cowling and Cole 2006, Cole and Cowling 2008). Additionally, MYC binds to the p300/CBP acetyltransferases (Figure 1.2) (Vervoorts, Luscher-Firzlaff et al. 2003). Recruitment of these acetyltransferases to the promoters of MYC-target genes results in increased histone acetylation and thus open chromatin allowing for the recruitment of other factors which influence transcription. In addition to its role in transcriptional initiation, MYC has been implicated in transcriptional elongation. Recent work showed RNA pol II paused at most cellular promoters, suggesting an added layer of transcriptional regulation (Guenther, Levine et al. 2007). After phosphorylation of Ser-5 on the C-Terminal Domain (CTD) of RNA pol II, transcription is frequently paused (Saunders, Core et al. 2006, Cole and Cowling 2008). However, MYC can recruit the positive transcription-elongation factor-b (P-TEFb) complex, which promotes transcriptional elongation via an additional phosphorylation event at Ser-2 on the CTD of RNA pol II (Cole and Cowling 2008).

In addition to its role as a transcriptional activator, MYC has been implicated in the repression of transcription (Gartel and Shchors 2003, Kleine-Kohlbrecher, Adhikary et al. 2006). In fact, as many as 25% of identified MYC transcriptional targets may be repressed rather than activated (Zeller, Jegga et al. 2003). However, unlike the ability of MYC to activate transcription, there does not appear to be a unifying mechanism by which MYC drives transcriptional repression. One mechanism by which MYC contributes to transcriptional repression is through the binding to a MYC-interacting zinc finger protein called zinc finger and BTB domain containing 17 (ZBTB17, or Miz1). Both INK4B (p15) and WAF1 are activated when Miz1 binds near the promoter region, but recent evidence has demonstrated that MYC can bind to Miz1, thus preventing Miz1 from binding DNA (Staller,

Peukert et al. 2001, Wu, Cetinkaya et al. 2003). In this way, MYC acts as an indirect repressor of transcription. Additionally, Li et al showed that MYC is able to target (not necessarily directly) a transcription initiator element (Inr) in adenovirus-2 major late promoter and thus repress transcription (Li, Nerlov et al. 1994). Inr elements are found in other genes, which may be targeted by MYC for transcriptional repression.

Bona fide transcriptional targets of MYC, whether repressed or activated, have been the source of some controversy in the field, particularly in the context of cancer where MYC is often deregulated. There are many reasons true transcriptional targets of MYC are difficult to identify. For example, MYC-MAX transcriptional activity on either synthetic promoters or suspected target genes is relatively weak compared to other activators, with a 2-5 fold increase in expression being the norm (Grandori and Eisenman 1997). Additionally, MYC's ability to either activate or repress transcription of a wide array of genes makes it difficult to distinguish direct from indirect transcriptional effects. Despite these challenges, many groups have attempted to identify MYC target gene sets with the ultimate goal of further characterizing MYC's role in both physiological and pathological contexts. Guo et al compared MYC null fibroblasts to the parental MYC wild-type rat fibroblasts using cDNA microarray analysis (Guo, Malek et al. 2000). Here, they found 198 genes which were responsive to physiologically expressed MYC, though this gene set was much larger than (and dissimilar from) the gene set identified following ectopic overexpression of MYC in the same cell lines (Guo, Malek et al. 2000). This finding suggested that supraphysiological levels of MYC may result in transcriptional alteration of a distinct set of genes. Using a similar approach, Perna et al identified nearly 300 genes which were induced by serum in a

MYC-dependent manner, and called them MYC-dependent serum-response (MDSR) genes (Perna, Faga et al. 2012). Here, they used ChIP-seq to confirm that MDSR genes were directly targeted by MYC. Numerous groups have since used an array-based approach to delineate a transcriptional program that is unique to MYC (Coller, Grandori et al. 2000, Schuhmacher, Kohlhuber et al. 2001, Menssen and Hermeking 2002, Watson, Oster et al. 2002, O'Connell, Cheung et al. 2003, Ji, Wu et al. 2011). Despite a fervent and widespread effort to elucidate the MYC transcriptional program, the resulting gene sets often have little overlap. Recent work has attempted to explain this finding by positing that MYC has no unique transcriptional program, but rather acts to amplify all actively transcribed genes (Lin, Loven et al. 2012, Nie, Hu et al. 2012). Using c-Myc activation, Nie et al identified responsive genes in B and T lymphocytes as well as mouse embryonic stem cells (Nie, Hu et al. 2012). The direction of response varied (roughly two-thirds of the genes were upregulated, while one-third were repressed), and the magnitude of change was both modest and variable across datasets. The authors could not rationalize the discordance of response between datasets, leading them to believe a unifying target set for Myc binding did not exist. Nie et al went on to show that MYC binds to open chromatin, and that MYC binding correlates with the expression levels of most active genes in the genome, as well as with RNA pol II binding (Nie, Hu et al. 2012). Lin et al made a similar finding, albeit in tumor cells (Lin, Loven et al. 2012). Specifically, Lin et al showed that MYC primarily occupies promoters of actively transcribed genes (as measured by co-occupancy with RNA pol II) in a lymphoma cell line with inducible MYC, and the increased MYC occupancy was shown to result in increased absolute levels of RNA. Additionally, MYC was found to primarily occupy the active promoters in small-cell lung cancer (SCLC), multiple myeloma (MM), and

glioblastoma multiforme (GBM) (Lin, Loven et al. 2012). Despite the evidence to suggest a general amplifier role for MYC, this is not the universally accepted model. Recent work in human cells and murine tumor models demonstrated that changes in MYC levels can activate and repress a unique set of target genes (Walz, Lorenzin et al. 2014). Here, Walz et al demonstrated that functionally distinct classes of target genes contain different E-box sequences in their respective promoters. Specifically, target genes involved in oncogenic properties such as migration, angiogenesis and metastasis showed a strong increase in MYC occupancy and expression upon MYC induction in U2OS cells, and the change in occupancy positively correlated with the fraction of non-consensus E-boxes in the promoter (Walz, Lorenzin et al. 2014). In contrast, the fraction of consensus (high-affinity) E-boxes negatively correlated with the change in MYC occupancy upon MYC overexpression, and these sequences were found enriched in promoters of genes that regulate normal cell growth. Importantly, the change in expression of the genes was proportional to the change in MYC occupancy at the promoter, rather than the overall MYC occupancy. Thus, Walz et al argued that elevated levels of MYC would bind to a unique set of target genes based on affinity for a given E-box sequence, and differentially increase expression of that gene set.

Despite MYC's reputation as a pervasive transcriptional regulator, a body of work is growing that suggests MYC has important biological functions that are independent of its role in transcription. For example, recent work has shown that the DNA-binding activity of MYC can be abrogated via mutation without a noticeable effect on cell proliferation or fibroblast transformation (Cowling, Chandriani et al. 2006). One transcription-independent role of MYC is the methylation of mRNA caps at the 5' end (Cowling and Cole 2007).

mRNA capping with a guanylyl group is required for protection from phosphatases and other nucleases as well as for translation of the mRNA to occur, though translation cannot occur efficiently unless the cap is methylated by an RNA methyltransferase (Rottman, Shatkin et al. 1974, Shatkin 1976, Cole and Cowling 2008). Thus, MYC acts to increase expression of certain genes through enhanced translation of the corresponding mRNAs. Another example of the transcription-independent role of MYC is the effect it has on DNA replication. Recent work demonstrated that MYC interacts with the pre-replicative complex in a human tumor cell line, and that MYC is required for efficient DNA replication even in the absence of transcription (Dominguez-Sola, Ying et al. 2007). Whereas any effect MYC had on genomic stability was previously thought to be an indirect result of its transcriptional role, this work provided the first evidence to combat this notion.

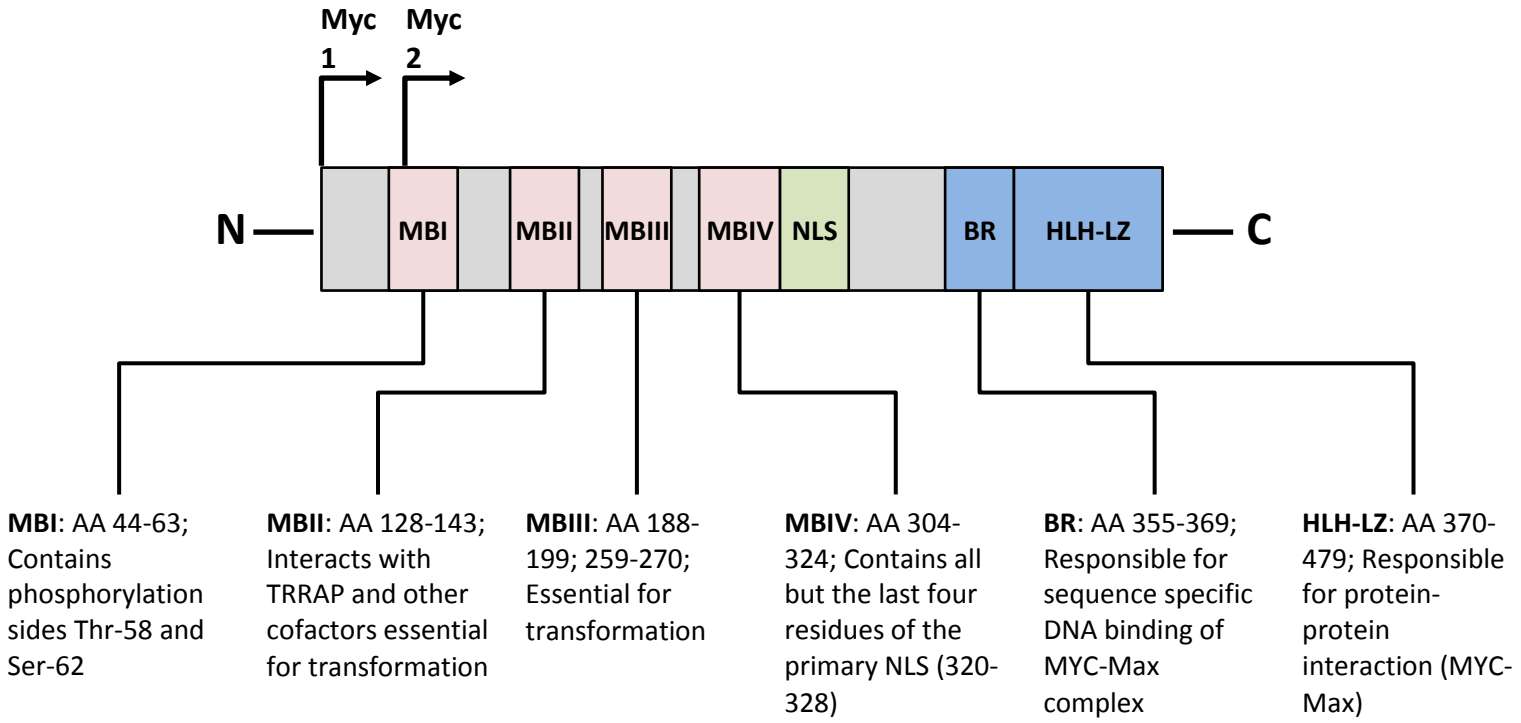


Figure 1.1: Topology of the MYC protein, including the location of all conserved MYC boxes (MBI – MBIV), the nuclear localization signal (NLS), the basic region (BR) and the helix-loop-helix Leucine zipper (HLH-LZ) region. All amino acid locations are based on the more common transcription start site (Myc-2), rather than the cryptic start site, which leads to the less abundant Myc-1.

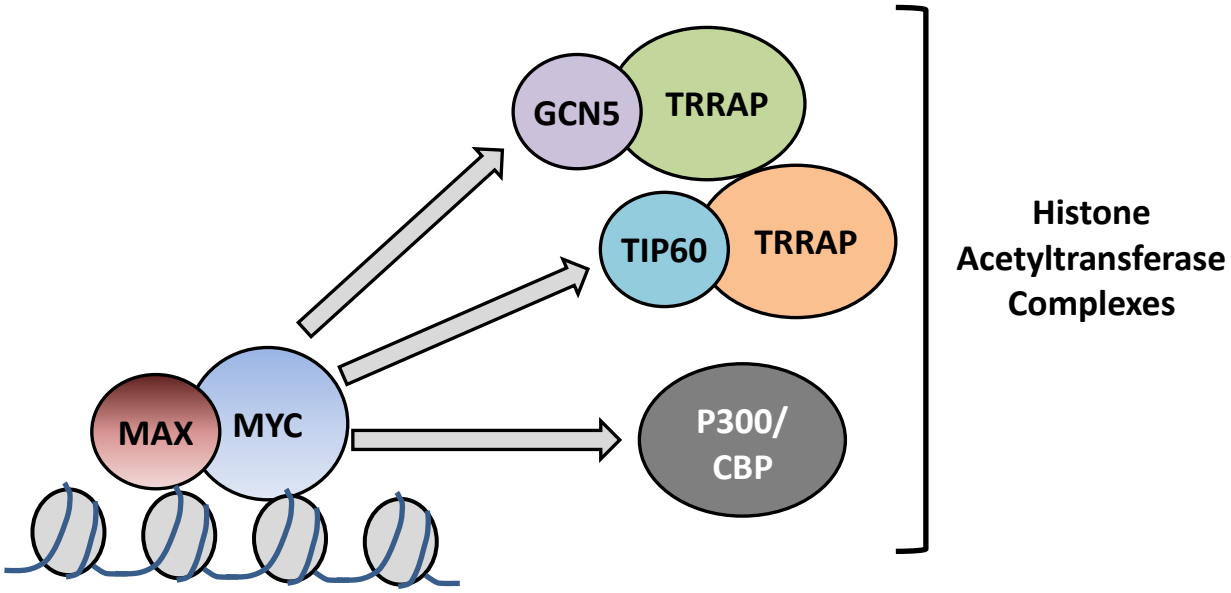


Figure 1.2: MYC Binding Schematic. The MYC-Max complex binds DNA, and through a typically MBII-dependent interaction, binds to one of the complexes pictured above. Recruitment of these acetyltransferases to promoters of MYC target genes results in increased histone acetylation and thus open chromatin, allowing for the recruitment of other factors which influence transcription.

1.3 The role of c-Myc in normal physiology

MYC is a short-lived phosphoprotein that has been implicated in nearly all facets of normal cell function. Specifically, MYC plays a crucial role in cell proliferation, regulation of differentiation, programmed cell death, metabolism and the regulation of stemness. Cell proliferation is largely under the control of cyclins and cyclin-dependent kinases (CDKs). While the cyclin and CDK families are large (21 CDK encoding genes and 29 cyclin encoding genes), only a subset of these proteins are involved in cell-cycle regulation (Bretones, Delgado et al. 2015). CDK1,2,4 and 6 as well as cyclin families A,B,E and D have been directly implicated in cell-cycle control. Shortly after the initial discovery of MYC, it was found that its protein expression correlated well with cell proliferation states. Indeed, MYC's ability to drive cell proliferation has been well documented, and includes direct regulation of key components of cell cycle progression (Amati, Alevizopoulos et al. 1998, Dang 1999, Eilers 1999). In quiescent cells, MYC expression is typically undetectable (Pelengaris, Khan et al. 2002). Upon mitogenic stimulation, MYC levels increase rapidly, peaking approximately three hours after stimulation (Sears 2004). At the beginning of G1 phase, high levels of MYC (the result of mitogenic stimulation) induce expression of the canonical MYC targets cyclin D2 and CDK4 (Coller, Grandori et al. 2000, Hermeking, Rago et al. 2000). Cyclin D2 and CDK4 form a complex and sequester kinase inhibitory protein 1 (KIP1, or p27), which normally inhibits formation of cyclin E-CDK2 complexes (Bretones, Delgado et al. 2015). The complex formation with their respective cyclins and thus activation of CDK2 and CDK4 leads to hyperphosphorylation of the retinoblastoma (Rb)

protein, which initiates the release and accumulation of E2F transcription factors (Bretones, Delgado et al. 2015). E2F transcription factors induce expression of proteins required for S phase, such as those proteins involved in DNA synthesis (Nevins 2001). One such gene is cyclin A2, which is synthesized and localized to the nucleus upon initiation of S phase (Girard, Strausfeld et al. 1991). CDK2 complexes with cyclin A2, and this complex is required for progression through S phase of the cell cycle. Finally, cyclins B1 and B2, which are also transcriptional targets of MYC, are induced and complex with CDK1 (Seo, Kim et al. 2008). CDK1-cyclin B complexes drive cells through mitosis, the final phase of the cell cycle (Sherr and Roberts 2004).

To confirm MYC's role in cell cycle progression, numerous groups have undertaken MYC overexpression and downregulation experiments. Forced expression of MYC in mammalian cells has been shown to result in several pro-cell cycle progression responses, including a reduced requirement for mitogenic signals, blocked exit from the cell cycle, accelerated cell division, and increased cell size (Sorrentino, Drozdoff et al. 1986, Karn, Watson et al. 1989, Iritani and Eisenman 1999). Recent work has clearly implicated MYC in cell proliferation using a tet-off conditional expression system. In this study, Schuhmacher et al demonstrated that the proliferation rates, the fraction of cells in S phase and the cell size all increase in a dose-dependent manner upon induction of MYC (Schuhmacher and Eick 2013). Experiments to biochemically inhibit MYC function provide similarly compelling evidence that MYC is directly involved in cell cycle progression. Specifically, the use of antisense transcripts to inhibit MYC function result in accelerated differentiation and, importantly, the inhibition of G1 progression (Prochownik, Kukowska et al. 1988). While MYC has a

reasonably well-defined role in cell cycle progression, it is unclear whether it is an absolute requirement. In fact, significant redundancy exists among many of the most crucial MYC-targets involved in cell cycle progression, including the cyclin D family, which has been shown to be dispensable for normal proliferation (Kozar, Ciemerych et al. 2004). Additionally, several groups have shown that MYC itself is dispensable for normal proliferation in both fibroblasts and human tumor cell lines (Mateyak, Obaya et al. 1997, Tidd, Giles et al. 2001).

In addition to its role in cell cycle regulation, MYC has been implicated in the inhibition of cell differentiation. Initially, MYC's role as a driver of cell proliferation was seen as incompatible with differentiation, though more recent reports have demonstrated MYC has the ability to more directly block differentiation. Specifically, La Rocca et al demonstrated that ectopic expression of MYC in primary quail myoblasts resulted in the inability to differentiate normally (La Rocca, Crouch et al. 1994). Further, the authors used mutational analysis to show that the leucine zipper (LZ) region was essential for the differentiation block, but dispensable for MYC's role in transformation. Thus, MYC was shown to block differentiation of muscle cells, and this function was found to be independent of MYC's ability to heterodimerize with Max and transform normal cells (La Rocca, Crouch et al. 1994). More recently, Ryan et al used a differentiation-deficient variant of a lymphoma cell line to study the role of MYC (Ryan and Birnie 1997). The differentiation-deficient cell line responds to a differentiation inducer by undergoing rapid growth arrest, but does not actually differentiate. This study showed that during growth arrest, the differentiation-deficient cell line still showed high levels of MYC relative to the parental cell line, which underwent

growth arrest and differentiation. Further, the use of antisense oligonucleotides in the growth-arrested cells was used to prove that MYC expression was required for the blockage of differentiation (Ryan and Birnie 1997). Thus, while high MYC expression is indeed incompatible with proliferative halt and subsequent differentiation, MYC also has the ability to directly influence the differentiation state of cells.

It is well understood that in order for a tumor to develop and progress, the unregulated proliferation must be complemented by evasion of apoptosis. Interestingly MYC, among other well-studied oncogenes, has been shown to have pro-apoptotic activity. Using Rat-1 fibroblasts with constitutive MYC expression, Evan et al showed that the cells undergo substantial cell death (Evan, Wyllie et al. 1992). The authors went on to show that the cell death occurred by apoptosis, and that this process was dependent upon MYC expression. Additionally, Evan et al used mutational analysis to determine which regions of the MYC protein were required for this pro-apoptotic effect. Similar to the regions necessary for MYC's transformative ability, the bHLH-LZ region and parts of the transactivation domain were both found to be essential for the pro-apoptotic role of MYC (Evan, Wyllie et al. 1992). In a separate study, Askew et al used an IL-3 dependent murine cell line that is known to downregulate MYC and known MYC targets upon IL-3 deprivation, resulting in G1 arrest (Askew, Ashmun et al. 1991). Here, the authors used a constitutive MYC expression vector to show that upon IL-3 withdrawal, these cells prematurely initiated apoptosis. Thus, in the absence of MYC expression, IL-3 withdrawal results in cell cycle arrest and eventual cell death. However, if MYC is artificially overexpressed in this setting, an accelerated apoptotic response occurs, suggesting MYC has a pro-apoptotic function in this context (Askew,

Ashmun et al. 1991). Some of the earliest evidence directly linking MYC to induced apoptosis came from studies elucidating MYC's role in cytochrome c release from mitochondria. Here, Juin et al show that MYC induces release of cytochrome c in a caspase-independent manner (Juin, Hueber et al. 1999). Cytochrome c then complexes with apoptotic protease-activating factor 1 (APAF1) to create the apoptosome, which in turn activates the caspase cascade that leads to cell destruction. Insulin-like growth factor 1 (IGF1), a pro-survival factor known to inhibit cytochrome c release from the mitochondria, was shown to inhibit MYC-induced apoptosis (Juin, Hueber et al. 1999). MYC's role in induced apoptosis was later found to be mediated through the conserved region myc box IV (MBIV) (Cowling, Chandriani et al. 2006).

MYC plays a defined role in the regulation of glycolysis and glutaminolysis, two essential metabolic processes for normal cells. Many genes required for the glycolytic pathway contain consensus E-box sequences in their promoters, and are known to be directly activated by MYC (Kim, Zeller et al. 2004). Osthus et al used MYC-transformed Rat1a fibroblasts to find upregulation of several key glycolytic genes, including glucose transporter 1 (GLUT1) (Osthus, Shim et al. 2000). The authors went on to show that upon adenoviral delivery of MYC *in vivo*, the same panel of glycolytic genes was induced, thus mimicking the result seen *in vitro*. The upregulation of GLUT1 proved to be functionally important, as MYC induced cells exhibited increased glucose uptake (Osthus, Shim et al. 2000). In addition to its role in glucose import and glycolysis, MYC is known to stimulate genes involved in glutamine metabolism. Wise et al recently demonstrated that MYC coordinates the expression of genes necessary for glutamine catabolism, which leads to metabolic

reprogramming of the cell and subsequent addiction to glutaminolysis (Wise, DeBerardinis et al. 2008). Gao et al later showed that MYC can indirectly induce expression of glutaminase, a key enzyme involved in glutaminolysis, through suppression of miR-23-a/b (Gao, Tchernyshyov et al. 2009). In further support of MYC's role in metabolic processes, acute deletion of MYC resulted in suppression of activation-induced glycolysis and glutaminolysis in T cells (Wang, Dillon et al. 2011).

Last, MYC has a well-established role in the stem cell properties of cells. Most notably, MYC is one of four genes which, when introduced via retrovirus, can reprogram somatic cells to pluripotency (Okita, Ichisaka et al. 2007). Additionally, MYC has been shown to regulate the stem cell properties in certain cell lineages, as recently demonstrated in hematopoietic stem cells (HSCs) (Wilson, Murphy et al. 2004). Further, immature HSCs are known to express MYC, and MYC family knockout (of MYC and MYCN) results in pancytopenia and rapid lethality, suggesting lack of MYC function results in the inability to proliferate and subsequently differentiate into the appropriate cell types (Laurenti, Varnum-Finney et al. 2008). Another recent report has implicated MYC in the regulation of self-renewal in bronchoalveolar stem cells (BASCs). Here, Dong et al showed that MYC is highly expressed in BASCs and developing lung, and suggest that MYC overexpression disturbs the balance between self-renewal and differentiation, which results in the expansion of the stem cell population (Dong, Sutor et al. 2011). It has been proposed that MYC's role in pluripotency is a function of its direct involvement in the regulation of the cell-cycle, though a more direct mechanism remains a possibility (Singh and Dalton 2009).

In addition to the key roles discussed above, MYC has also been implicated in protein synthesis, angiogenesis, and reactive oxygen species (ROS) production (Dang 1999, Guo, Malek et al. 2000, Meyer and Penn 2008). As a result of MYC's pervasive role in multiple facets of cell growth and proliferation, expression of MYC is tightly regulated under normal conditions. The stability of MYC protein is controlled through several phosphorylation events that occur on the amino-terminal end of the protein, within the conserved region MBI. Two phosphorylation sites, Thr-58 and Ser-62, are conserved across species and serve as the primary regulatory sites of MYC protein stability (Sears 2004). Ser-62 is a target of the extracellular receptor kinase (ERK), and Thr-58 is a target of glycogen synthase kinase (GSK-3β), making both phosphorylation sites targets of Ras-mediated signaling pathways. These phosphorylation sites were found to have opposing effects on MYC protein stability, as Ser-62 phosphorylation stabilizes MYC protein and Thr-58 destabilizes it (Sears, Nuckolls et al. 2000). After stimulation by growth factors, MYC levels increase and the Ras pathway is activated, leading to phosphorylation at Ser-62 and MYC protein stability. Ras activation also prevents phosphorylation at Thr-58 by activating Akt, which inhibits GSK-3β. Importantly, an improperly activated Ras pathway (as is seen in some cancers) results in enhanced MYC protein stability (Sears, Leone et al. 1999). Toward the end of the G1 phase of the cell cycle, Akt signaling declines, leading to an increase in GSK-3β and subsequent phosphorylation at Thr-58 and MYC degradation (Sears 2004). Of note, recent evidence has implicated protein phosphatase 2A (PP2A) in the regulation of MYC protein stability. Specifically, Sears et al demonstrated that PP2A directly interacts with the amino-terminal end of the MYC protein, and that Ser-62 is a target for dephosphorylation by PP2A (Sears

2004). Thus, both Ser-62 and Thr-58 are critically important regulatory residues on the MYC protein, the malfunction of which leads to altered MYC stability and function.

The bulk of the degradation of MYC protein is mediated by the proteasome, but cleavage by calpains plays an important role as well. Calpain-dependent cleavage is a calcium-dependent process that occurs primarily in the cytosol, where it has been shown to inactivate MYC by cleavage of the carboxy terminus. This process has been shown to result in a MYC cleavage product, called "MYC-nick", which is a 298-amino acid protein that has been implicated in the regulation of muscle differentiation (Conacci-Sorrell, Ngouenet et al. 2010). The primary mode of MYC degradation relies upon the proteasome, however. In this process, an E1 ubiquitin-activating enzyme activates ubiquitin, which is then transferred to an E2 ubiquitin conjugating enzyme. The E2 enzyme works in concert with the target-specific E3 ligase to facilitate the transfer of the ubiquitin molecule to a lysine on the target protein (Farrell and Sears 2014). Lys-48 on the added ubiquitin molecule is the target of further reactions, eventually generating the poly-ubiquitin chain that will be recognized by the 26S proteasome. A large number of E3 ubiquitin ligases that target MYC have been identified, including SCF^{Fbw7}, Skp2, and CHIP (Kim, Herbst et al. 2003, Welcker, Orian et al. 2004, Yada, Hatakeyama et al. 2004, Paul, Ahmed et al. 2013).

1.4 c-Myc alterations in human cancer

MYC has been shown to cooperate with another oncogene, Ras, to transform murine cells (Land, Parada et al. 1983). While two oncogenic events proved sufficient in murine cells,

human cells require additional oncogenic events for transformation (Hahn, Counter et al. 1999, Boehm, Hession et al. 2005). Thus, MYC deregulation in the context of cooperating genetic events has shown to be highly correlated, and in some cases causative in human tumors. In addition to the development of some human tumors, MYC deregulation has been implicated in the progression and risk stratification of diagnosed patients (Albihn, Johnsen et al. 2010). MYC is susceptible to several mechanisms of deregulation that have been observed in human cancer, including chromosomal translocation, overexpression and amplification. Mutations, while rare, have been reported. In instances of MYC deregulation, stimulation of MYC is no longer dependent on external signals, often resulting in unchecked growth and proliferation, a hallmark of cancer. The most notable example of MYC deregulation comes in the form of a chromosomal translocation that is causative in Burkitt's Lymphoma, a non-Hodgkin's B cell lymphoma (Taub, Kirsch et al. 1982). In this malignancy, chromosomes 14, 2 or 22 (which are the locations of the immunoglobulin heavy and light chain genes) are translocated with the *MYC* locus on chromosome 8, bringing the MYC gene in close proximity to the promoter of one of the immunoglobulin genes. This translocation results in a fusion gene that constitutively activates transcription of MYC (Albihn, Johnsen et al. 2010). As a result of this deregulation, Burkitt's Lymphoma has the highest proliferation rate observed for any human tumor.

Relative to translocation, amplification is a more ubiquitous mechanism of MYC deregulation in human cancer. MYC amplification has been observed in a wide variety of tumor types, including lung, breast, and medulloblastoma. In lung cancer, reports indicate up to 20% of non-small cell lung cancer and 30% of small-cell lung cancer diagnoses exhibit

MYC amplification (Albihn, Johnsen et al. 2010). Additionally, some reports suggest treatment may impact MYC copy numbers. Brennan et al studied 90 small-cell lung cancer patients and found that 8% of the untreated samples exhibited amplification of MYC (or a MYC family gene), whereas 28% of the treated samples exhibited amplification (Brennan, O'Connor et al. 1991). In a recent paper, MYC gain (defined as > 2 copies per nucleus) was observed in 20% of patients, and this gain proved to be an independent poor-prognosis factor for both disease free survival and overall survival (Seo, Yang et al. 2014). MYC amplification is seen at an even higher rate in breast cancer, where as many as 48% of primary samples have an amplification of the 8q24 region, though some reports suggest this number is much lower (Ioannidis, Mahaira et al. 2003). Importantly, several groups have investigated the prognostic value of MYC amplification in patient tumors. In breast cancer, MYC amplification correlates with progression from the *in situ* to the invasive stage of the carcinoma, making MYC amplification the first genetic alteration to do so (Robanus-Maandag, Bosch et al. 2003). Further, amplification of MYC has been observed in medulloblastoma, which is the most common malignant pediatric brain tumor seen in the clinic (Albihn, Johnsen et al. 2010). Using *in situ* hybridization, a recent study detected MYC mRNA in 31% of medulloblastoma cases studied, and showed a significant association between MYC expression and shorter patient survival times (Eberhart, Kratz et al. 2004). Additionally, a more recent report showed that MYC protein expression is correlated with survival in primary diffuse large B-cell lymphomas of the central nervous system (Tapia, Baptista et al. 2015). Here, the authors found elevated MYC protein expression in 43% of patients, and showed that this was correlated with poorer overall survival. Similar reports have identified MYC amplification in numerous other tumor types, including atypical

carcinoid lung cancer, cancer of the bladder, cervix, colon, prostate, liver, ovaries and kidneys, as well as large cell neuroendocrine carcinoma, melanoma, esophageal carcinoma, and gastric cancer (Vita and Henriksson 2006).

MYC amplification is often associated with protein overexpression, though this is not always the case. Recently, co-amplification of the long non-coding RNA PVT1 was shown to be a requirement for high MYC protein levels in 8q24-amplified human cancer cells (Tseng, Moriarity et al. 2014). Additionally, despite MYC translocation being causative in Burkitt's Lymphoma, a recent study of pediatric cases found MYC overexpression in only 91% of cases, suggesting some translocations may not result in protein overexpression (Frost, Newell et al. 2004). Thus, some human cancers exhibit genetic alteration of MYC without corresponding protein overexpression. Similarly, MYC mRNA or protein overexpression has been observed without corresponding DNA amplification or translocation. In a study of 136 primary breast carcinomas, Escot et al studied the copy number and mRNA expression of MYC(Escot, Theillet et al. 1986). Here, the authors found that half of the patients with a normal MYC gene copy number displayed elevated MYC mRNA levels (Escot, Theillet et al. 1986).

In contrast to many other proto-oncogenes, mutations in the MYC gene are quite rare. However, mutations are observed, particularly in the context of a translocated MYC gene, as is seen in Burkitt's Lymphoma. Bhatia et al screened the entire coding region of MYC in a panel of Burkitt's Lymphomas and found that 65% of cases displayed at least one amino acid substitution relative to wild type (Bhatia, Huppi et al. 1993). Interestingly, these mutations

typically occurred at phosphorylation sites, such as Thr-58. This finding suggests that the MYC mutations may affect the stability of the MYC protein, and thus likely have a pathogenic role in this malignancy. A summary of MYC deregulation in various cancer types can be found in Table 1.1.

Table 1.1

Tumor Type	*MYC Deregulation*	*Reference*
B-Cell ALL	*Rearrangement/Amplification (47-52%)*	*(Miranda Peralta, Valles Ayoub et al. 1991)*
Burkitt's Lymphoma	*Translocation (100%); Overexpression (91%)*	*(Boxer and Dang 2001, Albihn, Johnsen et al. 2010)*
Diffuse Large Cell Lymphoma	*Rearrangement/Translocation (6-16%)*	*(Kramer, Hermans et al. 1998, Vitolo, Gaidano et al. 1998, Akasaka, Akasaka et al. 2000, Frost, Newell et al. 2004)*
Multiple Myeloma	*Translocation (15%)*	*(Avet-Loiseau, Gerson et al. 2001)*
Primary Plasma Leukemia	*Rearrangement (13%)*	*(Avet-Loiseau, Gerson et al. 2001)*
Atypical Carcinoid Lung Cancer	*Amplification (17%)*	*(Gugger, Burckhardt et al. 2002)*
Bladder Cancer	*Amplification (33%)*	*(Sardi, Dal Canto et al. 1998)*
Breast Cancer	*Amplification (9-48%); Overexpression (45%)*	*(Albihn, Johnsen et al. 2010)*
Cervical Cancer	*Amplification (29%)*	*(Abba, Laguens et al. 2004)*
Colon Cancer	*Amplification (17%)*	*(Finley, Schulz et al. 1989, Smith, Myint et al. 1993)*

Gastric Cancer	***Amplification (15-30%); Overexpression (47%)***	***(Hajdu, Kozma et al. 1997, Hara, Ooi et al. 1998, Han, Kim et al. 1999, Koo, Kwon et al. 2000)***
Glioblastoma	***Overexpression of ANY family member (57-78%)***	***(Herms, von Loewenich et al. 1999, Hui, Lo et al. 2001)***
Hepatocellular Carcinoma	***Amplification (33%)***	***(Kawate, Fukusato et al. 1999)***
Large Cell Neuroendocrine Cancer	***Amplification (23%)***	***(Gugger, Burckhardt et al. 2002)***
Medulloblastoma	***Amplification (5-15%); Overexpression (31%)***	***(Albihn, Johnsen et al. 2010)***
Melanoma (nodular)	***Amplification (61%)***	***(Treszl, Adany et al. 2004)***
Melanoma (superficial spreading)	***Amplification (28%)***	***(Treszl, Adany et al. 2004)***
Esophageal Squamous Cell Carcinoma	***Amplification (10-30%)***	***(Bitzer, Stahl et al. 2003)***
Osteosarcoma	***Amplification (7-78%)***	***(Ladanyi, Park et al. 1993, Squire, Pei et al. 2003)***
Ovarian Cancer	***Amplification (40%); Overexpression (44%)***	***(Baker, Borst et al. 1990)***
Prostate Cancer	***Amplification (30-50%); Overexpression (70%)***	***(Fleming, Hamel et al. 1986, Buttyan, Sawczuk et al. 1987, Bubendorf, Kononen et al. 1999)***

Renal Clear Cell Carcinoma	***Amplification (8%)***	***(Kozma, Kiss et al. 1997)***
Lung Cancer	***Amplification (20%)***	***(Albihn, Johnsen et al. 2010)***

1.5 MYC induced models of human cancer

Given the frequency with which MYC is deregulated in human cancer, it is unsurprising that MYC induction has been a popular approach for researchers developing oncogene-induced models of cancer. The earliest studies showing MYC's role in transformation of normal cells came from a study that demonstrated MYC and Ras cooperate to transform primary embryo fibroblasts (Land, Parada et al. 1983). Since this initial study, MYC overexpression has been used as a driving oncogenic event in various cell and mouse models. One of the earliest models of MYC-induced cancer was developed by replacing the MYC promoter with a hormonally inducible mouse mammary tumor virus promoter (Stewart, Pattengale et al. 1984). In the MMTV-MYC model, spontaneous mammary adenocarcinomas developed in the founder mice during pregnancy. MYC overexpression was also used in lung cells in an effort to develop lung carcinomas. Ehrhardt et al used murine MYC under the control of a lung-specific promoter and found that mice developed bronchio-alveolar adenomas that eventually progressed to adenocarcinomas, albeit with incomplete penetrance (Ehrhardt, Bartels et al. 2001). However, Geick et al overexpressed MYC specifically in Clara Cells, which resulted only in bronchioalveolar hyperplasia, suggesting that MYC overexpression alone in these cells was not enough to initiate a tumor (Geick, Redecker et al. 2001). Constitutive MYC overexpression led to a significantly increased understanding of MYC's role in tumor initiation, but conditional models of MYC overexpression have also proven useful for studying tumorigenesis. The Eμ and MMTV models, among others, have been used to generate both Tet-on and Tet-off inducible systems. Using a Tet-off model, Felsher and Bishop induced MYC in hematopoietic cells to form T-

cell lymphoma and acute myeloid leukemia (Felsher and Bishop 1999). In a separate study, Boxer et al use a Tet-on system to generate breast adenomas upon induction of MYC expression (Boxer, Jang et al. 2004). One of the benefits of the inducible models was that they allowed researchers to determine whether MYC was important for initiation, maintenance, or both.

In addition to solid tumors, MYC overexpression also drives tumorigenesis in lymphoma models. In an effort to recreate the MYC translocation seen in Burkitt's Lymphoma, Adams et al fused MYC to an immunoglobulin enhancer element (Eμ), resulting in mice that developed both pre-B cell and mature B cell lymphomas (Adams, Harris et al. 1985). As this fusion protein was the only manipulation made, this study demonstrated that MYC overexpression was the only change needed to drive lymphomagenesis. In addition to the models discussed, MYC overexpression has been used to drive T-cell lymphoma, prostate adenocarcinoma, and pancreatic endocrine tumors (Morton and Sansom 2013). A summary of these findings can be found in Table 1.2.

While MYC overexpression alone can successfully drive tumorigenesis in many contexts, several models have been crossed with mice that lack a functional tumor suppressor gene, such as p53. Numerous groups have demonstrated that overexpression of MYC cooperates with loss of p53 in tumorigenesis. Blyth et al showed that mice which overexpressed MYC and had a homozygous null mutation in p53 were viable, but had an increased frequency and decreased latency of thymic lymphoma development (Blyth, Terry et al. 1995). However, a separate study suggested that this cooperative effect between MYC and p53 may be tissue-

specific. Elson et al used the MMTV-MYC model and showed that, when combined with a single p53 null allele, mice developed aggressive pre-T and T-cell lymphomas much more quickly than mice carrying either the p53 null allele or MYC alone (Elson, Deng et al. 1995). However, the authors went on to show that the presence of a p53 null allele in conjunction with MYC overexpression did not accelerate the development of mammary carcinomas. Additionally, only 25% of the mammary tumors that arose had lost their wild-type p53 allele, whereas 100% of the lymphomas had done so. In another study, Sato et al used an *in vitro* model based on immortalized human bronchial epithelial cells (HBECs) to study the importance of MYC in tumorigenesis (Sato, Larsen et al. 2013). Here, the authors demonstrated that MYC alone could not drive an *in* vitro model to tumorigenesis, but when overexpressed in the context of inactive p53 and oncogenic Ras, MYC could fully transform normal lung epithelial cells. These findings suggest that the cooperative relationship between MYC and p53 loss may not be universal.

Table 1.2

Model	*Tissue*	*Outcome*	*Reference*
Eμ-c-MYC	*Hematopoietic*	*Pre-B Cell Lymphoma; Mature B cell lymphoma*	*(Adams, Harris et al. 1985)*
CD2-c-MYC	*T cells*	*T cell lymphoma*	*(Stewart, Cameron et al. 1993)*
MMTV-c-MYC	*Mammary*	*Mammary adenocarcinoma*	*(Stewart, Pattengale et al. 1984)*
Probasin-c-MYC	*Prostate*	*Invasive adenocarcinoma*	*(Ellwood-Yen, Graeber et al. 2003)*
SP-C-c-MYC	*Lung alveolar epithelium*	*Hyperplasia, adenoma, carcinoma*	*(Ehrhardt, Bartels et al. 2001)*
UG-c-MYC	*Lung clara cells*	*Clara cell hyperplasia of the lung, T-Cell lymphoma*	*(Geick, Redecker et al. 2001)*
WAP-MYC	*Mammary*	*Mammary*	*(Schoenenberger,*

		adenocarcinoma	***Andres et al. 1988)***
Elastase-tv-a, Cdkn2a -/- + RCAS-c-MYC	***Pancreas***	***Pancreatic endocrine tumors***	***(Lewis, Klimstra et al. 2003)***

1.6 Overview of the efforts to target MYC in human cancer

Justifiably, the demonstrable role MYC plays in tumor initiation and maintenance has garnered it significant attention as a potential therapeutic target in many tumor types. However, drug developers have long viewed MYC as a challenging therapeutic target for several reasons. First, MYC expression is nearly ubiquitous in proliferating cells (including non-cancerous cells), and thus its inhibition might lead to unacceptable toxicity. Second, inhibition of MYC function will likely require the inhibition of its interaction with either Max or other cofactors. The protein surfaces that participate in the protein-protein interactions with MYC are relatively featureless, and thus lack structural motifs that would make suitable drug binding sites (Prochownik and Vogt 2010). Third, mutations in MYC are quite rare in human cancer, with certain lymphomas being the exception. Researchers cannot rely on the abundant experience in enzyme inhibitor design, as these principles often apply to mutated enzymes. PLX4032, for example, was developed to specifically target the mutated form of BRAF (Yang, Higgins et al. 2010). Gain of function mutations in kinases have long been used to distinguish the oncogenic form of the kinase from the normal form, thus limiting the widespread effect of drugs and their associated toxicity. The paucity of MYC mutations, particularly in solid tumors, makes this an unlikely approach to targeting MYC.

Despite these potential challenges, several methods have been used to target MYC, including antisense oligonucleotides, conventional RNA-interference (RNAi), pharmacological inhibition, and genetically engineered mouse models. The earliest

approach, the antisense strategy, involves generating antisense oligonucleotides targeted to the mRNA of specific genes. After hybridization to target mRNA, translation would be blocked, thus repressing protein expression of the target (Prins, de Vries et al. 1993). Harel-Bellan et al used this method in human T-cells and showed a reduction of MYC protein synthesis, as well as reduced entry into S phase of the cell cycle (Harel-Bellan, Ferris et al. 1988). Using the same approach, Prochownik et al showed a reduction in endogenous MYC levels in murine erythroleukemia cells, which correlated with accelerated differentiation and reduced progression through the G1 phase of the cell cycle (Prochownik, Kukowska et al. 1988). To further support this method in tumor cells, Wickstrom et al used this approach to target human promyelocytic leukemia cells and showed reduced MYC protein expression as well as reduced proliferation and colony formation (Wickstrom, Bacon et al. 1988, Wickstrom, Bacon et al. 1989). However, despite the promise shown with this approach in early studies, there were some drawbacks that provided an impetus for new method development. One of the chief challenges of the antisense approach is that oligonucleotides are delivered as single strands, and thus must be stable in the cell as a single strand. Further, as the single strands do not associate with any endogenous machinery in the cell, they must find their target mRNA unassisted, which can lead to inefficient inhibition of target translation.

A similar approach to antisense oligonucleotides that relied upon mRNA targeting was later developed, and broadly classified as RNA-interference, or RNAi. RNAi includes the use of both short interfering RNAs (siRNAs), and short hairpin RNAs (shRNAs). siRNAs were first synthesized and used to repress mammalian genes in 2001 (Elbashir, Harborth et

al. 2001). siRNAs are delivered as duplex RNA, thus increasing their stability relative to the single stranded antisense oligonucleotides. The duplex consists of a guide strand and a passenger strand. The duplex will incorporate into the RNA-induced silencing complex (RISC), at which point the passenger strand will be degraded, and the guide strand will base pair with a complementary sequence of mRNA, leading to message degradation of the target gene. siRNAs were used extensively to probe the functional importance of MYC in various tumor models. Prathapam et al used siRNAs against MYC (and other MYC family members) in ovarian cancer cells (Prathapam, Aleshin et al. 2010). Here, the authors demonstrated that siRNA-mediated knockdown of MYC resulted in decreased cell proliferation and induction of replicative senescence, which they argued was mediated through an upregulation of p27 (KIP1) (Prathapam, Aleshin et al. 2010). In another study, Liu et al used siRNAs against MYC in human myeloid leukemia cell lines (Liu, Zou et al. 2009). Here, the authors showed decreased proliferation and colony forming efficiency, as well as increased apoptosis. Despite the early success of siRNAs, there were some concerns. Shortly after the rise in popularity of this technology, it was reported that the siRNAs were not as specific as initially thought (Jackson, Bartz et al. 2003). Additionally, since the siRNAs are not integrated into the genome, the repressive effect was transient, making this approach inappropriate for long-term assays. The use of short-hairpin RNAs, or shRNAs, partially addresses these concerns. Unlike siRNAs, which can load directly onto the RISC, shRNAs are first processed by Drosha and then Dicer. Often delivered virally, shRNAs can be continuously produced by the host cell, whereas less than 1% of the siRNA duplex remains 48 hours after transfection (Rao, Vorhies et al. 2009). This property makes shRNA a much more appropriate approach for long-term assays. Several groups have used shRNAs

against MYC to study the functional importance of MYC in human tumors. Holien et al used this approach in multiple myeloma cell lines, and demonstrated induced cell death upon shRNA-mediated knockdown (Holien, Vatsveen et al. 2012). In a separate study, Wang et al used this approach in human tumor lines from diverse cancer types, including melanoma, osteosarcoma, lung carcinoma, ovary adenocarcinoma, prostate adenocarcinoma, glioma, Ewing's sarcoma, colon adenocarcinoma, breast adenocarcinoma, and large cell lung cancer (Wang, Mannava et al. 2008). Here, the authors demonstrated a reduction in MYC protein, and an inhibition of cell proliferation in all cell lines tested. Of note, the tumor cells underwent proliferative halt at varying stages of the cell cycle, and the outcome appeared to depend on the status of other cell cycle regulators (Wang, Mannava et al. 2008). Thus, both methods of RNAi have proven effective in inhibiting MYC function and assessing dependency in human tumor models.

While RNAi has remained a popular approach, the increased number of genetically engineered mouse models with inducible MYC has provided yet another useful tool. The reversibility of MYC-induced models of cancer allows researchers to determine whether the MYC-initiated tumors remain dependent on MYC for their tumorigenic phenotype. Tran et al generated a double conditional transgenic mouse model of lung adenocarcinoma and lymphoma (Tran, Fan et al. 2008). In this system, MYC expression could be induced in the lung using a Tet-on model. This allowed the authors to study both the effect of MYC deregulation in the lung, as well as the effect of highly specific MYC inhibition. Here, the authors showed that MYC induced lung tumors failed to regress completely upon MYC inactivation, but the frequency of complete regression was increased if both MYC and KRAS

were inhibited simultaneously (Tran, Fan et al. 2008). In a separate study, Jain et al generated a conditional transgenic model for MYC-induced development of osteogenic sarcoma (Jain, Arvanitis et al. 2002). Here, the authors used a Tet-off system to conditionally express MYC in murine lymphocytes *in vivo*. MYC induction resulted in osteogenic sarcomas in ~1% of mice, and upon MYC inactivation, the sarcomas differentiated into mature bone and exhibited sustained tumor regression (Jain, Arvanitis et al. 2002). In yet another conditional model, Shachaf et al generated a MYC-induced model of hepatocellular carcinoma. Here, the authors showed that MYC inactivation resulted in complete regression in some cases, and tumor dormancy in others (Shachaf, Kopelman et al. 2004). Thus, genetically engineered mouse models with inducible MYC have provided a useful tool for studying both MYC deregulation as well as the effects of inhibiting MYC in MYC-driven tumors.

To complement the RNAi and transgenic mouse approaches, a novel reagent was generated that could act as a MYC dominant negative when ectopically expressed in cells. This dominant negative mini-protein, called Omomyc, was originally generated by Soucek et al in 1998 (Soucek, Helmer-Citterich et al. 1998). Here, the authors identified the residues responsible for the specificity of binding between MYC and Max, and then mutated those residues on Myc such that they resembled their complementary residues on Max. The result was a mini-protein that spanned the bHLH-LZ domain, but was capable of dimerizing with MYC (importantly, endogenous MYC does not homodimerize). The Omomyc-MYC dimer exhibited low DNA binding affinity, thus significantly hindering the transcriptional activity of MYC (Soucek, Helmer-Citterich et al. 1998). Since this initial work, Omomyc has been

used in numerous cell-based and mouse-based models to investigate the functional importance of MYC in human tumors. Fukazawa et al used Omomyc in a limited number of non-small cell lung cancer cell lines to argue that only *KRAS* mutant cell lines with overexpressed MYC would respond to Omomyc *in vitro* (Fukazawa, Maeda et al. 2010). Subsequent work, however, showed in a genetically engineered Ras-driven model of lung cancer that all tumors would respond to metronomic MYC inhibition (Soucek, Whitfield et al. 2013). Omomyc has proven to be a useful and popular tool for the functional investigation of MYC in human tumors, but the potential use of Omomyc as a clinical entity has not been fully investigated.

The success seen with RNAi, genetically engineered mouse models, and novel dominant negative-mediated approaches yielded significant interest in the potential for pharmacological MYC inhibitors, including both direct and indirect inhibitors of MYC message, protein and/or transcriptional activity. In addition to the effort to develop novel MYC inhibitors, researchers began to investigate the potential anti-MYC properties that existing drugs may have. Some drugs, such as 10058-F4 and KSI-3716, were developed to directly target MYC, while others such as JQ1, SB218078, Trichostatin A, BI-2536 and diclofenac were developed for other purposes but later found to have anti-MYC properties (Yin, Giap et al. 2003, Delmore, Issa et al. 2011, Zhao, Bai et al. 2012, Gottfried, Lang et al. 2013, Khanna, Kauko et al. 2013, Ciceri, Muller et al. 2014, Jeong, Kim et al. 2014). The ultimate goal of developing a MYC targeted therapy was the ability to selectively target MYC dependent tumors, though to date, no MYC targeted therapy has been used successfully in humans. Thus, despite renewed interest in drugging MYC over the past

decade, there are still many challenges to overcome. While potency has been a significant challenge, the most effective pharmacological approach to MYC inhibition (i.e., direct or indirect inhibition) also remains unclear.

1.7 Overview of the other *MYC* family oncogenes

c-Myc (MYC) is a member of a family of proteins that also includes MYCN and MYCL. All family members share the same topographic makeup, including conserved MYC boxes, nuclear localization signal, basic region, HLH-LZ region, and an open reading frame (ORF) that spans the second and third exons (Albihn, Johnsen et al. 2010). Despite these similarities, there are many important functional differences in the family members.

Several years after the initial discovery of the cellular homolog of the *v-myc* oncogene, Kohl et al cloned a fragment of genomic DNA with homology to *v-myc*, but that was clearly distinct from *c-myc* (Kohl, Kanda et al. 1983). This sequence was found to be amplified between 25 and 700-fold in eight of nine human neuroblastoma cell lines, suggesting the discovery of a new oncogene which was called N-Myc (MYCN) (Kohl, Kanda et al. 1983). The MYCN gene is located on chromosome 2 (2p24), and its role as an oncogene was confirmed when it was used to transform rat embryo fibroblasts in culture (Yancopoulos, Nisen et al. 1985). These initial discoveries were followed by a more complete analysis in neuroblastoma, which showed MYCN amplification in as many as 40% of all cases, as well as the fact that the degree of MYCN amplification correlated well with advanced disease and poor patient survival (Brodeur, Seeger et al. 1984, Seeger, Brodeur et al. 1985). This

information proved important from a clinical standpoint, as neuroblastoma patients identified as having MYCN amplification are treated more aggressively than patients with normal MYCN levels (Look, Hayes et al. 1991). In addition to neuroblastoma, MYCN was found to be amplified in a wide variety of human tumors, including medulloblastoma, glioblastoma multiforme, retinoblastoma, alveolar rhabdomyosarcoma, small-cell lung cancer, prostate cancer and breast cancer (Lee, Murphree et al. 1984, Nau, Brooks et al. 1986, Mizukami, Nonomura et al. 1995, Aldosari, Bigner et al. 2002, Hodgson, Yeh et al. 2009, Beltran, Rickman et al. 2011, Tonelli, McIntyre et al. 2012). Given the frequency with which MYCN was found amplified in various tumors, several groups developed MYCN overexpression systems to model MYCN induced tumorigenesis. Using a Eμ-N-myc transgenic model, transgenic animals were shown to develop lymphoid tumors of pre-B and B cell origin (Dildrop, Ma et al. 1989, Rosenbaum, Webb et al. 1989, Sheppard, Samant et al. 1998). MYCN was later found to play an important role in many of the same functions as MYC, including development and transcriptional regulation.

Expression of MYCN is more tissue restricted than MYC. Early *in situ* hybridization experiments demonstrated that MYCN expression is restricted to developing kidney, hair follicles, and in several parts of the central nervous system (Mugrauer, Alt et al. 1988). In all cases, expression of MYCN was limited to early differentiation states, and declined at the onset of differentiation. Further, MYCN expression was decoupled from proliferation, as several rapidly proliferating cells showed very little expression, whereas post-mitotic (yet undifferentiated) cells in the brain expressed very high levels of MYCN (Mugrauer, Alt et al. 1988). Given the apparent role MYCN plays during development, the degree to which

MYCN is required for embryonic development was questioned. As expected, loss of MYCN leads to embryonic lethality at day 11.5 of gestation (Sawai, Shimono et al. 1991, Charron, Malynn et al. 1992, Moens, Auerbach et al. 1992). Loss of MYCN results in abnormalities in the heart, liver, kidney, limb bud, lungs and nervous system, all tissues where MYCN was previously shown to be expressed (Zimmerman, Yancopoulos et al. 1986, Stanton, Perkins et al. 1992, Sawai, Shimono et al. 1993). Additionally, MYCN knockout mice were shown to have extensive hepatic apoptosis in day 11.5 embryos (Giroux and Charron 1998). Further, MYCN is essential during normal brain development. Conditional disruption of MYCN in murine neuronal progenitor cells (NPCs) resulted in mice with ataxia, behavioral abnormalities, and tremors that correlate with a two-fold decrease in brain mass (Knoepfler, Cheng et al. 2002). The reduced brain mass is likely the result of decreased proliferation of neuronal cells, as targeted deletion of MYCN was shown to significantly reduce proliferating cells as measured by a decrease in S phase and mitotic cells (Knoepfler, Cheng et al. 2002).

The sequence homology between MYCN and MYC suggests the function of each protein will be comparable. Indeed, MYCN acts as a transcriptional regulator for a large number of genes in the human genome. Similar to MYC, MYCN forms a heterodimer with Max through an interaction with its bHLH-LZ region (Wenzel, Cziepluch et al. 1991). Additionally, MYCN exhibits the same sequence specificity as MYC, as the MYCN-MAX heterodimer binds E-boxes in the promoter regions of target genes. Unlike MYC, which has the highest affinity for the 5'-CACGTG-3' sequence, MYCN has the highest affinity for 5'-CATGTG-3' sequence, but can be bound to all other E-box sequences under MYCN-amplified conditions (Murphy, Buckley et al. 2009). Like MYC, the binding of MYCN to

DNA affects transcription through its ability to facilitate histone acetylation, and the ability of MYCN to bind DNA can be affected by E-box methylation patterns (Knoepfler, Zhang et al. 2006). Finally, MYCN has also been shown to repress some transcriptional targets through a variety of mechanisms, including through a physical interaction with DNA methyltransferases (Corvetta, Chayka et al. 2013).

Using similar approaches as with MYC, many groups have attempted to characterize the MYCN transcriptome. Given other similarities between MYC and MYCN, however, it remains to be seen whether a target gene set unique to MYCN exists. Of note is the discordance between the expression pattern of the MYC and MYCN, as well as the cancer types in which each gene has been implicated. As previously noted, MYCN expression is more tissue restricted than MYC, and the tumor types in which MYCN has been implicated are of neuroendocrine (e.g., small-cell lung cancer) or neural (medulloblastoma) origin. Given these differences, a transcriptional program unique to MYCN would not be unexpected.

The third member of the MYC family of genes is MYCL, located on chromosome 1 (1p32) (Nau, Brooks et al. 1985, Zelinski, Verville et al. 1988). MYCL was cloned from small-cell lung cancer DNA, and was shown to have homology to a small region of the other family members, MYC and MYCN (Nau, Brooks et al. 1985). A MYCL homologous sequence was identified in both mouse and hamster DNA, suggesting evolutionary conservation of the gene. In the initial study, MYCL was found to be 10-20-fold amplified in four SCLC cell lines as well as one SCLC patient tumor sample (Nau, Brooks et al. 1985).

Numerous follow-up studies confirmed this finding in SCLC cell lines, primary tumors and xenograft experiments (Johnson, Ihde et al. 1987, Gu, Linnoila et al. 1988, Takahashi, Obata et al. 1989, Noguchi, Hirohashi et al. 1990, Johnson, Brennan et al. 1992, Makela, Saksela et al. 1992, Rygaard, Vindelov et al. 1993). These studies reported the incidence of amplification in SCLC to be in the 5-10% range, though incidence as high as 50% has been shown in some studies, particularly when analyzing SCLC cell lines derived from patients who had been treated. In addition to amplification and overexpression, MYCL was shown to have an EcoRI restriction site polymorphism that could be used to distinguish between a long (L) and short (S) allele (Nau, Brooks et al. 1985). When studied across a large panel of SCLC, lung adenocarcinoma, neuroblastoma and retinoblastoma DNAs, the L allele was found to have a frequency of 0.51, whereas the S allele was found to have a frequency of 0.49 (Nau, Brooks et al. 1985). However, the prognostic value of MYCL allelic differences remains unclear. While most known for its role in SCLC, MYCL has also been found to be amplified or overexpressed in several other tumor types, including ovarian cancer and non-small cell lung cancer (Yamamoto, Shimizu et al. 1997, Wu, Lin et al. 2003).

Of the three family members discussed, MYCL has the most restricted pattern of tissue expression. MYCL is expressed in the kidney, the newborn lung, as well as the proliferative and differentiated tissue of the brain and neural tube (Hatton, Mahon et al. 1996). Zimmerman et al showed that during murine development, MYCL was expressed at its highest levels in newborn forebrain, hindbrain and kidney, and that the lung and intestines expressed much lower levels (Zimmerman, Yancopoulos et al. 1986). While there was a significant decrease in MYCL expression between the newborn and adult forebrain,

hindbrain and kidney, there was no observable difference in expression in adult lung when compared to newborn lung (Zimmerman, Yancopoulos et al. 1986). Thus, like MYCN, MYCL appears to have an early stage role in differentiation pathways. In contrast to MYC, MYCL was never detected in any of the pre-B or B-cell lines tested (Zimmerman, Yancopoulos et al. 1986). In further contrast to its other family members, MYCL knockout animals show no phenotype in any of the tissues analyzed (Hatton, Mahon et al. 1996). The lack of an obvious phenotype in the absence of MYCL activity argues against a unique and required role for MYCL in early development.

Like MYC and MYCN, MYCL acts as a transcriptional regulator through its ability to heterodimerize with Max. While the transcriptional program of MYCL likely overlaps with those of MYC and MYCN, some important differences exist, as evidenced by the inability of MYCL to activate the promoter of the well-established MYC target ornithine decarboxylase (ODC) (Bello-Fernandez, Packham et al. 1993, Nesbit, Tersak et al. 1999). Additionally, while MYC and MYCN were shown to both effectively transform rat fibroblasts, MYCL was shown to be only ~10% as effective in this task, though whether this is a function of a unique transcriptional program or topological differences in the gene remains unclear (Yancopoulos, Nisen et al. 1985, Birrer, Segal et al. 1988). A schematic of the different MYC family members can be found in Figure 1.3.

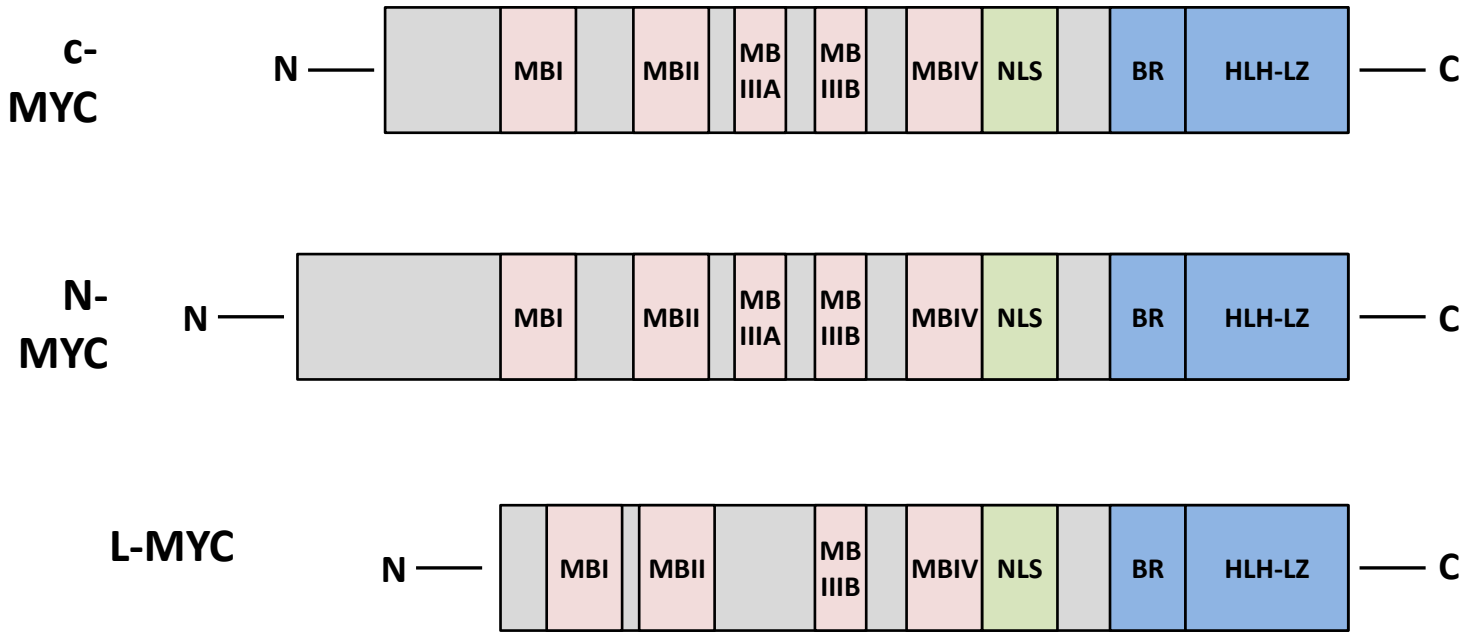


Figure 1.3: Topology of the MYC family members. All family members retain the primary nuclear localization signal (NLS), as well as the Basic Region (BR) and the helix-loop-helix-Leucine Zipper region (HLH-LZ). Additionally, all MYC boxes (MB) are conserved across family members, except MBIII-A, which is absent in L-MYC.

1.8 Hypothesis and specific aims

Given what is known about MYC's role in normal cell physiology and tumorigenesis, I hypothesize that MYC is required for maintenance of the tumorigenic phenotype in a subset of non-small cell lung cancers. I further hypothesize that if a subset of NSCLCs requires MYC, then the resistant subset will have acquired some change to allow those cells to persist in the absence of MYC's transcriptional activity.

1.8.1 Specific Aim One

To characterize MYC deregulation across human tumors and to determine whether our cell line panel recapitulates this deregulation

- **A.** Analyze available data from The Cancer Genome Atlas, including reverse phase protein array, RNA-Seq, and DNA copy number
- **B.** Analyze DNA copy number, mRNA and protein levels of MYC across the panel of NSCLC cell lines

1.8.2 Specific Aim Two

To assess the functional importance of MYC in Non-Small Cell Lung Cancer

A. Utilize purported pharmacological inhibitors of MYC function to probe a large panel of non-small cell lung cancer cell lines

B. Use a well-established MYC dominant negative protein, OMOMYC, to functionally probe MYC dependence in a subset of the NSCLC panel

C. Determine which, if any, of the pharmacological MYC inhibitors correlate with the more specific OMOMYC

1.8.3 Specific Aim Three

To characterize the molecular correlates of MYC-dependence, and to assess the validity of identified biomarkers or mechanisms of resistance

A. Characterize the morphology, gene expression, methylation pattern, and mutational profile of the tested NSCLC cell lines in order to identify correlations with MYC dependence

B. Use molecular correlates to probe potential mechanisms for resistance to MYC dependence

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Lung Cancer Cell Lines

All cell lines used in these studies were originally established in the John D. Minna and Adi F. Gazdar laboratories. The cultured Non-Small Cell Lung Cancer (NSCLC) cell lines were obtained from both the National Cancer Institute (NCI) and Hamon Cancer Center (HCC) libraries. Cells were cultured in RPMI-1640 media (Sigma Life Science, St. Louis, MO) supplemented with 5% Fetal Bovine Serum (FBS). RPMI-1640 supplemented with 5% FBS will be referred to as R5. Normal human bronchial epithelial cells (HBECs) were immortalized with ectopic expression of both CDK4 and hTERT, and were cultured in KSFM (Life Technologies, Carlsbad, CA) with the provided supplements (epidermal growth factor and bovine pituitary extract). All cells were incubated in NuAire (NuAire, Plymouth, MN) humidified incubators at 37°C at 5% CO_2. All cell lines were regularly tested for mycoplasma contamination (Bulldog Bio, Portsmouth, NH) and fingerprinted using a PowerPlex 1.2 kit (Promega, Madison, WI) to confirm the cell line identity.

2.1.2 NSCLC Tissue Microarray

Archived, formalin-fixed, paraffin-embedded tissues from surgically resected lung cancer specimens (acquired during lobectomies and pneumonectomies) containing tumor and adjacent normal epithelium tissues were obtained from the Lung Cancer Specialized Program of Research Excellence (SPORE) Tissue Bank at The University of Texas M. D. Anderson Cancer Center (Houston, TX), which has been approved by an institutional review board. The tissue specimens were histologically examined and classified using the 2004 World Health Organization classification system and 218 NSCLC samples (152 adenocarcinomas and 66 squamous cell carcinomas) were selected for our tissue microarray (TMA). TMAs were constructed using triplicate 1-mm diameter cores per tumor; each core included central, intermediate, and peripheral tumor tissue. Detailed clinical and pathologic information, including patient demographics, smoking history, smoking status, clinical and pathologic TNM stage, overall survival duration, and mutation status of several key oncogenes, was available for most cases.

2.2 Methods

2.2.1 Immunoblotting

Cell lysates were made using 0.1% SDS lysis buffer supplemented with protease and phosphatase inhibitors. Specifically, cell pellets were resuspended in 50-75 µl of lysis buffer and incubated on ice for 20 minutes. Tubes were centrifuged at 14,000 RPM for 15 minutes at 4°C, and the supernatant was collected. Protein content was quantified using the BioRad Protein Assay Dye Reagent Concentrate (BioRad, Hercules, CA). Lysates were

diluted into loading buffer and samples were boiled for 5 minutes before loading 40 µg per sample onto a gel. Cellular proteins were separated by 10% SDS/polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Millipore, Billerica, MA). The membrane was blocked for 1 hour at room temperature (RT) in a 5% milk in TBST solution, then incubated with a primary antibody overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA) for 2 hours at RT. Proteins were detected by enhanced chemiluminescence (Thermo Scientific, Waltham, MA). Primary antibodies used in this study can be found in Table 2.1.

Table 2.1: Primary Antibodies Used in this Study

Target	Antibody	Conditions
c-Myc (Western Blots)	**Santa Cruz sc-764**	**1:1K in 5% Milk in TBST Overnight at 4°C**
c-Myc (IHC)	**Epitomics (Abcam) ab32072**	**1:300. HIER Bond Solution #2, equivalent to EDTA pH 9.0**
Phospho-β-catenin	**Cell Signaling 9561**	**1:1K in 5% Milk in TBST Overnight at 4°C**
β-catenin	**Cell Signaling 9587**	**1:1K in 5% Milk in TBST Overnight at 4°C**
PARP	**Cell Signaling 9542**	**1:1K in 5% Milk in TBST Overnight at 4°C**
HSP90	**Santa Cruz sc-13119**	**1:1K in TBST Overnight at 4°C**
GAPDH	**Gene Tex GTX627408**	**1:1K in 5% Milk in TBST Overnight at 4°C**
Dvl2	**Cell Signaling 3216**	**1:1K in TBST Overnight at 4°C**

2.2.2 Colony Forming Efficiency (CFE) Assays

Cells were counted using a Beckman Coulter Z2 Particle Count and Size Analyzer, and plated at a density of 1,000 cells per well in a 6-well dish. Cells were then allowed 24 hours to attach, at which point fresh R5 supplemented with 2 µg/µl doxycycline (Sigma Life Science, St. Louis, MO) was added. Cells were then allowed to grow for 14 days, with the R5 + doxycycline being replaced every 2-3 days. At the end of the assay, cells were stained with Crystal Violet Staining Solution (50% ethanol, 0.5% Crystal Violet) for 40 minutes, rinsed gently in water and imaged. Colonies were counted if they consisted of >50 cells. For drug treatment assays, the same protocol was followed except instead of adding R5 supplemented with doxycycline, R5 with the relevant concentration of drug was added.

2.2.3 qRT-PCR

mRNA was isolated from cell pellets using the RNEasy Plus kit from Qiagen (Valencia, CA). mRNA was isolated robotically using the QIAcube, also from Qiagen. cDNA was generated using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Gene specific Taq-Man probes (Applied Biosystems, Foster City, CA) were utilized for quantitative analyses of mRNA transcript levels. The GAPDH gene was used as an internal reference to normalize input cDNA. PCR reactions were run using the ABI 7300 Real-time PCR System and analyzed with the included software (Applied Biosystems, Foster City, CA).

The delta-delta-CT method was used to calculate relative mRNA expression levels. qRT-PCR probes used in this study can be found in Table 2.2.

Table 2.2: qRT-PCR Probes Used in this Study

Gene Target	Identifier	Source
MYC	**Hs00153408_m1**	**Life Technologies # 4331182**
N-MYC	**Hs00232074_m1**	**Life Technologies # 4331182**
L-MYC	**Hs00420495_m1**	**Life Technologies # 4331182**
MYC Copy Number Assay	**Hs02758348_cn**	**Life Technologies # 4400291**
rs6983267 Allelic Discrimination	**C__29086771_20**	**Life Technologies # 4351379**
Omomyc	**Custom Sequence**	**Life Technologies 5313596-1**
GAPDH	**4352934-1107035**	**PE Applied Biosystems**
Taqman Copy Number Reference Assay RNaseP	**4401631**	**Life Technologies**
β-catenin	**Hs00355049_m1**	**Life Technologies # 4331182**

2.2.4 Microarray Analysis

Total RNA from cell lines was isolated using RNEasy kit, and robotically extracted on the QIAcube, both from Qiagen (Qiagen, Valencia, CA). Gene expression profiling on each sample was performed using Illumina HumanWG-6 V3 BeadArrays (for the parental lung cell lines GSE32036), and Illumina Human WG-6 V4 BeadArrays for the manipulated cell lines. Bead-level data were obtained and pre-processed using the R package mbcb for background correction and probe summarization. Pre-processed data were then quartile-normalized and log-transformed. Class comparison was performed using MATRIX 1.48 (manuscript in preparation).

2.2.5 Cell Cycle Analysis

50,000 cells were plated in 2 wells of a 6-well dish (per cell line). Cells were then allowed 24 hours to attach, at which point the media was replaced with fresh R5 supplemented with 2 µg/µl doxycycline (Sigma Life Science, St. Louis, MO). Cells were left to incubate for 72 hours, at which point they were trypsinized, pelleted and resuspended in PBS. The cell suspension was slowly pipetted into 2 ml of ice cold absolute ethanol. The suspension was incubated on ice for 15 minutes, after which the cells were pelleted. Vibrant Dye Cycle Green Stain (Life Technologies, Grand Prairie, TX) and the accompanying manufacturer's protocol were used to stain DNA. Cell cycle analysis was performed on a FACS-Calibur flow cytometer.

2.2.6 Xenograft Experiments

Cells at approximately 70% confluency were trypsinized, spun down and then suspended in PBS at a concentration of 2.5 million cells / 50 µl. Using a 27.5 gauge needle and syringe, cells were then injected into the right flank of 4-6 week old female NOD-SCID mice (5 mice per group). Tumors were monitored closely and allowed to engraft and grow to approximately 300 mm^3. At this point, the standard water supply was replaced with water containing 2 mg/ml doxycycline (Sigma Life Science, St. Louis, MO). Doxycycline-supplemented drinking water was replaced approximately every 3-4 days. Tumors were measured and tumor volume was calculated using the equation shown below. A schematic describing the xenograft experiments can be found in Figure 2.1.

$$V = (l * w^2) * 0.52$$

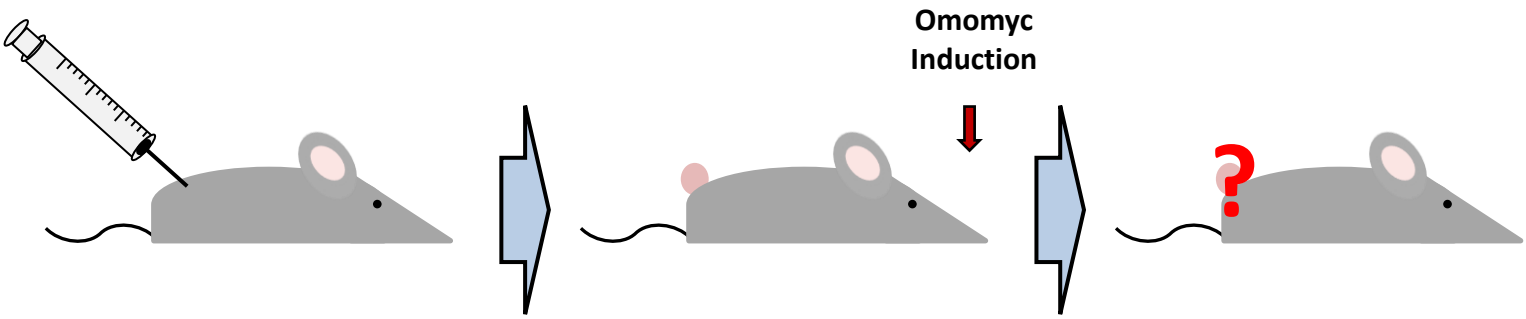


Figure 2.1: Schematic of the xenograft experiments performed. 2.5M cells diluted in 50 µl PBS were injected into the flank of female NOD-SCID mice. Tumors were allowed to engraft and grow to approximately 300 mm^3, at which point Omomyc was induced with doxycycline.

2.2.7 Allelic Discrimination

The genotype at the rs6983267 SNP was determined by amplifying (via PCR) a region of genomic DNA containing the SNP, running the product on a 1% agarose gel and then isolating and purifying the relevant fragment using the Qiagen Gel Purification Kit (Qiagen, Valencia, CA). The PCR fragment was then sequenced. Additionally, genomic DNA was tested using a commercially available allelic discrimination assay (Life Technologies, Grand Prairie, TX). The information for the allelic discrimination probe can be found in Table 3.

2.2.8 Immunohistochemistry (IHC)

Immunohistochemical (IHC) staining for c-Myc was performed on TMA samples as follows: 5 μm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffined, hydrated, heated in a Biocare decloaker for 30 minutes pretreated with Target Retreval Solution (Dako), and washed in Tris buffer. Peroxide blocking was performed with 3% H2O2 in methanol at room temperature for 15 minutes, followed by 35 minute incubations in Tris-buffered saline containing 15% FBS. Slides were incubated with the primary antibody (c-Myc 1:100) at room temperature for 65 minutes, washed with Tris-buffered saline, followed by incubation with Envision Dual Link+ Polymer-Labeled System (Dako) for 30 minutes. Staining was developed with chromogen substrate (Dako) for 5 minutes and then counterstained with hematoxylin, dehydrated, and mounted.

Expression was quantified using light microscopy (total magnification, 200x) and expression was quantified using a four-value intensity score (0, 1, 2, and 3) and the percent of IHC+ tumor cells (0-100%). Intensity scores were defined as follows: 0 = no appreciable staining; 1 = barely detectable staining; 2 = readily appreciable staining; and 3 = dark brown epithelial cell staining. An expression score was obtained by multiplying the intensity and reactivity extension values (range 0 – 300). Expression scores from samples stained with the c-Myc antibodies were dichotomized by their mean values into high or low staining categories.

2.2.9 Ranked KS Test

MYC target gene sets were downloaded from the Molecular Signatures Database v5.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp). To calculate pathways that were down regulated relative to the background distribution on an individual cell line basis, we used a modification of a Kolmogorov Smirnov test. We first determined the degree to which gene expression changes relative to control after application of the inhibitor by dividing treated expression values by their control expression and log2 transforming. Log2 transformed values were then converted to a ranked integer list. To determine the degree to which the values in a set are located towards the top of a ranked list, and thus upregulated relative to background, the following equation was used:

$$u = max_{j=1}^{t}\left[\frac{j}{t} - \frac{V(j)}{n}\right]$$

and to determine the degree to which a set is downregulated relative to background, the following equation was used:

$$u = max_{j=1}^{t}\left[\frac{V(j)}{n} - \frac{(j-1)}{t}\right]$$

where v(j) is the position of each gene in the gene set in the ordered list of genes, t is the total number of genes in the gene set, and n is the total number of genes assayed in the array.

To determine a p-value, 5000 permutations of randomized sorting of genes of genes of the same set size was performed, and u_{random} was calculated. The resulting p-value was determined to be:

$$p = \frac{\#\ instances\ u_{random} > u}{\#\ total\ permutation}$$

Our procedure is superior to a regular KS test in several ways. First, when comparing a large distribution to a small distribution in a regular KS test, the NULL hypothesis is biased towards being rejected. Second, a ranked KS test allows for the preferential ranking of sets that are separated from the background at the tails of the distribution.

2.2.10 TCGA Data Analysis

The TCGA data for copy number was downloaded from the TCGA data portal. The platform for SNP copy number is Affymetrix Genome-Wide Human SNP Array 6.0 with 1.9 million probes. For this data set, only level-3 data are available, which provide a table

of segment values. Thus, the segmented values were downloaded and mapped to individual genes. Copy number amplification was defined as >4 copies for MYC. Comparisons between expression and copy number values were made using a non-parametric T-test.

2.2.11 Reverse Phase Protein Array (RPPA)

Protein expression was analyzed by RPPA through a collaboration with the John Heymach laboratory (MD Anderson, Houston, TX), as part of the Specialized Program of Research Excellence (SPORE) research grant. RPPA protocol was conducted as previously described (Byers, Sen et al. 2009). Briefly, after lysates were isolated, quantified and diluted with SDS sample buffer, they were serially diluted (1:2 – 1:16) with SDS sample buffer. Next, 80% glycerol/2X PBS was added to each of the samples in an equal amount, and the samples were transferred to 384-well plates for analysis. RPPA arrays were printed on nitrocellulose coated glass FAST Slides by a GeneTAC G3 arrayer (Genomic Solutions) with 48 200 µM-diameter pins arranged in a 4X12 format. Automated Bio-Genex autostainer was used to block, and stain for primary and secondary antibodies, and signal was detected using the catalyzed signal amplification (CSA) system, Dako Cytomation (Carpinteria, CA). Between steps, each slide was washed with TBST. The slides were incubated with streptavidin-biotin complex and biotinyl-tyramide (for amplification) for 15 minutes each, streptavidin peroxidase for 15 min, and 3,3-diaminobenzidine tetrahydrochloride chromogen for 5 minutes. Spot images were quantified using imaging analysis with a Hewlett Packard Scanjet 8200 scanner with a 256-shade gray scale at 600

dots per inch. RPPA data were quantified using a SuperCurve method which detects changes in protein level by Microvigene software, VigeneTech (Carlisle, MA), and an R package which was developed at MDACC (Hu, He et al. 2007).

2.2.12 Proliferation Assays

Cells were counted as described above, and plated in a 12-well dish at a density of 5,000 cells per well. Cells were then allowed 24 hours to attach, at which point fresh R5 supplemented with 2 µg/µl doxycycline (Sigma Life Science, St. Louis, MO) was added. Cell counts were then taken in triplicate at days 3, 6 and 10. Doubling time was calculated using the equation shown below.

$$DT\ (days) = D * \ln(2)/\ln(C2/C1)$$

2.2.13 MTS Assay

Relative cell growth was measured using an MTS assay (MTS reagent was acquired from Promega, Madison, WI). 1,000 – 4,000 cells were plated in 50 µl R5 in each well of a 96-well microtiter plate. These cells were allowed 24 hours to attach, at which point an additional 50 µl R5 plus either a control (DMSO) or drug was added to the plate. 96 hours after drug treatment, 20 µl MTS assay reagent (which consists of the tetrazolium compound as well as phenazine methosulfate, or PMS) was added to each well of the plate. The plate was incubated at 37°C at 5% CO_2 for 1-4 hours, depending on when the formation of a

formazan product became visible. Absorbance of formazan, which occurs at 490 nm, was measured for each well of the plate using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA). The absorbance was used as a proxy for cell number, and the IC_{50} for each drug was calculated by determining the drug concentration at which the absorbance reached 50% of control.

2.2.14 High Throughput Drug Screen

Non-small Cell Lung Cancer Cell (NSCLC) lines were cultured in NSCLC culture medium (RPMI/L-glutamine medium (Invitrogen, Inc.), 1000 U/ml penicillin (Invitrogen, Inc.), 1 mg/ml streptomycin (Invitrogen, Inc.), and 5% fetal bovine serum (Atlanta Biologicals, Inc.) for dose-response studies as previously described (Kim, Mendiratta et al. 2013). For dose-response experiments, each cell line was plated on day 1 in 384-well plates at a cell density that would ultimately lead to ~70 – 80% confluency after a 96 hour incubation period following compound addition on day 2. After plating, cell lines were incubated overnight at 37°C in the presence of CO_2 (5%). On day 2, an Echo 555 (acoustic dispense; LabCyte, Inc.) was used to dispense the compounds and vehicle (DMSO) into assay plates containing the cell lines. Twelve (12) doses were added in triplicate for each compound with half-log dilutions between each dose. Dose ranges for these compounds were as follows: SB218078 (49.5 µM to 0.165 nM), JQ1 (49.5 µM to 0.165 nM), THZ1 (49.5 µM to 0.165 nM), and 10058-F4 (495 µM to 1.65 nM). Assay plates were incubated at 37°C for ~96 hours at 37°C in the presence of CO_2 (5%). At the end of the incubation period, each cell line was assayed using the Cell-Titer-Glo reagent (Promega, Inc.) and the data

was analyzed using the GeneData Screening™ software suite (GeneData, Inc.) as previously described (Kim, Mendiratta et al. 2013).

2.2.15 siRNA Transfection

Transfection conditions for lung cancer cell lines and immortalized human bronchial epithelial cell lines were optimized in the lab by monitoring lipid concentration, siRNA concentration and cell number. For 6-well dishes, cells were plated on day 0 at a density that would allow them to reach ~40% confluency on day 1. On day 1, 4 µl RNAiMAX lipid (Life Technologies, Carlsbad, CA) was diluted into 200 µl RPMI-1640 and incubated at room temperature for 5 minutes (this was done for each well). During this incubation, the siRNA was diluted into RPMI such that the final concentration in the well would be 20 nM. The siRNA dilution was added to the lipid dilution, and the two were allowed to complex for 20 minutes at room temperature. During the incubation, the media on the cells in the 6-well dish was replaced with fresh R5. After the 20 minute incubation, the siRNA:lipid solution was added to the wells in a drop-wise fashion. On day 2, one of the wells was trypsinized, counted, and plated in a new 6-well dish at 500-1,000 cells per well for a colony forming efficiency assay. The remaining 5 wells were allowed to grow for 48 hours, at which point they were trypsinized and pelleted for future mRNA extraction. siRNA sequences can be found in Table 2.3.

2.2.16 Lentivirus Production and Transduction

A pTRIPZ-OMOMYC-RFP plasmid (generated by Laura Soucek) was transformed into competent DH5α *E. coli*. Competent cells were thawed on ice for 10 minutes, at which point 0.1 µg of plasmid was added and incubated for 30 minutes. After incubation, cells were heat shocked at 42°C for 45 seconds, allowed 2 minutes on ice to recover, and then incubated in 800 µl SOC media at 37°C for one hour. After incubation, 50 µl of media was streaked onto an LB agar plate containing 100 µg/ml ampicillin and incubated overnight at 37°C. An ampicillin-resistant colony was isolated and expanded in culture for 16 hours on a shaker at 37°C. After expansion, the cells were spun down for 15 minutes at 4,000 RPM, and plasmid DNA was isolated using a Qiagen HiSpeed Plasmid Midi Kit (Qiagen, Valencia, CA). Lentivirus was generated by transfecting HEK-293T packaging cells with the OMOMYC containing plasmid, as well as two packaging plasmids (pMD.G-VSVG, pCMV-ΔR8.91). HEK-293T cells were grown to 50-70% confluency, and all three plasmids were transfected using transfection reagent FuGENE 6 (Roche Life Sciences, Basel, Switzerland). Appropriate viral precautions were taken from this point on. 16 hours after transfection, media was replaced with fresh R5. Viral supernatant was then collected every 24 hours for three cycles. Viral supernatant was pooled, filtered through a 0.45µm filter and stored as 1 ml aliquots at -80°C. Lung cancer cells were transduced with viral supernatant and 4 µg/ml polybrene (Sigma Life Science, St. Louis, MO). 16 hours after transduction, viral media was replaced with fresh R5. 24 hours later, R5 was replaced with fresh media supplemented with 1-2 µg/ml puromycin, and a stable cell line was generated after 4-5 days in selection. The same protocol was followed for pLKO-short hairpins. shRNA sequences can be found in Table 2.3.

Table 2.3: Summary of RNAi Reagents

Reagent	Sequence or Variant	Product ID
si-MYC	Hs_MYC-9 (NM_002467)	Qiagen SI03101847
si-N-MYC	Hs_MYCN-5 (NM_005378)	Qiagen SI03078222
si-L-MYC	Hs_MYCL1-9 (NM_005376)	Qiagen SI03111402
sh-MYC	CCGGCCTGAGACAGATCAGCAACAACTCGAGTTGTTGCTGATCTGTCTCAGGTTTTTG	TRCN0000039642
sh-N-MYC	CCGGCAGCAGCAGTTGCTAAAGAAACTCGAGTTTCTTTAGCAACTGCTGCTGTTTTT	TRCN0000020695
sh-L-MYC	CCGGCCTGTGCCACTAAACTACATTCTCGAGAATGTAGTTTAGTGGCACAGGTTTTT	TRCN0000020579
sh-β-catenin	CCGGAGGTGCTATCTGTCTGCTCTACTCGAGTAGAGCAGACAGATAGCACCTTTTTT	TRCN0000003843

2.2.17 MYC Level Prediction Analysis in Human Tumors

Using MATRIX 1.48 (manuscript in preparation), the top ten NSCLC cell lines ranked by MYC expression (as measured using Illumina HumanWG-6 V3 BeadArrays) were compared to the bottom ten NSCLC cell lines. The Log_2 ratio was calculated for all genes, and the top 100 differentially regulated genes (which were found to be statistically significant by a student's T-test) were used as a predictive signature. The average expression for each gene within the "MYC High" and "MYC Low" group was taken for all 100 genes. Next, the expression values for these 100 genes in each of the 275 tumor samples from the MDACC-275 panel were correlated to both the "MYC High" and "MYC Low" average. MDACC-275 tumors that displayed a higher correlation coefficient with the "MYC High" signature were labeled "High", and tumors that displayed a higher correlation coefficient with the "MYC Low" signature were labeled "Low."

CHAPTER THREE

CHARACTERIZING MYC IN HUMAN TUMORS

3.1 Introduction

MYC plays a role in nearly all biological processes essential for tumor cell survival and growth. In fact, since its discovery more than 30 years ago, MYC has been implicated in most of the hallmarks of cancer originally described by Hanahan and Weinberg, including resisting cell death, sustaining proliferative signals, activating invasion and metastasis, enabling replicative immortality, and inducing angiogenesis (Fujimoto and Takahashi 1997, Hanahan and Weinberg 2000, Nilsson and Cleveland 2003, Morrish, Isern et al. 2009, Rapp, Korn et al. 2009, Cho, Cho et al. 2010, Ma, Young et al. 2010, Singh, Singh et al. 2010, Hanahan and Weinberg 2011, Sotillo, Laver et al. 2011, Chen, Cai et al. 2013, Katanasaka, Kodera et al. 2013). Thus, MYC represents a potential keystone regulator involved in most, if not all essential aspects of tumor initiation and survival. It follows that deregulation of MYC could have wide ranging, possibly deleterious effects on cell growth and proliferation.

Unsurprisingly, MYC is known to be deregulated in a wide variety of cancers, including non-small cell lung cancer. Normal cells are typically protected against deregulated MYC through a variety of checkpoints, including cell cycle arrest and apoptosis, both of which can be initiated upon the pathologic overexpression of MYC. However, in the right genetic and epigenetic contexts, MYC deregulation can bypass these checkpoints and drive tumorigenesis. In many cases, restoration of MYC to physiologic levels can lead to the restoration of the aforementioned checkpoint mechanisms. Previous studies of MYC dependence in various tumor types have

shown a correlation between MYC deregulation, particularly amplification and/or protein expression, and the tumor's dependence on MYC. For example, Fukazawa et al used NSCLC cell lines as a model to study MYC dependence (Fukazawa, Maeda et al. 2010). Here, the authors used two NSCLC cell lines (one with high MYC, and one with low MYC protein levels), and found that only the cell line with high MYC protein showed reduced proliferation upon inhibition of MYC. The authors went on to show that if MYC levels were artificially increased in the "low MYC" cell line, that cell line would then show a negative growth response upon MYC inhibition. Thus, the authors argued that NSCLC cell lines with both mutated *KRAS* as well as overexpressed MYC were dependent on MYC for the maintenance of their tumorigenic phenotype. In a separate study in multiple myeloma, Holien et al used pharmacological inhibition of MYC to assess its functional importance in multiple myeloma cell lines as well as primary myeloma cells (Holien, Vatsveen et al. 2012). Here, the authors used a panel of six multiple myeloma cell lines, five of which expressed MYC. They treated all six cell lines with a drug which had been previously shown to inhibit MYC function, and showed that apoptosis was induced only in the five cell lines which expressed MYC (Holien, Vatsveen et al. 2012). Interestingly, the degree of sensitivity did not directly correlate with MYC levels, as the two cell lines with the highest MYC levels were not the most sensitive to drug treatment. The authors went on to measure MYC levels in primary myeloma cells and found that all patients exhibited levels of MYC mRNA equivalent to or higher than the cell lines tested. Here, the authors showed that while all primary cells were sensitive to the MYC inhibitor, the responses still varied. Further, the degree of sensitivity did not correlate with the endogenous MYC level, suggesting that MYC mRNA or protein would not be an appropriate biomarker for response to MYC inhibition in multiple myeloma (Holien, Vatsveen et al. 2012).

Evidence in the literature suggests that, in some cases, amplified or overexpressed MYC could serve as an effective biomarker for MYC dependence. However, it remains unclear if this will be the case across all tumor types. It follows that in order to determine if an understudied tumor type is dependent on MYC, and further that the level of MYC is an appropriate biomarker, one must first study the nature of MYC deregulation in that tumor type. Thus, the goal of this study was to fully characterize the deregulation of MYC in both human lung tumors samples as well as a non-small cell lung cancer cell line panel. To accomplish this goal, the DNA copy number, mRNA and protein level of MYC would be assessed in several human tumor datasets, as well as a panel of NSCLC cell lines. The genetic signature of the NSCLC cell lines with high levels of MYC would be used to determine if the cell line panel recapitulates the gene expression pattern of the patient samples with high MYC levels. Further, the prognostic value of the MYC levels would be assessed in the patient samples in order to determine if MYC levels were clinically relevant.

3.2 Results

3.2.1 Analysis of MYC Deregulation in Human Tumors

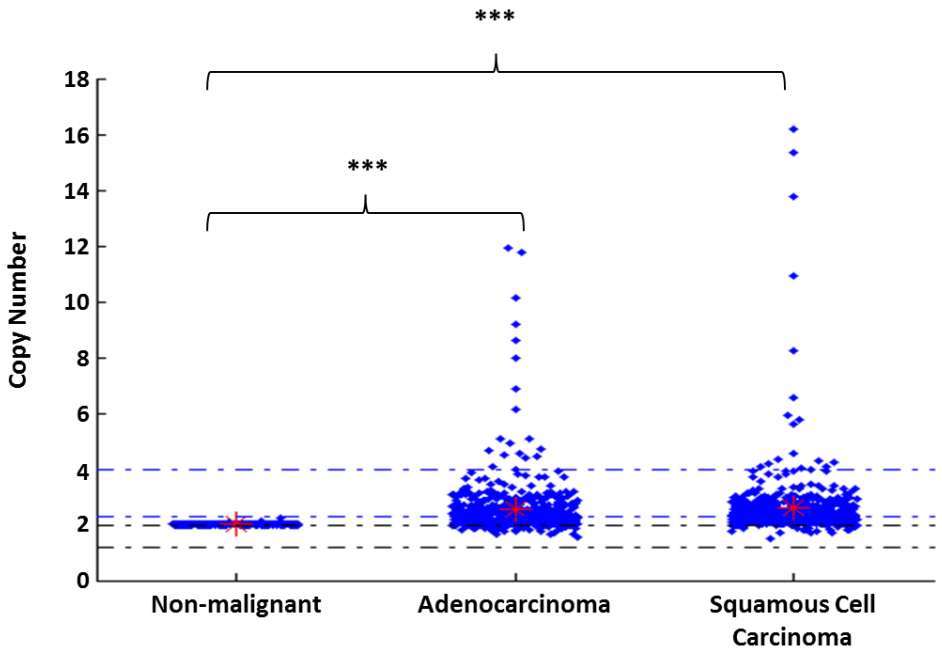
MYC is a tightly controlled transcriptional regulator. Transcription and expression of MYC change depending on numerous factors, including the level of differentiation and the proliferative state of the cell. Additionally, MYC can be deregulated in the context of human cancer, though this may not always be a driving event. Nevertheless, understanding the degree

of MYC deregulation in lung cancer is a necessary first step in understanding the functional role of MYC in this tumor type.

The Cancer Genome Atlas (TCGA) is a coordinated effort by the NIH to expedite the full characterization of the molecular underpinnings of human cancer. TCGA has made available patient and tumor information, including large-scale genome sequencing, for dozens of cancer types, including non-small cell lung cancer. To assess the relative levels of MYC in NSCLC patient samples, we analyzed the Copy Number Variation (CNV) for 1,509 patients and RNASeq data for 1,088 patients from TCGA. The analysis of available TCGA data revealed that 3-4% of lung cancer patients (both adenocarcinoma and squamous cell carcinoma) exhibit a genomic amplification of 4-fold or greater (Fig. 3.1). Both squamous cell carcinomas and adenocarcinomas display a wide distribution of MYC mRNA across the tumors in the panel. Additionally, squamous cell carcinomas show a statistically significant increase in MYC mRNA relative to normal tissue (Fig. 3.1). Surprisingly, adenocarcinomas exhibit lower levels of MYC mRNA as compared to normal tissue (Fig. 3.1). To further validate these results, we analyzed an independent panel of 218 lung cancer tissue samples (66 squamous cell carcinomas, 152 adenocarcinomas) for MYC protein levels. Similar to the TCGA data, the patient tumor samples showed dramatic variation in MYC protein expression. Again, squamous cell carcinomas showed a higher average MYC expression level than adenocarcinomas (Fig. 3.1). Figure 3.2 shows representative images of MYC expression in high and low-expressing NSCLC patient samples. To assess the clinical significance of MYC levels, the protein and mRNA levels were correlated with patient survival data for all data sets available. Interestingly, in all cases tested, elevated MYC had no prognostic relevance in the context of overall survival, and in only one

case was prognostic of poorer cancer free survival (CFS) (Fig. 3.3). Thus, despite frequent deregulation in lung cancer, the level of MYC does not necessarily predict a poor patient outcome.

A.


***
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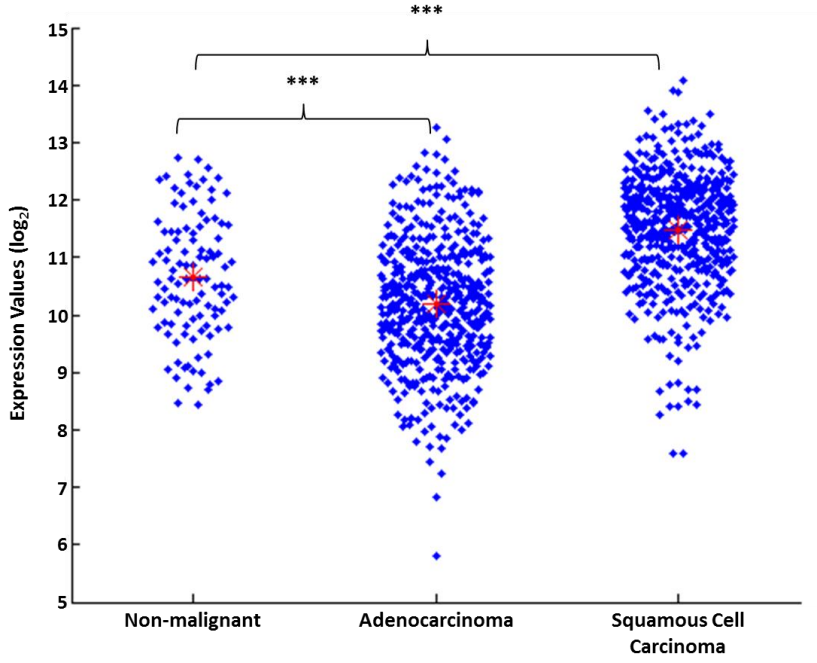
Non-malignant
Adenocarcinoma
Squamous Cell Carcinoma

B.


***
***
Expression Values (log2)
15
14
13
12
11
10
9
8
7
6
5
Non-malignant
Adenocarcinoma
Squamous Cell Carcinoma

C.

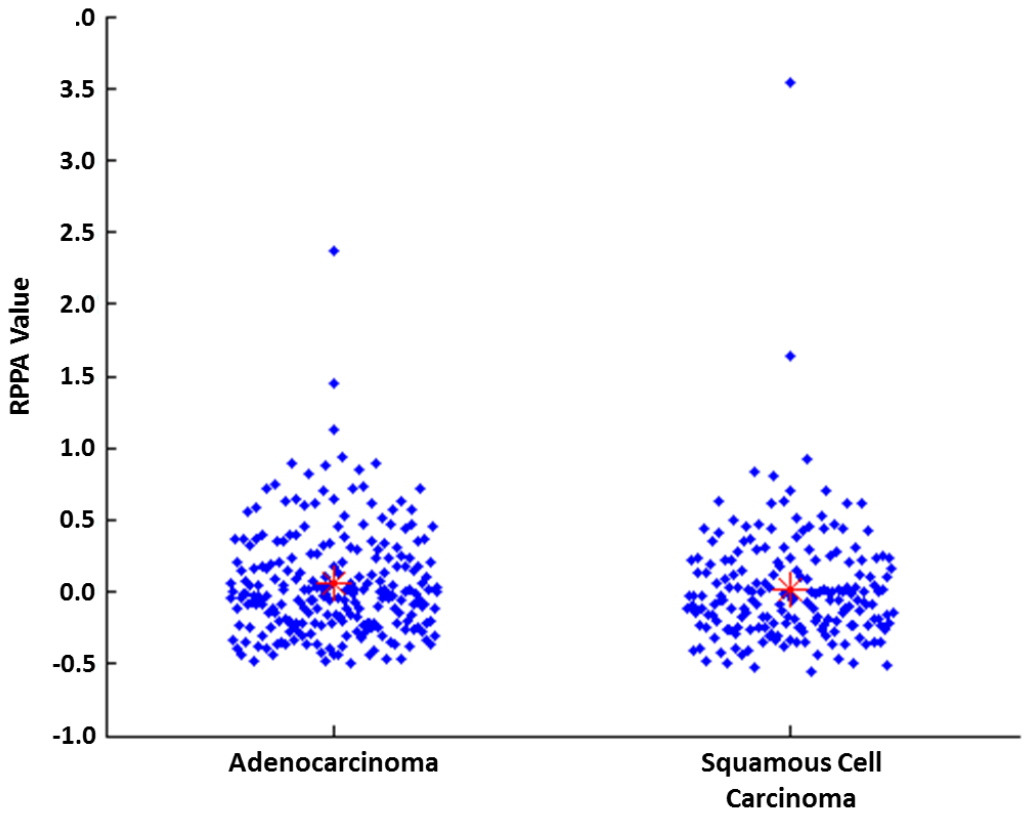
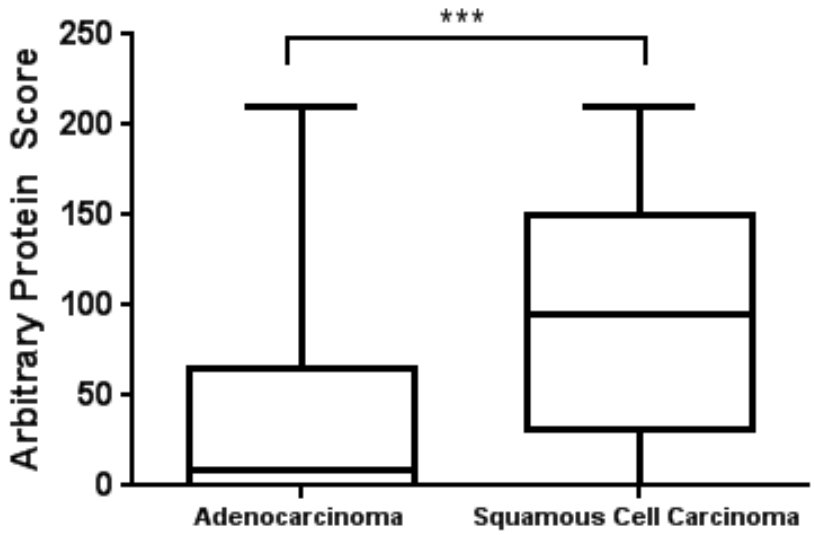


Figure 3.1: Summary of MYC deregulation in human tumors as reported in The Cancer Genome Atlas (TCGA). (A) Copy Number Variation (CNV) shows a statistically significant increase in MYC copy number in adenocarcinoma and squamous cell carcinoma as compared to non-malignant tissue. (B) RNA-Seq data shows lower expression of MYC mRNA in adenocarcinoma relative to non-malignant tissue, but higher expression of MYC mRNA in squamous cell carcinoma. (C) Shows a broad range of MYC protein expression in both adenocarcinoma and squamous cell carcinoma. *** = $p < 0.001$.

A.



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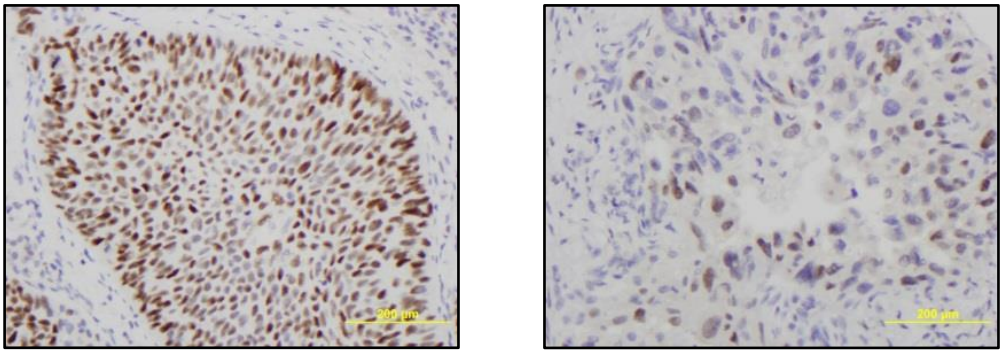
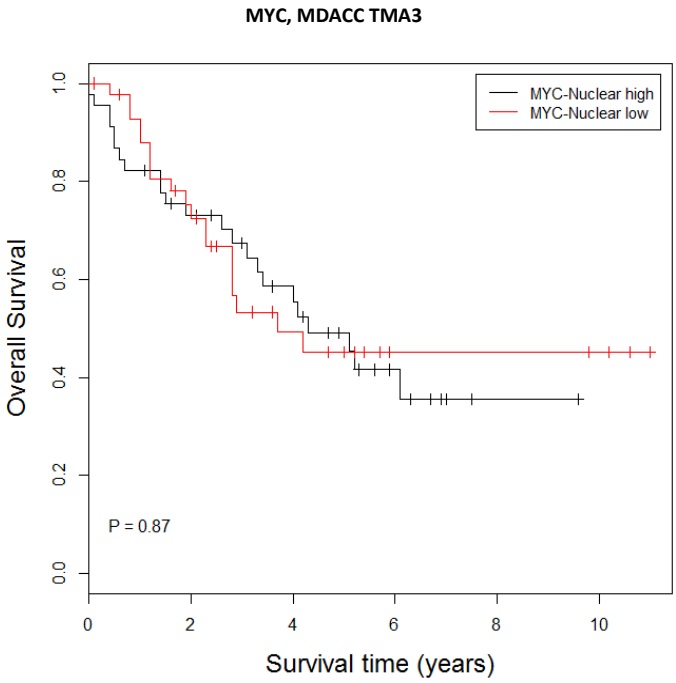


Figure 3.2: MYC immunohistochemistry in non-small cell lung cancer patient samples. (A) Shows the arbitrary protein score assigned to tumors (on a scale of 1-300) and shows that squamous cell carcinomas express a higher level of MYC than adenocarcinomas. (B) Shows representative IHC images of a "High" expressing and "Low" expressing NSCLC patient sample (adenocarcinoma). *** = $p < 0.001$.

A.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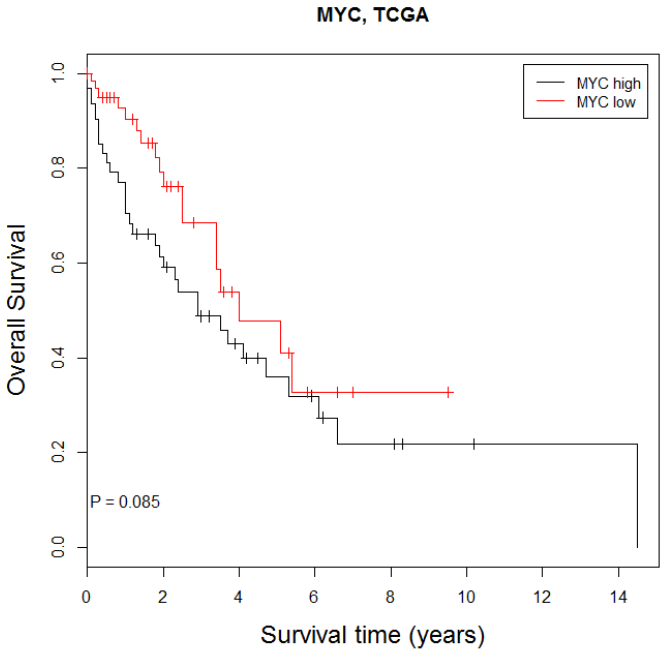
MYC-Nuclear high
MYC-Nuclear low
Overall Survival
P = 0.87
Survival time (years)

B.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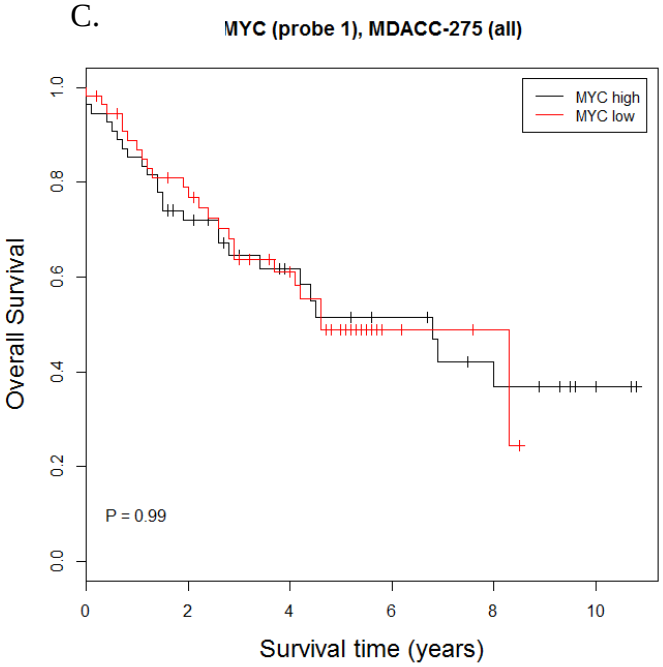
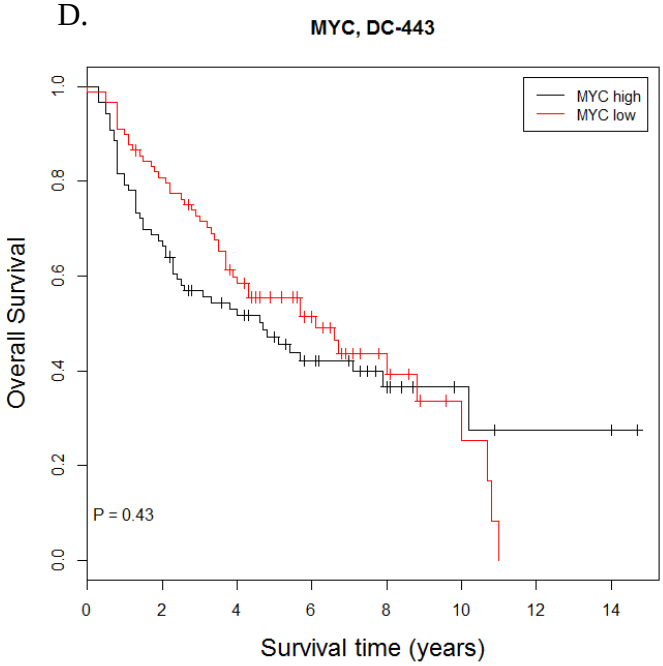
MYC high
MYC low
Overall Survival
P = 0.085
Survival time (years)

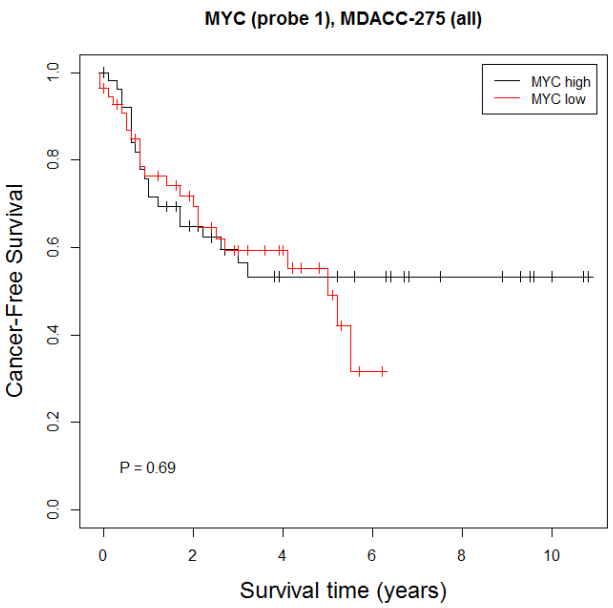
C.


MYC (probe 1), MDACC-275 (all)
MYC high
MYC low
Overall Survival
P = 0.99
Survival time (years)

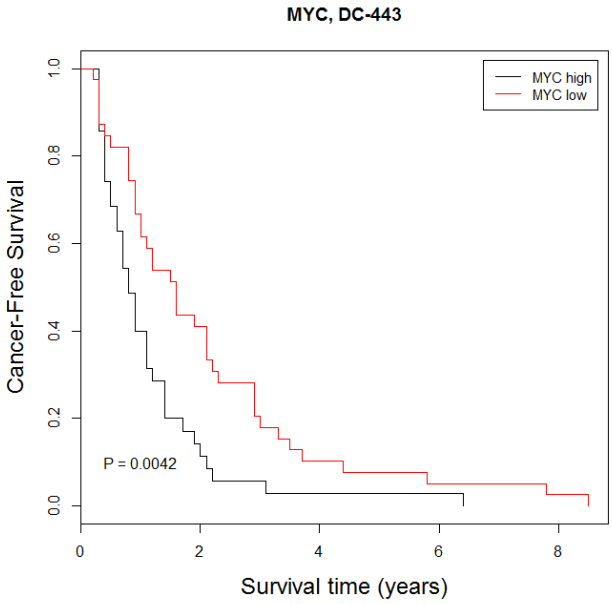
D.


MYC, DC-443
MYC high
MYC low
Overall Survival
P = 0.43
Survival time (years)

E.



F.



G.

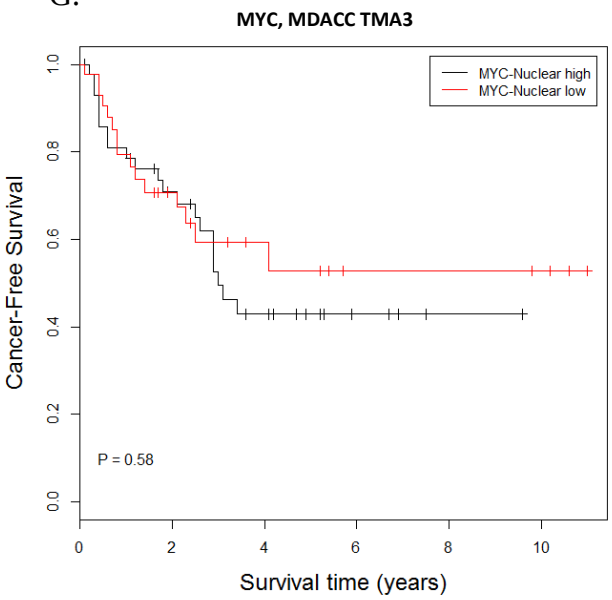


Figure 3.3: Survival analyses of High vs. Low MYC patients. MDACC TMA3 groups were divided into top and bottom quartile, all other data sets were divided into top and bottom quintile. MYC expression does not correlate with overall survival in the MDACC TMA3 (A), TCGA RNA-Seq (B), MDACC-275 qRT-PCR (C), or the DC-443 (D). MYC expression also doesn't correlate with cancer free survival in either the MDACC-275 qRT-PCR (E) or the MDACC TMA3 (G). Patients with high MYC in the DC-443 have a poorer cancer free survival than those with low MYC (F).

3.2.2 Analysis of MYC Deregulation in a Panel of NSCLC Cell Lines

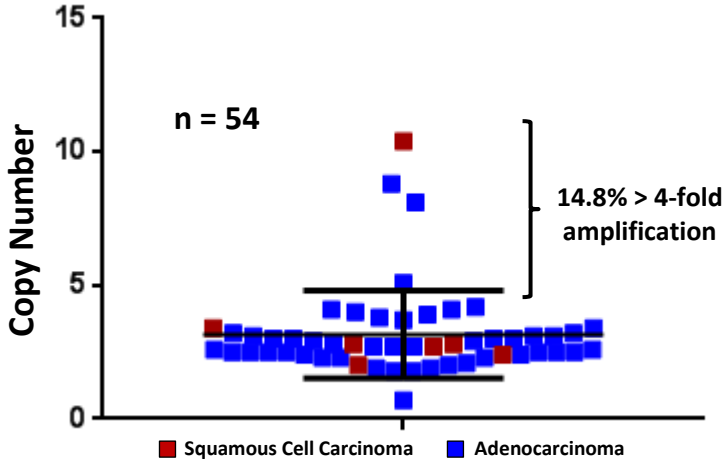
To validate the relevance of our lung cancer cell line panel for this study, we carried out a similar analysis of MYC levels in the NSCLC cell lines. Specifically, we characterized the genomic copy number of *MYC*, as well as mRNA and protein levels in the complete panel of NSCLC cell lines. Each of these analyses were conducted in a high-throughput manner, and then validated with a separate approach.

As measured by SNP copy number array, approximately 15% of the 54 NSCLC cell lines tested showed a copy number amplification of 4-fold or greater (Fig. 3.4). This number was significantly higher than what was observed in the TCGA data. However, this result is consistent with what is seen in the literature, where treatment with chemotherapy has been shown to correlate with an increased frequency of MYC amplification (Brennan, O'Connor et al. 1991). Whereas only about 17% of TCGA samples had prior treatment (106/568 adenocarcinoma; 73/494 squamous cell carcinoma), the lung cancer cell line panel was closer to 50%. The SNP array data was complemented with Taqman copy number assay across a subset of the panel, which showed 4/17 cell lines (~23%) to have a *MYC* copy number amplification of 4 or greater (Figure 3.4). Thus, the Taqman assay confirmed what was seen by SNP array. Next, the Illumina microarray platform was used to measure MYC mRNA levels across the panel of NSCLC cells. Similar to the RNA-Seq data from TCGA, the cell line panel displayed significant variation with respect to MYC mRNA levels (Figure 3.4). To validate this result, qRT-PCR was performed on a subset of the cell line panel. This yielded results consistent with

the microarray data, namely that the cell lines tested displayed a wide range of MYR mRNA levels (>20-fold differences) (Figure 3.4). Next, MYC protein levels were assessed across the cell line panel using reverse phase protein array, or RPPA (Figure 3.4). Again, the high-throughput RPPA data was validated using immunoblotting. In both the RPPA and immunoblots, the cell lines tested showed dramatically different levels of MYC protein (>20 fold differences) (Figure 3.4).

Last, to determine whether *MYC* amplification yielded increased levels of MYC mRNA or protein, the values from each assay were correlated. Interestingly, the correlation between any two datasets never exceeded 0.6, which implies the existence of additional mechanisms regulating MYC transcription, translation and stability. For example, a recent report demonstrated that *MYC* amplification only resulted in MYC protein overexpression if *PVT1* was co-amplified with the *MYC* locus (Tseng, Moriarity et al. 2014).

A.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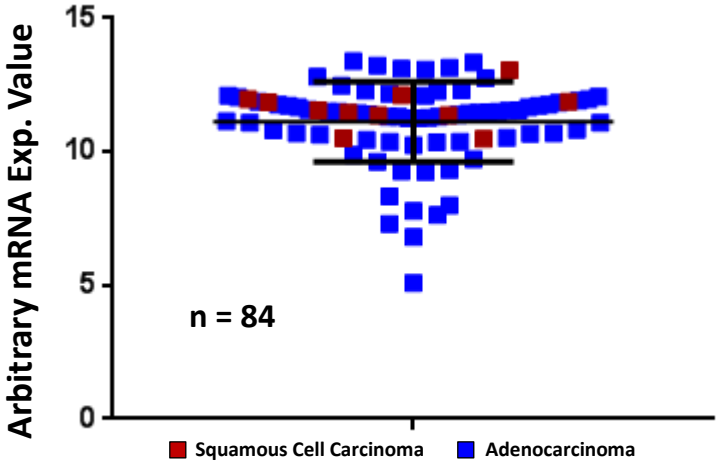
Squamous Cell Carcinoma
Adenocarcinoma

B.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0
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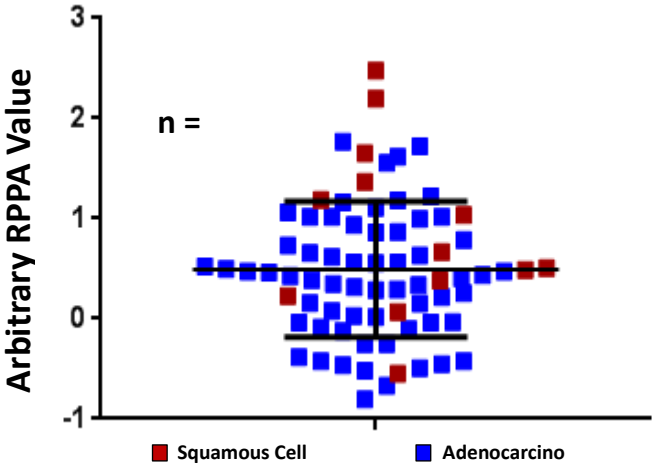
Squamous Cell Carcinoma
Adenocarcinoma

C.


Arbitrary RPPA Value
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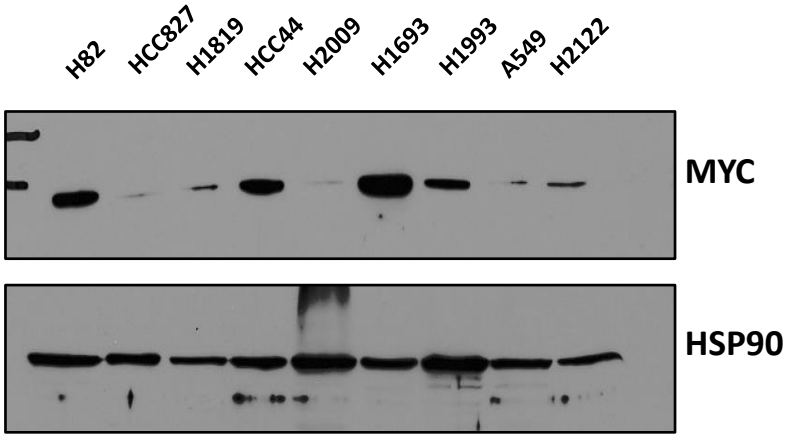
2
1
0
-1
n =
Squamous Cell
Adenocarcino

D.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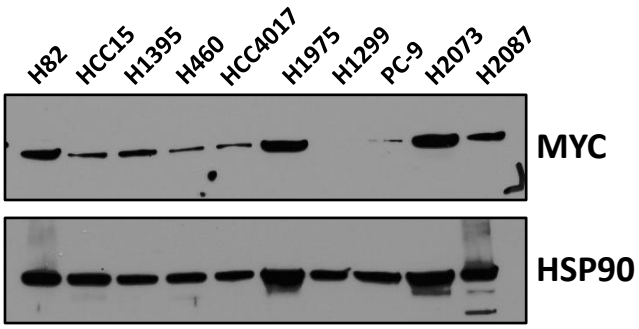
H1819
HCC44
H2009
H1693
H1993
A549
H2122
MYC
HSP90

E.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H460
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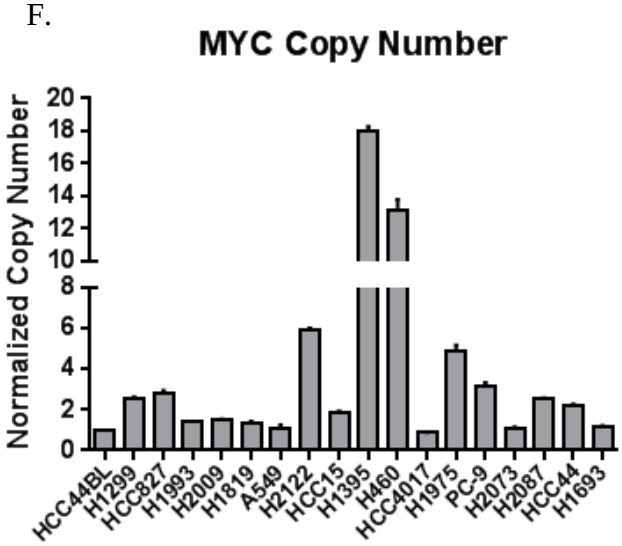
H1299
PC-9
H2073
H2087
MYC
HSP90

F.



G.

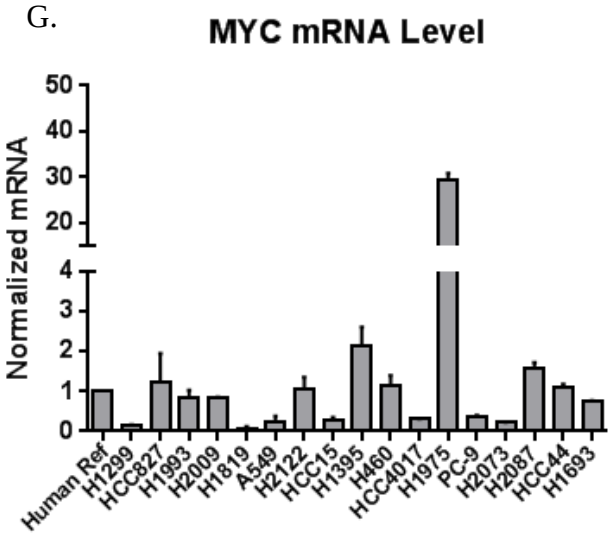
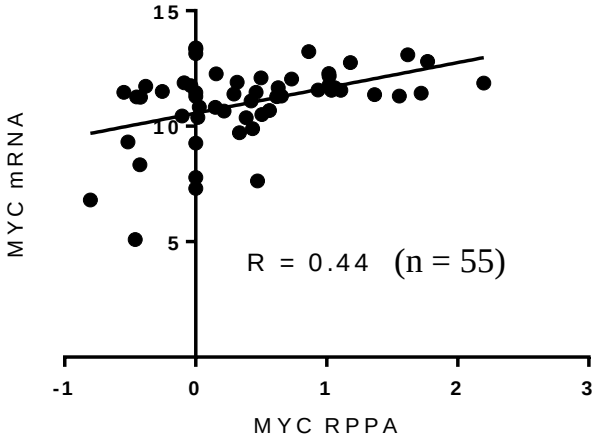
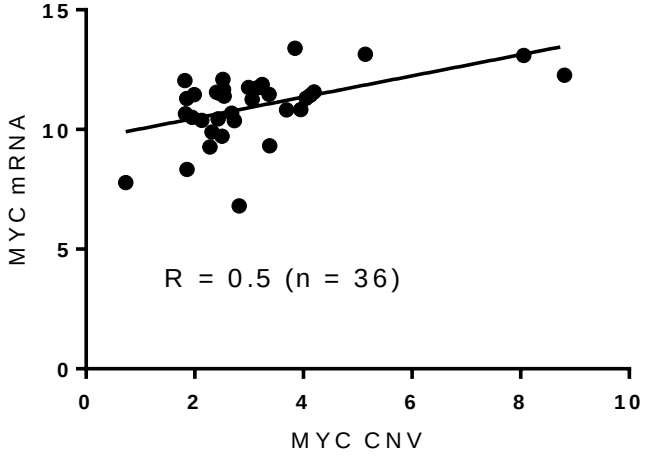


Figure 3.4: MYC Deregulation in NSCLC cell lines. (A) MYC copy number variation as measured by SNP copy number arrays. (B) MYC mRNA as measured by illumine microarray. (C) MYC protein expression as measured by RPPA. (D), (E) MYC protein expression as measured by immunoblot. (F) MYC Copy number as measured by Taqman Copy Number Assay. (G) MYC mRNA as measured by qRT-PCR. H82 was used as a positive control in the Western Blots, as it is known to have high expression of MYC.

A.



B.



C.

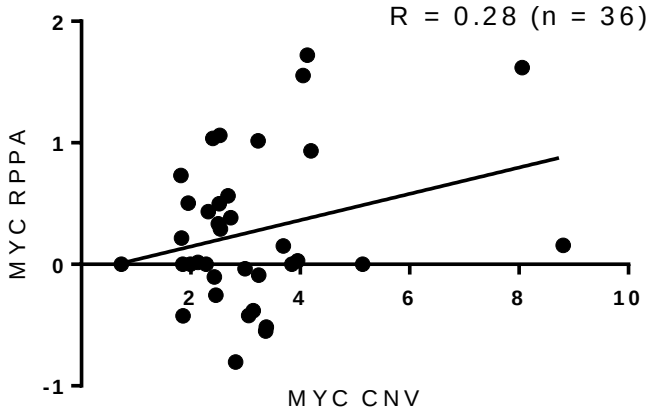


Figure 3.5: Correlation between MYC datasets in NSCLC Cell lines. (A) MYC mRNA vs. MYC RPPA. (B) MYC mRNA vs. MYC CNV. (C) MYC RPPA vs. MYC CNV.

3.2.3 Analyzing the Relationship between MYC Deregulation in Cells and in Human Tumors

In order to use a panel of NSCLC cell lines as a model system to better understand lung tumors, the cell line panel would need to accurately represent patient tumors. To this end, the gene expression profile of the cell line panel was compared to that of the human tumor samples. Specifically, a differential gene expression analysis was performed on the NSCLC cell lines with the highest (n = 10) and lowest (n = 10) MYC mRNA levels (Figure 3.6). The top 100 differentially regulated genes were then used to predict the MYC status of the panel of tumors obtained from MD Anderson Cancer Center (MDACC) as “MYC high” or “MYC low.” Existing microarray on the MDACC tumor panel was used to classify tumors as “MYC high” or “MYC low”, and this classification was compared to the prediction derived from the NSCLC gene signature. The gene signature derived from the NSCLC cell line panel successfully predicted the MYC status of the MDACC tumors approximately 68% of the time (Figure 3.7). Thus when bifurcated by MYC levels, the gene expression pattern of NSCLC cell lines accurately represents the gene expression pattern of lung tumor samples.

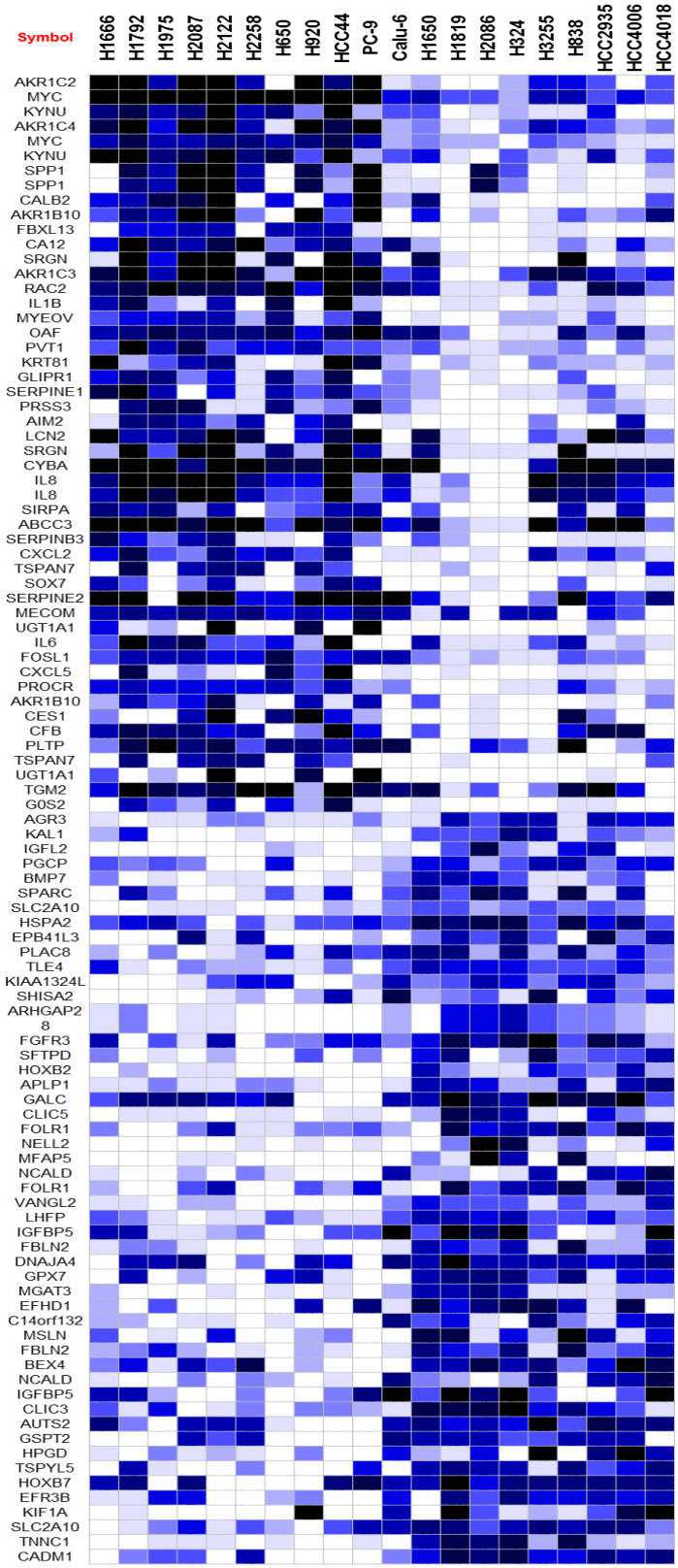


Figure 3.6: Differential gene expression analysis in NSCLC. The top 100 (50 upregulated, 50 downregulated) differentially regulated genes ($p < 0.05$) were determined by comparing the highest MYC expressing cell lines (left) with the lowest MYC expressing cell lines (right).

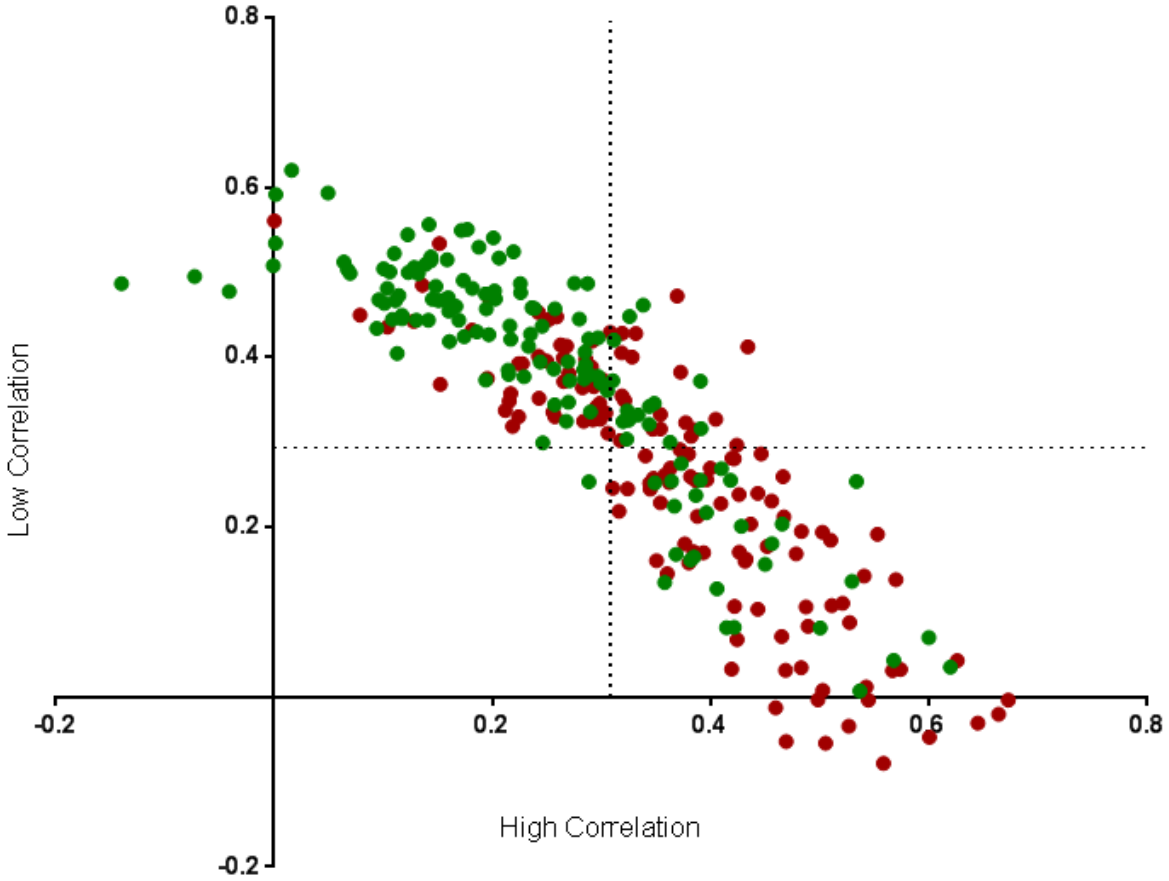


Figure 3.7: Classifying MYC status of patient samples. Each dot is a MDACC tumor (n = 275), and the correlation of the MDACC tumor and the "high" NSCLC signature is plotted against the correlation of the MDACC tumor and the "low" NSCLC signature. Green dots were classified as high and red as low using the MDACC microarray data on the tumors. The MYC status of 68% of the MDACC tumors was accurately predicted based on the correlations with the NSCLC MYC signature.

3.3 Discussion

Here, a thorough characterization of the degree of MYC deregulation in lung cancer has been provided. Specifically, MYC deregulation at the DNA, mRNA and protein level has been assessed in multiple panels of human lung tumors, as well as a large panel of non-small cell lung cancer cell lines. Further, the gene expression pattern (as it relates to MYC) has been compared between tumor samples and cell lines and found to be largely consistent.

In certain tumor settings such as lymphoma, MYC deregulation is known to be required for both the initiation and maintenance of the tumorigenic phenotype. In these settings, MYC is an important therapeutic target. Thus, the characterization of MYC deregulation in lung tumors, as well as NSCLC cell lines, very likely has important implications for cancer therapy. However, the relationship between clinical utility and MYC deregulation may not be as straight-forward as in lymphomas. For example, the *MYC* mutations seen in Burkitt's Lymphoma most often affect Thr-58 and Ser-62. Mutations in these residues result in stabilized mutant MYC protein, which has been shown to affect tumorigenesis in certain settings (Salghetti, Kim et al. 1999, Thomas and Tansey 2011, Wang, Cunningham et al. 2011). In the presence of such stabilizing mutations, MYC levels are sustained at elevated levels, and are no longer subject to the cyclic pattern of MYC expression mediated, in part, by rapid protein turnover. As a result, total elevated average levels of MYC may not be required for tumorigenesis. Rather, constant deregulation of MYC throughout the growth and proliferative phases may be sufficient to drive tumorigenesis (Dang 2012). Thus, total average levels of MYC, which is what was assessed in this chapter, may not be indicative of overall MYC deregulation in the cell. Additionally, total average MYC levels

may not necessarily indicated dependence on MYC for the maintenance of the tumorigenic phenotype.

Even if the total MYC levels are indicative of overall MYC deregulation in the cell, the functional significance of this remains unclear. As discussed in Chapter 1, recent evidence suggests that MYC, rather than regulating a unique transcriptional program, may just be a universal amplifier of actively transcribed genes. In other words, it is not clear whether tumors with deregulated MYC and that depend on this deregulation, require the transcription of a specific MYC target, or if they require general amplification of all actively transcribed genes. Understanding this aspect of MYC biology will have important therapeutic implications. For example, if MYC has a unique transcriptional program, there will likely be MYC target genes upon which the tumor depends. These target genes may provide more suitable drug targets than MYC itself. If, however, tumors with deregulated MYC depend on general amplification of transcription, then the potential list of therapeutic targets will be smaller. As discussed, the universal amplifier theory is not yet universally accepted. Chi Dang provided a recent critique, arguing that if MYC serves as a universal amplifier, rather than a regulator of a unique transcriptional program, then one would expect amplified transcription of whichever genes were already being transcribed. As Dang pointed out, this would entail an increase in transcription of both pro-growth and anti-growth genes, thus leaving the ratio, and theoretically the proliferative rate, unchanged (Dang 2013). To draw further contrast to the universal amplifier theory, Walz et al recently showed that MYC has a tumor-specific transcriptional program (Walz, Lorenzin et al. 2014). Regardless of which theory proves correct, the nature and consequences of MYC deregulation in lung cancer will likely have therapeutic implications. As such, a comprehensive

understanding of deregulation in this setting will be an important component of any work seeking to characterize the functional importance of MYC in human lung tumors.

CHAPTER FOUR

PROBING THE FUNCTIONAL IMPORTANCE OF MYC IN NSCLC

4.1 Introduction

As discussed in Chapter 1, much emphasis has been placed on MYC as a therapeutic target in various tumor types, yet the ability to target MYC in a clinical setting remains elusive. Despite this ongoing challenge, the past two decades have seen a rapid expansion in the understanding of the effects of MYC inhibition in human tumors. Most well-studied tumor types have been assessed for their dependence on MYC, and researchers have relied on a growing arsenal of approaches. These efforts have ranged from the antisense approach to carefully designed therapeutics.

While the antisense approach met with some success initially, it has largely been replaced by other methods due to the suboptimal stability and specificity of the single strand oligonucleotides. RNA-interference, however, has remained a popular approach to targeting MYC. siRNAs have been used extensively to target MYC in various settings, including ovarian cancer, leukemia and lung cancer. siRNA-mediated MYC inhibition *in vitro* has resulted in decreased cell proliferation and colony forming efficiency, as well as increased senescence and apoptosis (Liu, Zou et al. 2009, Prathapam, Aleshin et al. 2010). However, due to the short-lived effect on target gene message, siRNAs are not suitable reagents for long-term assays. Specifically, the transient effect does not lend itself well to studying sustained tumor regression or the development of resistance, both of which are critically important questions when investigating a potential therapeutic target. For longer-term MYC targeting, shRNAs have been

a common approach. While functionally similar to siRNAs, shRNAs have the added benefit of being continuously produced by the host cell, making them a useful reagent for long-term assays. shRNAs against MYC have been used in a variety of tumor types, and have shown reduced MYC protein as well as slowed proliferation rates (Wang, Mannava et al. 2008).

One of the most popular approaches to date has been the use of the dominant-negative mini-protein, Omomyc. As previously discussed, Omomyc is a mini-protein that encompasses the bHLH-LZ region of MYC, but has been mutated at key residues to resemble Max. As a result, Omomyc has the ability to dimerize with MYC, but this complex is unable to bind DNA and activate transcriptional targets of MYC. Thus, when ectopically expressed in either cells or animal models, Omomyc inhibits the normal transcriptional function of endogenous MYC. Omomyc has been used in a variety of models, both *in vitro* and *in vivo*. For example, Omomyc was used to argue that *KRAS* mutant NSCLC cell lines with overexpressed MYC were uniquely susceptible to MYC inhibition (Fukazawa, Maeda et al. 2010). Similarly, Omomyc has been used in genetically engineered mouse models of lung cancer to show that even transient MYC inhibition could lead to tumor regression (Soucek, Whitfield et al. 2013). Omomyc has proved to be a reliable inhibitor of MYC in laboratory models, and has contributed much to the understanding of MYC's role in tumor development and maintenance.

Despite the promise seen with Omomyc use in the laboratory, it is not a suitable reagent for use in humans. As a result, various drugs have been either developed or repurposed with the goal of targeting MYC in humans. The drugs that have been developed can act either directly or indirectly to inhibit MYC, and can do so at the mRNA or the protein level. Drugs that were

developed for other purposes and later found to have anti-MYC properties tend to fall into the "indirect" category, whereas drugs that were purpose-built for MYC targeting tend to do so directly. The most commonly used direct inhibitor of MYC function is 10058-F4, which works by inhibiting dimerization of MYC with its binding partner, Max. Originally discovered using a yeast two-hybrid approach, 10058-F4 was identified as one of several low-molecular weight compounds that block the interaction of MYC and Max (Yin, Giap et al. 2003). In this initial study, 10058-F4 inhibited cell cycle progression and prevented proliferation of fibroblasts in a MYC-dependent manner. Additionally, researchers found the drug induced apoptosis in multiple myeloma, another cancer reportedly driven by MYC (Holien, Vatsveen et al. 2012). This drug was later used in various tumor settings, including acute myeloid leukemia, where it elicited cell cycle arrest, apoptosis and differentiation (Huang, Cheng et al. 2006). Since its discovery more than ten years ago, 10058-F4 has been used in an array of studies on the effects of MYC inhibition. In addition to the effects on tumor cell growth and viability, the use of 10058-F4 has been instrumental in characterizing the effect of MYC on drug resistance, tumor metabolism and tumor progression (Xia, Guo et al. 2013, Zirath, Frenzel et al. 2013, Tsai, Wu et al. 2014). Despite such promising results *in vitro*, however, 10058-F4 was found to have a poor pharmacokinetic/pharmacodynamics profile, and as such has not been successfully used *in vivo* (Guo, Parise et al. 2009). Specifically, the poor antitumor activity of 10058-F4 was found to be the result of rapid metabolism and a low drug concentration in the tumor. As a result, researchers have turned their attention to drugs with better *in vivo* profiles that have been shown to indirectly affect MYC activity.

Inhibition of members of the BET family of proteins was recently posited as an effective method for indirect MYC inhibition. One member of the family, BRD4, has been shown to regulate transcription via a direct interaction with the positive transcription elongation factor complex b (P-TEFb) (Bisgrove, Mahmoudi et al. 2007). This finding, combined with the knowledge that MYC also interacts with P-TEFb, provided rationale for using the BET family of proteins as an indirect mechanism by which to target MYC itself. Filippakopoulos et al recently characterized a small-molecule inhibitor of the BET family of proteins, JQ1 (Filippakopoulos, Qi et al. 2010). JQ1 structurally resembles an acetylated lysine, and thus works to displace BET family members from chromatin by competitively binding to the acetyl-lysine binding pockets (Delmore, Issa et al. 2011). Recently, Delmore et al used multiple myeloma, a tumor type with a nearly 60% frequency of MYC deregulation, as a model system to screen for MYC dependence using JQ1. Here, the authors showed that the *MYC* locus is transcriptionally regulated by BET bromodomains. Further, the authors showed that MYC levels could be decreased in a time and dose-dependent manner using JQ1 (Delmore, Issa et al. 2011). The authors went on to assess the functional impact of JQ1 treatment, and showed an antiproliferative effect in a panel of multiple myeloma cell lines, though the effect varied across lines tested. Since this initial work, JQ1 has been used in several other tumor types, including non-small cell lung cancer. Lockwood et al screened JQ1 across a panel of NSCLC cell lines and found that a subset of the cell lines tested was exquisitely sensitive to treatment, exhibiting a significant decrease in proliferation and viability (Lockwood, Zejnullahu et al. 2012). However, in contrast to data in blood tumors, Lockwood et al argued that the effect seen in NSCLC was independent of MYC downregulation, and was instead dependent on the BRD4-mediated reduction of another oncogenic transcription factor, FOSL1. Still, other researchers continued to use JQ1 as a MYC inhibitor in solid tumors.

In 2013, Shimamura et al published a separate study of JQ1 efficacy, also across a panel of NSCLC cell lines (Shimamura, Chen et al. 2013). Here, the authors contradicted the results from Lockwood et al, arguing that JQ1 did depend on MYC downregulation in *KRAS* mutant NSCLC. However, the authors determined that this effect would be lost in the absence of functional LKB1, suggesting that the genetic context matters when determining MYC dependence using JQ1 (Shimamura, Chen et al. 2013). Thus, JQ1 appears to downregulate MYC levels in multiple tissue types, though this may be a context dependent phenomenon.

In addition to downregulating MYC via transcriptional repression, several drugs have been shown to downregulate MYC by altering protein stability. Recent work characterized a novel response to checkpoint kinase 1 (Chk1) inhibition that resulted in MYC protein destabilization. Khanna et al showed that Chk1 inhibition resulted in the induction of protein phosphatase 2A (PP2A) (Khanna, Kauko et al. 2013). This occurred via decreasing the transcription of cancerous inhibitor of PP2A (CIP2A). Induction of PP2A resulted in the dephosphorylation of Ser-62 on MYC, which destabilized the protein and thus inhibited MYC activity. The authors went on to show that SB218078, a small-molecule Chk1 inhibitor, could effectively inhibit MYC-induced proliferation in a human breast cell line (MCF10A) (Khanna, Kauko et al. 2013). Chk1 inhibition represents a previously unknown mechanism to indirectly inhibit MYC function, and this may be a viable approach to targeting MYC-driven cancers.

A more recent approach to pharmacologically targeting MYC, as well as other potent oncogenes, has been to target newly described “super enhancers.” Super-enhancers are regions with clustered single enhancers, and that contain abnormally high localization of transcriptional

coactivators, specifically Mediator (Med1) (Pott and Lieb 2015). Based on the cutoff for Med1 levels, Whyte et al demonstrated that fewer than 3% of the enhancer regions studied qualified as super-enhancers (Whyte, Orlando et al. 2013). Interestingly, super enhancers tend to associate with genes that control cell identity. Additionally, Hnisz et al recently demonstrated that cancer cells have the capability to generate super-enhancers at oncogenes or other genes that are important for the survival of the tumor (Hnisz, Abraham et al. 2013). Recently, a small molecule inhibitor of CDK7, THZ1, was found to have a potent effect against small-cell lung cancer cells that expressed super-enhancer associated transcription factors, including MYC (Christensen, Kwiatkowski et al. 2014). Specifically, THZ1 was found to dramatically reduce RNAPII-mediated gene transcription. Here, the authors showed that THZ1 elicited a potent anti-proliferative and apoptotic response in murine small cell lung cancer cells. They went on to show that super-enhancers were found to be associated with genes that had been amplified, including *MYC* and *MYCN*, and that these super-enhancer genes were disproportionately vulnerable to THZ1 treatment (Christensen, Kwiatkowski et al. 2014). However, the authors did not observe differential sensitivity in cell lines with genomic amplification of *MYC*. Thus, while MYC levels may not be a biomarker for THZ1 sensitivity, MYC is a gene that is uniquely susceptible to THZ1 treatment.

As described, a variety of approaches to inhibit MYC exists. Importantly, these approaches have not always led to repeatable results across research groups, as evidenced by the recent JQ1 data previously discussed. As a result, a multimodal approach to targeting MYC may be required in order to truly identify and characterize MYC dependence in a given tumor type. The goal of this study was to determine whether MYC is universally required for both the general

viability and the maintenance of the tumorigenic phenotype in NSCLC cell lines. In order to accomplish this goal, a variety of approaches was used, including both transient and stable RNAi, pharmacological inhibition, and a dominant-negative approach.

4.2 Results

4.2.1 RNAi-mediated Inhibition of MYC

As previously discussed, RNAi has been a popular approach for targeted inhibition of various targets. Given the success with which this approach has been used to target other genes, both siRNAs and shRNAs were used here to inhibit MYC function. siRNAs against MYC, MYCN and MYCL were transfected into four NSCLC cell lines, and the mRNA for each MYC family member was quantified. As shown in Figure 4.1, all siRNAs targeted their respective MYC family member, though to varying degrees. The siRNA against MYC effectively repressed MYC mRNA in all four cell lines. The siRNA against MYCN effectively repressed MYCN mRNA in the only cell line that expressed MYCN. The siRNA against MYCL was only effective in one of the four cell lines tested. Additionally, some of the siRNAs also targeted MYC family members non-specifically. The siRNA against MYCN also repressed MYCL levels in two of the four cell lines tested. Thus, despite some non-specific effects, the siRNAs against MYC and MYCN repressed their respective targets in all cases, whereas the siRNA against MYCL was less effective. Using these siRNAs, the colony forming efficiency (CFE) was measured after knockdown of each MYC family member. The siRNA against MYC elicited a statistically significant reduction in CFE in three of the four cell lines, whereas the siRNA

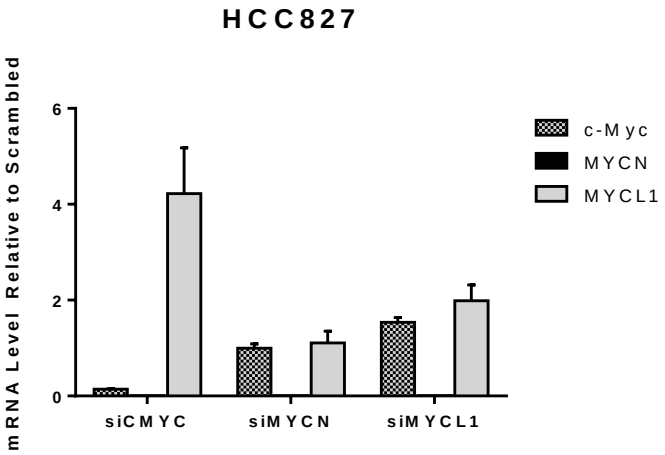
against MYCN did so in only one cell line (Figure 4.1). Interestingly, the cell line which showed a response to siMYCN does not appear to express MYCN at baseline, suggesting this may be an off-target effect. The siRNA against MYCL did not elicit a CFE reduction in any of the four cell lines.

Next, to bolster the siRNA data, the same four cell lines were functionally probed with shRNAs against the MYC family members. Here, the shRNAs were stably expressed in each of the four cell lines, and the knockdown efficiency of the targets was measured. The shRNA against MYC effectively repressed MYC mRNA in all four cell lines (Figure 4.1). However, shMYC also repressed MYCL in one of the four cell lines, suggesting an off-target effect. The shRNA against MYCN did not repress MYCN levels effectively in the one cell line that expressed MYCN, though MYCL levels were suppressed in one of the cell lines, suggesting yet another potential off-target effect. The shRNA against MYCL effectively repressed MYCL levels in two of the four cell lines tested, but also significantly repressed MYCN mRNA levels in the one cell line that expressed MYCN (Figure 4.1). Similar to the siRNA experiment, all cell lines were assessed via CFE assays to determine the functional significance of each MYC family member. Surprisingly, all three shRNAs agains the MYC family members elicited a statistically significant reduction in CFE in all four cell lines tested, with one exception (Figure 4.1). The shRNA against MYC had no effect on the CFE of one of the cell lines tested. This result is not consistent with the sometimes ineffective and inconsistent knockdown of target message.

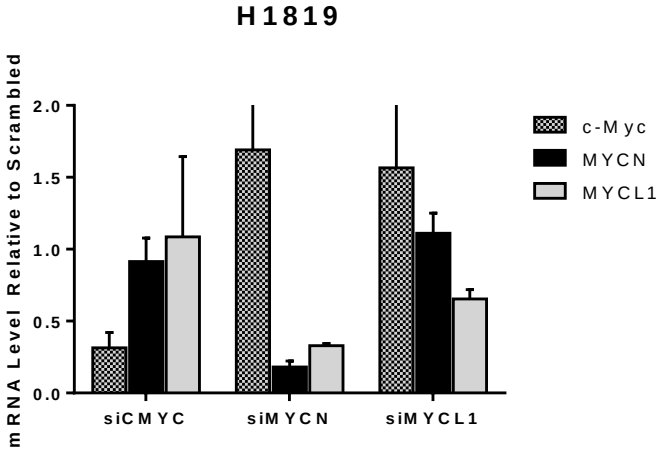
Thus, both traditional RNAi reagents were used to functionally assess the requirement of MYC family members in a small panel of NSCLC cell lines. While shRNAs elicited a CFE

reduction in all cell lines, this result was not consistent with the siRNA experiment. Further, the functional results for both the si- and shRNA experiments are not fully consistent with the knockdown verification. As a result, additional methods were pursued to validate these data.

A.


HCC827
mRNA Level Relative to Scrambled
6
4
2
0
siCMYC
siMYCN
siMYCL1
c-Myc
MYCN
MYCL1

B.


H1819
mRNA Level Relative to Scrambled
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1.0
0.5
0.0
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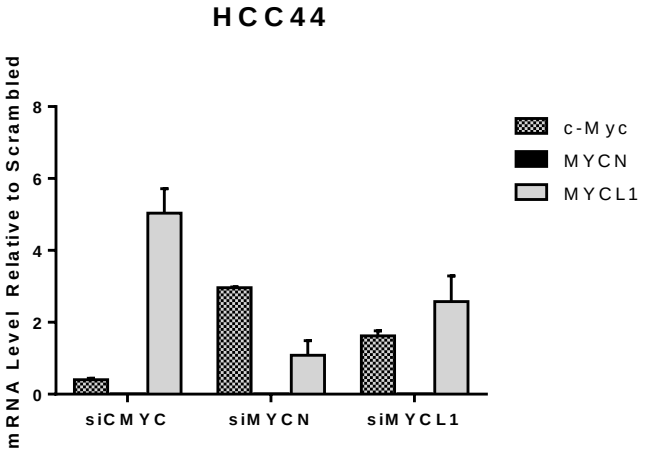
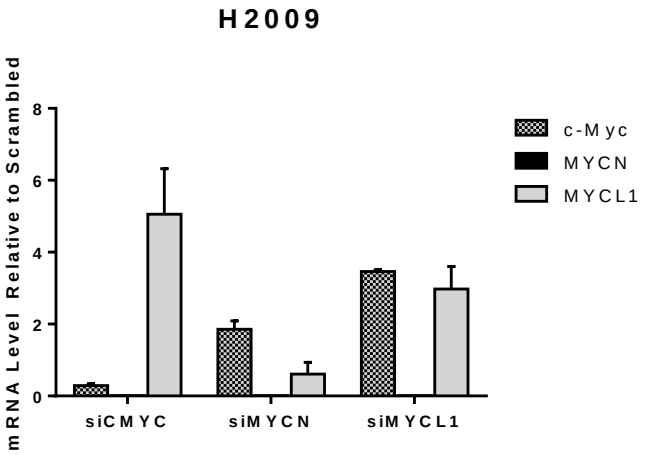
siMYCN
siMYCL1
c-Myc
MYCN
MYCL1

C.


HCC44
mRNA Level Relative to Scrambled
8
6
4
2
0
siCMYC
siMYCN
siMYCL1
c-Myc
MYCN
MYCL1

D.


H2009
mRNA Level Relative to Scrambled
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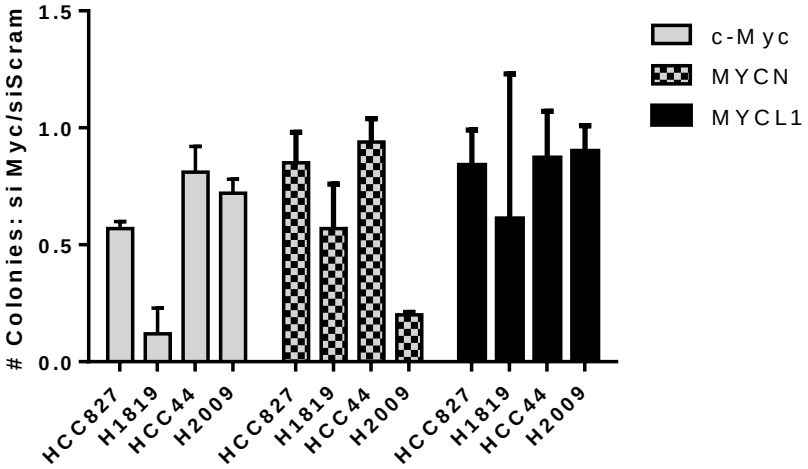
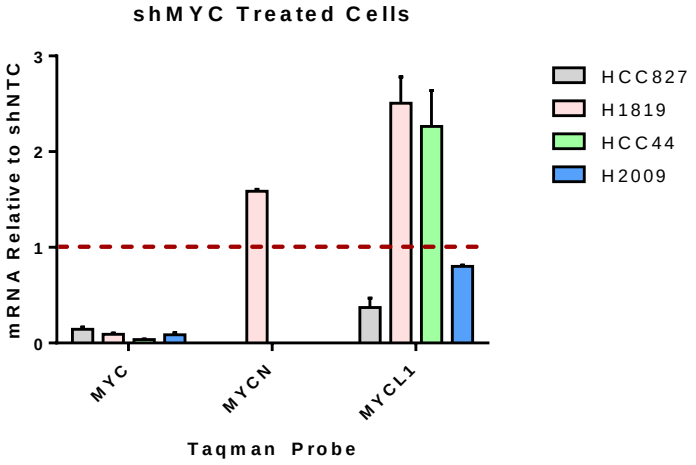
6
4
2
0
siCMYC
siMYCN
siMYCL1
c-Myc
MYCN
MYCL1

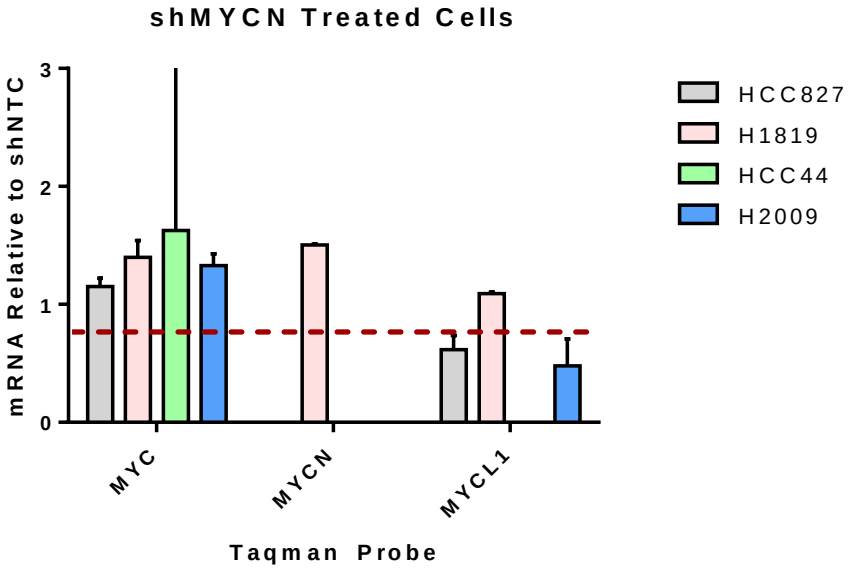
E.


Colonies: si Myc/siScram
1.5
1.0
0.5
0.0
HCC827
H1819
HCC44
H2009
HCC827
H1819
HCC44
H2009
HCC827
H1819
HCC44
H2009
c-Myc
MYCN
MYCL1

F.


shMYC Treated Cells
mRNA Relative to shNTC
3
2
1
0
MYC
MYCN
MYCL1
Taqman Probe
HCC827
H1819
HCC44
H2009

G.


shMYCN Treated Cells
mRNA Relative to shNTC
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MYC
MYCN
MYCL1
Taqman Probe
HCC827
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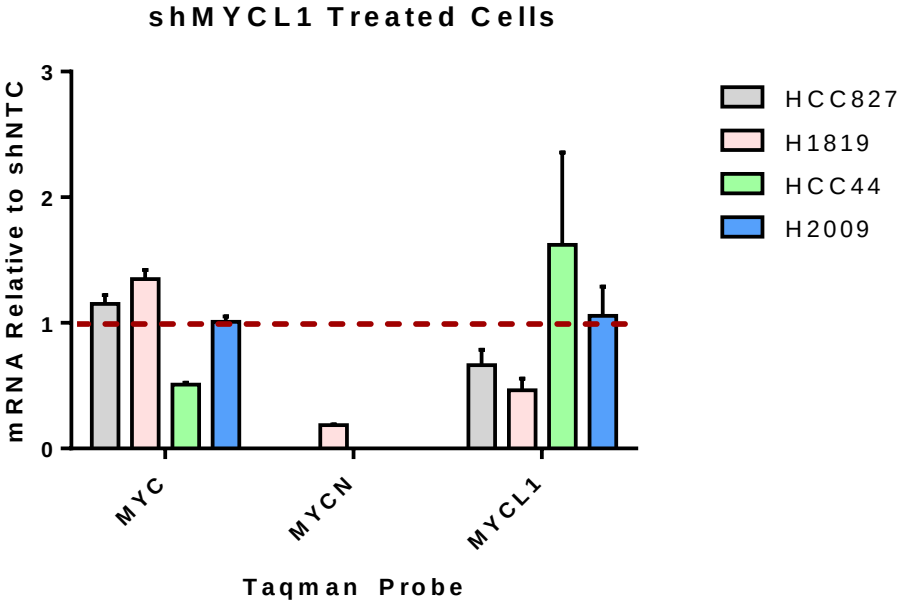
HCC44
H2009

H.


shMYCL1 Treated Cells
mRNA Relative to shNTC
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MYCN
MYCL1
Taqman Probe
HCC827
H1819
HCC44
H2009

I.

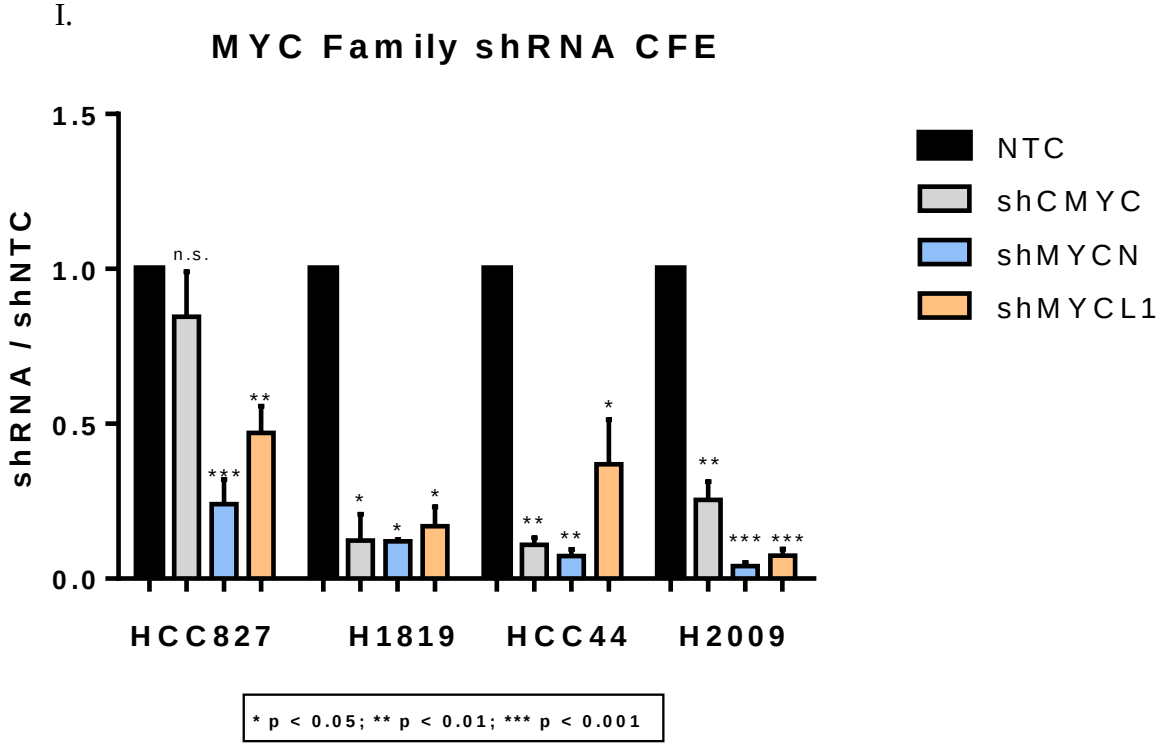


Figure 4.1: Summary of RNA-interference Targeting MYC. Confirmation of knockdown of the target using siRNA in (A) HCC827, (B) H1819, (C) HCC44, and (D) H2009. (E) CFE response after siRNA knockdown. mRNA of MYC family members after transduction with short-hairpin against MYC (F), MYCN (G), and MYCL1 (H). (I) CFE response after shRNA knockdown. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

4.2.2 Pharmacological Inhibition of MYC

Due to the frequent deregulation of MYC in human tumors, an ongoing effort exists to develop a drug to target MYC-driven cancers. Recently, several reports have claimed anti-MYC properties for a host of currently approved and late-stage development drugs. These reports have studied, among other drugs, Trichostatin A (TSA), ABT-263, and diclofenac. TSA is a histone deacetylase (HDAC) inhibitor. HDAC inhibitors have emerged as a novel anti-tumor class of drugs, though the molecular mechanism for their anti-tumor activity is unclear. A recent report demonstrated that when endometrial cancer cells were treated with TSA they displayed a downregulation of MYC, and subsequently exhibited a slowed growth rate and increased apoptosis (Zhao, Bai et al. 2012). ABT-263 is a Bcl-2 inhibitor and was recently shown to target MYC driven lymphomas more effectively than a direct MYC inhibitor (Sasaki, Kuroda et al. 2011). Diclofenac is a non-steroidal anti-inflammatory drug (NSAID), which has been shown to elicit potent anti-cancer effects, though these effects were thought to be the result of its classical mechanism of action (COX inhibition). However, a recent report showed that treatment with diclofenac resulted in reduced MYC expression and decreased melanoma, leukemia and carcinoma cell line proliferation. As a pilot study, these three drugs were tested across two NSCLC cell lines. While ABT-263 elicited a comparable response across the cell lines, TSA and diclofenac elicited completely opposite responses (Figure 4.2). Given the different mechanisms of action, this result is unsurprising, but underscores the need for a larger screen in order to determine under what circumstances MYC is required in NSCLC.

While the aforementioned pilot experiment used drugs that have been shown to have anti-MYC properties in a limited setting, more established inhibitors of MYC function would be required for a large-scale screen. To this end, four purported MYC inhibitors were chosen to screen a large panel of clinically and molecularly annotated NSCLC cell lines. 10058-F4, the lone direct inhibitor of MYC, as well as JQ1, SB218078 and THZ1, all indirect inhibitors, were chosen.

Using a luminescent cell viability assay, these four compounds were screened across a large panel of lung cancer and normal lung epithelial cell lines (83 cell lines total) (Figures 4.3 – 4.6; Table 4.1). These data show a >25-fold range in sensitivity for 10058-F4, a >700-fold range for JQ1, a >500-fold range for SB218078, and a >50-fold range for THZ1 (Figures 4.3 – 4.6). Since treatment with any one drug decreased viability in a subset of the panel, we sought to determine if the drugs affected the same cell lines. The AC50s for each drug in each cell line were plotted against each other and a Pearson correlation was calculated. Interestingly, none of the four drugs correlated well with each other, with the highest correlation coefficient being 0.24 (THZ1 vs. SB218078) (Figure 4.7). These data confirm that while the drugs did all elicit a response, they did not elicit the same response in the same cell lines.

To further investigate the effect of drug treatment on the tumorigenic phenotype, the colony forming efficiency (CFE) was measured in a subset (10) of the cell lines initially screened (Figure 4.8). First, a dose response curve was generated for 10058-F4 and JQ1 on a subset of the larger NSCLC cell line panel. Using this curve, a single dose was chosen to screen the CFE The

colony forming efficiency correlated well with the AC50, with both correlations yielding R^2 coefficients of >0.5 (Figure 4.8).

A.

IC50 (µM) via MTS Assay

Drug	Cell Line	
	HCC827	HCC44
ABT-263	5	4.3
Trichostatin-A	0.08	0.17
Diclofenac	0.13	0.07

Figure 4.2: Summary of MYC-targeting Drugs. ABT-263 elicits a comparable response in both cell lines, but Trichostatin-A (TSA) and diclofenac elicit inversely correlated responses.

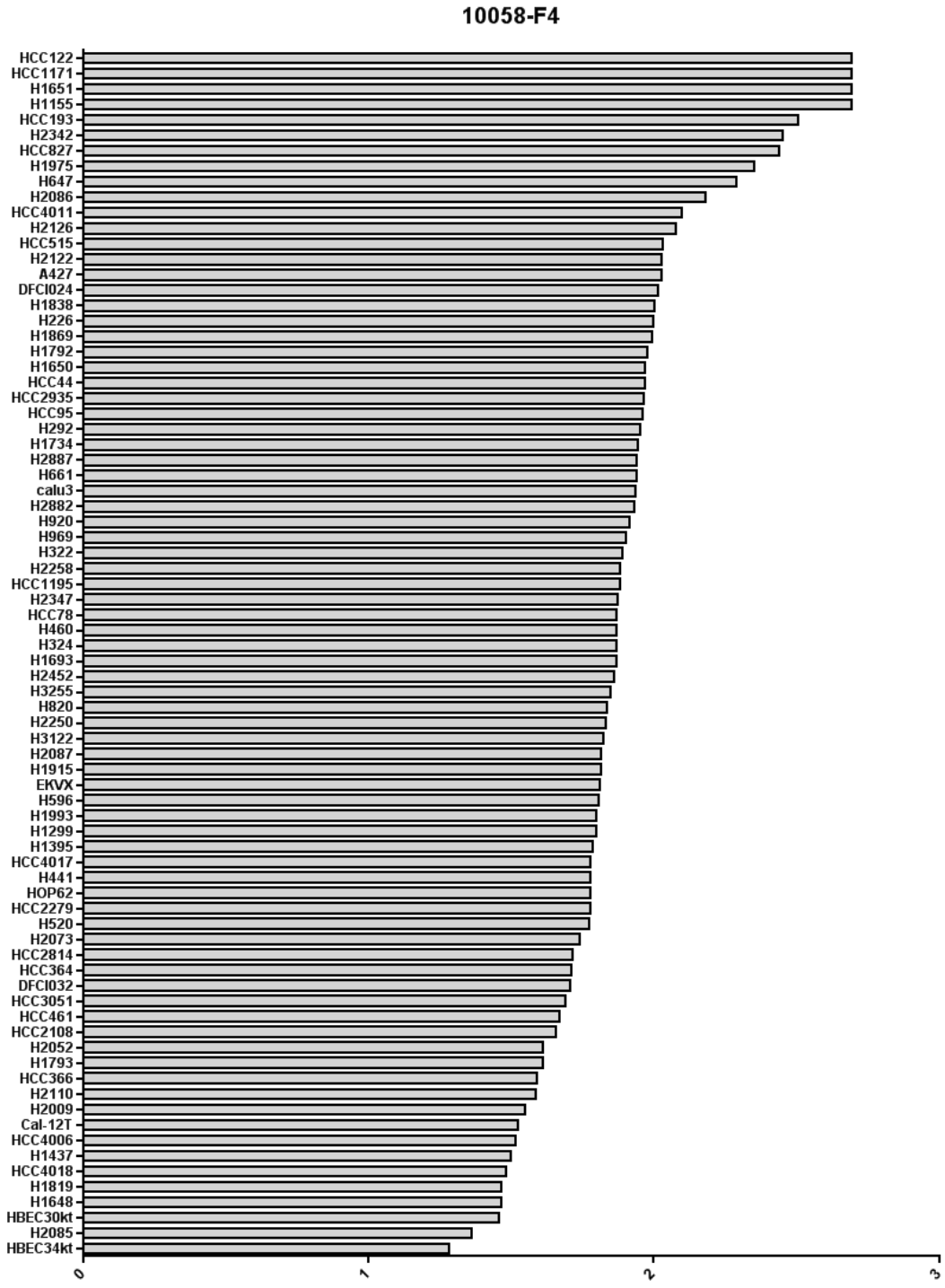


Figure 4.3: Log_{10} of the AC50 for cells treated with 10058-F4.

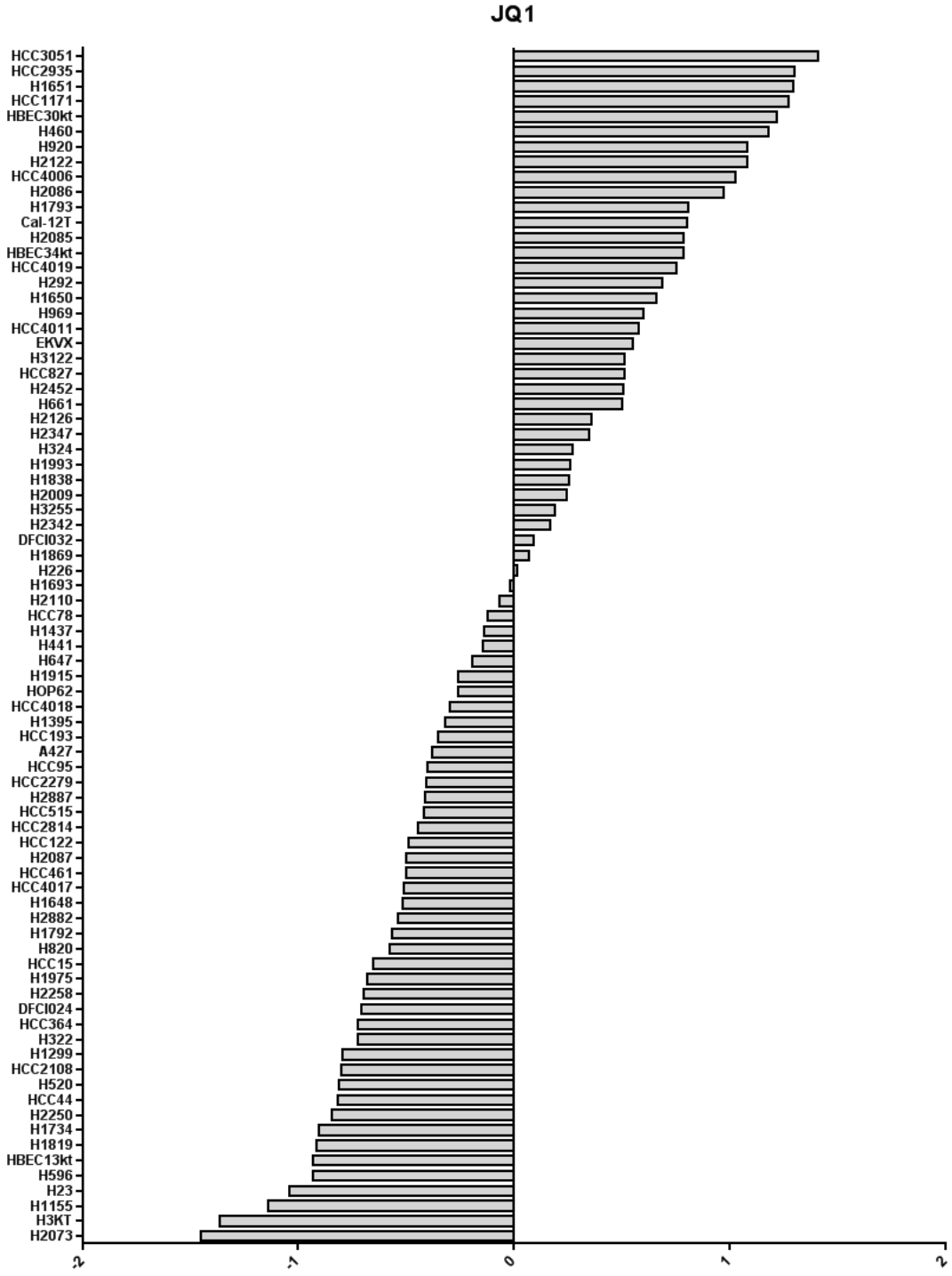


Figure 4.4: Log_{10} of the AC50 for cells treated with JQ1.

SB218078

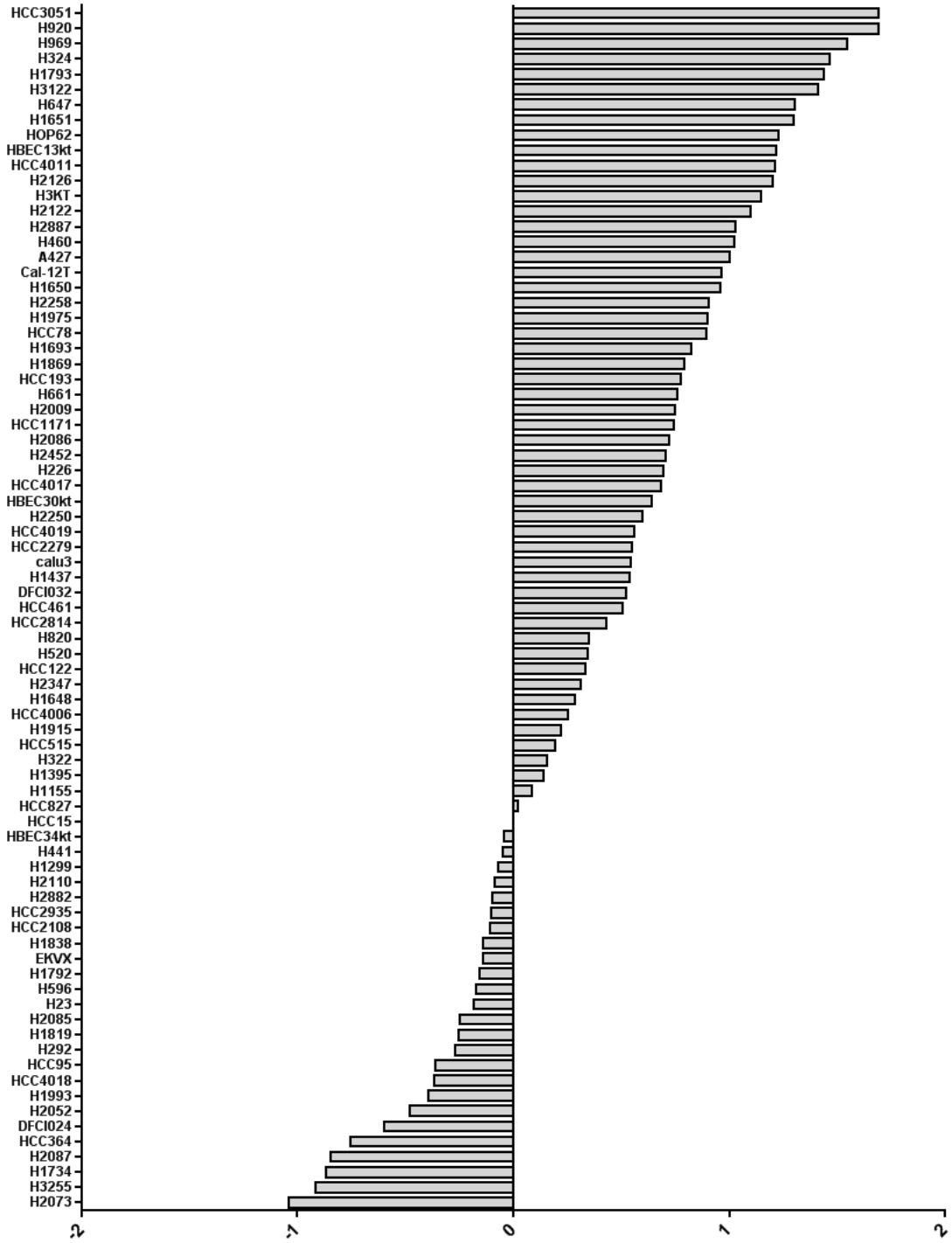


Figure 4.5: Log_{10} of the AC50 for cells treated with SB218078.

THZ1

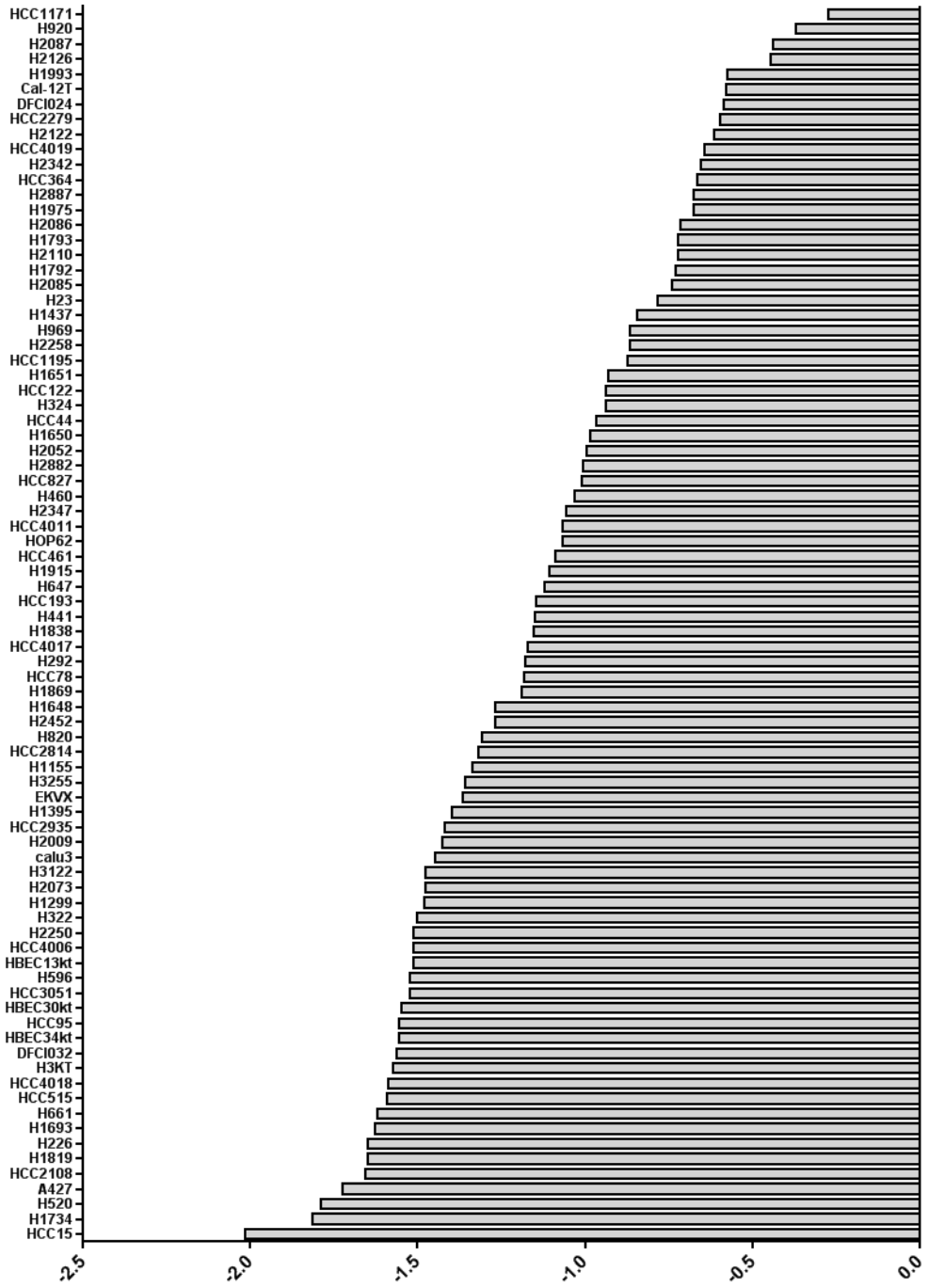


Figure 4.6: Log_{10} of the AC50 for cells treated with THZ1.

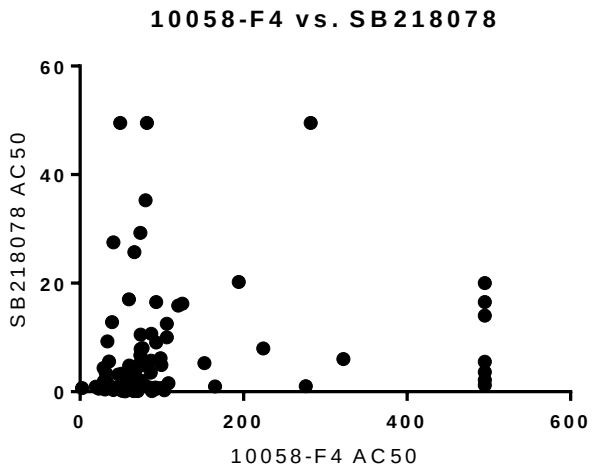
Table 4.1: Summary of MYC-targeting Drug Data

	AC50			
Cell	**10058-F4**	**JQ1**	**SB218078**	**THZ1**
HCC461	46.9	0.315	3.19	0.0814
HCC364	51.5	0.189	0.176	0.217
HCC2814	51.9	0.359	2.69	0.0481
Cal-12T	33.5	6.34	9.25	0.263
H2110	38.5	0.857	0.823	0.189
H820	68.3	0.265	2.23	0.0491
H647	194	0.641	20.2	0.0756
H1915	65.5	0.549	1.67	0.0785
H1793	40.8	6.42	27.5	0.19
H324	73.9	1.86	29.3	0.115
H661	86.8	3.19	5.72	0.0239
H1869	98.5	1.18	6.19	0.0648
HCC95	91.1	0.396	0.434	0.0278
H2122	106	12	12.5	0.243
H2126	120	2.28	15.9	0.358
H441	60	0.721	0.893	0.0711
H2882	85.2	0.291	0.799	0.0983
EKVX	64.4	3.55	0.722	0.0431
HCC827	276	3.24	1.05	0.098
HCC4019	495	5.66	3.61	0.228
HOP-62	59.9	0.548	17	0.0854
H2073	55.2	0.0353	0.0918	0.0334
H1993	62.8	1.82	0.403	0.266
H23	2.44	0.0911	0.654	0.165
H2087	65.5	0.317	0.142	0.365
H920	81.9	12.1	49.5	0.426
H3255	70.4	1.55	0.121	0.044
H1819	29.3	0.121	0.56	0.0224
H1693	73.8	0.955	6.71	0.0236
HCC4006	32.9	10.7	1.79	0.0307
HCC2935	92.2	19.9	0.791	0.0382
H1650	93.2	4.55	9.05	0.104
HCC2279	59.9	0.393	3.56	0.253
HCC4011	125	3.8	16.2	0.0856

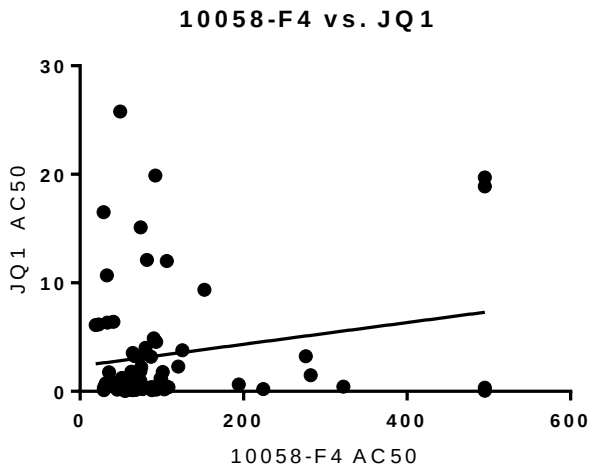
H1437	31.6	0.728	3.46	0.143
H1155	495	0.073	1.22	0.046
H2009	35.5	1.75	5.59	0.0374
HCC122	495	0.326	2.15	0.115
H2085	22.9	6.15	0.568	0.182
H2342	282	1.48	49.5	0.221
HCC366	39.1	11.3	12.8	0.0536
HCC1171	495	18.9	5.54	0.535
HCC15	165	0.223	0.998	0.00967
H3122	66.4	3.26	25.7	0.0334
H2887	87.3	0.387	10.7	0.211
HCC4017	60.1	0.307	4.84	0.0674
HCC3051	49.2	25.8	49.5	0.0299
HCC515	108	0.385	1.56	0.0256
HCC78	74.2	0.754	7.87	0.0656
H2258	76.5	0.201	8.03	0.136
H2250	68.1	0.144	3.96	0.0308
HCC44	93	0.152	16.5	0.108
DFCI032	51	1.24	3.31	0.0274
HCC2108	45.4	0.159	0.781	0.022
Calu-3	86.6	1.52	3.5	0.0357
HCC193	322	0.447	6	0.0714
H1975	224	0.21	7.97	0.211
H1838	101	1.8	0.726	0.07
HCC4018	30.5	0.503	0.43	0.0259
H226	99.6	1.04	4.93	0.0225
H322	77.8	0.189	1.44	0.0316
H1734	87.7	0.124	0.135	0.0154
H596	64.3	0.117	0.676	0.0301
H1792	95.3	0.271	0.7	0.187
H1299	62.8	0.161	0.847	0.0331
H2086	152	9.36	5.25	0.192
H1651	495	19.7	20	0.117
H520	59.5	0.155	2.2	0.0163
H1648	29.3	0.306	1.93	0.0538
H2347	74.8	2.23	2.05	0.0878
H1395	61	0.478	1.38	0.0399
DFCI024	103	0.196	0.254	0.26
H969	80	4.01	35.3	0.136
HCC1195	75.9	49.5	4.95	0.134
H2052	40.9	25.8	0.333	0.101

H292	90	4.89	0.54	0.0664
H460	74	15.1	10.5	0.0931
A427	106	0.418	10	0.0188
H2452	72.5	3.22	5.1	0.0537
HBEC34-KT	19.2	6.12	0.903	0.0278
HBEC13-KT	495	0.117	16.5	0.0307
HBEC30-KT	28.8	16.5	4.37	0.0284
HBEC3-KT	495	0.0432	14	0.0268

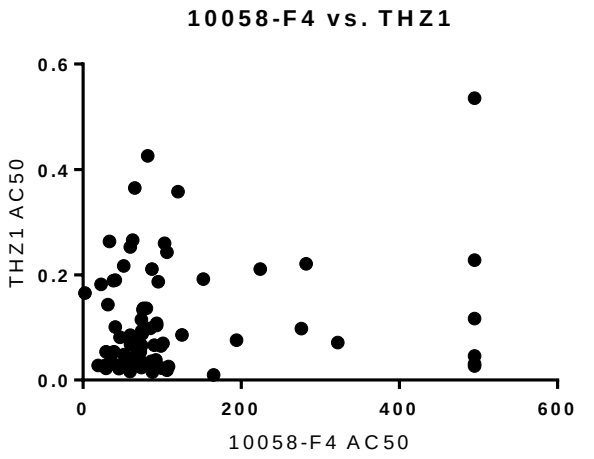
A.


10058-F4 vs. SB218078
SB218078 AC50
60
40
20
0
0
200
400
600
10058-F4 AC50

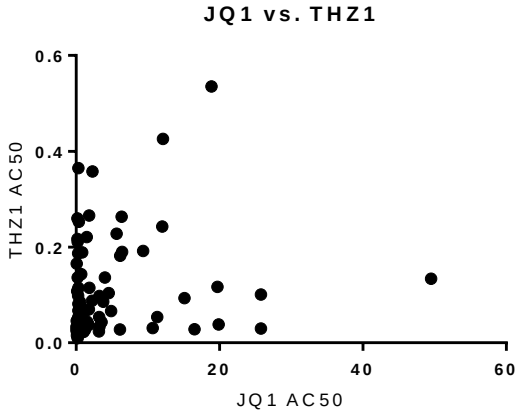
B.


10058-F4 vs. JQ1
JQ1 AC50
30
20
10
0
0
200
400
600
10058-F4 AC50

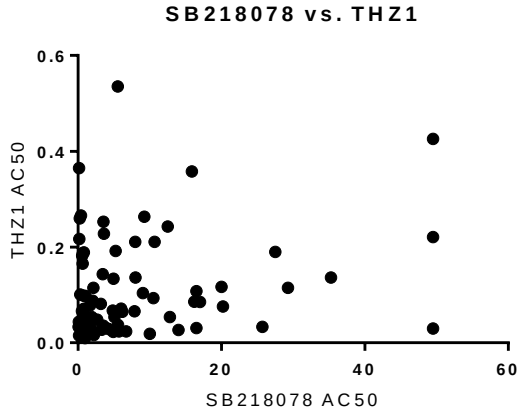
C.


10058-F4 vs. THZ1
THZ1 AC50
0.6
0.4
0.2
0.0
0
200
400
600
10058-F4 AC50

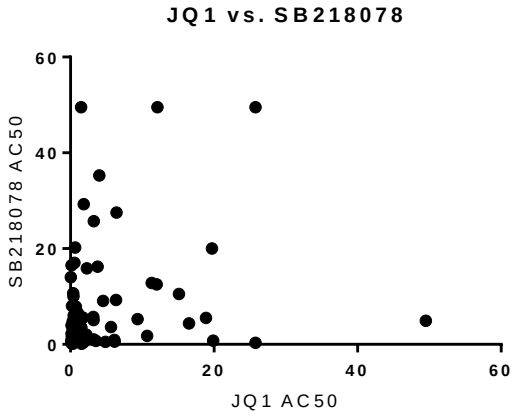
D.



E.



F.



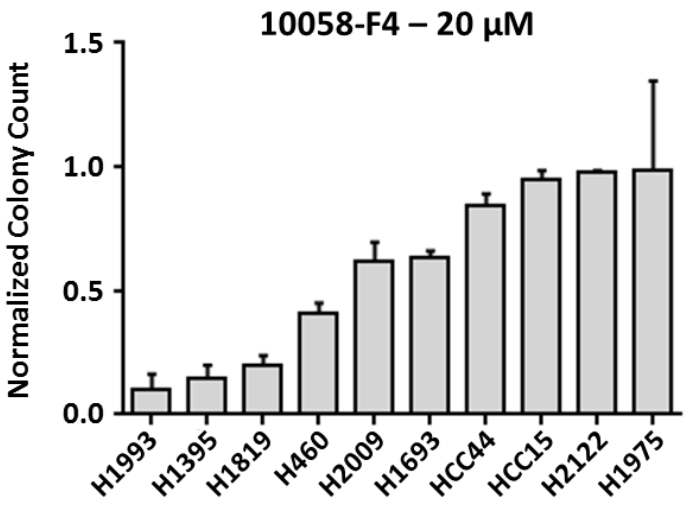
G.

Correlation Coefficients

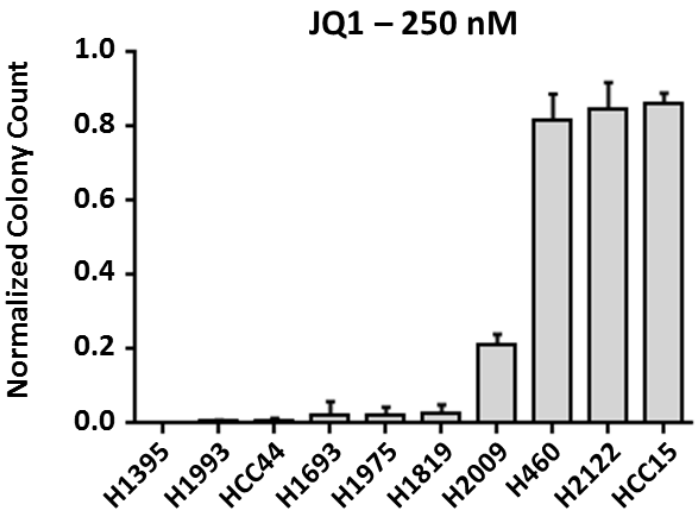
	10058-F4	JQ1	SB218078	THZ1
10058-F4		0.03	0.13	0.18
JQ1	0.04		0.22	0.17
SB218078	0.13	0.22		0.24
THZ1	0.18	0.17	0.24	

Figure 4.7: Correlation of different MYC-targeting drugs. (A) 10058-F4 vs. SB218078. (B) 10058-F4 vs. JQ1. (C) 10058-F4 vs. THZ1. (D) JQ1 vs. THZ1. (E) SB218078 vs. THZ1. (F) JQ1 vs. SB218078. (G) Summary table of all R values.

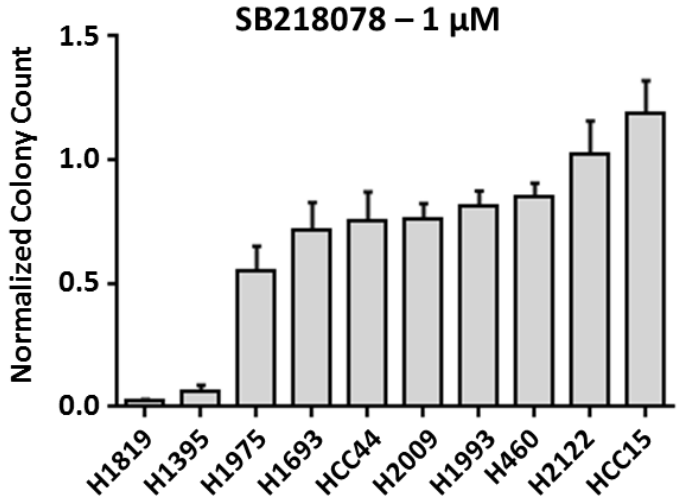
A.


10058-F4 – 20 µM
Normalized Colony Count
1.5
1.0
0.5
0.0
H1993
H1395
H1819
H460
H2009
H1693
HCC44
HCC15
H2122
H1975

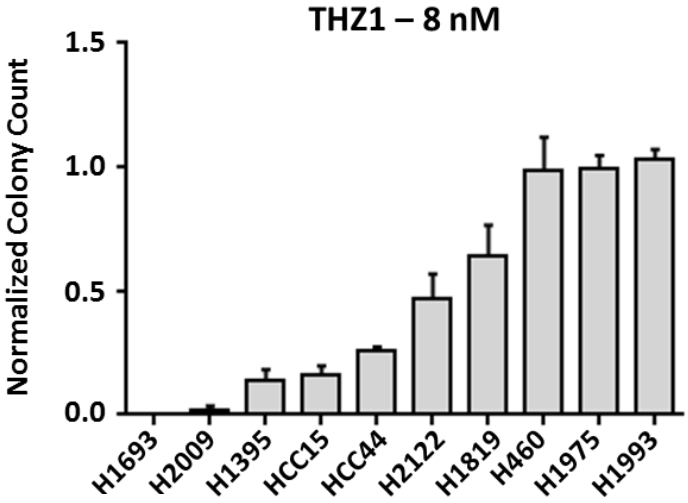
B.


JQ1 – 250 nM
Normalized Colony Count
1.0
0.8
0.6
0.4
0.2
0.0
H1395
H1993
HCC44
H1693
H1975
H1819
H2009
H460
H2122
HCC15

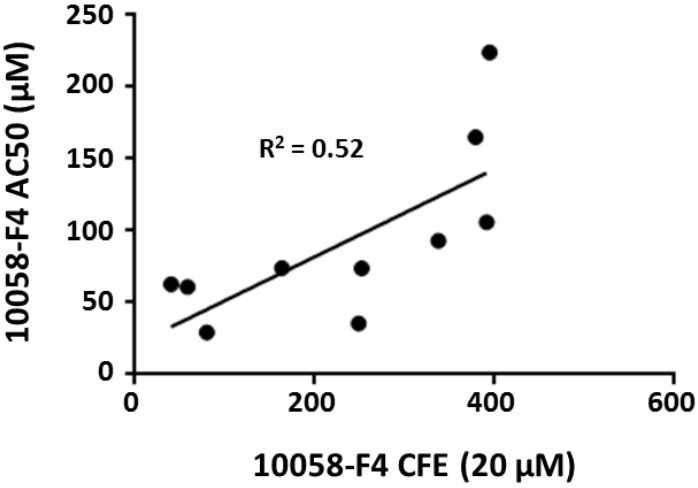
C.


SB218078 – 1 µM
Normalized Colony Count
1.5
1.0
0.5
0.0
H1819
H1395
H1975
H1693
HCC44
H2009
H1993
H460
H2122
HCC15

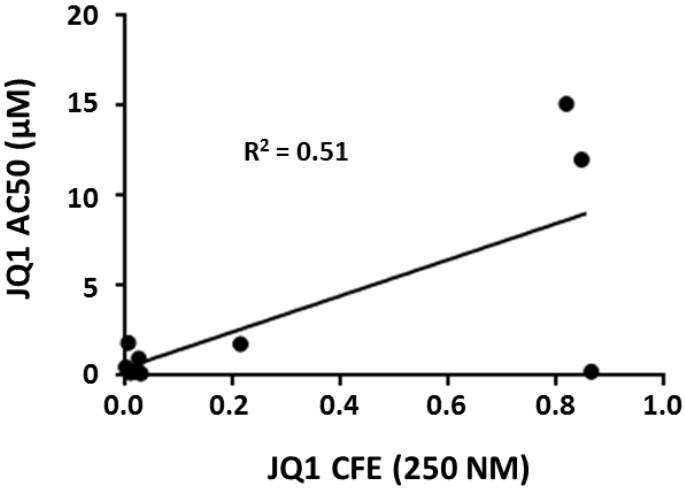
D.


THZ1 – 8 nM
Normalized Colony Count
1.5
1.0
0.5
0.0
H1693
H2009
H1395
HCC15
HCC44
H2122
H1819
H460
H1975
H1993

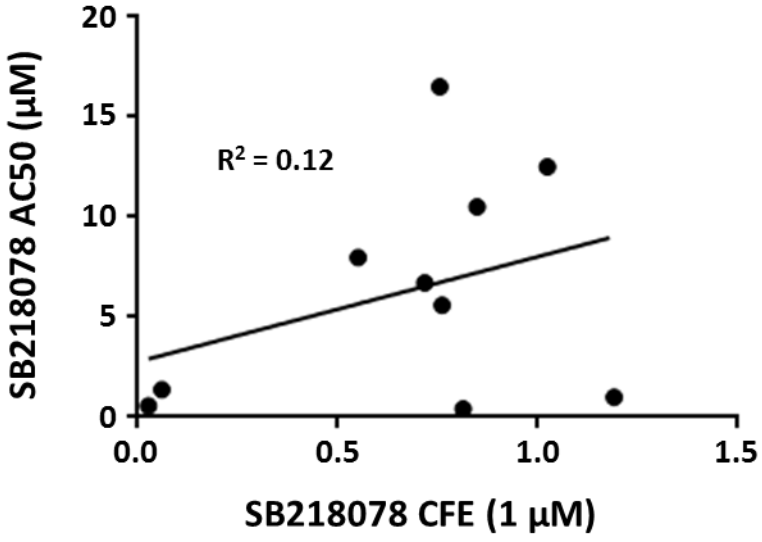
E.


10058-F4 AC50 (µM)
250
200
150
100
50
0
R² = 0.52
0
200
400
600
10058-F4 CFE (20 µM)

F.


JQ1 AC50 (µM)
20
15
10
5
0
R² = 0.51
0.0
0.2
0.4
0.6
0.8
1.0
JQ1 CFE (250 NM)

G.



H.

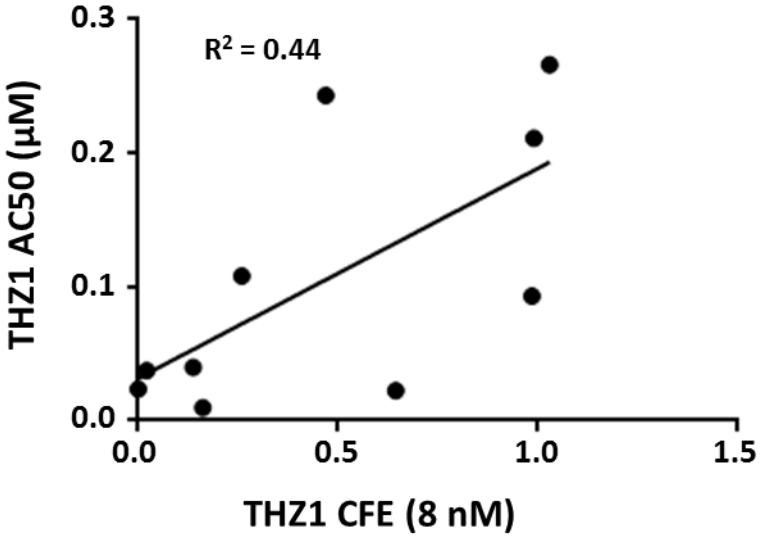


Figure 4.8: Clonogenic data for MYC targeting drugs on a panel of 10 NSCLC cell lines. CFE after treatment with (A) 20 µM 10058-F4, (B) 250 nM JQ1, (C) 1 µM SB218078, (D) 8 nM THZ1. Correlation between AC50 and single dose CFE for (E) 10058-F4, (F) JQ1, (G) SB218078 and (H) THZ1.

4.2.3 Dominant-negative-mediated Inhibition of MYC

Omomyc is a dominant negative mini-protein that binds to endogenous MYC, thus preventing MYC from binding to Max and initiating transcription of canonical MYC targets (Soucek, Helmer-Citterich et al. 1998, Savino, Annibali et al. 2011). This construct has been used to elicit an anti-MYC effect in various tumor models, including lung cancer (Fukazawa, Maeda et al. 2010, Soucek, Whitfield et al. 2013). Use of Omomyc first required verification that the protein could efficiently repress MYC activity. A doxycycline-inducible Omomyc construct was stably expressed in a panel of 8 NSCLC cell lines with the goal of functionally probing MYC dependence across the panel. The construct used contains an RFP marker under inducible control, yielding visual confirmation of construct expression (Figure 4.9). A Taqman primer-probe set was custom-designed to recognize Omomyc, but not endogenous MYC (Figure 4.9). Omomyc mRNA was quantified and shown to be equivalent across the panel of cell lines tested (Figure 4.9), thus confirming equivalent expression of the dominant-negative mini-protein across cells to be tested.

As MYC is a well-studied transcriptional regulator, we next sought to confirm that the dominant-negative mediated inhibition resulted in repression of MYC transcriptional activity. Upon induction of Omomyc, MYC levels do not exhibit a common response across cell lines tested. Several of the cell lines show an upregulation of MYC message (H1299, HCC44), while others show repression (H1693) (Figure 4.9). One possible explanation for the varying response of MYC to Omomyc induction is that some cell lines are able to activate potentially compensatory mechanisms to upregulate MYC transcription in response to the dominant-

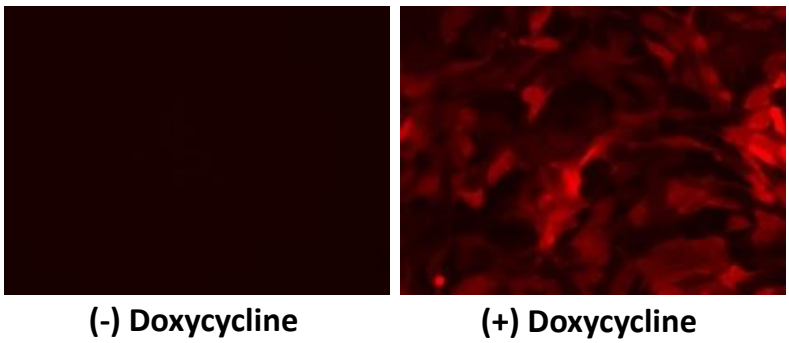
negative mediated MYC inhibition. To elucidate the effect of Omomyc expression on the transcriptional output, microarrays were performed before and after treatment with Omomyc, and expression of known MYC targets was assessed. MYC target gene sets were downloaded from the Broad Institute's Molecular Signatures Database (Subramanian, Tamayo et al. 2005). After induction of Omomyc in each of the cell lines tested, mRNA levels of MYC target genes were significantly repressed (Figure 4.9). Thus, despite having varying effects on the level of MYC in the cells, Omomyc induction represses MYC's transcriptional activity.

Next, to complement the MYC inhibitor screen, the dominant negative mini-protein Omomyc was used to functionally probe a subset of the NSCLC cell line panel. All 8 cell lines were functionally tested via colony forming efficiency assays. A subset of the cell lines showed significantly reduced colony forming efficiency in the presence of induced Omomyc (Figure 4.10). Next, we wanted to determine if response to Omomyc correlated with the response to either of the pharmacological MYC inhibitors screened. To this end, we correlated the CFE in the presence of Omomyc to the AC50 of 10058-F4, JQ1, SB218078 and THZ1. These data showed that only 10058-F4 positively correlated with Omomyc response (Figure 4.10). Lack of response to Omomyc could have been the result of a loss of the Omomyc construct. To test this, we isolated mRNA from cells following the CFE assay and quantified Omomyc message using qRT-PCR. In both the sensitive and resistant cell lines, Omomyc levels remained largely unchanged (Figure 4.10).

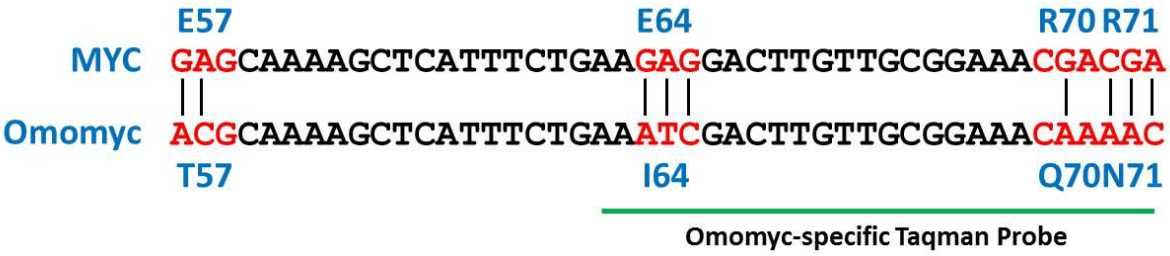
To determine whether the tumor microenvironment could compensate for the loss of MYC's transcriptional activity, xenograft experiments were performed on a subset of the cell lines

tested. Control or Omomyc-manipulated cell lines were injected subcutaneously into NOD-SCID mice and tumor growth was monitored following induction of Omomyc (Figure 4.11). Similar to the *in vitro* studies, the sensitive cell line showed slowed tumor growth *in vivo* upon the induction of Omomyc, whereas the resistant cell line showed no effect (Figure 4.11). Additionally, the final tumor weight in the sensitive cell line xenograft was significantly lower than control, whereas the resistant cell line xenograft showed no change (Figure 4.11). In HCC44, the tumors exhibit a moderately-well differentiated adenocarcinoma both before and after induction of Omomyc. Again, the lack of response in the resistant cell line xenograft could have been the result of a loss of the Omomyc construct. To test this, mRNA was isolated from tumors at the end of the experiment, and Omomyc mRNA was quantified using qRT-PCR. In both the sensitive and resistant cell line xenografts, the level of Omomyc mRNA remained unchanged as a result of the in vivo growth (Figure 4.11).

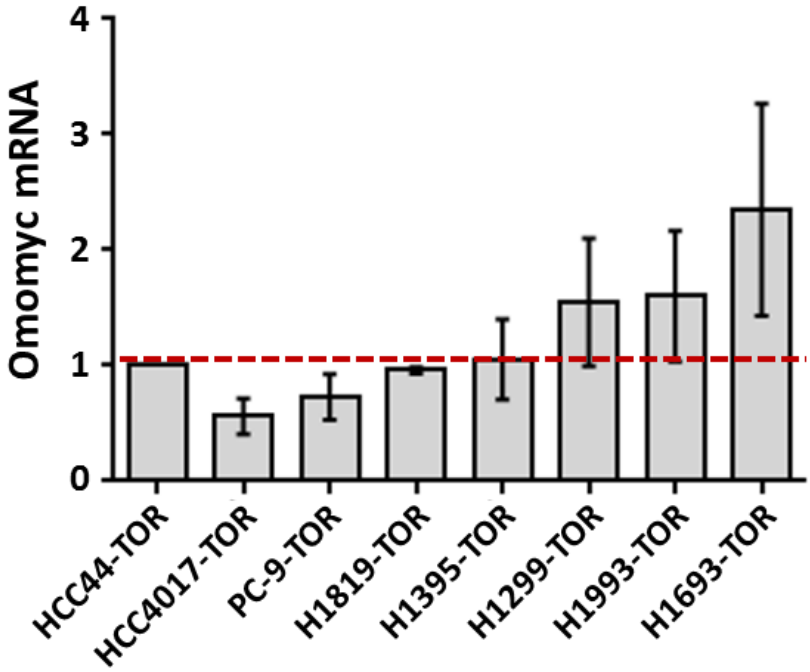
A.


(-) Doxycycline
(+) Doxycycline

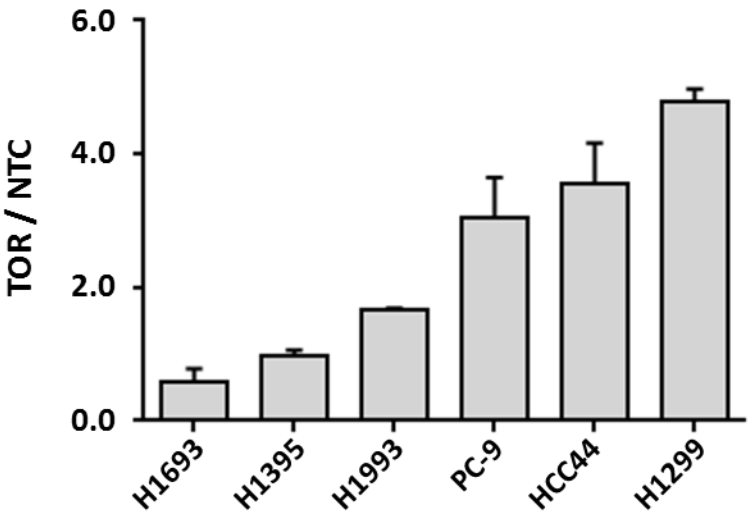
B.


E57
E64
R70 R71
MYC GAGCAAAAGCTCATTTCTGAAGAGGACTTGTTGCGGAAACGACGA
Omomyc ACGCAAAAGCTCATTTCTGAAATCGACTTGTTGCGGAAACAAAAC
T57
I64
Q70N71
Omomyc-specific Taqman Probe

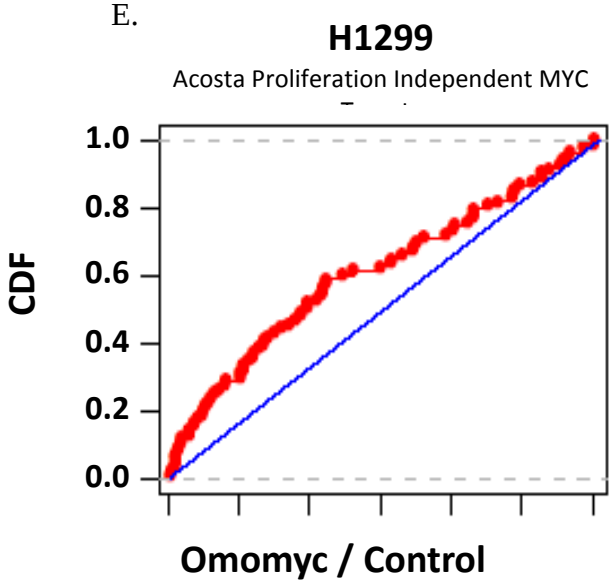
C.


Omomyc mRNA
0
1
2
3
4
HCC44-TOR
HCC4017-TOR
PC-9-TOR
H1819-TOR
H1395-TOR
H1299-TOR
H1993-TOR
H1693-TOR

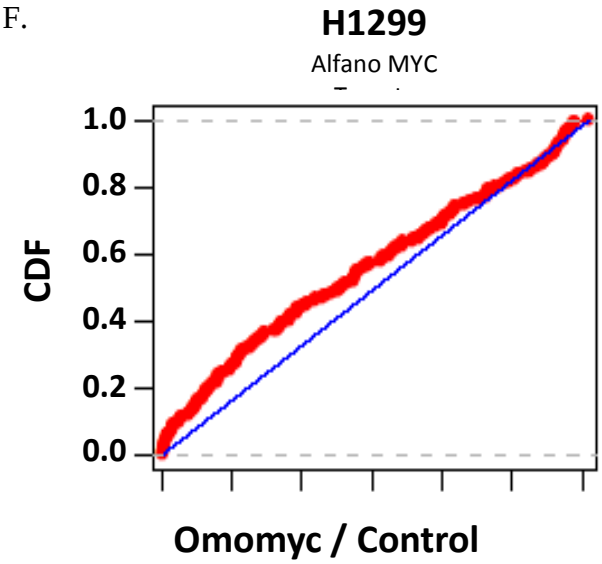
D.


6.0
4.0
2.0
0.0
TOR / NTC
H1693
H1395
H1993
PC-9
HCC44
H1299

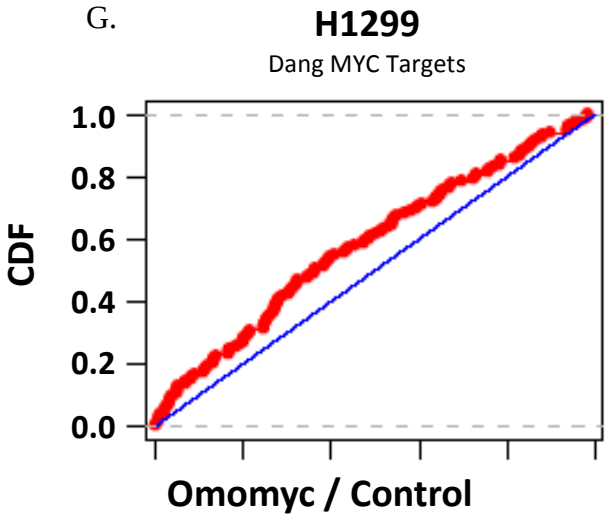
E.


H1299
Acosta Proliferation Independent MYC
1.0
0.8
0.6
0.4
0.2
0.0
CDF
Omomyc / Control

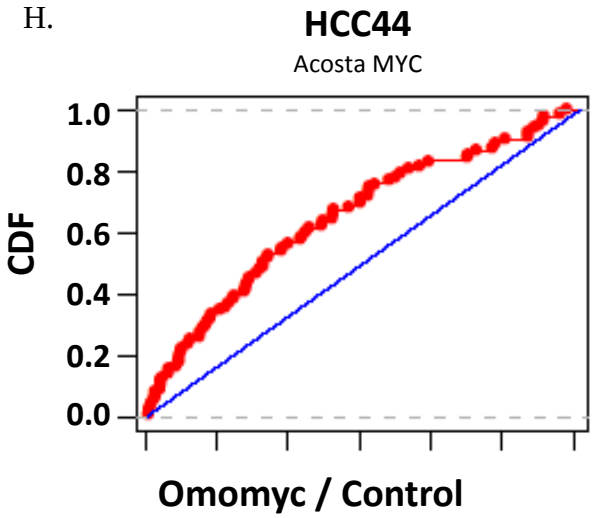
F.


H1299
Alfano MYC
1.0
0.8
0.6
0.4
0.2
0.0
CDF
Omomyc / Control

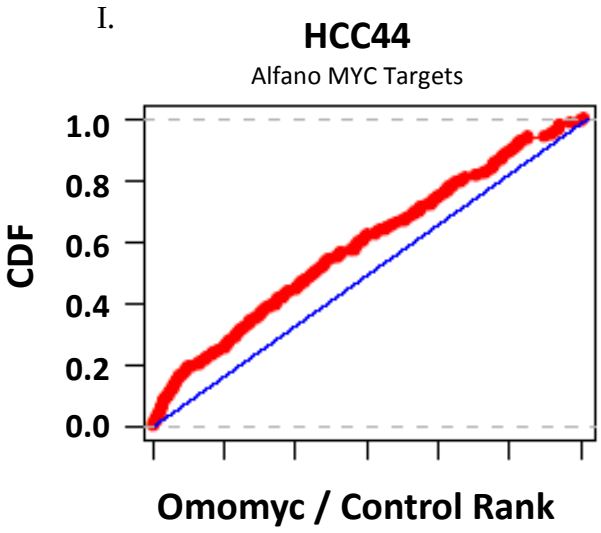
G.


H1299
Dang MYC Targets
1.0
0.8
0.6
0.4
0.2
0.0
CDF
Omomyc / Control

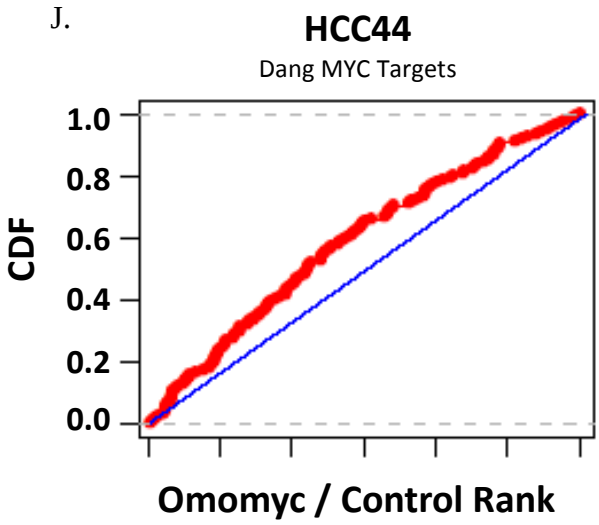
H.


HCC44
Acosta MYC
1.0
0.8
0.6
0.4
0.2
0.0
CDF
Omomyc / Control

I.



J.



K.

Gene Set	Cell Lines with $p \leq 0.05$
ACOSTA_PROLIFERATION_INDEPENDENT_MYC_TARGETS_UP	PC-9, HCC44, HCC4017, H1299, H1819
COLLER_MYC_TARGETS_UP	PC-9, HCC4017, H1299, H1819
ALFANO_MYC_TARGETS	PC-9, HCC4017, H1299
DANG_MYC_TARGETS_UP	HCC44, HCC4017, H1395

Figure 4.9: Validation of the Omomyc system. (A) RFP fluorescence visually confirms construct expression. (B) Taqman probe design for specific recognition of Omomyc mRNA. (C) Omomyc mRNA as measured by qRT-PCR. (D) qRT-PCR for MYC after Omomyc induction. (E) - (G) Show a CDF plot for known MYC target gene sets for H1299 after Omomyc induction. (H) – (J) Show a CDF plot for known MYC target gene sets for HCC44 after Omomyc induction. (K) Summary table of gene sets which are downregulated for each cell line tested.

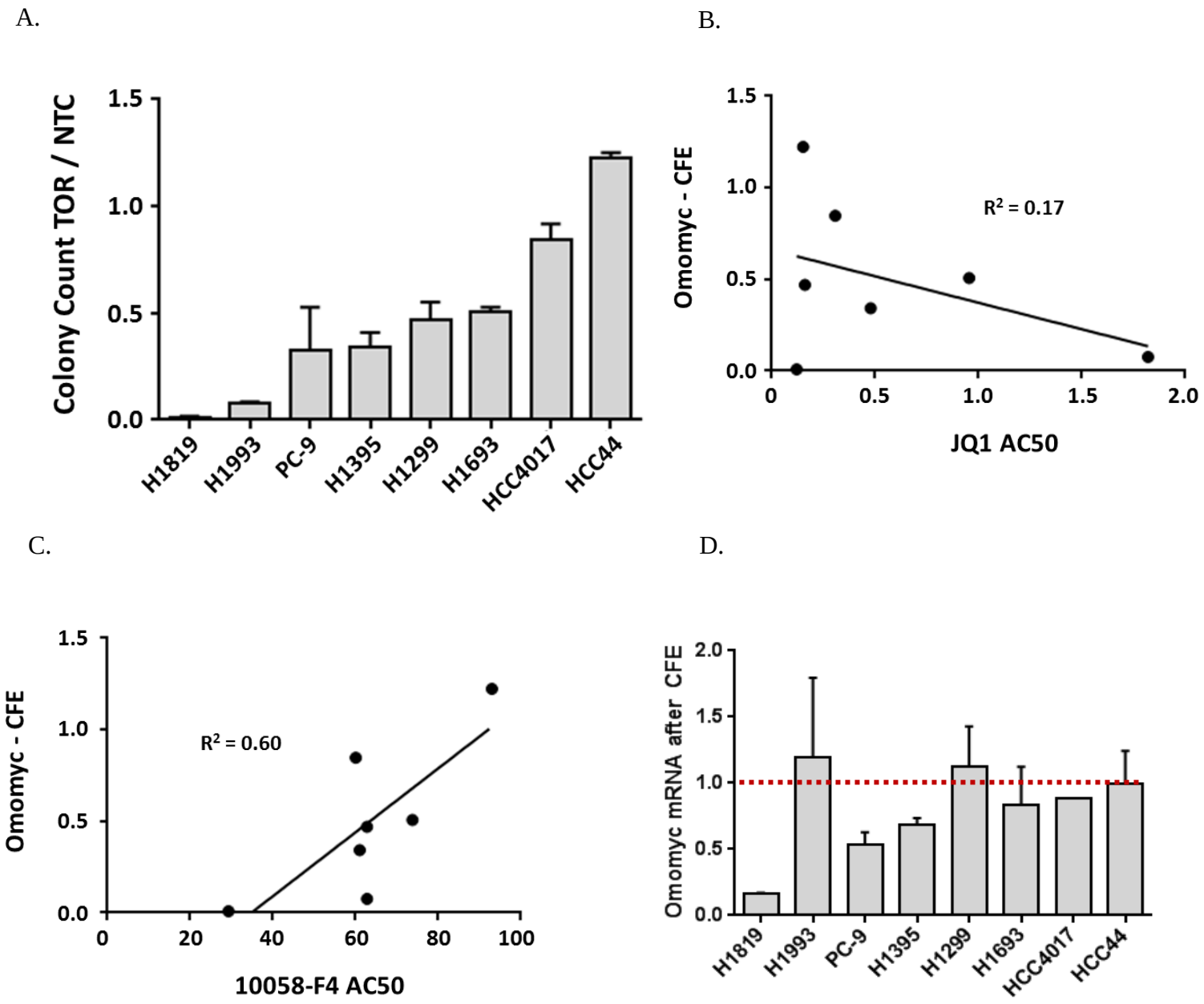


Figure 4.10: Functional Omomyc data and drug correlations. (A) CFE after induction of Omomyc in a panel of 8 NSCLC cell lines. (B) Correlation between Omomyc CFE and JQ1 AC50. (C) Correlation between Omomyc CFE and 10058-F4 AC50. (D) Omomyc levels after colony forming efficiency assay.

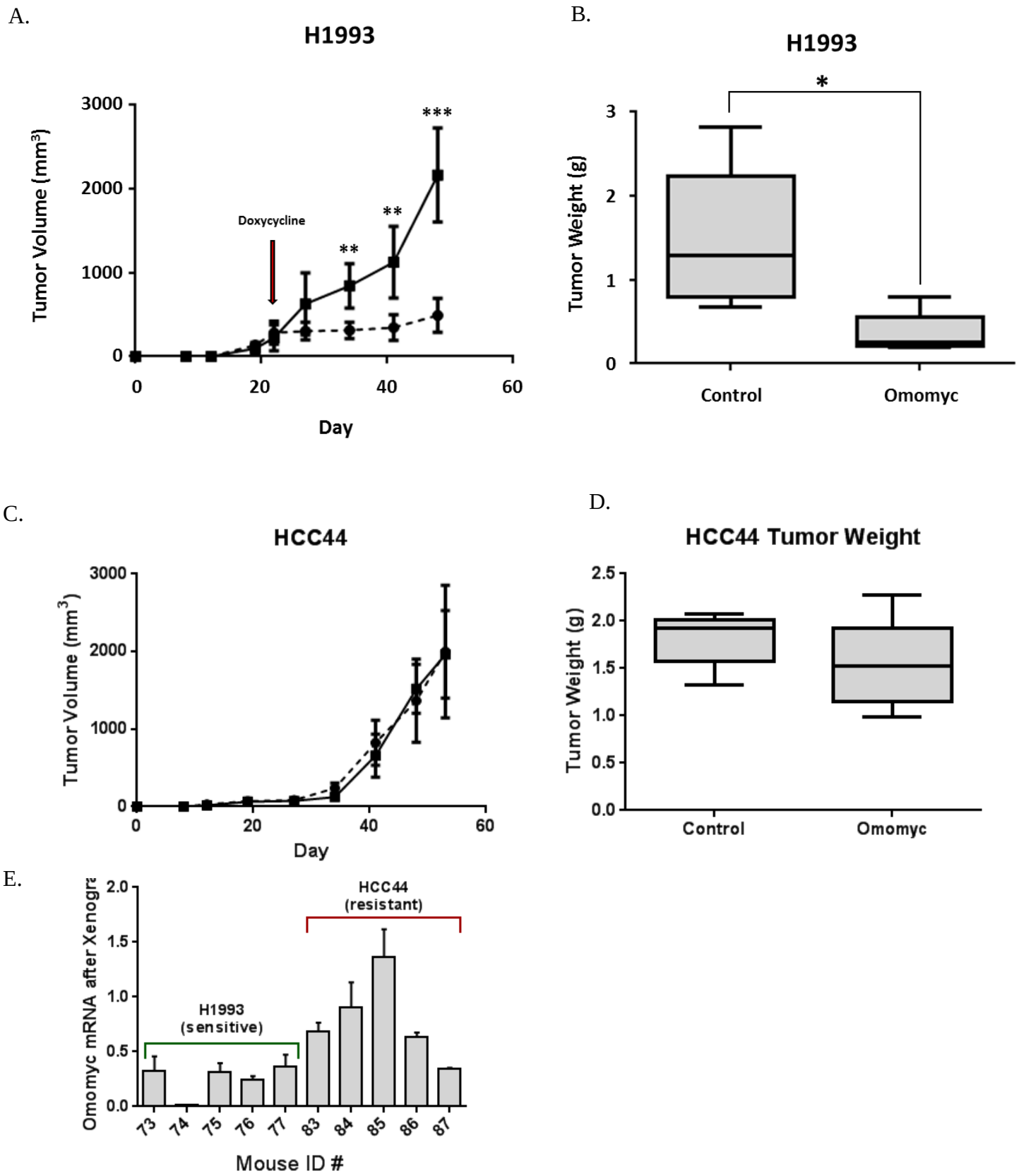


Figure 4.11: Omomyc xenograft data. (A) Tumor growth for Omomyc-sensitive cell line H1993. Solid line = control, dashed line = Omomyc. (B) Final tumor weight for the sensitive cell line +/- Omomyc. (C) Tumor growth for Omomyc-resistant cell line HCC44. Solid line = control, dashed line = Omomyc. (D) Final tumor weight for the resistant cell line +/- Omomyc. (E) Omomyc levels after xenograft experiment.

4.3 Discussion

Therapeutically targeting MYC has historically been a significant challenge, but the field has seen a resurgent effort following the discovery of the anti-MYC properties of several drugs, as well as the development of well-defined genetic models. Previous studies, including those in lung cancer, have shown continued dependence on MYC for the tumorigenic phenotype, though these studies have not been on a large scale and thus have not determined whether this dependence is universal. In fact, the degree to which a tumor depends on MYC activity appears to depend on the genetic context in many cases.

In order to advance MYC-targeted therapies through the clinic, a thorough understanding of whether MYC is a universal requirement in certain tumor types, or if MYC dependence is context-dependent, will be required. Here, we used several different approaches to assess MYC dependence in a large panel of NSCLC cell lines, including conventional RNA-interference, pharmacological MYC inhibition, and dominant-negative mediated MYC inhibition. RNAi was an appropriate starting point for this work, due to the ease of use and availability of well-validated reagents. While perhaps inappropriate for large-scale screening, this method yielded targeted inhibition of the various MYC family members, and the functional readout suggested that MYC dependence was not a universal characteristic in lung cancer. However, the degree of inhibition of the MYC family members was inconsistent across cell lines and reagents, casting doubt on the validity of this approach for a large-scale experiment. Specifically, the lack of correlation between the response to siRNA and shRNA suggests that at least one approach yielded a phenotype that is unrelated to the inhibition of MYC family members. Further, the

decreased viability without concurrent reduction in the RNAi target bolstered the off-target concern of this approach (e.g., MYCN knockdown in H2009). Nevertheless, the RNAi-mediated inhibition of MYC family members provided necessary proof of principle. Clearly, MYC (and its family members) was required for the viability and likely the tumorigenic phenotype in lung cancer, though this was not a universal requirement.

To further characterize the dependence on MYC in lung cancer, as well as the clinical relevance of this dependence, a complementary approach would be required. Over the past decade, a large number of drugs have been studied for their potential to elicit anti-MYC properties in various cancer settings. Thus, the next approach to inhibiting MYC in lung cancers was to use drugs that had been previously shown to exhibit anti-MYC properties (Trichostatin A, ABT-263 and diclofenac). Similar to the RNAi proof of principle experiments, the initial assays with these three putative MYC inhibitors elicited inconsistent results across cell lines tested. The inconsistency across reagents and cell lines provided a significant confounding factor to these studies. Clearly, a large-scale, multimodal approach would be required in order to truly characterize MYC-dependence in lung cancer. To this end, four well-established drugs with anti-MYC properties were screened across a panel of ~80 NSCLC cell lines each. While each drug elicited an anti-tumor response in a subset of cell lines, the drugs did not necessarily target the same cell lines. Despite the inconsistency in the individual datasets, the combined data set (RNAi and drug screens) provided a sufficient foundation for understanding the differential dependence on MYC in lung cancer. The data from this foundation were compared to the gold-standard MYC inhibitor, the dominant-negative protein Omomyc. Using this well-established, specific inhibitor of MYC family function, we were able to determine which of the drugs was

most likely eliciting an anti-tumor response in MYC dependent cancers. Unsurprisingly, the Omomyc dataset correlated best with the direct inhibitor, 10058-F4. The consistency between the targeted Omomyc approach and 10058-F4 bolsters the finding that the responsive subset of lung cancers represents a MYC-dependent subset. While the other drugs that were used may have clinical benefit, these data suggest that their effect is likely to be independent of MYC. Thus, a multimodal approach was used to identify differential MYC dependence in NSCLC.

Targeting MYC using drugs and well-characterized genetic models has provided great insight into MYC dependence in various tumors, but translating this information to the clinic will be significantly more challenging. Of particular concern is how to most effectively target MYC (i.e., upstream, downstream, or directly). Here, we demonstrate that directly targeting MYC yields the most consistent results. However, the poor potency and potential for toxicity has hindered the clinical development of direct MYC therapies. As a result, alternative approaches have been pursued, such as the indirect targeting of MYC and targeting downstream pathways of MYC in an effort to identify potential acquired vulnerabilities.

Targeting downstream targets of MYC through the use of large-scale RNAi screens in a system with overexpressed MYC have been used to identify therapeutic targets specific to tumors with high c-Myc expression (Kessler, Kahle et al. 2012, Toyoshima, Howie et al. 2012). Studies of this type typically identify either a MYC transcriptional target that represents an acquired vulnerability, or an unrelated target that has a synthetic-lethal relationship with overexpressed MYC. MYC affects transcription of a large number of genes, and many of these genes encode proteins which have been implicated in tumorigenesis (Robson, Pelengaris et al.

2006). Importantly, many of the encoded proteins participate in functionally redundant pathways, making the prospect of therapeutically targeting a single target in MYC-dependent cancers daunting. Further, the utility of identifying a novel synthetic lethal relationship with high MYC is limited to tumors where MYC dependence correlates with MYC overexpression. While other tumor types may fit this profile, it remains unclear whether this is the case in NSCLC. Targeting MYC through an indirect, upstream mechanism represents a viable therapeutic approach, as demonstrated by recent work targeting the BET family of proteins (Delmore, Issa et al. 2011). As discussed, the most notable inhibitor of BET family member BRD4 is JQ1, a drug that has been shown to repress MYC levels in multiple settings (Delmore, Issa et al. 2011, Mertz, Conery et al. 2011, Roderick, Tesell et al. 2014). A significant amount of preclinical evidence exists to support the notion of targeting MYC through BRD4, as evidenced by the advance of next-generation BET inhibitors into clinical trials (NCT02157636). However, repressing transcription of MYC via an upstream target may be an approach that is uniquely vulnerable to the activation of compensatory pathways. For example, a previous study demonstrated that the treatment of lung cancer cell lines with JQ1 can result in the upregulation of MYC mRNA and protein, though whether this upregulation could play a role in acquired resistance to JQ1 is unclear (Lockwood, Zejnullahu et al. 2012).

Given the limitations of indirectly targeting MYC (e.g., upstream or downstream), a direct approach may be ideal. Here, directly targeting MYC with both a drug and an exogenously expressed dominant negative protein has yielded a robust dataset that delineates the degree of MYC dependence in NSCLC. However, the potency of available MYC inhibitors is insufficient for clinical use. While these data provide important insight into MYC dependence in lung

cancer, a more potent therapeutic will be required if clinical success is to be achieved. Additionally, toxicity remains a concern with direct MYC inhibitors, as normal proliferating cells require MYC for survival and growth. However, as the efforts to develop an efficient MYC inhibitor intensify, so too do efforts to fully understand the broader effects of MYC inhibition. For example, recent work with Omomyc in a mouse model of KRAS-driven lung cancer demonstrated that the side effects associated with MYC inhibition are both mild and reversible, supporting the viability of a direct approach in the future (Soucek, Whitfield et al. 2013). Thus, this dataset provides essential context to the field of MYC dependence, and will be an important complementary piece once an effective MYC-targeting drug is developed.

CHAPTER FIVE

CHARACTERIZING MOLECULAR CORRELATES OF MYC DEPENDENCE

5.1 Introduction

MYC inhibition has resulted in tumor regression in multiple contexts, but a sustained response is not universally seen. In order for MYC to be effectively targeted in the clinic, a full characterization of the context in which MYC inhibition will elicit an anti-tumor response is required. Further, in several recent studies, researchers have reported acquired resistance to MYC inhibition that, at times, is conferred by the activation of a compensatory pathway. Such activation has been documented in the form of mutations, overexpression, or other signaling mechanisms. Understanding these mechanisms of either inherent or acquired resistance is an important hurdle in the targeting of MYC-driven tumors, as this knowledge may provide insights into the potential for combination therapy.

In many other studies of MYC dependence, MYC amplification or overexpression serves as an effective biomarker for response. A recent publication in lung cancer demonstrated that Omomyc-mediated MYC inhibition was effective in NSCLC, but only in cell lines with overexpressed MYC protein (Fukazawa, Maeda et al. 2010). Given this, the copy number and expression of MYC in NSCLC was the first molecular correlate to study, considering the degree of MYC deregulation described in Chapter 1.

In addition to MYC levels, the mutational landscape of the tumor likely plays a role in MYC dependence. In the aforementioned report on MYC dependence in lung cancers with overexpressed MYC, the authors also argued that this phenomenon was dependent on the presence of mutant *KRAS* (Fukazawa, Maeda et al. 2010). Additionally, Soucek et al recently reported that MYC inhibition could effectively target *KRAS* mutant lung cancers (Soucek, Whitfield et al. 2013). Yet, whether mutant *KRAS* is a necessary precondition for MYC dependence remains unclear. In fact, in other tumor types, mutant *KRAS* appears to confer resistance to MYC inhibition. In breast cancer, for example, D'Cruz et al recently showed that MYC amplification and overexpression is a marker of poor prognosis, but the mechanism by which MYC contributed to tumor progression was not clear (D'Cruz, Gunther et al. 2001). The authors further sought to understand how MYC-induced mammary adenocarcinomas might persist in the absence of MYC function. The authors showed that upon MYC inactivation, tumors lacking activating *ras* family mutations fully regressed, whereas tumors with *ras* family mutations did not, suggesting that mutations in this family could allow tumors to persist despite MYC inactivation (D'Cruz, Gunther et al. 2001). Yet another study, this one in lung adenocarcinoma, showed that in MYC induced tumors with mutant *KRAS*, MYC inactivation alone failed to induce complete regression (Tran, Fan et al. 2008). However, inhibition of both MYC and KRAS resulted in more frequent complete regression.

In addition to *KRAS* status, the mutational status of other oncogene and tumor suppressor genes will likely play a role in MYC dependence. For example, despite evidence that JQ1 does not rely on MYC downregulation in NSCLC, Shimamura et al recently demonstrated that JQ1 does downregulate MYC in NSCLC, but that this effect was dependent on mutant *KRAS* and

wild type *LKB1* (Lockwood, Zejnullahu et al. 2012, Shimamura, Chen et al. 2013). Thus, the mutational landscape clearly plays a role in mediating response to MYC inhibition, and understanding the impact of mutations will be an important part of characterizing MYC dependence in lung cancer.

Outside of the genetic landscape of tumor cells, there are other molecular features that are likely to play a role in MYC dependence. Recent work in MYC-induced mammary tumors showed that heterogeneity in the tumors allowed for the outgrowth of resistant tumor cells. Specifically, upon MYC inactivation, the tumors that persist are distinct from the primary tumors and exhibit a more mesenchymal phenotype (Leung, Andrechek et al. 2012).

A recent genome-wide association study identified a single-nucleotide polymorphism (SNP) in a gene desert upstream of the *MYC* locus (Tomlinson, Webb et al. 2007). The risk genotype at this SNP (rs6983267) was found to confer an increased risk of developing a number of cancers, including colorectal cancer, prostate, kidney, thyroid and larynx (Wokolorczyk, Gliniewicz et al. 2008). Later, this SNP was found to reside in an enhancer region that physically interacts with the *MYC* promoter, though the effect on MYC levels was unclear (Pomerantz, Ahmadiyeh et al. 2009, Wright, Brown et al. 2010, Sur, Hallikas et al. 2012).

Clearly, the genetic and morphologic context will be important determining factors of MYC dependence. As importantly, rapidly growing tumor cells possess the ability to adapt to MYC inhibition via the activation of compensatory pathways. This phenomenon has been observed in many tumor settings, including a recent report in lung cancer. Tran et al recently demonstrated

that MYC inactivation in lung tumors elicited regression in many cases (Tran, Fan et al. 2008). However, in the MYC-induced tumors that failed to regress upon MYC inactivation, persistent levels of phosphorylated Stat3 and Stat5 were observed. Stat3 and Stat5 are both downstream mediators of *KRAS*, which could explain why the authors were able to demonstrate more efficient tumor regression upon dual inhibition of MYC and RAS. Still, this finding suggests that the expected response to MYC inhibition can be evaded via persistent activation of a potentially compensatory pathway. Similarly, recent evidence in lymphoma implicates the Wnt pathway as another possible compensatory pathway. Lymphoma is a classically MYC-dependent tumor type, and MYC inhibition often results in complete and sustained regression (Choi, Li et al. 2014). In a recent report, Choi et al show that lymphomas often acquire activating mutations in β-catenin. The authors go on to show that, while tumors initially respond to MYC inhibition, tumor recurrence is common. However, simultaneous inhibition of both MYC and β-catenin resulted in sustained tumor regression, suggesting that an activated Wnt pathway could ultimately allow for the outgrowth of MYC-independent tumors, even in a previously MYC-dependent setting (Choi, Li et al. 2014).

Thus, the literature is ripe with examples of how tumors can escape MYC dependence. Importantly, these mechanisms do not necessarily translate across tumor types, a fact which underscores the importance of characterizing the correlates of MYC dependence in lung cancer. The goal of this study is to characterize the genetic and morphologic context of MYC dependence in NSCLC, and further to investigate any potential compensatory pathways that may allow MYC dependent tumors to survive.

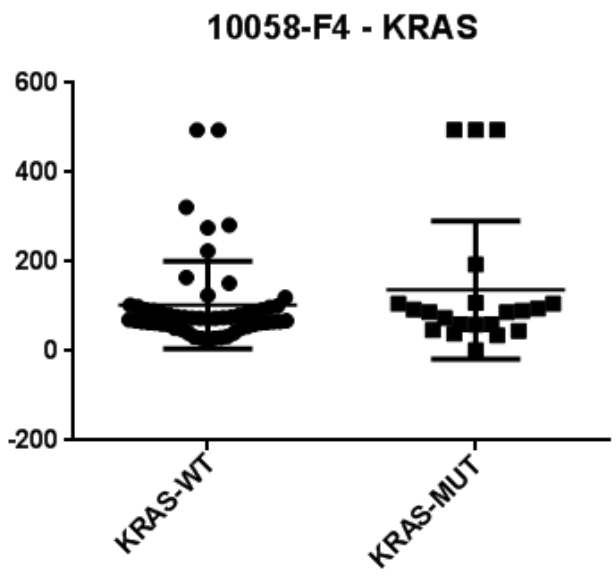
5.2 Results

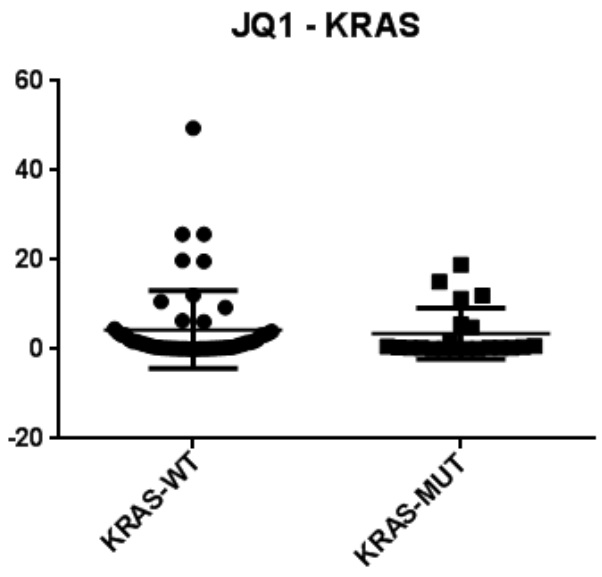
5.2.1 Correlating MYC Dependence with Oncogenotype

We next assessed the correlation between drug response and the mutational status of *KRAS* and *p53* in an effort to identify potential biomarkers for response. As discussed, the status of *KRAS* in MYC dependence studies has been the subject of conflicting results across tumor types. NSCLC cell lines were divided based on *KRAS* status, and the AC50 values for each of the four MYC inhibitors were compared. In the case of all four drugs, the AC50 values were not statistically different between wild-type and mutants, suggesting *KRAS* status does not impact response to either direct or indirect MYC inhibitors (Figure 5.1). Similarly, cell lines were divided based on their *p53* status, and showed no statistical difference in AC50 values for three of the four drugs screened (Figure 5.1). However, cell lines with wild-type *p53* exhibited an average AC50 for JQ1 3-fold higher than the average of their mutant counterparts.

Further, given the reported ability of these drugs to specifically target MYC-driven cancers, MYC copy number, mRNA and protein levels were correlated with drug response. MYC copy number (as measured by SNP arrays), mRNA level (as measured by microarray) and protein level (as measured by RPPA) were used to bifurcate the panel of NSCLC cell lines, and the AC50 values for the “high” and “low” groups were compared. Surprisingly, response to none of the four drugs correlated with either the copy number or endogenous levels of MYC (Figure 5.1).

A.


10058-F4 - KRAS
600
400
200
0
-200
KRAS-WT
KRAS-MUT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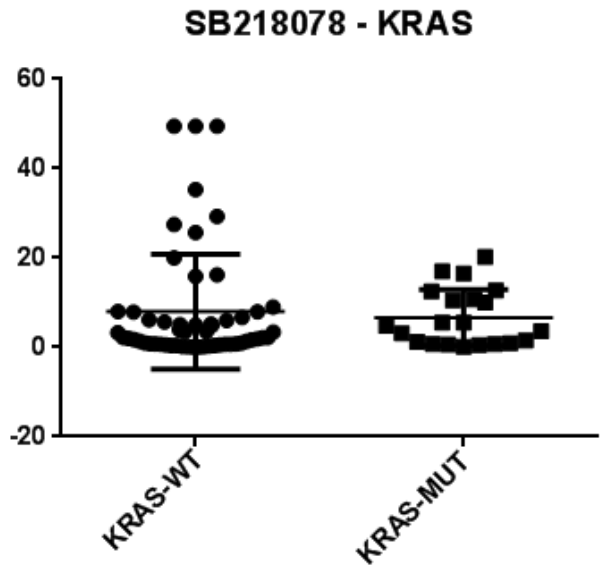
40
20
0
-20
KRAS-WT
KRAS-MUT


SB218078 - KRAS
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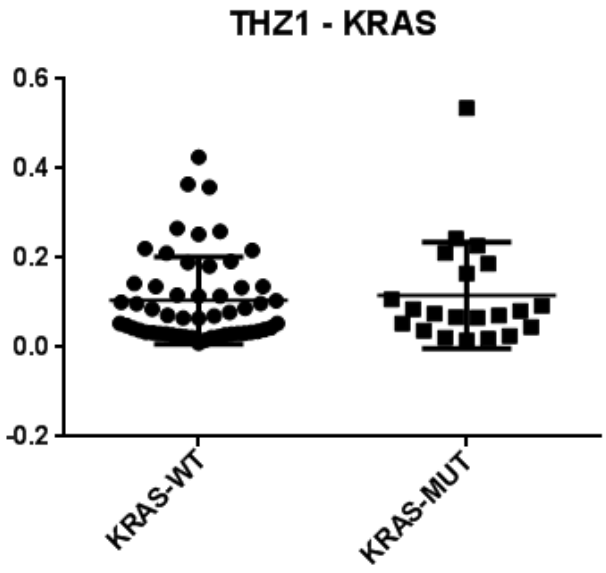
40
20
0
-20
KRAS-WT
KRAS-MUT


THZ1 - KRAS
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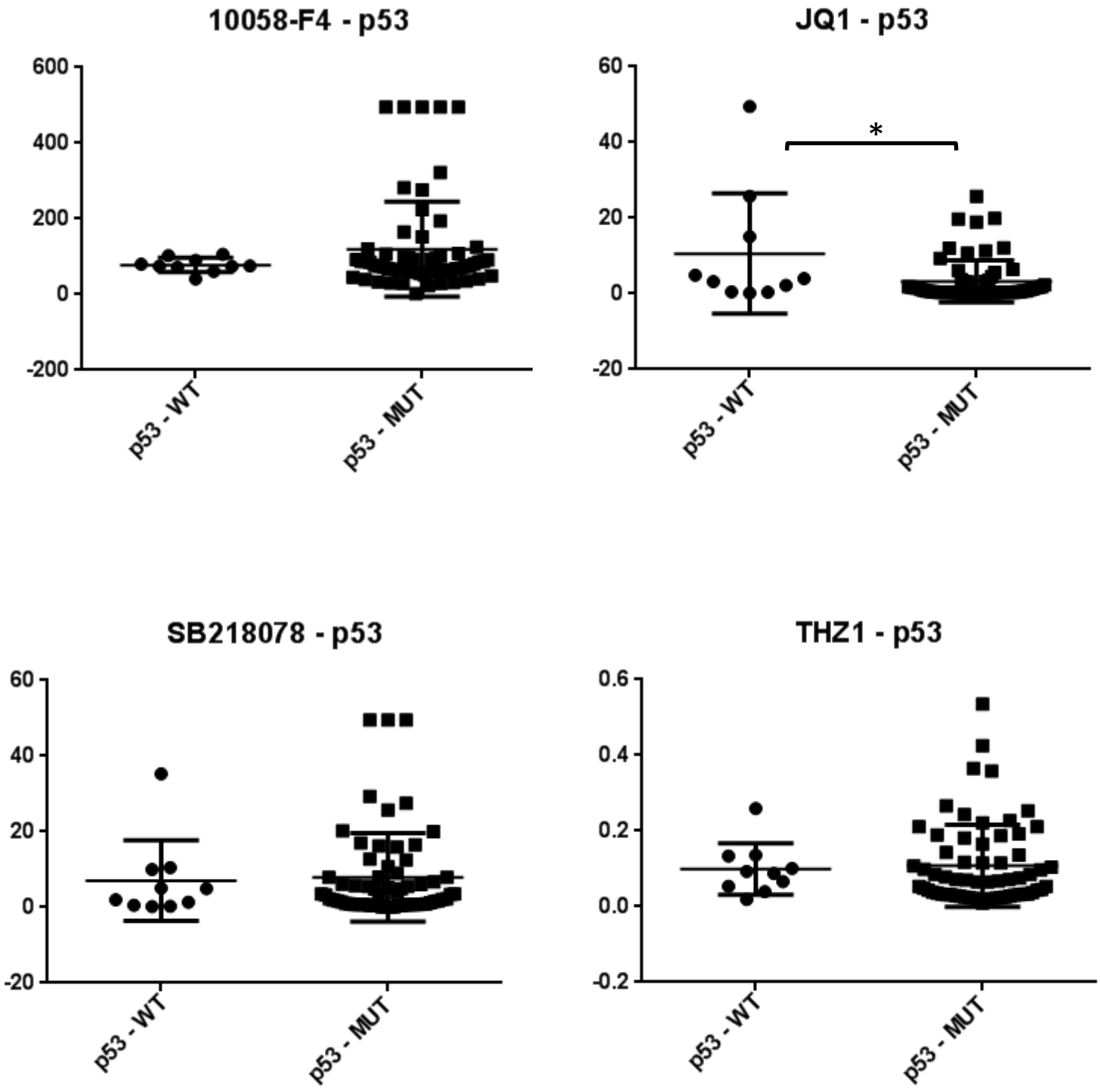
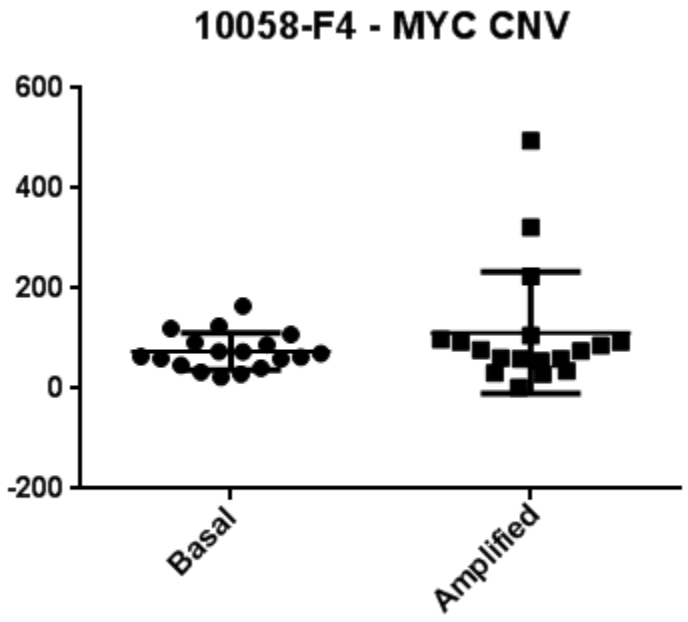
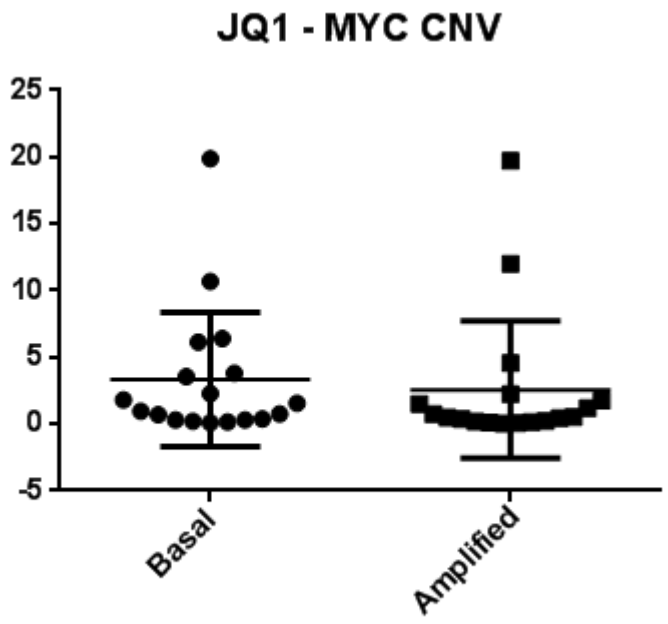
0.4
0.2
0.0
-0.2
KRAS-WT
KRAS-MUT

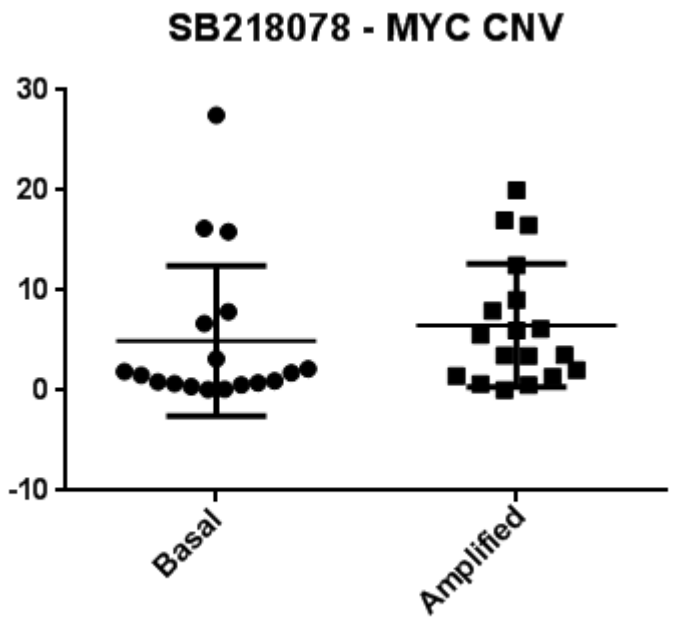
B.

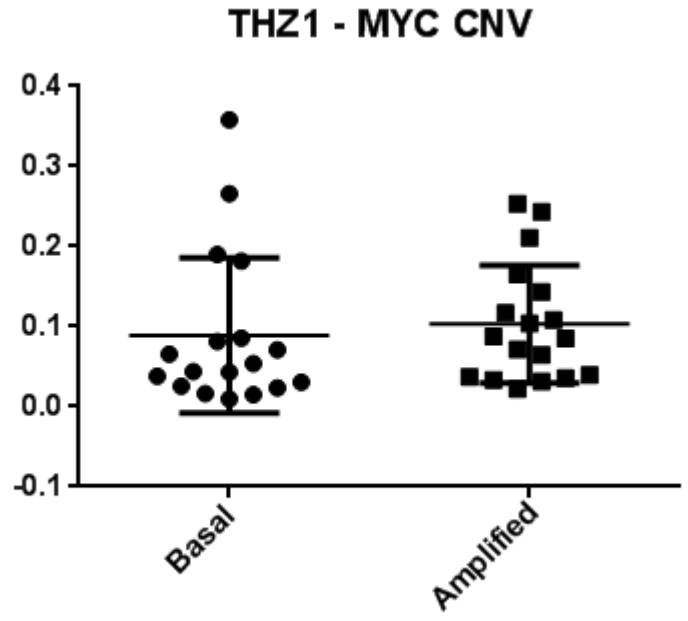

10058-F4 - p53
600
400
200
0
-200
p53 - WT
p53 - MUT
JQ1 - p53
60
40
20
0
-20
*
p53 - WT
p53 - MUT
SB218078 - p53
60
40
20
0
-20
p53 - WT
p53 - MUT
THZ1 - p53
0.6
0.4
0.2
0.0
-0.2
p53 - WT
p53 - MUT

C.

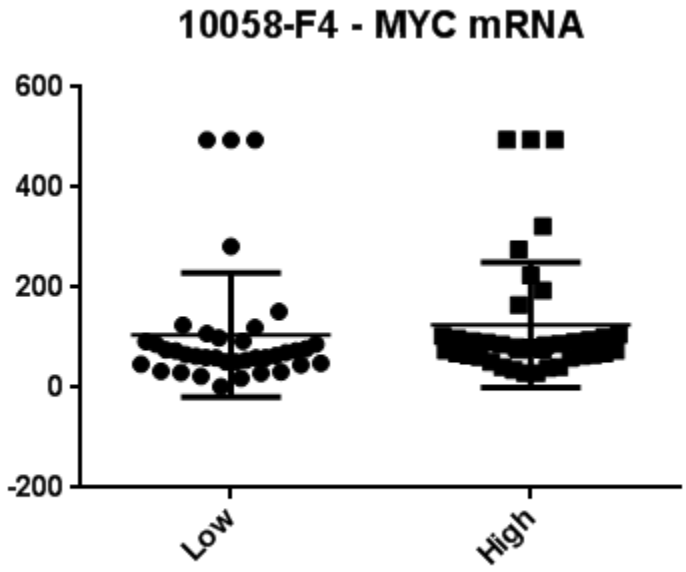

10058-F4 - MYC CNV
600
400
200
0
-200
Basal
Amplified

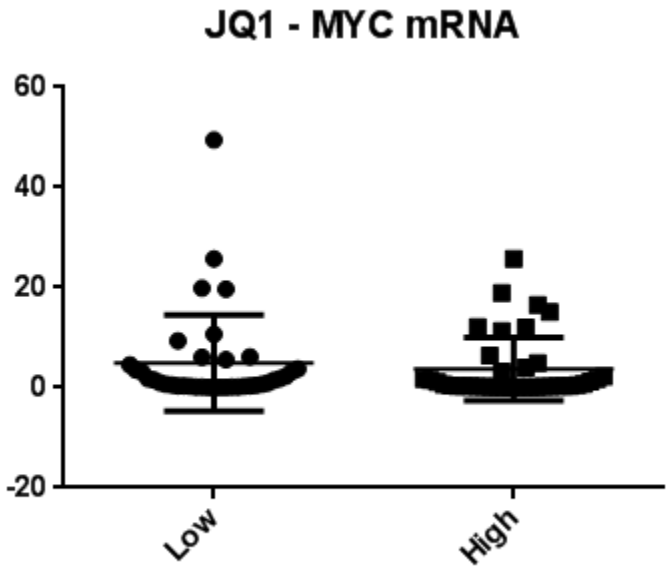

JQ1 - MYC CNV
25
20
15
10
5
0
-5
Basal
Amplified

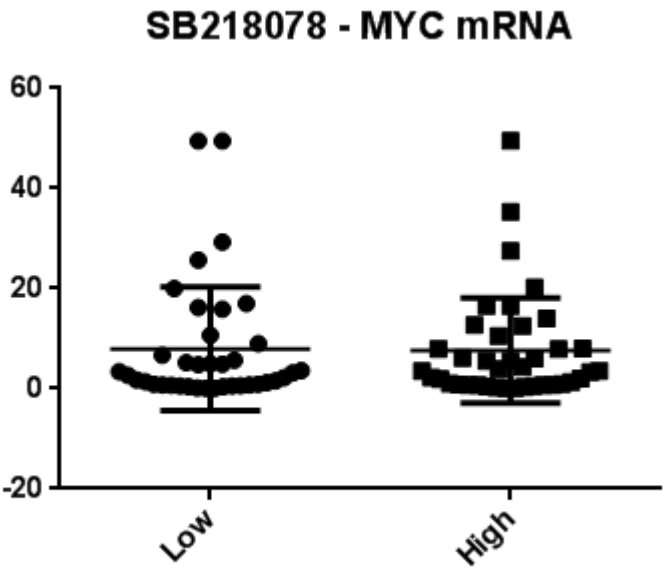

SB218078 - MYC CNV
30
20
10
0
-10
Basal
Amplified

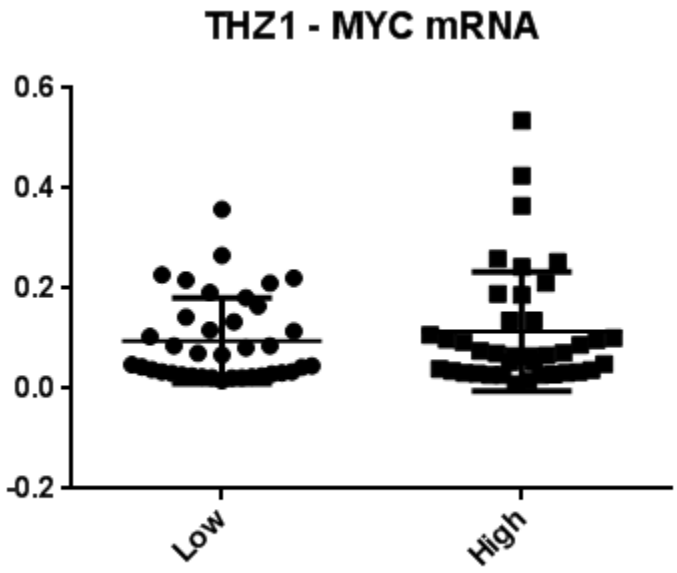

THZ1 - MYC CNV
0.4
0.3
0.2
0.1
0.0
-0.1
Basal
Amplified

D.


10058-F4 - MYC mRNA
600
400
200
0
-200
Low
High


JQ1 - MYC mRNA
60
40
20
0
-20
Low
High


SB218078 - MYC mRNA
60
40
20
0
-20
Low
High


THZ1 - MYC mRNA
0.6
0.4
0.2
0.0
-0.2
Low
High

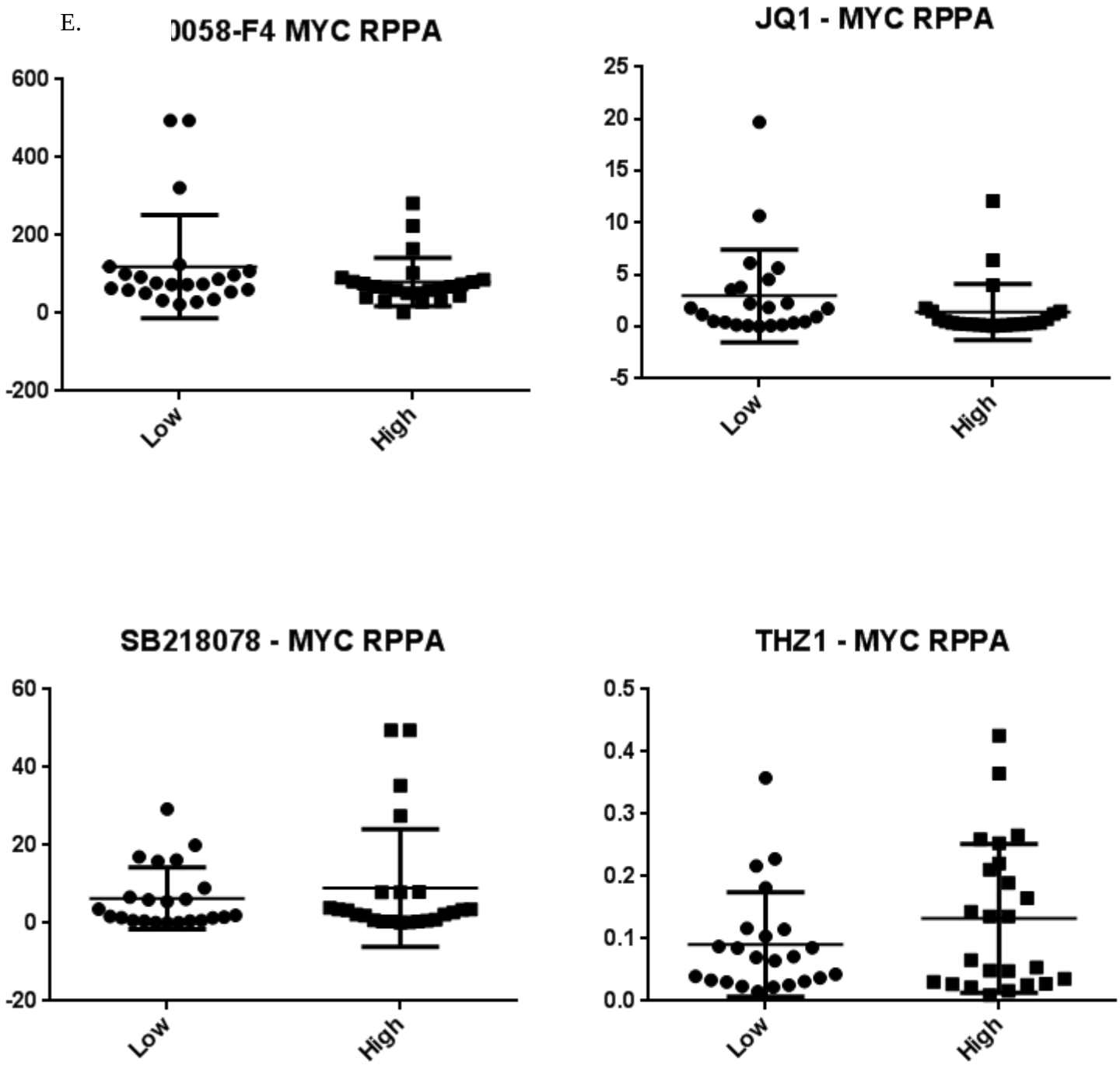


Figure 5.1: The impact of oncogenotype on drug response. (A) Cell lines bifurcated by KRAS status respond similarly to all drugs tested. (B) Cell lines bifurcated by p53 status respond similarly to 3/4 drugs tested, with JQ1 being the exception. (C) – (E) Cell lines bifurcated by MYC status respond similarly to all drugs. Y-Axis in all graphs is the AC50 for the corresponding drug.

5.2.2 Correlating MYC Dependence with Cell Morphology and a Well-studied SNP

Using the EMT signature developed by Byers et al, the panel of NSCLC cell lines was divided into epithelial and mesenchymal cell lines (Table 5.1) (Byers, Diao et al. 2013). In all four cases, the epithelial and mesenchymal subsets of cell lines exhibited similar drug responses, suggesting that the mesenchymal status of the NSCLC cell lines does not confer resistance to MYC inhibition (Figure 5.2).

Next, the impact of a polymorphism in an enhancer region upstream of *MYC* was assessed. First, the NSCLC cell line panel was genotyped at this polymorphism. Using a combination of sequencing and allelic discrimination assays, the NSCLC cell line panel was characterized for the genotype at this SNP (Table 5.2). Next, the cell lines were divided based on the genotype into a “risk” group (GG), or a “non-risk” group (TT/GT). The groups were compared for each of the four drugs that were screened, and in each case there was no statistically significant difference between groups (Figure 5.3). Thus, the genotype at a well-characterized polymorphism in a region that has been confirmed to interact with the *MYC* promoter does not affect response to MYC inhibition in NSCLC.

Table 5.1: Morphological and SNP Classification of Cell Lines

Cell Line	EMT (Byers)	SNP
H1819	E	GT
H1650	E	TT
H324	E	No Data
HCC4006	E	GG
H322	E	TT
HCC193	E	GG
H1693	E	GT
H2347	E	GT
H2009	E	GG
H2122	E	GG
H441	E	GT
H3255	E	GG
HCC2935	E	TT
H2086	E	No Data
H1395	E	TT
HCC515	E	GT
HCC4011	E	GT
H2126	E	GG
HCC4019	E	No Data
H2085	E	GG
DFCI032	E	No Data
HCC4018	E	No Data
H1993	E	GT
H820	E	No Data
HCC78	E	GT
H2087	E	GG
HCC2279	E	GG
Calu-3	E	GT
H1648	E	GG
H969	E	No Data
H1975	E	GG
H1437	E	GT
HCC95	E	GG
H647	E	No Data
HCC827	E	TT
HCC1171	E	GG
H3122	E	GT

HCC4017	E	GT
HCC1195	E	GG
H292	E	No Data
HCC364	M	No Data
H1734	M	GT
HCC461	M	GT
HCC44	M	GG
H1792	M	TT
EKVX	M	GG
H2073	M	GT
H1838	M	No Data
HCC2108	M	No Data
DFCI024	M	No Data
HCC15	M	GG
H23	M	TT
H920	M	No Data
H661	M	GT
H2882	M	GG
H1155	M	GT
HCC366	M	GG
H2887	M	GT
H226	M	GT
H1299	M	GG
H460	M	GG
H1869	No Data	GG
H2258	No Data	No Data
HCC2814	No Data	No Data
HCC3051	No Data	No Data
H596	No Data	No Data
H2052	No Data	GG
A427	No Data	No Data
H1651	No Data	TT
HOP-62	No Data	No Data
H1793	No Data	GT
H520	No Data	GT
H2342	No Data	No Data
H2250	No Data	No Data
Cal-12T	No Data	No Data
H2110	No Data	No Data
H1915	No Data	No Data
HCC122	No Data	No Data

H2452	No Data	No Data
HBEC34-KT	No Data	No Data
HBEC13-KT	No Data	No Data
HBEC30-KT	No Data	No Data
HBEC3-KT	No Data	No Data

A.

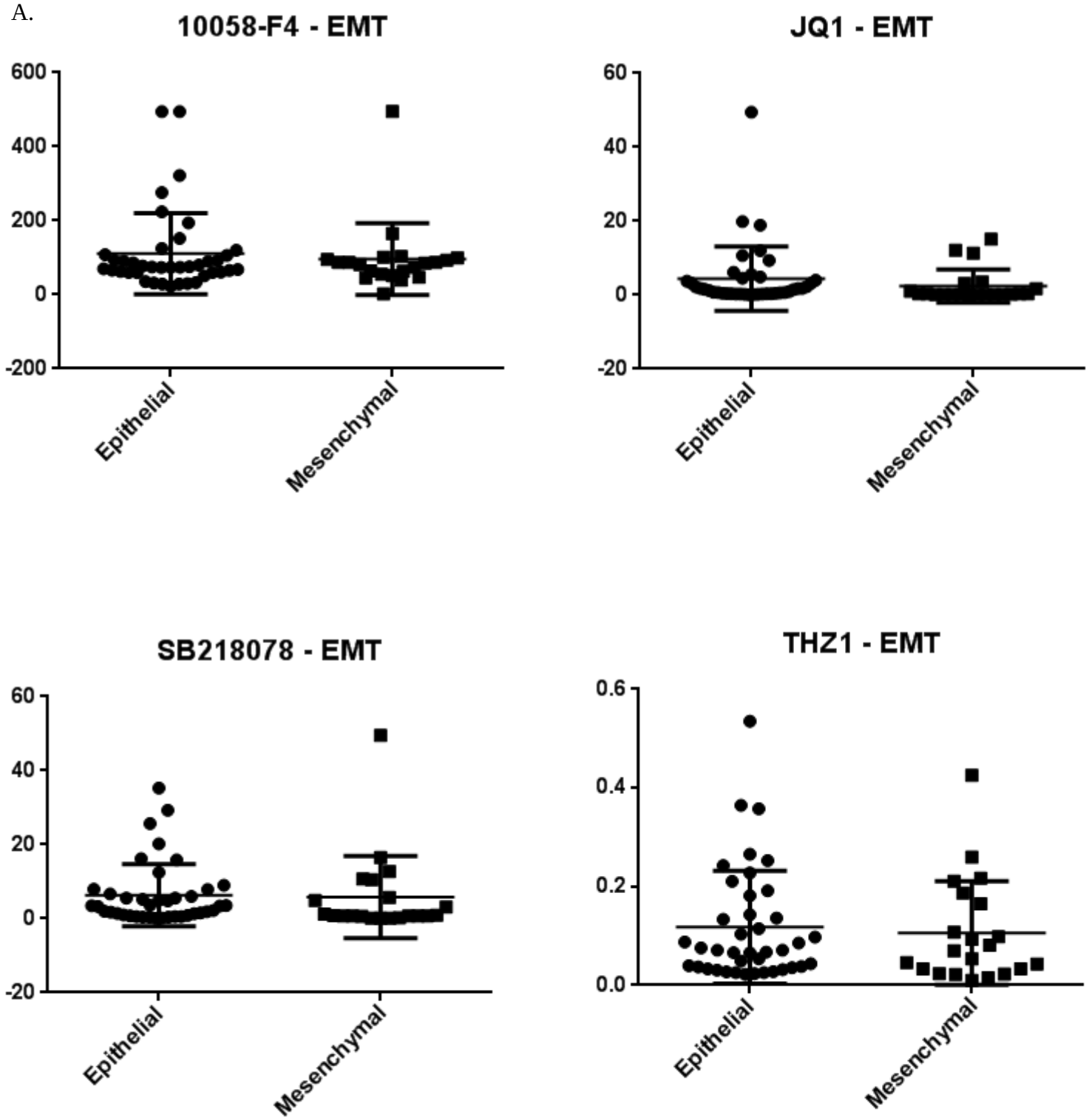


Figure 5.2: The impact of morphology on drug response. Cell lines bifurcated by epithelial vs. mesenchymal status respond similarly to all drugs tested. The Y-axis is the AC50 of the corresponding drug.

A.

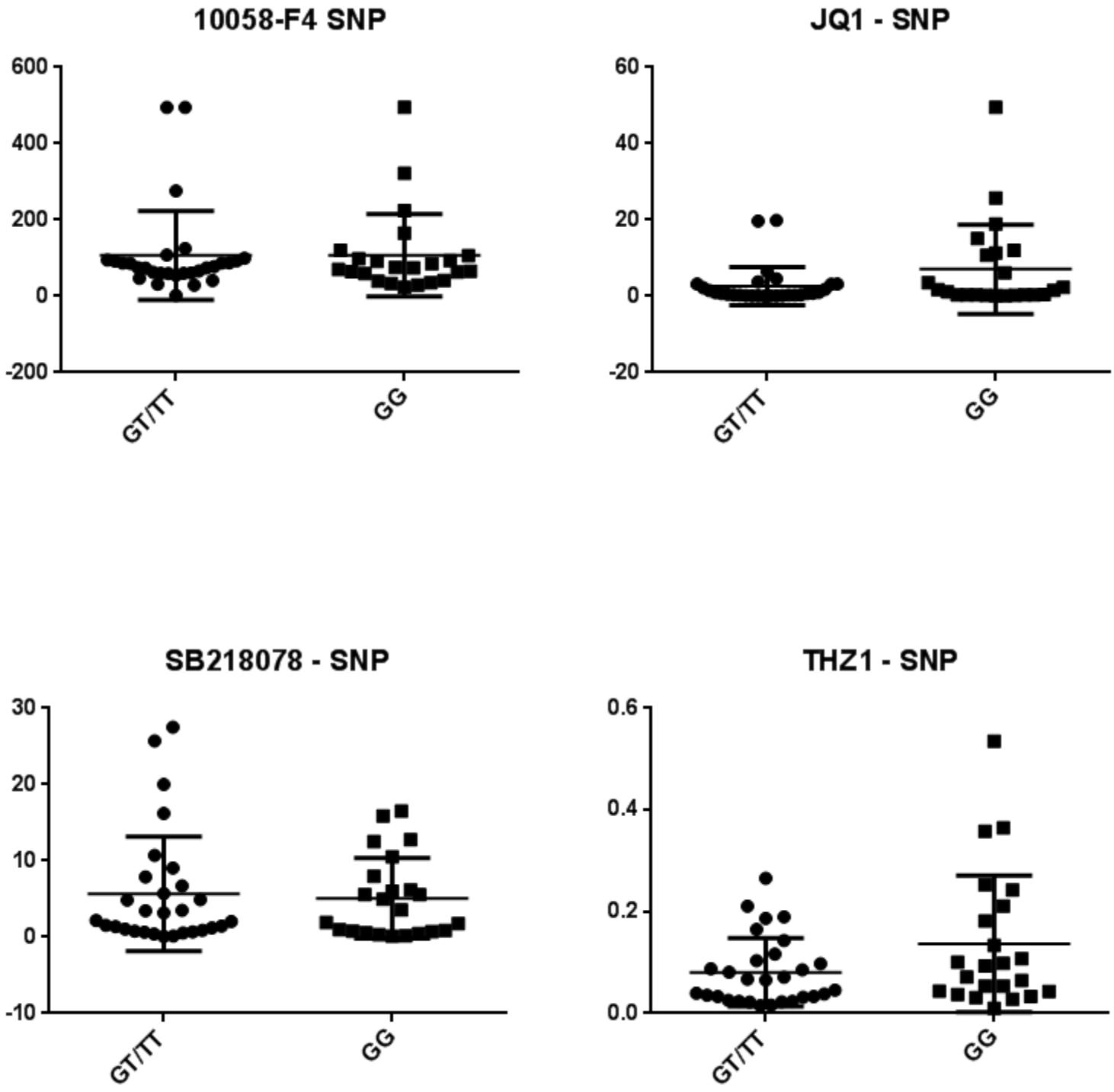


Figure 5.3: The impact of SNP genotype on drug response. Cell lines bifurcated by "Risk (GG)" vs. "Non-Risk (GT/TT)" allele status respond similarly to all drugs tested. The Y-axis is the AC50 of the corresponding drug.

5.2.3 Characterizing the Response of the Wnt Pathway upon MYC Inhibition

As previously discussed, activation of the Wnt pathway can confer resistance to MYC inhibition in a MYC-induced lymphoma model. However, it remains unclear whether the Wnt pathway can provide a more universal escape to MYC dependence, or if this is a phenomenon that is specific to lymphomas. To determine if, like in lymphoma models, the Wnt pathway is implicated in resistance, immunoblotting was performed on phosphorylated β-catenin after Omomyc-mediated MYC inhibition. After Omomyc induction, the level of phosphorylated (inactive) β-catenin increased in H1993, suggesting Wnt pathway activity was repressed by Omomyc in the most sensitive cell line (Fig. 5.4). However, in the cell lines that were more resistant to Omomyc than H1993 (H1693, H1299, HCC44), a significant reduction in phosphorylated β-catenin was observed upon induction of Omomyc, suggesting an activation of the Wnt pathway (Fig. 5.4). To investigate whether Wnt-pathway activation played a functional role in resistance to MYC inhibition, we used short-hairpin mediated knockdown of β-catenin in conjunction with Omomyc-mediated MYC inhibition in a subset of cell lines. In all three cell lines tested, knockdown of β-catenin was confirmed and resulted in further sensitization to MYC inhibition, even in the most resistant cell line tested (Fig. 5.5). To determine the therapeutic relevance of this phenotype, we combined the previously tested MYC inhibitor (10058-F4) with a PORCN inhibitor (Wnt-C59) which has been shown to inhibit Wnt pathway activity. Whereas treatment with 10058-F4 mimicked the effects of Omomyc and Wnt-C59 showed no obvious effect, the combination of 10058-F4 and Wnt-C59 elicited a dramatic reduction in colony forming efficiency in the cell lines tested (Fig. 5.6). Together, these data indicate that the Wnt pathway is activated upon MYC inhibition, and that this activation is functionally important.

A.

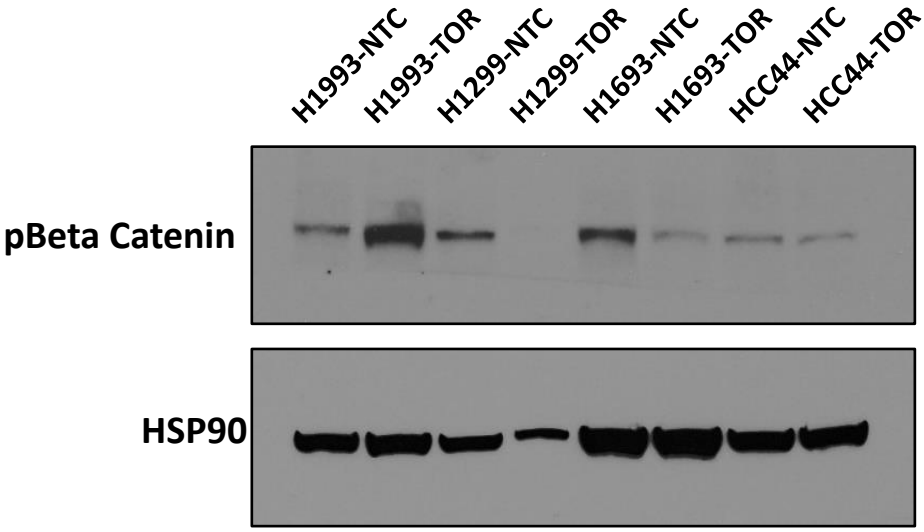
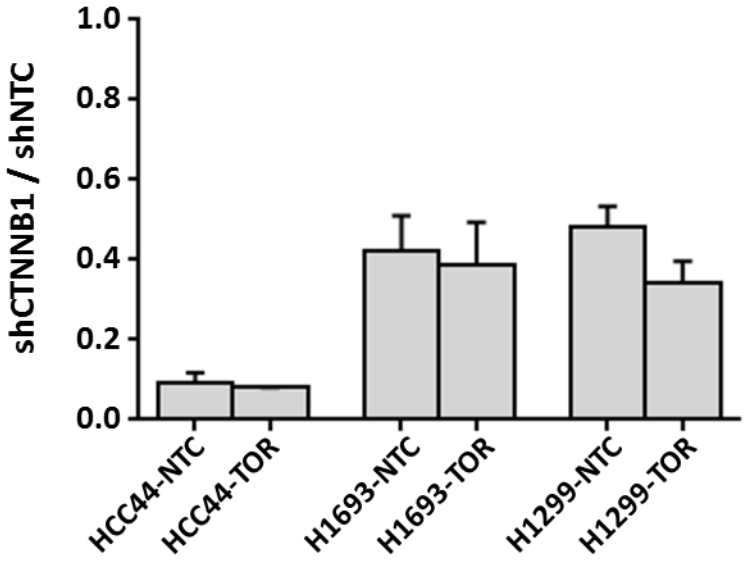


Figure 5.4: The effect of MYC inhibition on the Wnt pathway. (A) Inactive phospho-β-catenin exhibits a variable response upon Omomyc induction in a subset of NSCLC cell lines. NTC = Non targeting control, TOR – TRIPZ-Omomyc-RFP. All cell lines were induced with doxycycline for 72 hours.

A.



B.

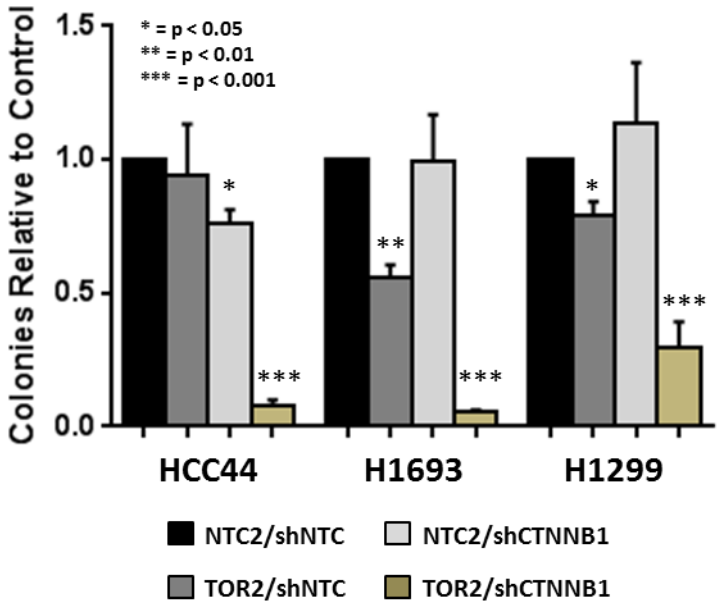
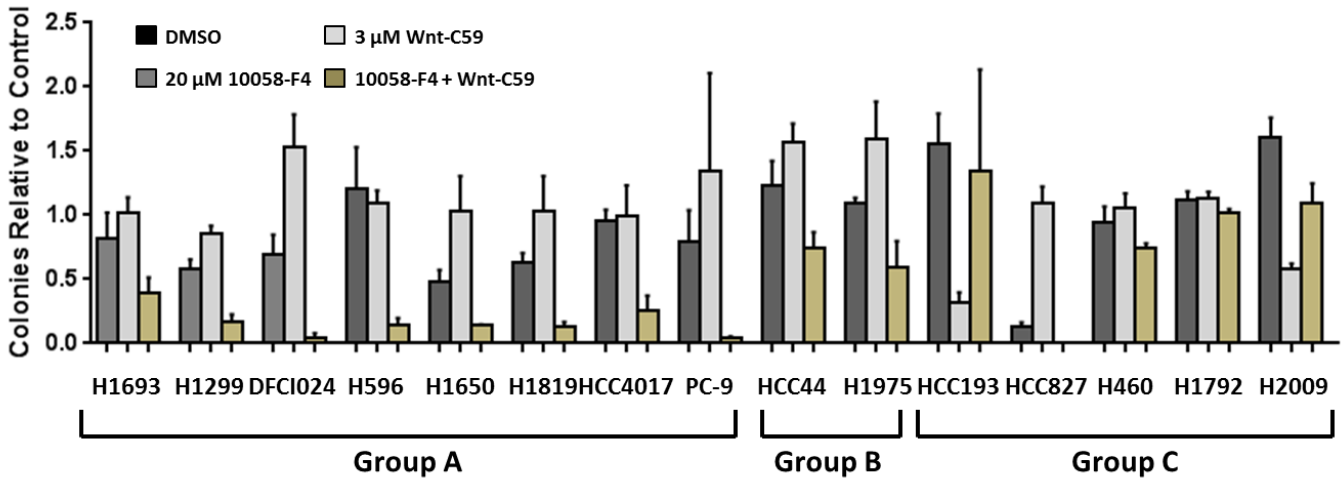


Figure 5.5: Combining Omomyc with Wnt pathway inhibition. (A) Confirmation of knockdown efficiency for the short-hairpin against β-catenin. (B) Colony forming efficiency for Omomyc induction, β-catenin knockdown, or both. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. NTC = Non targeting control. TOR = TRIPZ-Omomyc-RFP.

A.



B.

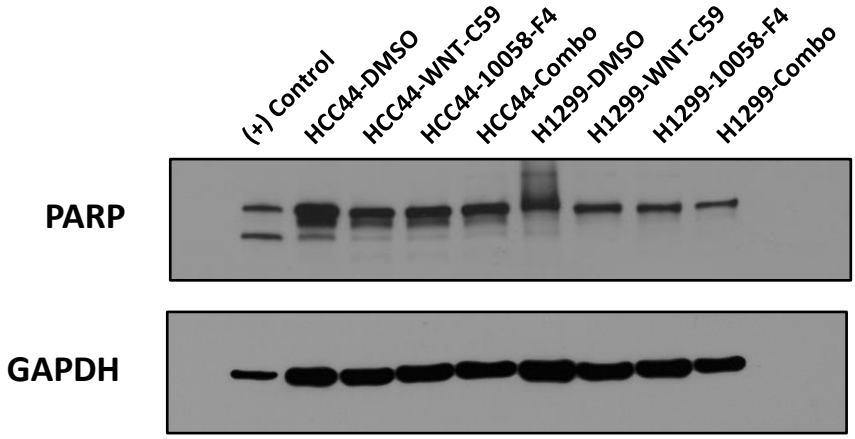


Figure 5.6: Rationale for combination therapy in NSCLC. (A) CFE assay after treatment with either the MYC inhibitor 10058-F4, the Wnt inhibitor Wnt-C59, or both. (B) Immunoblot for PARP after the same treatment regimen, showing no induction of cleaved PARP. Group A = Cell lines with statistically significant sensitization. Group B = Cell lines with statistically significant but biologically modest sensitization. Group C = Cell lines with no sensitization.

5.3 Discussion

Here, an analysis of the oncogenotype and drug response pattern of a large panel of NSCLC cell lines showed no statistically significant correlation, and yielded no clinically useful biomarker for response. Specifically, the response of the cell lines to the direct inhibitor 10058-F4 did not differ depending on MYC copy number, mRNA levels, protein levels or the mutational status of either *KRAS* or *p53*. Similarly, the response of the cell lines to the indirect MYC inhibitors SB218078 and THZ1 did not depend on any of these molecular characteristics. Interestingly, when bifurcated by *p53* status, wild-type cell lines treated with JQ1 exhibit a 3-fold higher AC50 than their mutant counterparts, though this may be largely driven by several outliers. Bifurcation by *KRAS* status, *MYC* copy number or endogenous MYC mRNA or protein levels yields no statistically significant differences in response to JQ1.

Next, in addition to the mutation status of several key genes and the endogenous levels of MYC, the response to MYC inhibition was correlated with both morphology (epithelial vs. mesenchymal) as well as the genotype at a well-characterized polymorphism in an enhancer region upstream of the *MYC* locus. Neither morphology nor SNP genotype showed a statistically significant correlation with the response to any of the four drugs screened. While the relationship between the SNP and MYC levels remains controversial, the result showing no correlation between MYC dependence and morphology stands in contrast to reports in the literature that state that cells which are able to grow out independently of MYC exhibit a primarily mesenchymal phenotype.

Finally, an analysis into potentially compensatory pathways was conducted. As previously discussed, activation of the Wnt pathway has been shown to confer resistance to MYC inhibition in lymphomas. While the mutation pattern of Wnt pathway components did not correlate with response to MYC inhibition in the NSCLC cell line panel, activation of the Wnt pathway upon MYC inhibition did prove functionally important. Dual inhibition of MYC and the Wnt pathway resulted in a more potent anti-tumor response, which suggests a combinatorial approach may be an effective therapeutic regimen in some lung cancers. Such a combinatorial approach might prevent the outgrowth of resistant tumor cells that might otherwise result from the activation of the compensatory Wnt pathway.

Acquired resistance represents a significant clinical problem, as response to targeted therapies often begins to wane after the first year of treatment. This resistance may arise as the result of either a secondary mutation or the upregulation of a compensatory pathway. In the case of EGFR-mutant lung tumors that stop responding to erlotinib, for example, a T790M mutation, activation of downstream signaling as is seen in the PI3K pathway or the amplification of the MET oncogene can all lead to resistance to tyrosine kinase inhibitors (Engelman and Janne 2008). While the acquisition of a new mutation (e.g., in the T790 gatekeeper residue) may prove difficult to target, the upregulation of a compensatory pathway potentially provides a complementary therapeutic approach. In the case of MET amplification conferring resistance to EGFR inhibitors, inhibiting MET restores sensitivity to the EGFR inhibitors, thus providing rationale for simultaneously targeting multiple oncogenic pathways to achieve a lasting response (Engelman, Zejnullahu et al. 2007). In this case, targeting a compensatory mechanism after the development of drug resistance may confer a clinical benefit. An alternative approach is to

target multiple pathways at the outset of treatment in order to prevent the development of resistance, but the success of this approach is predicated on an understanding of how resistance to a given targeted therapy will develop. Thus, precedent exists for targeting multiple oncogenic pathways either simultaneously or sequentially in order to achieve a more sustained clinical benefit. Such combinatorial approaches may be required in order to maximize the response to clinical regimens based on the use of targeted therapies.

The emergence of targeted therapies represents a major advance in the armamentarium of clinicians. When used in the appropriate patient population, the anti-tumor effects of such therapies can be profound, as is seen in the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (e.g., erlotinib, gefitinib) to treat non-small cell lung cancer patients with tumors bearing a somatic mutation in EGFR. While the response to MYC inhibition will depend on the development of a sufficiently potent inhibitor, the potential clinical benefit of a MYC-targeted therapy is clear from the data presented in Chapter 4. Like with EGFR inhibitors, understanding potential mechanisms for resistance may allow for the development of a more effective therapeutic regimen. As discussed, Choi et al implicate Wnt pathway activation as a potential mechanism for resistance to MYC inhibition (Choi, Li et al. 2014). MYC-driven lymphoma models have been used to show that tumors initially regress upon withdrawal of MYC, but much like the clinical response to single-agent targeted therapy, the tumors will usually recur. In addition to their dependence on MYC, lymphomas often acquire activating mutations in CTNNB1, and these tumors were shown to be dependent on β-catenin for their survival (Choi, Li et al. 2014). Similar to MYC, withdrawal of β-catenin would elicit tumor regression initially, but recurrence was highly likely. However, simultaneous inhibition of both

MYC and β-catenin resulted in more rapid tumor regression than targeting either alone, and the combination successfully prevented the recurrence of resistant lymphomas (Choi, Li et al. 2014). Thus, rationally targeting multiple oncogenic pathways may provide more effective tumor control, limit the outgrowth of resistant tumors, and ultimately lead to improved patient survival. This finding is supported by the data in our study, which suggests Wnt-pathway inhibition can work to sensitize NSCLC tumors to MYC inhibition. The data presented provide important insights into a potential mechanism of resistance to MYC inhibition. This finding, once sufficiently proven in *in vivo* models, should guide clinical practice.

CHAPTER SIX

CONCLUSIONS AND ONGOING STUDIES

6.1 Conclusion

MYC is one of the most commonly deregulated oncogenes in human cancer. Deregulation at the DNA, mRNA and protein level has been observed, and this deregulation has been shown to be critically important for the initiation and maintenance of the tumorigenic phenotype in many settings. MYC has thus garnered significant attention as a potential clinically actionable therapeutic target. In fact, mounting evidence suggests that targeting MYC in the clinic may be an effective therapeutic approach for many tumor types. However, therapeutically targeting MYC has historically been a significant challenge. Further, the context in which MYC is required is not yet clear. While previous studies have demonstrated dependence on MYC for the tumorigenic phenotype, these studies have not been on a large enough scale to determine whether this dependence is a universal characteristic. In order to advance MYC targeted therapies into the lung cancer clinic, a thorough understanding of whether MYC is a universal requirement in this tumor type will be required.

The objective of this study was to characterize the degree of MYC deregulation in human non-small cell lung cancer, and to determine whether (and in what context) MYC is required for the maintenance of the tumorigenic phenotype. To this end, the most salient findings include:

1. Non-small cell lung cancer patient samples and a large cell line panel display MYC deregulation at the DNA, mRNA and protein levels. Despite dramatic variation in *MYC*

copy number, mRNA or protein expression, these values do not serve any prognostic value for lung cancer patients (Chapter 3).

2. Each of the four purported MYC inhibitors elicits an anti-growth response in a subset of NSCLC cell lines, but these drugs do not target the same subset of cell lines. However, targeting MYC with the direct inhibitor 10058-F4 produces a result consistent with targeted genetic inhibition, which led to the identification of a truly "MYC-dependent" subset of lung cancers (Chapter 4).

3. The mutation status of *KRAS*, *p53* or the mutation status or copy number of *MYC* does not predict sensitivity to MYC inhibition, nor do endogenous levels of MYC mRNA or protein. However, Wnt pathway activation upon inhibition of MYC is functionally important, as dual inhibition of MYC and the Wnt pathway results in a more potent anti-tumor response in *in vitro* assays (Chapter 5).

In conclusion, our findings suggest MYC inhibition represents a viable treatment strategy in a subset of non-small cell lung cancers, though more potent small molecule inhibitors will likely be required. Additionally, like other targeted therapy approaches, inhibition of MYC may require a complementary therapy in order to prevent the outgrowth of resistant cancer cells. Our data using inhibitors of both MYC and the Wnt pathway, as well as genetically mediated inhibition of each pathway, suggest simultaneous inhibition of MYC and the Wnt pathway may be an optimal therapeutic approach in some lung cancers.

6.2 Ongoing and Future Studies

This study yielded important insights into the differential dependence on MYC in non-small cell lung cancer, as well as the potential for the Wnt pathway to confer resistance to targeted MYC inhibition. In order to develop a more comprehensive understanding of how to successfully target MYC in the clinic and prevent resistance, two paths of inquiry should be further explored. First, a more potent inhibitor of MYC function must be developed. Here, a small molecule was used for *in vitro* assays, though this molecule is unsuitable for *in vivo* studies. To fully understand the potential of clinically targeting MYC in human cancer, extensive *in vivo* experiments with a clinically relevant MYC inhibitor will be required. As previously discussed, targeting MYC directly has been a significant challenge in the field. However, given the novel developments regarding the lower-than-expected side effects of MYC inhibition in normal tissue as well as advances in medicinal chemistry make the development of a direct MYC inhibitor a near-term possibility.

Second, the mechanistic link between MYC inhibition and Wnt pathway activity must be further elucidated. Here, Omomyc-mediated MYC inhibition resulted in a variable response of the Wnt pathway. In some cell lines, the pathway was repressed, while in others the pathways was activated. As demonstrated by the functional experiments in Chapter 6, this activation proved to be functionally significant in approximately 50% of cases. However, the mutational profile provides no biomarker for which tumors would benefit from a dual inhibition of MYC and Wnt, and which tumors would not. The effect MYC has on the Wnt pathway may be tissue specific. For instance, while MYC has been shown to inhibit the Wnt pathway in colon cancer, a

recent report demonstrated that MYC activates the Wnt pathway as well as endogenous TCF activity by suppressing the Wnt inhibitors DKK1 and SFRP1 (Cowling and Cole 2007). Thus, a more thorough understanding of the mechanism by which MYC inhibition alters the Wnt pathway is required, as this understanding would provide much-needed insight into this compensatory pathway. To this end, the following experiments are proposed:

1. Increase the number of cells for which Wnt pathway alterations are assessed following Omomyc induction. Expanding the list of cell lines studied will help flesh out any potential biomarkers for Wnt pathway response to MYC inhibition, which may in turn provide insights into the mechanism.

2. Functionally investigate the genes which are differentially expressed between the cell lines sensitized by Wnt inhibition and those which are not sensitized (Figure 6.1). This analysis will determine which, if any of the differentially regulated genes are functionally involved in the response of the Wnt pathway to MYC inhibition.

3. Acute overexpression of MYC in either NSCLC cell lines or immortalized bronchial epithelial cells, followed by gene expression profiling in order to determine what, if any, effect on Wnt pathway components is observed.

These experiments will provide a more thorough understanding of the relationship between MYC and Wnt in human NSCLC. Understanding this relationship is a necessary first step to effectively targeting these pathways in the clinic.

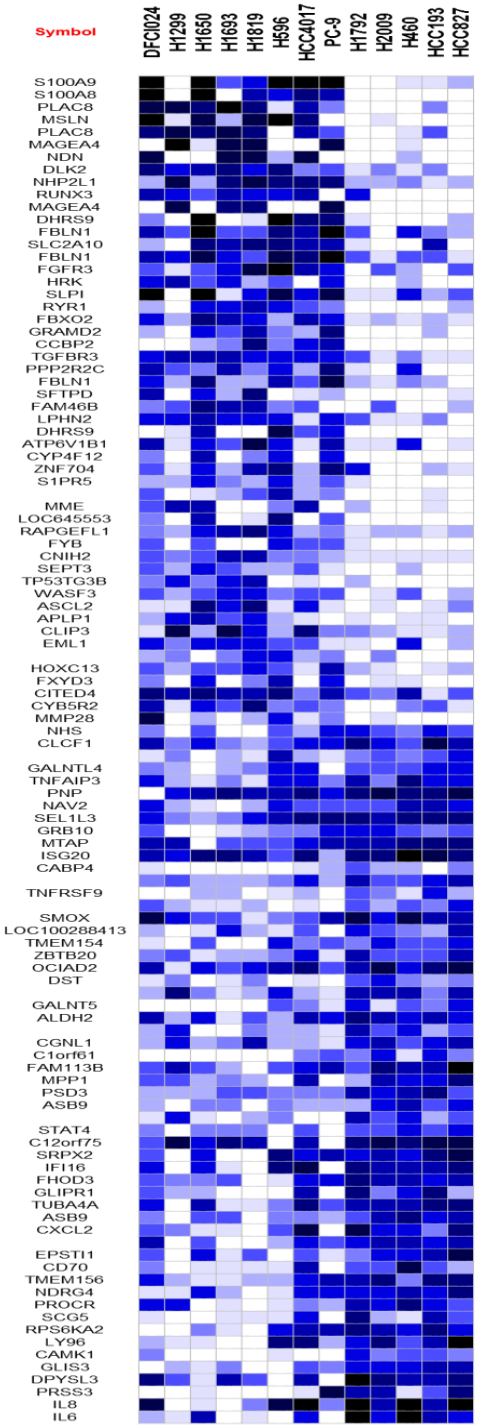


Figure 6.1: Differential gene expression for Wnt sensitized cell lines. Here, the differential expression pattern was determined by comparing Group A to Group C (as classified in Figure 5.6).

APPENDIX A: TCGA TUMOR DESCRIPTION

TUMOR	HISTOLOGY	MYC - RPPA	MYC - RNA SEQ	P53	KRAS	MYC
TCGA-33-6737	Squamous	3.5343	8.477	[c.332T>A; p.L111Q; MS], [c.850A>C; p.T284P; MS]	No Mutation	No Mutation
TCGA-44-7667	Adenocarcinoma	2.370874	7.679	No Mutation	No Mutation	No Mutation
TCGA-63-7023	Squamous	1.640017	9.819	No Data	No Data	No Data
TCGA-49-4514	Adenocarcinoma	1.449355	8.91	[c.524G>A; p.R175H; MS]	No Mutation	No Mutation
TCGA-69-7765	Adenocarcinoma	1.133794	10.513	[c.499C>T; p.Q167*; NS]	No Mutation	No Mutation
TCGA-75-6207	Adenocarcinoma	0.941555	11.757	[c.574C>T; p.Q192*; NS]	No Mutation	No Mutation
TCGA-66-2794	Squamous	0.917886	9.288	[c.833C>G; p.P278R; MS]	No Mutation	No Mutation
TCGA-78-7542	Adenocarcinoma	0.900785	10.399	[c.455delC; p.P153fs; Del-FS]	No Mutation	No Mutation
TCGA-86-7701	Adenocarcinoma	0.889617	10.563	[c.880G>T; p.E294*; NS]	No Mutation	No Mutation
TCGA-44-7672	Adenocarcinoma	0.877808	9.929	No Mutation	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-55-7728	Adenocarcinoma	0.852988	9.051	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-43-6143	Squamous	0.829953	9.421	[c.641A>G; p.H214R; MS]	No Mutation	No Mutation
TCGA-78-7540	Adenocarcinoma	0.823758	8.26	[c.817C>T; p.R273C; MS]	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-37-4133	Squamous	0.809256	8.903	[c.652_654 delGTG;	No Mutation	No Mutation

				p.V218del; Del-IF]		
TCGA-50-6590	Adenocarcinoma	0.737937	12.341	No Mutation	No Mutation	No Mutation
TCGA-91-6831	Adenocarcinoma	0.725557	8.095	[c.1001G>T; p.G334V; MS]	No Mutation	No Mutation
TCGA-50-5931	Adenocarcinoma	0.721285	11.943	[c.273G>A; p.W91*; NS]	No Mutation	No Mutation
TCGA-91-6848	Adenocarcinoma	0.720603	10.487	[c.467G>C; p.R156P; MS]	No Mutation	No Mutation
TCGA-53-7813	Adenocarcinoma	0.71718	9.263	No Mutation	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-60-2698	Squamous	0.710171	8.493	[c.637C>T; p.R213*; NS]	No Mutation	No Mutation
TCGA-77-7139	Squamous	0.70878	9.83	No Data	No Data	No Data
TCGA-05-4422	Adenocarcinoma	0.70496	12.529	No Mutation	No Mutation	No Mutation
TCGA-64-1678	Adenocarcinoma	0.640807	10.295	[c.428_467 delTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCG; p.VQLWVDSTPPPGTR143fs; Del-FS], [c.672_splice; p.E224_splice; SNP-SS]	No Mutation	No Mutation
TCGA-55-6969	Adenocarcinoma	0.638818	10.93	[c.818G>T; p.R273L; MS]	No Mutation	No Mutation
TCGA-44-6777	Adenocarcinoma	0.633202	10.02	[c.734G>T; p.G245V; MS]	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-75-7031	Adenocarcinoma	0.630425	12.128	[c.844_849 delCGGCGC; p.RR282del; Del-IF]	No Mutation	No Mutation

TCGA-37-4135	Squamous	0.628633	7.237	[c.202G>T; p.E68*; NS]	No Mutation	No Mutation
TCGA-90-7769	Squamous	0.628438	9.697	No Data	No Data	No Data
TCGA-63-7021	Squamous	0.615865	8.044	No Data	No Data	No Data
TCGA-34-5239	Squamous	0.612864	8.055	[c.375_splice; p.T125_splice; SNP-SS]	No Mutation	No Mutation
TCGA-94-7943	Squamous	0.609915	10.002	No Data	No Data	No Data
TCGA-50-6593	Adenocarcinoma	0.607265	10.185	No Mutation	No Mutation	No Mutation
TCGA-50-5933	Adenocarcinoma	0.605467	12.182	[c.473G>T; p.R158L; MS]	No Mutation	No Mutation
TCGA-75-6205	Adenocarcinoma	0.604485	11.922	No Mutation	No Mutation	No Mutation
TCGA-78-7158	Adenocarcinoma	0.582381	13.266	No Mutation	No Mutation	No Mutation
TCGA-44-7669	Adenocarcinoma	0.570423	11.374	[c.375_splice; p.T125_splice; SNP-SS]	No Mutation	No Mutation
TCGA-44-6146	Adenocarcinoma	0.56509	9.761	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-97-7552	Adenocarcinoma	0.561965	10.312	No Mutation	No Mutation	No Mutation
TCGA-70-6723	Squamous	0.532481	8.629	[c.559_splice; p.G187_splice; SNP-SS]	No Mutation	No Mutation
TCGA-91-6829	Adenocarcinoma	0.528137	9.082	[c.216delC; p.P72fs; Del-FS]	No Mutation	No Mutation
TCGA-50-5939	Adenocarcinoma	0.510148	12.392	[c.853G>A; p.E285K; MS]	No Mutation	No Mutation
TCGA-60-2712	Squamous	0.507956	8.538	No Mutation	No Mutation	No Mutation
TCGA-85-7698	Squamous	0.501271	8.72	No Data	No Data	No Data
TCGA-39-5028	Squamous	0.474062	9.824	[c.833C>T; p.P278L; MS]	No Mutation	No Mutation

TCGA-50-6591	Adenocarcinoma	0.471637	10.664	[c.161delT; p.F54fs; Del-FS]	No Mutation	No Mutation
TCGA-44-6775	Adenocarcinoma	0.471067	9.531	[c.503A>T; p.H168L; MS]	No Mutation	No Mutation
TCGA-43-5670	Squamous	0.465969	8.457	No Data	No Data	No Data
TCGA-38-6178	Adenocarcinoma	0.465764	9.533	[c.848G>C; p.R283P; MS]	No Mutation	No Mutation
TCGA-55-6970	Adenocarcinoma	0.464476	10.462	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-22-5491	Squamous	0.459696	9.577	[c.452C>G; p.P151R; MS]	No Mutation	No Mutation
TCGA-60-2709	Squamous	0.454823	7.6	No Mutation	No Mutation	No Mutation
TCGA-78-7166	Adenocarcinoma	0.449046	9.273	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-64-1677	Adenocarcinoma	0.448547	11.121	[c.142_151 delGACGATATTG; p.DDIE48fs; Del-FS], [c.475G>C; p.A159P; MS]	No Mutation	No Mutation
TCGA-66-2795	Squamous	0.446893	8.921	[c.473G>T; p.R158L; MS]	No Mutation	No Mutation
TCGA-34-5927	Squamous	0.444974	8.486	[c.548C>G; p.S183*; NS]	No Mutation	No Mutation
TCGA-77-7141	Squamous	0.443597	9.091	No Data	No Data	No Data
TCGA-44-5643	Adenocarcinoma	0.438478	12.807	[c.107delC; p.P36fs; Del-FS]	No Mutation	No Mutation
TCGA-33-4582	Squamous	0.432819	8.119	[c.832C>T; p.P278S; MS]	No Mutation	No Mutation
TCGA-43-7656	Squamous	0.42649	8.26	No Data	No Data	No Data
TCGA-34-	Squamous	0.419509	#N/A	No Data	No Data	No Data

2605						
TCGA-21-5787	Squamous	0.40442	8.956	[c.527G>A; p.C176Y; MS]	No Mutation	No Mutation
TCGA-44-6776	Adenocarcinoma	0.400406	8.468	No Mutation	[c.35G>A; p.G12D; MS]	No Mutation
TCGA-91-6847	Adenocarcinoma	0.399697	10.323	[c.329G>T; p.R110L; MS]	No Mutation	No Mutation
TCGA-75-6206	Adenocarcinoma	0.386796	9.947	No Mutation	[c.38G>A; p.G13D; MS]	No Mutation
TCGA-33-4566	Squamous	0.378655	8.977	[c.490A>G; p.K164E; MS]	No Mutation	No Mutation
TCGA-67-3771	Adenocarcinoma	0.376573	11.455	[c.380C>G; p.S127C; MS]	No Mutation	No Mutation
TCGA-78-7153	Adenocarcinoma	0.372042	12.39	No Mutation	No Mutation	No Mutation
TCGA-34-2596	Squamous	0.367693	9.015	[c.913A>T; p.K305*; NS]	No Mutation	No Mutation
TCGA-95-7567	Adenocarcinoma	0.367128	10.921	[c.613T>C; p.Y205H; MS]	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-75-7030	Adenocarcinoma	0.364233	10.619	No Mutation	[c.35G>A; p.G12D; MS]	No Mutation
TCGA-78-7145	Adenocarcinoma	0.361045	11.35	[c.475G>C; p.A159P; MS]	[c.34G>T; p.G12C; MS], [c.35G>A; p.G12D; MS]	No Mutation
TCGA-37-5819	Squamous	0.356232	7.304	[c.725G>T; p.C242F; MS]	No Mutation	No Mutation
TCGA-67-6217	Adenocarcinoma	0.354532	9.463	No Mutation	No Mutation	No Mutation
TCGA-39-5040	Squamous	0.352245	9.012	No Data	No Data	No Data
TCGA-67-6216	Adenocarcinoma	0.351062	10.536	No Mutation	No Mutation	No Mutation
TCGA-55-7911	Adenocarcinoma	0.347122	11.629	[c.432G>T; p.Q144H;	[c.35G>T; p.G12V;	No Mutation

				MS]	MS], [c.262A>T; p.K88*; NS]	
TCGA-44-6778	Adenocarcinoma	0.346613	11.567	[c.694A>T; p.I232F; MS]	No Mutation	No Mutation
TCGA-55-6982	Adenocarcinoma	0.338819	10.103	No Mutation	No Mutation	No Mutation
TCGA-44-3396	Adenocarcinoma	0.336867	9.779	[c.857A>G; p.E286G; MS]	No Mutation	No Mutation
TCGA-86-7955	Adenocarcinoma	0.327523	11.412	No Mutation	No Mutation	No Mutation
TCGA-55-5899	Adenocarcinoma	0.31677	12.066	[c.456delG; p.P153fs; Del-FS]	No Mutation	No Mutation
TCGA-77-6843	Squamous	0.311986	9.129	No Data	No Data	No Data
TCGA-49-6745	Adenocarcinoma	0.311525	11.803	No Mutation	No Mutation	No Mutation
TCGA-66-2758	Squamous	0.309396	8.177	[c.373A>C; p.T125P; MS]	No Mutation	No Mutation
TCGA-44-6147	Adenocarcinoma	0.307869	10.221	[c.329G>T; p.R110L; MS]	No Mutation	No Mutation
TCGA-66-2770	Squamous	0.30257	7.816	[c.800G>C; p.R267P; MS]	No Mutation	No Mutation
TCGA-50-6595	Adenocarcinoma	0.299182	11.022	[c.524G>A; p.R175H; MS], [c.548C>G; p.S183*; NS]	No Mutation	No Mutation
TCGA-64-5778	Adenocarcinoma	0.298245	9.475	[c.716A>G; p.N239S; MS]	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-43-2578	Squamous	0.295343	7.403	No Mutation	No Mutation	No Mutation
TCGA-22-1000	Squamous	0.287587	7.355	No Data	No Data	No Data
TCGA-56-5897	Squamous	0.283628	8.245	[c.637C>T; p.R213*; NS]	No Mutation	No Mutation
TCGA-22-5483	Squamous	0.277118	7.81	No Data	No Data	No Data

TCGA-91-6849	Adenocarcinoma	0.271489	11.346	No Mutation	No Mutation	No Mutation
TCGA-44-5645	Adenocarcinoma	0.265208	8.762	[c.1060C>T; p.Q354*; NS]	No Mutation	No Mutation
TCGA-39-5024	Squamous	0.254796	6.974	[c.920_splice; p.A307_splice; SNP-SS]	No Mutation	No Mutation
TCGA-55-7910	Adenocarcinoma	0.253406	8.674	[c.358A>G; p.K120E; MS]	[c.37G>T; p.G13C; MS]	No Mutation
TCGA-56-7730	Squamous	0.248181	8.179	No Data	No Data	No Data
TCGA-78-7148	Adenocarcinoma	0.243708	9.901	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-56-7221	Squamous	0.2401	8.968	No Data	No Data	No Data
TCGA-97-7546	Adenocarcinoma	0.238751	10.521	No Mutation	No Mutation	No Mutation
TCGA-77-7335	Squamous	0.238615	6.755	No Data	No Data	No Data
TCGA-34-2608	Squamous	0.238534	7.201	[c.473G>T; p.R158L; MS]	No Mutation	No Mutation
TCGA-77-7142	Squamous	0.237765	7.36	No Data	No Data	No Data
TCGA-50-5936	Adenocarcinoma	0.226971	10.703	No Mutation	No Mutation	No Mutation
TCGA-55-6543	Adenocarcinoma	0.226511	10.381	No Mutation	No Mutation	No Mutation
TCGA-22-5489	Squamous	0.2259	7.507	[c.916C>T; p.R306*; NS]	No Mutation	No Mutation
TCGA-60-2719	Squamous	0.222633	7.297	[c.577C>G; p.H193D; MS]	No Mutation	No Mutation
TCGA-39-5022	Squamous	0.221512	7.437	No Mutation	No Mutation	No Mutation
TCGA-85-7696	Squamous	0.219762	8.799	No Data	No Data	No Data
TCGA-34-5929	Squamous	0.217546	7.906	[c.844C>T; p.R282W; MS]	No Mutation	No Mutation
TCGA-73-4675	Adenocarcinoma	0.214849	9.216	No Mutation	No Mutation	No Mutation

TCGA-21-1070	Squamous	0.2111	8.132	No Mutation	No Mutation	No Mutation
TCGA-44-7670	Adenocarcinoma	0.206522	10.935	[c.1010G>T; p.R337L; MS]	No Mutation	No Mutation
TCGA-34-5232	Squamous	0.205472	8.303	[c.841G>T; p.D281Y; MS]	No Mutation	No Mutation
TCGA-49-6767	Adenocarcinoma	0.20321	10.781	[c.991C>T; p.Q331*; NS]	No Mutation	No Mutation
TCGA-78-7167	Adenocarcinoma	0.198738	10.486	No Mutation	[c.34G>T; p.G12C; MS], [c.35G>T; p.G12V; MS]	No Mutation
TCGA-39-5034	Squamous	0.193766	7.174	No Data	No Data	No Data
TCGA-95-7039	Adenocarcinoma	0.189535	10.528	[c.818G>T; p.R273L; MS]	[c.99T>A; p.D33E; MS]	No Mutation
TCGA-95-7948	Adenocarcinoma	0.178805	11.012	No Mutation	No Mutation	No Mutation
TCGA-78-7149	Adenocarcinoma	0.17738	10.944	[c.743G>T; p.R248L; MS]	No Mutation	No Mutation
TCGA-60-2704	Squamous	0.177097	8.085	No Data	No Data	No Data
TCGA-97-7554	Adenocarcinoma	0.171976	10.487	[c.745A>T; p.R249W; MS]	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-78-7152	Adenocarcinoma	0.171705	12.181	No Mutation	No Mutation	No Mutation
TCGA-49-4486	Adenocarcinoma	0.169765	10.158	No Mutation	No Mutation	No Mutation
TCGA-78-7159	Adenocarcinoma	0.16321	8.645	No Mutation	No Mutation	No Mutation
TCGA-85-7950	Squamous	0.162955	7.725	No Data	No Data	No Data
TCGA-44-7660	Adenocarcinoma	0.161844	11.359	[c.1006G>T; p.E336*; NS]	No Mutation	No Mutation
TCGA-56-5898	Squamous	0.154818	10.254	[c.488A>G; p.Y163C; MS]	No Mutation	No Mutation
TCGA-60-	Squamous	0.151264	7.621	No Data	No Data	No Data

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TCGA-50-6673	Adenocarcinoma	0.148818	10.568	[c.1009C>T; p.R337C; MS]	No Mutation	No Mutation
TCGA-75-6214	Adenocarcinoma	0.147729	11.882	[c.469G>T; p.V157F; MS]	No Mutation	[c.198G>T; p.K66N; MS]
TCGA-97-7553	Adenocarcinoma	0.14535	9.856	[c.844C>T; p.R282W; MS]	No Mutation	No Mutation
TCGA-77-6844	Squamous	0.135242	8.574	No Data	No Data	No Data
TCGA-75-7027	Adenocarcinoma	0.131759	9.186	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-77-6845	Squamous	0.131431	9.382	No Data	No Data	No Data
TCGA-35-4123	Adenocarcinoma	0.127752	12.102	[c.438G>A; p.W146*; NS]	No Mutation	No Mutation
TCGA-05-5715	Adenocarcinoma	0.126342	8.71	No Mutation	No Mutation	No Mutation
TCGA-55-6979	Adenocarcinoma	0.118129	10.187	[c.431A>T; p.Q144L; MS]	No Mutation	No Mutation
TCGA-66-2791	Squamous	0.115402	7.767	[c.734G>T; p.G245V; MS]	No Mutation	No Mutation
TCGA-34-5231	Squamous	0.113546	9.971	No Mutation	No Mutation	No Mutation
TCGA-21-5782	Squamous	0.112623	8.876	[c.584T>G; p.I195S; MS]	No Mutation	No Mutation
TCGA-21-5786	Squamous	0.106832	8.921	[c.469G>T; p.V157F; MS]	No Mutation	No Mutation
TCGA-44-7662	Adenocarcinoma	0.104257	10.333	[c.202G>T; p.E68*; NS]	No Mutation	No Mutation
TCGA-05-5420	Adenocarcinoma	0.103627	10.557	No Mutation	No Mutation	No Mutation
TCGA-34-7107	Squamous	0.10226	9.386	No Data	No Data	No Data
TCGA-64-5815	Adenocarcinoma	0.098878	10.92	No Mutation	[c.182A>T; p.Q61L; MS]	No Mutation
TCGA-66-2788	Squamous	0.098245	7.394	[c.375_splice; p.T125_spli	No Mutation	No Mutation

				ce; SNP-SS]		
TCGA-39-5030	Squamous	0.093902	6.867	No Mutation	No Mutation	No Mutation
TCGA-91-6835	Adenocarcinoma	0.092477	10.041	No Mutation	No Mutation	No Mutation
TCGA-93-7348	Adenocarcinoma	0.089931	9.371	No Mutation	No Mutation	No Mutation
TCGA-21-1071	Squamous	0.089158	7.684	No Mutation	No Mutation	No Mutation
TCGA-50-5045	Adenocarcinoma	0.083191	9.851	[c.625A>T; p.R209*; NS]	No Mutation	No Mutation
TCGA-55-7573	Adenocarcinoma	0.069094	9.41	[c.404G>T; p.C135F; MS]	No Mutation	No Mutation
TCGA-44-6145	Adenocarcinoma	0.068654	9.782	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-22-5474	Squamous	0.066775	7.906	[c.701A>C; p.Y234S; MS]	No Mutation	No Mutation
TCGA-78-7146	Adenocarcinoma	0.064815	12.314	[c.749C>T; p.P250L; MS]	No Mutation	No Mutation
TCGA-60-2710	Squamous	0.063056	7.743	[c.731G>A; p.G244D; MS]	No Mutation	No Mutation
TCGA-78-7535	Adenocarcinoma	0.060637	8.498	[c.574C>T; p.Q192*; NS]	No Mutation	No Mutation
TCGA-44-3917	Adenocarcinoma	0.06063	#N/A	No Data	No Data	No Data
TCGA-50-5932	Adenocarcinoma	0.060534	9.497	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-78-7154	Adenocarcinoma	0.053731	11.284	[c.811G>A; p.E271K; MS]	No Mutation	No Mutation
TCGA-79-5596	Squamous	0.052957	9.32	No Data	No Data	No Data
TCGA-56-6546	Squamous	0.050857	6.631	[c.535C>T; p.H179Y; MS]	No Mutation	No Mutation
TCGA-55-6712	Adenocarcinoma	0.050297	10.392	[c.1015delG; p.E339fs; Del-FS]	No Mutation	No Mutation
TCGA-78-	Adenocarci	0.042607	9.234	[c.809T>G;	No	No

7536	noma			p.F270C; MS]	Mutation	Mutation
TCGA-05-4410	Adenocarcinoma	0.040192	10.162	[c.842A>T; p.D281V; MS]	No Mutation	No Mutation
TCGA-73-7499	Adenocarcinoma	0.037586	9.177	[c.586C>T; p.R196*; NS]	No Mutation	No Mutation
TCGA-63-7022	Squamous	0.032762	7.766	No Data	No Data	No Data
TCGA-60-2695	Squamous	0.031416	8.225	No Data	No Data	No Data
TCGA-49-4494	Adenocarcinoma	0.027273	8.877	No Mutation	No Mutation	No Mutation
TCGA-33-6738	Squamous	0.027235	6.183	No Data	No Data	No Data
TCGA-05-5429	Adenocarcinoma	0.0267	10.111	No Mutation	No Mutation	No Mutation
TCGA-95-7944	Adenocarcinoma	0.026687	10.545	[c.487T>G; p.Y163D; MS]	No Mutation	No Mutation
TCGA-90-6837	Squamous	0.024836	8.252	No Data	No Data	No Data
TCGA-39-5029	Squamous	0.024504	9.102	[c.637C>T; p.R213*; NS]	No Mutation	No Mutation
TCGA-55-6978	Adenocarcinoma	0.022394	11.042	No Mutation	No Mutation	No Mutation
TCGA-50-5049	Adenocarcinoma	0.022159	10.394	[c.892G>T; p.E298*; NS]	No Mutation	No Mutation
TCGA-63-5128	Squamous	0.022135	9.212	[c.959_968 delAGAAAC CACT; p.K320fs; Del-FS]	No Mutation	No Mutation
TCGA-80-5608	Adenocarcinoma	0.01787	9.192	No Mutation	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-44-3918	Adenocarcinoma	0.015911	10.505	No Mutation	No Mutation	No Mutation
TCGA-91-6830	Adenocarcinoma	0.015646	9.715	No Mutation	No Mutation	No Mutation
TCGA-22-5479	Squamous	0.015187	7.984	No Data	No Data	No Data
TCGA-39-5037	Squamous	0.013043	7.577	[c.535C>G; p.H179D;	No Mutation	No Mutation

				MS]		
TCGA-67-6215	Adenocarcinoma	0.012696	9.765	No Mutation	No Mutation	No Mutation
TCGA-44-6148	Adenocarcinoma	0.012475	10.462	No Mutation	No Mutation	No Mutation
TCGA-78-7163	Adenocarcinoma	0.012351	7.805	No Mutation	No Mutation	No Mutation
TCGA-91-6828	Adenocarcinoma	0.011804	11.033	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-85-6175	Squamous	0.010613	8.116	No Mutation	No Mutation	No Mutation
TCGA-22-5478	Squamous	0.008108	6.951	[c.839G>T; p.R280I; MS]	No Mutation	No Mutation
TCGA-85-6561	Squamous	0.006259	8.895	[c.848G>C; p.R283P; MS]	No Mutation	No Mutation
TCGA-21-1080	Squamous	0.005661	10.208	No Data	No Data	No Data
TCGA-44-7671	Adenocarcinoma	0.004142	10.657	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-39-5027	Squamous	0.0035	7.503	[c.808T>A; p.F270I; MS]	No Mutation	No Mutation
TCGA-49-6761	Adenocarcinoma	8.78E-05	11.681	No Mutation	[c.35G>A; p.G12D; MS]	No Mutation
TCGA-51-6867	Squamous	0	8.237	No Data	No Data	No Data
TCGA-60-2708	Squamous	0	8.837	[c.461delG; p.G154fs; Del-FS]	No Mutation	No Mutation
TCGA-78-7156	Adenocarcinoma	0	10.084	No Mutation	No Mutation	No Mutation
TCGA-66-2782	Squamous	0	8.922	[c.859G>T; p.E287*; NS]	No Mutation	No Mutation
TCGA-22-5471	Squamous	0	7.63	[c.742C>T; p.R248W; MS]	No Mutation	No Mutation
TCGA-75-6211	Adenocarcinoma	0	10.444	[c.205_206insG; p.C69fs; Ins-FS]	No Mutation	No Mutation
TCGA-50-	Adenocarci	-0.00091	8.381	[c.814G>A;	No	No

6594	noma			p.V272M; MS]	Mutation	Mutation
TCGA-63-6202	Squamous	-0.00441	8.435	[c.473G>T; p.R158L; MS]	[c.183A>T; p.Q61H; MS]	No Mutation
TCGA-60-2711	Squamous	-0.0058	6.483	[c.792_793insA; p.L264fs; Ins-FS]	No Mutation	No Mutation
TCGA-60-2713	Squamous	-0.00711	6.121	[c.217delG; p.V73fs; Del-FS]	No Mutation	No Mutation
TCGA-97-7938	Adenocarcinoma	-0.00825	11.396	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-44-7659	Adenocarcinoma	-0.00931	10.089	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-73-4670	Adenocarcinoma	-0.00952	11.116	No Mutation	[c.436G>C; p.A146P; MS]	No Mutation
TCGA-66-2778	Squamous	-0.01571	8.288	No Mutation	No Mutation	No Mutation
TCGA-85-6560	Squamous	-0.0163	4.644	[c.405C>G; p.C135W; MS]	No Mutation	No Mutation
TCGA-73-4659	Adenocarcinoma	-0.02072	10.317	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-18-5595	Squamous	-0.02107	9.376	No Mutation	No Mutation	No Mutation
TCGA-78-7150	Adenocarcinoma	-0.02256	9.997	[c.473G>T; p.R158L; MS]	No Mutation	No Mutation
TCGA-85-7844	Squamous	-0.02266	7.165	No Data	No Data	No Data
TCGA-68-7756	Squamous	-0.0228	8.738	No Data	No Data	No Data
TCGA-53-7624	Adenocarcinoma	-0.02817	10.267	No Mutation	No Mutation	No Mutation
TCGA-35-4122	Adenocarcinoma	-0.03068	10.452	[c.746G>T; p.R249M; MS]	No Mutation	No Mutation
TCGA-44-6779	Adenocarcinoma	-0.03163	11.503	[c.394A>G; p.K132E; MS]	No Mutation	No Mutation
TCGA-21-	Squamous	-0.03183	8.247	[c.273G>A;	No	No

5784				p.W91*; NS]	Mutation	Mutation
TCGA-64-5779	Adenocarcinoma	-0.03219	9.358	No Mutation	No Mutation	No Mutation
TCGA-95-7043	Adenocarcinoma	-0.03423	10.558	[c.473G>T; p.R158L; MS]	No Mutation	No Mutation
TCGA-38-4627	Adenocarcinoma	-0.03541	10.031	No Mutation	No Mutation	No Mutation
TCGA-69-7763	Adenocarcinoma	-0.0357	10.053	No Mutation	No Mutation	No Mutation
TCGA-21-1076	Squamous	-0.03702	8.011	[c.488A>G; p.Y163C; MS]	No Mutation	No Mutation
TCGA-55-7815	Adenocarcinoma	-0.04086	9.901	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-50-5946	Adenocarcinoma	-0.04117	9.576	[c.472delC; p.R158fs; Del-FS]	No Mutation	No Mutation
TCGA-49-6742	Adenocarcinoma	-0.04428	10.318	No Mutation	No Mutation	No Mutation
TCGA-75-6203	Adenocarcinoma	-0.04693	8.88	No Mutation	No Mutation	No Mutation
TCGA-35-3615	Adenocarcinoma	-0.05066	10.308	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-22-5473	Squamous	-0.05173	8.858	[c.892G>T; p.E298*; NS]	No Mutation	No Mutation
TCGA-71-6725	Adenocarcinoma	-0.05203	9.197	No Mutation	No Mutation	No Mutation
TCGA-43-3394	Squamous	-0.05394	8.704	[c.942delC; p.S314fs; Del-FS]	No Mutation	No Mutation
TCGA-05-4398	Adenocarcinoma	-0.05547	10.804	[c.314G>A; p.G105D; MS]	No Mutation	No Mutation
TCGA-66-2785	Squamous	-0.05568	7.417	No Mutation	No Mutation	[c.961C>G; p.Q321E; MS]
TCGA-60-2697	Squamous	-0.05695	8.547	No Data	No Data	No Data
TCGA-39-5019	Squamous	-0.05758	8.568	[c.811G>T; p.E271*; NS]	No Mutation	No Mutation
TCGA-39-	Squamous	-0.05831	9.41	[c.523C>G;	No	No

5016				p.R175G; MS]	Mutation	Mutation
TCGA-44-4112	Adenocarcinoma	-0.0588	11.191	No Mutation	No Mutation	No Mutation
TCGA-18-5592	Squamous	-0.05915	9.873	[c.536A>T; p.H179L; MS]	No Mutation	No Mutation
TCGA-05-4418	Adenocarcinoma	-0.06053	10.148	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-86-7711	Adenocarcinoma	-0.06226	10.731	[c.527G>T; p.C176F; MS]	No Mutation	No Mutation
TCGA-44-2665	Adenocarcinoma	-0.06277	10.664	No Mutation	No Mutation	No Mutation
TCGA-78-7160	Adenocarcinoma	-0.06554	10.742	No Mutation	[c.35G>A; p.G12D; MS]	No Mutation
TCGA-64-1679	Adenocarcinoma	-0.06842	10.073	[c.734G>T; p.G245V; MS]	No Mutation	No Mutation
TCGA-60-2716	Squamous	-0.07055	9.269	No Data	No Data	No Data
TCGA-49-4488	Adenocarcinoma	-0.07232	10.952	[c.375_splice; p.T125_splice; SNP-SS]	No Mutation	No Mutation
TCGA-39-5031	Squamous	-0.07583	8.408	[c.309C>A; p.Y103*; NS]	No Mutation	No Mutation
TCGA-39-5035	Squamous	-0.08027	8.183	[c.473G>A; p.R158H; MS]	No Mutation	No Mutation
TCGA-44-5644	Adenocarcinoma	-0.0814	10.918	[c.326T>G; p.F109C; MS]	No Mutation	No Mutation
TCGA-86-7713	Adenocarcinoma	-0.08172	10.364	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-33-4586	Squamous	-0.08534	7.799	[c.743G>A; p.R248Q; MS]	No Mutation	No Mutation
TCGA-78-7537	Adenocarcinoma	-0.08759	9.016	No Mutation	No Mutation	No Mutation
TCGA-66-2790	Squamous	-0.08807	6.75	No Data	No Data	No Data
TCGA-22-	Squamous	-0.0886	9.445	[c.469G>T;	No	No

5477				p.V157F; MS]	Mutation	Mutation
TCGA-39-5021	Squamous	-0.08901	8.148	[c.423C>G; p.C141W; MS]	No Mutation	No Mutation
TCGA-69-7761	Adenocarcinoma	-0.09005	9.66	No Mutation	No Mutation	No Mutation
TCGA-69-7760	Adenocarcinoma	-0.09106	11.111	[c.808T>G; p.F270V; MS]	No Mutation	No Mutation
TCGA-66-2766	Squamous	-0.09244	8.455	[c.578A>T; p.H193L; MS]	No Mutation	No Mutation
TCGA-50-5941	Adenocarcinoma	-0.09295	10.439	[c.216delC; p.P72fs; Del-FS]	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-77-7337	Squamous	-0.09538	8.667	No Data	No Data	No Data
TCGA-49-4506	Adenocarcinoma	-0.09677	11.686	No Mutation	No Mutation	No Mutation
TCGA-90-7767	Squamous	-0.09997	6.8	No Data	No Data	No Data
TCGA-43-5668	Squamous	-0.10005	6.066	[c.818G>T; p.R273L; MS]	No Mutation	No Mutation
TCGA-97-7937	Adenocarcinoma	-0.1038	10.09	[c.734G>T; p.G245V; MS]	No Mutation	No Mutation
TCGA-78-7147	Adenocarcinoma	-0.10543	9.386	[c.708C>A; p.Y236*; NS]	No Mutation	No Mutation
TCGA-50-6597	Adenocarcinoma	-0.10743	6.836	No Mutation	No Mutation	No Mutation
TCGA-55-7574	Adenocarcinoma	-0.10916	11.013	[c.1036G>T; p.E346*; NS]	No Mutation	No Mutation
TCGA-66-2769	Squamous	-0.1135	7.601	No Data	No Data	No Data
TCGA-22-5482	Squamous	-0.11359	8.804	[c.376_splice; p.Y126_splice; SNP-SS]	No Mutation	No Mutation
TCGA-43-7658	Squamous	-0.11389	7.15	No Data	No Data	No Data
TCGA-85-7843	Squamous	-0.11434	8.803	No Data	No Data	No Data
TCGA-22-	Squamous	-0.11466	7.872	[c.329G>T;	No	No

5480				p.R110L; MS]	Mutation	Mutation
TCGA-05-4244	Adenocarcinoma	-0.11544	10.798	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-05-5423	Adenocarcinoma	-0.11646	9.823	[c.585C>G; p.I195M; MS], [c.746G>T; p.R249M; MS]	No Mutation	No Mutation
TCGA-55-1596	Adenocarcinoma	-0.11791	9.346	[c.841G>T; p.D281Y; MS]	No Mutation	No Mutation
TCGA-46-6026	Squamous	-0.11894	9.282	[c.754_762 delCTCACC ATC; p.LTI252del; Del-IF]	No Mutation	No Mutation
TCGA-75-5122	Adenocarcinoma	-0.12025	11.069	No Mutation	No Mutation	No Mutation
TCGA-86-6562	Adenocarcinoma	-0.12073	9.775	No Mutation	No Mutation	No Mutation
TCGA-77-6842	Squamous	-0.12704	6.611	No Data	No Data	No Data
TCGA-60-2714	Squamous	-0.13349	8.374	No Data	No Data	No Data
TCGA-50-5944	Adenocarcinoma	-0.13767	9.479	[c.853G>T; p.E285*; NS]	No Mutation	No Mutation
TCGA-91-6840	Adenocarcinoma	-0.14373	7.227	[c.659A>G; p.Y220C; MS]	No Mutation	No Mutation
TCGA-44-3398	Adenocarcinoma	-0.14406	9.678	No Mutation	No Mutation	No Mutation
TCGA-52-7812	Squamous	-0.14419	7.863	No Data	No Data	No Data
TCGA-66-2783	Squamous	-0.14448	8.056	[c.994_splice; p.I332_splice; SNP-SS]	No Mutation	No Mutation
TCGA-44-6144	Adenocarcinoma	-0.14889	#N/A	[c.1010G>T; p.R337L; MS]	No Mutation	No Mutation
TCGA-52-7810	Squamous	-0.15127	9.42	No Data	No Data	No Data
TCGA-85-	Squamous	-0.15147	8.651	No Data	No Data	No Data

6798						
TCGA-95-7947	Adenocarcinoma	-0.15531	7.45	[c.614A>G; p.Y205C; MS]	No Mutation	No Mutation
TCGA-94-7557	Squamous	-0.15553	8.228	No Data	No Data	No Data
TCGA-49-4512	Adenocarcinoma	-0.15692	8.384	No Mutation	No Mutation	No Mutation
TCGA-64-1676	Adenocarcinoma	-0.15978	11.175	[c.686_687 delGT; p.C229fs; Del-FS]	No Mutation	No Mutation
TCGA-34-5236	Squamous	-0.16281	7.974	[c.395A>G; p.K132R; MS]	No Mutation	No Mutation
TCGA-05-4434	Adenocarcinoma	-0.16389	11.532	No Mutation	No Mutation	No Mutation
TCGA-94-7033	Squamous	-0.16481	8.45	No Data	No Data	No Data
TCGA-22-1011	Squamous	-0.16792	7.282	[c.811G>A; p.E271K; MS]	No Mutation	No Mutation
TCGA-55-1594	Adenocarcinoma	-0.16872	10.78	[c.497C>G; p.S166*; NS]	No Mutation	No Mutation
TCGA-66-2781	Squamous	-0.17049	8.965	No Mutation	No Mutation	No Mutation
TCGA-66-2800	Squamous	-0.17178	8.872	[c.734G>T; p.G245V; MS]	No Mutation	No Mutation
TCGA-46-6025	Squamous	-0.17429	9.181	[c.514G>T; p.V172F; MS]	No Mutation	No Mutation
TCGA-22-4605	Squamous	-0.17851	8.283	No Data	No Data	No Data
TCGA-44-2656	Adenocarcinoma	-0.18137	11.955	[c.217delG; p.V73fs; Del-FS]	No Mutation	No Mutation
TCGA-85-7697	Squamous	-0.18303	8.789	No Data	No Data	No Data
TCGA-18-4721	Squamous	-0.18556	9.071	No Mutation	No Mutation	No Mutation
TCGA-55-6971	Adenocarcinoma	-0.19039	9.862	No Mutation	No Mutation	No Mutation
TCGA-34-2609	Squamous	-0.19159	#N/A	No Data	No Data	No Data
TCGA-70-	Squamous	-0.19569	8.349	No	No	No

6722				Mutation	Mutation	Mutation
TCGA-49-6743	Adenocarcinoma	-0.19668	9.153	No Mutation	No Mutation	No Mutation
TCGA-22-4591	Squamous	-0.19979	6.921	No Mutation	No Mutation	No Mutation
TCGA-50-5942	Adenocarcinoma	-0.20227	11.044	No Mutation	No Mutation	No Mutation
TCGA-56-6545	Squamous	-0.20566	8.214	[c.469G>T; p.V157F; MS]	No Mutation	No Mutation
TCGA-33-4589	Squamous	-0.2069	8.157	No Data	No Data	No Data
TCGA-50-6592	Adenocarcinoma	-0.20758	10.936	[c.745A>G; p.R249G; MS]	No Mutation	No Mutation
TCGA-86-7953	Adenocarcinoma	-0.20762	9.483	[c.342_358 delGCATTCTGGGACAGCCA; p.LHSGTAK114fs; Del-FS]	[c.35G>A; p.G12D; MS]	No Mutation
TCGA-64-5781	Adenocarcinoma	-0.21067	10.588	No Mutation	No Mutation	No Mutation
TCGA-35-5375	Adenocarcinoma	-0.21139	9.938	[c.722C>T; p.S241F; MS]	No Mutation	No Mutation
TCGA-50-5051	Adenocarcinoma	-0.21769	10.103	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-50-5044	Adenocarcinoma	-0.21851	9.977	No Mutation	No Mutation	No Mutation
TCGA-49-6744	Adenocarcinoma	-0.22101	9.852	No Mutation	No Mutation	No Mutation
TCGA-64-1681	Adenocarcinoma	-0.2218	8.82	[c.538G>A; p.E180K; MS]	No Mutation	No Mutation
TCGA-44-3919	Adenocarcinoma	-0.22191	10.122	No Mutation	No Mutation	No Mutation
TCGA-52-7811	Squamous	-0.22222	8.598	No Data	No Data	No Data
TCGA-38-4625	Adenocarcinoma	-0.22227	12.214	[c.818G>T; p.R273L; MS]	No Mutation	No Mutation
TCGA-78-7162	Adenocarcinoma	-0.22229	9.781	No Mutation	No Mutation	No Mutation
TCGA-66-	Squamous	-0.22301	9.055	[c.743G>T;	No	No

2777				p.R248L; MS]	Mutation	Mutation
TCGA-60-2726	Squamous	-0.22365	7.569	No Mutation	No Mutation	No Mutation
TCGA-80-5607	Adenocarcinoma	-0.22433	10.265	No Mutation	No Mutation	No Mutation
TCGA-50-5066	Adenocarcinoma	-0.2244	10.93	[c.537T>A; p.H179Q; MS]	No Mutation	No Mutation
TCGA-49-4487	Adenocarcinoma	-0.22685	8.928	[c.230C>T; p.P77L; MS]	No Mutation	No Mutation
TCGA-85-7710	Squamous	-0.22751	8.258	No Data	No Data	No Data
TCGA-05-4420	Adenocarcinoma	-0.22974	8.053	[c.839G>T; p.R280I; MS]	No Mutation	No Mutation
TCGA-96-7544	Squamous	-0.22993	8.511	No Data	No Data	No Data
TCGA-44-7661	Adenocarcinoma	-0.23398	11.601	[c.637C>T; p.R213*; NS]	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-34-5234	Squamous	-0.23523	7.15	No Mutation	No Mutation	No Mutation
TCGA-05-4426	Adenocarcinoma	-0.23719	10.362	[c.464C>T; p.T155I; MS]	No Mutation	No Mutation
TCGA-63-7020	Squamous	-0.23758	8.213	No Data	No Data	No Data
TCGA-60-2721	Squamous	-0.23887	7.489	[c.610G>T; p.E204*; NS]	No Mutation	No Mutation
TCGA-05-4384	Adenocarcinoma	-0.23898	9.913	[c.614A>G; p.Y205C; MS]	No Mutation	No Mutation
TCGA-39-5011	Squamous	-0.23944	7.223	No Data	No Data	No Data
TCGA-80-5611	Adenocarcinoma	-0.24048	10.929	No Mutation	No Mutation	No Mutation
TCGA-66-2727	Squamous	-0.24058	8.207	[c.701A>G; p.Y234C; MS]	No Mutation	No Mutation
TCGA-49-4501	Adenocarcinoma	-0.2416	9.37	No Mutation	No Mutation	No Mutation
TCGA-66-2737	Squamous	-0.24386	7.83	No Data	No Data	No Data
TCGA-78-7539	Adenocarcinoma	-0.24543	10.414	No Mutation	[c.34G>T; p.G12C;	No Mutation

					MS]	
TCGA-22-4607	Squamous	-0.24762	8.615	[c.527G>T; p.C176F; MS]	No Mutation	No Mutation
TCGA-78-7633	Adenocarcinoma	-0.24881	10.182	No Mutation	No Mutation	No Mutation
TCGA-60-2696	Squamous	-0.25037	10.299	No Data	No Data	No Data
TCGA-37-4141	Squamous	-0.25067	6.003	[c.725G>T; p.C242F; MS]	No Mutation	No Mutation
TCGA-49-4505	Adenocarcinoma	-0.2512	11.008	[c.830G>T; p.C277F; MS]	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-92-7340	Squamous	-0.25166	8.227	No Data	No Data	No Data
TCGA-21-1078	Squamous	-0.25466	6.961	No Mutation	[c.353G>C; p.C118S; MS]	No Mutation
TCGA-63-5131	Squamous	-0.25599	6.831	[c.473G>T; p.R158L; MS]	No Mutation	No Mutation
TCGA-73-7498	Adenocarcinoma	-0.25964	8.869	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-60-2724	Squamous	-0.26336	9.799	[c.730G>T; p.G244C; MS]	No Mutation	No Mutation
TCGA-91-6836	Adenocarcinoma	-0.26445	11.42	[c.1027G>T; p.E343*; NS]	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-22-5472	Squamous	-0.26454	7.911	No Mutation	No Mutation	No Mutation
TCGA-85-7699	Squamous	-0.2668	7.82	No Data	No Data	No Data
TCGA-05-4250	Adenocarcinoma	-0.26866	12.47	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-05-5425	Adenocarcinoma	-0.26995	9.611	[c.202G>T; p.E68*; NS]	No Mutation	No Mutation
TCGA-21-1083	Squamous	-0.27066	8.709	No Data	No Data	No Data
TCGA-73-4676	Adenocarcinoma	-0.27223	11.077	[c.527G>T; p.C176F; MS]	No Mutation	No Mutation
TCGA-33-4532	Squamous	-0.2786	8.778	[c.711G>T; p.M237I;	No Mutation	No Mutation

				MS]		
TCGA-78-7161	Adenocarcinoma	-0.28104	9.155	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-44-2661	Adenocarcinoma	-0.28352	9.254	No Mutation	No Mutation	No Mutation
TCGA-21-1072	Squamous	-0.28765	8.816	No Data	No Data	No Data
TCGA-05-4417	Adenocarcinoma	-0.28956	10.33	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-73-4658	Adenocarcinoma	-0.29541	11.13	No Data	No Data	No Data
TCGA-34-5241	Squamous	-0.29547	7.76	No Mutation	No Mutation	No Mutation
TCGA-60-2706	Squamous	-0.29865	8.454	No Data	No Data	No Data
TCGA-21-1082	Squamous	-0.30006	7.79	No Data	No Data	No Data
TCGA-86-7954	Adenocarcinoma	-0.30149	9.113	[c.743G>T; p.R248L; MS]	No Mutation	No Mutation
TCGA-86-6851	Adenocarcinoma	-0.30212	9.1	[c.774A>T; p.E258D; MS]	No Mutation	No Mutation
TCGA-05-4396	Adenocarcinoma	-0.30322	8.668	[c.416delA; p.K139fs; Del-FS]	No Mutation	No Mutation
TCGA-21-1075	Squamous	-0.30441	8.216	No Data	No Data	No Data
TCGA-38-4626	Adenocarcinoma	-0.30782	11.018	No Mutation	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-22-5492	Squamous	-0.31726	5.861	[c.661G>T; p.E221*; NS]	No Mutation	No Mutation
TCGA-75-5125	Adenocarcinoma	-0.31783	10.262	[c.388C>T; p.L130F; MS]	No Mutation	No Mutation
TCGA-98-7454	Squamous	-0.32499	7.723	No Data	No Data	No Data
TCGA-38-4629	Adenocarcinoma	-0.32582	11.076	[c.602_603insT; p.S201fs; Ins-FS]	No Mutation	No Mutation
TCGA-78-7155	Adenocarcinoma	-0.33037	5.795	[c.1010G>T; p.R337L;	No Mutation	No Mutation

				MS]		
TCGA-78-7143	Adenocarcinoma	-0.33083	9.139	No Data	No Data	No Data
TCGA-60-2725	Squamous	-0.33671	6.88	No Mutation	No Mutation	No Mutation
TCGA-37-3792	Squamous	-0.34158	7.254	No Data	No Data	No Data
TCGA-43-6770	Squamous	-0.34657	8.011	[c.614A>G; p.Y205C; MS]	No Mutation	No Mutation
TCGA-77-7465	Squamous	-0.34694	7.472	No Data	No Data	No Data
TCGA-05-4402	Adenocarcinoma	-0.34699	9.057	[c.518_533 delTGAGGCGCTGCCCCCA; p.VRRCPH173fs; Del-FS]	No Mutation	No Mutation
TCGA-37-4129	Squamous	-0.35214	4.685	No Data	No Data	No Data
TCGA-50-7109	Adenocarcinoma	-0.3523	8.881	No Mutation	No Mutation	No Mutation
TCGA-83-5908	Adenocarcinoma	-0.35232	11.298	[c.824G>T; p.C275F; MS]	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-33-4547	Squamous	-0.35246	7.358	[c.811G>A; p.E271K; MS]	No Mutation	No Mutation
TCGA-21-1081	Squamous	-0.35278	8.907	No Mutation	No Mutation	No Mutation
TCGA-44-2664	Adenocarcinoma	-0.35659	#N/A	No Data	No Data	No Data
TCGA-97-7941	Adenocarcinoma	-0.35705	11.02	No Mutation	[c.35G>C; p.G12A; MS]	No Mutation
TCGA-50-5930	Adenocarcinoma	-0.35855	9.499	[c.785G>T; p.G262V; MS]	No Mutation	No Mutation
TCGA-44-2668	Adenocarcinoma	-0.35954	10.345	[c.856G>T; p.E286*; NS]	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-64-1680	Adenocarcinoma	-0.36293	10.257	No Mutation	No Mutation	No Mutation
TCGA-37-3789	Squamous	-0.36406	9.294	[c.193A>T; p.R65*; NS]	No Mutation	No Mutation
TCGA-22-	Squamous	-0.36505	8.739	No Data	No Data	No Data

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TCGA-77-7463	Squamous	-0.36684	8.62	No Data	No Data	No Data
TCGA-50-5068	Adenocarcinoma	-0.37078	9.667	[c.646G>A; p.V216M; MS]	No Mutation	No Mutation
TCGA-99-7458	Adenocarcinoma	-0.37812	9.34	No Mutation	[c.34G>C; p.G12R; MS]	No Mutation
TCGA-55-6981	Adenocarcinoma	-0.37967	9.96	No Mutation	No Mutation	No Mutation
TCGA-05-4430	Adenocarcinoma	-0.38241	9.586	[c.746G>T; p.R249M; MS]	No Mutation	No Mutation
TCGA-66-2768	Squamous	-0.38288	8.375	[c.488A>G; p.Y163C; MS]	No Mutation	No Mutation
TCGA-75-6212	Adenocarcinoma	-0.38608	8.549	No Mutation	No Mutation	No Mutation
TCGA-66-2767	Squamous	-0.38752	6.327	[c.783_splice; p.S261_splice; SNP-SS]	No Mutation	No Mutation
TCGA-69-7764	Adenocarcinoma	-0.39428	8.166	No Mutation	No Mutation	No Mutation
TCGA-90-7766	Squamous	-0.40039	8.086	No Data	No Data	No Data
TCGA-55-1592	Adenocarcinoma	-0.4005	8.89	[c.730G>T; p.G244C; MS]	No Mutation	No Mutation
TCGA-60-2720	Squamous	-0.40157	7.05	[c.659A>G; p.Y220C; MS]	No Mutation	No Mutation
TCGA-05-4397	Adenocarcinoma	-0.40623	7.889	[c.744delG; p.R249fs; Del-FS]	No Mutation	No Mutation
TCGA-77-7338	Squamous	-0.40759	8.329	No Data	No Data	No Data
TCGA-34-5928	Squamous	-0.4228	7.602	[c.785G>T; p.G262V; MS]	No Mutation	No Mutation
TCGA-50-5072	Adenocarcinoma	-0.4245	10.027	No Mutation	No Mutation	No Mutation
TCGA-49-4507	Adenocarcinoma	-0.42522	10.644	No Mutation	No Mutation	No Mutation
TCGA-05-4249	Adenocarcinoma	-0.4287	9.999	No Mutation	[c.34G>T; p.G12C;	No Mutation

					MS]	
TCGA-91-7771	Adenocarcinoma	-0.43043	10.077	No Mutation	No Mutation	No Mutation
TCGA-21-1079	Squamous	-0.43262	8.515	No Data	No Data	No Data
TCGA-64-5774	Adenocarcinoma	-0.43462	8.761	No Mutation	[c.34G>T; p.G12C; MS]	No Mutation
TCGA-44-2657	Adenocarcinoma	-0.43849	9.402	No Mutation	No Mutation	No Mutation
TCGA-22-0940	Squamous	-0.44369	8.785	No Data	No Data	No Data
TCGA-44-6774	Adenocarcinoma	-0.45555	10.993	[c.733G>T; p.G245C; MS]	No Mutation	No Mutation
TCGA-05-4427	Adenocarcinoma	-0.45559	10.788	[c.841G>A; p.D281N; MS]	[c.35G>T; p.G12V; MS]	No Mutation
TCGA-34-2604	Squamous	-0.47327	#N/A	No Data	No Data	No Data
TCGA-05-4405	Adenocarcinoma	-0.47668	10.327	[c.524G>A; p.R175H; MS], [c.830G>T; p.C277F; MS]	No Mutation	No Mutation
TCGA-56-7222	Squamous	-0.48076	10.15	No Data	No Data	No Data
TCGA-53-7626	Adenocarcinoma	-0.48139	11.3	[c.711G>A; p.M237I; MS]	No Mutation	No Mutation
TCGA-49-4510	Adenocarcinoma	-0.48812	10.642	No Mutation	[c.35G>A; p.G12D; MS]	No Mutation
TCGA-22-5485	Squamous	-0.49868	8.584	[c.486_487insATC; p.162_163insI; Ins-IF]	No Mutation	No Mutation
TCGA-22-4596	Squamous	-0.49928	8.941	No Data	No Data	No Data
TCGA-43-6771	Squamous	-0.50999	8.186	No Mutation	No Mutation	No Mutation
TCGA-43-2581	Squamous	-0.52567	8.465	No Data	No Data	No Data
TCGA-66-2773	Squamous	-0.54978	9.031	No Mutation	No Mutation	No Mutation

APPENDIX B: NSCLC CELL LINE SUMMARY TABLE

Cell	Histology	MYC RPPA	MYC mRNA	MYC CNV	p53	KRAS
H1819	Adenocarcinoma	-0.8034561	6.805	2.82	[c.597+1G>T; SS] (CPRIT-NGS)	WT
H1869	Squamous	-0.5487679	11.467	3.37	[ATG to ATA] (NCI-Navy), [c.G711A; p.M237I; Likely Som] (CPRIT-NGS Unpaired)	WT
H1650	Adenocarcinoma	-0.5173082	9.323	3.38	[c.672_673 49 insertion(Insertion) AA:p.(Insertion-Frameshift)] (Gazdar), [c.276-2A>G; SS; Likely Som] (CPRIT-NGS Unpaired)	WT
H324	Adenocarcinoma	-0.462183	5.096	0	[AGG to AGC] (NCI-Navy)	WT
HCC364	Adenocarcinoma	-0.4509281	11.263	0	0	WT
HCC4006	Adenocarcinoma	-0.4244754	8.33	1.86	[c.613T>C ; AA: p. Y205H (Substitution - Missense)] (Gazdar), [c.T613C; p.Y205H] (CPRIT-NGS Unpaired)	WT
H322	Adenocarcinoma	-0.4216446	11.252	3.06	[CGG to CTG(miss)] (NCI-Navy)	WT
HCC193	Adenocarcinoma	-0.3815119	11.725	3.14	[c.G743A; p.R248Q; MS] (CPRIT-NGS)	WT
H1734	Adenocarcinoma	-0.2540735	11.509	2.46	[CGT to CTT] (NCI-Navy), [c.G818T; p.R273L; Likely Som] (CPRIT-NGS Unpaired)	[c.37G>T; p.G13C; MS; Likely Som] (COSMIC-v67), [c.G37T; p.G13C; Likely Som] (CPRIT-NGS Unpaired)
H1693	Adenocarcinoma	-0.1032877	10.438	2.43	[c.597+1G>T; SS] (CPRIT-NGS), WT (NCI-Navy)	WT

H2347	Adenocarcinoma	-0.0886914	11.878	3.24	WT	WT (COSMIC-v67), [c.G57T; p.L19F; MS] (CPRIT-NGS), WT (Gazdar)
H2009	Adenocarcinoma	-0.0356444	11.756	2.99	[c.818G>T; p.R273L; MS; Likely Som] (COSMIC-v67), [c.818G>T; p.R273L; MS; Hom; Som] (COSMIC-v67), [c.G818T; p.R273L; MS] (CPRIT-NGS), [CGT to CTT] (NCI-Navy)	[c.35G>C; p.G12A; MS; Likely Som] (COSMIC-v67), [c.35G>C; p.G12A; MS; Likely Som] (COSMIC-v67), [c.35G>C; p.G12A; MS; Som] (COSMIC-v67), [c.G35C; p.G12A; MS] (CPRIT-NGS), [12GCT] (Gazdar)
HCC461	Adenocarcinoma	0	11.296	1.85	0	[12GAT] (Gazdar)

H2122	Adenocarcinoma	0	13.143	5.14	[c.131G>T; p.C44F; MS; Likely Som] (COSMIC-v67), [c.248G>T; p.C83F; MS; Likely Som] (COSMIC-v67), [c.47A>T; p.Q16L; MS; Likely Som] (COSMIC-v67), [c.47A>T; p.Q16L; MS] (COSMIC-v67), [c.527G>T; p.C176F; MS; Likely Som] (COSMIC-v67), [c.47A>T; p.Q16L; MS; Het; Som] (COSMIC-v67), [c.527G>T; p.C176F; MS; Het; Som] (COSMIC-v67), [c.G131T; p.C44F; MS] (CPRIT-NGS), [c.A47T; p.Q16L; MS] (CPRIT-NGS)	[c.34G>T; p.G12C; MS; Hom; Likely Som] (COSMIC-v67), [c.34G>T; p.G12C; MS; Likely Som] (COSMIC-v67), [c.34G>T; p.G12C; MS; Likely Som] (COSMIC-v67), [c.G34T; p.G12C; MS] (CPRIT-NGS), [12TGT] (Gazdar)
H441	Adenocarcinoma	0	11.453	1.99	[c.194G>T; p.R65L; MS; Likely Som] (COSMIC-v67), [c.473G>T; p.R158L; MS; Likely Som] (COSMIC-v67), [c.77G>T; p.R26L; MS; Likely Som] (COSMIC-v67), [CGC to CTC] (NCI-Navy)	[c.35G>T; p.G12V; MS; Likely Som] (COSMIC-v67), [c.35G>T; p.G12V; MS; Likely Som] (COSMIC-v67), WT (COSMIC-v67), [12TGT] (Gazdar)

H3255	Adenocarcinoma	0	9.271	2.28	[c.560_672del113(Delation) AA:p.(Delation-Frameshift)] (Gazdar), [c.163-1G>A; SS; Likely Som] (CPRIT-NGS Unpaired)	WT
HCC2935	Adenocarcinoma	0	7.779	0.73	[c.659A>G (Subsstitution) ; AA: p. Y220C(Substitution-Missense)] (Gazdar)	WT
HCC44	Adenocarcinoma	0	13.395	3.85	[c.G524T; p.R175L; MS] (CPRIT-NGS), [c.C281G; p.S94X; NS] (CPRIT-NGS)	[c.G34T; p.G12C; MS] (CPRIT-NGS), [12TGT] (Gazdar)
H1792	Adenocarcinoma	0	13.335	0	[GT to AT(spl don)] (NCI-Navy)	[c.34G>T; p.G12C; MS; Likely Som] (COSMIC-v67), [c.34G>T; p.G12C; MS; Som] (COSMIC-v67), [12TGT] (Gazdar)
H2086	Adenocarcinoma	0	7.306	0	[TAT-TCT (missense)] (NCI-Navy), [c.A659G; p.Y220C; Likely Som] (CPRIT-NGS Unpaired)	WT
EKVX	Adenocarcinoma	0.01712643	10.383	2.13	[c.214G>T; p.E72*; NS; Hom; Som] (COSMIC-v67), [c.331G>T; p.E111*; NS; Hom; Som] (COSMIC-v67), [c.610G>T; p.E204*; NS; Hom; Som] (COSMIC-v67)	WT

H1651	Adenocarcinoma	0.02864278	10.83	3.95	[TGC to TAC] (NCI-Navy), [c.G527A; p.C176Y; Likely Som] (CPRIT-NGS Unpaired)	WT
H2073	Adenocarcinoma	0.15104291	10.813	3.69	[c.447C>G; p.C149W; MS] (COSMIC-v67), [c.726C>G; p.C242W; MS] (COSMIC-v67), [c.C330G; p.C110W; MS] (CPRIT-NGS), [c.726C>G (Substitution) AA:p.C242W(Substitution - Missense)] (Gazdar), [TGC to TGG] (NCI-Navy)	WT
H1395	Adenocarcinoma	0.15713145	12.27	8.81	WT	WT
HCC515	Adenocarcinoma	0.21720115	10.651	1.83	[c.C580T; p.L194F; MS] (CPRIT-NGS)	[c.G38A; p.G13D; MS] (CPRIT-NGS), [13GAC] (Gazdar)
HCC4011	Adenocarcinoma	0.29201274	11.388	2.54	[c.75_96del22(Delation) AA:p.(Delation-Frameshift)] (Gazdar)	WT
H1838	Adenocarcinoma	0.31617404	11.908	0	[c.G818T; p.R273L; Likely Som] (CPRIT-NGS Unpaired)	WT
H2126	Adenocarcinoma	0.33415449	9.723	2.5	[c.184G>T; p.E62*; NS; Hom; Som] (COSMIC-v67), [c.G184T; p.E62X; NS] (CPRIT-NGS), WT (NCI-Navy)	WT

HOP-62	Adenocarcinoma	0.38462838	10.367	2.73	[c.394-2A>G; p.?; Hom; Som] (COSMIC-v67), [c.673-2A>G; p.?; Hom; Som] (COSMIC-v67)	[c.34G>T; p.G12C; MS; Het; Som] (COSMIC-v67), [c.34G>T; p.G12C; MS; Som] (COSMIC-v67)
HCC4019	Adenocarcinoma	0.42370105	11.088	0	[c.380C>T(Subsstitution) ; AA: p. S127F(Substitution-Missense)] (Gazdar)	[12TGT] (Gazdar)
H2085	Adenocarcinoma	0.43450991	9.894	2.32	[c.A659G; p.Y220C; Likely Som] (CPRIT-NGS Unpaired)	WT
DFCI032	Adenocarcinoma	0.4615207	11.472	0	[c.G527T; p.C176F; Likely Som] (CPRIT-NGS Unpaired)	WT
HCC4018	Adenocarcinoma	0.47159891	7.626	0	[c.G993C; p.Q331H; MS] (CPRIT-NGS)	WT
H1793	Adenocarcinoma	0.49914107	12.098	2.52	[AGA to TGA (stop)] (NCI-Navy), [c.A625T; p.R209X; NS; Likely Som] (CPRIT-NGS Unpaired), [c.G818A; p.R273H; Likely Som] (CPRIT-NGS Unpaired)	WT
H520	Squamous	0.50467894	10.503	1.95	[TGG to TGA (stop)] (NCI-Navy), [c.G438A; p.W146X; NS; Likely Som] (CPRIT-NGS Unpaired)	WT
H1993	Adenocarcinoma	0.56258948	10.683	2.68	[c.447C>G; p.C149W; MS] (COSMIC-v67), [c.726C>G; p.C242W; MS] (COSMIC-v67), [c.C726G; p.C242W; MS] (CPRIT-NGS), [TGC to TGG] (NCI-Navy)	WT

HCC2108	Adenocarcinoma	0.61886904	11.279	0	[c.G65T; p.G22V; MS] (CPRIT-NGS)	[c.A183C; p.Q61H; MS] (CPRIT-NGS), WT (Gazdar)
H820	Adenocarcinoma	0.63074663	11.672	0	[1,c.849C>G (Substitution) AA:p.R283R(Substitution - coding sillent) 2,c.850A>C (Substitution) AA:p.T284P(Substitution - Missense)] (Gazdar), [ACA to CCA(miss)] (NCI-Navy)	WT
H2342	Adenocarcinoma	0.65502836	11.304	0	[c.A659G; p.Y220C; Likely Som] (CPRIT-NGS Unpaired)	WT
HCC78	Adenocarcinoma	0.73213547	12.048	1.82	[c.C722T; p.S241F; Likely Som] (CPRIT-NGS Unpaired)	WT
H2087	Adenocarcinoma	0.86404685	13.232	0	[c.469G>T; p.V157F; MS; Hom; Som] (COSMIC-v67), [c.G469T; p.V157F; MS] (CPRIT-NGS), [GTC to TTC(miss)] (NCI-Navy)	WT
HCC2279	Adenocarcinoma	0.93495195	11.569	4.2	[c.701A>G (Substitution) AA:p.Y234C(Substitution - Missense)] (Gazdar), [c.A701G; p.Y234C; Likely Som] (CPRIT-NGS Unpaired)	WT
Calu-3	Adenocarcinoma	1.01692778	11.759	3.23	[c.G711T; p.M237I; Likely Som] (CPRIT-NGS Unpaired)	WT
H2250	Adenocarcinoma	1.01717746	12.284	0	[c.G481A; p.A161T; MS] (CPRIT-NGS), [GCC to ACC] (NCI-Navy)	WT

DFCI024	Adenocarcinoma	1.01925967	12.169	0	WT	WT (COSMIC-v67), [c.34G>T; p.G12C] (Gazdar), [c.G34T; p.G12C; Likely Som] (CPRIT-NGS Unpaired)
HCC15	Squamous	1.03622971	11.554	2.4	[c.A776T; p.D259V; MS] (CPRIT-NGS)	WT
H1648	Adenocarcinoma	1.06125232	11.672	2.53	[TTG to TTtG Δ1bp] (NCI-Navy)	WT
H969	Adenocarcinoma	1.10779826	11.554	0	WT	WT
H2258	Adenocarcinoma	1.18058191	12.758	0	[c.G481A; p.A161T; MS] (CPRIT-NGS)	WT
HCC2814	Squamous	1.36575118	11.365	0	0	0

H23	Adenocarcinoma	1.55460285	11.304	4.05	[c.459G>C; p.M153I; MS; Likely Som] (COSMIC-v67), [c.738G>C; p.M246I; MS; Likely Som] (COSMIC-v67), [c.459G>C; p.M153I; MS; Hom; Som] (COSMIC-v67), [c.738G>C; p.M246I; MS; Hom; Som] (COSMIC-v67), [ATG to ATC(miss)] (NCI-Navy), [c.G738C; p.M246I; Likely Som] (CPRIT-NGS Unpaired)	[c.34G>T; p.G12C; MS; Likely Som] (COSMIC-v67), [c.34G>T; p.G12C; MS; Het; Som] (COSMIC-v67), [c.34G>T; p.G12C; MS; Likely Som] (COSMIC-v67), WT (COSMIC-v67), [12TGT] (Gazdar), [c.G34T; p.G12C; Likely Som] (CPRIT-NGS Unpaired)
H1975	Adenocarcinoma	1.619964	13.099	8.06	[c.G818A; p.R273H; Likely Som] (CPRIT-NGS Unpaired)	WT
H1437	Adenocarcinoma	1.72095742	11.437	4.13	[c.800G>C; p.R267P; MS; Hom; Som] (COSMIC-v67), [c.G800C; p.R267P; MS] (CPRIT-NGS), [CGG to CCG(miss)] (NCI-Navy)	WT
H920	Adenocarcinoma	1.7691938	12.812	0	[c.559-575 17bp deletion (frameshift)] (Gazdar), [AG to AC (spl acc site)] (NCI-Navy), [c.163-1G>T; SS; Likely Som] (CPRIT-NGS Unpaired)	WT

HCC95	Squamous	2.19787618	11.864	0	[c.1003delC; p.R335fs; Del-FS] (CPRIT-NGS)	WT
Cal-12T	NSCLC	No Data	No Data	#N/A	0	0
H2110	NSCLC	No Data	No Data	#N/A	0	0
H647	Adenosquamous	No Data	11.638	#N/A	[344bp intron insertion at c.782] (Gazdar), [GT to TT; spl don; inclu 5' i7 to STOP] (NCI-Navy), [c.386+1G>T; SS; Likely Som] (CPRIT-NGS Unpaired)	[c.38G>A; p.G13D; MS; Likely Som] (COSMIC-v67), [13GAC] (Gazdar), [c.G38A; p.G13D; Likely Som] (CPRIT-NGS Unpaired)
H1915	Large Cell (poorly diff)	No Data	No Data	#N/A	[AAG to TAG (stop)] (NCI-Navy)	WT
H661	Large Cell	No Data	10.318	#N/A	[AGT to ATT] (NCI-Navy), [c.G473T; p.R158L; Likely Som] (CPRIT-NGS Unpaired), [c.G644T; p.S215I; Likely Som] (CPRIT-NGS Unpaired)	WT
H2882	NSCLC	No Data	11.525	#N/A	[c.203_225del; p.68_75del; Del-FS] (CPRIT-NGS)	WT
HCC827	Adenocarcinoma (BAC features)	No Data	11.566	#N/A	[c.257_259del; p.86_87del; Del-IF] (CPRIT-NGS), [c.652_654 del GTG(Deletion); AA: p.V218 (Deletion -In frame)] (Gazdar)	WT

H1155	Large Cell	No Data	7.88	#N/A	[c.818G>A; p.R273H; MS; Likely Som] (COSMIC-v67), [CGT to CAT(miss)] (NCI-Navy)	[c.183A>T; p.Q61H; MS; Likely Som] (COSMIC-v67), [c.183A>T; p.Q61H; MS; Likely Som] (COSMIC-v67), [c.183A>T; p.Q61H; MS; Som] (COSMIC-v67), [61CAT] (Gazdar)
HCC122	Adenocarcinoma	No Data	No Data	#N/A	[c.A536T; p.H179L; MS] (CPRIT-NGS)	WT
HCC366	Adenosquamous	No Data	11.782	#N/A	[c.A659G; p.Y220C; MS] (CPRIT-NGS)	[c.A183T; p.Q61H; MS] (CPRIT-NGS), WT (Gazdar)
HCC1171	NSCLC (poorly diff)	No Data	11.474	#N/A	[c.A740T; p.N247I; Likely Som] (CPRIT-NGS Unpaired)	[12TGT] (Gazdar)
H3122	NSCLC	No Data	8.916	#N/A	[c.A854T; p.E285V] (CPRIT-NGS Unpaired)	WT
H2887	NSCLC	No Data	11.423	#N/A	[c.C380T; p.S127F; MS] (CPRIT-NGS)	[c.G35T; p.G12V; MS] (CPRIT-NGS), [12TGT] (Gazdar)

HCC4017	Large Cell	No Data	11.399	#N/A	[c.C437A; p.P146H; MS] (CPRIT-NGS), [c.833C>A;p. P278H] (Gazdar)	[c.G34T; p.G12C; MS] (CPRIT-NGS), [12TGT] (Gazdar)
HCC3051	Large Cell	No Data	10.838	#N/A	[c.C451T; p.P151S; Likely Som] (CPRIT-NGS Unpaired)	WT
H226	Squamous Mesothelioma	No Data	11.464	#N/A	[CGC to CTC] (NCI-Navy)	WT
H596	Adenosquamous	No Data	12.673	#N/A	[GGC to TGC] (NCI-Navy), [c.G734T; p.G245V; Likely Som] (CPRIT-NGS Unpaired)	WT
H1299	Large Cell	No Data	12.63	#N/A	[HD] (Gazdar)	WT
HCC1195	Adenocarcinoma (mixed)	No Data	10.855	#N/A	WT	WT
H2052	Mesothelioma	No Data	11.88	#N/A	WT	WT
H292	Muco-epidermoid carcinoma	No Data	12.295	#N/A	WT	[c.34G>A; p.G12S; MS; Het; Likely Som] (COSMIC-v67), WT (COSMIC-v67)

H460	Large Cell	No Data	12.378	#N/A	WT	[c.?; p.Q61H; MS; Likely Som] (COSMIC-v67), [c.183A>T; p.Q61H; MS; Likely Som] (COSMIC-v67), WT (COSMIC-v67), [c.?; p.Q61H; MS; Likely Som] (COSMIC-v67), [c.183A>T; p.Q61H; MS; Hom; Som] (COSMIC-v67), [c.183A>T; p.Q61H; MS; Likely Som] (COSMIC-v67), [c.183A>T; p.Q61H; MS; Som] (COSMIC-v67), [61CAT] (Gazdar)

A427	0	No Data	No Data	#N/A	WT	[c.35G>A; p.G12D; MS; Likely Som] (COSMIC-v67), [c.35G>T; p.G12V; MS; Som] (COSMIC-v67), [Mut] (Gazdar), [c.G35A; p.G12D; Likely Som] (CPRIT-NGS Unpaired)
H2452	Mesothelioma	No Data	No Data	#N/A	WT	WT
HBEC34-KT	Immortalized Normal	No Data	11.42	#N/A	No Data	No Data
HBEC13-KT	Immortalized Normal	No Data	11.718	#N/A	No Data	No Data
HBEC30-KT	Immortalized Normal	No Data	12.373	#N/A	No Data	No Data
HBEC3-KT	Immortalized Normal	No Data	12.542	#N/A	No Data	No Data

APPENDIX C: MYC TARGET GENE SETS

ACOSTA_PROLIFERATION_INDEPENDENT_MYC_TARGETS_UP	ALFANO_MYC_TARGETS	COLLER_MYC_TARGETS_UP	DANG_MYC_TARGETS_UP
AATF	AATF	AHCY	ADRA1B
AIM1L	ABCA3	AK4	AIMP2
BLMH	ABCC5	ASS1	AKAP1
C19orf2	ABCF2	C1QBP	APEX1
C7orf68	ACTR1A	CCND2	BAX
CCNE1	ADCY3	CEBPZ	BCAT1
CD320	ADD3	CKS2	CAD
CECR5	ADM	EIF5A	CBX3
CSE1L	AES	FABP5	CCKBR
CTPS	AFAP1	FBL	CCNB1
CUTC	AGRN	FKBP4	CCNC
DDX28	AK4	G0S2	CCND2
DHCR7	AKAP1	GPI	CCT5
DUSP9	AKAP12	GRPEL1	CDC25A
EIF3J	ANXA1	HDGF	CDC25C
EIF4A3	ANXA7	HSPD1	CDK4
EXPH5	ARL6IP1	IARS	CEBPZ
FBXO41	ARPC5	NAMPT	CKS2
GCDH	ASB13	NCL	CSDA
GCSH	ATF7	ODC1	CSTB
GLB1L2	ATP1B1	POLR2H	CTSC
GLOD4	ATP8A1	PPIF	DBI
GLS	ATP8B1	SLC16A1	DDX10
GNPDA1	B3GNT1	TFRC	DDX18
JAG2	BLM	TRAP1	DDX5
KIAA0090	BLMH		DKC1
KIAA0664	BMP4		DPY30
KIAA0930	C10orf26		E2F1
LAS1L	C12orf24		E2F3
LEPR	C18orf1		EIF2S1
LOC730101	CACNB2		EIF3C
LPL	CAMKK2		EIF4A1
LYPLA1	CCL2		EIF4B

MAST2	CCNA1		EIF4E
MATK	CCNI		ELAVL1
MINA	CD1D		EMP1
MPZL1	CD59		ENDOG
MRPL4	CDC42EP3		ENO1
MTMR4	CDK2AP2		EXOSC8
MTRR	CDKN1C		FASN
NOLC1	CDX1		FOSL1
NQO1	CELSR3		FXN
NR1D2	CLCN2		GAPDH
NRTN	CLCN7		GGH
NUP210	CLK1		GNL3
NUP214	COPB1		H2AFZ
ODC1	CPN1		HMGA1
OPN3	CPNE3		HNRNPA1
OXCT1	CRIP2		HNRNPA2B1
PARP1	CSE1L		HSP90AA2
PDCD11	CSRP1		HSPA4
PEBP1	CTH		HSPA8
PEX5	CTNND1		HSPA9
PRPF19	CTSK		HSPD1
PSMG1	CXCR7		HSPE1
PTK7	CYR61		ID2
PWP1	CYTIP		IGKC
RAB3A	DAB2		IMPA2
RCHY1	DDB2		IRF9
REPIN1	DDX17		LDHA
RGS14	DDX21		LTA4H
RGS16	DHCR7		MAT2A
RRP1B	DKK3		METTL1
SKP2	DLST		MGST1
SLC17A7	DNAJB6		MINA
SLC19A2	DNAJC11		MIR155HG
SLC27A2	DSG1		MRPL10
SLC29A1	DUSP2		MRTO4
SLC39A4	E2F5		MSH2
SLC39A6	EBNA1BP2		MTHFD1
SLC5A3	EEF1A1		MYCT1
SORD	EHD1		NAP1L1
SPG21	EIF2B1		NBN
STEAP1	EMP1		NCL

STUB1	ENPEP		NME1
TMEM161A	EPHA1		NME2
TRIAP1	FARSA		NPM1
TSPO	FGA		NRAS
UCK2	FILIP1L		NUP155
VPS13C	FKBP4		ODC1
WDR74	FLOT1		PA2G4
XPO6	FRZB		PAX3
ZNF395	FYN		PCNA
ZNF460	GAD2		PHB
	GAS6		PINK1
	GLDC		POLD2
	GLOD4		PPAT
	GNAI2		PPIA
	GNPDA1		PPID
	GOSR1		PRDX3
	GPD1L		PREP
	GPR143		PRPS2
	GREM1		PSMB1
	GSN		PSMG1
	HERPUD1		PTMA
	HMGB3		PYCR1
	HOXC9		RCC1
	HS3ST1		RFC2
	HSPA1L		RPL10
	ID3		RPL13
	IGFBP1		RPL19
	IMP4		RPL22
	IMPDH1		RPL26
	IRF1		RPL27
	ITGA6		RPL27A
	ITGA7		RPL3
	ITGB5		RPL32
	KARS		RPL5
	KCNH2		RPL9
	KCNN4		RPP30
	KCTD12		RPS13
	KIAA0368		RPS16
	KIAA0930		RPS17
	KIF3C		RPS19
	LAPTM4A		RPS20

	LARGE		RPS5
	LARP1		RPS6KA2
	LASP1		SHMT1
	LRRC17		SLC25A3
	LTBP1		SLC39A6
	LY6E		SMN1
	MAG		SNRPB
	MAP1B		SNRPD3
	MARCKS		SRM
	MCL1		SRSF1
	MDN1		SRSF2
	METAP1		SRSF7
	MGLL		SURF6
	MMADHC		TERT
	MME		TESK1
	MMP17		TFRC
	MSTN		TIMM10
	MTIF2		TK1
	MVP		TOMM22
	MXI1		TOP1
	MYBL2		TP53
	MYC		TXN
	MYCBP		TYMS
	MYL12A		UBE2C
	MYL6		UCHL1
	MYLK		UPRT
	NBL1		UXT
	NHP2L1		XIAP
	NOP56		
	NPAS1		
	NPC2		
	OXCT1		
	PAFAH1B3		
	PAICS		
	PCCA		
	PCDH8		
	PCYT1B		
	PDCD11		
	PDIA5		
	PDLIM4		
	PES1		

	PEX5		
	PGAM2		
	PHLDA1		
	PICALM		
	PLAU		
	PLAUR		
	PLEC		
	PLK4		
	PNP		
	PON1		
	PPAT		
	PPIF		
	PPP1R15A		
	PPP6C		
	PPPDE2		
	PSMG1		
	PTPN12		
	PTPRN2		
	PXN		
	PYCR1		
	PYGB		
	QDPR		
	QSOX1		
	RAB5B		
	RAP1GAP		
	RAPGEF2		
	RASSF4		
	RBBP7		
	RBM5		
	RBP1		
	RCC1		
	RER1		
	RND2		
	RRP12		
	RSL1D1		
	RSL24D1		
	RUNX3		
	RUVBL1		
	S100A10		
	SDC1		
	SDC4		

	SDCBP		
	SLC12A1		
	SLC16A1		
	SLC20A1		
	SLC39A6		
	SLPI		
	SMPD1		
	SMPD4		
	SMTN		
	SNRPD1		
	SOX9		
	SPARC		
	STC1		
	STMN1		
	STOM		
	SURF2		
	SVIL		
	SYNGR2		
	TACC2		
	TARBP1		
	TATDN2		
	TCF19		
	TCF7L2		
	TCOF1		
	TFAP4		
	TGFB1I1		
	TGFB2		
	TGM2		
	TIMP2		
	TOMM40		
	TP53BP2		
	TRAP1		
	TRIP6		
	TTLL12		
	TUFM		
	UBE2K		
	UCK2		
	VCL		
	VCP		
	XK		
	ZEB1		

	ZNF177		

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VITAE

Patrick was born in Chicago, Illinois, on February 16, 1985, son of Steve and Nora Dospoy. He has three brothers: Christopher, Kevin and Sean. Patrick graduated from Brother Rice High School in 2003. That same year he was awarded a full tuition scholarship to the University of Chicago, in conjunction with the Chicago Fire Department. He enrolled in The College at the University of Chicago and began his undergraduate study in Biological Sciences. He began his research career under the supervision of Dr. Elizabeth McNally, who was the Director of the Cardiovascular Institute at the University of Chicago. In 2007, Patrick graduated with a Bachelor of Arts from The College and continued his research in the laboratory of Dr. McNally. In 2008 he accepted a position as an Analyst with a life sciences consulting firm (ClearView Healthcare Partners) in Boston, MA. In 2010, Patrick rejoined the research community as he began his graduate work at the University of Texas Southwestern Medical Center. He performed his dissertation research under the guidance of Dr. John D. Minna within the Cancer Biology program of the Graduate School for Biomedical Sciences at UTSW and successfully defended his thesis in July, 2015.

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