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The role of the D-element in Myc biology

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Danny Nejad Khalil

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Abstract of the Dissertation

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The oncogene *c-Myc* encodes a modular transcription factor that is deregulated in various human cancers. While much of the protein has been well characterized, a central region known as the D-element has not. In order to gain a better understanding of the role of this region, I conducted screens to identify a protein that potentially interacts with it and mediates its biological activity. I have found the product of the *eno1* gene to be such a factor, as it physically associates with the D-element, decreases Myc stability, and attenuates Myc's ability to drive neoplastic transformation in mammalian cells. Furthermore, there is a significant correlation between *c-Myc* and *eno1* copy number in human cancer samples, suggesting that this interaction has clinical importance.

for Malka

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List of abbreviations

Ade, adenine
ATPase1, a subunit of the proteasome's 19S regulatory particle
BrdU, bromodeoxyuridine
CD, Cytochalasin D
c-Myc, cellular Myc gene
co-IP, co-immunoprecipitation
DBD, DNA binding domain
DMF, Dimethylformamide
DMSO, Dimethyl sulfoxide
DΔMb3, the D-element deleted for Myc box III
E2, ubiquitin-conjugating enzyme
E3, ubiquitin ligase
E-box, enhancer box
EGFP, enhanced green fluorescent protein
eno1, enolase1α gene
Enolase, enolase 1α
GFP, green fluorescent protein
HA, hemagglutinin
HDAC, histone deacetylase
His, histidine
IB, immnoblot
IP, immuneprecipitation, immunoprecipitate
IRES, internal ribosome entry site
Leu, leucine,
MbI, Myc box I (conserved region within Myc, amino acids 44-63)
MbII, Myc box II (conserved region within Myc, amino acids 128-143)
MbIII, Myc box III, (conserved region within Myc, amino acids 188-199)
MBP-1, Myc-promoter-binding protein 1
MG132, chemical proteasome inhibitor
Myc, the 64kD protein product of the *c-Myc* gene
NIH3T3: mouse-embryo-fibroblast cell line
NLS, nuclear localization signal
ONPG, ortho-Nitrophenyl-β-galactoside
PAGE, polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
ROMA: Representational Oligonucleotide Microarray Analysis

SDS, sodium dodecyl sulfate
shRNA, short haipin RNA
shRNA, short hairpin RNA
TAD, transcriptional activation domain
TLS, tumor lysis syndrome
Trp, tryptophan
U20S, Human osteosarcoma cell line
ZPS, ZsProSensor

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CHAPTER 1: Introduction

Myc is a 64kD transcription factor. It is the predominant product of the human *c-Myc* gene (*1*). Originally discovered as the homologue of a viral gene associated with avian blood cancer (*2*), Myc has emerged, in the subsequent three decades, as one of the most studied and yet most enigmatic human oncogenes. Myc is reported to be amplified and upregulated in a wide array of human cancers (*3*) and Myc overexpression is the defining genetic lesion of Burkitt's lymphoma, one of the most aggressive human cancers (*4*).

c-Myc is tightly regulated at all levels of expression: transcription, transcript stability, translation, and protein stability (*5-7*). It is the last that is the focus of this study, particularly the role of Myc protein stability as it relates to the initiation and progression of cancer.

Myc structure

The human *c-Myc* gene is located at chromosomal locus 8q24. It is composed of three exons. Translation beginning in the second exon produces the

predominant product, the 64kD Myc protein. Once the gene is transcribed the transcript is tightly regulated (8) and has a half-life of approximately 20 minutes.

Like most transcription factors, Myc is a modular protein. Its transcriptional activation domain (TAD) is comprised of the N-terminal 143 amino acids (figure 1). Myc's TAD functions primarily by recruiting cofactors that facilitate gene activation and repression (9). These co-factors are mainly recruited at the two conserved regions within the TAD known as Myc box I (MbI) and Myc box II (MbII), the latter of which appears to be essential for nearly all Myc functions. As is the case with many transcription factors, Myc's TAD overlaps with a region, known as the degron, which confers instability to the protein. Myc's TAD appears to lack a three-dimensional structure; a feature which may facilitate Myc's ability to bind multiple other proteins.

The C-terminal region of Myc is its DNA-binding domain (DBD). It is composed of a basic region, a helix-loop-helix, and a leucine zipper. The leucine zipper binds Max and the heterodimer binds, in turn, to consensus elements known as enhancer boxes (E-boxes) in the promoter of target genes (10, 11). While there is no known crystal structure corresponding to Myc's TAD, the crystal structure of Myc's DBD has been solved (12). The basic region forms an α-helix that is continuous with the N-terminal α-helix of the helix-loop-helix domain while the leucine zipper forms an α-helix that is continuous with the C-

terminal α-helix of the helix-loop-helix domain. It is the leucine zipper in the center of the DBD that presumably binds consensus sequences in genomic DNA.

Outside of the TAD and DBD there are two other important elements of Myc structure. Myc's nuclear localization signal, which is necessary for its nuclear import, is located just N-terminal to its DBD. Lastly, a central region know as the D-element has been found to be a second region of the protein that confers instability. Within the D-element, there is one final conserved region: Myc box III (MbIII).

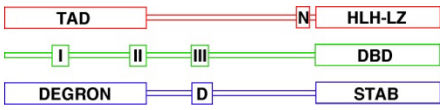

Figure 1: Myc. The cartoon shows the relationship between functional elements in Myc (top), conserved sequences (middle) and stability determinants (bottom).

Myc's molecular function

During three decades of research into Myc biology it has been proposed that Myc has both transcriptional and non-transcriptional functions (13). Today Myc's role as a transcription factor is well established, whereas possible non-transcriptional roles are still debated (14-17).

As a transcription, factor Myc regulates expression of 10-20% of the human genome (18-22). Initially Myc's role in transcriptional regulation was deduced from its localization to the nucleus and the fact that it could activate transcription at a specific promoter (23). Subsequent data suggested that Myc is a modular transcription factor with a distinct TAD and DBD (24). The finding that each of these regions is necessary for Myc to transform cells (25-27) suggested that Myc's ability to act as a transcription factor in inseparable from its role in transformation. Today we have clearer evidence that Myc regulates the expression of multiple different genes.

There are multiple specific mechanisms by which Myc induces gene activation, and more are currently being identified. Perhaps the most well-established general mechanism by which Myc activates transcription is by recruitment of factors that alter chromatin structure. One such factor is TRRAP, a protein essential for Myc to induce transformation (28), which in turn recruits a component of the histone acetyltransferase complex SAGA (29). Myc has also been shown to bind chromatin remodeling components such as INI1 (30), a component of the human SWI-SNF complex, as well as components of the basal transcription machinery (31).

In addition to Myc's ability to activate target genes, Myc is also able to repress a distinct set of target genes. Interestingly, the first gene shown to be repressed by Myc was *c-Myc*. This conclusion grew out of the finding that in

malignant cells from Burkitt's lymphoma Myc expression from the endogenous locus was almost entirely repressed (32-37), suggesting that the product of the aberrantly overexpressed *c-Myc* allele was inhibiting the non-translocated allele. Today there is a long and growing list of genes that are known to be repressed by Myc, and it appears that Myc depends on an interaction with the zinc-finger-containing protein Miz1 to repress most of these genes (38). It is thought that Myc causes gene repression in much the same way that it causes gene activation, namely by recruiting proteins that act on the chromatin. Recently it has been shown by our lab that Myc manifests its repressive function, in part, by recruiting histone deacetylases (39).

Myc and physiology

The fact that Myc is involved in essential physiologic cellular processes can be predicted from the fact that it is retained, and ubiquitously expressed, in higher organisms despite the risks of malignancy that it confers. Indeed, Myc deletion has been found to be lethal within the first eleven days of mouse embryo development (40) as Myc is critical at multiple stages in organ development (41) in addition to its role in the mature tissues.

Myc has been associated with cellular proliferation (42, 43), apoptosis (44), differentiation (45), self-renewal in stem cells (46, 47), global protein (48) and nucleic acid (49) synthesis, cell-size regulation (50), mitochondrial biogenesis (3), cellular migration (51), energy metabolism (52, 53), regulation of the cell's redox status (53), regulation of specific microRNAs (54), as well as other processes and pathways (3). Furthermore, Myc is able to regulate the expression of genes transcribed by RNA polymerase I (55, 56), II (20), and III (57). All in all, Myc's target genes are not limited to any specific functionality or even to a specific means of transcription.

Myc and pathology

Considering the diverse cellular processes coordinated by Myc, it is not surprising that Myc's role in disease is complex. Many of the pathways that are normally regulated by Myc go awry when Myc is deregulated.

While Myc has been associated with non-neoplastic diseases (58-60), Myc's potency as an oncogene has meant that the overwhelming focus of research on Myc's role in disease has centered on its role in cancer. Furthermore, there is almost no cellular function associated with Myc that does not at least have implications for the formation and progression of cancer. For example, the role that Myc plays in augmenting proliferation and protein production is seized upon

in cancer to drive tumor growth and supply the growing cells with the material they need to thrive. Myc's ability to induce apoptosis is often downregulated or circumvented in cancer (61). Myc's ability to drive neovascularization (62), providing tumors with oxygen and nutrients, is important for the growth many cancers. And Myc's ability to reduce cell-to-cell adhesion, and thereby promote migration, is seen as vital to metastasis (63), often the most destructive aspect of cancer progression.

Unlike some proto-oncoproteins, Myc does not require mutation to become oncogenic as evidenced, for example, by the fact that many Burkitt's lymphomas express unmutated Myc (64). This is not to say, however, that mutations in Myc cannot contribute to the molecule's oncogenicity. T58A, a mutation in Myc which is frequently found in Myc-driven blood cancers (65) and known to increase Myc stability (7), has been shown to increase Myc's oncogenic capacity by, at least in part, attenuating Myc's ability to induce apoptosis (61).

Perhaps the most telling empirical indication of Myc's role in cancer is provided by a survey of cancers in which Myc is deregulated. Myc is often associated with blood cancers, and although the effects of Myc are clearly decisive in many of them, there are many other types of cancer in which Myc deregulation has been implicated. For example, *c-Myc* was recently found to be the most frequently amplified gene in lung cancers (66). Up to 48% of breast cancers have an amplification of Myc, and approximately 45% of breast cancers

overexpress Myc (67-72). One third of liver cancers have also been shown to have Myc amplifications (73). This partial list of the diverse types of cancer in which Myc is affected draws attention to the fact that Myc deregulation can transcend profound differences in cancer type.

Myc and disease treatment

Myc's prominent place in the etiology of cancers has meant that its role in disease treatment cannot be separated from its role in malignancy, and this will likely continue as cancer treatment continues to move away from broadly cytotoxic therapies towards therapies that hone in on specific molecules and even on specific functions of molecules. Considering that Myc is involved in both proliferation and apoptosis (74), an ideal treatment would block the former and enhance the latter.

Therapies that reduce Myc activity would be particularly attractive, as multiple Myc-driven tumors have regressed subsequent to Myc deprivation in inducible animal systems. Such regression has been seen in models of pancreatic (75), blood (76), bone (77), liver (78, 79), and breast cancers (80, 81). In terms of off-target effects, animal models have shown that Myc inhibition affects physiologically regenerating tissues, not unlike conventional chemotherapies (82).

The therapeutic targeting of Myc has been approached from multiple angles. Antisense oligonucleotides designed to target and destroy Myc mRNA have been developed and have shown some promise in human cancer cells (83, 84). Antisense technology has been exploited to target Myc via peptide nucleic acids and morpholinos – molecules that use synthetic polymers in place of sugar-phosphate backbones to increase their stability and bioavailability (85-88). Myc-targeting morpholinos have even shown limited adverse effects in preliminary clinical trials (89). Synthetic oligonucleotides designed to disrupt Myc expression have been able to reduce cancer-cell growth in tissue culture (90, 91). So-called 'decoy oligonucleotides' designed to mimic Myc consensus sites, thereby competing with Myc target genes in the genome for a limited number of Myc molecules, have shown similar effects in tissue culture (92). Finally, therapies have been designed to facilitate the degradation of the Myc protein. For example, a chimera of Max and the ubiquitin ligase (E3) CHIP has been designed to prime Myc for degradation by the proteasome (93).

Unfortunately, these potential treatments of Myc-driven cancer have not progressed beyond the first phase of clinical trials. Perhaps one reason for this is that most such therapies make a crucial assumption that appears no longer to be supported by basic science. It is often taken for granted that reduced Myc levels correspond to reduced Myc function. There is, however, a growing body of evidence indicating that this is not always the case – not for transcription factors

in general (94), and not for Myc specifically (95). An ideal therapy would therefore target a specific pathway that affects Myc function with distinct and well-defined effects, not only on Myc levels, but also on Myc-dependent processes such as apoptosis and proliferation. While the work presented in this thesis does not assess therapeutics, it aims to identify such a pathway.

A role for Myc in the treatment of an entirely distinct set of maladies has emerged in the last three years. Myc has been found to be one of only a handful of genes important for inducing pluripotency in adult human cells (96). This makes Myc central to a new group of clinical problems ranging from Huntington's disease to spinal cord trauma, cases that can potentially be addressed with stem-cell therapy. Our knowledge of Myc's place in cancer biology is not independent of Myc's role in stem-cell induction; in fact the two are necessarily tied together. The fact that Myc is an oncogene complicates its use in generating stem cells for use in patients, and highlights the importance of identifying pathways that tease apart different aspects of Myc biology.

Myc stability

Myc is degraded rapidly by the ubiquitin-proteasome system (7). The half-life of soluble Myc is approximately thirty-five minutes (7, 95, 97). Deletion

studies (98) have indicated that Myc's TAD confers instability to the protein whereas Myc's DBD confers stability (figure 1).

To date there are four known ubiquitin ligases that act on Myc: skp2, fbw7, hectH9, and trim32. Skp2 has been shown to facilitate degradation and to augment Myc activity (95). Fbw7 has been shown to increase the rate of Myc degradation and to diminish its activity (99, 100). The role of fbw7 may, however, be masked by Usp28 an enzyme that deubiquitylates Myc and thereby attenuates fbw7's ability to act on it (101). Initially it was reported that hectH9 activates Myc function without affecting its stability (102). A subsequent study, however, has shown that the product of *c-Myc* is indeed destabilized by hectH9 (103), a finding that is consistent with my own data. The most recent addition to the list of ubiquitin ligases that act on Myc is trim32, an E3 that appears to facilitate Myc degradation (104).

This list of E3s that target Myc is only a partial list of proteins reported to affect Myc's activity and stability, but it highlights two important considerations in Myc biology: First, one cannot take for granted that a protein that reduces Myc stability will reduce Myc activity. Second, there is a high likelihood that a protein that affects Myc stability will, in some way, affect its activity as well.

The D-element

An important and intriguing region within Myc known as the D-element plays a critical role in Myc stability. Although the D-element is outside the classical Myc degron (figure 1) it acts to confer instability to Myc such that deletion of the D-element increases Myc stability more than twofold (98). The deletion of this element, however, does not reduce Myc's ubiquitylation as would be expected if it were the binding site for a traditional E3. In fact, deletion of the D-element causes a striking increase in Myc's ubiquitylation (98). A mechanism whereby the D-element recruits a factor that facilitates the degradation of Myc at a post-ubiquitylation step would be one possible hypothesis to account for this phenomenon.

The ability of the D-element to confer instability to Myc is cell-type specific, suggesting that a cell's genetic background affects processes that act on Myc through this element. Yet although such processes are cell-type specific the mechanism that enables them is highly conserved – even in yeast, an organism that lacks Myc (98). Therefore, it appears that the processes and molecules acting on Myc endogenously at the D-element are likely ancient ones that evolved before Myc did.

MbIII, the conserved part of the D-element (figure1), has been shown to regulate Myc's activation and repression of target genes. Deletion of this

conserved region reduces Myc's ability to fully activate various target genes as well as Myc's ability to repress target genes. Deletion of MbIII diminishes Myc's ability to transform, without affecting Myc's ability to drive proliferation but by increasing the levels of apoptosis induced by Myc (105). In a mouse lymphoma system in which the hematopoietic system is reconstituted to express exogenous genes (106), it was found that deletion of MbIII increases Myc-driven tumor latency and decreases tumor penetrance (105). Together these data indicate that MbIII augments Myc's ability to induce neoplastic transformation by negatively regulating Myc's pro-apoptotic function.

While there are several protein-binding partners known to interact with the other conserved regions of Myc there is only one known to associate with MbIII. Our lab has found that histone deacetylase 3 (HDAC3) binds MbIII and is, at least partially, responsible for the role of MbIII in gene repression (39). However, it is unlikely that this region manifests its effect on Myc stability and oncogenicity through HDAC3 alone. It is therefore of interest to identify other factors that bind the D-element and affect Myc function.

The purpose of this study

Few other cellular proteins have received as much sustained interest as Myc. After three decades of research much is known about this transcription factor, yet there are still issues of practical importance that elude us: Can Myc's ability to induce apoptosis be separated from its ability to induce proliferation? Can Myc's oncogenicity be attenuated in humans while preserving its physiologic functions? An answer to the first question would have important ramifications for the development of therapies that target Myc in cancer. An answer to the second question would shed light on the side effects of such a therapy, as well as on the role of Myc in generating induced pluripotent stem cells for safe clinical use.

A focal point of Myc biology with the potential to address both of these questions is Myc's D-element. Yet research into this region of Myc has lagged behind that of other regions of the protein. It was therefore the purpose of this study to ask the following question: How does the D-element manifest its role in Myc biology?

Knowing that Myc functions primarily by protein recruitment, I decided to begin with an unbiased screen for proteins that interact with this region of Myc. The next step was to prioritize the results of this screen. Considering that functional readouts of Myc activity can be unreliable, I decided to take advantage of Myc's short half-life and the relationship of Myc activity to stability. Based on all of this, my hypothesis was that a protein that interacts with Myc's D-element is likely to affect its stability, and that the proteins that have the greatest effect on

Myc stability would likely have an important effect on Myc's oncogenicity. Moreover, being that this region is important for some but not all of Myc's functions (in contrast to, for example, MbII or the DBD) a binding partner that is recruited to the D-element could have an effect on a subset of Myc's functions making it a promising potential tool for understanding specific aspects of Myc biology.

Method

To identify a protein that interacts with the D-element and affects Myc stability I screened for candidates that bind the D-element and then screened these candidates for an effect on Myc stability. However, in order to introduce an additional layer of stringency and to hone in on proteins that specifically affect Myc levels, I screened the candidates for an effect on Myc levels before assessing their effect on Myc stability.

There were thus three levels of screening: First, a yeast-two-hybrid screen for sequences that interact with the D-element; second a steady-state screen to determine how the candidate binding partners affect Myc levels; and finally focused stability screen to determine the remaining candidates' effect on Myc turnover (figure 2). The library of candidates assayed in the two-hybrid screen

included a total of 22 million clones; and this screen ultimately yielded 39 candidate genes. These 39 candidate genes are filtered down to seven by the subsequent steady-state screen. And the stability screen showed that only one gene among the remaining candidates – *eno1* – had a significant effect on Myc turnover.

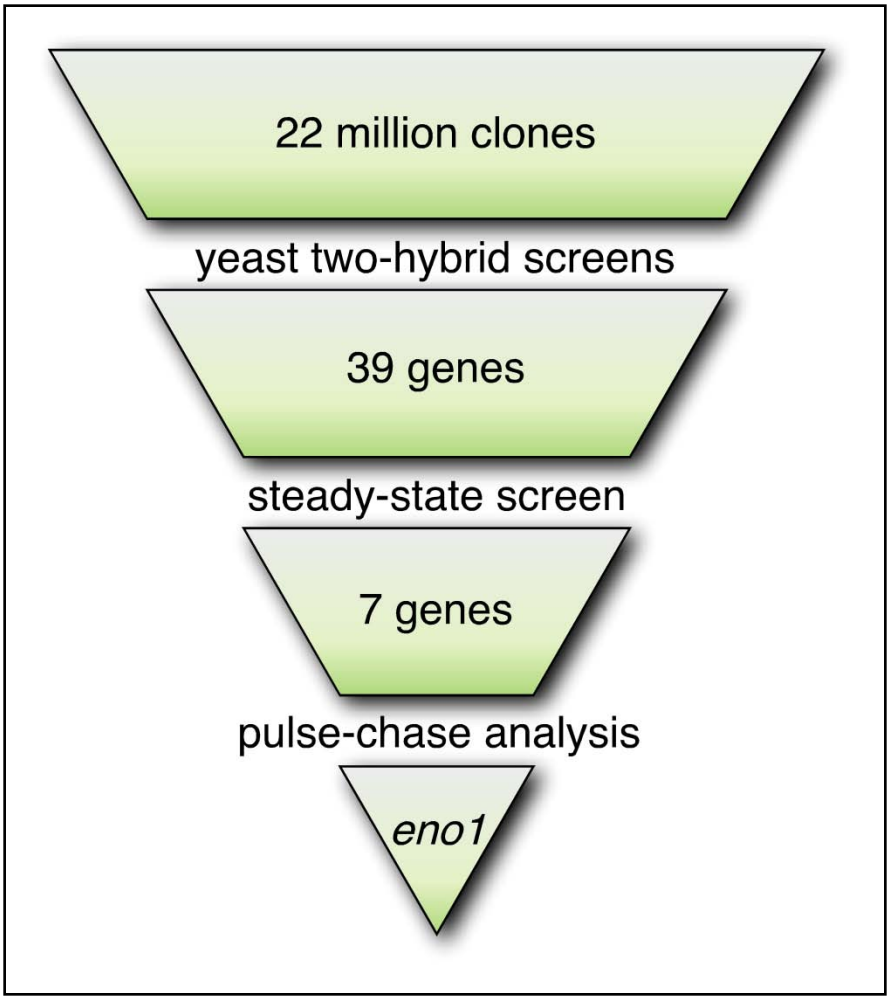

Figure 2: Schematic of screening steps. From the approximately 22,000,000 initial clones represented in the two-hybrid cDNA libraries, 39 candidate genes were identified. These candidates were then sub-screened in a steady-state RNAi-based screen from which seven emerged as genes thought to affect Myc levels. These genes were then tested by pulse-chase analysis, which yielded a single candidate: the *eno1* gene.

CHAPTER 2: Yeast-two-hybrid screen

Background:

Although it was not screened for directly, one of the ultimate goals for this project was to identify a protein that not only binds Myc's D-element and affects its activity and stability, but one that does so in a manner that affects Myc's ability to transform cells. Since these are fairly stringent criteria I began with a screen that would yield a relatively high number of candidate interacting partners (i.e., an assay with high sensitivity) from the outset so that the list of candidates could be filtered subsequently. A yeast-two-hybrid screen, therefore, was selected as a technique that is unbiased and that generally casts a wide net.

Purpose:

The purpose of this screen was to identify proteins that potentially interact with Myc's D-element.

Methodology:

The two-hybrid system

The yeast-two-hybrid system was based around the Gal4 transcriptional activator. The 'baits' were fused to the Gal4 DBD whereas the 'preys' were fused to the Gal4 TAD. A successful interaction between a bait and a prey would draw the Gal4 DBD to the Gal4 TAD and thereby activate reporter genes downstream of the Gal upstream activation sequence. These reported genes could then be selected for.

The baits & preys

I performed the two-hybrid screen two times, each with two bait sequences fused to Gal4 DBD. The first such sequence was a segment encompassing the D-element corresponding to amino acids 167-217 of Myc: GHSVCSTSSLYLQDLSAAASECIDPSVVFPYPLNDSSSPKSCASQDSSAFS. The second bait sequence was the above portion of Myc deleted for MbIII (DΔMb3). MbIII, amino acids 188-199 of Myc (ECIDPSVVFPYPL), is the most highly conserved region of the D-element. It would therefore be important to determine if a protein that binds the D-element relies on MbIII.

However, regardless of whether a candidate relies on MbIII for an interaction with the D-element it is likely that one such binding partner would, at least partially, account for the role of the D-element in Myc proteolysis. This initial hypothesis is based on the fact that Myc is a modular transcription factor that depends on protein recruitment, not only to affect its activity, but also to affect its stability.

While it was important to perform an unbiased screen, I wanted to assure that proteins likely to interact with Myc in tissues where Myc is important physiologically and pathologically were represented in the screen. Myc is necessary for the development of the healthy embryonic hematopoietic system, and the role of Myc in neoplasia of the hematopoietic system is of central significance to such diseases. I therefore performed one screen with a cDNA library derived from a human bone marrow (representing approximately 9 million independent clones). A separate screen was performed with a cDNA library derived from human cervical cancer (HeLa) cells (representing approximately 13 million independent clones). Each library, pretransformed into yeast, was purchased from Clontech. It should be noted that the cDNA derived from human bone marrow was unpurified and therefore included cells from systems other than the hematopoietic system, such as cells from adipose, endothelial, and mesenchymal tissue.

The baits were cloned into the plasmid pGBKT7, such that the Gal4 DBD would be fused to the amino terminus of each bait, and transformed into the haploid MATa yeast strain AH109. The cDNA libraries had been cloned into the plasmid pGAD subsequent to oligo(dT) priming, such that the Gal4 TAD would be fused to the amino terminus of each prey sequence, and were pretransformed into the haploid MATα yeast strain Y187.

Reporter genes:

A successful interaction between a bait and a prey would draw the Gal4 DBD to the Gal4 TAD thereby reconstituting the Gal4 modular yeast transcriptional activator. This reassembled molecule would thus become transcriptionally competent and bind, intact, to Gal upstream activation sequences 5' of four reporter genes: *ade*, *his*, *mel1*, and *lacZ*. *Mel1* expresses α-galactosidase, an enzyme that is secreted from the cell and degrades the sugar x-α-galactose forming an end-product that causes colonies to appear blue. *LacZ* encodes β-galactosidase which can convert ortho-Nitrophenyl-β-galactoside (ONPG) to o-nitrophenol, a product that absorbs light with a wavelength of 420nm, thus allowing one to quantify an interaction by measuring the levels of this product spectrophotometrically.

Matings & selection

Mating between AH109 yeast containing the bait in the pGBKT7 construct and Y187 yeast containing the prey in the pGAD construct were conducted in liquid YPAD media for 24hrs with gentle shaking. Cells were then pelleted, resuspended, and plated onto plates lacking tryptophan (to select for yeast containing pGBKT7), leucine (to select for yeast containing pGAD) as well as adenine and histidine (as *ade* and *his* are two of the reporter genes). 20mM of 3-aminotriazole (3-AT) and 40ng/mL of x-α-galactose were included in the media for reasons described below.

The absence of *ade* expression in cells on media lacking adenine will cause the accumulation of a precursor that causes cells to turn red. Therefore, in order to eliminate colonies in which a weak interaction is taking place, I avoided colonies with any visual trace of red or pink color.

Leaky *his* expression is a phenomenon that can lead to the selection of false-positives in screens such as this one. In order to minimize this problem we seeded cells on medium containing a competitive inhibitor, 3-AT, of the product of the *his* gene. I initially titrated several concentrations of 3-AT such that I could eliminate background growth (in this case, from a negative control interaction between lamin and p53), but still detect a positive interaction (in this case, from a positive-control interaction between p53 and SV40 large-T antigen). Ultimately 20mM of 3-AT proved optimal.

Lastly, the medium contained 40ng/mL of x-α-galactose so that I could select for colonies that process this sugar and become blue in color. This sugar was diluted in dimethylformamide, applied to plates, and spread with glass beads once the medium had solidified and dried.

I could thus select for the expression of three of the four reporter genes after the cells were mated and the colonies formed. *His* expression was suggested by the colonies ability to grow on medium lacking histidine and containing 20mM of 3-AT. Expression of *mel1* was suggested by the formation of blue colonies in the presence of x-α-galactose; and expression of *ade* was suggested by the absence of any red color in the colonies (particularly after cells were replated onto medium lacking x-α-galactose so that the blue color would not obscure any red coloration).

Plasmid recovery

Colonies that expressed the reporter genes *ade*, *his*, and *mel1* were selected and grown in 5mL cultures until saturation. Plasmids were then recovered from yeast using the Zymoprep kit from Zymo Research. The plasmids were then used to transform competent bacteria that were subsequently grown on ampicillin-containing medium to select for the presence of the plasmid encoding the candidate sequence (pGAD). Bacteria were grown overnight in a 96-well block that was then spun down to pellet the bacteria. Pellets were submitted

directly to the Cold Spring Harbor sequencing facility to reveal the identity of each candidate sequence.

Results:

Interacting sequences

Candidate genes

Preliminary data were collected before I joined the lab by a postdoctoral fellow, Andreas Herbst, who performed a two-hybrid screen using the same two baits that I used and a cDNA library from HeLa cells. He identified three candidates: enolase1α, a glycolytic enzyme; Ump1/POMP, a protein involved in proteasome maturation; and ETFA, a protein involved in mitochondrial metabolism.

My two rounds of subsequent screening, using cDNA libraries from HeLa cells and bone marrow cells respectively, yielded a total of 39 candidates. These candidates can be grouped into categories that are not mutually exclusive (figure 2, bottom) that emphasize the fact that over one third of them are involved in either protein stability or transcription.

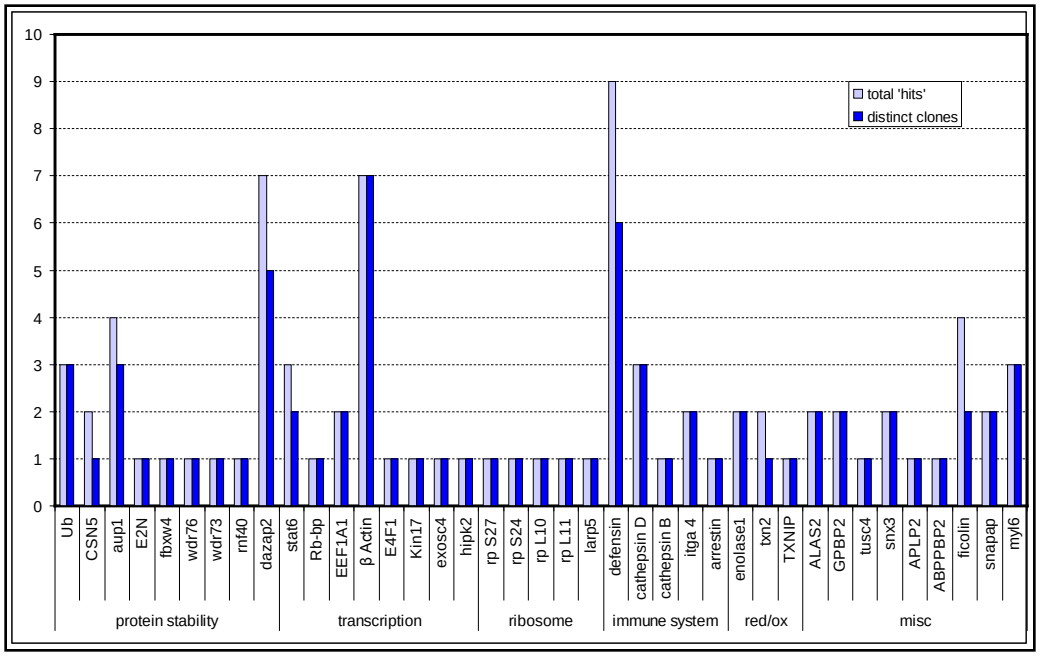

Figure 3: Pooled results of both two-hybrid screens. Total number of interactions per gene (light blue) and total number of distinct clones per interaction (dark blue) are indicated. The candidates are divided into six non-mutually-exclusive categories based on known or predicted functionality.

It was important to determine which repeated interactions resulted from overrepresented clones in the library and which represented a number of distinct clones corresponding to overlapping regions of a given gene (the latter being more likely to represent a real physical interactions). I therefore determined the number of distinct clones corresponding to each gene in addition to the total number of 'hits' for each candidate gene (figure 2).

β-actin yielded the greatest number of distinct clones. All seven of the recovered plasmids corresponded to different clones expressing overlapping

region of this gene. This was particularly interesting as β-actin has often been reported to be in the nucleus (107-113) and to be specifically involved in transcription (114-122). Considering that cytochalasin D (CD), an inhibitor of actin polymerization, also appears to affect actin's roles in transcription (123-130) I decided to test the effect of CD on Myc target gene expression using an inducible-Myc system. In addition, I tested a panel of actin inhibitors (including CD) for changes in endogenous Myc steady-state levels by Western blot. Both sets of experiments yielded no discernable effect on Myc status (data not shown).

Among the other candidates from the screens, four (*aup1*, *dazap2*, *eno1*, and *myl6*) arose at least once in each of the latter two screens. Only one of the four, *eno1*, encodes a protein that is reported to localize to the nucleus (131). Furthermore, *eno1* was the only gene to emerge in Andreas Herbst's screen as well as both of my screens.

Candidate domains

In addition to scoring the genes that interacted with the D-element in the two-hybrid screen I was also able to score the domains interacted. Four such regions arose repeatedly: the WD40, RING, UBR, and CUE domains.

WD40 repeats, which frequently mediate protein-protein interactions, bound the D-element six times in the two-hybrid screens. These six interactions represented six distinct clones from four distinct genes (*gnb2*, *fbw4*, *wdr76*,

rbbp4). While any one of these genes may be physically interacting with the D-element, the fact that this domain interacts with the D-element in the context of different genes suggests the possibility that other genes, not detected in this screen, might be interacting with the D-element through their WD40 domains.

The RING domain, which is a component of various ubiquitin ligases, arose in the screens two times, once as part the gene ring finger protein 40 (*rnf40*) and once as part of the ubiquitin ligase E3C (*UBE3C*) gene.

The UBR domain is a component of special ubiquitin ligases that recognize residues that signal degradation (degrons) at the amino terminus of various proteins. The UBR box arose seven times, with five distinct clones. Surprisingly, all interactions arose from sequences produced when the *DAZAP2* gene was translated out of frame (frame +2). The fact that this protein sequence, which presumably does not exist in nature, has extremely high homology to known UBR box domains suggests two possibilities. One, that the D-element interacts with another UBR domain that is a component of another protein. Or, that *DAZAP2* can undergo alternate translation initiation in a different frame to produce this protein sequence that is able to interact with the D-element.

Finally, the CUE domain, a ubiquitin binding motif, interacted with the D-element four times via three distinct clones, all of which were a part of *aup1* (ancient ubiquitous protein 1) another gene with unknown function. The fact that this domain repeatedly binds the D-element in the two-hybrid screen, and that the

D-element contains a lysine residue, raises the possibility that the D-element is ubiquitylated.

To test this possibility I mutated the one lysine in the D-element and quantified the strength of the interaction by spectrophotometrically quantifying the end-product of ONPG degradation, o-nitrophenol, which corresponds to the expression level of the *lacZ* reporter gene. I detected no significant difference in the strength of the interaction with *aup1* between the wild-type D-element and the D-element with lysine mutated.

Conclusions and significance:

The two-hybrid screens were successful in that they identified a number of different proteins that may bind the D-element. While I was cognizant of the specific properties of each potential binding partner (e.g., the role of β-actin in chromatin remodeling) and how they could account for the function of the D-element, it was important not to be prejudiced by what is already known about each candidate. That is, while I performed some experiments to test hypotheses suggested by specific interactions, no candidates were eliminated before the next stage of screening.

Overview

The two-hybrid libraries included 22 million clones (13 million from HeLa-cell cDNA and 9 million from bone-marrow cDNA). The screens yielded a total of 39 candidate genes. Considering that the D-element destabilizes Myc, and the more general connection between transcription factor stability and activity, I planned to screen these candidates for an effect on Myc stability. Being that it is difficult to rapidly and reliably screen for effects on protein stability, I decided to perform an intermediate screen on steady-state levels next. After a relatively high-throughput, quantitative screen of the 39 candidates' effect on Myc steady-state levels I then assayed the remaining candidates' effect on Myc stability using the pulse-chase method (figure 2).

It is important to note that this method is not relying on sequential *confirmatory* screens (i.e., the screens are not intended to confirm one another). That is, one screen will not indicate whether any of the results of the previous screen were false positives. For example, the steady-state screen indicated that 32 of the 39 candidates from the two-hybrid screen likely do not affect Myc stability; it did not indicate whether candidates from the two-hybrid screen actually interact with the D-element. Likewise, the pulse-chase analysis showed that six of the seven candidates from the steady-state screen do not have a dramatic effect on

Myc turnover, it did not show whether candidates from the steady-state screen affect Myc levels.

This means that candidates that were not ultimately characterized in this project may have interesting roles in Myc biology. A number of candidates among the thirty-nine from the two-hybrid screen, particularly the seven that seemed to affect Myc levels in the steady-state screen (including *rnf40*, which is of special interest) may indeed act on Myc through the D-element. The goal of this work, however, was to identify a candidate that binds the D-element and affects Myc steady-state levels as well as stability.

CHAPTER 3: RNAi-based screen for genes that affect Myc steady-state levels

Background:

Knowing that I would go on to screen candidates' effect on Myc turnover, I sought an intermediate screening step to eliminate some candidates from the two-hybrid screen in a rapid, quantitative, and inexpensive manner. In 2004 Silva *et al.* described a technique in which short hairpin RNAs (shRNAs) could be rapidly screened for their effect on the steady-state levels of a protein that is degraded by the proteasome (132). Since Myc proteolysis is mediated by the ubiquitin proteasome, I decided to attempt to adapt this system to identify proteins that affect Myc stability.

Purpose:

The goal is to screen the candidates from the two-hybrid screens for an effect on Myc steady-state levels, in order – ultimately – to identify a protein that affects Myc proteolysis.

Methodology:

This screen was initially developed to assay the levels of ZsProSensor (ZPS). ZPS is composed of ZsGreen, a green fluorescent protein from coral *Zoanthus* species, fused to the amino-terminus of the mouse ornithine decarboxylase degron. This degron allows ZPS to be rapidly degraded by the proteasome. ZPS was expressed from the pZsProSensor-1 plasmid, commercially available from Clontech. In order to screen for changes in ZPS levels, pZsProSensor-1, and two other plasmids, were transiently transfected into HEK 293T (human embryo kidney) cells. The two other plasmids expressed dsRed and one of multiple shRNAs.

The dsRed protein is a red fluorescent protein from *Discosoma* species. It is highly stable, and is included to emit a red fluorescent signal against which the green fluorescent signal from ZPS can be normalized. This normalization is performed to correct for variance in green signal that cannot be attributed to changes in intracellular changes in ZPS steady-state levels, such as fluctuations in transfection efficiency and cell number.

The third plasmid was pSM2c, and it expressed one of a number of shRNAs from the U6 RNA Polymerase-III promoter. The pSM2c plasmids are commercially available from Open Biosystems. The shRNA molecules targeted specific genes, such as components of the proteasome. The control hairpin, used

to establish the baseline, was designed to target the firefly luciferase gene. A hairpin designed to target the proteasome, for example, would cause accumulation of ZPS. The buildup of ZPS would produce an increased green fluorescent signal which would then be normalized to the constant red signal.

The first step in adapting this system to measure changes in Myc steady-state level was to express GFP-tagged Myc and ensure that the instability of Myc is not disrupted by the presence of this fluorophore. Fortunately such a construct had already been generated and tested in our lab. The pCGN-EGFP-Myc plasmid encodes HA-tagged Enhanced Green Fluorescent Protein (EGFP) fused to Myc. EGFP is a variant of GFP from Aquorea victoria modified to increase fluorescence. In this construct EGFP is fused to the N-terminus of Myc and the HA tag is fused to the N-terminus of EGFP. Pulse-chase experiments (as described in Chapter 4) have shown that Myc's short half-life is maintained in EGFP-Myc.

Next, we confirmed that gross changes in Myc levels could be seen visually as changes in green versus red fluorescence. To test this I treated cells expressing EGFP-Myc and dsRed with 25μM of the proteasome inhibitor MG132 for 24hrs and compared this to a control sample treated with vehicle (DMSO). I then examined the cells under a fluorescent microscope and was able to visualize changes in green fluorescence (corresponding to increased Myc levels) as red

levels remained essentially unchanged (figure 4). Quantitatively, this would be scored as an increase in the green to red fluorescence with MG132 treatment.

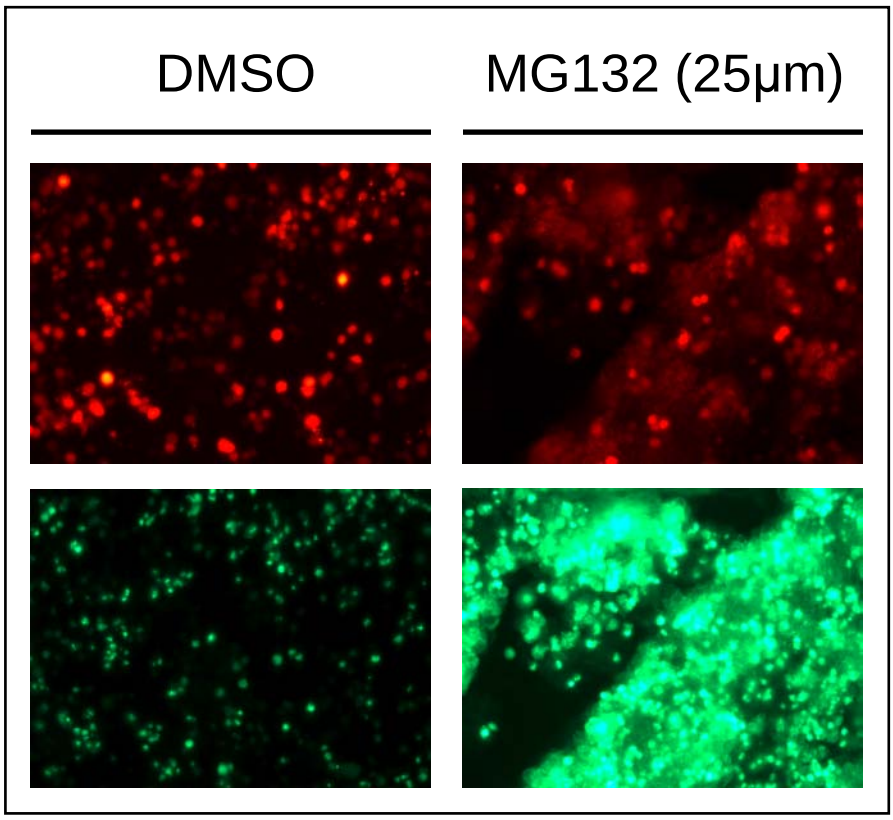

Figure 4: MG132 increases green, but not red, fluorescence in human cells transfected with EGFP-Myc and dsRed. HEK293T Cells were transfected with 10ng of pCGN-EGFP-Myc and 13.3ng of the dsRed1 plasmid. Cells were then treated with 25μM MG132 or vehicle (DMSO) for 24hrs.

Having established that levels of EGFP-Myc are responsive to proteasome inhibition whereas dsRed levels remain essentially unchanged, I then attempted to quantify this information from cells assayed on a 96-well plate. To do this I used a Perkin Elmer/Wallac Victor 2 multilabel counter (model # 1420). This plate

reader is capable of detecting fluorescence throughout a range of spectra from individual wells of a 96-well plate. The apparatus was set to detect fluorescence from EGFP (excitation 488nm, emission 507nm) and dsRed (excitation 545nm, emission 620nm) in each well of a standard-sized (8 x 12cm) 96-well plate.

One initial concern, however, with this system is that emissions from one fluorophore might 'bleed' into the spectrum of the other. In order to test this possibility, cells were transfected with dsRed and EFGP-Myc individually to determine if green signal would erroneously be detected from cells expressing dsRed, or red signal erroneously from cells expressing EGFP-Myc. Our data indicate that this would not be a significant problem as only cells expressing both EGFP-Myc and dsRed registered a signal in both spectra (figure 5).

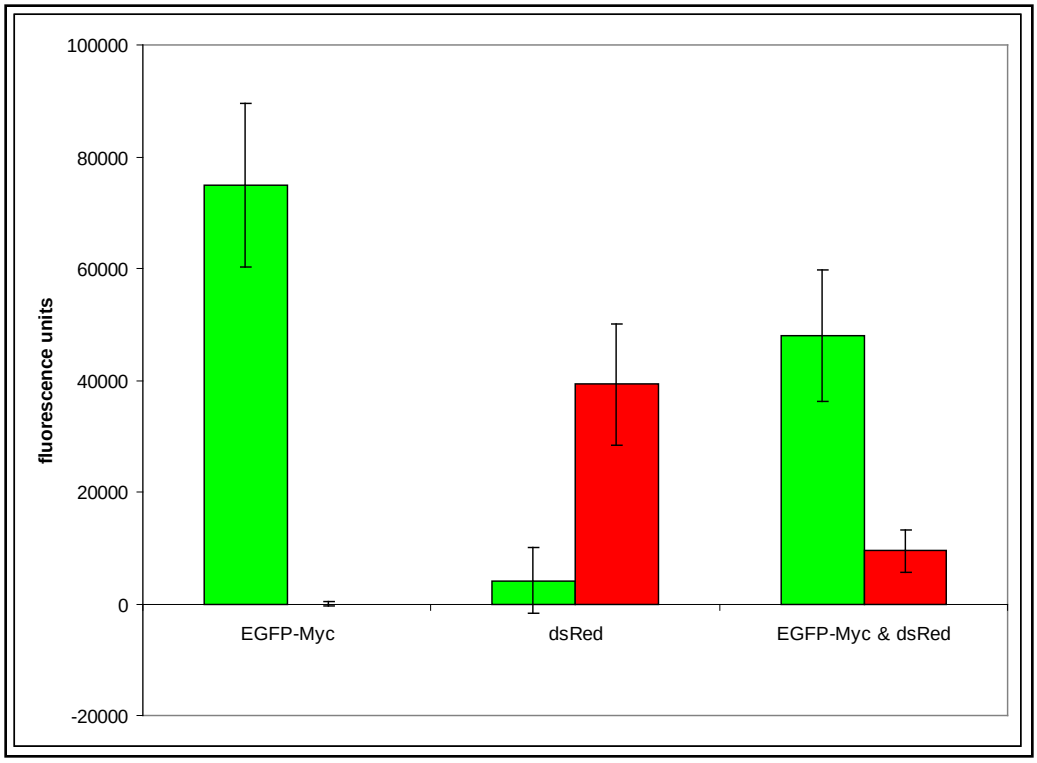

Figure 5: Green and red fluorescence detected distinctly. HEK293T cells were transfected with 10ng of pCGN-EGFP-Myc (left) 13.3ng the dsRed1 (center) and both together (right) and analyzed with a Perkin Elmer/Wallac Victor 2 multilabel counter (model # 1420) 24hrs later.

At this stage it was important to assure that the system could read out changes in Myc levels in response, not only to chemical inhibition of the proteasome as with MG132, but also to genetic inhibition of the proteasome. To test this, I transfected EGFP-Myc-expressing cells with an shRNA against the ATPase1 subunit of the proteasome. I included an shRNA against firefly luciferase as a negative control and MG132 treatment as a positive control. This

set was tested side-by-side with cells expressing ZPS instead of EGFP-Myc (figure 6). These data indicate that genetic and chemical inhibition of proteasomal degradation affect Myc levels in a manner, and to a degree, that can be read out quantitatively as changes in green to red fluorescence.

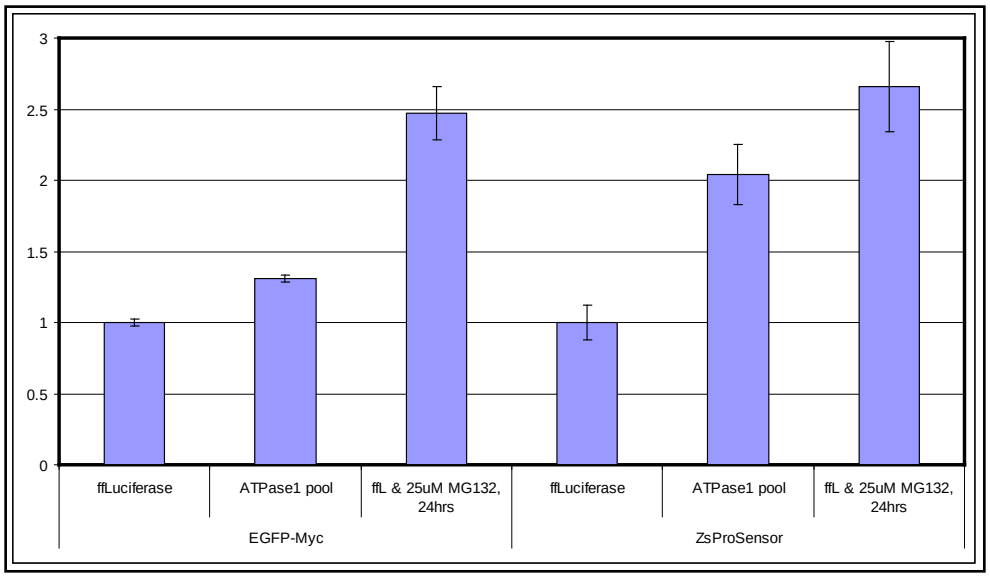

Figure 6: Genetic and chemical inhibition of the proteasome affects EGFP-Myc and ZsProSensor similarly. HEK293T cells were transfected with 13.3ng dsRed, 10ng of pCGN-EGFP-Myc or 10ng of ZsProsensr-1, and 33ng of pSM2c-ffLucferase or 33ng of pooled pSM2c plasmids expressing shRNAs against ATPase1. One sample of cells transfected with EGFP-Myc, and one sample of cells transfected with ZPS, were treated with 2525μM MG132 for 24hrs.

Having established that this system can be adapted to measure changes in Myc steady-state levels, I was able to begin screening the candidates from the two-hybrid screens. This steady-state screen was performed on 96 well-plates plated with HEK 293T cells at a concentration of 20,000 cells per well. 48 hours

later cells were transfected with 10ng EGFP-Myc, 33ng of pooled pSM2c plasmids expressing shRNAs against a given candidate gene, and 13.3ng of the plasmid expressing dsRed per well (figure 7). 24 hours later the medium was changed and 48 hours after this the media was replaced with phosphate buffered saline (PBS) and read immediately with the plate reader.

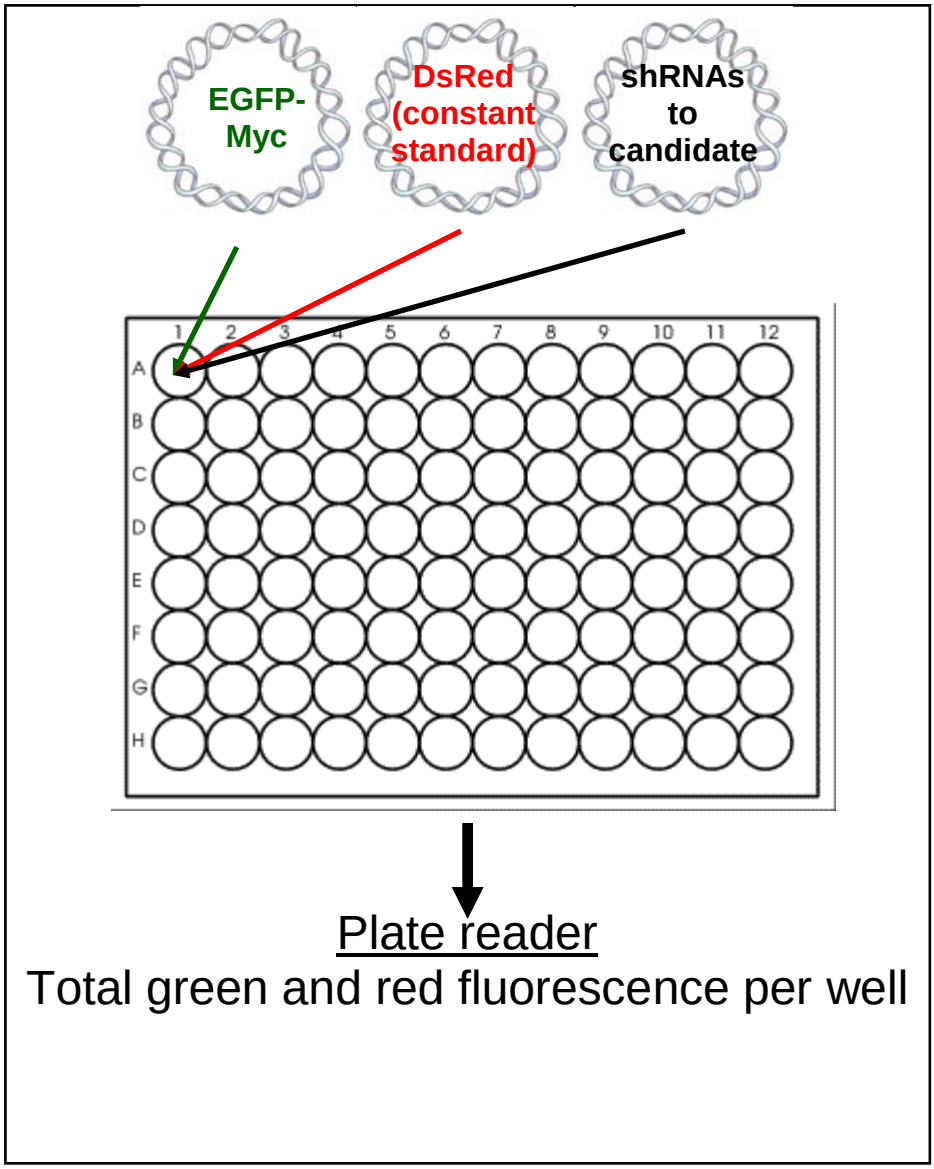

Figure 7: Setup of steady-state-screen. Each well is transfected with plasmids expressing EGFP-Myc (10ng pCGN-EGFP-Myc), dsRed (13.3ng dsRed1), and shRNAs against candidate genes from the two-hybrid screens (33ng total of a pool of pSM2c plasmids).

Results:

The results of the steady-state screen are shown in figure 8. This experiment was performed with a number of controls. The lowest green to red ratio was detected with an shRNA against Myc. The highest green to red ratio was detected with MG132 treatment (25μM for 24hours). HectH9 and Pml1 were also included as controls as both are known to facilitate Myc degradation (103, 133) and, as expected, hairpins against each one raised Myc levels significantly.

Each sample was assayed a total of nine times (n=9) and the five hairpins that increased Myc levels most – txn2 , *eno1*, hectH9, pml1, and rnf40 – yielded a p value < 0.01 as compared to the luciferase control. The hairpin that decreased Myc levels most was against Myc itself, and it also did this in a statistically-significant manner ($p < 0.01$).

Thioredoxin2 (txn2) is a highly conserved enzyme that that acts as an antioxidant capable of reducing reactive oxygen species within in the cell. Txn2 is itself reduced by thioredoxin reductase in an NADPH-dependent reaction. A single clone corresponding to txn2 emerged twice in my second yeast-two-hybrid screen. Txn2 has not previously been reported to bind to Myc.

RING finger protein 40 (Rnf40) is a ubiquitin ligase with a high degree of homology to bre1, a eukaryotic E3 known to ubiquitylate histone H2B. Importantly, Rnf40 has been shown to act as a transcriptional coactivator (134). Rnf40 has been reported to bind the Rb tumor suppressor (135), but it has not

been reported to bind Myc. Rnf40 emerged one time from my second yeast-two-hybrid screen.

HectH9 and Pml1 were included as controls as both have been shown to mediate the degradation of the product of the *c-Myc* gene. Finally, enolase1α is a glycolytic enzyme with multiple pleiotropic functions. It emerged one time in each of the three yeast two-hybrid screens. I will elaborate on its function in chapter 4 as it would emerge as the most promising candidate screened in this work.

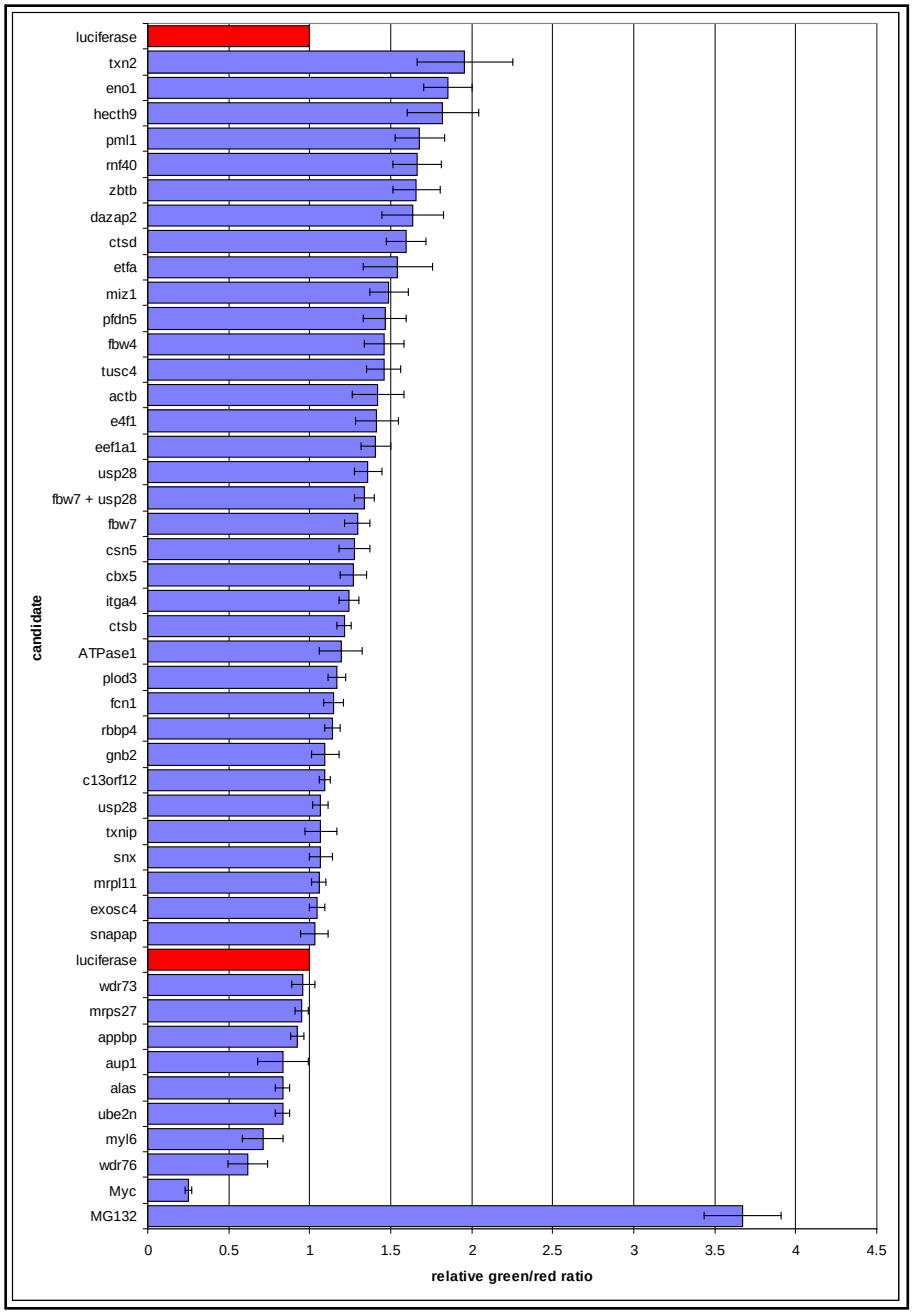

Figure 8: Results of steady-state screen. Each sample was transfected with dsRed1, pCGN-EGFP-Myc, and pooled shRNAs targeting a single gene expressed from pSM2c (as in figure 6). A control sample expressing an shRNA to firefly Luciferase is represented by a red bar. Controls include shRNAs targeting PML1, HectH9, Fbw7, Usp28, and Myc. An additional control (expressing an shRNA against firefly Luciferase) was treated with 25µM MG132 for 24hrs.

Conclusions and significance:

Overall, the results of this steady-state screen are encouraging. Reproducible, statistically-significant, changes in Myc levels are detected with knockdown of the various candidates, and the controls are generally having expected effects. For example, *c-Myc* shRNA lowers detectable Myc levels more than any other hairpin, and MG132 treatment raises Myc levels more than any hairpin.

An important caveat, however, is that the ability of the shRNAs to knockdown candidate genes has not been tested in advance. It is thus possible that certain hairpins are not effectively knocking down their target genes. Off-target effects of the hairpins are also possible. When available, more than one hairpin was pooled together to knockdown a given gene. Nearly all genes were knocked down with more than one shRNA increasing the chance of successful gene knockdown.

The steady-state screen strongly suggested that txn2 , *eno1,* and rnf40 are promising as candidate genes for mediating Myc degradation. As a known ubiquitin ligase (136) that acts as a transcriptional coactivator (134), rnf40 was particularly interesting. In total seven candidates that appeared to affect Myc levels in this screen (*txn2, eno1, rnf40, ctsd, fbw4, tusc4* and *myl6*) were assayed in the next stage – the pulse chase analysis.

It is important to note that failure to affect Myc steady-state levels in this assay does not mean that a given candidate is not actually binding the D-element. It is entirely possible that a protein binds the D-element without affecting Myc steady-state levels, such a protein would not proceed further in this series of screens.

Future uses of the steady-state screen

Having established that this system can be used to assess rapidly Myc levels there are multiple additional genes that can be tested, and multiple ways to enhance the system itself. For example, at this stage it would be informative to screen a panel of proteins known to be, or predicted to be, involved in proteolysis such as ubiquitin conjugating enzymes (E2s), ubiquitin ligases (E3s), deubiquitylating enzymes, proteins with ubiquitin binding domains, etc. The most recent E3 thought to act on Myc was only identified as this thesis was being written (104), and it seems highly likely that there are many additional proteins that affect Myc stability to be characterized, and this screen can be a tool with which to identify some of them.

One issue that will emerge as this screen is expanded is how to differentiate between shRNAs that affect proteasomal degradation in general from those that specifically affect Myc stability. For example, the results of my screen indicate that several shRNAs affect Myc levels but I cannot yet be sure that this is

a Myc-specific effect as opposed to a general one, and this will be tested once the candidates are narrowed down further. There is, however, a way to adapt the screen so these two effects can be distinguished by the screen itself. By introducing another proteasomal substrate fused to a third fluorophore, such as a yellow fluorescent protein, one can measure general proteasome function by following the levels of this proteasome substrate in addition to the levels of Myc in any given sample, for any given shRNA. If an shRNA increases the yellow as well as the green signal it is likely a general inhibitor of the proteasome, if it increases only the green signal it would likely be Myc-specific.

Finally, it may be possible to enhance the sensitivity of this screen by treating cells with cycloheximide (CHX), a chemical that blocks protein translation. By using CHX to inhibit protein synthesis, short-lived proteins will likely be degraded and eliminated from the cell soon after treatment, whereas proteins that are stabilized would remain. Thus the fluorescent signal from stabilized proteins would remain high relative to the red signal, while the signal from the unstable proteins would be diminished. This procedure, however, would take some initial adjustment to establish the optimal cycloheximide concentration and the duration of treatment.

CHAPTER 4: Screening for changes in Myc stability in response to candidate knockdown

Background:

Pulse-chase analysis was selected as the final step in screening the remaining seven candidate genes. It was selected as a direct and quantitative method of assaying protein stability. Moreover, Myc's short half-life of approximately thirty-five minutes made this system particularly tractable.

The pulse-chase method relies on the principle that the decay of a protein can be monitored quantitatively by measuring the quantity of radioactivity emitted from the protein at various time points after a pulse of radiolabeled amino acids have been administered to the cell.

This assay is perhaps the least intrusive and most direct method of measuring protein stability in cells. As opposed to assays that measure protein steady-state levels, the pulse-chase assay allows us to follow a particular bolus of a protein as it is turned over. Another popular method of assaying protein stability utilizes cycloheximide (CHX) to halt all protein synthesis prior to assaying the levels of the protein at various time points after CHX treatment.

There are, however, complications that this method introduces. For example, when cycloheximide is used *all* protein synthesis is inhibited. This means that levels of short-lived proteins that affect the stability of the protein of interest are artificially diminished and can no longer act normally on the protein of interest.

While pulse-chase analysis has fewer complicating aspects, it is not a completely unobtrusive method of measuring protein stability. For example, this technique generally requires a period of amino-acid deprivation before radiolabeled amino acids are administered. After this 'pulse' the medium is removed and cells are flooded with far greater concentrations of non-radiolabeled amino acids to terminate the pulse period. It is conceivable that amino-acid deprivation and subsequent fluctuations in amino-acid levels can affect the stability of the measured protein. Nevertheless, compared to the alternatives, pulse-chase analysis remains the most reliable method of directly assaying protein stability.

Purpose:

The objective is to assay directly Myc stability in response to shRNA-mediated knockdown of the remaining seven candidate genes (*txn2, eno1, rnf40, ctsd, fbw4, tusc4* and *myl6*).

Methodology:

The pulse-chase assay was performed as described by Salghetti *et al.* (7), with slight changes. Each sample in this assay was transfected with three plasmids. They were: pCGN3HAM-Myc, pCGN-EGFP-NLS, and one or more pSM2c vectors expressing a hairpin against each candidate gene. The pCGN3HAM-Myc expressed Myc with three copies of the so-called HAM tag on its N-terminus. The HAM-tag is a modified version of the HA tag containing additional methionine residues to increase the signal intensity when radiolabeled methionine residues are incorporated (137). The pCGN-EGFP-NLS construct encodes a nuclear localization signal with EGFP on its N-terminus, and an HA on the N-terminus of EGFP. The pSM2c plasmid was the same one used in the steady-state screen.

To begin the pulse-chase assay, human osteosarcoma (U2OS) cells were plated onto each of four 6cm plates per sample. Twenty-four hours later each plate was transfected with 1µg of pooled pSM2c plasmids expressing shRNAs against each candidate gene, 1µg of HAM-tagged Myc in the pCG vector, and 100ng of a second pCG vector expressing EGFP-HA-NLS. 24 hours after transfection cells were starved of methionine and cysteine for 30min and then radiolabeled with 80µL of methionine/cysteine (~10mCi/mL, from MP Biomedicals) for 40min. Medium was then removed and replaced with non-

radiolabeled medium containing 2mM methionine and 2mM cysteine. Cells were harvested with a cell-scraper at 0, 30, 60, and 90min, pelleted, and immediately frozen in liquid nitrogen. The following morning each sample was resuspended in 800µL TNN buffer (50mM tris pH 7.5, 250mM NaCl, 5mM EDTA, and 0.5% NP-40 alternative) with protease inhibitors Pefabloc (0.4mg/mL), Leupeptin (10µg/mL), Pepstatin (10µg/mL), and Aprotinin (5µg/mL). Cells were then rotated for 1hr at 4°C and subsequently spun for 5min to pellet the insoluble material.

The IP was performed with the soluble fraction (~800µL). An initial pre-clear step was performed by adding 50µL of a 1:1 combination of protein-A and protein-G agarose (A/G) and rotating for 1hr at 4°C to reduce background binding to the beads. HA and HAM-tagged proteins were recovered with the mouse monoclonal antibody 12CA5 which recognizes both tags. After rotation for 1hr with the antibody 50µL of A/G beads were added to the samples which were then rotated for another hour, washed 3 times and boiled in protein loading buffer. Samples were then run on an 8% SDS-PAGE gel overnight at 40v and subsequently visualized by phosphorimager.

EGFP-HA-NLS was included as a constant standard against which the signal from HAM-Myc could be normalized. Since this protein is highly stable, any decay that occurred during the course of the experiment was considered negligible. The presence of a signal against which the signal from HAM-Myc

could be normalized was particularly important as many of the hairpins caused cell-death and therefore caused the cell-number to vary unpredictably. Being able to correct the signal from Myc to a constant signal that directly correlated with cell number allows us to correct for this.

In 2002, Tworkowski *et al*. found that two distinct pools of Myc exist in mammalian cells. One pool, the soluble fraction, is degraded rapidly. The other pool, the insoluble fraction, is far more stable having a half-life of approximately two hours (97). In this experiment, we decided to consider only the unstable, soluble fraction for two reasons. First, the steady-state screen had suggested that six of the seven candidate shRNAs to emerge might increase Myc stability (the exception being shRNA against myl6). Since an increase in Myc stability would be more pronounced in the unstable fraction than in the stable fraction I decided to assay the former. The second reason for this choice was that a greater degree of Myc decay could be observed during the course of the experiment when analyzing the soluble fraction.

Results:

Use of the control luciferase hairpin, which should not target any human genes, established the baseline Myc half-life in this system of approximately

39min (figure 9). Knockdown of two candidate genes repeatedly affected Myc half-life. Expression of an shRNA against the ubiquitin ligase rnf40 extended Myc's half-life to approximately 48min, while knockdown of the glycolytic enzyme enolase extended Myc half-life to approximately 78min. The effect of rnf40 knockdown was just short of statistical significance ($p = 0.0659$). Knockdown of eno1, however, was highly significant ($p < 0.0001$).

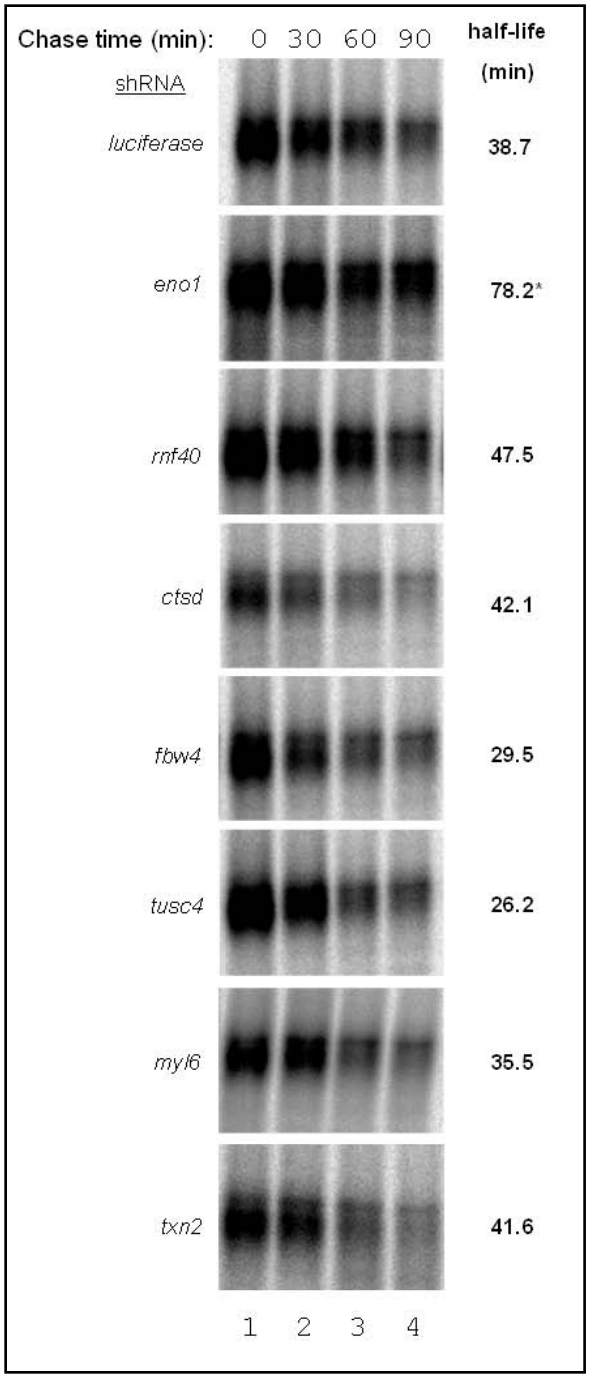

Figure 9: Knockdown of *eno1* increases Myc half-life. U2OS cells were transfected with 1µg of pCGN3HAM-Myc, 1µg of pooled pSM2c plasmids expressing shRNAs against a given gene (indicated in left column) and 100ng of pCGN-EGFP-NLS. Cells were harvested at 0, 30, 60, and 90 minutes after the initiation of the chase period. * = $p < 0.0001$ as compared to the luciferase control.

Having established *eno1* as the most promising gene at this point I tested the *eno1* shRNAs for both their ability to reduce enolase expression and for their ability to raise Myc steady-state levels (figure 10). There are two distinct products of the *eno1* gene (figure 11), enolase1α and MBP-1 (as will be discussed in the final chapter). Our results indicate that each of the two hairpins that I used reduced both enolase1α and MBP-1 levels, and increased Myc steady-state levels.

Conclusions and significance:

This phase of the project represented the final stage in screening the original 39 candidates from the yeast-two-hybrid screens. While two candidates – rnf40 and *eno1* – appeared interesting, the former did not prove to be having a statistically significant effect while the effect latter was clearly more pronounced.

Knockdown of *eno1* increases Myc half-life by approximately two-fold in a consistent, highly statistically-significant manner. The *eno1* gene has thus emerged as the only one to contain sequences that interact with the D-element in the yeast-two-hybrid screens, to affect Myc levels in the steady-state screen, and to affect Myc's stability as indicated by the pulse-chase assay. Since these different effects are not necessarily dependent on one another, *eno1* can now be implicated in three different aspects of Myc biology. I have confirmed that

knockdown of this gene increases Myc steady-state levels (figure 10) and I will go on to confirm that the product of this gene physically associates with Myc's D-element. First, though, we will look more closely at the *eno1* gene and consider why it may play an intriguing role in regulating Myc.

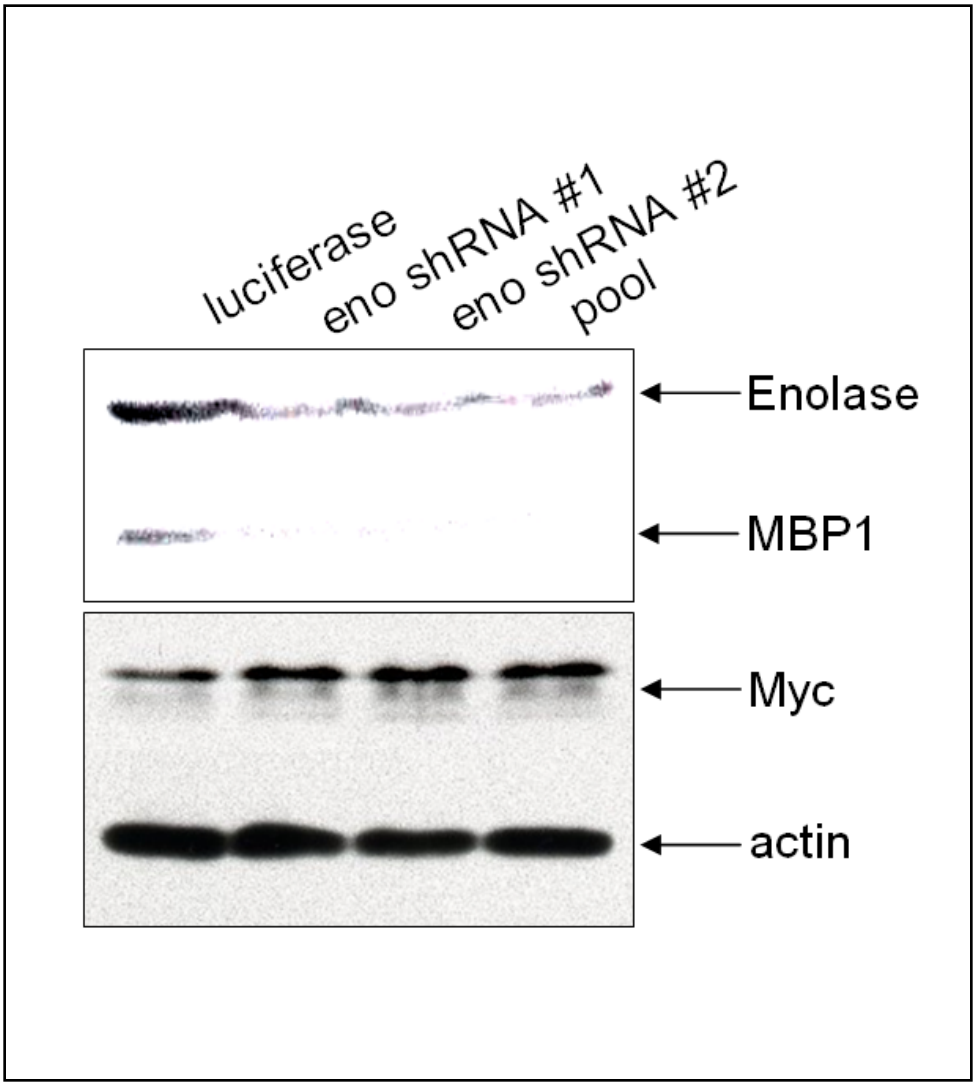

Figure 10: Knockdown of *eno1* decreases enolase and MBP-1 levels, and increases Myc levels. HEK293T cells were transfected with 1µg of firefly luciferase, pSM2c-eno#1, pSM2c-eno#2, and 500ng of pSM2c-eno#1 combined with 500ng of pSM2c-eno#2 (pool).

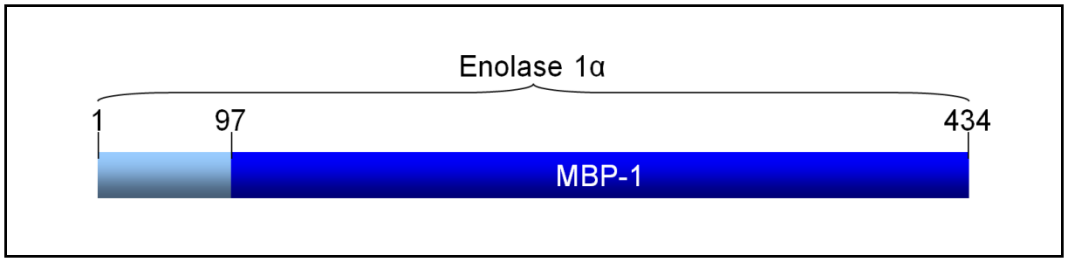

Figure 11: Schematic of the *eno1* gene. Translation of the full-length gene encodes enolase1α, τ-crystallin, and a plasminogen receptor. Alternate translation initiation at codon 97 yields Myc-promoter binding protein 1 (MBP-1).

CHAPTER 5: Myc and enolase/MBP-1 physically interact

Background:

At this point, we have confirmed two of the three effects for which we had initially screened. Simple Western blotting of endogenous Myc in cells expressing the shRNAs against *eno1* has confirmed that the *eno1* gene affects endogenous Myc steady-state levels (figure 10). And pulse-chase experiments have shown that Myc's rapid turnover is inhibited when *eno1* is knocked down (figures 9 and 12). Having done this it is important that I confirm the original two-hybrid data that motivated the subsequent experiments, namely that enolase/MBP-1 binds the D-element.

Purpose:

The purpose of this set of experiments is to confirm that enolase/MBP-1 physically associates with Myc via the D-element.

Methodology:

Exogenous Co-IP

The physical association between enolase/MBP-1 and Myc was tested by overexpressing the respective proteins via transient transfection, immunoprecipitating (IPing) Myc and immunoblotting (IBing) the potential binding partner. After a series of preliminary experiments indicating that full-length enolase specifically binds Myc, I conducted larger co-IP experiments to ask the following questions: Does either MBP-1 or enolase preferentially bind Myc? Is the interaction diminished by deletion of the D-element? And, is the interaction diminished by deletion of the most highly conserved region within the D-element, MbIII?

To address these questions I used the following plasmids: pCGN-Myc, pCGN-MycΔD-element (MycΔD), pCGN-MycΔMbIII (MycΔMbIII), and pCGN-EGFP; as well as pCGT-enolase, pCGT-MBP-1, and pCGT-Max. The pCGN vectors express proteins with an N-terminal HA tag, and the pCGT vectors express proteins with an N-terminal T7-tag. GFP was included as a negative control, as it should not interact with either product of the enolase gene, and Max was included as a positive control as it should bind tightly to the C-terminal DBD of Myc.

The exogenous co-IP experiment was performed in U2OS cells. Each sample was assayed in one 10cm plate. Cells were plated at an initial cell-density of approximately 40%. Twenty-four hours later cells were transfected with 500ng of a pCGN plasmid, and 500ng of a pCGT plasmid. Forty-eight hours later cells were washed and harvested with a cell-scraper. Cells were then pelleted and resuspended in 800uL per sample of KCl-TNN (50mM tris pH 7.5, 170mM KCl, 5mM EDTA, and 0.5% NP-40 alternative) with protease inhibitors Pefabloc (0.4mg/mL), Leupeptin (10µg/mL), Pepstatin (10µg/mL), and Aprotinin (5µg/mL). Cells were then rotated for 1hr at 4°C and subsequently spun for 5min to pellet the insoluble material. The IP was performed on the soluble fraction.

The mouse monoclonal antibody 12CA5, which recognizes the HA tag, was used to recover the protein expressed from the pCGN-vector. After rotating the samples with the antibody for 1hour at 4°C 50µL of a 1:1 combination of protein-A and protein-G agarose was added to each sample which was subsequently rotated for an additional hour. When this was complete, the beads were washed five times and then boiled in 100µL of protein loading buffer. Samples were run out on a 10% SDS-PAGE gel and probed for the T7 tag (Co-IP), for Myc (experimental input), and for GFP (control input).

Endogenous Co-IP

In order to determine if endogenous Myc can be seen to bind either endogenous enolase or MBP-1, a co-IP of these proteins was performed. This experiment included five samples; each sample consisted of U2OS cell-lysates from five 15-cm plates. Once the cells reached confluence they were harvested by scraping and resuspended in 1mL of KCl-TNN with the protease inhibitors Pefabloc, Leupeptin, Pepstatin, and Aprotinin as in the exogenous co-IP experiment. The IP procedure was performed as described for the exogenous co-IP.

Five samples were assayed. The first two samples were negative controls; sample 1 was IPed with 2μg of unconjugated rabbit anti-mouse IgG (rαm), and sample 2 was IPed with beads alone. The second two samples were positive controls; sample 3 was IPed with 2μg of the rabbit polyclonal anti-Max antibody C-124 (Santa Cruz), and sample 4 was IPed with 2μg of the rabbit polyclonal anti-Myc antibody N262 (Santa Cruz). The fifth sample was IPed with 10μL of rabbit antiserum raised against a peptide sequence common to enolase and MBP-1.

Results:

Exogenous Co-IP

As described above, Max was included in the exogenous co-IP as a positive control for Myc binding. The fact that Max IPed with wild-type Myc and the Myc deletion mutants, but not with the GFP control (figure 12, compare lanes 3, 6, 9, and 12), indicates that this assay can detect Myc-specific interactions.

An interaction between ectopically expressed wild-type Myc and both enolase and MBP-1 was detected in this experiment (figure 12, lanes 1 and 2). These data also indicate that Myc interacts more strongly with MBP-1 than enolase does as more MBP-1 than enolase is IPed with Myc despite higher levels of enolase in the input. Deletion of the D-element shows no enolase binding and greatly reduces MBP-1 binding to Myc (figure 12, lanes 4 and 5). The residual MBP-1 binding to the D-element suggests that deletion of the D-element is not sufficient fully to disrupt the interaction; this may be the result of MBP-1 binding the D-element as well as an additional region of Myc. Interestingly, the deletion of MbIII does not appear to disrupt Myc's interaction with either MBP-1 or enolase (figure 12, lanes 7 and 8) suggesting that enolase and MBP-1 bind a region within the D-element but outside of MbIII.

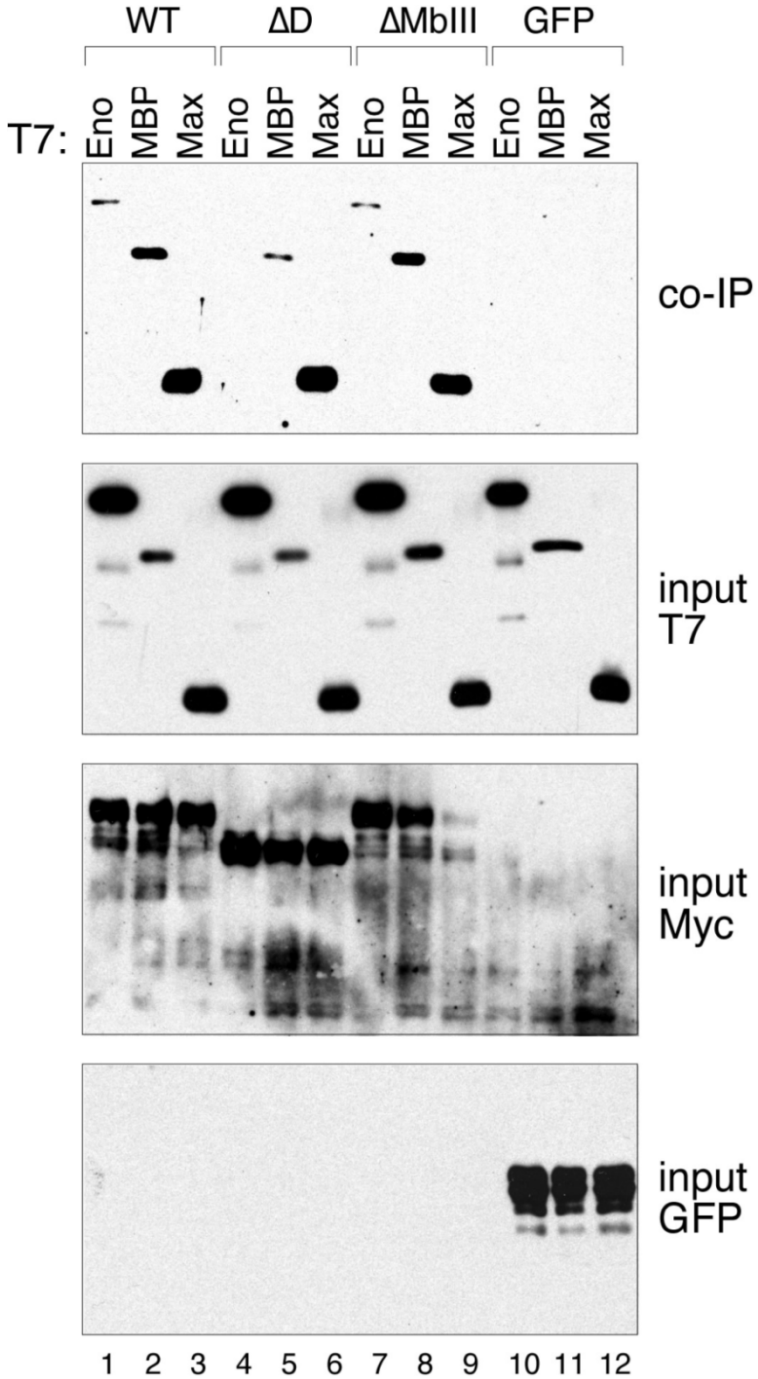

Figure 12: Myc preferentially associates with MBP-1 in a D-element-dependent manner. Sample sets were transfected with 500ng of either pCGN-Myc, pCGN-MycΔD, pCGN-MycΔMbIII, or pCGN-EGFP. Each set was cotransfected with either pCGT-enolase, pCGT-MBP-1, or pCGT-Max. Co-IP samples were IPed with 12CA5 and IBed with anti-T7 antibody.

Endogenous Co-IP

The endogenous co-IP was performed with two negative and two positive controls. The sample in lane 1 (figure 13) was IPed with an anti-mouse IgG antibody, and the sample in lane 2 included no antibody (beads alone). These two samples indicated no detectable background Myc binding. The next two samples were IPed with an antibody against Max and an antibody against Myc respectively, both pulled down much Myc, with the latter precipitating more than the former, as expected. Finally, the anti-enolase/MBP-1 antibody failed to precipitate Myc, and thus failed to show an interaction between endogenous Myc and endogenous enolase. There are several reasons why this might be the case.

First, endogenous MBP-1, which is localized to the nucleus as opposed to enolase which is localized to the cytoplasm, is a low abundance protein. Thus, if only a fraction of this protein is binding Myc and the protein is in low abundance to begin with it may be impossible to detect an interaction using this method. Second, there may be a mechanism that shields low levels of endogenous Myc from being bound and acted upon by enolase/MBP-1.

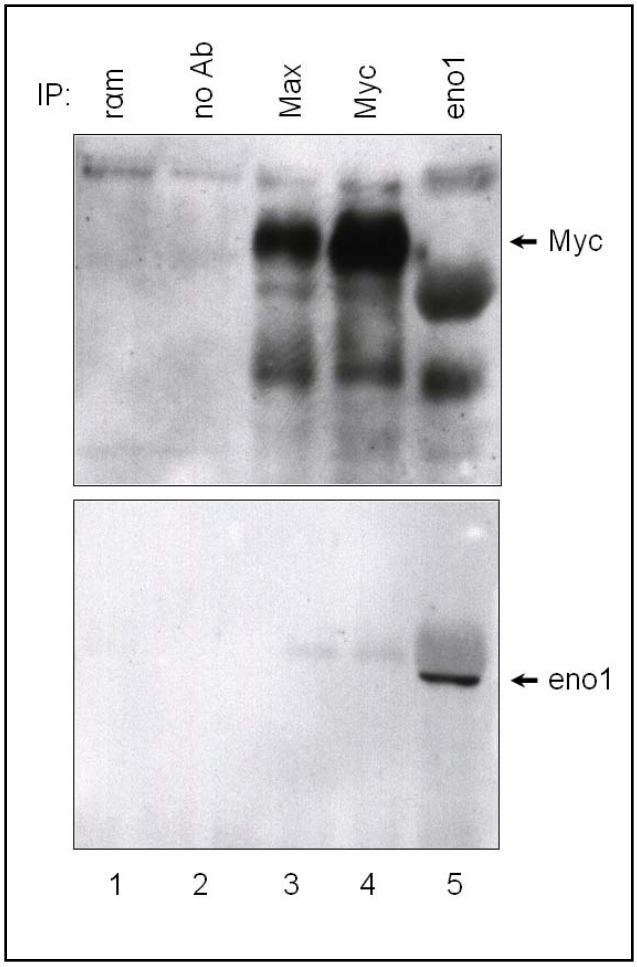

Figure 13: Endogenous Co-IP fails to show interaction between Myc and enolase/MBP-1. U2OS lysates were IPed with either rαm, beads alone, C-124, N262, or enolase anti-serum. Samples were subsequently probed for Myc and enolase respectively.

Conclusions and significance:

The exogenous Co-IP confirmed that enolase/MBP-1 interacts with Myc, the initial hypothesis suggested by the two hybrid screens. The finding that this interaction is dependent, at least in part, on the D-element is also consistent with

the two-hybrid data. I was surprised to find that the most highly conserved part of the D-element, MbIII, was dispensable for the physical interaction. This does not mean, however, that the role of enolase/MBP-1 in Myc biology is independent of MbIII, as MBP-1 may bind Myc to affect a change in Myc at MbIII which is nearly adjacent to its binding site.

It was also interesting to find that more MBP-1 than enolase is IPed with Myc, despite the higher abundance of enolase in the input. In fact, considering that more Max than MBP-1 was present in the input, it is possible that Myc was physically associating with MBP-1 to a greater extent than it was with Max, a protein with an extremely high affinity for Myc. This possibly was even more pronounced in some of the preliminary co-IP experiments.

In any case, there appears to be a strong physical association between Myc and MBP-1 that is diminished when full-length enolase is substituted for MBP-1. This may be the result of an N-terminal domain is present in enolase, but not in MBP-1, that inhibits the interaction.

The finding that there is still residual binding between MBP-1 and Myc when the D-element is deleted suggests that another region of Myc may also be binding to MBP-1. If this were the case, it would not be unprecedented for a protein that mediates Myc destruction. The ubiquitin ligase skp2, for example, has been shown to physically associate with two separate regions of Myc (95).

The fact that this co-IP was performed in cells means that additional factors were present that may be mediating the interaction between enolase/MBP-1 and Myc. It also means that the interaction may not be direct. A co-IP between purified Myc and purified enolase/MBP-1 would shed light on whether the interaction is indeed direct.

I was disappointed to find that I could not visualize a physical association between endogenous Myc and endogenous enolase/MBP-1 despite numerous attempts and the high quantity of cell-lysate that I used. The inability to detect the interaction may be a result of the fact that MBP-1 is simply a low abundance protein, and if only a small fraction of it is binding Myc in cells then one would expect to have difficulty measuring it. The cell may also have evolved a mechanism to shield endogenous Myc from the activity of MBP-1. If there were the case it would suggest that the cell has developed this mechanism to protect against the effects of pathologically high Myc levels.

CHAPTER 6: How enolase/MBP-1 affects Myc stability

Background:

The initial pulse-chase analysis that was used to screen the final seven candidates, as presented in chapter 4, indicated that knockdown of *eno1* increases Myc stability (figure 14). This result, however, leaves open some basic questions. Does the *eno1* gene act specifically on Myc to increase its stability, or does it have a broader effect on protein stability? Is full-length enolase necessary to affect Myc stability, or is catalytically-inactive MBP-1 sufficient to affect this change? Finally, do the products of *eno1* facilitate ubiquitylation of Myc as other proteins that affect Myc stability do, or do they affect Myc stability without affecting its ubiquitylation?

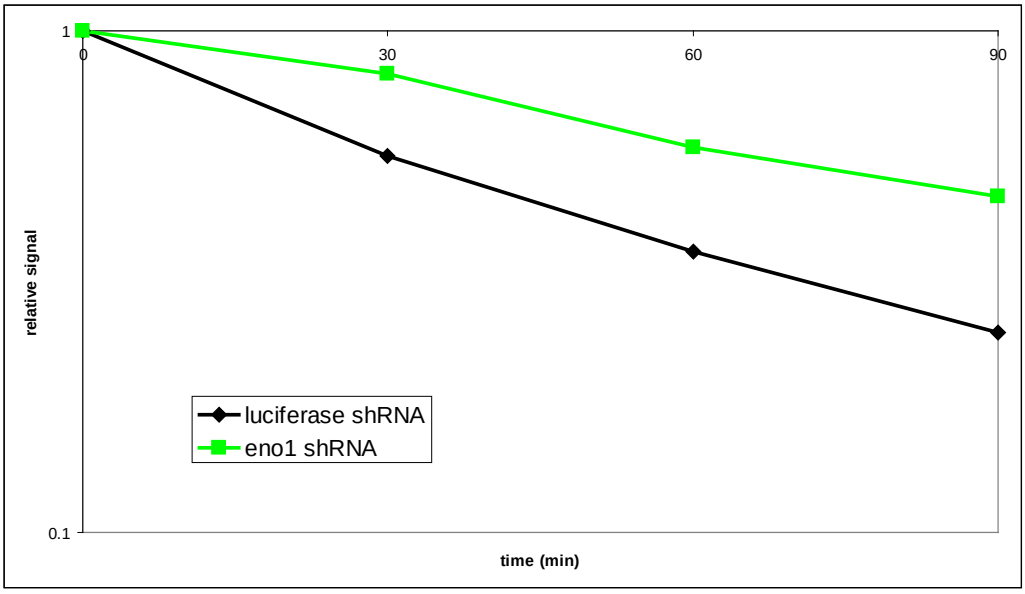

Figure 14: Knockdown of *eno1* slows Myc decay. U2OS cells were transfected with 1µg of pCGN3HAM-Myc, 100ng of pCGN-EGFP-NLS, and 1µg of either pSM2c-eno1 or pSM2c-luciferase. Cells were harvested at 0, 30, 60, and 90 minutes following the initiation of the chase period.

Purpose:

The purpose of this set of experiments was to address, on the basic level, how the enolase gene affects Myc stability.

Methodology:

Two techniques are presented in this chapter: the pulse-chase assay and the ubiquitylation assay. The pulse chase assay discussed in this chapter was performed as described in chapter 4.

The ubiquitylation assay was used to detect changes in Myc's ubiquitylation status as MBP-1 and enolase were overexpressed in cells. It was performed by transfecting cells with a plasmid, pMT107, that expresses his-tagged ubiquitin (138) as well as plasmids that express and Myc, MBP-1, and enolase. Cells were initially seeded onto 10cm plates, and 24hrs later the cells were transfected with 1.5µg of pMT107, 250ng of pCGN-Myc, and 500ng of either pCGT-enolase, pCGT-MBP-1 (the pCGT vector expresses the encoded protein with a T7 tag on its N-terminus), or dsRed as a control. Thirty-six hours later cells were lysed, lysates were enriched for ubiquitylated protein with nickel beads, and the samples were run on SDS-PAGE gels for Western blot analysis.

Results:

Before characterizing the way in which the products of *eno1* affect Myc stability, it was important to determine that the effect was a specific one. To

determine this I utilized a peptide consisting of six tandem copies of the so-called VN8 sequence (6xVN8). VN8 is an eight amino-acid sequence found within the TAD of the viral VP16 transcription factor that is known to be rapidly degraded by the proteasome. I expressed 6xVN8 while co-expressing an shRNA against either enolase or luciferase (figures 15 and 16). No detectable change in VN8 decay was caused by the *eno1* shRNA suggesting that enolase knockdown does not have a global affect on proteasome-mediated degradation.

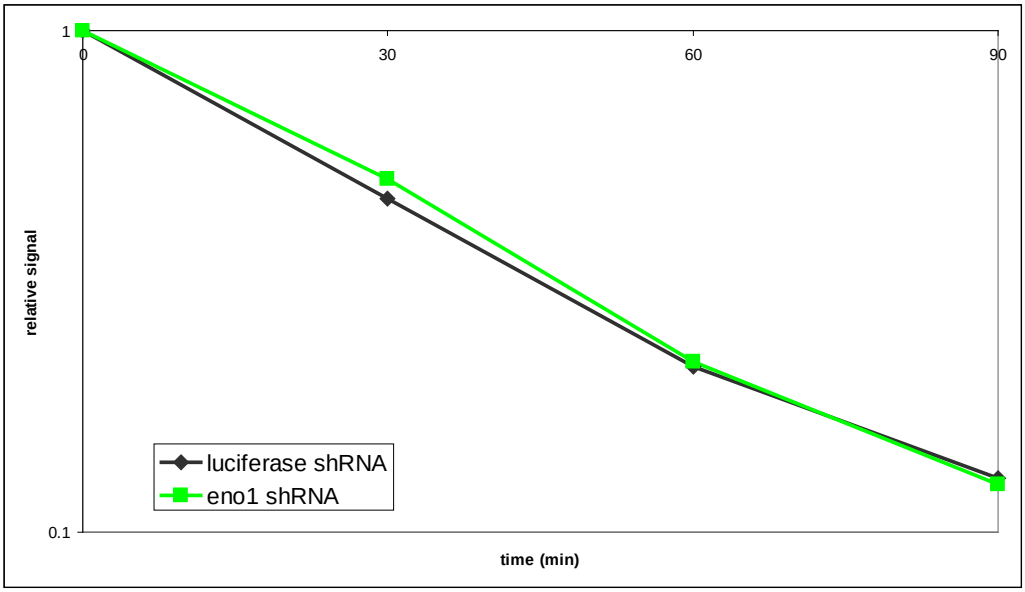

Figure 15: Knockdown of *eno1* does not affect VN8 decay. U2OS cells were transfected with 1µg of pCGN-6xVN8, 100ng of pCGN-EGFP-NLS, and 1µg of either pSM2c-eno1 or pSM2c-luciferase. Cells were harvested at 0, 30, 60, and 90 minutes following the initiation of the chase period.

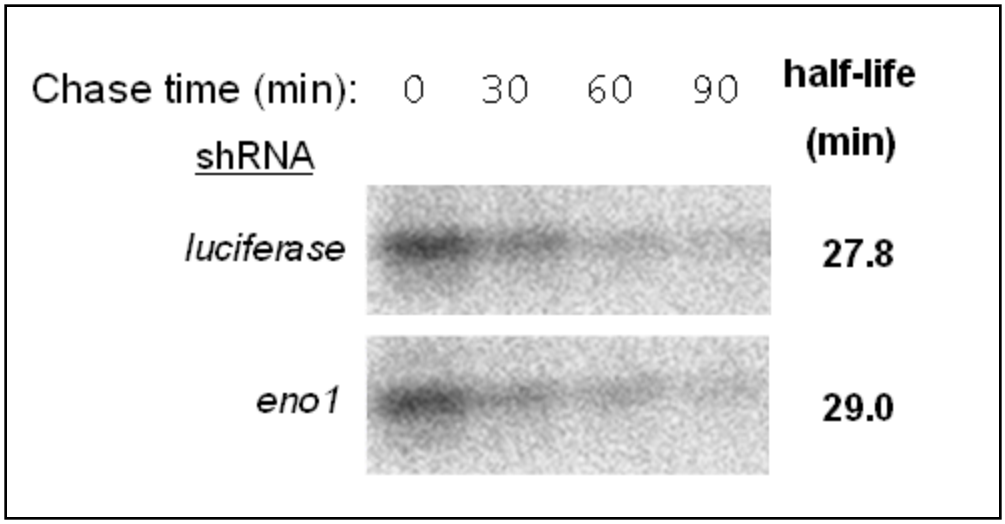

Figure 16: Knockdown of *eno1* does not affect VN8 decay. U2OS cells were transfected with 1µg of pCGN-6xVN8, 100ng of pCGN-EGFP-NLS, and 1µg of either pSM2c-eno1 or pSM2c-luciferase. Cells were harvested at 0, 30, 60, and 90 minutes following the initiation of the chase period.

Next, I asked if MBP-1 is sufficient to rescue the rapid degradation of Myc. I addressed this question by combining enolase knockdown with MBP-1 overexpression and performing a pulse-chase assay to determine if overexpressed MBP-1 could rescue the defect in Myc turnover caused by *eno1* knockdown. This indeed proved to be the case as cells expressing an *eno1* shRNA no longer exhibited a defect in Myc turnover when MBP-1 was overexpressed (figure 17, 16).

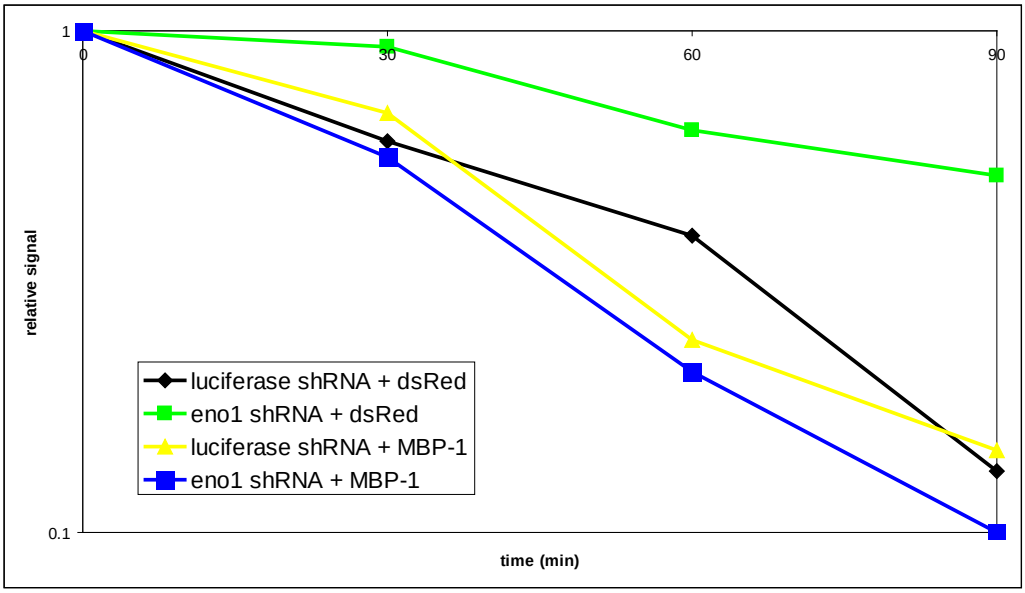

Figure 17: Overexpression of MBP-1 is sufficient to rescue Myc instability. U20S cells were expressing shRNA against either *luciferase* or *eno1* and are overexpressing either dsRed or MBP-1. Cells were harvested at 0, 30, 60, and 90 minutes following the initiation of the chase period.

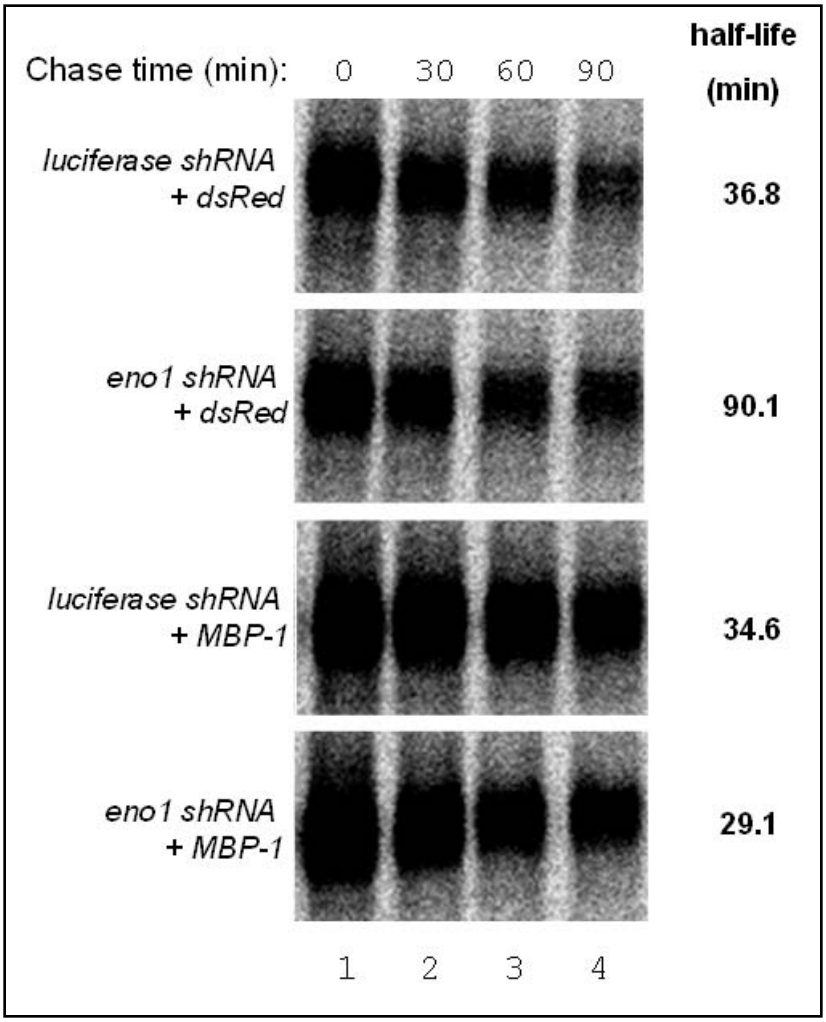

Figure 18: Overexpression of MBP-1 is sufficient to rescue Myc instability. U20S cells were expressing shRNA against either *luciferase* or *eno1* and are overexpressing either dsRed or MBP-1. Cells were harvested at 0, 30, 60, and 90 minutes following the initiation of the chase period.

Finally, I asked if either MBP-1 or full length enolase affect Myc's ubiquitylation status (figure 19). Interestingly, neither protein dramatically affected the ubiquitylation of Myc, suggesting that these proteins affect Myc stability without causing increased Myc ubiquitylation. These results are

consistent with the finding that the deletion of the D-element does not diminish Myc's ubiquitylation.

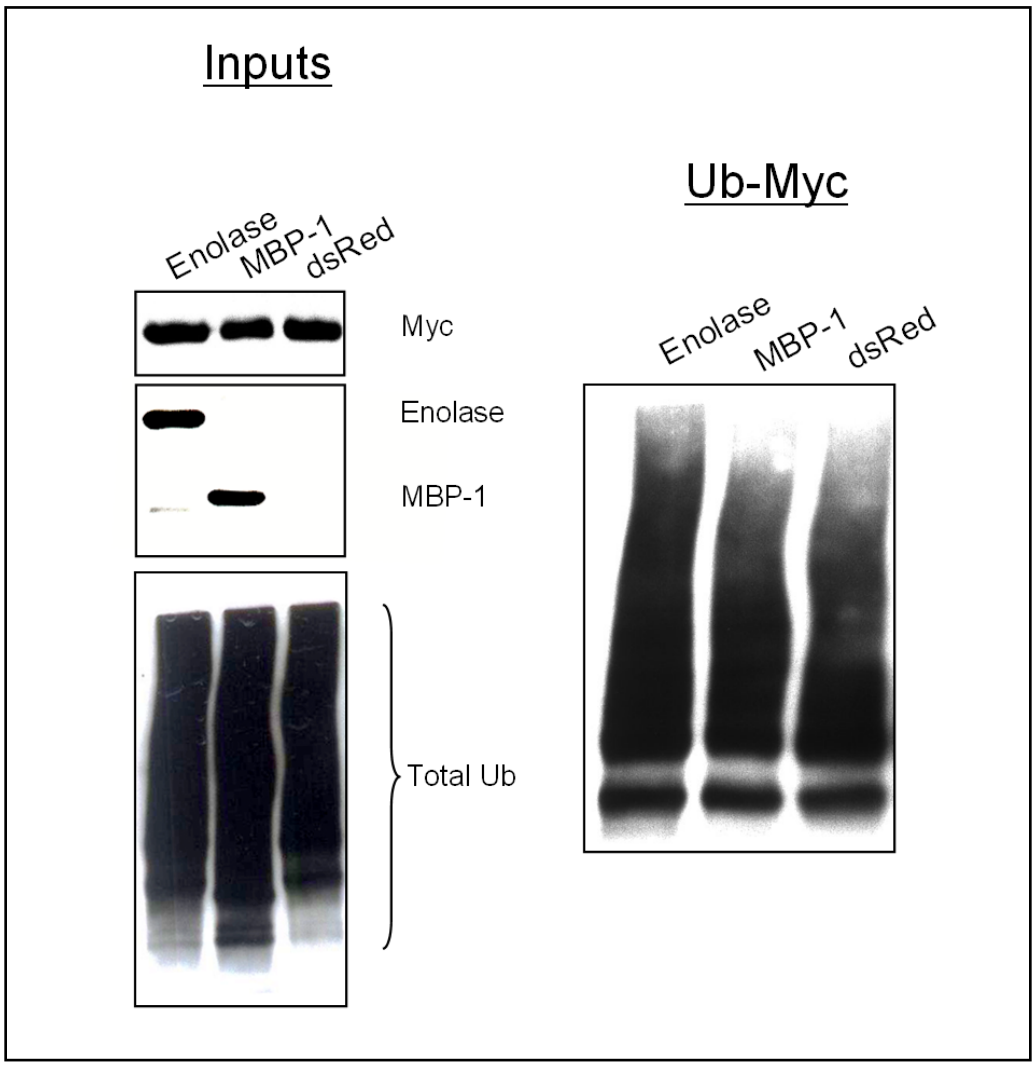

Figure 19: Myc's ubiquitylation status is unchanged with overexpression of MBP-1 and enolase. Cells transfected with pMT107 and pCGN-Myc are cotransfected with either pCGT-Eno, pCGT-MBP-1 or dsRed-1. Nickel beads are used to immobilize Ub-tagged proteins.

Conclusions and significance:

The results presented in this section suggest a model of how *eno1* affects Myc turnover that is consistent with some of our preliminary hypotheses. Having established that the effect of *eno1* knockdown is Myc-specific, and not global, I then saw that MBP-1 was sufficient to rescue the effect of the hairpin. This is particularly interesting as there is a precedent for MBP-1 binding a target protein (Mek5α) and facilitating its degradation. The unusual phenomenon by which MBP-1 affects Myc stability without affecting its ubiquitylation corresponds to the ubiquitylation-independent degradation of Mek5α that MBP-1 is said to mediate (139).

In addition to correlating with existing data about MBP-1, these data are also consistent with what is known about the D-element. Deletion studies have suggested that the D-element may function by recruiting a factor to degrade Myc after Myc has already been ubiquitylated, and the data presented in this chapter suggest that MBP-1 may be such a factor.

CHAPTER 7: MBP-1 attenuates Myc's ability to transform cells

Background:

Myc's identity as an oncogene is defined by its ability to induce neoplastic transformation (the set of changes that a cell undergoes to become malignant). One method of measuring such transformation is the soft-agar assay. This technique is a quantitative way to assay two of the key features of transformation: anchorage-independent growth and loss of contact inhibition. This technique is also highly responsive to the effects of Myc, as Myc is sufficient to drive colony formation in this system (140).

The role of *eno1* in mediating Myc destruction suggests that MBP-1 and/or enolase would attenuate Myc's ability to transform. This is not the only possible hypothesis suggested by these data, as some proteins that mediate Myc degradation can augment its activity; it is however the simplest.

If this hypothesis is validated, and enolase/MBP-1 attenuates Myc's ability to transform, this can be attributable to a number of possible mechanisms. These include increased apoptosis, quiescence, senescence, or contact inhibition; or

decreased proliferation or anchorage independence. And while it is not the goal of this work to identify the exact mechanism, I will begin to address a possible mechanism for any change in Myc's ability to transform. I am particularly interested in determining if enolase/MBP-1 affects Myc-induced apoptosis, for two reasons. First, the most conserved part of the D-element (MbIII) has been shown to negatively regulate Myc-induced apoptosis. And second, MBP-1 has been shown to inhibit the growth of cancer cells by increasing apoptosis in these cells.

Purpose:

The purpose of this section is to determine if enolase or MBP-1 affects Myc's ability to transform cells on soft-agar.

Methodology:

The transformation assays described in this chapter were performed together with Abhishek Chakraborty, a fellow graduate student in the Tansey lab who has extensive experience with these techniques.

The following plasmids were used for retroviral infection in the assays described in this chapter: MIG-Myc was used to overexpress Myc from the potent MSCV promoter, and to express GFP from an internal ribosome entry site (IRES). The pBabe-hygro vector was used to express MBP-1 and enolase1α respectively, from a relatively weak LTR promoter. The MLP vector expressing each of two hairpins against *eno1* (eno#1 and eno#2 respectively) was used to achieve knockdown of enolase and MBP-1. The shRNA sequences from pSM2c, used in the experiments described in Chapter 3, were cloned into in the MLP vector so that they would be under the control of an RNA Polymerase II-dependent promoter which is better suited for expressing an shRNA from a stably integrated locus. Finally, pBabe-pure-bcl2 was used to overexpress bcl2 after stable integration.

The soft-agar experiments described in this section were performed in 6-well plates using stably transduced NIH3T3 cells (a mouse-embryo-fibroblast cell line). Cells were plated within a layer of agar that was set atop a basal layer of agar containing no cells. The double layer of agar was then topped with liquid media that was replaced every four days for the duration of the experiment.

The basal layer was prepared by first boiling 3.2% agar in PBS. This solution was then diluted 1:4 with pre-warmed DMEM containing 10% calf serum. This mixture was then aliquoted at 2.5mLs per well. The plates were then left at room temperature for 15 minutes, then moved to 4°C for 15 minutes, and

then warmed to 37°C until the cell-containing layer was prepared. This top layer of agarose was prepared by mixing the 0.8%-agarose media described above with media containing 20,000 cells per mL at a 1:1 ratio. This mixture was then plated at 2mL per well atop the basal layer of cell-free agarose media. Plates were again left at room temperature for 15 minutes, then moved to 4°C for 15 minutes, and finally left at 37°C overnight. 1mL of liquid DMEM with 10% calf serum was added to each sample the following morning.

Two additional assays were performed to supplement the data from the soft-agar experiments. The first such assay was a simple cell-growth assay to test how the relationship between *c-Myc* and *eno1* affects the balance between cell-growth and cell-death. This assay was performed by plating an equal number of cells (/mL) from each sample onto a 10cm plate. When the first sample reached confluence, all cells were fixes with methanol, stained with crystal violet, and washed 5 times with PBS. Plates were then scanned and subsequently lysed, to release the dye which could then be quantified by measuring the optical density at 550nm. The second assay measured apoptosis induced in samples treated with 0.5µg/mL doxorubicin, a DNA-damaging agent that causes single- and double-strand breaks, for 24hrs. For the apoptosis assay, NIH3T3 cells were initially seeded onto 6-cm plates at a density of /mL, after 24hrs cells were treated with doxorubicin. After doxorubicin treatment, adherent and floating cells were harvested, fixed with methanol, stained with propidium iodide, and analyzed by

flow cytometry. 10,000 cells were analyzed per sample, and apoptosis was scored as the percent of cells accumulating in the sub-G1 phase of the cell cycle.

Results:

The first soft-agar experiment was performed to determine the effect of MBP-1 and enolase overexpression on the ability of Myc to drive colony formation. Six samples were divided into two sets of three, the first set was transduced with an empty MIG vector, and the second set was transduced with MIG-Myc. The first sample in each set was transduced with an empty pBabe-hygro vector, the second with pBabe-MBP-1, and the third with pBabe-empty. Results are shown in figure 20 and 21 (Western blot, figure 22). These data indicate that the number of Myc-driven colonies to form on soft agar is diminished to approximately 20% of initial levels when either enolase or MBP-1 are co-expressed with Myc.

I was unable to IB overexpressed MBP-1 and enolase, most likely because their expression was low. Being expressed in the pBabe vectors both these proteins were expressed of an LTR promoter in order to express them at near-endogenous levels. The result was that they could not be visualized by Western blot. Having seen a biological outcome of MBP-1 and enolase overexpression,

and the reverse outcome when MBP-1 and enolase are knocked down (figures 23-25), one can be confident that these proteins were being expressed.

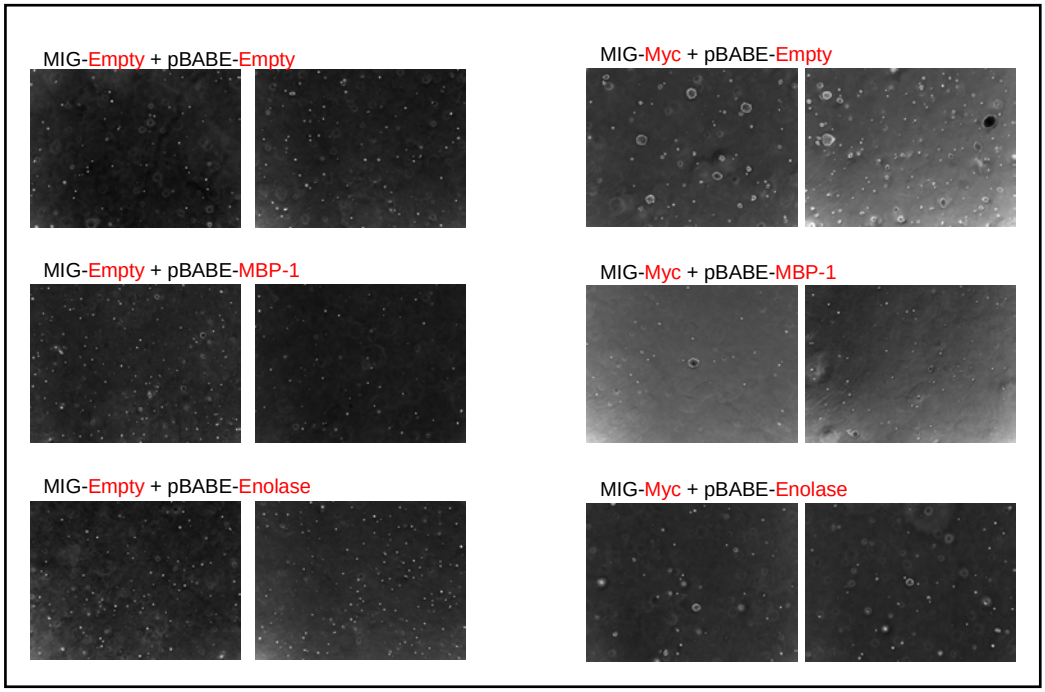

Figure 20: MBP-1 and enolase reduce the number of Myc-driven colonies on soft agar. NIH-3T3 cells were transduced with MIG-Empty or MIG-Myc together with pBabe-Empty, pBabe-MBP-1, or pBabe-Enolase.

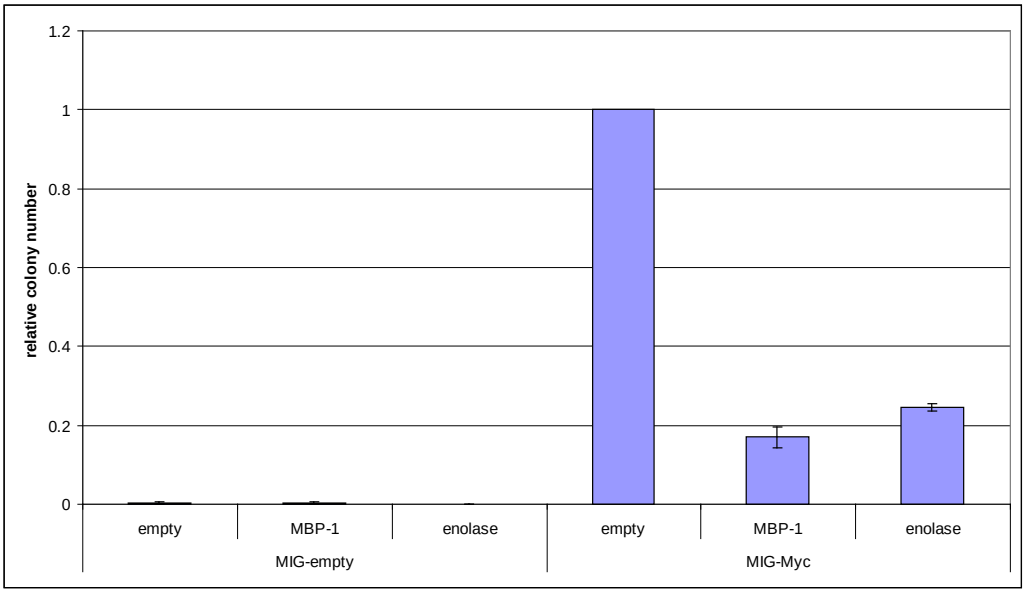

Figure 21: MBP-1 and enolase reduce the number of Myc-driven colonies on soft agar. NIH-3T3 cells were transduced with MIG-Empty or MIG-Myc together with pBabe-Empty, pBabe-MBP-1, or pBabe-Enolase.

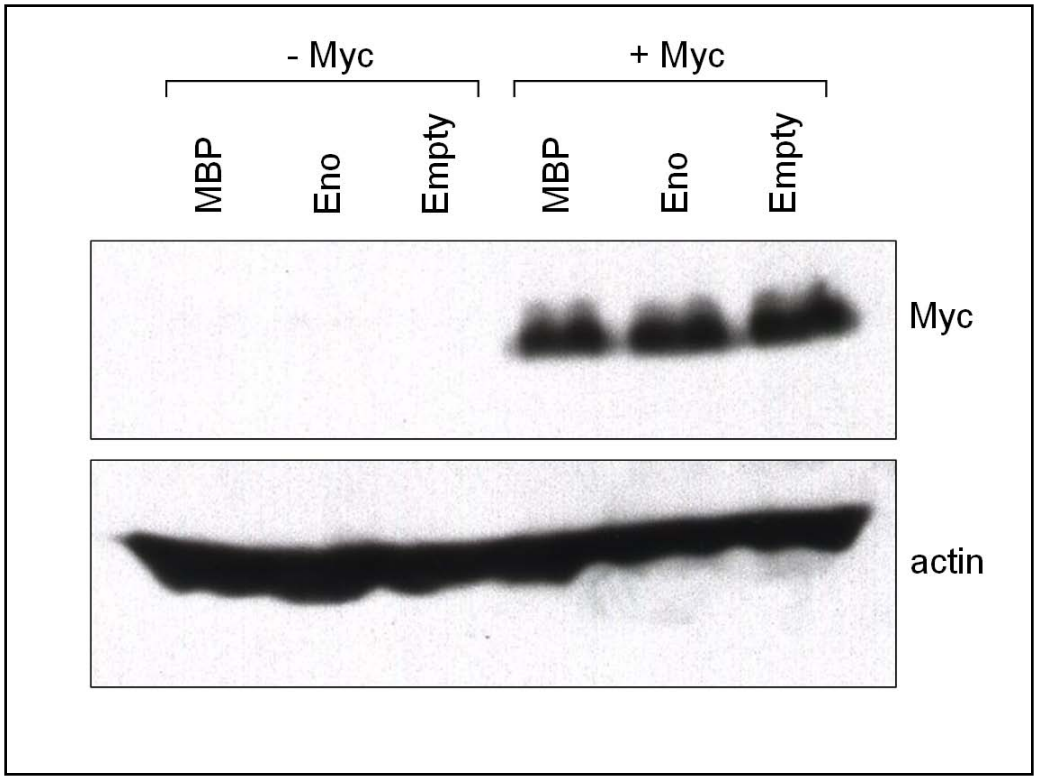

Figure 22: Protein expression in NIH-3T3 cells shown in figure 20. HA-tagged Myc was IBed with anti-HA-HRP (3F10) and actin is IBed with anti-actin antibody AC-15.

Next, I asked if knockdown of *eno1* would affect the number of Myc-driven colonies. To do this, cells were again divided into two sets, one set without Myc overexpression and the other set with Myc overexpression. Each set was co-transduced with vectors expressing each of two plasmids encoding an shRNA against the *eno1*. Results are shown in figure 23 and 24 (Western blot, figure 25). These data indicate that, even in cells expressing extremely high levels of ectopic Myc, the number of colonies can still be increased by knocking down enolase and MBP-1 by targeting the *eno1* gene.

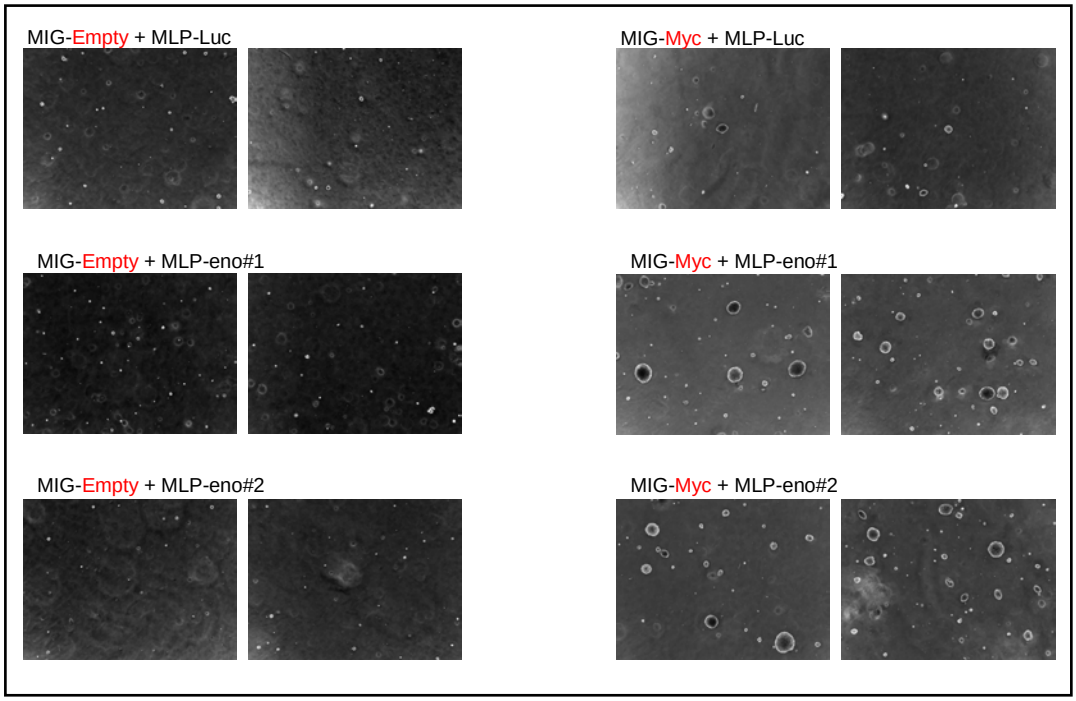

Figure 23: Knockdown of *eno1* increases the number of Myc-driven colonies on soft agar. NIH-3T3 cells were transduced with MIG-Empty or MIG-Myc together with MLP-Luciferase, MLP-eno#1, or MLP-eno#2.

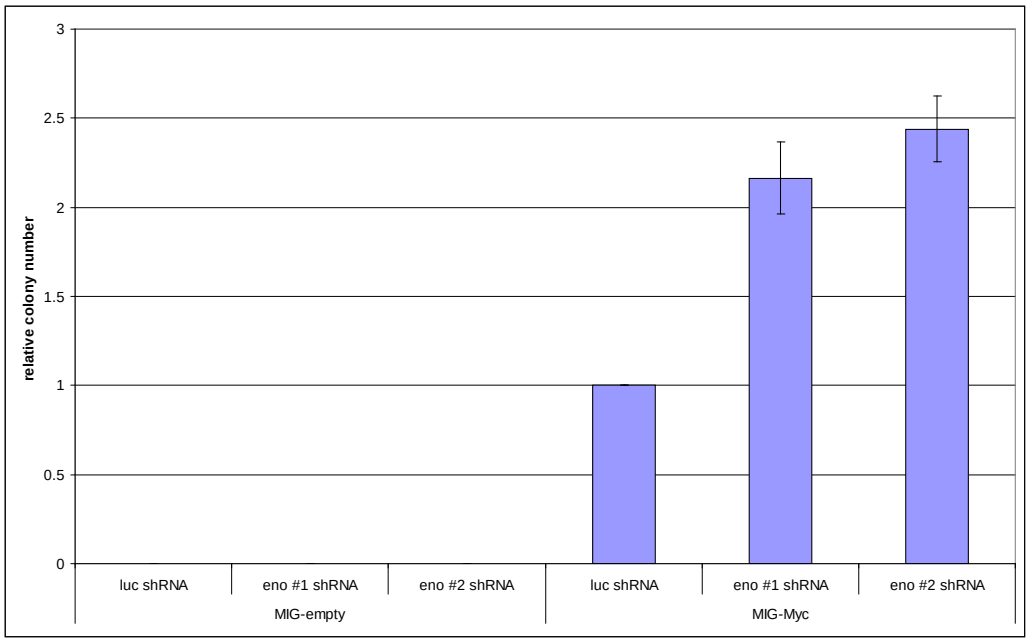

Figure 24: Knockdown of *eno1* increases the number of Myc-driven colonies on soft agar. NIH-3T3 cells were transduced with MIG-Empty or MIG-Myc together with MLP-Luciferase, MLP-eno#1, or MLP-eno#2.

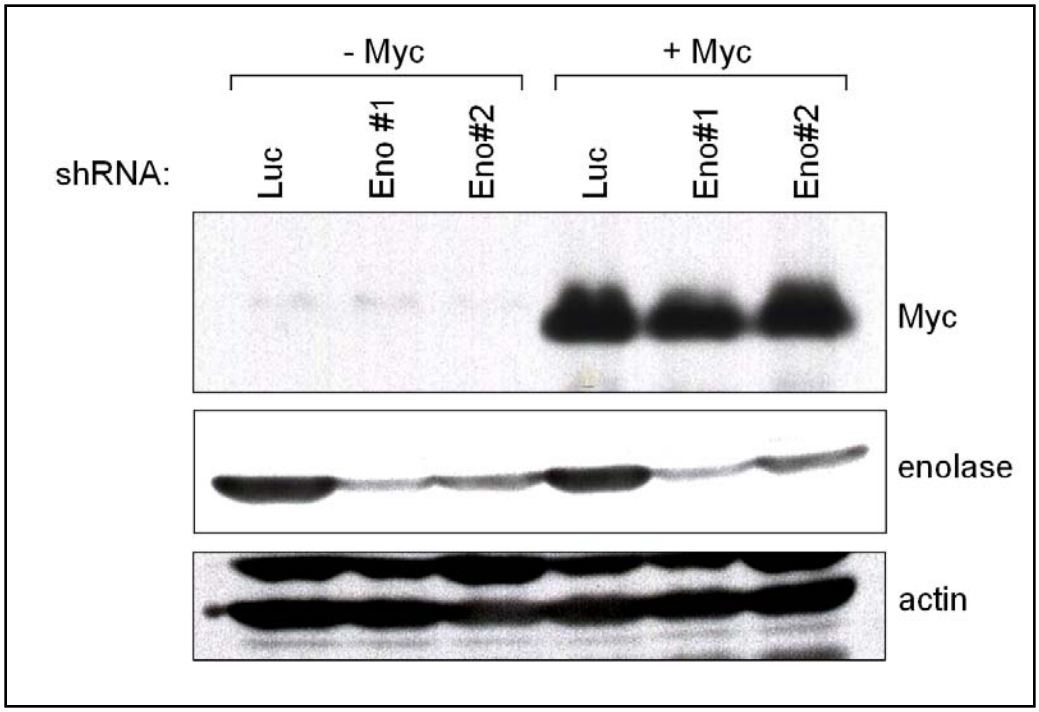

Figure 25: Protein expression in NIH-3T3 cells shown in figure 23. HA-tagged Myc was IBed with anti-HA-HRP (3F10), enolase was IBed with anti-enolase antiserum, and actin is IBed with anti-actin antibody AC-15.

Having seen that overexpression of MBP-1 and enolase can reduce the number of Myc-driven colonies, and that knockdown of *eno1* increases the number of Myc-driven colonies I asked is this effect is attributable to differences in apoptosis. Specifically, I asked if overexpression of MBP-1 reduces the number Myc-driven colonies by increasing the rate of apoptosis. To address this question I overexpressed Myc with and without MBP-1 overexpression, as in figure 20, but I included bcl2 overexpression to block apoptosis. Results are shown in figure 26 and 27 (Western blot, figure 28). These data indicate that bcl2 overexpression does not block MBP-1's ability to reduce Myc-driven colony

formation, suggesting that MBP-1 reduces Myc's ability to transform without increasing the rate of apoptosis.

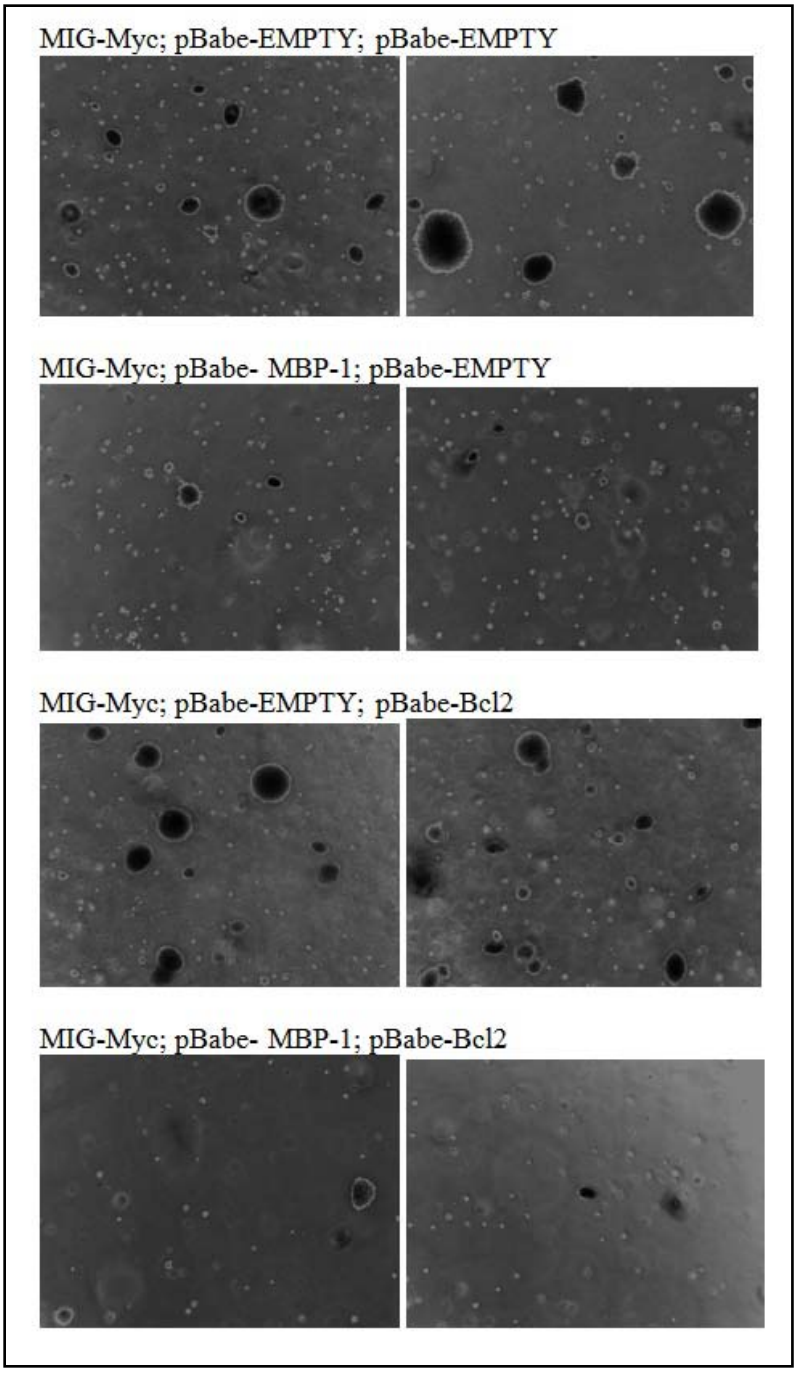

Figure 26: Bcl2 does not block MBP-1's ability to reduce Myc-driven colony formation on soft agar. NIH-3T3 cells were transduced with MIG-Myc together with pBabe-empty or pBabe-MBP-1, and pBabe-empty or pBabe-Bcl2.

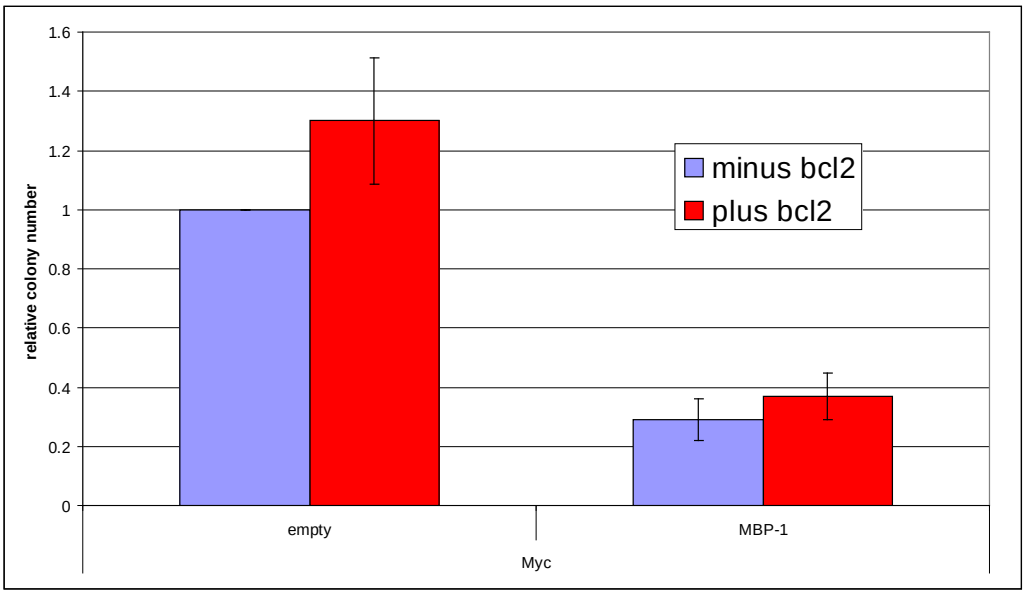

Figure 27: Bcl2 does not block MBP-1's ability to reduce Myc-driven colony formation on soft agar. NIH-3T3 cells were transduced with MIG-Myc together with pBabe-empty or pBabe-MBP-1, and pBabe-empty or pBabe-Bcl2.

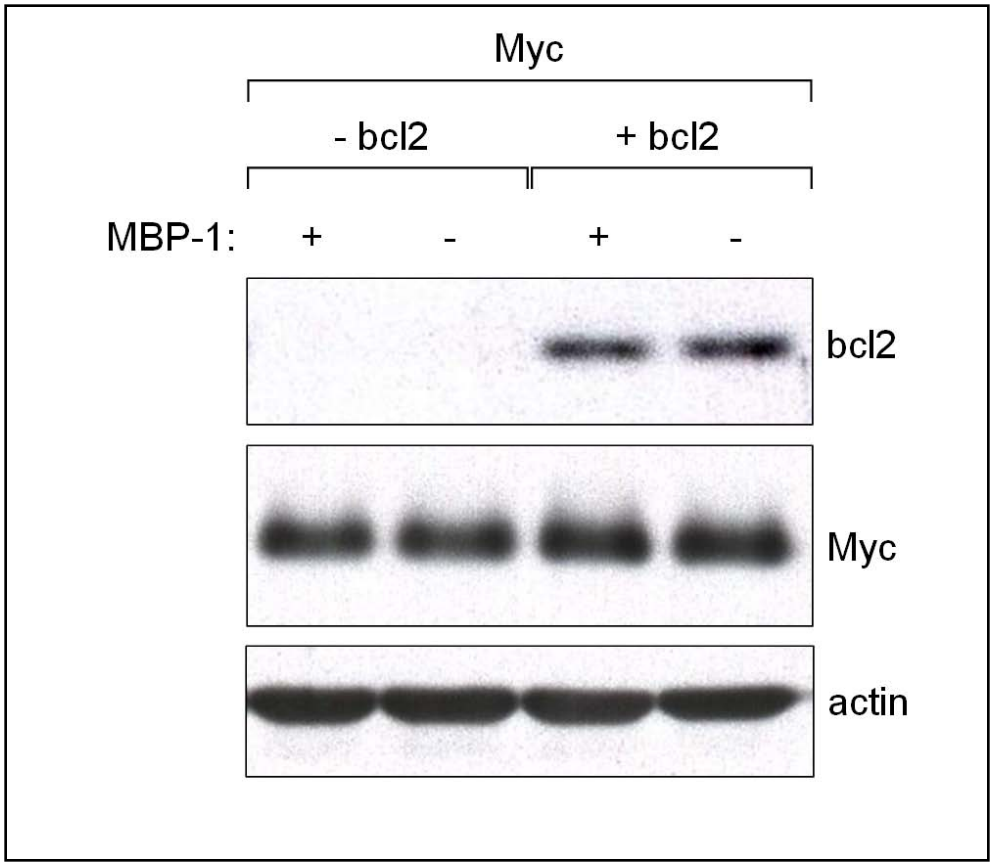

Figure 28: Protein expression in NIH-3T3 cells shown in figure 26. Bcl2 was IBed with mouse monoclonal anti-bcl2 antibody sc-509 (Santa Cruz), HA-tagged Myc was IBed with anti-HA-HRP (3F10), and actin is IBed with anti-actin antibody AC-15.

To confirm that MBP-1 does not affect Myc's ability to induce apoptosis NIH3T3 cells were transduced with Myc, MBP-1, Enolase, an shRNA against *eno1*, and combinations of these (figure 29). These data indicate that the samples were responsive to Myc, as cells transduced with Myc responded to doxorubicin treatment with approximately eight-fold more apoptosis than cells without Myc. Transducing cells with enolase, MBP-1, and a hairpin against *eno1*, however had no significant affect on the number of Myc-overexpressing cells undergoing apoptosis in response to doxorubicin.

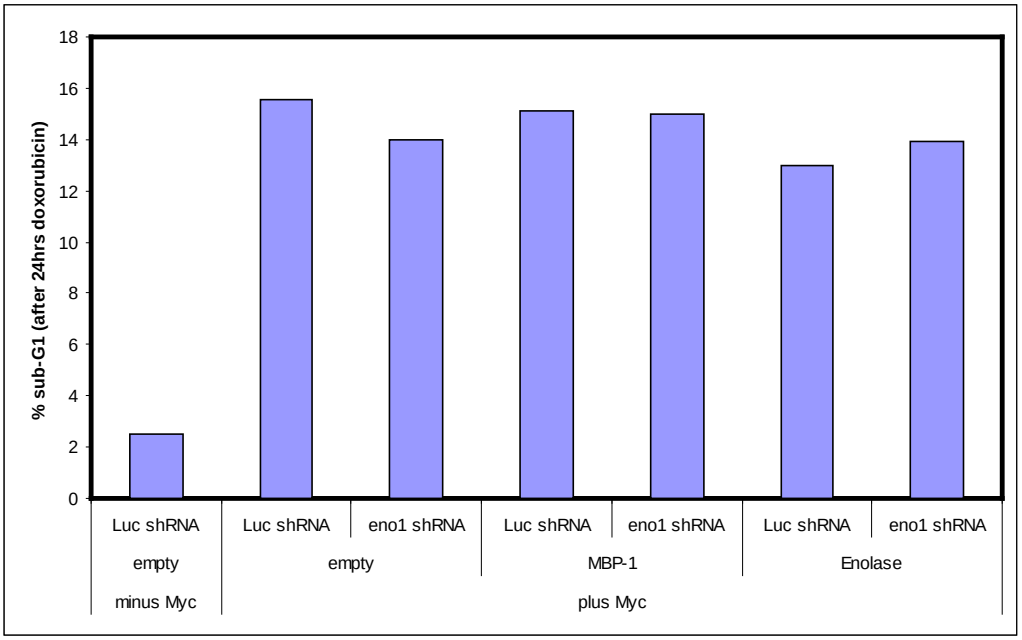

Figure 29: MBP-1 and enolase do not alter Myc's apoptotic response to doxorubicin. NIH3T3 cells were transfected with MIG-empty or MIG-Myc, pBabe-empty pBabe-MBP-1 or pBabe-enolase, and MLP-luciferase or MLP-eno1. Cells were treated with 0.5µg/mL doxorubicin for 24hrs.

These data indicate that changes in apoptotic rates are likely not responsible for the effect of the products of the *eno1* gene on Myc-driven colony formation. In general, differences in the number of colonies that form on soft agar can be attributed to differences in apoptosis, proliferation, quiescence, senescence, contact inhibition, or anchorage independence. At this point, changes in apoptotic rate can be ruled out as a likely mechanism by which enolase and MBP-1 affect Myc's ability to transform. In order to rule out changes in anchorage independence and contact inhibition I performed a simple crystal-violet assay on cells grown on plastic with liquid media (in which all living cells are stained with the violet dye). If the differences on soft-agar can be recapitulated in this assay, we can rule out the role of anchorage independence and contact inhibition in being responsible for these differences. And indeed, this is what is observed (figure 30 and 31).

In this experiment we see that when endogenous *eno1* is knocked down the number of cells increases (compare samples 8, 10, and 12 to samples 7, 9, and 11), although growth is limited by the fact that some plates are nearly confluent. We also observe that overexpression of MBP-1 and enolase reduce the number of cells (compare samples 9 and 11 to sample 7). The results seen on soft agar can thus be recapitulated by simply assaying cell number, suggesting that the role of MBP-1 and enolase on Myc-driven colony formation is independent of changes in anchorage independence and contact inhibition. The affect of MBP-1 and enolase

in this system is therefore likely due to changes in quiescence, senescence, or proliferation.

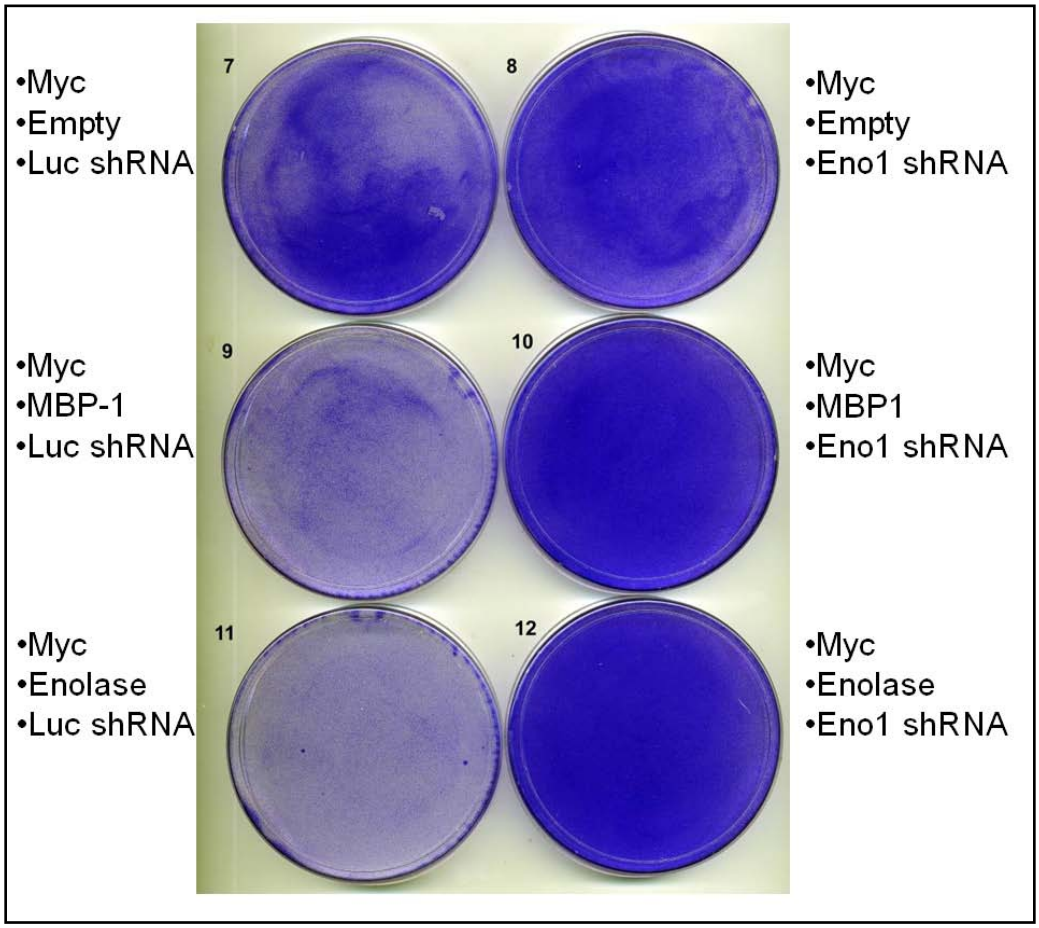

Figure 30: MBP-1 reduces cell number in cells overexpressing Myc. NIH3T3 cells were transfected with MIG-Myc, pBabe-empty pBabe-MBP-1 or pBabe-enolase, and MLP-luciferase or MLP-eno1. Cells were then stained with crystal-violet.

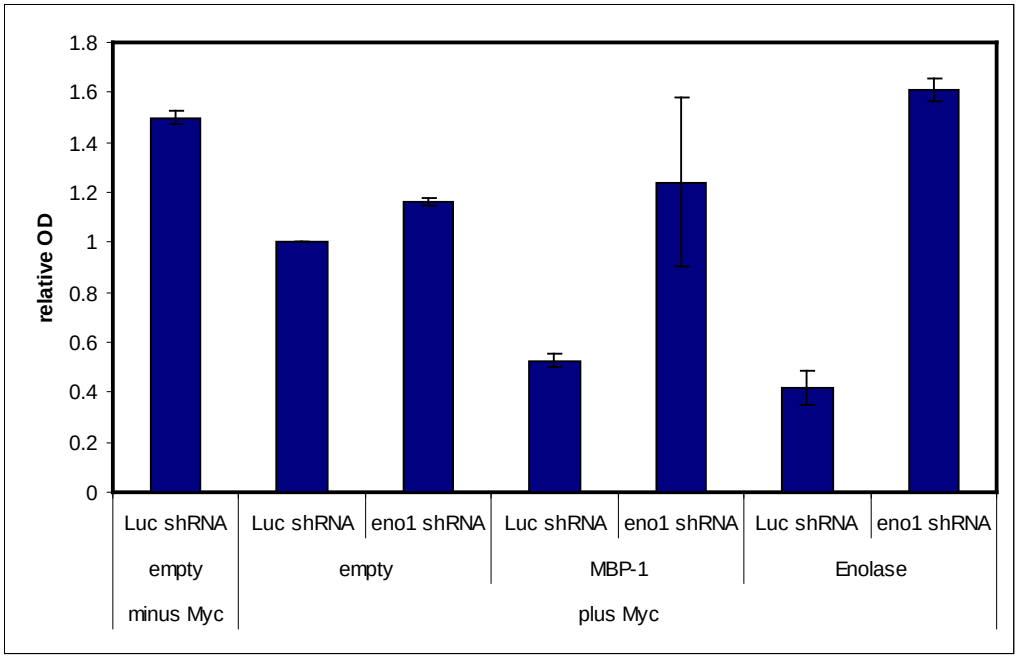

Figure 31: Quantified results from figure 30. NIH3T3 cells were transfected with MIG-Myc, pBabe-empty pBabe-MBP-1 or pBabe-enolase, and MLP-luciferase or MLP-eno1. Cells were then stained with crystal-violet and then lysed. Optical density was measured at OD 595.

Conclusions and significance:

The initial experiment presented in this chapter, the soft-agar assay in which MBP-1 and enolase were overexpressed, yielded results that were expected. Considering that MBP-1 and enolase facilitate Myc degradation, it is not surprising that they inhibit Myc-induced transformation.

I did not, however, predict the results of the second experiment, in which *eno1* was knocked down. In all of the soft-agar experiments presented in this chapter, Myc was highly overexpressed (see figures 22, 25, and 28) by the potent

MSCV promoter. It is therefore surprising that the number of Myc-driven colonies can be further increased by genetic manipulation. The fact that *eno1* knockdown had precisely this effect suggests that endogenous enolase/MBP-1 acts to limit the oncogenicity of Myc, even when the oncoprotein is artificially overexpressed. This has important implications for the evolution of cancer (as will be discussed in the next chapter) and for the role of enolase/MBP-1 in cancer treatment (as will be discussed in the Chapter 9).

The fact that the effect of *eno1* cannot be attributed to changes in apoptosis was also surprising. Considering that MBP-1 has been shown to induce apoptosis in cancer cells suggested that it might act to augment Myc's pro-apoptotic activity. The finding that this appears not to be the case also has important implications for cancer therapy, particularly with regard to a clinical phenomenon known as tumor lysis syndrome. This is a syndrome in which rapid, chemotherapy-induced cell death poses a risk to the life of certain cancer patients (including Burkitt's lymphoma patients). This too will be discussed further in the Chapter 9.

Finally, we must ask if one can account for the data presented in this chapter with what is already known about the role of MBP-1 and enolase. These proteins are known to repress Myc transcription (MBP-1 is an acronym for Myc-promoter binding protein 1), and so perhaps they are attenuating Myc's ability to drive colony formation simply by repressing Myc transcription and not by directly

binding to Myc and mediating its destruction. This is most likely not the case for the following reason: MBP-1, and enolose1α as well, have been shown to bind to Myc's primary promoter (known as P2) and act there to repress Myc transcription. This promoter is not present in the exogenously overexpressed *c-Myc* used to induce colony formation in the experiments presented here. This ectopic Myc is under the control of the MSCV promoter, and is therefore most likely not mediated by the factors that normally regulate Myc transcription.

CHAPTER 8: *eno1* is frequently deleted in cancers with amplified Myc

Background:

Having seen that enolase and MBP-1 attenuate Myc's ability to transform cells in a soft-agar assay, we asked if enolase/MBP-1 and Myc have a relationship in human cancers. Based on the data presented in the previous chapter, particularly the finding that reduced levels of enolase and MBP-1 augment Myc's ability to drive transformation, we predicted that reduced levels of enolase and MBP-1 would confer an advantage to cancer cells overexpressing Myc.

The fact that *eno1* is normally upregulated by Myc (141-143), and that *c-Myc* is normally repressed by MBP-1 and enolase (144-146) makes it difficult to determine precisely how expression of *eno1* is deregulated in cancers overexpressing Myc. For this reason, I decided to focus on gene copy-number variation in cancer instead of differences in gene expression. Because of the data showing that reduced enolase and MBP-1 levels increase Myc's ability to transform, my hypothesis was that deletion of *eno1* would confer a growth advantage to cancer cells with increased levels of Myc. Thus, in terms of gene

copy number, the prediction was that cancers with Myc amplifications would be more likely to have *eno1* deletions (i.e., a reduced number of *eno1* alleles) than cancers without Myc amplifications.

To test this hypothesis I worked with Jude Kendall of Michael Wigler's Lab at Cold Spring Harbor who had access to gene copy-number data from various human cancer samples. These data had been collected using a technique called Representational Oligonucleotide Microarray Analysis (ROMA) in which genomic DNA is harvested from healthy and cancer cells. The genomic DNA is then digested with a restriction enzyme (often BglII) and the resulting fragments are subsequently ligated to adapter oligonucleotides. Fragments are then amplified via PCR with adapter-specific primers. The amplified fragments are labeled with a fluorophore and hybridized to a microarray with probes representing locations across the genome. Often, genomic fragments from a normal sample and from a cancer sample, each labeled with a different fluorophore, are analyzed simultaneously to allow for direct comparison of malignant and non-malignant genomes. The method will allow us to separate cancer samples into those with amplified Myc and those without amplified Myc so that we can compare the number of *eno1* deletions in each set.

It should be noted that many cancers have aneuploid genomes (i.e., they are other than 2n). Therefore amplifications and deletions can refer to the gain and loss (partial or complete) of multiple copies of a given gene.

Purpose:

The goal is to determine if cancers with amplified *c-Myc* are more likely than cancers without this amplification to harbor *eno1* deletions.

Methodology:

The initial step in considering copy-number relationships between *c-Myc* and *eno1* was to gather data on as many cancers as possible. In this case, we were able to access data on cancers of the following tissues and organs: lung, liver, ovary, breast, pancreas, and colon. For each cancer, and for all available cancers combined, we asked how many bore Myc amplifications, and how many bore *eno1* deletions. For the purposes of this study, a signal for a given genetic locus in a cancer sample greater than 1.1-fold above the signal from the same locus in a non-cancer sample was considered an amplification. Similarly, a signal for a given genetic locus in a cancer signal that was less than 0.9-fold that of a non-cancer sample was considered a deletion.

Regions considered amplified or deleted for *eno1* could be of any size (ranging from less than 5megabases to whole-chromosome-arm copy number

changes). Regions considered amplified or deleted for *c-Myc* were divided into two categories: non-focal and focal. Non-focal copy-number changes for Myc, as for enolase, could be of any size. Focal copy number changes for Myc were considered 5megabases or less.

Once the number of cancers with amplifications and deletions of *c-Myc* and *eno1* was determined, the cancer samples from each tissue and organ was divided into two populations: those with *c-Myc* amplification (whether focal or not focal) and those without *c-Myc* amplifications. We then determined the number of *eno1* deletions in each population, and asked if the differences were statistically significant.

Results:

Nearly all analyzed cancers showed trends consistent with the initial hypothesis that cancers with *c-Myc* amplifications are more likely to carry *eno1* deletions than cancers without *c-Myc* amplifications. Not all cancer types, however, yielded statistically significant differences.

145 ovarian cancers were analyzed. 78 carried *c-Myc* amplifications among which 19 harbored *eno1* deletions (24%). 67 did not carry *c-Myc* amplifications among which 9 harbored *eno1* deletions (13%). 50 pancreatic cancers were analyzed. 26 carried *c-Myc* amplifications among which 3 harbored

eno1 deletions (11%). 24 did not carry *c-Myc* amplifications among which 1 harbored *eno1* deletions (4%). Lastly, 163 colon cancers were analyzed. 77 carried *c-Myc* amplifications among which 26 harbored *eno1* deletions (34%). 86 did not carry *c-Myc* amplifications among which 28 harbored *eno1* deletions (32%). These three cancer types showed a greater percentage of *eno1* deletions in cancers with amplified *c-Myc*; they did not, however, yield differences that were statistically significant.

While ovarian, pancreatic, and colon samples failed to yield statistically significant results, although the trends were consistent with the original hypothesis, data from three other cancer samples did show statistically significant differences. Samples from the breast, lung, and liver showed a statistically significant increase in the number of *eno1* deletions in cancers with *c-Myc* amplifications, as compared to cancers without *c-Myc* amplifications (figure 20). Focal Myc amplifications were considered for breast cancer samples, while non-focal Myc amplifications were considered for samples from lung and liver cancer. The fourth pair of pie charts in figure 20 (‘all available’) represent pooled data, in which non-focal Myc amplifications were considered, from ovarian, pancreatic, colon, breast, lung, and liver cancer.

This set of data indicates that cancers in which *c-Myc* is deleted are particularly likely to contain deletions in *eno1*, suggesting that cooperation between *c-Myc* amplification and *eno1* loss occurs in human cancer cells.

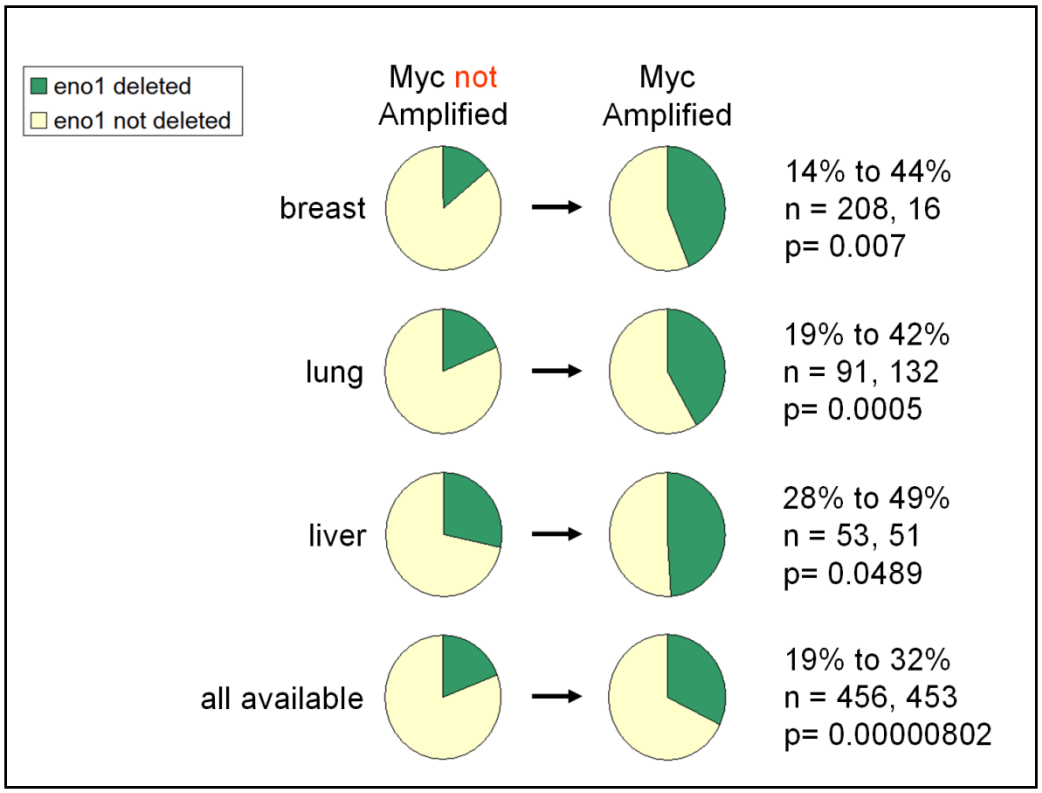

Figure 32: Human cancers with *c-Myc* amplifications are more likely to bear *eno1* deletions. Myc amplifications in breast cancers are focal (see text), whereas Myc amplifications in lung and liver cancers are non-focal. 'All available' includes samples from ovarian, pancreatic, colon, breast, lung, and liver cancers.

Conclusions and significance:

The first and most important caveat with regard to this copy-number data is that any inference to be drawn from it is purely correlative. Because of the mutual regulation of enolase/MBP-1 and Myc at the transcriptional level (i.e.,

MBP-1 is reported to repress *c-Myc* transcription and Myc is reported to activate *eno1* transcription), it was preferable to consider difference at the level of gene copy number. These data that are necessary correlative as amplifications often correspond with increased expression and that deletions often correspond with decreased expression.

Experimental transformation data, presented in chapter 7, indicates that reduced enolase levels (as obtained with *eno1* knockdown) increase Myc-induced transformation even when Myc is highly overexpressed. This would predict that *eno1* loss in Myc-overexpressing human cancers would provide a powerful growth advantage. In terms of gene copy number, this predicts that cancer cells with amplified *c-Myc* are more likely to become more aggressively oncogenic once *eno1* is lost than cancers without amplified *c-Myc*.

There are two possibilities for how this may occur in vivo. First, Myc amplifications may generate preneoplastic cells that subsequently become fully malignant when *eno1* is deleted. Second, a subset of cells – or even a single cell – within a cancer that is overexpressing Myc may lose *eno1* and then become aggressive enough to grow to predominate within the cancer. In either case, once such a cancer has developed, its cells would carry both the *c-Myc* amplification and the *eno1* deletion, as we observe.

While the data presented in this chapter are consistent with a model in which enolase/MBP-1 mediates Myc degradation thereby attenuating Myc's

oncogenicity (a model consistent with the data presented in this thesis), these data are also consistent with a model in which enolase/MBP-1 acts as a transcriptional repressor of *c-Myc*. In this latter model, loss of *eno1* might cooperate with *c-Myc* amplification in cancer cells, as transcriptional repression of amplified Myc would be relieved by *eno1* loss. This too could explain why *eno1* loss is particularly advantageous to cancer cells with amplified *c-Myc*. There are, however, no data showing that relieving Myc's transcriptional repression with *eno1* loss confers a growth advantage to cells already overexpressing Myc. In chapter 7 I present data that enolase/MBP-1 reduction cooperates with Myc overexpression in a manner that cannot be explained by transcriptional repression (as Myc expression in these cells is under the control of an ectopic promoter).

CHAPTER 9: Discussion

The enolase gene

Enolase

The *eno1* gene, which is reported to be a Myc target gene (141, 142), expresses two proteins: enolase1α (enolase) and MBP-1. The enolase protein is 47kD and is present in the cytoplasm of most tissues where it catalyses the penultimate, non-regulated, step of glycolysis in which 2-phosphoglycerate is converted to phosphoenolpyruvate and water. Interestingly, this same product of the *eno1* gene is τ-crystallin, a structural protein in the lens of the eye in vertebrates (147-154). This protein has another distinct function at the surface of various cells where it acts as a plasminogen receptor facilitating fibrinolysis (155-158).

An alternate translation initiation product of the enolase transcript, beginning at codon 97, produces a 38kD catalytically-inactive protein known as Myc-promoter binding protein 1 (MBP-1, figure 11). This protein was initially identified as one that binds the primary *c-Myc* promoter (P2) to repress Myc transcription, and has since been reported to repress the transcription of HIV

genes expressed from the viral LTR promoter (159-163). While it has been suggested that MBP-1 acts as a repressor by disrupting the formation of the pre-initiation complex at promoters, its ability to repress genes has also been associated with its ability to recruit HDACs (160). MBP-1 has also been shown to promote the regression of lung and prostate cancer cell growth (164, 165). And MBP-1 has been said to facilitate the degradation of the oncogene Mek5α via the proteasome, yet without affecting its ubiquitylation status.

MBP-1, Enolase, and Myc proteolysis

The piece of data that initially interested me in the D-element showed that deletion of this region significantly increases Myc stability (98). Curiously, this deletion did not cause Myc to accumulate in a hypo-ubiquitylated state as would be expected if the D-element were the binding site for a traditional ubiquitin ligase. Knowing that Myc is degraded by the proteasome, this strongly suggested that that the D-element mediates the proteasomal degradation of Myc independently of ubiquitylation.

Ultimately, after three stages of screening, I identified a protein that binds the D-element and mediates the degradation of Myc without affecting its ubiquitylation.

At that point the remainder of my thesis work was centered on the *eno1* gene. The fact that *eno1* encodes both enolase and MBP-1 added a layer of complexity to the subsequent experiments. While these experiments do not rule out the possibility that full-length enolase can act on Myc, MBP-1 certainly appears sufficient to manifest the effects of the *eno1* gene on Myc activity and stability. MBP-1 is sufficient to rescue Myc instability when combined with an *eno1* shRNA (figure 17). MBP-1 is sufficient to attenuate Myc's ability to transform (figures 20, 21, 23, and 24). And MBP-1 is sufficient to reduce cell number is cells overexpressing Myc (figure 30, compare plates 7 and 9). Moreover, MBP-1 physically associates with Myc more tightly than enolase does (figure 12). Considering that enolase is a cytoplasmic protein and that MBP-1 is nuclear one, one can speculate that *eno1* is acting on Myc primarily through MBP-1.

This is a key conclusion, as MBP-1 thus becomes perhaps one of the first proteins known to regulate both the transcription and the degradation of another protein.

MBP-1's molecular mechanism of action

The data presented in this thesis do not fully explain the molecular mechanism by which MBP-1 mediates Myc destruction. The data shown in figure 19 suggest that MBP-1 mediates Myc degradation through a mechanism other than that of a traditional E3, how then does it do this? We can only speculate.

One piece of information, that was gained fortuitously, can aid us in this speculation. As I was characterizing the effect of enolase and MBP-1 on Myc turnover, I tested the effect of enolase and MBP-1 overexpression. While I found that overexpression of these proteins did not affect Myc stability (compared to a control sample overexpressing dsRed), I noticed that both MBP-1 and enolase appeared on the gel when Myc was IPed (figure 33). This was encouraging as it confirmed that Myc physically associates with MBP-1 and enolase (and, although I do not know the input levels, it seems to suggest that MBP-1 binds Myc more tightly than enolase does as more MBP-1 than enolase is pulled down).

However, in addition to simply reinforcing the finding that these proteins physically associate, this gel also provides some new information. By measuring the decay of the bands corresponding to MBP-1 and enolase (and comparing them to the lower band which is the GFP normalization control described in chapter 4) we can determine the half-life – specifically – of the MBP-1 and enolase that

associate with Myc (figure 34). These data indicate that the half-life of Myc-associated enolase and Myc-associated MBP-1 are both approximately 60 minutes. Thus MBP-1 and enolase, molecules that mediate Myc instability, are unstable themselves.

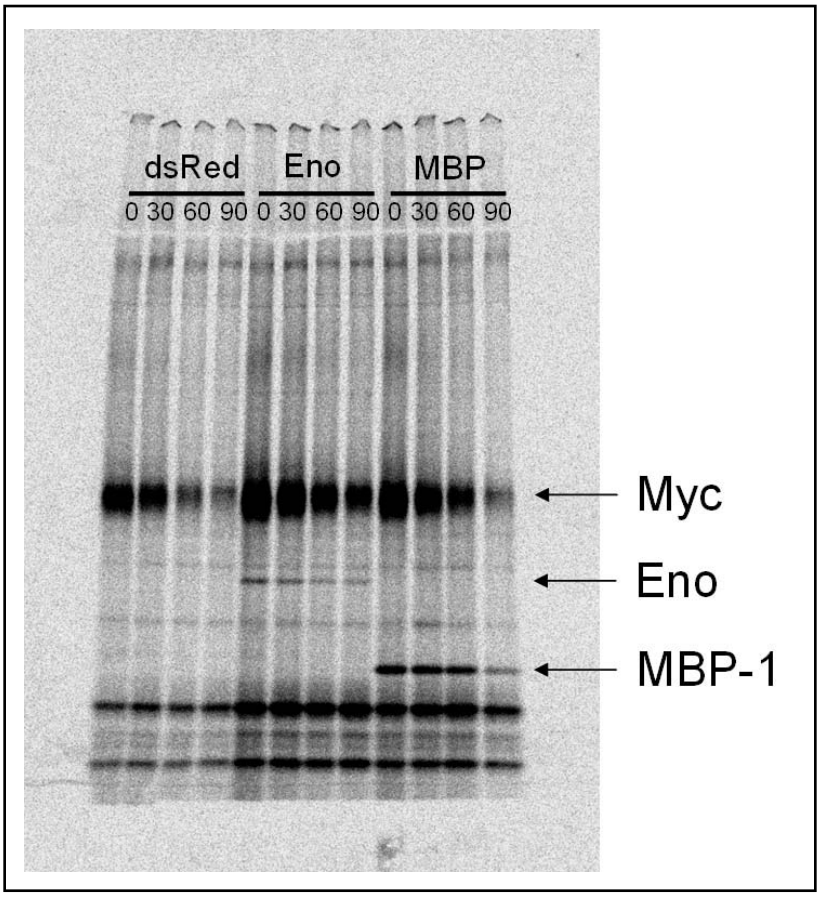

Figure 33: Enolase and MBP-1 associate with Myc in pulse chase assay. U2OS cells were transfected with 1µg of pCGN3HAM-Myc, 100ng pCGN-EGFP-NLS, and 1µg of dsRed1 pCGT-Enolase or pCGT-MBP1. Cells were harvested at 0, 30, 60, and 90 minutes after the initiation of the chase period.

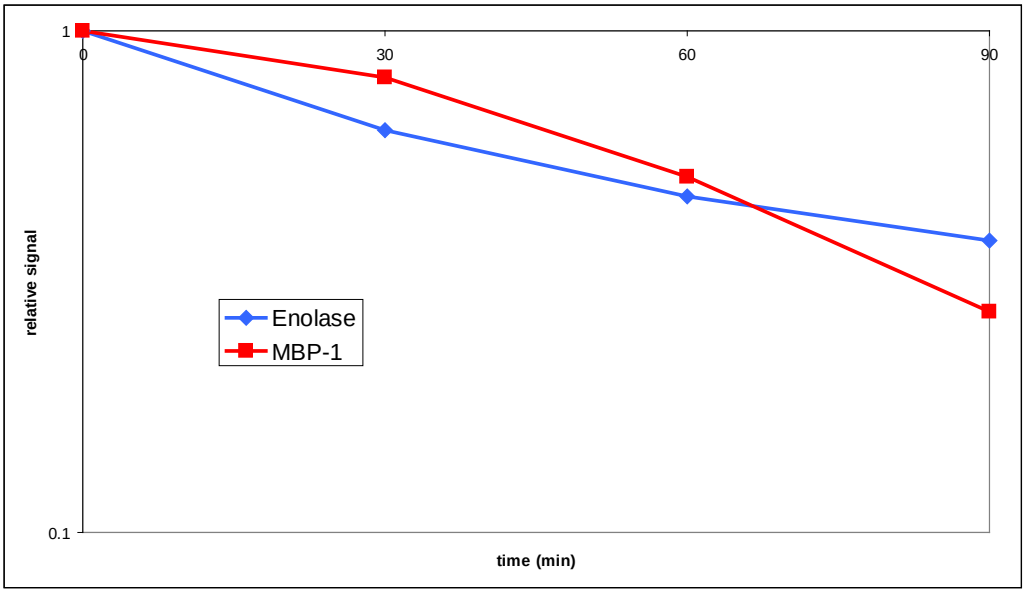

Figure 34: Quantified decay of Myc-associated MBP-1 and enolase. U2OS cells were transfected with 1µg of pCGN3HAM-Myc, 100ng pCGN-EGFP-NLS, and 1µg of dsRed1 pCGT-Enolase or pCGT-MBP1. Cells were harvested at 0, 30, 60, and 90 minutes after the initiation of the chase period.

Furthermore, while human enolase has not been shown to associate with the proteasome, yeast enolase has been shown to bind RPN10 a component of the 19S regulatory particle of the proteasome (166). Based on this interaction in yeast, and the fact that Myc-associated MBP-1 is rapidly degraded, we can speculate that MBP-1 binds the D-element and recruits the proteasome directly to the Myc-MBP-1 complex which is then degraded by the proteasome. Considering that overexpression of MBP-1 does not affect Myc ubiquitylation, we can also speculate that MBP-1 does not cause Myc to be ubiquitylated but rather that it

mediates the degradation of Myc once it has already been ubiquitylated (figure 35).

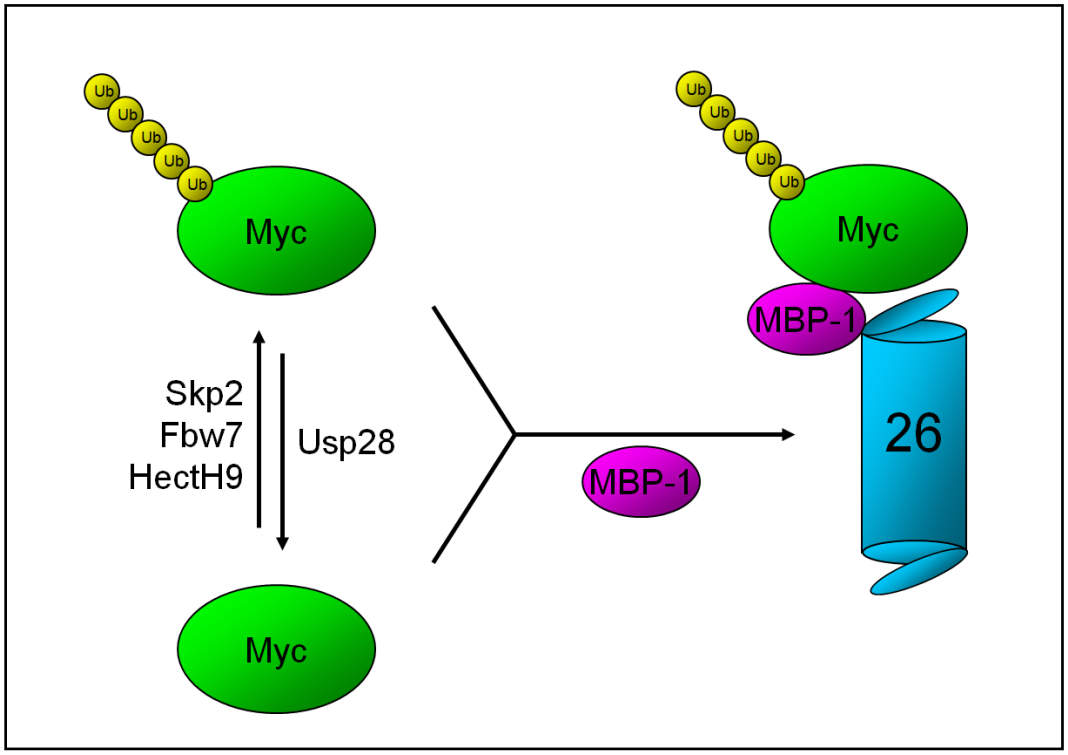

Figure 35: A model in which MBP-1 recruits Myc directly to the 26S proteasome subsequent to its ubiquitylation.

It is also theoretically possible that MBP-1 recruits Myc to the proteasome without itself being degraded. If this were the case the degradation signal on MBP-1, perhaps even its ubiquitylation, might signal the degradation of the Myc to which it is bound as there is a precedent for a degron on one molecule mediating the degradation of the protein to which it is bound (167). If this were

the case, it could explain how Myc is degraded by the proteasome without being ubiquitylated.

Finally, from a broader perspective, MBP-1 has now been shown to mediate the degradation of two oncoproteins: Mek5α and Myc. It has also been shown to repress Myc transcription. It therefore appears the MBP-1 has evolved a set of diverse functions to protect the cell against neoplastic transformation.

MBP-1 and glucose metabolism

Enolase1α catalyses the penultimate step of glycolysis, the conversion of 2-phosphoglycerate to phosphoenolpyruvate and water. While MBP-1 retains the catalytic residues of enolase, it retains almost none of its catalytic activity. Thus MBP-1, the product of *eno1* that is sufficient for many of the actions of this gene on Myc that are described in this thesis, does not play a significant direct role in glycolysis.

Almost all cancers upregulate glycolysis via the so-called Warburg effect, a phenomenon that is not fully understood. And Myc specifically upregulates glycolysis by regulating various target genes. It is therefore unlikely that MBP-1's action on Myc does not effect, or is not effected by, glycolysis.

Based on the model presented in this paper MBP-1 would be predicted to affect glycolysis in two ways. By facilitating Myc degradation and thereby attenuating its activity, Myc would no longer be fully able to regulate target genes in such a way that increases the rate of glycolysis. Second, by attenuating transformation and probably cancer progression as well, MBP-1 would be expected to reduce the Warburg effect.

Does MBP-1, however, play a more direct role on glycolysis? Can MBP-1 somehow communicate to Myc the status of glycolysis in the cell? Can Myc use MBP-1 to affect the rate of glycolysis? These are all complex questions that can be addressed in many ways. In my opinion it is best to begin by defining the effect of MBP-1 on glycolysis. Perhaps MBP-1 competes with catalytically-active full-length enolase and thereby slows glycolysis, or alternatively MBP-1 maybe shuttled to the nucleus so quickly that it has no bearing on glycolysis. In any case, once the role of MBP-1 in glycolysis is determined, we can then ask how this functionality (if it exists) is affected by Myc, a protein whose role in glycolysis is better defined.

MBP-1 and cancer treatment

The fact that MBP-1 mediates Myc degradation and reduces its ability to transform cells in vitro does not necessary imply that it plays an important role in

Myc-driven cancers. There are a number of proteins that can facilitate Myc degradation that have not been shown to counteract the effects of Myc in neoplastic cells. There are, however, reasons to expect that MBP-1 is one protein that can modulate Myc activity in a way that has implications for cancer progression and therefore cancer treatment.

Myc exists at relatively low levels in most healthy adult cells. In cancer, however, Myc expression can be elevated to the point that it becomes one of the most abundant proteins in the cell (as it is in Burkitt's lymphoma). In light of this, it is not difficult to imagine how proteins that normally regulate Myc turnover would be overwhelmed in such a setting, unable to blunt Myc's ability to drive the cancer. This effect of extremely high Myc levels, however, does not appear to overwhelm the ability of MBP-1 to attenuate Myc's oncogenicity. As the transformation data in figures 20-28 show, even when extremely high levels of Myc are present (at least enough to drive transformation) MBP-1 can still blunt the ability of Myc to transform cells. Perhaps the most important piece of information among the transformation data is the funding that knockdown of endogenous *eno1* leads to an increase in the ability ectopic Myc to transform cells in this system. This implies that even when (or perhaps, particularly when) Myc is highly overexpressed, the endogenous products of *eno1* still act to keep it in check. The finding that cancers with amplified *c-Myc* are more likely to carry

eno1 deletions, suggests that this effect is recapitulated in human neoplasms (figure 32, and discussed in chapter 8).

In terms of therapy, these findings imply the possibility that pharmaceuticals designed to mimic, to fix, or to strengthen the action of MBP-1 on Myc would reduce tumor growth even in cancers with Myc expressed many times higher than it is normally.

One of the initial reasons for investigating Myc's D-element was to identify a process that affects some, but not all, of Myc's functions. As it happens, MBP-1 appears to counteract Myc's oncogenicity without affecting Myc-induced apoptosis. At first, this seems to imply that MBP-1 is not an optimal point at which to target Myc-driven cancers, as increased apoptosis would be desirable in such malignancies. There is, however, an important case in which reducing Myc's oncogenicity without increasing apoptosis would be preferred therapeutically.

Many of the most aggressive cancers are also the most responsive to chemotherapy, as rapidly growing cells are killed preferentially by many DNA-damaging small molecules. In highly aggressive cancers – particularly Burkitt's Lymphoma – rapid cell death in response to chemotherapy causes a number of metabolic disturbances, collectively known as tumor lysis syndrome (TLS), that can lead to fatal acute renal failure among other sequelae (168-173). The

development of a therapy that reinforces the effect of MBP-1 on Myc, therefore, has the potential to reduce the tumor burden in cancers such as Burkitt's lymphoma while avoiding TLS. Such a therapy may be best suited for combination regimens with traditional anti-cancer treatments.

MBP-1 and the paradigm of Myc regulation

In light of data on Mek5α turnover (139), Myc appears to be the second protein whose post-ubiquitylation stability is mediated by MBP-1. While several proteins have been shown to ubiquitylate Myc, MBP-1 is the first shown to affect the degradation of ubiquitylated Myc specifically. This therefore represents a new layer in the regulation of a protein that is controlled at the level of transcription, transcript stability, translation, and protein stability.

This is not, however, the only unique aspect of the relationship between MBP-1 and Myc. In 1991 MBP-1 was reported to be a protein that regulates Myc transcription (145). Having now found that this same protein regulates Myc stability, we have identified the only protein known to regulate both Myc transcription and proteolysis. To the best of our knowledge, MBP-1 is the first protein shown to regulate another protein at both ends of its life-cycle: transcription and proteolysis.

CHAPTER 10: Future perspectives

It is important to determine if the results of the transformation assays can be recapitulated in animal models. For example, it would be informative to assess the role of enolase and MBP-1 on Myc-driven lymphomas using the lymphoma assay, described in the introduction, in which the hematopoietic system of sub-lethally irradiated mice is reconstituted with stem cells expressing genes of interest.

Additionally, it would be worthwhile to determine if the ability of MBP-1 to reduce cell growth (as demonstrated in figure 30) is necessarily dependent on the presence of Myc. This can be addressed with Myc-null cells with and without stably integrated Myc, as they would allow one to ask if ectopic MBP-1 depends on Myc to manifest its effect on cell growth.

Finally, it would be useful to determine if full-length enolase is sufficient, as MBP-1 is, to attenuate Myc's ability to transform cells in the soft-agar system. Being that the exogenous enolase that was expressed in the transformation assay presented in chapter 7 (figure 20, bottom right panels) also expresses MBP-1 as an alternate translation product it is not possible to know whether the effect that is observed is the result of full-length enolase or the MBP-1 that is co-expressed.

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