# Identification of Proteins that Regulate c-Myc Stability and Function

Dissertation by:

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Presented to the Department of Medical and Molecular Genetics

and the Oregon Health and Sciences

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

August 2008

School of Medicine

Oregon Health & Science University

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CERTIFICATE OF APPROVAL

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# List of Abbreviations

| | |
|---|---|
| 3AT | 3-Amino-1,2,4-Triazle |
| AD | Activation Domain |
| ATP | Adenosine Triphosphate |
| B | Basic Region |
| B56 | PP2A Regulatory B Subunit/ B' Family/PR65 |
| B-Myc | Brain Myc |
| BCS | Bovine Calf Serum |
| CAT | Chloramphenicol Acetyl Transferase |
| Cdc | Cell division cycle |
| Cdk9 | Cyclin dependent kinase 9 |
| ChIP | Chromatin Immunoprecipitation |
| CPRG | Chlorophenolred-β-D-galactopyranoside |
| c-Myc | Cellular Myc |
| DB | DNA Binding domain |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ERK | Extracellular Receptor Kinase |
| FBS | Fetal Bovine Serum |
| GSK3β | Glycogen Synthase Kinase 3β |
| HAT | Histone acetyltransferase |
| Hbp1 | High Mobility Group box protein 1 |
| HDAC | Histone deacetylase |
| His | Histidine |

**List of Abbreviations**

| | |
|---|---|
| HLH | Helix Loop Helix |
| HMG | High Mobility Group |
| INR | Initiator element |
| JNK | Jun N-terminal Kinase |
| LEF | Lymphoid Enhancer Binding Factor |
| Leu | Leucine |
| L-Myc | Lung Myc |
| LZ | Leucine Zipper |
| MAPK | Mitogen Activated Protein Kinase |
| MB1 or MBI | Myc box I |
| MB2 or MBII | Myc box II |
| MB3 or MBIII | Myc box III |
| MB4 or MBIV | Myc box IV |
| MEF | Mouse Embryo Fibroblast |
| METTL7B | Methyltransferase-Like 7B |
| NLS | Nuclear Localization Signal |
| N-Myc | Neuronal Myc |
| P | Phosphorylation |
| p107 | Retinoblastoma-like 1 |
| p130 | Retinoblastoma-like 2 |
| P63 | Proline 63 |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PCR | Polymerase Chain Reaction |

**List of Abbreviations**

| | |
|---|---|
| PI3K | Phosphoinositol-3-kinase |
| Pin1 | Prolyl Isomerase 1 |
| PP2A | Protein Phosphatase 2A |
| pRB | Retinoblastoma protein |
| qRT-PCR | Quantitative Real-Time PCR |
| REF | Rat Embryo Fibroblast |
| S62 | Serine 62 |
| SCF | Skp/Cullin/F-box |
| SD | Standard Deviation |
| SDS | Sodium Dodecyl Sulfate |
| shRNA | Short Hairpin RNA |
| siRNA | Small Interfering RNA |
| S-Myc | Suppressor Myc |
| T58 | Threonine 58 |
| TAD | Transactivational Domain |
| TCF | T Cell Specific Factor |
| Trp | Tryptophan |
| Ura | Uracil |
| v-Myc | viral Myc |
| WNT | Wingless |
| WT | Wildtype |
| βgal | β-galactoside |

# Acknowledgements

First and foremost I extend my deepest gratitude to my mentor, Rosalie Sears. She has taught me more than I could have possibly imagined and demonstrated how exciting and rewarding science can be. She has also shown me that I can be a successful woman in science while still being a devoted wife and mother. In addition to being a wonderful mentor, she is also a good friend who has helped me through some difficult periods in my life and I will always be thankful to her.

Secondly, I thank my committee: Dr. Melissa Wong, Dr. Richard Goodman, and Dr. Mike Liskay. I deeply appreciate the support and guidance they have given me through the years. I would also like to thank Dr. Stefan Lanker and Dr. Hua Lu for their extensive input on my work, and the members of the MMG office staff who worked tirelessly to make sure I kept on track.

Over the years I have been extremely fortunate to have had numerous talented co-workers, all of whom I am happy to call my friends. Melissa Cunningham and Kristi Piehl were instrumental in guiding my early graduate career, and they were always patient with me, for which I am extremely grateful. Maria Siri, Brian Laraway and Charlie Morgan always made the lab environment fun, which was especially comforting on the more difficult days. William DeWitt and Moon Yoon spent many hours working with me and I want to thank them for their assistance. Training and working with them increased the confidence I have in myself as a scientist and I will always be grateful to them for that. Dr. Suman Malempati, Dr. Peggy Chang, Dr. Xiaoyan Wang, Dr. Sarah Byers, and more recently, Dr. Amy Farrell, were always available for thoughtful discussion and I am extremely appreciative of all their support. In the last few months,

in addition to Amy, I was lucky to work with two newer additions to the Sears Lab, Karyn Taylor and Colin Daniel. They have helped me in immeasurable ways and I appreciate everything they have done. My fellow graduate students, Deanne Tibbitts and Xiaoli Zhang have both been great friends and I have enjoyed navigating the trials of graduate school together. I would also like to give a special thanks to Hugh Arnold. Over the last seven years Hugh has become one of my closest friends. He has taught me so much about science and life, and I feel that I owe many of my successes to him.

In addition to Hugh, I am so fortunate to have a number of close friends who have made the last years so much easier, including his wonderful wife, Alicia, and their incredible daughter, Marley. I would like to thank my fellow graduate students Kevin Friedman, Brenda Polster, as well as Carmen Baca-Jones, John Jones and their amazing daughter, Maya, for the many hours of science discussions, softball, game nights and margaritas. I would also like to thank two of my best friends from home, Jennifer Namba and Heather Chavez, who gently pushed to me to finish so I can rejoin them.

I am very blessed to have a very large and supportive family, all of whom have helped me in some way throughout my graduate career. I would especially like to thank my parents, Manuel Escamilla and Teal Taylor. They have shaped me into the person I am today and their support has been unlimited as they have helped me through this process. I will always be grateful to them. I would also like to thank my beautiful sisters, Susan Elder and Samantha Heckman. They are always there for me I am very lucky to have them in my life

Finally, I thank my wonderful husband, Colin. He has supported me and loved me every step of the way, regardless of my disposition, and I could not have made it this

far without him. Words can not describe how thankful I am that he is a part my life and I look forward to our many years together after this amazing journey.

# Abstract

c-Myc is a powerful oncoprotein whose expression is misregulated in a wide variety of human tumors. Studies of conditional c-Myc transgenic mouse models have demonstrated that loss of c-Myc from tumors may induce tumor regression. Therefore expanding our understanding of the regulation of c-Myc function and its degradation is critical to developing new targeted cancer therapies. In this study I use *Saccharomyces cerevisiae*, the budding yeast, as a model system to examine c-Myc degradation, as well as to identify novel c-Myc binding partners. Specifically, I found that c-Myc is phosphorylated at Threonine 58 (T58) and Serine 62 (S62) in an interdependent and sequential manner in yeast cells, as has been reported to occur in mammalian cells. Additionally, phosphorylation at these sites regulates c-Myc stability in yeast as in mammalian cells. Furthermore, yeast orthologs of mammalian proteins that regulate phosphorylation and dephosphorylation events on S62 and T58 in mammalian cells, appear to largely play a role in regulating the phosphorylation events on these sites in yeast. This is an important finding because it supports the discoveries made in mammalian cells. Additionally, it has been demonstrated that alterations in phosphorylation of T58 and S62 change the affinity c-Myc has for binding proteins that regulate its stability. It has also been reported that phosphorylation at these sites alter c-Myc activity. Therefore, this study also validates the use of *S. cerevisiae* to screen for novel c-Myc interacting proteins that regulate c-Myc stability and/or function. In my yeast two-hybrid screen I identified ten potential new c-Myc interacting proteins that appear to bind within the transactivational domain (TAD) of c-Myc, three of which have

been validated to interact in mammalian cells. One of these proteins, HMG Box Protein1 (HBP1), has been described as a tumor suppresser protein. Therefore, I characterized the interaction between HBP1 and c-Myc. Specifically, I found that HBP1 can bind to both the TAD and C-terminus of c-Myc in mammalian cells. This interaction appears to prevent c-Myc from binding the promoters of its target genes, thereby inhibiting c-Myc transactivational activity. This is a significant finding because it further solidifies the role of HBP1 as a tumor suppressor protein and it increases our understanding of the regulation of activity of the c-Myc oncoprotein.

# Chapter One:

# Introduction

## c-Myc

The oncoprotein c-Myc has been shown to play a critical role in many aspects of cancer (reviewed in (Lutz, Leon et al. 2002)). Cancer is a complex genetic disease that affects millions of people worldwide. The established molecular hallmarks of cancer include self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, unlimited replicative potential and tissue invasion and metastasis (Hanahan and Weinberg 2000). Given that Myc proteins regulate these processes, the Myc family of proteins is an important avenue of study.

The first described Myc family member, cellular Myc or c-Myc, was discovered as a result of its homology to the avian myelocytomatosis viral protein, v-Myc (Vennstrom, Sheiness et al. 1982). Other Myc family members include L-Myc, N-Myc, B-Myc and s-Myc. c-Myc is the most widely studied of all the members and is expressed in all mammalian tissues. c-Myc is most well known as a transcription factor of the basic helix loop helix family; however it is a weak transcription factor often only inducing gene expression two-fold (reviewed in (Patel, Loboda et al. 2004). Despite this, the activity of Myc family members is crucial in numerous cell cycle processes. Loss of Myc proteins has been shown to result in the inhibition of cell cycle progression as well as cell growth and can lead to accelerated differentiation (Mateyak, Obaya et al. 1997; Mateyak, Obaya et al. 1999; de Alboran, O'Hagan et al. 2001; Trumpp, Refaeli et al. 2001; Knoepfler, Cheng et al. 2002). Additionally, Myc proteins are important for embryonic development as *c-myc* homozygote knockout mice die at embryonic day 10.5 and *n-myc* knockout mice die at day 11.5 (Sawai, Shimono et al. 1991; Charron, Malynn et al. 1992; Davis,

Wims et al. 1993). In contrast, L-Myc knockout mice have no phenotype suggesting at least partial redundancy in the function of these proteins (Hatton, Mahon et al. 1996).

**Transcriptional regulation by the Myc/Max/Mad network**. c-Myc has been shown to activate transcription of endogenous and synthetic promoters in both yeast and mammalian cells. This transcriptional activity is dependent on the interaction between c-Myc and the basic helix-loop-helix leucine zipper (bHLH-LZ) protein Max (Amati, Dalton et al. 1992; Kretzner, Blackwood et al. 1992; Gu, Cechova et al. 1993). Max is a small, ubiquitously expressed protein that heterodimerizes with a number of bHLH-LZ proteins as discussed below. c-Myc and Max heterodimerize through their HLH-LZ regions and together bind E-box elements (CACGTG) in target gene promoters. Here c-Myc recruits histone acetyltransferase activity through its interaction with co-factors, resulting in transcriptional activation (McMahon, Van Buskirk et al. 1998; Liu, Tesfai et al. 2003) (Figure 1.1A). c-Myc/Max target genes include those that encode cell cycle regulators, cell growth regulators, metabolic proteins and apoptotic proteins (reviewed in (Dang 1999; Dang, O'Donnell et al. 2006)). The cellular effects of c-Myc-induced transcription include cellular proliferation and cell growth, induction of apoptosis in the absence of growth factors, and the inhibition of differentiation.

In addition to c-Myc, Max was found to heterodimerize to a variety of other proteins, specifically Mga, Mnt and the Mad family of proteins (Mad1, Mxi1, Mad3 and Mad4) (Ayer, Kretzner et al. 1993; Zervos, Gyuris et al. 1993; Hurlin, Queva et al. 1995; Hurlin, Queva et al. 1997; Hurlin, Steingrimsson et al. 1999). Heterodimerization of these proteins with Max also results in binding to E-box elements. However, in contrast

to c-Myc/Max complexes, these protein complexes inhibit expression of target genes. Mnt and the Mad family of proteins bind the scaffolding proteins Sin3 (Ayer, Lawrence et al. 1995; Schreiber-Agus, Chin et al. 1995; Hurlin, Queva et al. 1997). This protein acts as a co-repressor and can bind histone deacetylases resulting in chromatin remodeling and the repression of gene transcription (Laherty, Yang et al. 1997) (Figure 1.1C). Mga can also recruit chromatin modifying complexes. Specifically it has been shown that Mga recruits the histone methyltransferase Hp1γ (Ogawa, Ishiguro et al. 2002). This protein complex methylates histone 3 lysine 9, which is a transcriptional inhibition signal. Therefore these proteins antagonize Myc function by preventing gene transcription of c-Myc target genes.

In addition to activating transcription of target genes, c-Myc can also repress gene transcription. c-Myc repressed genes include the growth arrest gene *gadd45* and *c/ebpα* gene, which encodes a protein that regulates differentiation of pre-adipocytes (Li, Nerlov et al. 1994; Amundson, Zhan et al. 1998). The best characterized mode of c-Myc-mediated repression occurs at INR elements. Specifically, c-Myc has been shown to bind the transcription factor Miz1 through the c-Myc bHLH-LZ region (Peukert, Staller et al. 1997). This interaction does not prevent Myc/Max heterodimerization as triplexes have been found (Staller, Peukert et al. 2001). Miz1 recruits Myc/Max heterodimers to its binding sites and here repression is conferred. While initially this repression was thought to be passive, it has been reported that c-Myc can recruit the DNA methyltransferase Dnmt3a to INR elements while bound to Miz1 (Brenner, Deplus et al. 2005) (Figure 1.1B). This results in the methylation of the target promoter and inhibition of transcription. Specifically this was reported to occur at the p21 promoter. However, c-

Myc has also been shown to be recruited to the p15 promoter in a Miz1-dependent fashion, therefore recruitment of Dnmt3a may occur here as well (Staller, Peukert et al. 2001). These proteins are cyclin-dependent kinase inhibitors therefore their repression is important to c-Myc-driven cellular proliferation. At this time it is unknown how c-Myc preferentially recruits co-activators or co-repressors to the correct promoters.

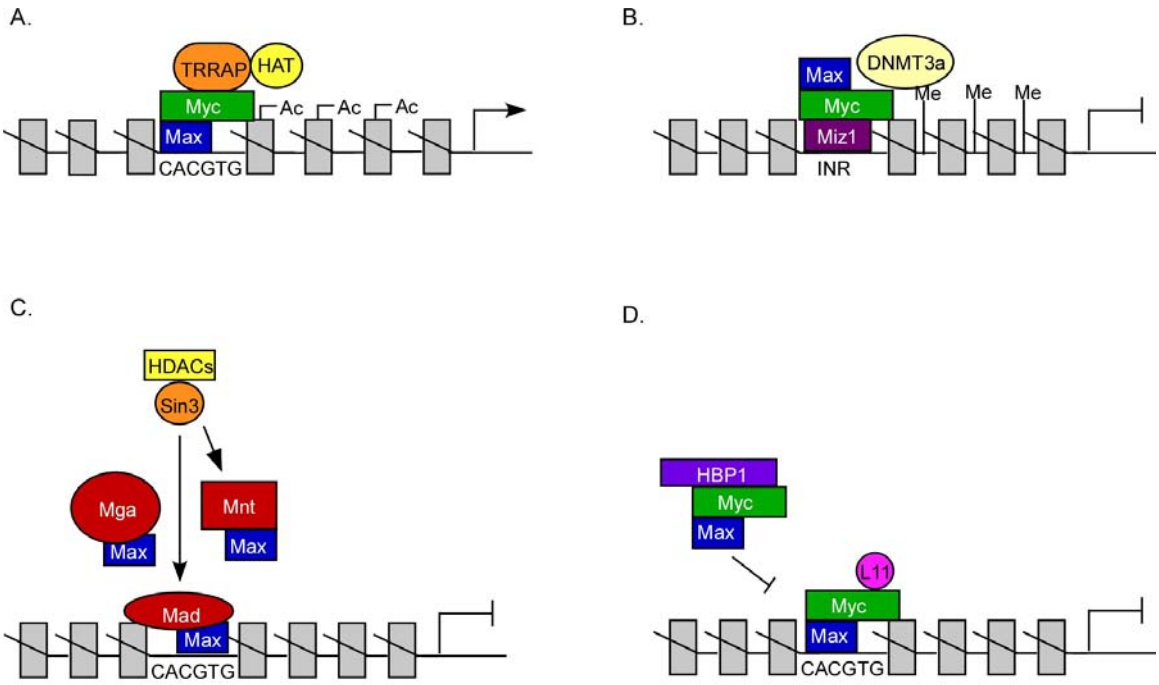



**Figure 1.1: Transcriptional regulation by the Myc/Max/Mad network of proteins.** (A) c-Myc/Max heterodimers activate gene transcription. c-Myc/Max heterodimers bind E-box elements in target gene promoters where they recruit co-activators, such as TRRAP. This results in the acetylation of histones and the activation gene transcription. (B) c-Myc/Max heterodimers can repress gene transcription at INR elements. c-Myc binds the transcription factor Miz1 and is recruited to Miz1 target genes. There the methyltransferase Dnmt3a binding c-Myc resulting in the methylation of DNA and the inhibition of gene transcription. (C) c-Myc function is antagonized by Mga, Mnt and the Mad family of transcription factors. Mga, Mnt and the Mad family of transcription factors heterodimerize with Max and bind E-box elements. Here they recruit co-repressors such as the scaffolding protein Sin3. In turn Sin3 recruits histone deacetylases (HDACs) resulting in the inhibition of gene transcription. (D) c-Myc binding proteins can inhibit c-Myc-driven gene activation. The ribosomal protein L11 can bind to the c-Myc protein and prevent the recruitment of critical co-factors while the HMG box transcription factor HBP1 can bind c-Myc and prevent its binding to target gene promoters (see text for additional details).

**c-Myc protein contains important regulatory domains**. Like most transcription factors c-Myc is a modular protein (Figure 1.2). The C-terminus harbors the basic region followed by a helix-loop-helix (HLH) and leucine zipper (LZ). The HLH and LZ are important for binding of Max, while the basic region forms contacts with DNA (Blackwood and Eisenman 1991; Prendergast and Ziff 1991). DNA binding of c-Myc is dependent upon its dimerization with Max (Blackwood and Eisenman 1991). Dimerization with Max is required for the transcriptional and transforming activity of c-Myc, as well as its apoptotic function, illustrating the need for direct DNA binding for c-Myc function (Amati, Dalton et al. 1992; Amati, Brooks et al. 1993; Amati, Littlewood et al. 1993; Bissonnette, McGahon et al. 1994).

The N-terminus of c-Myc is also required for c-Myc transactivational and transforming activity. The N-terminal 143 amino acids can induce transcription when fused to a heterologous DNA binding domain suggesting that it contains a functional transactivation domain (TAD) (Kato, Barrett et al. 1990). Located within the TAD are two regions highly conserved between both Myc family members and Myc orthologs, termed Myc Box I (MBI) (amino acids 45-63) and Myc Box II (MBII) (amino acids 128-143). MBII is essential for a majority of Myc functions as deletion mutants are unable to inhibit differentiation, induce apoptosis or cooperate with Ras to induce transformation (Stone, de Lange et al. 1987; Evan, Wyllie et al. 1992). Deletion of MBII also dramatically reduces the ability of c-Myc to induce transcription (Oster, Mao et al. 2003), however a MBII deletion mutant is only partially defective in its ability to induce cellular proliferation (Bush, Mateyak et al. 1998; Nikiforov, Chandriani et al. 2002). As

discussed below MBII is required for the interaction of c-Myc with a number of important co-factors that help mediate transactivation by c-Myc.

MBI harbors two phosphorylation sites, Threonine 58 (T58) and Serine 62 (S62), which have opposing effects on c-Myc protein stability (Sears, Nuckolls et al. 2000). Specifically phosphorylation of S62 stabilizes c-Myc protein while phosphorylation of T58 destabilizes the protein. These interdependent and sequential phosphorylation events regulate the ability of c-Myc to bind proteins that control its stability, including the scaffolding protein Axin1 and the F-box protein, Fbw7 ((Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004); Arnold et al, 2008 submitted for publication). Additionally phosphorylation at these sites may alter c-Myc activity. Specific regulation of these sites and their effects on c-Myc stability and function will be discussed below.

Two other conserved domains reside in the central region of the protein. Myc box III (amino acids 188-199) appears to be important for transcriptional repression, apoptosis, transformation and lymphomagenesis while Myc Box IV (amino acids 313-337) has been implicated in apoptosis, transformation and DNA-binding by Myc (Herbst, Hemann et al. 2005; Cowling, Chandriani et al. 2006; Kurland and Tansey 2008). Very little work has been done to elucidate how these regions specifically affect Myc activity. Further studies will be needed to confirm the described functions.


**Figure 1.2: Schematic of the c-Myc protein.**
Functionally important c-Myc domains are shown. At the c-terminus resides the nuclear localization signal (NLS), the basic region (b), the helix-loop-helix (HLH) and leucine zipper (LZ). In the N-terminus of c-Myc resides the transactivational domain (TAD) which harbors two highly conserved regions termed Myc box I (MB1) and Myc box II (MB2). Two additional highly conserved regions, Myc box III (MB3) and Myc box IV (MB4) reside within the middle of the protein. Two conserved phosphorylation sites that critical for regulating c-Myc stability, Threonine 58 (T58) and Serine 62 (S62), reside within MB1.

**c-Myc interacts with a wide variety of proteins that modulate its function.** c-Myc appears to interact with a variety of proteins. A number of these proteins can increase c-Myc activity and therefore are important co-factors. Specifically c-Myc has been shown to bind the ATM-related protein, TRRAP or transactivation/transformation domain associated protein (McMahon, Van Buskirk et al. 1998). TRRAP is a core subunit of histone acetyltransferase complexes and recruitment to c-Myc binding cites results in the activation of c-Myc target genes (Bouchard, Dittrich et al. 2001; Frank, Schroeder et al. 2001; Nikiforov, Chandriani et al. 2002) by in turn recruiting either Gcn4 or Tip60 complexes to induce acetylation of histones (McMahon, Wood et al. 2000; Frank, Parisi et al. 2003). TRRAP binds c-Myc in MBII, a region required for almost all of c-Myc functions, as described above. In addition to TRRAP, c-Myc has also been shown to interact with INI1, a member of the chromatin remodeling Swi/Snf complex, further

suggesting that chromatin remodeling in an important aspect of c-Myc-mediated gene activation (Cheng, Davies et al. 1999).

Other c-Myc co-activators include the cyclin dependent kinase Cdk9 and the F-box protein Skp2 (Eberhardy and Farnham 2002; Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003). Specifically Cdk9 interacts with the TAD of c-Myc and is recruited to c-Myc target gene promoters. There, Cdk9 phosphorylates the C-terminal domain of RNA polymerase II thereby enhancing transcriptional elongation (Eberhardy and Farnham 2002). Skp2 also binds within the transactivational domain of c-Myc, specifically within MBII. Skp2 has an additional binding site within the bHLH-LZ domain of c-Myc. Ubiquitination of c-Myc mediated by Skp2 increases both transcriptional activation and degradation of c-Myc (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003).

In addition to co-activators, a number of proteins have been described to bind c-Myc and inhibit its activity (Figure 1.1D). Recently the ribosomal protein L11 was described as a c-Myc interacting protein and a negative regulator of c-Myc transactivation (Dai, Arnold et al. 2007). In a collaborative effort, we demonstrated that L11 inhibited c-Myc-mediated transactivation of a reporter gene. Additionally, L11 could inhibit c-Myc induced expression of endogenous c-Myc target genes, by preventing interaction between c-Myc and its co-factor TRRAP. Other proteins that bind to c-Myc and inhibit its function include p107 and, as described in chapter four, the HMG box protein HBP1 (Beijersbergen, Hijmans et al. 1994; Gu, Bhatia et al. 1994).

**c-Myc protein stability and function is regulated by phosphorylation of Threonine 58 and Serine 62**. Given the importance of maintaining physiological levels throughout the cell cycle, it is not surprising that c-Myc is regulated at all levels, including transcriptionally, translationally and post-translationally (Kelly, Cochran et al. 1983; Jones and Cole 1987; Luscher and Eisenman 1990; Sears, Leone et al. 1999). c-Myc levels are kept low to non-existent in quiescent cells; however, following induction of the cell cycle, *c-myc* mRNA and protein increase dramatically and then decline to a baseline level (reviewed in (Cole and Nikiforov 2006)). While induction of *c-myc* gene expression by growth factors is partially responsible for this increase in c-Myc protein, changes in c-Myc protein stability are also a contributing factor. Work by our lab and others has focused on the posttranslational control of c-Myc stability. This work has resulted in the identification of two sequential and interdependent phosphorylation events on two highly conserved residues, T58 and S62. Phosphorylation at these sites has opposing effects on c-Myc protein stability as described below.

Both phosphorylation of S62 and T58 are regulated by Ras-activated signaling pathways (Figure 1.3). Specifically, following mitogen stimulation, S62 is phosphorylated by Ras-activated ERKs (Seth, Gonzalez et al. 1992; Pulverer, Fisher et al. 1994). This singly S62 phosphorylated form of c-Myc is a more stable form of the protein. It is important to note that S62 can also be phosphorylated by cyclin dependent kinases and JNK kinases (Lutterbach and Hann 1994; Noguchi, Kitanaka et al. 1999). Ras activity also prevents phosphorylation of T58 by GSK3β. GSK3β activity is inhibited by the Phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway downstream of Ras, which negatively regulates GSK3β activity through an inhibitory phosphorylation

event (Cross, Alessi et al. 1995). In late G1, when Ras activity declines, the inhibition of GSK3β is relieved and it is then free to phosphorylate c-Myc on T58 (Lutterbach and Hann 1994; Pulverer, Fisher et al. 1994). GSK3β is a processive kinase and phosphorylation of S62 is a prerequisite for phosphorylation of T58 (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000). This dually phosphorylated c-Myc protein is a substrate for the peptidyl prolyl isomerase, Pin1, which is believed to catalyze a *cis* to *trans* isomerization at the bond proceeding S62 (Yeh, Cunningham et al. 2004). The stabilizing S62 phosphate is then removed by the *trans*-specific phosphatase, PP2A (Yeh, Cunningham et al. 2004; Arnold and Sears 2006). PP2A is a trimeric complex consisting of a structural (A) subunit, a catalytic (C) subunit and a regulatory (B) subunit which dictates substrate specificity. Work in our lab identified B56α as the regulatory subunit that identifies c-Myc for dephosphorylation by PP2A (Arnold and Sears 2006). The singly T58 phosphorylated form of c-Myc is then believed to be targeted for multi-ubiquitination by the E3 ligase $SCF^{FBW7}$ and then degraded by the 26S proteasome (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004). Recently work in our lab has described a degradation complex, where the scaffolding protein Axin1 mediates binding of c-Myc, GSK3β, PP2A and Pin1, thereby facilitating degradation of c-Myc (Arnold et al. 2008, submitted for publication).

Mutations at T58 that render this site unphosphorylatable are found in the viral protein v-Myc as well as in c-Myc alleles isolated from human Burkitt's lymphomas (Papas, Kan et al. 1985; Bhatia, Huppi et al. 1993). Mutations at T58 result in increased transformation activity in the presence of RAS when compared to wildtype Myc in Rat embryo fibroblasts (Pulverer, Fisher et al. 1994). While mutations at this site stabilize

the c-Myc protein and therefore can lead to increased c-Myc activity (Yeh, Cunningham et al. 2004), it has also been found that mutations in and around this site impair the ability of c-Myc to induce apoptosis (Chang, Claassen et al. 2000; Conzen, Gottlob et al. 2000; Hemann, Bric et al. 2005). Induction of apoptosis in the absence of growth factors by oncogenes is an important fail-safe mechanism. Inhibition of apoptosis, often by mutation of p53, is important in tumor formation. Therefore, it is not surprising that mutations that inhibit this function of c-Myc are more oncogenic. At this time it is not fully understood how the mutant form of c-Myc inhibits induction of apoptosis but it may be due to its ability to induce expression of apoptotic proteins. Specifically, Hemann et al. showed that c-Myc mutated on and around T58 was unable to induce expression of the pro-apoptotic BH3-only protein, Bim (Hemann, Bric et al. 2005).

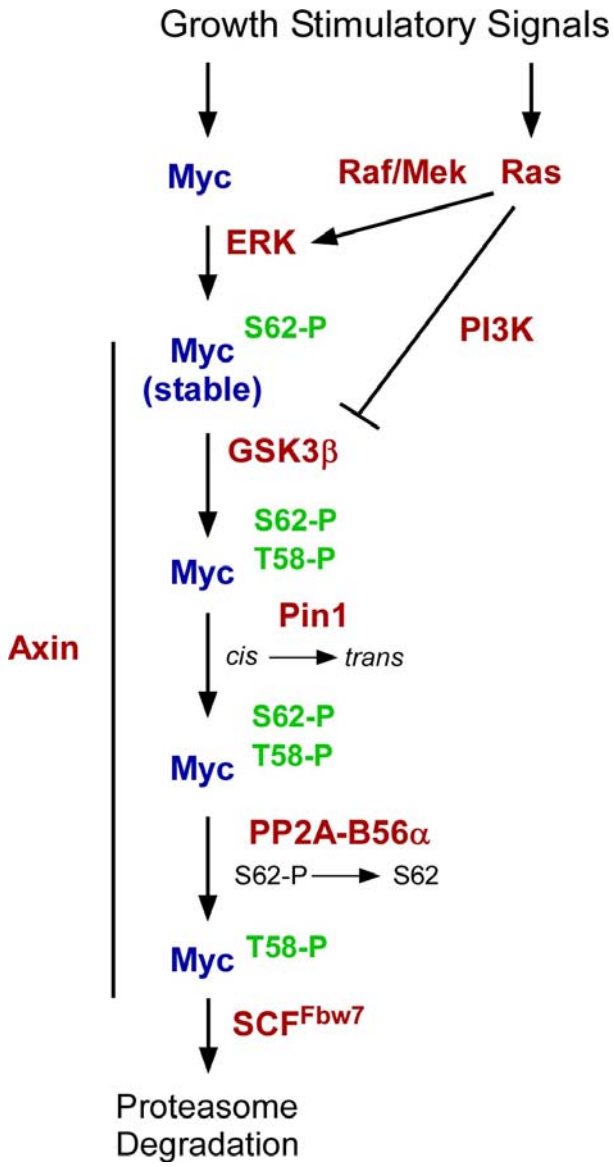



**Figure 1.3: Summary of the pathways controlling c-Myc phosphorylation and ubiquitin-mediated degradation.**

Following activation by growth stimulatory signals, Ras activates the Raf/MEK/ERK and PI3K/Akt pathways. This results in the phosphorylation at S62 of c-Myc and inhibition of phosphorylation at T58. In late G1, Ras activity decreases resulting in activation of the GSK3β kinase and phosphorylation of c-Myc at T58. Pin1 catalyzes a *cis* to *trans* isomerization at the bond preceding proline 63, followed by dephosphorylation of S62 by the PP2A phosphatase. Binding of GSK3β, PP2A and Pin1 to c-Myc is coordinated by the scaffolding protein Axin1. Singly phosphorylated c-Myc at T58 is targeted for multi-ubiquitination by its E3 ligase, $SCF^{FBW7}$ and degraded by the 26S proteasome. See text for additional details.

**c-Myc is a potent oncoprotein and a possible therapeutic target**. The *c-myc* gene resides on chromosome 8q24, which is found translocated to an immunoglobin enhancer in Burkitt's lymphoma, resulting in constitutive c-Myc expression in B cells (reviwed in (Boxer and Dang 2001)). In addition, c-Myc protein expression is elevated in 70% of all human tumors, including breast cancer, colon cancer and ovarian cancer (reviewed in (Nesbit, Tersak et al. 1999)). Overexpression of c-Myc, N-Myc and L-Myc family members have all been linked to different tumor types. The tumorigenic potential of c-Myc can be seen in cell culture where c-Myc can drive proliferation unchecked, inhibit differentiation and transform cells (Evan, Wyllie et al. 1992).

A large number of studies using transgenic animals have been performed to examine the tumorigenic potential of c-Myc *in vivo*. One of the most interesting themes that has arisen from conditional c-Myc models of tumorigenesis is that inactivation of c-Myc results in regression of tumors in a wide variety of tumor types, including lymphomas, breast adenomas and hepatocellular carcinomas (reviewed in (Arvanitis and Felsher 2005; Arvanitis and Felsher 2006). Depending on the specific tumor type, this regression can be a result of cell cycle arrest, differentiation and/or apoptosis. More recently, cellular senescence was also described to occur in response to c-Myc inactivation in different tumor types (Wu, van Riggelen et al. 2007). In some cases tumors became independent of c-Myc expression by acquiring new mutations. For example, in a transgenic model of c-Myc induced breast adenocarcinoma, a majority of the tumors that either did not regress or relapsed had activating mutations in K-ras or H-ras (D'Cruz, Gunther et al. 2001; Boxer, Jang et al. 2004). Despite this, conditional transgenic models suggest that c-Myc may be an excellent target for the treatment of

some human tumors. In support of this, it has recently been demonstrated that inhibition of Myc activity by prevention of Myc/Max heterodimerization can both prevent tumorigenesis, as well as induce regression of established lung adeonocarcinomas in an activated K-ras mouse model. Additionally, while inhibition of Myc activity in normal adult tissues was shown to cause adverse phenotypes in tissues that are rapidly turned over, including skin, testis and gut, these side effects were well tolerated and reversible (Soucek, Whitfield et al. 2008). These results may suggest that while Myc activity is essential in proliferating tissue, short periods of Myc inhibition may be beneficial in promoting tumor regression.

Understanding how both c-Myc stability and function is regulated is key to deciphering ways to target this protein or others that regulate it, in order to prevent tumorigenesis or promote tumor regression. My research has focused on studying both of these important aspects of c-Myc regulation. Specifically, I showed that the degradation pathway that regulates Myc stability occurs in yeast (Chapter 2). This is an important finding because this conservation exemplifies the importance of this regulatory pathway and suggests that other proteins, both yeast and mammalian, may be targeted for degradation in a similar manner. Additionally, this study further illustrates the importance of yeast as a model system to study mammalian protein degradation and it suggests use of yeast as tool to identify other proteins degraded in a similar fashion. In addition to studying c-Myc degradation in yeast, I also used yeast as a tool to identify novel c-Myc binding proteins that may be important in regulating c-Myc stability or function (Chapter 3). In this study I isolated HBP1, which I have characterized as a novel c-Myc interacting protein and a negative regulator of c-Myc activity (Chapter 4).

## HBP1

The HMG Box Protein1 (HBP1) is a ubiquitously expressed transcription factor most widely known for its ability to repress transcription. HBP1 has important roles in regulating cell cycle progression, differentiation and senescence, and it is emerging as an important tumor suppressor protein. Mammalian HBP1 was first isolated in a screen for mammalian proteins that could rescue a potassium channel defect in yeast (Lesage, Hugnot et al. 1994). While HBP1 could partially and specifically rescue the potassium channel defect, the 514 amino acid sequence revealed no hydrophobic domains that would suggest it to be a transmembrane protein. The authors observed significant homology between the C-terminus of HBP1 to the High Mobility Group (HMG) box domain, a known DNA binding domain of a family of transcription factors, and therefore they suggested this to be the molecular function of HBP1.

**HBP1 has multiple functional domains and binding partners.** HBP1 contains a number of important functional motifs (Figure 1.4). As stated above, HBP1 contains a C-terminal HMG box. The ~ 75 amino acid domain is found in a large family of proteins with diverse cellular functions (reviewed in (Stros, Launholt et al. 2007)). This family can be further broken down into two subfamilies. The first subfamily is non-sequence specific DNA binding proteins and the majority of family members contain more than one HMG box. The second subfamily, to which HBP1 belongs, consists mainly of proteins that harbor one HMG box that can bind specific DNA sequences. Other members of this subfamily include the sex determining factor, SRY and the Wnt signaling proteins TCF1 and LEF1 (Sinclair, Berta et al. 1990; Travis, Amsterdam et al.

1991; van de Wetering, Oosterwegel et al. 1991). The HMG box helps mediate DNA binding of these proteins by attaching to the minor groove of DNA and creating a DNA bend. The amount of bend appears to vary between HMG proteins and is dependent on the HMG box amino acid sequence (Love, Li et al. 1995; Murphy, Zhurkin et al. 2001). This bend also allows for binding of multi-protein complexes to DNA, making the HMG box proteins important transcriptional regulators.

HBP1 harbors two conserved RB binding motifs; LXCXE and IXCXE. These motifs are found in both viral and cellular proteins that bind to the pocket domain of the retinoblastoma (Rb) family members, pRB, p130 and p107 (Defeo-Jones, Huang et al. 1991; Dowdy, Hinds et al. 1993; Ewen, Sluss et al. 1993). Rb family members are important cell cycle regulators, most widely know as negative regulators of the E2F family of transcription factors. pRb is important in regulating cell cycle arrest as well as mediating cellular senescence. Two groups independently isolated HBP1 as a protein that interacts with p130 in a yeast two hybrid screen and demonstrated that HBP1 can interact with pRb or p130 through the conserved RB binding motifs (Lavender, Vandel et al. 1997; Tevosian, Shih et al. 1997). In contrast, HBP1 binding to p107 was not observed in mammalian cells. Mutation of both pRB binding motifs is required to completely abolish binding of these pocket proteins to HBP1. Furthermore, mutation of the pocket domain of p130 or pRb prevents binding of these proteins to HBP1 (Tevosian, Shih et al. 1997). Binding of pRb to HBP1 appears to be important for some but not all the transcriptional activities of HBP1 as discussed below.

In addition to RB family members, two other proteins have been shown to interact with HBP1. Xiu et al. isolated HBP1 in a yeast two-hybrid screen as a protein that

interacts with the p38 MAP kinase (Xiu, Kim et al. 2003). p38 MAPK has been shown to play important roles in regulating inflammation, apoptosis, cell cycle arrest, cell differentiation and senescence (reviewed in (Zarubin and Han 2005)). p38 was found to phosphorylate HBP1 at Serine 401. This phosphorylation event delays degradation of HBP1 by the 26S proteasome, increasing its half-life over two-fold. It is important to note that phosphorylated and unphosphorylated HBP1 had similar abilities to repress transcription when expressed at equal levels, indicating that phosphorylation does not alter the activity of the HBP1 protein (Xiu, Kim et al. 2003). HBP1 has also been shown to interact with the scaffolding co-repressor Sin3 (Swanson, Knoepfler et al. 2004). Sin3 binds a number of proteins including those known to antagonize c-Myc function, specifically Mnt and the Mad family of repressors (Ayer, Lawrence et al. 1995; Schreiber-Agus, Chin et al. 1995; Hurlin, Queva et al. 1997). The Sin3 binding site was mapped to amino acids 362-398 of HBP1 (see Figure 1.4). The histone deacetylase HDAC1, a protein known to associate with Sin3 and an important transcriptional repressor, could be co-immunoprecipitated with HBP1, suggesting that these proteins form a repression complex (Swanson, Knoepfler et al. 2004). Furthermore, these data suggest that chromatin remodeling may be an important aspect of HBP1 function.

Given that the HBP1 binding partners, Sin3 and pRB recruit histone deacetylases (HDACs) to chromatin thereby inhibiting gene expression (Laherty, Yang et al. 1997; Zhang, Iratni et al. 1997; Brehm, Miska et al. 1998; Ferreira, Magnaghi-Jaulin et al. 1998; Magnaghi-Jaulin, Groisman et al. 1998), it is not surprising that HBP1 is best known as a transcriptional repressor (Figure 1.5A). In addition, a repression domain that has been reported to be mapped to the middle of the protein between amino acids 199 and

400. Tevosian et al. showed that fusing the N-terminal 393 amino acids to the LEF1 HMG box could confer repression of a reporter plasmid containing HPB1 binding sequences (Tevosian, Shih et al. 1997). Since LEF1 itself is able to bind to, but not repress transcription from HBP1 sites, this demonstrates that HBP1 contains an independent repression domain. Additionally, it has been shown that the N-terminal 220 amino acids of HBP1 are unnecessary for repression of different HBP1 targets, as deletion mutants lacking the N-terminus could still inhibit expression from different reporter constructs (Sampson, Haque et al. 2001; Berasi, Xiu et al. 2004). Transcriptional repression by HBP1 is important in regulating both cell cycle progression as well as differentiation as described below.

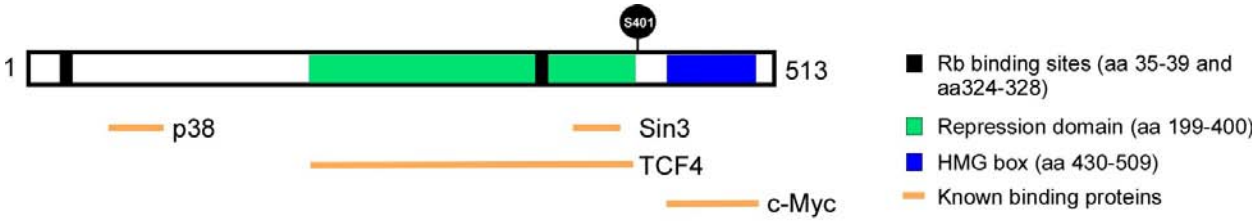



**Figure 1.4: HBP1 functional domains and binding partners.**
The C-terminus of HBP1 harbors the HMG box, required for DNA binding while the central region of the protein contains the repression domain. HBP1 has two pRB interaction motifs as wells as binding sites for the Sin3 scaffolding protein and the MAPK p38. p38 phosphorylates HBP1 at Serine 401. HBP1 can bind the transcription factors TCF4 and c-Myc and inhibit their transactivational activity (see text for details).

**Transcriptional Repression by HBP1 results in cell cycle inhibition**. Thus far only a small number of HBP1 target genes have been described, but they all give insight into the

overall effect of HBP1 expression. For example, the first described HBP1 target gene was *n-myc* (Tevosian, Shih et al. 1997). The *n-myc* promoter was found to contain three HBP1 binding motifs with a consensus sequence of A/T-C/G-A-A-T-G-G-G. A reporter gene driven by the *n-myc* promoter was efficiently repressed by HBP1. This repression required DNA binding by HBP1, as a HMG box triple point mutant that is unable to bind DNA was no longer able to confer repression. Additionally, repression of *n-myc* by HBP1 in part, requires pRB binding. Like c-Myc, N-Myc is an important regulator of cell cycle progression and the inhibition of *n-myc* expression by HBP1 illustrates that HBP1 may be a key cell cycle inhibitor. In support of this hypothesis, the authors showed that expression of HBP1 in a C2 muscle cell line resulted in an almost five-fold decrease in cells in S phase (Tevosian, Shih et al. 1997). This was the first evidence indicating that HBP1 is a key regulator of the cell cycle.

Another described HBP1 target is the *p47phox* gene, which contains six high affinity HBP1 binding sites (TTCATTCATTCA) (Berasi, Xiu et al. 2004). This gene encodes for a cytoplasmic subunit of NADPH oxidase complex which is responsible for producing reactive oxygen species (ROS). HBP1 reduced expression of a reporter gene driven by the *p47phox* promoter and it was shown that both DNA binding by HBP1 as well as the repression domain was required for this function. Additionally, ectopic HBP1 bound the endogenous *p47phox* promoter and reduced superoxide levels in a cell line. Interestingly, the expression of the HMG box of HBP1 alone resulted in a dominant negative effect. To date, this is the only gene where this effect of the HMG box has been observed. Additionally, reduction in superoxide levels through repression of *p47phox* by HBP1 contributed to cell cycle inhibition by HBP1 (Berasi, Xiu et al. 2004).

In addition to binding its targets directly, HBP1 indirectly represses transcription through interaction with other transcription factors (Figure 1.5B). It is in this way that HBP1 can inhibit Wnt signaling, an important signaling pathway that regulates expression of multiple genes, including those that regulate cell cycle progression (Sampson, Haque et al. 2001). In the absence of Wnt ligands, the transcription factor β-catenin is phosphorylated by GSK3β and targeted for ubiquitination and degradation by the 26S proteasome (Hart, de los Santos et al. 1998; Ikeda, Kishida et al. 1998; Latres, Chiaur et al. 1999; Liu, Kato et al. 1999; Winston, Strack et al. 1999). In response to Wnt signaling, phosphorylation by GSK3β is inhibited and β-catenin is free to accumulate and translocate to the nucleus where it binds with TCF/LEF-1 and induces transcription of target genes (Li, Yuan et al. 1999; Fukumoto, Hsieh et al. 2001). These genes include *c-jun*, *cyclin D1*, and notably *c-myc* (He, Sparks et al. 1998; Mann, Gelos et al. 1999; Tetsu and McCormick 1999). Sampson et al. examined the relationship between HBP1 and Wnt signaling and found that HBP1 could prevent the expression of a reporter gene driven by a promoter containing LEF1/TCF sites. Expression of HBP1 was found to inhibit transcription of the β-catenin/TCF/LEF target genes, *cyclin D* and *c-myc*, in a colon carcinoma cell line with a constitutively active Wnt pathway. This repression was found to be due to the binding of HBP1 to the HMG box transcription factor TCF4. This binding did not affect the ability of TCF4 to bind β-catenin; however binding of DNA by the β-catenin/TCF4 complex was inhibited. HBP1 was found to bind TCF4 at two regions; within the TCF4 HMG box and in an undescribed N-terminal region. The negative regulation of the cell cycle proteins, c-Myc and Cyclin D, by HBP1-mediated

inhibition of Wnt signaling further elucidates the role of HBP1 as a negative regulator of the cell cycle.

In addition to negatively regulating the cell cycle, HBP1 may be important in regulating muscle cell differentiation through its negative regulation of the MyoD family of transcription factors. One of the first observations regarding HBP1 was that its mRNA increased upon differentiation of muscle and adipose cells (Lesage, Hugnot et al. 1994). Shih et al, further examined the relationship between HBP1 and muscle cell differentiation by stably expressing low levels of HBP1 in the C2 muscle cell line (Shih, Tevosian et al. 1998). Interestingly, it was shown that expression of HBP1 resulted in the block of terminal differentiation due to the inhibition of expression of the basic helix-loop-helix (bHLH) proteins MyoD and myogenin. Expression of Myf5, a bHLH protein that is functionally upstream of MyoD and myogenin was not affected. These transcription factors activate gene expression by binding E-boxes in target gene promoters and have been shown to be crucial to normal muscle differentiation (reviewed in (Berkes and Tapscott 2005)). It is currently thought that these family members form a signaling cascade where Myf5 activates transcription of MyoD (reviewed in (Rawls and Olson 1997)). Shih et al. found that in the presence of HBP1, re-expression of MyoD or Myogenin could restore differentiation, suggesting that these proteins are functionally downstream of HBP1 (Shih, Tevosian et al. 1998). HBP1 was found to inhibit transcriptional activation by the MyoD family from both a natural target promoter as well as synthetic E-box promoter. However, the authors noted that this was not due to direct DNA binding to MyoD family consensus sites as HBP1 was unable to bind E-boxes in a gel shift assay. The direct mode of inhibition is currently unknown as HBP1 was able to

bind MyoD family members *in vitro* but no binding was observed in cell culture. It is possible that the interaction was transient and difficult to examine. Therefore it is possible that like TCF4, HBP1 binds the MyoD family members thereby preventing them from binding DNA and activating transcription. Likewise, the authors hypothesized that HBP1 prevents differentiation by inhibiting Myf5 function and preventing activation of MyoD and Myogenin promoters (Shih, Tevosian et al. 1998). Interestingly, increased expression of pRb was able to restore MyoD family transcriptional activity as well as differentiation in the presence of HBP1. Shih et al. hypothesized that when pRB levels are low in early differentiation and HBP1 levels are high, HBP1 induces a necessary cell cycle arrest prior to differentiation. However, terminal differentiation can not occur until the ratio of pRB to HBP1 switches creating a differentiation checkpoint.

**HBP1 can act as a transcriptional activator**. While HBP1 is commonly regarded as a transcriptional repressor, in some cases HBP1 has been reported to have the capacity to activate transcription (Figure 1.5C). Lavender et al. first suggested that HBP1 might harbor a masked activation domain (Lavender, Vandel et al. 1997). They found when amino acids 37-120 of Rat HBP1 were fused to a Gal4 DNA binding domain this region of HBP1 was able to significantly activate expression of a reporter gene. Indeed, HBP1 has been shown to induce expression of specific genes (Lemercier, Duncliffe et al. 2000; Lin, Zhao et al. 2001). For example, HBP1 was reported to activate histone H1(0) (Lemercier, Duncliffe et al. 2000; Lin, Zhao et al. 2001). This is a linker histone that is specifically expressed during differentiation (reviewed in (Zlatanova and Doenecke 1994)). HBP1 was identified in a yeast one-hybrid screen as a protein that binds to a

highly conserved region of the H1(0) promoter and it was subsequently shown to induce transcription of a reporter gene under the control of this promoter. Interestingly pRB increased this activation capacity to four-fold suggesting in this case these two proteins are acting cooperatively. It is important to note that the putative activation domain previously described was unable to induce expression from the H1(0) promoter, suggesting that other regions of the protein are necessary for this activation function. While it is unknown what regions are important at this time it will be important to further elucidate them in order to understand regulation by HBP1.

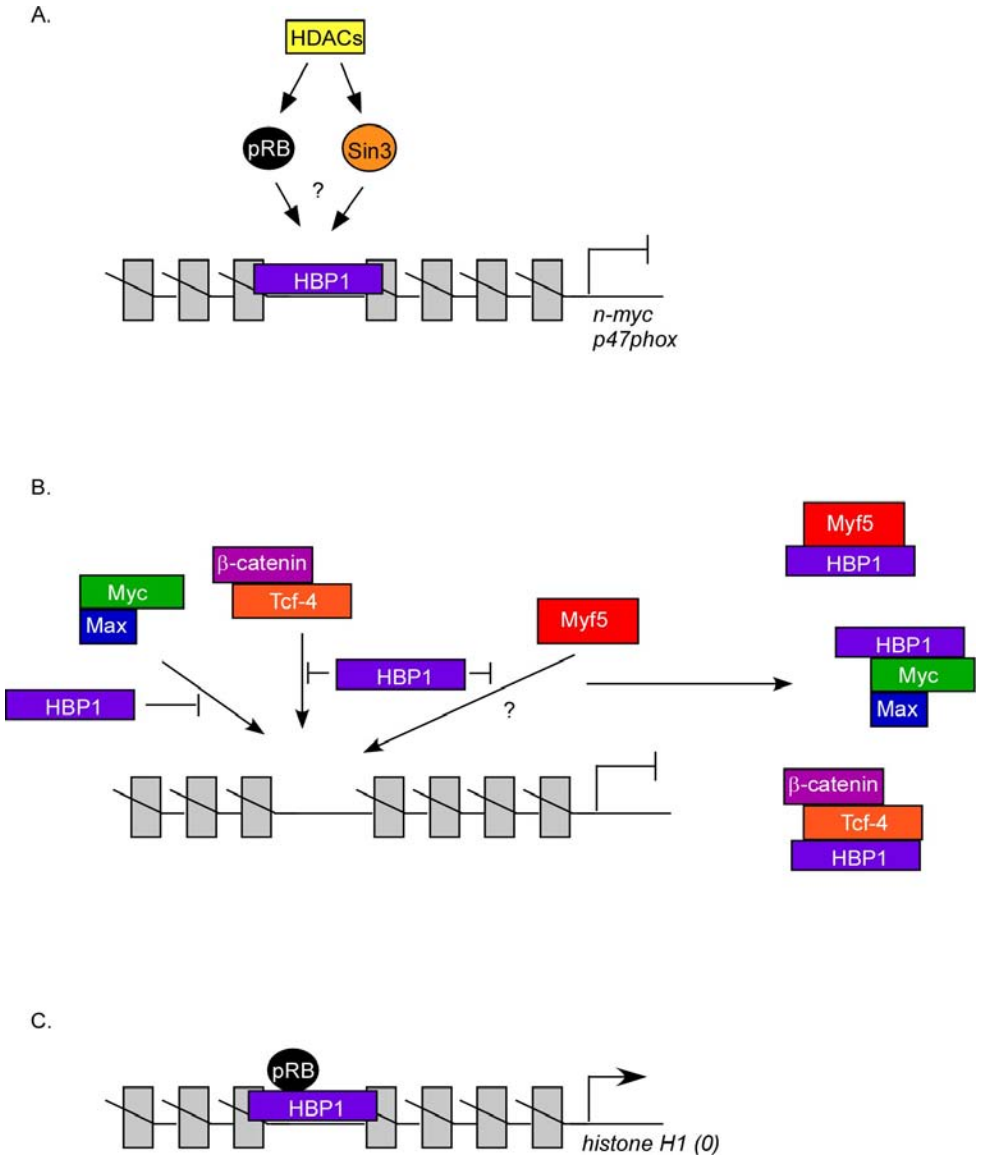



**Figure 1.5: Potential modes of HBP1 activity.**

(A) Sequence-specific repression by HBP1. HBP1 binds to DNA directly and represses transcription of its target genes. The exact mechanism of repression is currently unknown; however, HBP1 may recruit its co-factors, pRB and Sin3 to DNA, both of which have been shown to interact with histone deacetylases. (B) HBP1 inhibits DNA binding by different transcription factors. HBP1 was shown to interact with TCF4 and prevent its interaction with DNA (Sampson, Haque et al. 2001). HBP1 can also inhibit Myf5 activity through an unknown mechanism; however, it is possible that HBP1 can prevent DNA interaction by Myf5 (see text for details). In Chapter 4 we show that HBP1 inhibits c-Myc activity by preventing c-Myc interaction with DNA. (C) HBP1 can act as a transcriptional activator. It was shown that HBP1 induces expression of the histone H1(0) gene and interaction with pRB enhances this activation (Lemercier, Duncliffe et al. 2000), however the exact mode of activation is unknown.

**HBP1 is a tumor suppressor protein**. Growing evidence suggests that HBP1 is a tumor suppressor protein. A number of reports have shown that HBP1 can delay S phase in a variety of cell types including adipose, muscle, hepatocytes and myeloid cells (Tevosian, Shih et al. 1997; Shih, Xiu et al. 2001; Yao, Works et al. 2005). HBP1 inhibits the Wnt pathway, which is often misregulated in cancer (Sampson, Haque et al. 2001). Additionally, the HBP1 gene is located on chromosome 7q31.1, a region lost in many different cancer types (Zenklusen, Thompson et al. 1994; Zenklusen, Thompson et al. 1995; Zenklusen, Weitzel et al. 1995; Driouch, Briffod et al. 1998; Liang, Fairman et al. 1998; Koike, Tasaka et al. 1999). Recently HBP1 has been shown to play a role in oncogene-induced senescence, a mechanism thought to act as a stop-gag to tumorigenesis (reviewed in (Dimri 2005)). While previous reports had shown that oncogene-induced senescence was common in cell culture, it has only recently been shown to occur *in vivo* in premalignant tumors (Braig, Lee et al. 2005; Chen, Trotman et al. 2005; Collado, Gil et al. 2005; Michaloglou, Vredeveld et al. 2005). Zhang et al. examined the role of HBP1 in RAS-induced premature senescence and found HBP1 to be an important downstream effector of RAS but upstream of pRb, an essential regulator of cellular senescence (Zhang, Kim et al. 2006). The ability of HBP1 to bind pRb was necessary for inducing senescence but not sufficient, as a mutant form of HBP1 that is unable to bind DNA was unable to induce senescence, indicating that DNA binding by HBP1 is also important. This suggests that either direct transcriptional activation or repression by HBP1 is required for this effect. However, the exact role of HBP1 in this process is currently unknown.

More recently research has focused on the role of HBP1 in cancer cell lines. Yao et al. found that stable expression of HBP1 in the K562 leukemia cell line delayed cell proliferation. When the cells were grown in soft agar both cell number and colony size were reduced compared to control cells, suggesting a block in some of the tumorigenic properties of these cells. The authors observed a block in G1 in the K562 cells in response to HBP1 expression. In agreement with this observation, expression of the important cell cycle regulators, *cyclin D1* and *D3*, was decreased in K562 cells expressing HBP1, compared to a control cell line. Additionally, the expression of the cyclin dependent kinase inhibitor p21 and the transcription factor c/EBPα was increased in HBP1 expressing cells. The delay in cell cycle expression observed in response to HBP1 was also accompanied by differentiation of the cells to the erythroid or megakaryocytic lineages. In agreement with this, mRNA levels of *c-myc* and *c-myb*, whose expression is known to decline during differentiation, were both decreased (Yao, Works et al. 2005). It is interesting to note that both p21 and c/EBPα genes have been shown to be repressed by c-Myc (Freytag and Geddes 1992; Wu, Cetinkaya et al. 2003). At this time it is unknown if these proteins were upregulated in response to decreased c-Myc expression in this system. Taken together these results suggest that HBP1 can reduce the tumorigenic potential of leukemic cells.

Finally, recent work has examined the loss of HBP1 in both breast cancer cell lines and in human breast tumor samples. Paulson et al. found 7 HBP1 variants in 10 patient samples (Paulson, Rieger-Christ et al. 2007). These variants occurred in 13% of the patient samples examined. Interestingly, despite examining different breast cancer types, all mutants were found in infiltrating ductal carcinoma, the most commonly

diagnosed form of breast cancer. All mutants were defective in their ability to repress Wnt signaling, a commonly activated pathway in breast cancer (reviewed in (Howe and Brown 2004)). Additionally, *hbp1* mRNA levels were examined in invasive breast cancer patient samples and over half the samples tested had reduced HBP1 expression (15 out of 22) (Paulson, Rieger-Christ et al. 2007). To examine the effect of reduced HBP1 levels in breast cancer cells, HBP1 was knocked down in the MDA-MB-231 breast cancer cell line using shRNA. This knockdown resulted in increased cell invasion as measured by boyden chamber assays, as well as increased colony size and number when the cells were grown in soft agar (Kim, Zhang et al. 2006; Paulson, Rieger-Christ et al. 2007). Additionally, when MDA-MB-231 cells expressing shRNA to HBP1 were xenograft to nude mice the resulting tumors were decreased both in number and size compared to a control xenograft (Paulson, Rieger-Christ et al. 2007). Taken together, recent data suggests that the HBP1 protein is important in preventing tumorigenesis in a number of cell types.

# Chapter Two:

# A Conserved Pathway that Controls c-Myc Protein Stability Through Opposing Phosphorylation Events Occurs in Yeast

Published in Journal of Biological Chemistry Febuary 23, 2007

## Abstract:

The c-Myc transcription factor is a key regulator of cell proliferation and cell fate decisions. c-Myc overexpression is observed in a variety of human tumors, revealing the importance of maintaining normal levels of c-Myc protein. c-Myc protein stability in mammalian cells is controlled by interdependent and sequential phosphorylation and dephosphorylation events on two highly conserved residues, Serine 62 and Threonine 58. Here we show that these sequential phosphorylation and dephosphorylation events, and their effect on c-Myc stability also occurs in the model system *Saccharomyces cerevisiae*. These results suggest the presence of a conserved pathway in yeast that controls protein turnover in response to a specific phospho-degron sequence. These findings have implications regarding conserved pathways for regulated protein degradation and validate the use of genetically tractable yeast for the study of the turnover of proteins, such as c-Myc, that contain this phospho-degron motif.

## Introduction:

The *c-myc* proto-oncogene encodes a helix-loop helix transcription factor that is involved in a number of crucial cellular processes, including cell proliferation, cell growth, differentiation and apoptosis. c-Myc heterodimerizes with its partner protein, Max, and together they regulate transcription at E-box sequences (CAC A/GTG) of a variety of important genes. Recently, it has been reported that c-Myc also regulates transcription of PolI and PolIII- dependent genes (Arabi, Wu et al. 2005; Grewal, Li et al. 2005). Given that c-Myc is involved in many vital cellular activities, it is not surprising that it is highly regulated at several levels, including transcription, mRNA stability, translation and protein stability (Kelly, Cochran et al. 1983; Jones and Cole 1987; Luscher and Eisenman 1990; Sears, Leone et al. 1999). A number of animal models have shown that misregulation of c-Myc can result in tumorigenesis (Arvanitis and Felsher 2005). Indeed, overexpression of c-Myc is observed in over 70% of human cancers. This can involve amplification or translocation of the *c-myc* gene (Popescu and Zimonjic 2002). However, these genetic changes are observed only in a minority of the cases, suggesting that other mechanisms, such as a change in c-Myc protein stability, may play a role in tumorigenesis.

We and others have previously reported that the stability of c-Myc protein in mammalian cells is controlled by sequential phosphorylation and dephosphorylation events on two highly conserved residues, Threonine 58 and Serine 62 (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). Phosphorylation at these sites has opposing effects on c-Myc protein stability. An initial event, phosphorylation at residue Serine 62 by Ras-activated ERKs, stabilizes c-Myc, while a

subsequent phosphorylation at residue Threonine 58 by the Glycogen Synthase Kinase 3β (GSK3β), destabilizes c-Myc. Prior to degradation, a *cis* to *trans* isomerization at the bond proceeding Serine 62 is catalyzed by the peptidyl prolyl isomerase, Pin1, allowing the stabilizing Serine 62 phosphate to be removed by the *trans*-specific phosphatase, PP2A. Singly Threonine 58 phosphorylated c-Myc can then be targeted for multi-ubiquitination by the E3 ligase $SCF^{Fbw7}$ and degraded by the 26S proteasome (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004).

*Saccharomyces cerevisiae* is frequently used as a model system to study mammalian proteins because of the ease of genetic manipulation, rapid doubling time, and the presence of conserved orthologs between a number of yeast and mammalian proteins, including a number of proteins required for proteasome-mediated degradation. For these reasons, it is an excellent model system to study interactions, function and turnover of mammalian proteins. Indeed, a number of groups have used the budding yeast, *S. cerevisiae*, to study the ubiquitination and subsequent destruction of important mammalian cell cycle proteins (Flinn, Busch et al. 1998; Salghetti, Kim et al. 1999; Strohmaier, Spruck et al. 2001; Herbst, Salghetti et al. 2004). Likewise, c-Myc half-life has been studied in yeast cells and found to very short (Salghetti, Kim et al. 1999; Kim, Herbst et al. 2003). Additionally, it has been reported that c-Myc protein can be stabilized in this system by mutating core components of the yeast SCF E3 ubiquitin ligase complex. Specifically, c-Myc half-life has been shown to be increased in yeast strains containing mutations in Cdc53, Skp1 or the F-box protein, Grr1 (Kim, Herbst et al. 2003). Interestingly, many of the mammalian proteins that regulate c-Myc phosphorylation and protein stability were conserved from yeast.

**A Conserved Pathway that Controls c-Myc Protein Stability Through Opposing Phosphorylation Events Occurs in Yeast**

In this study we show that yeast orthologs of key proteins in the signaling pathways that control c-Myc phosphorylation are functionally conserved. These proteins are involved in the same sequential and interdependent phosphorylation and dephosphorylation events at Serine 62 and Threonine 58 in c-Myc that occur in mammalian cells. Additionally, these phosphorylation events have the same consequence on c-Myc protein stability. This is an important finding because it supports discoveries made in mammalian cells, it emphasizes the importance of this signaling pathway in regulating protein turnover through a conserved phospho-degron motif, it validates the use of the yeast *S. cerevisiae* as a model system to study c-Myc turnover, and it implicates this pathway in the degradation of yeast proteins and other mammalian proteins.

## Results:

**Interdependent phosphorylation of c-Myc at Threonine 58 and Serine 62 occurs in yeast.** We and others have previously used phospho-peptide mapping and phospho-specific antibodies to demonstrate an inter-relationship between phosphorylation at Serine 62 (S62) and Threonine 58 (T58) (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000). Specifically, S62 phosphorylation is required prior to T58 phosphorylation, and T58 phosphorylation facilitates subsequent S62 dephosphorylation (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). In order to perform a yeast two-hybrid assay to identify new proteins that interact with phospho-T58 and phospho-S62, we first investigated whether mammalian c-Myc is phosphorylated at either of these sites in yeast. We expressed c-Myc$^{WT}$ or one of two c-Myc phosphorylation mutants, c-Myc$^{T58A}$ or c-Myc$^{S62A}$, in a wildtype yeast strain using the GAL1 promoter. This promoter is induced by addition of galactose to the yeast media. Following a one-hour induction of c-Myc$^{WT}$ or the c-Myc mutants from the GAL1 promoter, total cell lysates from yeast expressing c-Myc$^{WT}$, c-Myc$^{T58A}$ or c-Myc$^{S62A}$ were visualized by western blot using antibodies specific for phosphorylated Threonine 58 or phosphorylated Serine 62. Specificity of the T58 phospho-specific antibody has been previously documented (Sears, Nuckolls et al. 2000). We recently generated new S62 phospho-specific antibodies (see methods). Specificity of these antibodies were demonstrated by ELISA (data not shown) and western blotting with wildtype and mutant c-Myc expressed in mammalian cells (Arnold and Sears 2006) and yeast (here). Using LI-COR technology we can double label for total c-Myc and phospho-c-Myc. This technology allows us to accurately quantitate the ratio of phospho-S62 or phospho-T58 c-Myc to total c-Myc (see methods). As shown in

Figure 2.1A, c-Myc$^{WT}$ is both T58 and S62 phosphorylated, with a weaker signal detected by the anti-phosopho-S62 antibody (lane 1). The relative difference in T58 and S62 phosphorylation detected with the phospho-specific antibodies, is consistent with phosphopeptide mapping results showing a reduced amount of S62 phosphorylation and increased T58 phosphorylation under conditions where c-Myc is unstable (Sears, Nuckolls et al. 2000). In contrast, c-Myc$^{T58A}$, which lacks phosphorylation at T58, showed a large increase, over 10-fold, in S62 phosphorylation levels compared to c-Myc$^{WT}$ (Figure 2.1A, lane 2). This increase in S62 phosphorylation with the c-Myc$^{T58A}$ mutant is also observed in mammalian cells, both by phospho-peptide mapping and phospho-specific antibodies, and is due to T58 phosphorylation-dependent S62 dephosphorylation (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). Lastly, c-Myc$^{S62A}$ does not appear to be phosphorylated on either site in yeast cells (Figure 2.1A, lane 3). This is again consistent with observations in mammalian cells in which c-Myc$^{S62A}$ lacks phosphorylation at T58, forming the basis for the reported dependent relationship between S62 and T58 (Lutterbach and Hann 1994; Chang, Claassen et al. 2000; Sears, Nuckolls et al. 2000). These results are striking because they demonstrate the conservation of a pathway controlling two interdependent relationships between the S62 and T58 phosphorylation sites: 1) the hierarchical phosphorylation of S62 followed by T58, and 2) the role of T58 phosphorylation in promoting S62 dephosphorylation.

Given that the interdependent phosphorylation of c-Myc at S62 and T58 occurs in yeast, we next asked if phosphorylation at these sites also controls c-Myc protein stability in yeast as it does in mammalian cells. In order to study the stability of c-Myc protein in

yeast, we used a galactose induction/glucose shut off system. Specifically, addition of galactose to yeast cells activates expression of genes under control of the GAL1 promoter while addition of glucose rapidly shuts down the promoter. Galactose was added to yeast cells carrying a plasmid with either *c-myc*$^{WT}$ or the phosphorylation mutants under the control of this promoter to induce expression. After one hour, expression was terminated by addition of glucose and samples were taken at various time points for western blot analysis. c-Myc protein levels were quantified and normalized to the control Cdc28 protein and c-Myc half-life was calculated. As shown in Figure 2.1B, c-Myc$^{WT}$ exhibited a short half-life in a wildtype yeast strain of 8.3 minutes, with a mean half-life of 9.5 ± 1.6 minutes based on multiple independent experiments. This short half-life has previously been reported for c-Myc in both yeast cells and mammalian cells (Hann and Eisenman 1984; Flinn, Busch et al. 1998; Salghetti, Kim et al. 1999; Kim, Herbst et al. 2003), and it is consistent with the levels of detected T58 and S62 phosphorylation seen in Figure 2.1A. In contrast, c-Myc$^{T58A}$ showed a marked increase in half-life to 34.1 minutes, with a mean half-life of 33.9 ± 1.7 minutes. This approximate four-fold increase over wildtype c-Myc is consistent with results in mammalian cells where c-Myc$^{T58A}$ is 4-6 times more stable compared to c-Myc$^{WT}$ (Salghetti, Kim et al. 1999; Sears, Nuckolls et al. 2000). The c-Myc$^{S62A}$ mutant demonstrated an intermediate mean half-life of 16.1 ± 1.5 minutes. The half-life of c-Myc$^{S62A}$ in mammalian cells is either reported to be short like c-Myc$^{WT}$, or somewhat longer, but less than c-Myc$^{T58A}$, consistent with our observations in yeast (Sears, Nuckolls et al. 2000; Yada, Hatakeyama et al. 2004). Taken together our results not only show that the interdependent phosphorylation of T58 and S62 occurs in yeast, but also that phosphorylation of c-Myc at these sites appears to

control c-Myc protein stability in yeast cells. Since there are no known functional yeast homologues to mammalian c-Myc, these results suggest to us that a conserved pathway that controls protein degradation through a conserved phospho-degron may be present in yeast.

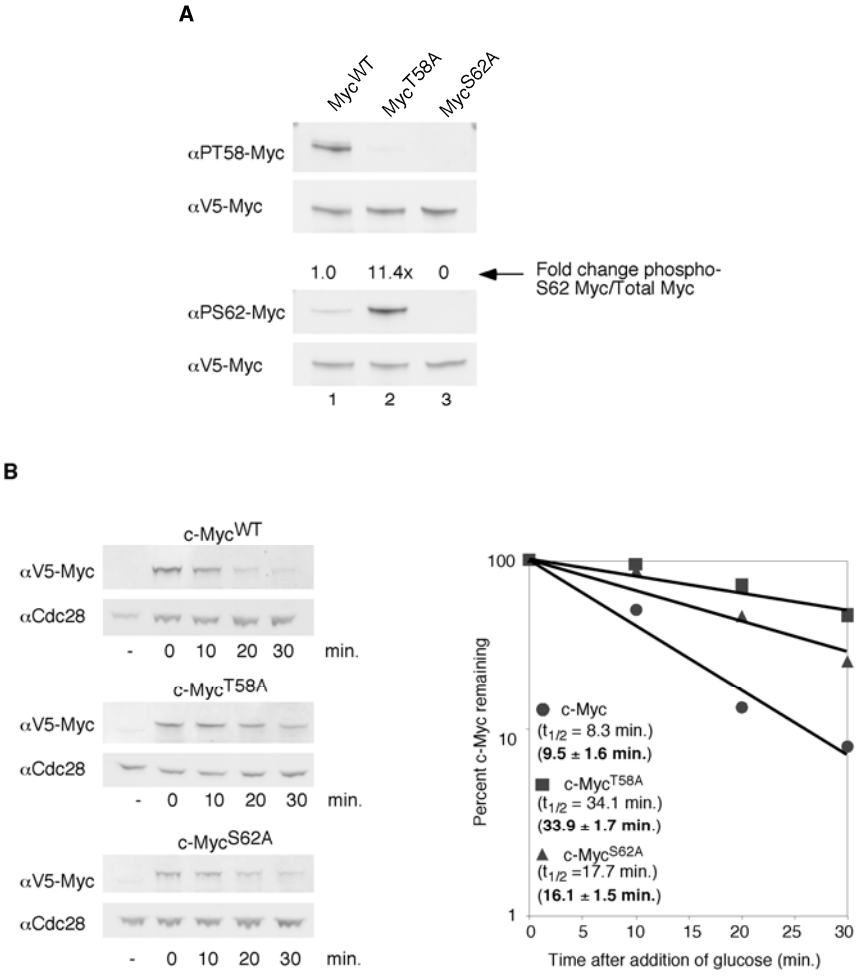



**Figure 2.1: Interdependent phosphorylation of c-Myc at T58 and S62 in yeast.**

(A) V5-tagged Myc$^{WT}$, Myc$^{T58A}$ or Myc$^{S62A}$ expression was induced from a GAL1 promoter in the BY4741 yeast strain by addition of galactose for one hour at 30°C. Equal cell numbers were visualized by western blot analysis with αV5, αP-Ser 62, or αP-Thr 58 using dual probing and overlay with the Odyssey Imaging System. Protein levels were quantitated and ratios of phosphorylated c-Myc to total c-Myc were calculated as described in methods. Fold change compared to c-Myc$^{WT}$ is shown. (B) V5-tagged Myc$^{WT}$, Myc$^{T58A}$ or Myc$^{S62A}$ expression was induced for one hour in the BY4741 yeast strain at 30°C by addition of galactose. Glucose was added to inhibit expression of c-Myc. Cells were harvested at the timepoints indicated after glucose addition and lysed in SDS sample buffer. Equal cell numbers were visualized by western blot analysis with αV5 and αCdc28. c-Myc protein levels and Cdc28p levels were quantitated by the Odyssey Imaging System and c-Myc levels were normalized to total protein as determined by the amount of Cdc28 protein. c-Myc levels at each time point are shown as a percent of the first time point and are plotted on a semi-log graph. Best-fit lines were calculated using Microsoft Excel. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold.

**Proteins controlling c-Myc S62 and T58 phosphorylation and c-Myc stability in mammalian cells are conserved in yeast**. The previous results suggest that functional homologues to mammalian proteins that target c-Myc for degradation are conserved in yeast. Indeed, many of the key proteins that have been implicated in controlling c-Myc T58 and S62 phosphorylation and regulating c-Myc protein stability in mammalian cells have *S. cerevisiae* orthologs (Table 2.1). One of the advantages to working in yeast is its easy genetic manipulation. Thus, strains that are mutant in each of these proteins are available. We made use of these strains to ask whether deletion or mutation of yeast orthologs of proteins which destabilize c-Myc in mammalian cells affect c-Myc phosphorylation in yeast, and whether this correlates with changes in protein stability.

**Table 2.1: Mammalian proteins known to regulate c-Myc protein stability and their *S. cerevisiae* orthologs**

| **Mammalian Protein** | **Effect on c-Myc Protein stability** | ***S. cerevisiae* orthologs** |
|---|---|---|
| ERK kinase | Stabilizing (Sears, Leone et al. 1999) | Kss1 |
| Gsk3β kinase | Destabilizing (Sears, Nuckolls et al. 2000) | Rim11 |
| Pin1 isomerase | Destabilizing (Yeh, Cunningham et al. 2004) | Ess1 |
| PP2A C subunit | Destabilizing (Yeh, Cunningham et al. 2004; Arnold and Sears 2006) | Ppph21 |
| PP2A B subunit (B' type) | Destabilizing (Arnold and Sears 2006) | Rts1 |
| F-box hCdc4 (Fbw7) | Destabilizing (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004) | Cdc4 |
| F-box Skp2 | Destabilizing (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003) | Grr1 |

**The yeast kinases Kss1p and Rim11p facilitate phosphorylation of c-Myc.** Kss1p is a yeast mitogen-activated protein kinase (MAPK) that has high sequence homology to the mammalian ERK kinase, and Rim11p is the yeast serine/threonine kinase that shares a conserved function to the GSK3β kinase (Boulton, Yancopoulos et al. 1990; Puziss, Hardy et al. 1994). In mammalian cells, ERK can phosphorylate c-Myc on S62, stabilizing the protein, while GSK3β is responsible for phosphorylation c-Myc on T58, thereby destabilizing c-Myc protein. Both *KSS1* and *RIM11* are non-essential yeast genes, presumably due the presence of additional MAPK-like and GSK3-like kinases (Elion, Brill et al. 1991; Bianchi, Plyte et al. 1993; Hardy, Wu et al. 1995; Casamayor, Khalid et al. 1996).

We asked whether the yeast kinase, Kss1p affects the phosphorylation of c-Myc at S62 in yeast. To examine this, we expressed V5-tagged c-Myc in a wildtype strain and a strain lacking the Kss1p gene (*Δkss1*) from the GAL1 promoter for one hour. Cells were lysed in SDS sample buffer and whole cell lysates were run on an SDS-PAGE gel. c-Myc protein was visualized by double labeling with one of the c-Myc phospho-antibodies, as well as an antibody to the V5 tag to measure total c-Myc. The intensity of each band was quantified and ratios of phosphorylated c-Myc to total c-Myc were calculated relative to c-Myc phosphorylation in the wildtype strain. As shown in Figure 2.2A, c-Myc expressed in the *Δkss1* strain is decreased for phosphorylation at S62, compared to that of c-Myc expressed in a wildtype strain. This decrease in S62 phosphorylation is presumably due to the loss of Kss1p. Residual phosphorylation of S62 is likely due to other MAP-like kinases is the cell. This result suggests that Kss1p

plays a role in phosphorylating c-Myc at S62 in yeast cells, as ERK does in mammalian cells.

We next examined whether the presence of Rim11p affects phosphorylation of T58 in yeast. To test this, V5-tagged c-Myc$^{WT}$ was again expressed from the GAL1 promoter for one hour in both a yeast strain lacking *RIM11* (*Δrim11*) and an isogenic wildtype control strain. Whole cell lysates were prepared in SDS sample buffer and run on an SDS-PAGE gel. c-Myc protein was visualized by double labeling with one of the c-Myc phospho-specific antibodies and anti-V5 (Figure 2.2B). We observed a substantial decrease in T58 phosphorylation and an increase in S62 phosphorylation in the *Δrim11* strain compared to the wildtype control. This decrease in T58 phosphorylation is consistent with observations in mammalian cells where inhibition of GSK3β results in a decrease in phospho-T58 c-Myc (Sears, Nuckolls et al. 2000; Gregory, Qi et al. 2003). Likewise, the increase in S62 phosphorylation is consistent with the role of T58 phosphorylation in facilitating PP2A-mediated dephosphorylation of S62 seen in mammalian cells (Yeh, Cunningham et al. 2004). Thus, the inter-relationship between the two phosphorylation sites is again observed. There is some residual phosphorylation of T58 in the *Δrim11* yeast strain. This is presumably due to the presence of three other GSK3-like kinases in the yeast (Bianchi, Plyte et al. 1993; Hardy, Wu et al. 1995; Casamayor, Khalid et al. 1996). This result suggests that Rim11p plays a significant role in phosphorylating c-Myc at residue 58 in yeast just as GSK3β does in mammalian cells.

We next asked whether loss of Kss1p and/or Rim11p affects the half-life of c-Myc in yeast. As shown in Figure 2.1, mutation of the S62 residue of c-Myc results in a small increase in half-life compared to c-Myc$^{WT}$. Therefore, if the Kss1 kinase

phosphorylates c-Myc at S62 we would expect a similar result from loss of Kss1p. To test this, c-Myc expression was induced in the *Δkss1* strain for one hour and following termination of induction, cells were collected at the indicated timepoints. As shown in Figure 2.2C, the half-life of c-Myc in a wildtype strain was 11.7 minutes while the half-life in the *Δkss1* strain was 12.2 minutes. Based on multiple experiments the mean half-life of c-Myc in the *Δkss1* yeast strain was 15.7 ± 3.1 minutes. This is similar to the c-Myc$^{S62A}$ mutant mean half-life, which was measured to be 16.1 ± 1.5 minutes (see Figure 2.1B). The small increase in stability with loss of phosphorylation at S62 is also consistent with previously published results in mammalian cells and suggests that without phosphorylation at S62 or at T58 c-Myc is degraded by an alternate E3 ligase (Yada, Hatakeyama et al. 2004).

On the other hand, mutation of T58 to a non-phosphorylatable residue results in enhanced S62 phosphorylation and substantial stabilization of c-Myc (Figures 2.1A and 2.1B). Thus, loss of Rim11p may result in a similar stabilization of c-Myc. To test this, c-Myc was expressed from a GAL1 promoter for one hour in the *Δrim11* strain. Following addition of glucose to stop induction of the promoter, samples were taken at various timepoints. c-Myc protein levels and half-lives are shown in Figure 2.2C. The half-life of c-Myc expressed in the *Δrim11* background was 28.4 minutes. Based on multiple independent experiments the mean half-life of c-Myc in the *Δrim11* strain is 28.1 ± 1.0 minutes, a threefold increase when compared to mean c-Myc half-life in a wildtype strain. These results demonstrate that in *S. cerevisiae*, Rim11p participates in the phosphorylation of c-Myc at T58, and that this phosphorylation leads to c-Myc destabilization, similar to the activity of GSK3β on c-Myc in mammalian cells.

Since c-Myc is not a yeast protein, it is important to ask whether these yeast kinases are able to directly phosphorylate mammalian c-Myc protein. In order to answer this question we preformed an *in vitro* kinase assay. Briefly, V5-6xhis-c-Myc expression was induced in either the *Δkss1* strain or the *Δrim11* strain and c-Myc protein was extracted using nickel agarose. We used c-Myc protein from these strains because (1) we have already shown them to have low PS62 or low PT58, respectively (see Figures 2.2A and 2.2B) and (2) in the case of Rim11-mediated phosphorylation, to ensure that c-Myc was properly primed with a phosphate at S62, since GSK3β kinases are reported to be processive kinases. c-Myc protein was eluted from the nickel agarose and incubated with either the Kss1 or Rim11 kinase in the presence of $^{32}$P-labeled ATP. As a negative control nickel agarose was incubated with non-induced yeast lysates, and those elutions were also incubated with either the Kss1 or Rim11 protein (Figure 2.2D, lanes 1 and 3). The samples were analyzed by SDS-PAGE and autoradiography. As shown in Figure 2.2D, c-Myc extracted from the *Δkss1* strain is phosphorylated in the presence of purified Kss1 (lane 2), while c-Myc extracted from the *Δrim11* strain is phosphorylated in the presence of purified Rim11 (lane 4). As an additional control, c-Myc alone was incubated with $^{32}$P-labeled ATP to ensure that additional kinases were not being co-purified with Myc (lane 5). This data demonstrates that the Kss1 and Rim11 yeast kinases can directly phosphorylate mammalian c-Myc protein.

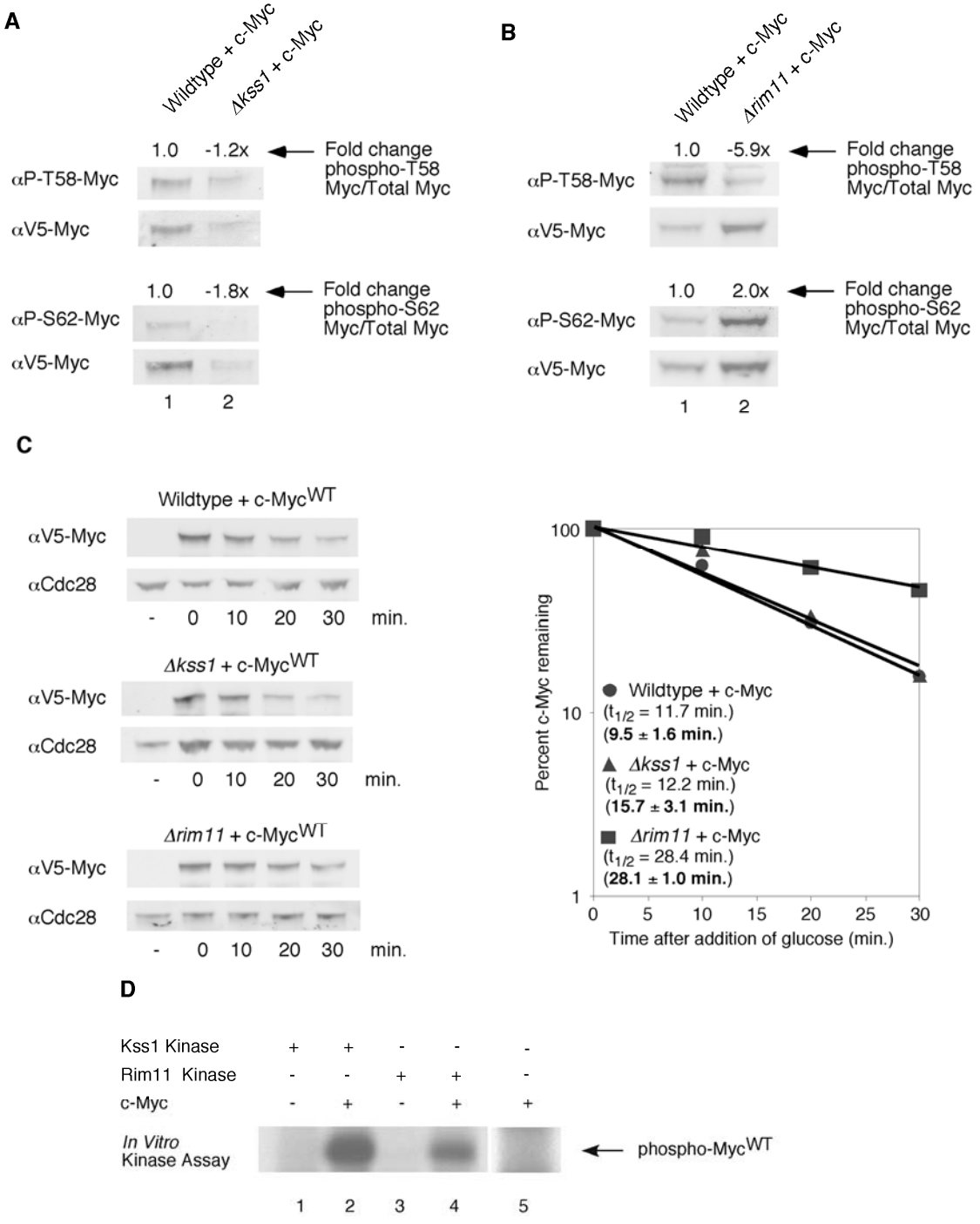



**Figure 2.2: The yeast kinases Kss1 and Rim11 phosphorylate c-Myc and thereby control c-Myc protein stability.**

(A) V5-tagged c-Myc$^{WT}$ was expressed in the BY4741 and *Δkss1* yeast strains for one hour at 30°C. Cells were lysed in SDS sample buffer. Equal cell numbers were analyzed by western blotting with the indicated antibodies as described in Figure 2.1A. Ratios of phosphorylated c-Myc$^{WT}$ to total c-Myc$^{WT}$ were calculated. Fold change of ratios in the

*Δkss1* strain compared to the BY4741 strain are shown. (B) V5-tagged c-Myc$^{WT}$ was expressed in the BY4741 and *Δrim11* yeast strains for one hour at 30°C. Cells were lysed in SDS sample buffer. Equal cell numbers were analyzed by western blotting as described in Figure 2.1A. Ratios of phosphorylated c-Myc$^{WT}$ to total c-Myc$^{WT}$ were calculated. Fold change of ratios in the *Δrim11* strain compared to the BY4741 strain are shown. (C) V5-tagged c-Myc$^{WT}$ expression was induced in the BY4741, *Δkss1* or *Δrim11* strain for one hour at 30°C. Following addition of glucose, cells were harvested at the timepoints indicated and cells were lysed in SDS sample buffer. Western Blotting and quantitation were performed as described in Figure 2.1B. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold. (D) Lysates from *Δkss1* or *Δrim11* strains expressing or not expressing V5-6xhis tagged c-Myc protein were incubated with Nickel agarose to purify c-Myc. Kss1 and Rim11 kinases were purified from yeast strains with knocked-in TAP tags using calmodulin purification as described in methods. c-Myc or lysates not expressing c-Myc were eluted from the Nickel agarose and then incubated with either the immobilized TAP-Kss1 or TAP-Rim11 kinases in the presence of $^{32}$P-labeled ATP (lanes 1-4). As an additional control c-Myc protein was incubated with $^{32}$P-labeled ATP in the absence of either TAP-tagged kinase (lane 5). Representative results are shown.

**c-Myc isomerization by Ess1 is not a significant limiting step in c-Myc degradation in yeast.** Ess1p is the yeast homologue of the peptidyl-prolyl isomerase, Pin1. In mammalian cells, Pin1 recognizes c-Myc phosphorylated at T58 and catalyzes a *cis* to *trans* isomerization of the bond preceding Proline 63 in c-Myc. This isomerization is thought to underlie the role of T58 phosphorylation in facilitating the dephosphorylation of S62 by the PP2A phosphatase, which dephosphorylates residues when the proceeding proline is in *trans*. In Pin1-null mouse embryo fibroblasts c-Myc S62 phosphorylation is increased and degradation is inhibited (Yeh, Cunningham et al. 2004). *ESS1* is an essential yeast gene and cells mutated for this gene arrest in M phase of the cell cycle (Lu, Hanes et al. 1996). It is also a highly conserved gene and mammalian Pin1 can substitute for Ess1 protein function in yeast (Lu, Hanes et al. 1996).

We asked if the yeast peptidyl-prolyl isomerase, Ess1p, affects phosphorylation, and consequently protein stability of c-Myc in yeast. Since *ESS1* is an essential yeast gene, a temperature-sensitive yeast mutant strain with an H164R mutation in the active site of the enzyme was used (Lu, Hanes et al. 1996; Wu, Wilcox et al. 2000). This strain arrests in M phase, however with slow kinetics. c-Myc expression was induced in the *ess1*$^{H164R}$ strain at the restrictive temperature of 37°C for three hours. Approximately 85% of the cells showed mutant phenotype microscopically (data not shown). Whole cell lysates were run on a gel and the western blot was duel labeled with antibodies specific for phospho-S62 or phospho-T58 and anti-V5 (Figure 2.3A). Interestingly, c-Myc expressed in the *ess1*$^{H164R}$ strain showed only a small increase in T58 or S62 phosphorylation compared to the similarly treated wildtype strain. We also examined c-Myc half-life in the *ess1*$^{H164R}$ at the restrictive temperature, 37°C, and compared this to an isogenic wildtype control strain under the same temperature but with the addition of the microtubule inhibitor, nocodazole, to control for M phase arrest in the *ess1*$^{H164R}$ strain. c-Myc half-life overall did not appear to be significantly longer in the *ess1*$^{H164R}$ strain (13.8 minutes) compared to the wildtype strain arrested with nocodazole (12.5 minutes, Figure 2.3B). Based on multiple experiments the mean half-life of c-Myc in a nocodazole-arrested wildtype strain was 13.0 ± 2.0 minutes while the mean overall half-life of c-Myc in the *ess1*$^{H164R}$ strain was 13.8 ± 1.3 minutes. Although the overall half-life for c-Myc did not appear to be significantly affected by loss of Ess1p, it is interesting to note that we consistently observed a biphasic decay, where there is an initial decrease in c-Myc levels, which then levels off between ten and twenty minutes in the *ess1*$^{H164R}$ strain (dashed lines). This suggests that loss of Ess1p does affect a subset of c-Myc protein,

which could explain why we only saw a small increase in phosphorylation in c-Myc in the *ess1*$^{H164R}$ strain. It may be that a significant portion of c-Myc already exists in the *trans* conformation in the yeast cells and therefore does not require Ess1p activity for dephosphorylation or degradation. However, after this portion is degraded a remaining subset of c-Myc protein exists in the *cis* conformation, and it is this population that continues to be stable out to at least 60 minutes in the *ess1*$^{H164R}$ strain (data not shown).

To confirm the previous results and ensure that the biphasic nature of c-Myc decay was not specific to the *ess1*$^{H164R}$ yeast strain, we also tested c-Myc half-life in another *ESS1* temperature-sensitive strain with a A144T mutation in the substrate-binding pocket (Wu, Wilcox et al. 2000). As shown in Figure 2.3C, c-Myc expressed in the *ess1*$^{A144T}$ stain has a half-life of 16.1 minutes with an overall mean half-life of 16.6 ± 1.8 minutes. Consistent with our observations in the *ess1*$^{H164R}$ strain, this is not a significant increase compared to c-Myc half-life in a wildtype strain under the same conditions (13.0 ± 2.0 minutes). However, again we observe a biphasic decay of c-Myc in this yeast strain (dashed lines), suggesting that this phenotype may be common to all *ESS1* mutants and reflect a requirement for ESS1 to degrade a subset of c-Myc protein.

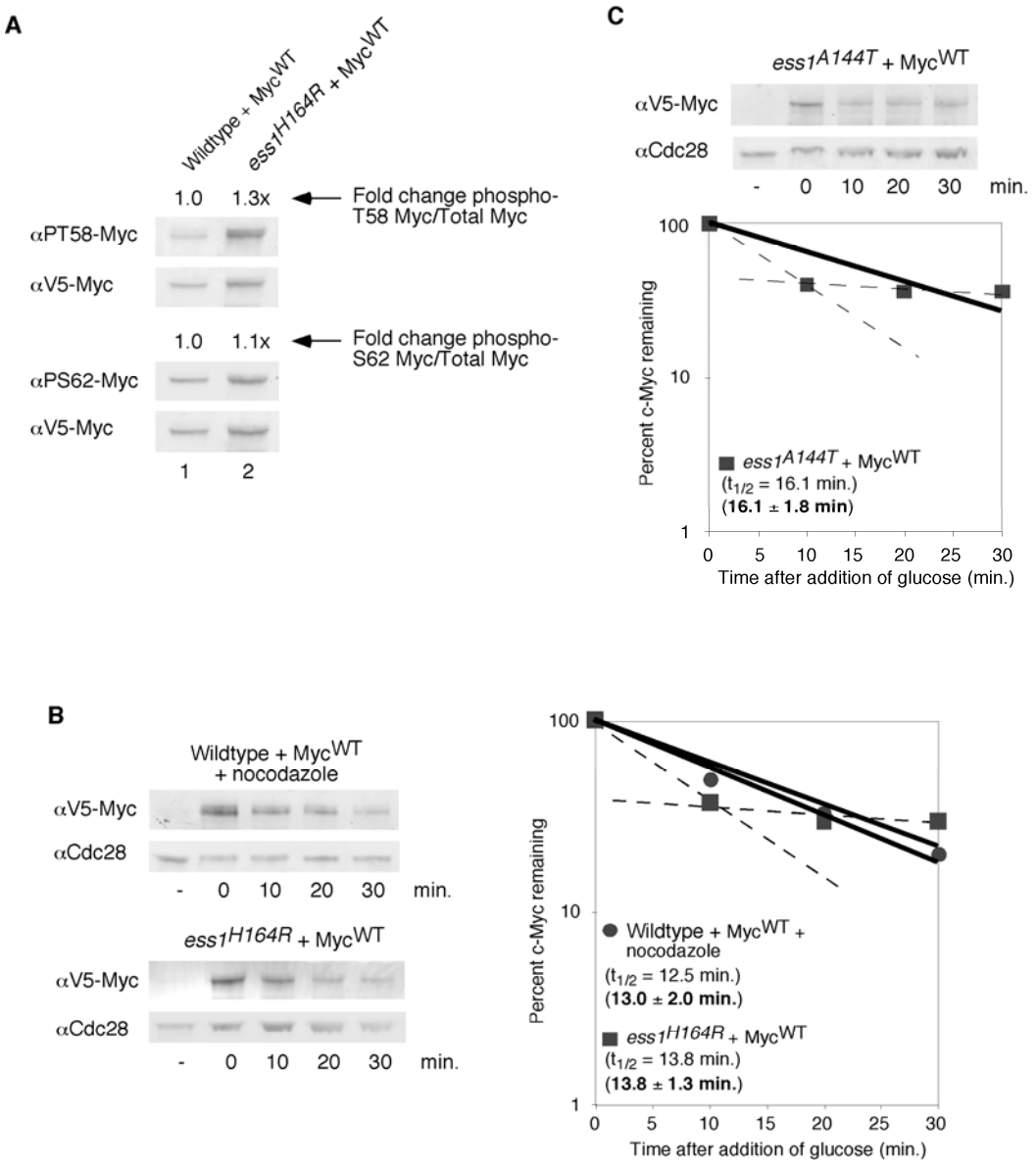



**Figure 2.3: Mutation of the yeast peptidyl prolyl isomerase, Ess1, does not significantly affect c-Myc phosphorylation or overall stability.**

(A) W303 and *ess1*$^{H164R}$ cells were grown in 2% raffinose medium at 30°C overnight. Cells were diluted to $OD_{600}$ = 0.3, nocodazole was added to the W303 strain and cells were grown at 37°C for 4 hours. Galactose was added and c-Myc$^{WT}$ expression was induced for 3 hours at 37°C. Following addition of glucose, cells were harvested and lysed in SDS sample buffer. Equal cell numbers were visualized by western blot analysis with the indicated antibodies and quantitated as described in Figure 2.1A. Ratios of phosphorylated c-Myc$^{WT}$ to total c-Myc$^{WT}$ were calculated. Fold change of ratios in the *ess1*$^{H164R}$ strain compared to the W303 strain are shown. (B) c-Myc$^{WT}$ expression in the

W303 or *ess1*$^{H164R}$ yeast strain was induced as described above. Following addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blot analysis and quantitation were performed as described in Figure 2.1B. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold. Dashed lines indicate the biphasic degradation of c-Myc in the *ess1*$^{H164R}$ strain. (C) c-Myc expression was induced in the *ess1*$^{A144T}$ yeast strain as described in A. Following addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blot analysis and quantitation were performed as described in Figure 2.1B. Experiments were repeated three or more times and representative data is shown. Mean half-life is indicated in bold. Dashed lines indicate the biphasic degradation of c-Myc in the *ess1*$^{A144T}$ strain.

**Yeast PP2A activity facilitates c-Myc S62 dephosphorylation and degradation.** Protein phosphatase 2A (PP2A) is a heterotrimeric enzyme that dephosphorylates Serine 62 of c-Myc thereby destabilizing the protein in mammalian cells (Yeh, Cunningham et al. 2004; Arnold and Sears 2006). PP2A is a *trans*-specific phosphatase, which explains the role of prior isomerization by Pin1 (Zhou, Kops et al. 2000). The PP2A holoenzyme is composed of a catalytic (C) subunit, which is encoded by two genes in *S. cerevisiae*, *PPH21* and *PPH22*, a scaffolding (A) subunit, encoded by the *TPD3* gene in *S. cerevisiae*, and a substrate-recognizing regulatory (B) subunit, of which there are two in yeast. *RTS1* encodes the B' family ortholog while *CDC55* encodes the B family ortholog (Healy, Zolnierowicz et al. 1991; Csortos, Zolnierowicz et al. 1996).

In mammalian cells, S62 phosphorylation is associated with c-Myc stabilization and removal of the S62 phosphate by PP2A occurs prior to polyubiquitination and degradation. Previous studies in mammalian cells have shown that inhibiting PP2A activity by addition of okadaic acid or SV40 small T antigen results in increased c-Myc half-life (Yeh, Cunningham et al. 2004). To determine if yeast PP2A activity affects the phosphorylation of Serine 62, we used a mutant strain lacking one of the two main PP2A

catalytic subunits in *S. cerevisiae, PPH21*. We asked whether loss of Pph21p (*Δpph21*) changes the amount of c-Myc phosphorylation at Serine 62 compared to that of c-Myc in a wildtype yeast strain. As shown in Figure 2.4A, c-Myc in the mutant strain has increased Serine 62 phosphorylation compared to c-Myc expressed in the wildtype strain. This is presumably due to a reduction in the ability of the cell to dephosphorylate Serine 62. The increase in S62 phosphorylation in the *Δpph21* strain is modest, likely due to redundancy by the other PP2A C subunit.

We next examined c-Myc half-life in the *Δpph21* strain. As shown in Figure 2.4B, c-Myc half-life in this mutant strain was increased approximately two-fold (19.1 minutes) compared to c-Myc half-life in a wildtype control strain (7.8 minutes). Based on multiple independent experiments, the mean half-life of c-Myc in the *Δpph21* strain was 18.0 ± 2.2 minutes compared to a mean half-life of 9.5 ± 1.6 minutes in the isogenic control. We also examined c-Myc protein stability in a strain lacking the yeast ortholog to the PP2A substrate-recognizing subunit from the B' family, *RTS1* (*Δrts1*). We have recently reported that the B' family subunit, B56α, is responsible for targeting PP2A to c-Myc for dephosphorylation in mammalian cells (Arnold and Sears 2006). As shown in Figure 2.4B, c-Myc expressed in the *Δrts1* strain had a little more than a two-fold increase in half-life compared to c-Myc expressed in the wildtype control, with a mean half-life of 22.1 ± 3.1 minutes. The modest effect in these PP2A mutant yeast strains is likely due to multiple PP2A C and B subunits. Taken together this data suggests that PP2A in yeast does dephosphorylate c-Myc at S62, and this leads to destabilization of c-Myc protein. We also tested c-Myc half-life in a strain deleted for the PP2A A subunit. However, we obtained variable results from quite stable (34.3 minutes) to unstable (data not shown).

This is likely due to spurious activity by the C subunit in the absence of the structural A subunit as previously reported (Arnold and Sears 2006).

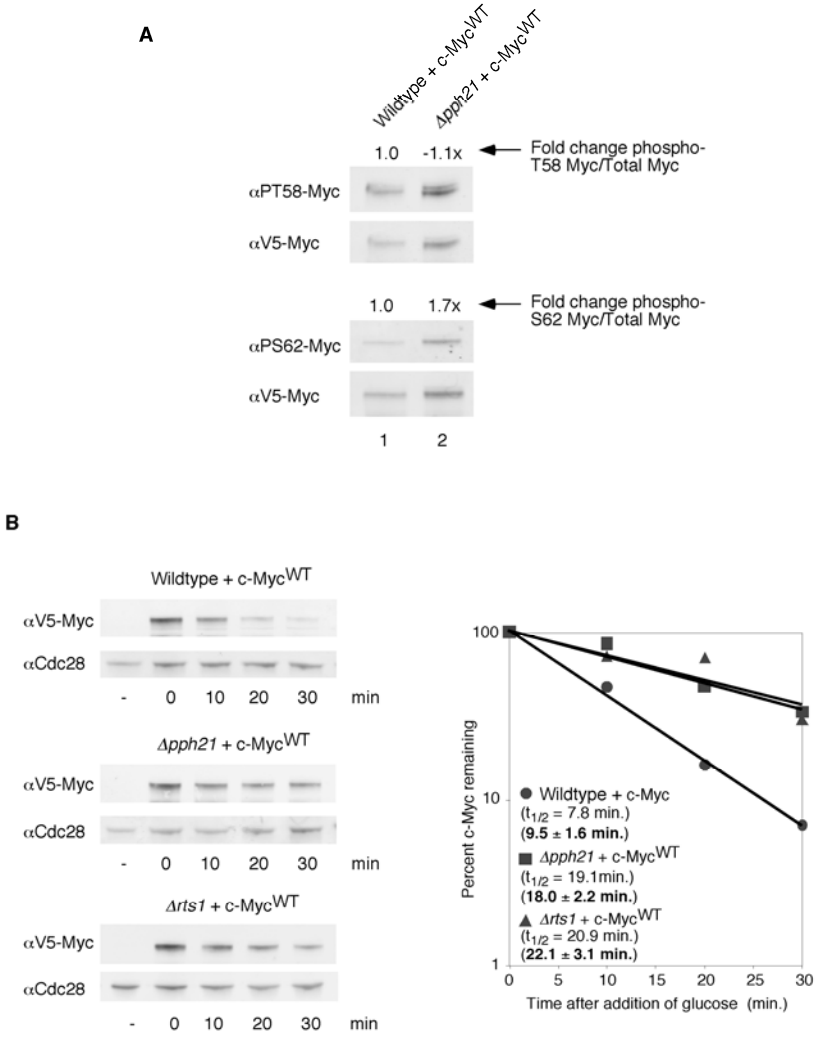



**Figure 2.4: Loss of yeast PP2A activity increases c-Myc S62 phosphorylation and c-Myc half-life.**

(A) c-Myc$^{WT}$ expression was induced at 30°C for one hour in the BY4741 or *Δpph21* yeast strains. Following addition of glucose, cells were harvested and lysed in SDS sample buffer. Equal cell numbers were visualized by western blot analysis with the indicated antibodies and quantitated as described in Figure 2.1A. Ratios of phosphorylated c-Myc$^{WT}$ to total c-Myc$^{WT}$ were calculated. Fold change of ratios in the *Δpph21* strain compared to the BY4741 strain are shown. (B) c-MycWT expression was induced for one hour at 30°C in the BY4741, *Δpph21* or *Δrts1* yeast strain. Following addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blot analysis and quantitation were performed as described in Figure 2.1B. Experiments were repeated three or more times and representative data is shown. Mean half-lives are indicated in bold.

**The yeast F-box protein, Cdc4p, does not appear to be involved in controlling c-Myc degradation.** The ubiquitination machinery is highly conserved from yeast to mammals. The ubiquitin ligase complex, SCF, which targets phospho-proteins for ubiquitination, has been shown in yeast to target c-Myc and other mammalian cell cycle proteins for ubiquitination and subsequent degradation (Strohmaier, Spruck et al. 2001; Kim, Herbst et al. 2003). The SCF complex is composed of four subunits, Skp1, Cdc53, Roc1 and a variable F-box protein, which determines substrate specificity. In mammalian cells, the F-box protein, Skp2 is reported to target c-Myc through MBII and the C-terminal domains in a phosphorylation independent manner (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003). In contrast, the F-box protein, Fbw7, has been shown to target c-Myc that is phosphorylated at T58, suggesting that it is the E3 ligase which degrades c-Myc in response to regulated phosphorylation of S62 and T58 (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004).

Fbw7 is also referred to as hCdc4 since it is the structural homologue of the *S. cerevisiae* F-box protein, Cdc4 (Strohmaier, Spruck et al. 2001). Therefore to examine c-Myc half-life in the absence of functional Cdc4, we expressed c-Myc in the temperature-sensitive *cdc4-1* strain from the GAL1 promoter for two hours at the restrictive temperature. It has been reported that *cdc4-1* cells arrest at the G1/S transition, therefore, c-Myc protein stability was also measured in an isogenic wildtype control strain at the restrictive temperature with the addition of α-factor to arrest the cells at G1 phase of the cell cycle (Goh and Surana 1999). Mutant phenotype at the restrictive temperature was verified microscopically (data not shown). As shown in Figure 2.5A, c-Myc is equally unstable in both the *cdc4-1* strain and the wildtype control strain with the half-lives of

11.0 minutes and 12.6 minutes, respectively. This result is consistent with previous reports showing that c-Myc is not stabilized in a yeast strain mutant for Cdc4p; however, this is a surprising result given the structural homology with Fbw7/hCdc4 (Kim, Herbst et al. 2003).

The structural homology between Fbw7 and yeast Cdc4 is based on similar WD40 repeats (Figure 2.5B) (Strohmaier, Spruck et al. 2001). However, despite the fact that these proteins are structurally related their substrates do not have similar cell cycle roles. In mammalian cells, Fbw7 targets cyclin E and c-Jun, in addition to c-Myc; all of which are proteins known to drive cell cycle progression (Strohmaier, Spruck et al. 2001; Wei, Jin et al. 2005). In contrast, yeast Cdc4p targets the yeast cell cycle inhibitor, Sic1p, for ubiquitin-mediated degradation (Bai, Sen et al. 1996; Feldman, Correll et al. 1997) (Figure 2.5B). This suggests that while these proteins are structurally similar, they may not be functional homologues and it also argues for the presence of another yeast F-box protein capable of targeting c-Myc.

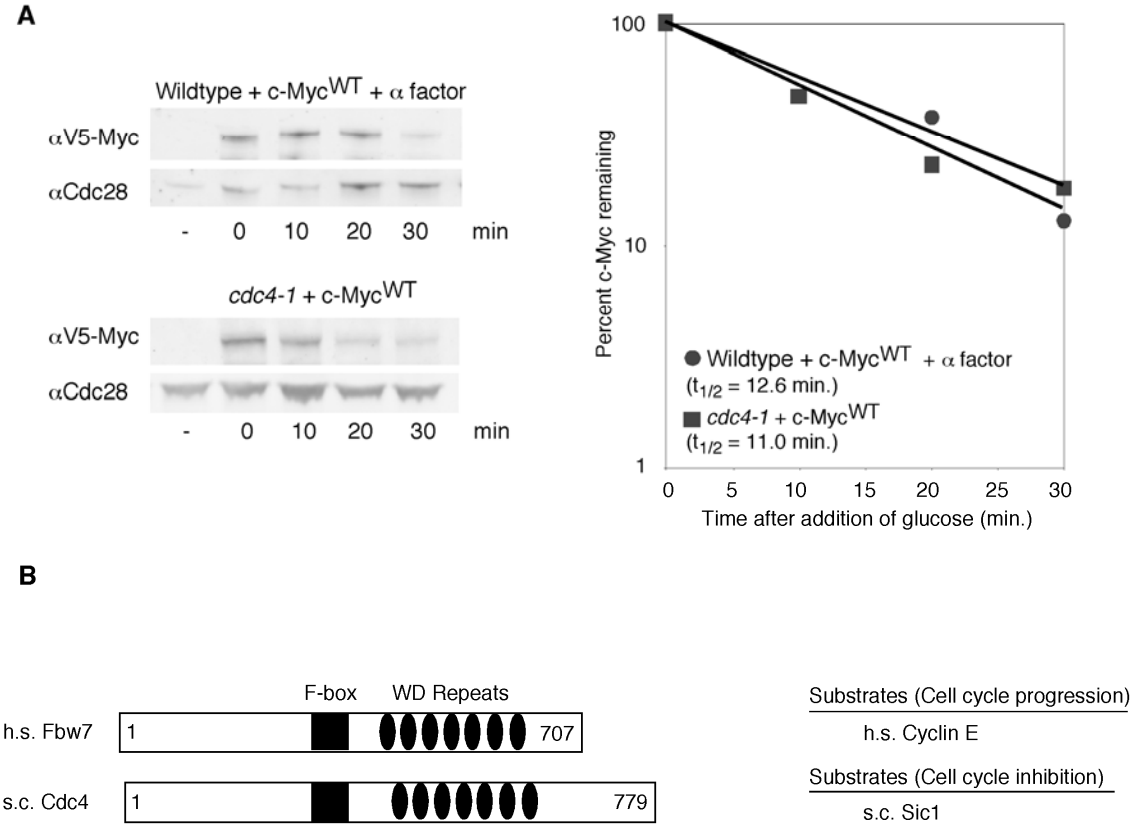



**Figure 2.5: Mutation of the Fbw7 structural homolog, Cdc4, does not result in c-Myc stabilization.**

(A) W303 cells, in the presence of α factor, and cdc4-1 cells were grown in 2% raffinose medium at 37°C for two hours. c-Myc expression was induced for one hour at 37°C by addition of galactose. Following termination of expression by addition of glucose, cells were harvested at the indicated timepoints and lysed in SDS sample buffer. Western blotting and quantitation were performed as described in Figure 2.1B. (B) Schematics of the human F-box protein, Fbw7, and the S. cerevisiae F-box protein, Cdc4. Key structural domains are indicated, as well as target proteins of the F-boxes that are important in regulating the cell cycle (see text).

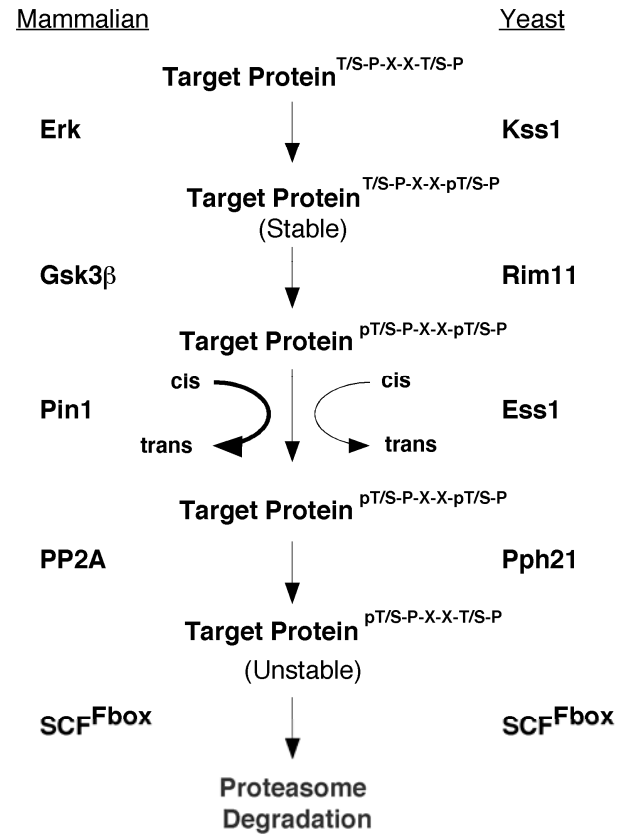
## Discussion:

Previously, we and others have shown that phosphorylation of c-Myc at Serine 62 and Threonine 58 is sequential and interdependent and that these phosphorylation events have opposing effects on c-Myc protein stability, presumably by influencing the ability of c-Myc to bind its E3 ligase (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004). Many investigators have used the genetically tractable budding yeast *S. cerevisiae* as a model system to study the ubiquitin-mediated degradation of mammalian proteins. This is because the ubiquitin-proteasome pathway is highly conserved from mammals to yeast. Several groups have examined the half-life of c-Myc in the *S. cerevisiae* and found it to be very short. However, it has never been determined whether c-Myc could be phosphorylated at either S62 or T58 in yeast. Additionally, while there are yeast orthologs to the mammalian proteins that phosphorylate c-Myc, it was unknown whether these proteins could phosphorylate mammalian c-Myc in the complex manner observed in mammalian cells. Here we have shown that c-Myc is phosphorylated at T58 and S62 in yeast and these phosphorylation events are interrelated like they are in mammalian cells. Specifically, phosphorylation of T58 required prior phosphorylation of S62 and S62 dephosphorylation was facilitated by T58 phosphorylation, just as in mammalian cells. This is a striking observation and points to the importance and conserved nature of the signaling pathway that can control phosphorylation of the T58-S62 phospho-domain found in mammalian c-Myc.

**Phosphorylation by conserved proteins controls c-Myc stability in yeast.** We have shown that phosphorylation of c-Myc at S62 and T58 occurs in yeast through a conserved signaling pathway which is likely to target a specific phospho-degron motif similar to that found in c-Myc (Figure 2.6A). As in mammalian cells, phosphorylation at S62 stabilizes c-Myc and phosphorylation at T58 destabilizes c-Myc. This suggests that the mode of recognition of c-Myc by an E3 ubiquitin ligase and its subsequent proteasomal degradation is conserved in yeast. The T58 and S62 residues are located in a highly conserved region of c-Myc designated Myc Box I (MBI). Consistent with our findings, it has previously been reported that deletion of MBI, or a region encompassing MBI, results in increased c-Myc protein stability in yeast (Flinn, Busch et al. 1998; Salghetti, Kim et al. 1999; Herbst, Hemann et al. 2005).

Serine 62 phosphorylation by ERK stabilizes c-Myc in mammalian cells. However, without S62 phosphorylation T58 cannot be phosphorylated and c-Myc degradation is likely mediated by an alternate E3 ligase not involving these phosphorylation sites, thus a somewhat longer half-life is observed in mammalian cells with the S62A mutant as well as in *Δkss1* yeast with wild-type c-Myc. Our data supports a role for yeast Rim11p in mediating T58 phosphorylation similar to GSK3β, leading to c-Myc destabilization. Likewise, Pph21p/Rts1p appears to dephosphorylate S62 similar to PP2A-B56α and destabilizes c-Myc. Our data, however, did not support a strong role for Ess1p in regulating c-Myc turnover in yeast, nor do we observe a role for the Fbw7 structural homologue, Cdc4p, in regulating c-Myc ubiquitin-mediated degradation. The F-box protein, Grr1p, is, however, reported to play a role in c-Myc degradation in yeast, as described below.

A




B

| Protein | Species | Sequence |
|---|---|---|
| c-Myc | H. Sapiens | $^{58}$tpplsp$^{62}$ |
| c-Jun | H. Sapiens | $^{239}$tpplsp$^{243}$ |
| Bcl-2 | H. Sapiens | $^{70}$splqtp$^{75}$ |
| KLF7 | H. Sapiens | $^{137}$tpplsp$^{142}$ |
| CyclinE | H. Sapiens | $^{380}$tppqsg$^{385}$ |
| Cdc6 | S. Cerevisiae | $^{39}$tpessp$^{44}$ $^{368}$tpttsp$^{373}$ |
| Ash1 | S. Cerevisiae | $^{465}$sppqsp$^{475}$ |
| Elm1 | S. Cerevisiae | $^{30}$sppitp$^{35}$ |

**Figure 2.6: A conserved pathway to control protein degradation.**
(A) Summary of the conserved pathway that controls protein stability through a specific phospho-degron present in c-Myc, in mammalian cells and yeast cells. (B) Other mammalian and yeast proteins with predicted phospho-degron sequence that would be affected by this pathway (see text for details).

**The requirement for c-Myc isomerization is not conserved in yeast.** While the phosphorylation and dephosphorylation events that control c-Myc protein stability appear to be conserved from yeast to mammals, isomerization by a peptidyl prolyl isomerase does not appear to be a limiting step in c-Myc degradation in yeast cells, as it is in mammalian cells. Since the dephosphorylation step leading towards c-Myc degradation

is conserved in yeast, there are two possibilities that would explain why Ess1p does not appear to be required for c-Myc destruction, 1) yeast PP2A does not require c-Myc to be in the *trans* conformation prior to dephosphorylation or 2) c-Myc is already in the *trans* conformation allowing for dephosphorylation of S62 by PP2A without Ess1p activity. The former is a less likely explanation since it has been previously shown that Pin1 and PP2A have a reciprocal genetic interaction in yeast, in that Pin1 has been able to partially rescue a yeast temperature sensitive mutant that lacks PP2A activity and overexpression of one of the yeast PP2A catalytic subunits almost completely rescues an Ess1 temperature sensitive mutation (Zhou, Kops et al. 2000). Thus, while there is accumulation of the *cis* conformation of c-Myc in mammalian cells prior to its degradation, in yeast cells the majority of c-Myc may remain in the *trans* conformation. Given that in mammalian cells, MAP kinases phosphorylate Ser/Thr-Pro motifs in the *trans* conformation, and PP2A requires Pin1 to efficiently dephosphorylate S62, it is likely that an intermediary *trans* to *cis* isomerization step exists in mammalian cells but not in yeast (Weiwad, Kullertz et al. 2000; Zhou, Kops et al. 2000). At this time it is unknown which enzyme catalyzes this initial conversion, although Pin1 is a likely candidate; however, it appears that this step may not occur in yeast, perhaps because c-Myc is not required to be a functioning transcription factor in yeast.

**Structurally related E3 ligase F-box proteins in mammals, Fbw7, and yeast, Cdc4, are not functionally conserved.** Another interesting aspect of this work is the lack of stabilization of c-Myc in a yeast strain mutated for the F-box Cdc4, the reported yeast homologue to mammalian Fbw7 (Strohmaier, Spruck et al. 2001). This observation has

been previously reported, Kim *et al* has shown that mutation of the F-box protein Grr1, but not Cdc4, results in the stabilization of c-Myc in yeast (Kim, Herbst et al. 2003). This led to the finding that the mammalian F-box, Skp2, which has a similar structure to Grr1, can target c-Myc for ubiquitination in mammalian cells. At the same time, Skp2 can also enhance c-Myc transcriptional activity (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003). However, it was also shown that the Skp2-c-Myc interaction was via MBII, a second highly conserved region in c-Myc, and through the c-Myc C-terminal domain; and recognition was not phosphorylation dependent (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003). Shortly after this, Fbw7 was identified as a second mammalian F-box that targets c-Myc for ubiquitination, and binding of Fbw7 to c-Myc was dependent on the phosphorylation of T58 in MBI (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004). Since we now have shown that these phosphorylation events are conserved and they are important for controlling c-Myc stability in yeast, the question arises why does mutation of the Fbw7 homologue, Cdc4, not result in c-Myc stability in yeast? While c-Myc contains a reported optimal Cdc4 phospho-degron (CPD) (Orlicky, Tang et al. 2003), some of Cdc4p substrates in yeast, like Sic1p, require multiple phosphorylation events for recognition, and perhaps a singly T58 phosphorylated c-Myc is not well recognized (Verma, Annan et al. 1997; Nash, Tang et al. 2001).

In yeast cells, Grr1p targets the G1 cyclin, Cln2p, for degradation (Barral, Jentsch et al. 1995). Since mammalian Fbw7 has similar substrates, such as Cyclin E, it is possible that Grr1p, but not Cdc4p, may be more functionally homologous to Fbw7. At this time it is unknown whether recognition of c-Myc for ubiquitination by Grr1p occurs through phosphorylation at T58 in yeast cells, but our data strongly suggests that either

Grr1p or another yet unknown F-box targets c-Myc for ubiquitination through this phospho-residue in yeast cells.

**A conserved pathway to control protein degradation.** In this work we have shown a number of similarities between the complex control of c-Myc protein stability in mammalian cells and in the model system *S. cerevisiae.* This conservation supports the importance of this pathway that targets c-Myc for degradation and it validates the use of *S. cerevisiae* as a model system to study c-Myc. Additionally, this work suggests that this pathway may act on other target proteins with a similar phosphorylation consensus sequence of T/S-P-X-X-T/S-P in both mammalian and yeast cells, as illustrated in Figure 2.6A.

Figure 2.6B shows a selected list of mammalian and yeast proteins with this potential phospho-degron sequence. As we and others have shown in mammalian cells, and now in yeast cells, the sequence TPPLSP of c-Myc controls c-Myc stability presumably by changing its affinity for its E3 ligase (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004; Yeh, Cunningham et al. 2004). In yeast cells the transcription factor Ash1, the kinase Elm1, and the pre-replication complex protein, Cdc6, all contain a similar phospho-degron sequence. While little work has been done elucidating the control of stability of Elm1 and Ash1, there has been a great deal of work dedicated to describing the mechanism of Cdc6 degradation. It has been shown that there are three modes of degradation for Cdc6 (Drury, Perkins et al. 2000). Two of the modes, which occur in either S Phase or M phase, appear to be controlled by the two sequences indicated in Figure 2.6B. For example, Perkins et al. demonstrated that mutation of T368

of Cdc6 completely stabilizes the Cdc6 protein while mutation of S372 of Cdc6 resulted in a partially stable protein (Perkins, Drury et al. 2001). These results are very similar to our observations of mutations of the corresponding residues of c-Myc (see Figure 2.1B), suggesting that this pathway in yeast may normally target Cdc6 for degradation.

There are also other proteins that may be targeted by this degradation pathway in mammalian cells, these include Kruppel-like factor KLF7, the anti-apoptotic protein Bcl-2, the cell cycle protein Cyclin E and the proto-oncogene c-Jun. While we were unable to find any data concerning regulation of KLF7 stability, it has been shown that ERK-dependent phosphorylation of Thr74 of Bcl-2 has a stabilizing effect on the protein (Breitschopf, Haendeler et al. 2000). This is similar to the effect of the ERK-dependent phosphorylation of S62, the corresponding residue of c-Myc in the phospho-degron sequence. Cyclin E does not have a perfect consensus sequence, however, its degradation is controlled by many of the same players as c-Myc, including GSK3β and FBW7 (Strohmaier, Spruck et al. 2001; Welcker, Singer et al. 2003). Additionally, it has recently been shown that PIN1 may be important in regulating Cyclin E levels, although at this time it appears to require phosphorylated S284, the residue that corresponds to c-Myc S62, for binding to PIN1 (Yeh, Lew et al. 2006). This is in contrast to c-Myc, which requires a phosphorylation at T58 for PIN1 binding (Yeh, Cunningham et al. 2004). Like c-Myc, c-Jun contains the sequence TPPLSP. Recently it was shown that T239 of c-Jun is phosphorylated by GSK3β and mutation of this site results in a stabilized protein, just as when the corresponding T58 is mutated in c-Myc (Zhou, Kops et al. 2000). Both proteins have also been reported to be substrates of the F-box $SCF^{Fbw7}$(Nateri, Riera-Sans et al. 2004; Welcker, Orian et al. 2004; Yada, Hatakeyama et

al. 2004). While testing all of these proteins is not within the scope of this paper, currently studies are underway to determine if this degradation pathway controls the stability of other mammalian and yeast proteins.

# Chapter Three:

# Use of a Yeast Two-Hybrid Assay to Identify c-Myc-interacting Proteins

## Abstract:

c-Myc is a potent oncogene whose expression is deregulated in 70% of human tumors. c-Myc functions as a transcription factor when bound to its partner protein Max. Together the c-Myc/Max heterodimer binds E-box elements and regulates that transcription of numerous proteins whose downstream effects are important in cellular proliferation, cell growth and metabolism, angiogenesis and apoptosis. In addition to Max, c-Myc has been found to bind a number of other proteins that act as co-activators or co-repressors, including the histone acetyltransferase, TRRAP, and the methyltransferase Dnmt3a, respectively. Additionally, a number of proteins have been identified that bind to c-Myc and inhibit its function either by blocking its activity or by destabilizing the protein. Many of these proteins have been shown to interact with the transactivational domain of c-Myc. In order to further understand the regulation of c-Myc we set out to identify new c-Myc interacting proteins through a yeast two-hybrid assay. Through a series of screens we identified the following proteins as those that interact within the transactivational domain of c-Myc: the ribosomal proteins L3 and L12, the undescribed methyltransferase METTL7B and the HMG box transcription factor HBP1.

## Introduction:

Identifying the binding partners of a given protein is essential to understanding the regulation and function of that protein. Over the years numerous binding partners have been described for the oncoprotein c-Myc (reviewed in (Sakamuro and Prendergast 1999)). c-Myc is a basic helix-loop-helix transcription factor whose functions include driving cell proliferation and cell growth, inhibiting differentiation and sensitizing cells to apoptosis in response to a lack of growth factors. Given these important roles in maintaining cell cycle progression, it is not surprising that c-Myc expression is deregulated in up to 70% of human tumors, supporting the need to further examine regulation of c-Myc.

The most well-known binding partner of c-Myc is the basic helix-loop-helix leucine zipper (bHLH-LZ) protein Max. c-Myc and Max heterodimerize through their HLH-LZ domain, and together they bind target gene promoters through E-box elements (CACGTG), and induce transcription of these genes (Blackwood and Eisenman 1991; Blackwood, Kretzner et al. 1992). Binding of Max also appears to be required for non-consensus sequence binding by c-Myc, where c-Myc is recruited to promoters through protein-protein interactions with other transcription factors (Staller, Peukert et al. 2001; Mao, Watson et al. 2003; Wu, Cetinkaya et al. 2003). This is the most common mode of c-Myc induced repression. It is currently believed that Myc/Max heterodimers regulate gene expression of up to ~15% of the genome (reviewed in (Patel, Loboda et al. 2004)).

Like most transcription factors c-Myc is a modular protein. As described above, the C-terminus contains the basic helix-loop-helix/leucine zipper domain, which is required for DNA binding and Max dimerization. In the N-terminus resides an

approximately 150 amino acid transactivation domain. Located within this domain are two highly conserved regions known as Myc box I (MBI) and Myc box II (MBII). MBI harbors two interdependent phosphorylation sites that have opposing effects on c-Myc stability (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). Specifically phosphorylation at Serine 62 (S62) stabilizes c-Myc while phosphorylation at Threonine 58 (T58) requires prior S62 phosphorylation and destabilizes the protein. Phosphorylation of c-Myc at these sites have been shown to change affinity of binding to proteins that regulate its stability including the F-box protein, Fbw7, which targets c-Myc for ubiquitination and degradation by the 26S proteasome (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004; Yeh, Cunningham et al. 2004; Arnold and Sears 2006). In addition to controlling stability, phosphorylation at these sites may affect c-Myc activity. It was demonstrated that a c-Myc mutant that can not be phosphorylated at T58 is abrogated in its ability to induce apoptosis (Chang, Claassen et al. 2000; Conzen, Gottlob et al. 2000; Hemann, Bric et al. 2005). Additionally, we have found that mice with one copy of a $Myc^{T58A}$ knockin allele at the ROSA26 locus expressing $Myc^{T58A}$ in T cells have increased tumor incidence compared to mice with two copies of the $c\text{-}Myc^{WT}$ allele at the ROSA26 locus, despite similar protein levels (Sarah Byers, unpublished data), further suggesting differences in activity of these two forms of c-Myc. It is important to note that T58 is often mutated in Burkitt's and AIDS-related lymphomas as well as in four different viral v-Myc proteins isolated from transforming retroviruses (Papas, Kan et al. 1985; Bhatia, Huppi et al. 1993), thus illustrating the relevance of this mutation in cancer.

MBII has been shown to be required for all of c-Myc biological functions (Stone, de Lange et al. 1987; Conzen, Gottlob et al. 2000). This region of c-Myc binds a number of co-activators, including TRRAP, a core component of two histone acetyltransferase complexes, which induces chromatin remodeling thereby activating transcription of c-Myc target genes (McMahon, Van Buskirk et al. 1998; McMahon, Wood et al. 2000; Park, Kunjibettu et al. 2001). Additionally, the F-box protein Skp2 binds both MBII and the C-terminus of c-Myc (Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003). In contrast to the F-box protein Fbw7, ubiquitination of c-Myc by Skp2 increases its transactivational activity, thereby acting as a co-activator for c-Myc. In addition to co-activators, proteins that inhibit c-Myc activity also bind MBII. In a collaborative effort with Mushui Dai in Dr. Lu's lab, the ribosomal protein L11 was identified as a c-Myc interacting protein as well as an inhibitor of c-Myc activity (Dai, Arnold et al. 2007). Specifically it was shown that L11 interacts with MBII of c-Myc and prevents binding of TRRAP, thereby inhibiting c-Myc activity. These observations illustrate the importance of MBII in regulating c-Myc activity.

Many c-Myc interacting proteins were identified as such through interaction in yeast two-hybrid assays. The yeast two-hybrid assay is an *in vivo* assay that relies on reporter gene activation to by a reconstituted transcription factor (Fields and Song 1989). This reconstituted transcription factor is made up of a dimer consisting of two fusion proteins: (1) a protein of interest fused to a DNA binding domain (DB), which is commonly referred to as the "bait", and (2) a protein of interest fused to an activation domain (AD), commonly referred to as the "prey". Interaction between the two proteins results in the formation of a functional transcription factor which activates the

transcription of chromosomally integrated reporter genes that contain promoters with the relevant DNA binding sites (Figure 3.1A).

In order to identify new proteins that interact with c-Myc and that are important in regulating either c-Myc function or stability we performed a yeast-two hybrid assay using amino acids 1-382 of c-Myc as bait, where the HLH-LZ region of c-Myc was replaced with the Gal4 DNA binding domain. We utilized a mating strategy to rescreen possible interactors to determine those proteins that interacted within the transactivational domain of c-Myc. A number or interesting proteins were identified including two ribosomal proteins, L3 and L12, the methyltransferase, METTL7B and the HMG-box protein, HBP1.

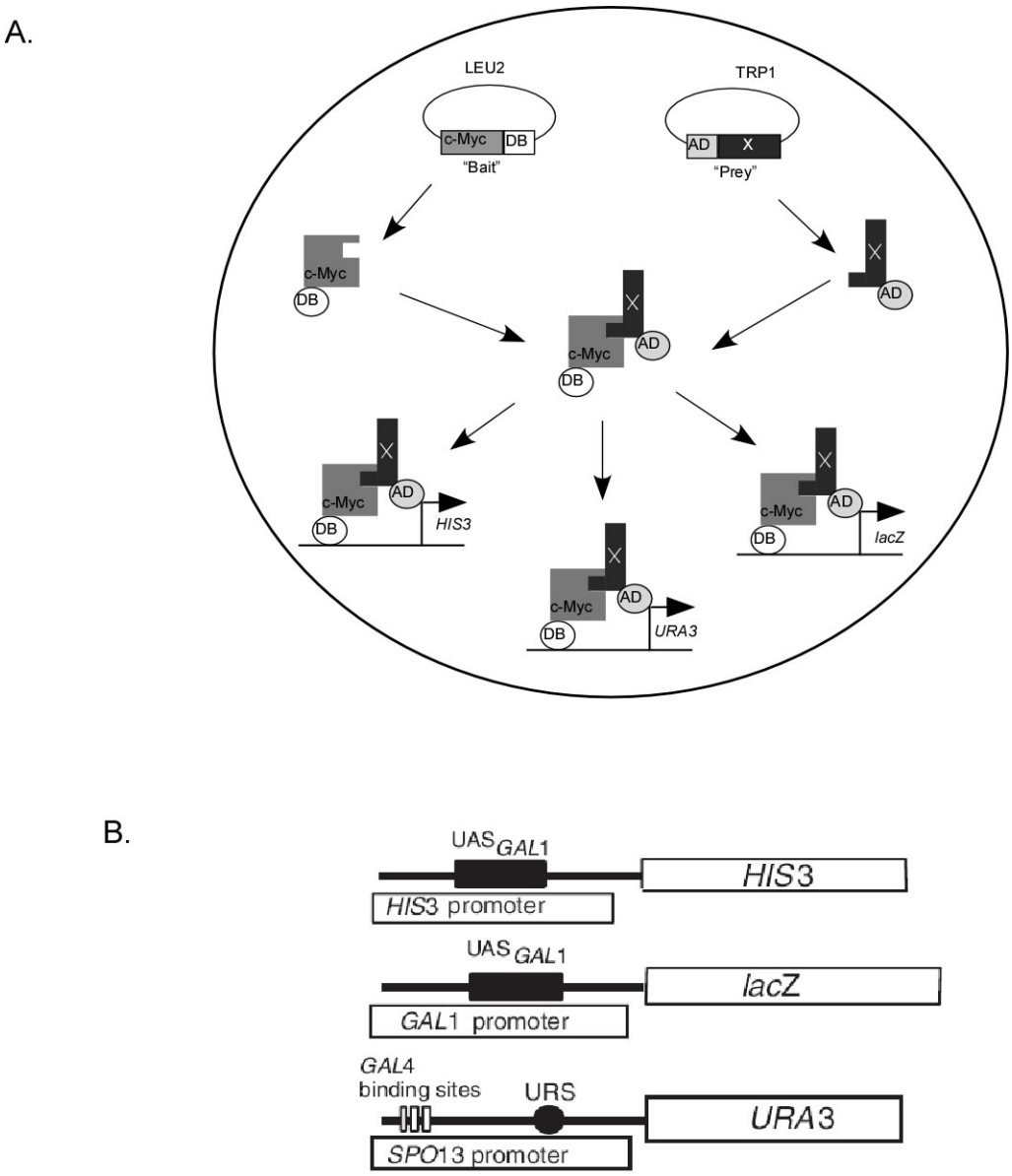



**Figure 3.1: Screening for c-Myc interacting proteins in a yeast two-hybrid assay with three reporter genes.**

(A) MaV203 yeast cells containing the "bait" and "prey" plasmids which encode for the c-MycGAL4DB fusion protein and an unknown protein fused to the GAL4 AD, respectively. Interaction between these proteins reconstitutes an active transcription factor which can bind GAL4 binding sequences in the promoters of three chromosomally integrated reporter genes. Figure adapted from "Proquest Two-Hybrid System with Gateway Technology Instruction Manual". Manual is available on Invitrogen website (www.invitrogen.com). (B) Structure of the three chromosomal integrated reporter genes and their promoters located in the MaV203 yeast strain. (www.invitrogen.com).

## Results:

**Cloning of the c-Myc bait plasmid for the Yeast Two Hybrid Assay.** We made use of a yeast two-hybrid system to identify novel binding partners of the oncoprotein c-Myc. We wanted to use as close to full-length c-Myc protein as possible for bait, as we believed that this would identify proteins not previously isolated by yeast two-hybrid assay, given that other groups had used fragments of c-Myc protein in prior yeast two-hybrid screens (Shrivastava, Saleque et al. 1993; Bannasch, Madge et al. 2001). However, since c-Myc can activate transcription on its own it was important to take this into consideration when designing our yeast two-hybrid bait. Previously it was shown that deletion mutants of c-Myc, when fused to a GAL4 DNA binding domain (GAL4 DB), have varying degrees of transcriptional activity in a CAT reporter assay (Kato, Barrett et al. 1990). The authors showed that deleting the C-terminus of c-Myc at amino acid 372, decreased transcriptional activation to six percent of full-length c-Myc. Due to this observation we decided to delete a small region of the C-terminus of c-Myc and replace it with the GAL4 DB, in order to keep as much of the protein as possible while potentially still dampening intrinsic c-Myc transactivation activity. Since cloning c-Myc into the pDBLeu plasmid would result in an N-terminal fusion protein we first cloned the GAL4DB into pBluescript (Stratagene), followed by insertion of c-Myc (see methods). The c-Myc/GAL4DB construct was removed from pBluescript vector by restriction digest and ligated into the pDBLeu backbone. The resulting plasmid should express a c-Myc fragment that is C-terminally truncated at amino acid 382 and C-terminally fused to GAL4DB. This plasmid was used as the bait plasmid for the yeast two-hybrid screen.

**Transformation of the yeast strains and testing of self-activation.** The MaV203 yeast strain (Invitrogen) was used for our yeast two-hybrid screen. This strain contains a single copy of three different reporter genes stably integrated within the yeast genome; specifically *HIS3*, *lacZ* and *URA3* (see Figure 3.1B). In addition to the three reporter genes this strain contains three auxotrophic mutations; *leu2* and *trp1* to allow for selection of the bait and prey plasmids, as well as *his3* to allow for screening of expression from the *HIS3* reporter dependent on a reconstituted transcription factor. The MaV203 strain was first transformed with the c-Myc bait plasmid using the lithium acetate method (Gietz, Schiestl et al. 1995). Following transformation with the c-Myc bait plasmid, the resulting strain was transformed with the plasmid pEXP-AD502 (Invitrogen). This plasmid acts as a negative control since it contains a GAL4 activation domain without being fused to another protein. In order to reduce the number of false positives, the resulting strain was tested for self-activation. This is particularly important given that c-Myc may still retain its transactivational activity. *HIS3* encodes the gene for the enzyme imidazole glycerol phosphate dehydratase, which is required for histidine biosynthesis. This enzyme can be inhibited in a dose dependent manner with 3-Amino-1,2,4-Triazle (3AT). We titrated the *HIS3* activity on plates lacking histidine but containing 3AT to a point where growth of the MaV203 yeast strain expressing c-Myc/Gal4DB plus the empty activation domain is inhibited. Specifically, four single colonies were patched on to a plate lacking both leucine (Leu) and tryptophan (Trp), in order to retain both the plasmids, and the plate was incubated for 24 hours at 30ºC. This plate was then replica plated onto plates lacking leucine, tryptophan and histidine (His) and containing 10 mM, 25 mM or 100mM 3AT. After another 24 hour incubation these

plates were replica cleaned and then further incubated for 48 hours. While there was still growth of the yeast strain expressing c-MycGAL4DB at 10mM 3AT, at 25 mM 3AT this strain was no longer able to grow. This concentration of 3AT was added to all plates lacking histidine for the remainder of the screen to help prevent isolation of yeast clones where c-Myc is inducing transcription in the absence of the GAL4DB.

The MaV203 strain transformed only with the c-MycGAL4DB was then transformed with a pre-made human liver cDNA library where all cDNAs are fused to the GAL4 Activation Domain (AD) (Proquest). Previous tests had determined the number of transformants resulting from transforming the MaV203 yeast strain containing the c-MycGAL4DB plasmid with one microgram of library DNA to be $2.7 \times 10^5$. We expanded this 20-fold to screen $5.4 \times 10^6$ transformants. The yeast transformation was plated over 104 150mm -Leu-Trp-His plus 25mM 3AT plates. Following a 24 hour incubation at 30ºC, the plates were replica cleaned and then incubated for an additional 48 hours. Following this incubation, 643 single clones were streaked on -Leu-Trp plates to isolate single colonies from each clone. Each of these clones potentially contains a cDNA expressing a c-Myc interacting protein. To help determine if these are true c-Myc interacting proteins the yeast clones were screened as described below.

**Initial Screen for c-Myc interacting proteins.** The 643 colonies were screened for activation of the three reporter genes, *His3*, *Ura1* and *lacZ*, by examining the ability of the isolated yeast clones to grow on plates lacking histidine or uracil. Additionally, *lacZ* expression was examined by either filter assays or the more sensitive CPRG assay (see methods). Interestingly, almost all yeast clones grew on plates lacking uracil regardless

of their ability to grow on plates lacking histidine. This may suggest that c-Myc was able to transactivate this promoter in the absence of interaction. Therefore, those colonies that grew on plates lacking histidine and that were found to express *LacZ* were considered positive for containing a potential c-Myc interacting protein. It is important to note that none of the clones had strong growth on the -His plates or showed a substantial color change in either assay reading *LacZ* expression. This suggests that any interaction with c-Myc may be weak or transient. Therefore any colonies that showed induction of the reporter genes over that of a negative control of a yeast clone containing c-MycGAL4DB plus empty vector were further considered. While this increases the chances of isolating false positives (clones that show activation of the reporter genes in the absence of a reconstituted transcription factor) it ensures that weak interactions are not missed. From this first screen we isolated 190 yeast clones containing cDNA library plasmids expressing possible c-Myc interacting proteins.

**Mating Screen.** Given the large number of potential c-Myc interacting proteins we wanted to re-screen those clones we identified in hopes of both eliminating false positives as well as narrowing down the number or proteins we continued to examine. We are particularly interested in identifying those proteins that regulate either c-Myc stability and/or function. c-Myc protein stability is regulated by two phosphorylation sites within Myc box I (Sears, Nuckolls et al. 2000). Phosphorylation of these sites alters binding of proteins required to destabilize c-Myc (Welcker, Orian et al. 2004; Yada, Hatakeyama et al. 2004; Arnold and Sears 2006) (Arnold, Zhang et al., 2008 submitted for publication). Additionally, a number of proteins that enhance or inhibit c-Myc activity bind MBII

(McMahon, Van Buskirk et al. 1998; Wood, McMahon et al. 2000; Kim, Herbst et al. 2003; von der Lehr, Johansson et al. 2003; Dai, Arnold et al. 2007). Both MBI and MBII reside in the transactivation domain of c-Myc, therefore we chose to focus on proteins that bind within this region. To rescreen clones for those that bind within the transactivational domain (TAD) we made use of a mating strategy (Figure 3.2). Briefly, the positive yeast clones were streaked onto a plate lacking tryptophan, but containing cycloheximide. This results in growth of only those yeast clones that have retained the cDNA plasmid, which expresses tryptophan, but that have spontaneously lost the c-Myc bait plasmid, which contains a dominant $CYH2^S$, conferring sensitivity to cycloheximide in the MaV203 strain. Therefore, yeast clones only harboring the cDNA plasmid should grow. We then made use of the fact that yeast can grow both as haploid and diploid cells. Following isolation of the clones containing only cDNA plasmids, the yeast cells were then mated to a MaV103 yeast strain that had been previously transformed with either the original c-Myc bait plasmid or a bait plasmid where the TAD of c-Myc has been deleted. The MaV103 strain is genetically identical to the MaV203 strain with the exception that it is mating type *MATa*, the opposite of MaV203 which is *MAT*$\alpha$. The resulting diploid yeast clones were screened for expression of the *HIS* reporter gene to determine interaction. Following this screen we isolated 42 clones that showed expression of the *HIS* reporter gene in the presence of either c-Myc bait protein. We found 38 clones that only showed expression of the reporter gene when the original c-Myc bait protein was present but not in the presence of the c-Myc bait protein where the transactivational domain had been deleted, suggesting that these clones harbor cDNA plasmids which express proteins that may interact with c-Myc in its transactivational domain. Since these

proteins may be potentially important for regulation of c-Myc we next determined their identity.

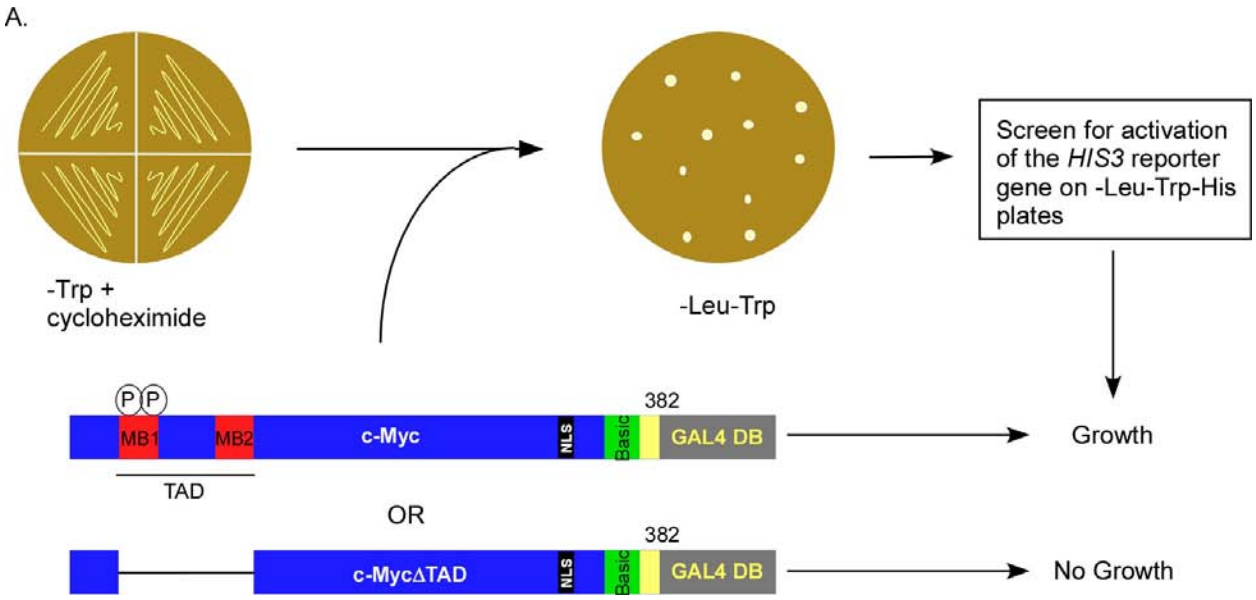



**Figure 3.2: Schematic of mating strategy used to rescreen potential c-Myc interacting proteins**

(A) Clones isolated from yeast two-hybrid screen as harboring c-Myc interacting proteins were rescreened for interaction with a c-Myc mutant lacking the transactivational domain (TAD) using a mating strategy to reintroduce the bait proteins (see text for details). Clones were screened for induction of the *HIS3* reporter gene and those clones that induced growth with the c-Myc bait plasmid but not a bait plasmid where the TAD had been deleted were further considered.

**Isolation of DNA and sequencing results.** We first isolated the cDNA plasmids that were positive for expressing proteins that interacted with the transactivational domain of c-Myc. Specifically, total DNA was isolated from the positive yeast clones and transformed into bacteria cells. The cells were plated on Luria Broth plates containing

Ampicillin. This results in growth of only those bacterial cells containing the cDNA plasmid since this plasmid encodes an Ampicillin resistance gene. Plasmid DNA was then isolated from the bacteria and sequenced. A majority of the plasmids isolated from the mating screen as those that may interact in the TAD of c-Myc contained cDNA sequences that were either outside of the open reading frame of a gene or out of frame with the N-terminal GAL4AD, indicating that they were false positives. However, ten of the cDNAs encoded in-frame proteins (Table 3.1). Of particular interest to us are the ribosomal proteins L3 and L12, the methyltransferase METTL7B and the HMG box protein HBP1. We also sequenced a subset of the plasmids that were isolated as those that interacted with both bait plasmids and found two in-frame sequences (Table 3.1).

**Table 3.1: c-DNA isolated in yeast two-hybrid screen as those expressing c-Myc interacting proteins**

| Clone ID | Interaction with c-Myc TAD? | cDNA | NCBI Accession Number |
|---|---|---|---|
| 1-C | Yes | Homo sapiens peptidase inhibitor, clade G (serpinG1) | NM_000062 |
| 2 - F | No | Homo sapiens solute carrier family 39 (zinc transporter) member 14 (SLC39A14) | NM_001128431 |
| 14 - A | No | Homo sapiens HMG-box transcription factor 1 (HBP1) | NM_012257 |
| 21-C | Yes | Homo sapiens nuclear receptor subfamily 4, group A, member 1 (NR4A1) | NM_002135 |
| 22-H | No | Homo sapiens ATP synthase H+ transporting mitochondrial F1 complex, B polypeptide | NM_001686 |
| 30-G | No | Homo sapiens methyltransferase-like 7B (METTLT7B) | NM_152637 |
| 33-G | No | Homo sapiens ribosomal protein L12 | NM_000976 |
| 34-D | No | Homo sapiens transferrin (TF) | NM_001063 |
| 39-C | No | Homo sapiens Alcohol dehydrogenase Iβ (class I), beta polypeptide (ADH1B) | NM_000668 |
| 76-I | No | Homo sapiens ribosomal protein L3 | NM_000967 |
| 88-A | No | Homo sapiens dihydropyrimidinase (DYPS) | NM_001385 |
| 88 - C | No | Homo sapiens ATP synthase 8- mitochondria genome | NP_536847 (protein) |

**c-Myc interacts with L3 and L12.** In order to confirm interactions isolated by the yeast two-hybrid assay it is important to show interaction in mammalian cells. Therefore, we asked whether c-Myc can interact with the ribosomal protein L12 in 293 cells. L12 is a member of the large subunit of the ribosome and it has been shown to be upregulated in human hepatocellular carcinomas (Kondoh, Shuda et al. 2001). We cloned L12 out of the human liver cDNA library by PCR using primers that created an N-terminal Flag tag, and then inserted the construct into a mammalian expression vector (see methods). We

then co-expressed c-Myc and Flag-L12 in 293 cells and assayed for binding by co-immunoprecipitation. As shown in Figure 3.3A, when L12 is immunoprecipitated from 293 lysates by Flag antibody we observe binding of c-Myc. However, in the absence of L12 expression there is no c-Myc pulldown. This supports binding of c-Myc by the ribosomal protein L12 and suggests that a functional interaction may exist. In addition, our collaborator, Mushui Dai, has examined the ability of a variety of ribosomal proteins to bind to c-Myc. As shown in Figure 3.3B, he found that L3 can also bind c-Myc, again confirming another interaction isolated by our yeast two-hybrid screen. Interestingly, L3 also appeared to decrease soluble ectopic c-Myc proteins levels (see upper Lysate panel). It is unknown at this time whether L3 affects total ectopic c-Myc levels or endogenous c-Myc levels. However, it was found that overexpression of L11 could increase ectopic c-Myc levels by relocalizing c-Myc protein to the nucleolus, which resulted in a decrease in soluble c-Myc protein. Additionally, knockdown of L11 resulted in increased *c-myc* RNA levels (Dai, Arnold et al. 2007). Therefore, in addition to binding c-Myc protein, ribosomal proteins may also regulate c-Myc expression and localization.

Together, the interactions between c-Myc and the ribosomal proteins L3 and L12 help validate our use of the yeast two-hybrid screen to identify proteins that interact with c-Myc and suggest that other interactions we isolated may be relevant. While examining all of these proteins is beyond the scope of this study, we do believe that a number of these proteins may be functionally relevant interactors as discussed below. Additionally, we have more extensively characterized the interaction between c-Myc and a protein isolated in our screen, HBP1, discussed in Chapter 4.

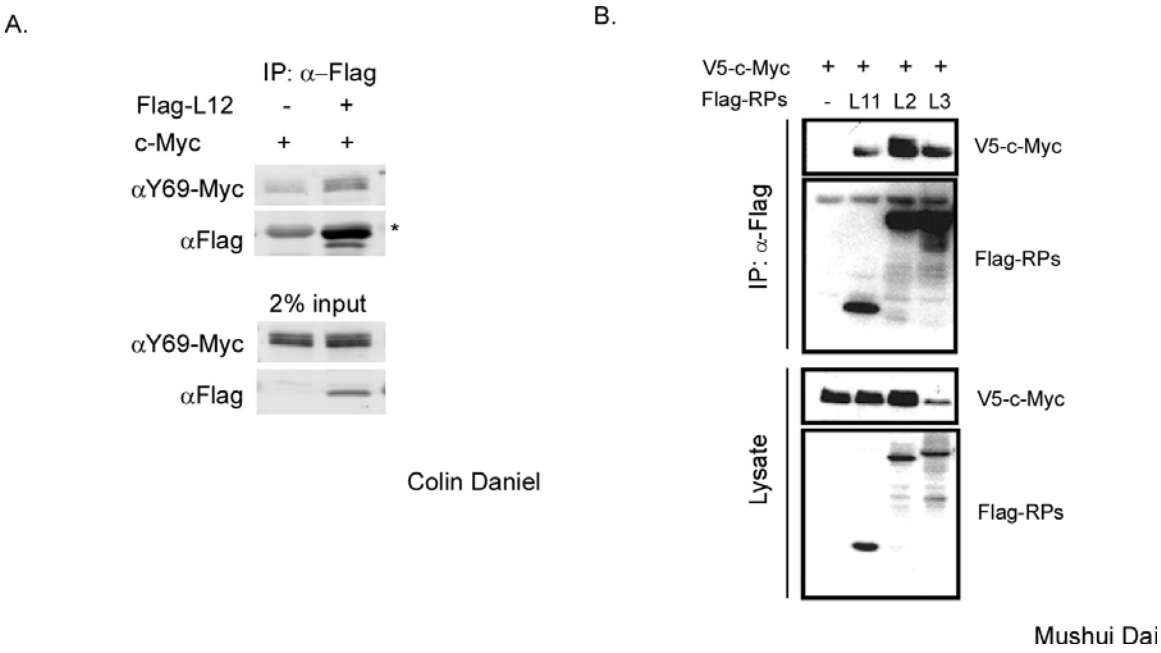



**Figure 3.3: c-Myc interacts with the ribosomal proteins L12 and L3**

(A) Ectopically expressed L12 co-immunoprecipitates with ectopic c-Myc. 293 cells were transiently transfected with CMV-driven V5-c-Myc and Flag-L12 or empty vector. CMV-βgal was also transfected to assess transfection efficiency. Lysates were collected and volumes were adjusted based on βgal activity. Flag-tagged proteins were immunoprecipitated with Flag coupled beads. Inputs and immunoprecipitates were analyzed by western blotting with the indicated antibodies. * designates light chain which co-migrates with L12. (B) Ectopically expressed L3 interacts with ectopic c-Myc. Cells were transiently transfected with CMV-driven V5-c-Myc and one of the indicated ribosomal proteins. Cleared lysates were immunoprecipitated with αFlag. Inputs and immunoprecipitates were analyzed by western blotting with the indicated antibodies. The L11-c-Myc interaction has been previously documented (Dai, Arnold et al. 2007).

## Discussion

Here we describe a yeast two-hybrid screen used to identify novel interacting proteins of the oncoprotein c-Myc. The c-Myc protein, c-terminally truncated at amino acid 382, was used as "bait" and a human liver cDNA library was used as "prey". From our initial screen we isolated a large number of yeast clones containing possible c-Myc interacting proteins, therefore we re-screened the potential positives for interaction with either the original bait plasmid or a bait plasmid lacking the c-Myc transactivational domain. This allowed us to identify and disregard potential false positives by validating induction of the reporter gene. Additionally this screen allowed us to identify those proteins that interact within the transactivational domain of c-Myc. From this screen we identified ten novel c-Myc interacting proteins, a subset of which will be discussed below.

**A Yeast Two-Hybrid screen with c-Myc resulted in many false positive interactions.** The most confounding aspect of our screen was the large amount of false positive interactions we isolated. False positives are defined as those clones that harbor both bait and prey plasmids and induce reporter genes, but the bait and prey proteins do not physically interact. False positives most often arise due to self activation; where a GAL4AD fusion protein can bind the reporter gene promoters or can bind proteins other than the GAL4DB fused protein at the promoter of reporter genes or the GAL4DB fused protein can induce transcription on its own. Therefore growth on knockout plates occurs in the absence of a reconstituted transcription factor. Other instances of false positives occur due to changes in the yeast due to expression of either the bait or prey plasmids or random mutations with in the reporter genes themselves.

Given that c-Myc and its family members have intrinsic activation activity it is possible that these proteins can activate transcription of the reporter genes within the yeast two-hybrid system in the absence of interaction with a GAL4AD fusion protein. Many groups have employed different techniques to circumvent this possible problem. Some have made use of c-Myc protein fragments that do not include the transactivational domain. For instance, Shrivastava et al. isolated the zinc finger protein Ying-yang-1 (YY1) from a yeast two-hybrid assay using the bHLH/LZ region of c-Myc as bait (Shrivastava, Saleque et al. 1993). Additionally, Bannasch et al. used amino acids 177-456, which reside outside the transactivational domain, and isolated another zinc finger protein, Yaf2, as a N-Myc interacting protein (Bannasch, Madge et al. 2001). Another innovative technique made use of the transactivation activity of c-Myc to identify interaction proteins. The transrepression yeast two-hybrid assay identifies those proteins that interact with c-Myc transactivation domain and block its activity by assaying for repression of a reporter gene. This technique was used to identify JPO2 as a c-Myc interacting protein (Huang, Ho et al. 2005). However, this assay used the N-terminal domain of c-Myc for bait and may have missed interactions that lie outside this region.

We chose to use amino acids 1-382 of c-Myc as bait in our screen in order to (1) increase the probability of proper folding of the c-Myc protein and (2) increase the possibility of isolating proteins that interact with different c-Myc functional domains. Given that we used a c-Myc bait protein with an intact transactivation domain it is possible that a number of false positives were isolated due to the intrinsic transactivational activity of c-Myc. However, it is important to note that if this were solely the case we should have observed a skewing in the number of clones isolated in

our mating screen. If the TAD of c-Myc was responsible for inducing transcription of the reporter genes we would have expected to isolate a great number of clones from our mating screen, as those that had reporter gene expression in the presence of the c-MycGAL4DB plasmid but not the c-MycΔTADGAL4DB plasmid. However, we isolated roughly an equal number of clones from both conditions. Therefore, it is likely that we had a difficulty discerning between weak and transient c-Myc interactions and false positives.

**Methyltransferases are important players in the Myc network**. Despite the large number of false positives, we did isolate a number of c-Myc interacting proteins that may have functional relevance. We are especially interested in the methyltransferase, METTL7B, was first isolated in a screen for secreted or transmembrane proteins (Clark, Gurney et al. 2003). While the function of this protein has not been examined, it does share homology to the S-adenoymethal-dependent methyltransferases. This superfamily of proteins use the methyl donor S-adenosylmethionine and have been shown to methylate small molecules, lipids, proteins and nucleotides (reviewed in (Martin and McMillan 2002)). Interestingly, both DNA and histone methyltransferases have been shown to modulate the activity of c-Myc and members of the c-Myc network. Specifically, it has been shown that the histone methyltransferase Hp1γ, interacts with the Myc agonist, Mga, in a complex with E2F-6 and Max in quiescent cells (Ogawa, Ishiguro et al. 2002). Hp1γ can be recruited to both Myc-responsive and E2F-responsive genes where it catalyzes the methylation of lysine 9 of histone H3 resulting in the inhibition of transcription. In addition, c-Myc itself has been shown to interact with the DNA

methyltransferase, Dnmt3a (Brenner, Deplus et al. 2005). Dnmt3a can be recruited with c-Myc to INR elements of gene promoters through the binding of c-Myc to the transcription factor Miz-1. This can result in DNA methylation of these promoters, thereby inhibiting transcription. This was shown to be the mode of c-Myc-dependent inhibition of expression of the cyclin dependent kinase p21. Therefore, Dnmt3a acts as a co-repressor for c-Myc. Given the evidence that both histone and DNA methyltransferases act as co-factors for the Myc network of proteins we hypothesize that METTL7B may be important for c-Myc activity. Further work will be needed to validate that this protein is a true c-Myc interacting protein, as well as to confirm our hypothesis.

**Ribosomal proteins may regulate c-Myc function and are important in tumorigenesis.** In our screen we also identified the ribosomal proteins L3 and L12 as c-Myc interacting proteins. These proteins are members of the large 60S subunit of the ribosome. While ribosomal proteins are often considered as false positive in yeast two-hybrid screens we believed these to be true c-Myc interacting proteins for a variety of reasons. For instance, in a collaborative effort with our lab, c-Myc was shown to interact with the ribosomal protein L11 (Dai, Arnold et al. 2007). We showed that L11 could inhibit c-Myc induced transactivation. Specifically, L11 prevented recruitment of c-Myc co-activators by competing for a common binding site, MBII, which resides in the transactivational domain of c-Myc. L11 is also a direct target of c-Myc suggesting the presence of an inhibitory feedback loop (reviewed in (Dai, Sears et al. 2007)). Like L11, L12 and L3 are target genes of c-Myc, perhaps suggesting the presence of other feedback

loops (Guo, Malek et al. 2000; Kim, Li et al. 2000; Schuhmacher, Kohlhuber et al. 2001; Menssen and Hermeking 2002; Mao, Watson et al. 2003).

In addition to playing a direct role in c-Myc activity, there is growing evidence that alterations in expression of ribosomal proteins result in tumorigenesis (reviewed in (Ruggero and Pandolfi 2003), making interactions with the oncoprotein c-Myc potentially interesting. It has been shown that ribosomal proteins can act as either tumor suppressors or oncoproteins. Specifically, it has been shown that a number of ribosomal proteins are upregulated in hepatocellular carcinoma, including L12 (Kondoh, Shuda et al. 2001). Additionally, an insertional mutagenesis screen in zebrafish revealed that decreased expression of a number of ribosomal proteins resulted in neuronal sheath tumors (Amsterdam, Sadler et al. 2004). Further studies are required to elucidate the relevance of the interactions between L3 and/or L12 and c-Myc. It is important to note that not all ribosomal proteins have the ability to interact with c-Myc (((Dai, Arnold et al. 2007) and Mushui Dai, personal communication). Therefore, it will be important to investigate why these particular ribosomal proteins interact with c-Myc. We believe that understanding the significance of the interaction of c-Myc with these proteins, along with METTL7B, will be important to further understanding c-Myc function and its role in tumorigenesis.

# Chapter Four:

# The Tumor Suppressor Protein HBP1 Negatively Regulates c-Myc Activity

Submitted for publication January 2009

## Abstract:

c-Myc is an important transcription factor that regulates cellular proliferation, cell growth, differentiation and apoptosis. The c-Myc protein heterodimerizes with its partner protein, Max, and together they regulate transcription of a wide variety of genes at E-box sequences (CAC A/GTG). A number of transcriptional co-factors for c-Myc have been described that have binding sites within highly conserved regions of the c-Myc transactivational domain (TAD). In addition to harboring binding sites for different co-activators, the TAD of Myc also contains phosphorylation sites that control c-Myc stability. Mutations in and around these sites have been implicated in tumorigenesis. Given the importance of the TAD of c-Myc we set out to identify new proteins that interact with c-Myc in this region using a yeast two-hybrid assay. The HMG-box protein, HBP1, was identified in our screen as a protein that interacts with full-length c-Myc but not a c-Myc mutant lacking the TAD. HBP1 is a transcriptional repressor and has been shown to negatively regulate the cell cycle. Recently, a correlation between HBP1 underexpression and breast cancer relapse has been described, suggesting that HBP1 is an important tumor suppressor protein. We have found that HBP1 binds c-Myc in cells and expression of HBP1 inhibits c-Myc transactivational activity. Expression of HBP1 appears to prevent c-Myc from binding its target promoters resulting in decreased endogenous expression of c-Myc target genes.

## Introduction:

c-Myc is a transcription factor whose wide range of functions includes promoting cellular proliferation and cell growth, inhibiting differentiation, and inducing apoptosis under growth restrictive conditions. c-Myc activity is essential for cell cycle progression as cells deleted for c-Myc cease to proliferate and exit the cell cycle (Mateyak, Obaya et al. 1997). Additionally, c-Myc function is required for normal animal development as mice homozygously deleted for *c-myc* die at embryonic day 10.5 (Davis, Wims et al. 1993). Given its extensive role in cell proliferation and cell growth, it is not surprising that *c-myc* is a potent oncogene and deregulated c-Myc expression is observed in 70% of all human tumors (reviewed in (Nesbit, Tersak et al. 1999)).

The c-Myc protein is a basic helix-loop-helix transcription factor which heterodimerizes with its partner protein, Max. Together c-Myc and Max regulate transcription of a number of significant genes including those that encode for cell cycle regulators, such as Cdc25 and E2F2, cell growth regulators, such as eIF4e, and apoptotic proteins, such as Bax (Galaktionov, Chen et al. 1996; Jones, Branda et al. 1996; Sears, Ohtani et al. 1997; Mitchell, Ricci et al. 2000). However, the transactivational activity of c-Myc is relatively weak, initially being reported to increase transcription approximately three-fold (Kretzner, Blackwood et al. 1992). In addition to its activation capacity, c-Myc has also been shown to repress gene transcription. For example, c-Myc and Max have been shown to bind the transcription factor Miz1 and prevent transcription from INR elements present in the promoters of the cyclin dependent kinase inhibitors p15 and p21 (Staller, Peukert et al. 2001; Wu, Cetinkaya et al. 2003).

The transactivational domain (TAD) of c-Myc is critical for both the activating and repressing activities of the protein. The TAD contains two highly conversed domains known as Myc Box I (MBI) and Myc box II (MBII). MBI harbors two phosphorylation sites, Threonine 58 (T58) and Serine 62 (S62), which have been shown to regulate c-Myc stability (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). In addition, phosphorylation of these sites may alter c-Myc function. For instance, it has been demonstrated that an unphosphorylatable point mutant at T58 is abrogated in its ability to induce apoptosis (Chang, Claassen et al. 2000; Conzen, Gottlob et al. 2000; Hemann, Bric et al. 2005). Therefore, it is not surprising that mutations in and around these sites have been implicated in tumorigenesis (Bhatia, Huppi et al. 1993).

MBII is important for recruiting a number of co-regulators to c-Myc target genes. For example, c-Myc recruits histone acetyltransferase activity to target gene promoters by binding the protein TRRAP, a core component of both the GCN5 and TIP 60 histone acetyltransferase complexes (McMahon, Van Buskirk et al. 1998; McMahon, Wood et al. 2000; Park, Kunjibettu et al. 2001). These complexes catalyze the acetylation of histones, thereby allowing for transcription of c-Myc target genes. Additionally, in a collaborative effort, we have recently shown that the ribosomal protein L11 inhibits c-Myc transactivational activity (Dai, Arnold et al. 2007). Therefore, both positive and negative regulators of c-Myc activity bind through MBII.

We used a yeast two-hybrid assay to identify new proteins that interact with c-Myc in its TAD. The HMG-box protein, HBP1, was identified in this screen as a novel c-Myc interacting protein. HBP1 was first identified in a screen for mammalian proteins that rescued a potassium channel defect in yeast (Lesage, Hugnot et al. 1994). Since then

it has been described as a binding partner of pRB and a transcriptional repressor (Lavender, Vandel et al. 1997; Tevosian, Shih et al. 1997; Sampson, Haque et al. 2001; Berasi, Xiu et al. 2004). HBP1 has been shown to repress gene expression both by preventing transcriptional activators from binding their target genes as well as by its direct, sequence-specific DNA binding. HBP1 direct target genes include the *p47phox* and *n-myc* genes (Tevosian, Shih et al. 1997; Berasi, Xiu et al. 2004). Additionally, HBP1 has been shown to negatively regulate Wnt signaling in the absence of DNA binding (Sampson, Haque et al. 2001). Specifically, HBP1 binds the transcription factor TCF4 and prevents it from binding its target genes, including *c-myc* and *cyclin D*. Given its suppression of important cell cycle regulators it is not surprising that overexpression of HBP1 has been shown to induce cell cycle arrest in a number of different cells types (Tevosian, Shih et al. 1997; Shih, Xiu et al. 2001; Yao, Works et al. 2005).

Recent evidence suggests that HBP1 is a tumor suppressor protein. HBP1 maps to chromosome 7q31.1, a region that has been reported to be frequently deleted in numerous types of cancer (Zenklusen, Thompson et al. 1994; Zenklusen, Thompson et al. 1995; Zenklusen, Weitzel et al. 1995; Driouch, Briffod et al. 1998; Liang, Fairman et al. 1998; Koike, Tasaka et al. 1999). Additionally, HBP1 is an important effector in oncogene-induced premature senescence, a tumor suppressing mechanism (Zhang, Kim et al. 2006). Finally, a number of natural HBP1 mutants occur in human breast cancer and underexpression of HBP1 is correlated with poor prognosis (Paulson, Rieger-Christ et al. 2007). Here we show that HBP1 interacts with c-Myc and prevents c-Myc-mediated transcription. This inhibition appears to be due to HBP1 inhibiting the binding

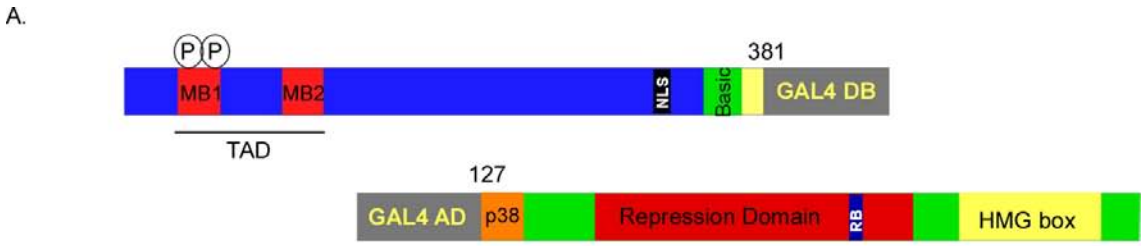
of c-Myc to its target promoters. Given that HBP1 inhibits *c-myc* expression this maybe a secondary mechanism for HBP1-mediated down regulation of c-Myc activity.

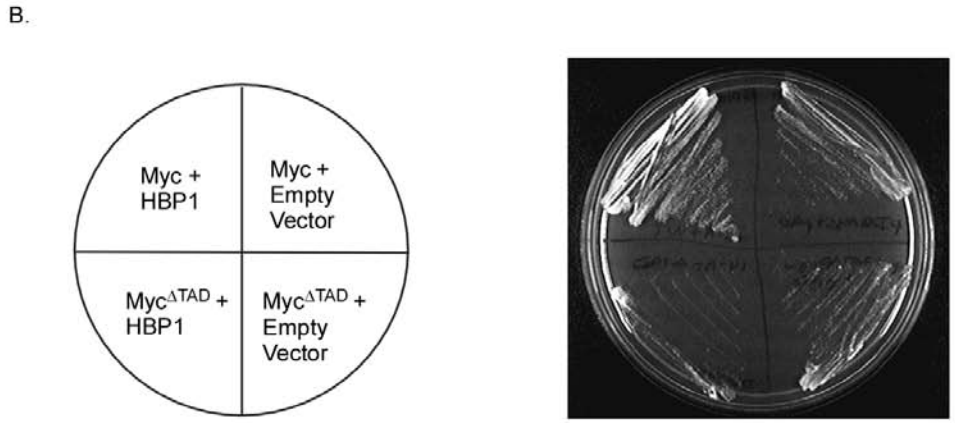
## Results:

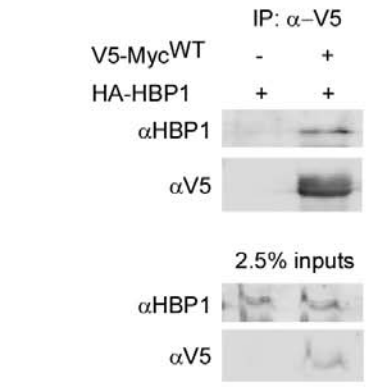
**Identification of HBP1 as a c-Myc-interacting protein.** In order to identify new proteins that interact with c-Myc and that may help regulate c-Myc activity we used a yeast two-hybrid assay. Since c-Myc is a transcription factor with an established transactivational domain we wanted to diminish intrinsic c-Myc activity in order to only identify true protein-protein interactions in the yeast two-hybrid assay. It has been previously shown that a C-terminally truncated c-Myc protein fused to a GAL4 DNA binding domain (GAL4DB) had greatly diminished transactivation activity in a CAT reporter assay (Kato, Barrett et al. 1990). Therefore we truncated the C-terminus of c-Myc at amino acid 382 and fused the protein to a GAL4DB (Figure 4.1A). This c-Myc fusion construct was cloned into the pDBLeu yeast expression plasmid. The resulting bait plasmid was transformed in a MaV203 yeast strain followed by a second transformation with a human liver cDNA liver library. Approximately $5.4x10^6$ transformants were screened for expression of the *HIS3* reporter gene. Colonies were isolated and further screened for expression of two reporter genes.

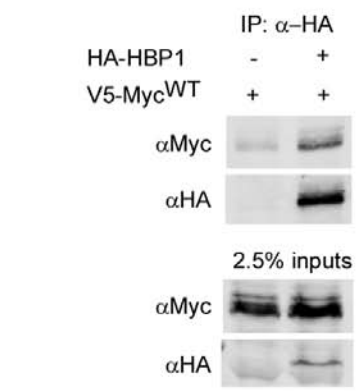
We were particularly interested in identifying those proteins that bind within the c-Myc transactivation domain (TAD). Both Myc box I and Myc box II reside within this region and these domains have been shown to be important for both c-Myc stability and c-Myc function. To narrow down the clones to only those that express the GAL4 activation domain (GAL4AD) fusion proteins that interact within the TAD of c-Myc we used a mating strategy as described in Chapter 3. Through this process we isolated a cDNA encoding amino acids 127-514 of the HMG box protein 1 (HBP1) as a putative c-Myc interacting protein (Figure 4.1A). Expression of this cDNA with the c-Myc bait

plasmid resulted in growth on yeast plates lacking histidine (Figure 4.1B). Conversely, yeast expressing both the *hbp1* cDNA and the c-MycΔTAD bait gene showed no growth on this plate, suggesting that HBP1 can not interact with this mutant form of c-Myc.


A.
P P
MB1
MB2
NLS
Basic
381
GAL4 DB
TAD
127
GAL4 AD
p38
Repression Domain
RB
HMG box


B.
Myc + HBP1
Myc + Empty Vector
Myc^ΔTAD + HBP1
Myc^ΔTAD + Empty Vector


C.
IP: α–V5
V5-Myc^WT - +
HA-HBP1 + +
αHBP1
αV5
2.5% inputs
αHBP1
αV5


D.
IP: α–HA
HA-HBP1 - +
V5-Myc^WT + +
αMyc
αHA
2.5% inputs
αMyc
αHA

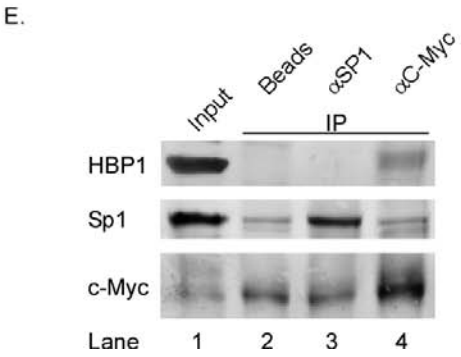

E.
Input
Beads
αSP1
αC-Myc
IP
HBP1
Sp1
c-Myc
Lane 1 2 3 4

**Figure 4.1: HBP1 is a c-Myc interacting protein.**
(A) Schematic of "bait" and "prey" constructs isolated in yeast two-hybrid assay. Amino acids 1-382 of c-Myc was fused to a GAL4 DNA binding domain (GAL4DB) and used to screen for novel interacting proteins. cDNA encoding amino acids 127-514 of HBP1 was isolate in the screen as a positive interaction. Important features and domains of each protein are indicated (see text). (B) HBP1 and c-Myc interact in yeast cells. Yeast cells containing cDNA expressing HBP1 were mated to yeast containing either c-MycGAL4DB or c-MycΔTADGAL4DB and were plated on -Leu-Trp-His + 25 mM 3AT plates to assess expression of the *HIS3* reporter gene. As a control, yeast containing the pEXPAD-502 plasmid which harbors a GAL4 activation domain (GAL4AD) without a fused cDNA was mated to yeast containing either bait plasmid. (C) Ectopically expressed HBP1 co-immunoprecipitates with ectopic c-Myc. 293 cells were transiently transfected with CMV-driven V5-c-Myc and EF-1α-driven HA-HBP1 or empty vector. CMV-β-gal was also transfected to assess transfection efficiency. Lysates were collected and immunoprecipitation volumes were adjusted based on β-gal activity. V5-tagged proteins were immunoprecipitated with anti-V5. Inputs and immunoprecipitates were analyzed by western blotting with the indicated antibodies. (D) Ectopically expressed c-Myc co-immunoprecipitates with ectopic HBP1. 293 cells were transiently transfected with HA-HBP1 and V5-Myc or empty vector. CMV-β-gal was again transfected to assess transfection efficiency. Lysates were collected and immunoprecipitation volumes were adjusted based on β-gal activity. HA-tagged proteins were immunoprecipitated with anti-HA. Inputs and immunoprecipitates were analyzed by western blotting with the indicated antibodies. (E) Endogenous HBP1 interacts with endogenous c-Myc but not the transcription factor Sp1. 293 cleared lysates were immunoprecipitated with agarose conjugated C-33 anti-Myc antibody for c-Myc pulldown or agarose conjugated anti-SP for SP1 pulldown. Control immunoprecipitation was done with protein G agarose. Input and immunoprecipitation were analyzed by western blotting with the indicated antibodies.

**HBP1 interacts with c-Myc in mammalian cells.** HBP1 has been reported to be a transcriptional repressor both by binding specific DNA sequences and by its direct binding to other transcription factors (Tevosian, Shih et al. 1997; Sampson, Haque et al. 2001; Berasi, Xiu et al. 2004). Therefore, we next asked whether HBP1 interacts with c-Myc in cell culture by performing co-immunoprecipitation analyses using full-length V5-tagged c-Myc and full length-HA-tagged HBP1. Specifically, HEK293 cells were transiently transfected with HA-HBP1 and either V5-c-Myc or empty vector. V5-c-Myc

was immunoprecipitated from cell lysates using an antibody to the V5 tag and immunoprecipitates were analyzed by western blotting with antibodies to HBP1 and the V5 tag. As shown in Figure 4.1C, HBP1 is co-immunoprecipitated with c-Myc but is not pulled down in the absence of transfected c-Myc. Inputs were also run to show equal protein expression. To confirm this interaction we preformed the reverse co-immunoprecipitation and found that c-Myc immunoprecipitated with HBP1 (Figure 4.1D). Finally, we examined whether the endogenous proteins interact in cells culture. 293 lysates were incubated with antibodies to c-Myc, the transcription factor Sp1, or beads alone. As shown in Figure 4.1E, immunoprecipitation of endogenous c-Myc co-immunoprecipitated endogenous HBP1. No HBP1 pulldown was observed in the beads alone or Sp1 control immunoprecipitations where only a small amount of non-specific c-Myc binding was detected. Together these data suggests that HBP1 interacts with c-Myc in mammalian cells.

**HBP1 inhibits c-Myc induced expression of reporter genes.** Given that HBP1 has a described positive role in cell cycle arrest and differentiation we asked whether HBP1 negatively regulates c-Myc activity (Tevosian, Shih et al. 1997; Shih, Tevosian et al. 1998; Lemercier, Duncliffe et al. 2000; Sampson, Haque et al. 2001; Shih, Xiu et al. 2001; Berasi, Xiu et al. 2004; Yao, Works et al. 2005). We first tested this by examining whether HBP1 affected the ability of c-Myc to induce transcription of a reporter gene. A reporter plasmid containing four E-boxes proximal to a minimal SV40 promoter driving luciferase was transiently transfected into 293 cells with empty vector control, V5-c-Myc, HA-HBP1 or both V5-c-Myc and HA-HBP1. CMV-βgal was also co-transfected in

order to control for transfection efficiency. Luciferase units were normalized to βgal activity and luciferase activity is shown relative to empty vector control transfection (Figure 4.2A). c-Myc induced expression of luciferase approximately four-fold over background levels. This activation is consistent with previous reports (Hurlin, Queva et al. 1997). While HBP1 had no effect on luciferase expression on its own, it decreased expression two-fold in the presence of c-Myc, indicating that overexpression of HBP1 can repress c-Myc induced transcription.

We next asked whether the c-terminal region of HBP1 containing the HMG box is necessary for HBP1-mediated repression of c-Myc activity. The HMG box is the DNA binding domain of HBP1 and has previously been shown to be required for sequence-specific repression and activation by HBP1 (Tevosian, Shih et al. 1997; Lemercier, Duncliffe et al. 2000; Berasi, Xiu et al. 2004). However, mutation of the HMG box did not prevent HBP1 from inhibiting Wnt signaling (Sampson, Haque et al. 2001). To determine whether the HMG box of HBP1 is required for HBP1-mediated inhibition of c-Myc-induced expression we cloned a HBP1 mutant with the N-terminal 429 amino acids which lacks its HMG box (HBP1ΔC). This mutant form of HBP1 was unable to inhibit c-Myc-mediated expression of luciferase when driven by E-boxes (Figure 4.2A). It has been previously reported that HBP1 is unable to bind E-boxes, suggesting another important function of the HBP1 C-terminus for its repression of c-Myc (Shih, Tevosian et al. 1998). It is important to note that we observed no appreciable effect on a control plasmid in any luciferase assay (Figure 4.2A).

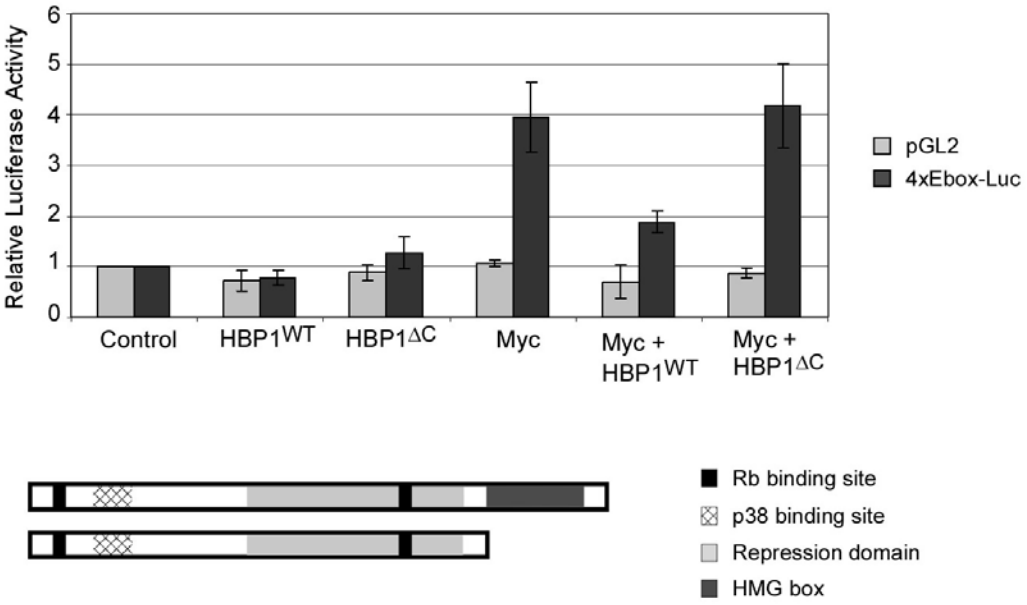
In the previously described assays, *c-myc* expression is controlled by a CMV promoter. Since HBP1 has been reported to inhibit expression from the CMV promoter

we also examined protein levels in these assays (Tevosian, Shih et al. 1997). Whole cell lysates from 293 cells were run on an SDS-Page gel and a western blot was probed for both V5-c-Myc and HA-HBP1. As shown in Figure 4.2B, we saw no appreciable change in c-Myc protein levels when HBP1 was overexpressed, indicating that changes in c-Myc levels is not the cause of the decreased luciferase expression we observed in Figure 4.2.

In addition to examining the ability of HBP1 to inhibit expression of a synthetic promoter we also examined the ability of HBP1 to inhibit c-Myc-mediated activation of luciferase under control of the natural E2F2 promoter, which harbors three c-Myc binding sites (Sears, Ohtani et al. 1997). Using this reporter plasmid we have observed two to ten-fold induction of luciferase activity in the presence of c-Myc (Sears, Ohtani et al. 1997; Yeh, Cunningham et al. 2004; Arnold and Sears 2006). REF52 cells were transfected with the E2F2-luciferase reporter vector along with either empty vector, HA-HBP1 alone, V5-c-Myc alone or V5-c-Myc and HA-HBP1. Transfected cells were serum starved for 48 hours to reduce endogenous activation of the E2F2 promoter. Cells were harvested and luciferase and βgal activity was measured. Shown in Figure 4.2C is luciferase activity normalized to βgal and relative to the empty vector control transfection. With expression of c-Myc there is an approximate four-fold increase in luciferase activity. HBP1 had no significant effect on the E2F2 promoter alone, however in the presence of c-Myc, HBP1 reduces expression of luciferase to background levels, suggesting that HBP1 can inhibit c-Myc induced expression from a natural promoter. We again tested the effect of the C-terminal deletion mutant of HBP1 on c-Myc-induced transcription. In contrast to what we observed with the E-box-Luc reporter plasmid, HBP1ΔC retained some ability to repress transcription from the E2F2 promoter, decreasing luciferase

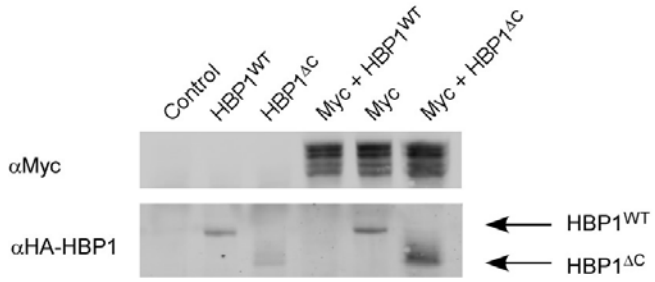
activity approximately two-fold (Figure 4.2C). This difference is most likely due to the complexity of this promoter which can be regulated by a number of proteins, including the AP-1 transcription factor and the E2F proteins (Sears, Ohtani et al. 1997; Shen, Uray et al. 2008). Taken together these results suggest that HBP1 can represses c-Myc activity and this repression partially relies on an intact HMG box.

A.


Relative Luciferase Activity
0
1
2
3
4
5
6
Control
HBP1WT
HBP1ΔC
Myc
Myc + HBP1WT
Myc + HBP1ΔC
pGL2
4xEbox-Luc


Rb binding site
p38 binding site
Repression domain
HMG box

B.


Control
HBP1WT
HBP1ΔC
Myc + HBP1WT
Myc
Myc + HBP1ΔC
αMyc
αHA-HBP1
HBP1WT
HBP1ΔC

C.

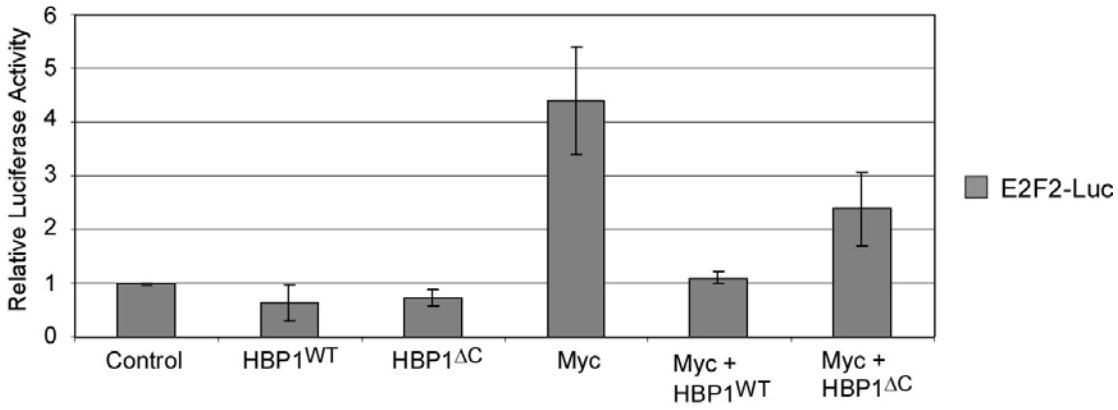

Relative Luciferase Activity
0
1
2
3
4
5
6
Control
HBP1WT
HBP1ΔC
Myc
Myc + HBP1WT
Myc + HBP1ΔC
E2F2-Luc

**Figure 4.2: HBP1 inhibits c-Myc induced transcription.**
(A) HBP1 inhibits c-Myc-induced transcription from a synthetic E-box promoter. 293 cells were transiently transfected with either control pGL2 or 4xEbox-luciferase and CMV-β-gal, together with V5-c-Myc and/or HA- HBP1 expression vectors as indicated. 18-20 hours post-transfection cells were serum starved in DMEM supplemented with 0.2% serum for 48 hours. Cleared lysates were analyzed for β-gal and luciferase activity. Luciferase activity was normalized to transfection efficiency, as measured by β-gal activity, and is shown relative to empty vector control transfected cells. The average of three independent experiments is shown with standard deviations. A schematic of the HBP1 proteins used in this experiment are shown. (B) Expression of HBP1 does not affect expression of ectopic c-Myc. Whole cell lysates from experiment (A) were run on a SDS-PAGE gel and were visualized by western blotting using the indicated antibodies. (C) HBP1 inhibits c-Myc induced transcription from the E2F2 promoter. REF52 cells were transiently transfected with CMV-βgal, E2F2-luciferase and V5-c-Myc and/or HA-HBP1 expression vectors. 18-20 hours post-transfection cells were serum starved in DMEM supplemented with 0.1% serum for 48 hours. Cleared lysates were assayed for β-gal activity and luciferase activity. Luciferase activity was normalized to transfection efficiency, as measured by β-gal activity, and is shown relative to empty vector control transfected cells. The average of four independent experiments is shown with standard deviations.

**Knockdown of HBP1 protein increase c-Myc transactivational activity**. We next asked whether knockdown of HBP1 altered c-Myc activity. We first examined the effect of HBP1 knockdown on c-Myc protein levels in 293 cells. HBP1 overexpression has been previously reported to decrease c-Myc expression in a number of cell types (Sampson, Haque et al. 2001; Kim, Zhang et al. 2006), therefore, we wanted to determine if knockdown of HBP1 would increase c-Myc protein, thereby increasing c-Myc activity. Cells were transiently transfected with shRNA to HBP1 or a scramble control. Whole cell lysates were visualized by western blot using antibodies against HBP1 or c-Myc and antibodies against the protein loading controls β-actin or Cdk2. Both HBP1 and c-Myc protein levels were quantitated and normalized to total protein. Percent protein relative to the scramble control is shown (Figure 4.3A). We observed no appreciable effect on c-

Myc protein levels in response to a significant knockdown of HBP1. Therefore, we proceeded to examine the effect of HBP1 knockdown on c-Myc-mediated transcription. 293 cells were transfected with 4xEbox-luc, CMV-βgal and either shRNA to HBP1 or a scramble control. 48 hours post-transfection cells were harvested and βgal and luciferase activities were measured. As shown in Figure 4.3B, we observed an approximate 1.7-fold increase in luciferase activity when HBP1 protein is reduced. This modest effect on luciferase activity is most likely due to the fact that this assay was performed in actively cycling cells where c-Myc activity is already high. However, our results suggest that endogenous HBP1 has a role in inhibiting c-Myc activity.

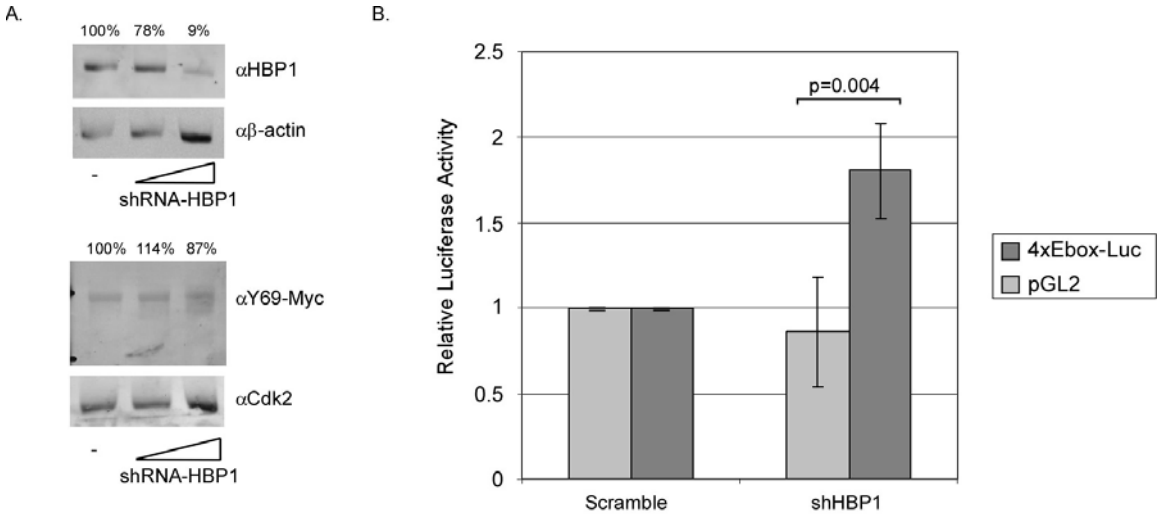



**Figure 4.3: Knockdown of HBP1 results in increased c-Myc transcriptional activity.** (A) Knockdown of HBP1 does not significantly change c-Myc protein levels. 293 cells were transiently transfected with either a scramble control, or 2ug, or 10ug of shRNA directed to HBP1. Whole cell lysates were run on an SDS-PAGE gel and protein was analyzed by western blotting with the indicated antibodies. Blots were scanned with a LI-COR Odyssey Infrared Imager (Lincoln, Nebraska) to visualize proteins. Protein levels were quantitated using LI-COR Odyssey Infrared Imager software version 1.2. c-Myc and HBP1 protein levels were normalized to total protein as measured by β-actin or Cdk2 protein levels. Normalized c-Myc and HBP1 protein levels were calculated as a percentage of levels with transfected scramble shRNA. (B) Knockdown of HBP1 results in increased expression of a synthetic reporter gene driven by E-boxes. 293 cells were transiently transfected with CMV-β-gal, either pGL2 or 4xEbox-luc, and either a scramble shRNA control or shHBP1. 48 hours post-transfection, cells were collected and lysates were assayed for βgal and luciferase activity. Luciferase activity was normalized to transfection efficiency, as measured by βgal activity, and is shown relative to levels from scramble shRNA transfections. The average of four independent experiments ± S.D. is shown.

**HBP1 represses transcription induced by c-Myc point mutant proteins.** Based on our yeast two-hybrid assay it appears that HBP1 binds within the TAD of c-Myc (Figure 4.1B). Therefore, we next asked if HBP1 is able to inhibit expression induced by the c-Myc unphosphorylatable point mutants, c-Myc$^{T58A}$ and c-Myc$^{S62A}$. The T58 and S62 residues reside in MBI, which is located within the TAD and phosphorylation at these

sites results in opposing effects on c-Myc stability. Specifically, phosphorylation at S62 stabilizes c-Myc protein while phosphorylation at T58 destabilizes c-Myc protein (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). Point mutations at these sites have varying effects on c-Myc stability. Specifically, the T58A mutation increases c-Myc stability 4-6 fold, while S62A mutants have low to intermediate stability (Salghetti, Kim et al. 1999; Sears, Nuckolls et al. 2000; (Yada, Hatakeyama et al. 2004). Additionally, phosphorylation at these sites may be important in regulating c-Myc function. It has been shown that point mutants at T58 have reduced ability to induce apoptosis (Chang, Claassen et al. 2000; Conzen, Gottlob et al. 2000; Hemann, Bric et al. 2005). Additionally, a S62A c-Myc point mutant was found to be unable to be recruited to the promoter of the c-Myc target gene γ-GCS (Benassi, Fanciulli et al. 2006). Given this variation in c-Myc function by the c-Myc point mutants we examined whether HBP1 could inhibit transcription induced by these mutants. We transiently co-transfected REF52 cells with CMV-βgal, E2F2-luciferase and one of the c-Myc point mutants in the presence and absence of HA-HBP1. As shown in Figure 4.4, the c-Myc point mutants had varying degrees of transactivational activity on this promoter; however, in all cases HBP1 overexpression decreased this activity. Therefore, in regards to the E2F2 promoter it does not appear that HBP1 differentially affects the activity of c-Myc point mutants in MBI. However, at this time we cannot rule out that there may be target specific effects with these point mutants, and this will need to be examined in future studies.

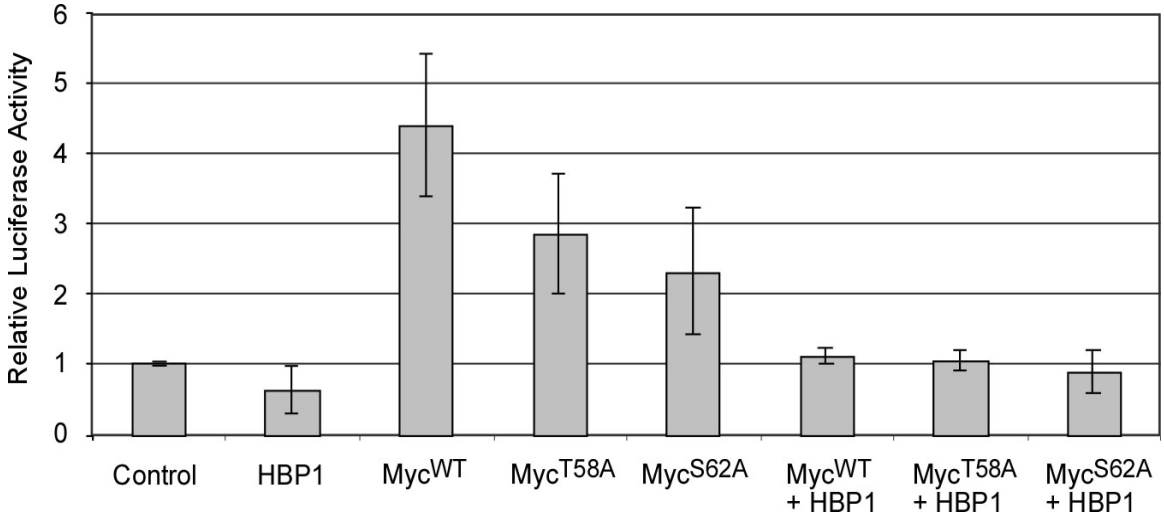
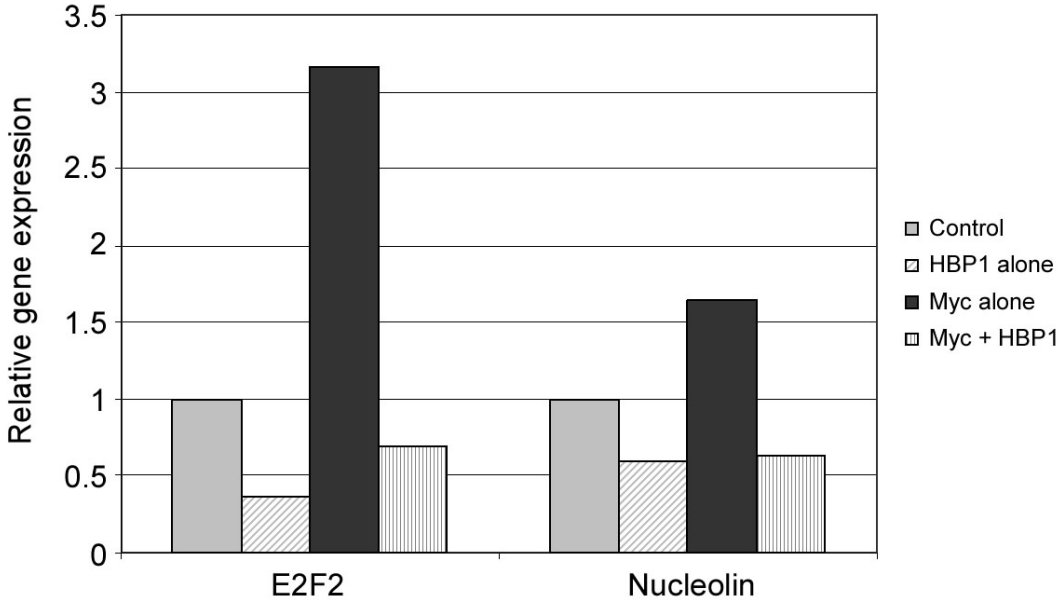



**Figure 4.4: HBP1 inhibits transactivation by c-Myc MBI point mutants.**
REF52 cells were transiently transfected with CMV-β-gal, E2F2-luciferase, HA- HBP1 and/or V5-c-Myc expression vectors as indicated. 18-20 hours post-transfection cells were serum starved in DMEM supplemented with 0.1% serum for 48 hours. Cells were collected and cleared lysates were assayed for βgal activity and luciferase activity. Luciferase activity was normalized to transfection efficiency, as measured by βgal activity, and is shown relative to empty vector control transfected cells. The average of three independent experiments is shown with standard deviations.

**HBP1 negatively regulates expression of endogenous c-Myc target genes.** Since we have shown that HBP1 can negatively regulate c-Myc-mediated expression of reporter genes, we next asked whether expression of HBP1 inhibits expression of known endogenous c-Myc target genes. To examine this, we transiently transfected 293 cells with empty vector, V5-c-Myc alone, HA-HBP1 alone, or V5-c-Myc plus HA-HBP1. Total RNA was extracted and analyzed by quantitative RT-PCR. We examined the expression of *e2f2* and *nucleolin*, both described target genes of c-Myc (Sears, Ohtani et al. 1997; Greasley, Bonnard et al. 2000). As shown in Figure 4.5A, overexpression of c-Myc resulted in a three-fold and 1.7-fold induction of *e2f2* and *nucleolin* expression,

respectively. However, overexpression of HBP1 in the presence of overexpressed c-Myc inhibited expression of both genes beyond that of control levels. Interestingly, HBP1 alone was able to reduce expression of *e2f2* and *nucleolin* approximately two-fold and this is presumably due to HBP1 inhibition of endogenous c-Myc. However, we can not rule out effects that HBP1 may have on other transcriptional activators as well as on endogenous c-Myc levels in this assay. We again examined the effect HBP1 had on ectopic c-Myc protein levels (Figure 4.5B). In agreement with our previous data, we did not observe a decrease in ectopic c-Myc protein levels in the presence of HBP1 expression. Therefore, our preliminary data suggest that HBP1 is able to inhibit c-Myc induction of endogenous target genes and that this inhibition appears to be due to a direct effect on c-Myc activity and not c-Myc protein levels.

A




B.

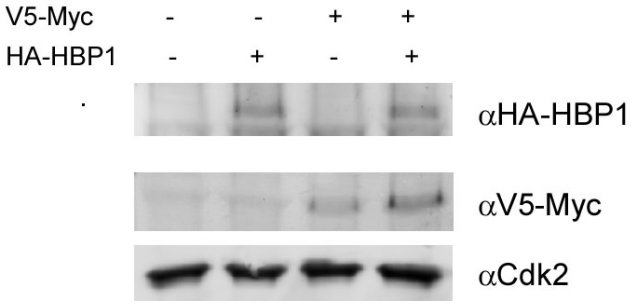



**Figure 4.5: HBP1 inhibits expression of endogenous c-Myc target genes.**

(A) HBP1 inhibits c-Myc induced transcription of *e2f2* and *nucleolin.* 293 cells were transiently transfected in duplicate with empty vectors, HA-HBP1, V5-c-Myc or both V5-c-Myc and HB-HBP1 as indicated. One set of transfected cells were used as described below. The second set of transfected cells were collected 48 hours post-transfection and RNA was isolated. cDNA was generated and used for quantitative PCR using primers to *e2f2*, *nucleolin* and *gapdh*. *E2f2* and *nucleolin* message levels were normalized to total RNA message as measured by *gapdh* and are shown relative to empty vector control transfection. (B) 48 hours post-transfection cells were collected in SDS sample buffer and whole cell lysates were run on an SDS-PAGE gel. Protein levels were analyzed by western blotting with the indicated antibodies.

**HBP1 prevents c-Myc from binding target gene promoters.** In order to determine how HBP1 inhibits c-Myc induced transcription we examined c-Myc protein binding to promoters of its target genes in the presence of HBP1. It has previously been demonstrated that HBP1 inhibits TCF4-induced gene expression by preventing binding of TCF4/β-catenin complexes to DNA (Sampson, Haque et al. 2001). To examine if a similar mechanism exists for inhibition of c-Myc activity, we performed chromatin immunoprecipitation (ChIP) assays. 293 cells were transfected with either V5-c-Myc alone or V5-c-Myc and HA-HBP1. Following crosslinking, cells were sonicated and ectopic c-Myc protein was immunoprecipitated using an antibody to the V5 tag. Co-immunoprecipitated DNA fragments were examined by PCR using primers to the *e2f2* and *nucleolin* promoters. Additionally, primers within the *gapdh* gene were used as a negative control. As shown in Figure 4.6A, immunoprecipitation of c-Myc resulted in increased pulldown of both the *e2f2* and *nucleolin* promoters as compared to immunoprecipitation with a control antibody (compare lanes 1 and 2). Interestingly, when c-Myc and HBP1 are co-expressed, c-Myc binding to the *e2f2* and *nucleolin* promoters appears to decrease significantly (compare lanes 2 and 4). This result was reproducible and representative PCR is shown. Again to show that that this effect was not due to a change in c-Myc protein levels, inputs were collected prior to immunoprecipitation and run on a SDS-PAGE gel. Ectopic c-Myc and HBP1 levels were visualized by western blotting using antibodies to the V5 and HA tags, respectively. As shown in Figure 4.6B, c-Myc protein levels are similar in both the presence and absence of ectopic HBP1 expression suggesting that the effects on c-Myc promoter binding are not a result of decreased c-Myc protein. These results suggest that HBP1 reduces

expression of c-Myc target genes by preventing c-Myc from binding the target gene promoters. Again, this result is similar to HBP1-mediated inhibition of TCF4/β-catenin activity further indicating that, in addition to its sequence-specific mediated repression, this may be another important mechanism of HBP1-induced repression.

To confirm the previous result we asked whether knockdown of HBP1 affected binding of edogenous c-Myc to its target promoters. 293 cells were transfected with siRNA against HBP1 or control siRNA. c-Myc and associated DNA were co-immunoprecipitated with an antibody against c-Myc and PCR was performed using primers to *e2f2* and *nucleolin* promoters. *Gapdh* primers were again used as a control. Preliminary results are shown in Figure 4.6C. Due to the difficulty in immunoprecipitating endogenous c-Myc, we were unable to observe increased binding compared to an IgG control pulldown in cells that were transfected with the control siRNA (Figure 4.6C, lanes 1 and 2). However, when cells were transfected with a siRNA pool guaranteed to significantly knockdown HBP1, we observed an increase in c-Myc binding to its target promoters relative to the IgG pulldown, demonstrating that more c-Myc is binding to the promoters of these genes with decreased HBP1 knockdown (Figure 4.6C, lanes 3 and 4). Additional experiments will need to be performed in order to confirm this result.

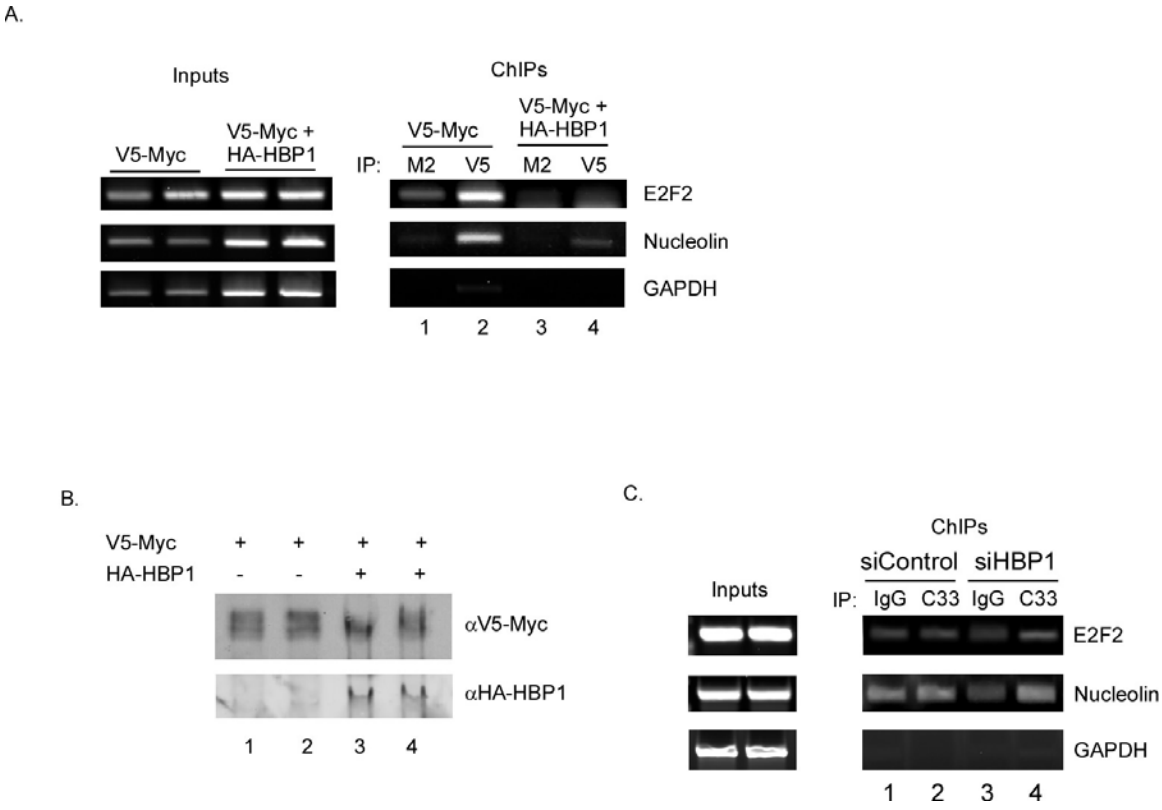



**Figure 4.6: HBP1 inhibits binding of c-Myc to its target gene promoters.**

(A) Expression of HBP1 decreases the levels of c-Myc protein at its target promoters. 293 cells were transiently transfected with CMV-β-gal along with V5-c-Myc alone or V5-c-Myc and HA-HBP1 as indicated. 48 hours post-transfection cells were crosslinked in formaldehyde, collected and sonicated to shear DNA. Cleared lysates were immunoprecipitated with anti-V5 to immunoprecipitate c-Myc or anti-Flag for a control immunoprecipitation. DNA-protein complexes were uncrosslinked and DNA was purified. Primers to the *e2f2* and nucleolin promoters, as well as internal *gapdh* primers were used to amplify DNA. (B) Ectopic expression of HBP1 does not affect ectopic c-Myc protein levels. Cellular lysates from the above immunoprecipitation were run on an SDS-PAGE gel and proteins were analyzed by western blotting with the indicated antibodies. (c) Knockdown of HBP1 increases endogenous c-Myc binding to its target promoters. 293 cells were transiently transfected with either control siRNA or siRNA to HBP1. Cells were crosslinked, collected and sonicated as described above. Lysates were immunoprecipitated with either control IgG or the C33 antibody to c-Myc. DNA-protein complexes were uncrosslinked and DNA was purified and used as a template for PCR with primers to the *e2f2* and nucleolin promoters, as well as internal *gapdh* primers.

**c-Myc binds to the C-terminus of HBP1**. To further understand the mechanism behind HBP1 induced repression of c-Myc we examined the regions of interaction between the two proteins. We first examined which regions of HBP1 were required for c-Myc binding. To do this we used the HBP1ΔC mutant, described above, and we generated a HBP1 deletion mutant lacking the repression domain, HBP1ΔREP. These mutants were chosen since we had previously determined that the N-terminus of HBP1 was not required for interaction with c-Myc in the yeast two-hybrid assay (see Figure 4.1A). Additionally, it was previously shown that TCF4 bound within the HBP1 repression domain, and given the apparently similar mode of HBP1-mediated repression of TCF4 and c-Myc, we were interested in determining if c-Myc binds HBP1 in the same region (Sampson, Haque et al. 2001). c-Myc was expressed in the presence of either wildtype HA-HBP1, one of the HBP1 mutants, or empty vector and CMV-βgal. HBP1 complexes were immunoprecipitated using an antibody against the HA tag from cleared lysates that had been adjusted for transfection efficiency, as measured by βgal activity. As shown in Figure 4.7A, immunoprecipitation of full-length HBP1 and HBP1ΔREP resulted in pulldown of c-Myc. However, HBP1ΔC was unable to co-immunoprecipitate c-Myc suggesting that this is the region of interaction. This is consistent with our previous data showing that this mutant was unable to repress c-Myc-driven transcription (see Figure 4.2A). This data also demonstrates that the regions of interaction between c-Myc and HBP1 are different than for TCF4 and HBP1.

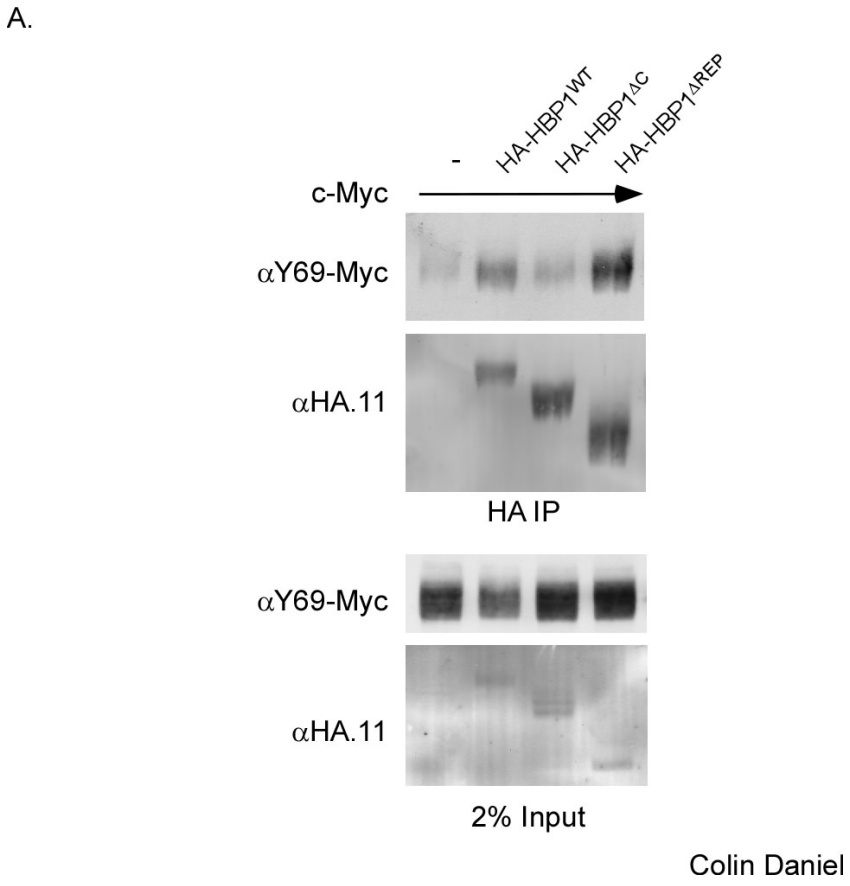



**Figure 4.7: c-Myc interacts with the C-terminus of HBP1**

293 cells were transiently transfected with CMV-βgal, V5-c-Myc and either empty vector or one of the HA-HBP1 expression vectors. Cleared lysates were immunoprecipitated with anti-HA. Inputs and immunoprecipitated complexes were analyzed by western blotting with the indicated antibodies. Immunoprecipitation was performed by Colin Daniel.

**Multiple domains of c-Myc are required for binding to HBP1.** Next we examined the regions of c-Myc responsible for interaction with HBP1. As we believe HBP1 interacts with the TAD of c-Myc based on our yeast two-hybrid assay (Figure 4.1B), we used this mutant as well as c-Myc mutants where MBI or MBII have been deleted (Figure 4.8A). These mutants, as well as wildtype V5-c-Myc were transiently transfected into

293 cells in the presence of HA-HBP1 and CMV-βgal. c-Myc and c-Myc mutants were immunoprecipitated from cleared lysates that had been adjusted for transfection efficiency with an antibody against the V5 tag. As shown in Figure 4.8B, deletion of either MBI or MBII resulted in decreased HBP1 binding to c-Myc when compared to its interaction with full-length c-Myc, while deletion of the TAD appeared to abolish binding (compare lanes 2, 4, 6 and 8), confirming that the TAD of c-Myc is required for binding of HBP1 to c-Myc. However, these results do not suggest specific binding of HBP1 to either MBI or MBII. We did occasionally observe some binding of HBP1 to the ΔTAD c-Myc deletion mutant (data not shown), therefore, we asked whether the C-terminal region deleted in the yeast two-hybrid construct was also important for HBP1 binding. We created c-Myc deletion mutants that lacked the C-terminal 57 amino acids, which harbor the leucine zipper and most of the helix-loop-helix region (Figure 4.8A). These regions are critical for Max dimerization and therefore DNA binding (Blackwood and Eisenman 1991). We found that deletion of this C-terminal region of c-Myc also appears to prevent binding of HBP1 to c-Myc protein even with an intact N-terminal domain (Figure 4.8B, lanes 3, 5, 7 and 9). Therefore it appears that the C-terminus of c-Myc is also important for its ability to interact with HBP1 in mammalian cells. It is possible that HBP1 can bind weakly to these C-terminal deletion mutants and therefore we are unable to detect this interaction by co-immunoprecipitation, but with the sensitivity of the yeast two-hybrid system we could observe the interaction in that assay. Taken together this data suggests that both the transactivational domain, as well as the C-terminus of c-Myc, are important for HBP1 binding. Interestingly, HBP1 was found to bind to two regions of TCF4, an undescribed N-terminal region as well as the HMG box

of TCF4 (Tevosian, Shih et al. 1997). This result may also suggest a mechanism for HBP1-mediated inhibition of c-Myc activity as discussed below.

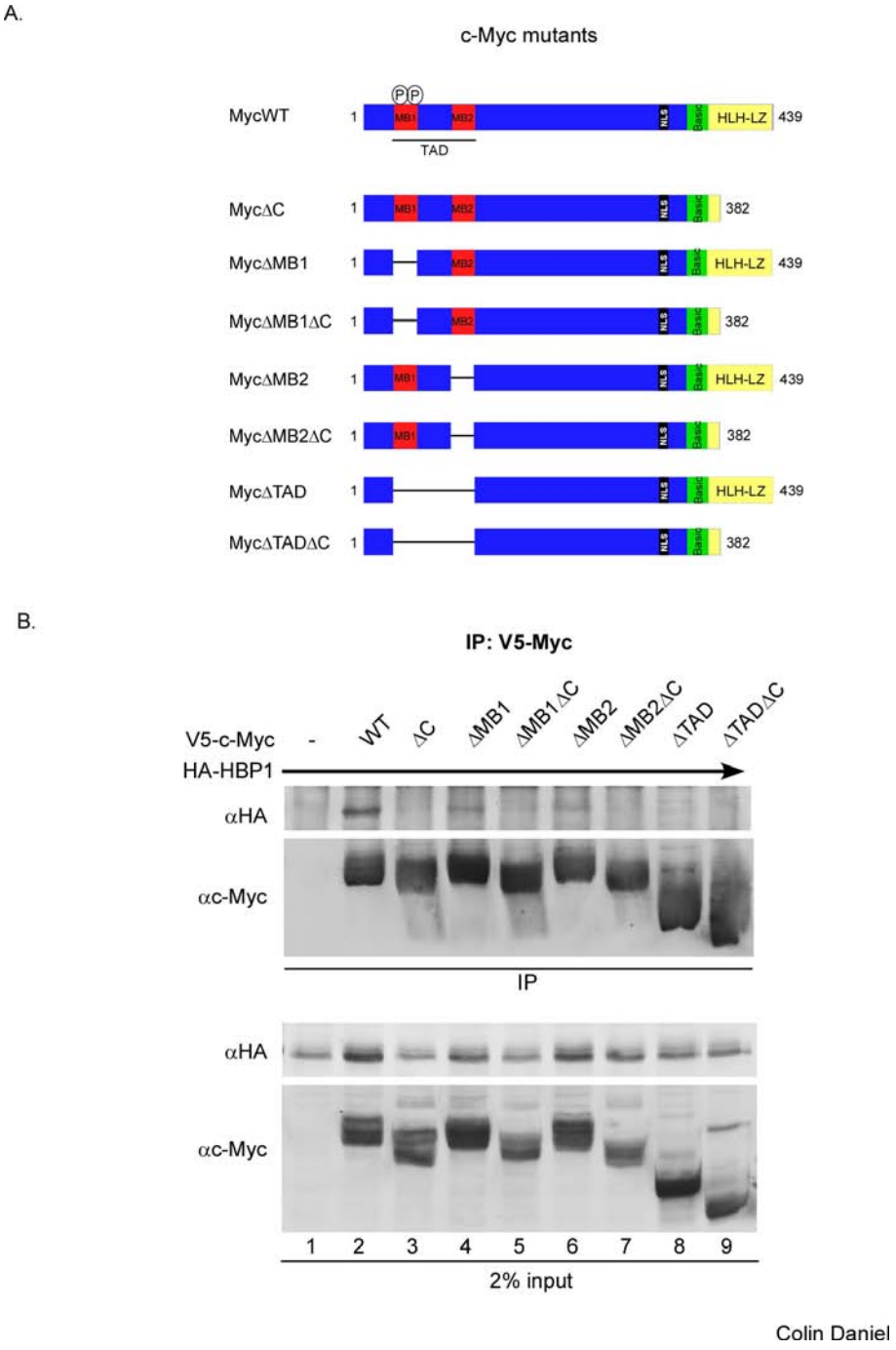



**Figure 4.8: Multiple c-Myc domains are required for binding to HBP1**

(A) Schematic of full-length c-Myc and c-Myc deletion mutants. c-Myc functional domains are indicated. (B) HBP1 interacts with the transactivational domain and c-terminus of c-Myc. 293 cells were transiently transfected with CMV-βgal and HBP1, together with empty vector or one of c-Myc expression vectors. Cleared lysates were immunoprecipitated with anti-V5. Inputs and immunoprecipitated complexes were analyzed by western blotting with the indicated antibodies. Immunoprecipitation was performed by Colin Daniel.

## Discussion:

**Summary of Results**. c-Myc activity drives cellular proliferation and cell growth, inhibits differentiation, and can induce apoptosis in the absence of growth factors. Unchecked c-Myc activity results in tumorigenesis in a number of different cell types, therefore, proper regulation of c-Myc levels and activity is critical for maintaining cells in a differentiated state. In this work we have identified HBP1 as a novel c-Myc interacting protein through a yeast two-hybrid assay. HBP1 is regarded as a tumor suppressor protein and its expression has been shown to inhibit cell cycle progression in multiple cell types (Tevosian, Shih et al. 1997; Shih, Xiu et al. 2001; Yao, Works et al. 2005). Additionally, HBP1 appears to be an important mediator of both oncogene-induced senescence and differentiation (Shih, Tevosian et al. 1998; Shih, Xiu et al. 2001; Yao, Works et al. 2005; Zhang, Kim et al. 2006). Specifically, it has been shown that expression of HBP1 results in the induction of differentiation in leukemic cells, and in pre-muscle cells, HBP1 initiates a necessary cell cycle arrest prior to differentiation (Shih, Tevosian et al. 1998; Yao, Works et al. 2005). Given that the activities of HBP1 oppose those of c-Myc, a potent oncogene and regulator of cell cycle progression, we believe this to be a significant and important interaction.

Here we show that overexpression of HBP1 inhibits c-Myc induced transcription from both a synthetic and natural promoter (Figure 4.2), while reduction of HBP1 protein increase c-Myc transactivation activity (Figure 4.4). HBP1 appears to prevent c-Myc-induced transcription by preventing binding of c-Myc to its target gene promoters (Figure 4.6), and thereby reduces endogenous expression of these genes (Figure 4.5). HBP1

binds c-Myc both in the transactivational domain as well as the HLH-LZ region, perhaps giving a basis for prevention of c-Myc binding to promoters, discussed below.

**HBP1-mediated inhibition of DNA binding by c-Myc**. As previously stated, we found that overexpression of HBP1 reduced c-Myc binding to its target promoters. There are multiple possible mechanisms to explain HBP1-mediated prevention of DNA binding by c-Myc. One possibility is that HBP1 may itself bind promoters of c-Myc target genes, or be recruited there by other proteins, and induce chromatin remodeling, thereby preventing c-Myc from accessing its target genes. HBP1 is a known binding partner of pRB, which itself can recruit histone deacetylases, inducing chromatin condensation (Brehm, Miska et al. 1998; Luo, Postigo et al. 1998; Magnaghi-Jaulin, Groisman et al. 1998). pRB is recruited to DNA by the E2F transcription factor family and many Myc target gene promoters contain both E-boxes and adjacent E2F binding sites, suggesting that pRB could recruit HBP1 to Myc target genes (Sears, Ohtani et al. 1997; Suzuki, Adachi et al. 1998; Bolognese, Forni et al. 2006). Additionally, HBP1 itself can recruit the co-repressor SIN3, which in turn can recruit histone deacetylases (Swanson, Knoepfler et al. 2004). While this mechanism does not account for the binding of HBP1 to c-Myc, it may represent another mechanism of HBP1-mediated repression of c-Myc target genes, as a mutant form of HBP1 unable to bind c-Myc can still confer some repression of expression from the *e2f2* promoter (Figure 4.2C). Therefore, HBP1 may have multiple mechanisms for inhibiting expression of the same gene. Other possibilities for HBP1-mediated repression of c-Myc activity include either prevention of the c-Myc/Max heterodimer from binding DNA or prevention of the heterodimerization itself.

Both of these remain viable possibilities as HBP1 appears to binds c-Myc in the DNA binding/Max heterodimerization domain and this possibility will need to be examined in future studies.

**Inhibition of c-Myc activity by HMG box proteins**. c-Myc was found to bind HBP1 in its C-terminal domain, which contains the DNA binding region of HBP1, the HMG box. It has been previously reported that c-Myc binds another HMG box protein, SSRP1 (Bunker and Kingston 1995). SSRP1 is a member of the FAcilitates Chromatin Transcription (FACT) complex which displaces histones to allow for Pol II- driven transcription (Belotserkovskaya, Oh et al. 2003). SSRP1 was identified in a screen for proteins that interacted with the bHLH-LZ region of c-Myc. In that screen the authors isolated a region of SSRP1 which included its HMG box. While the authors were unable to detect binding in cell culture, they did find that overexpression of SSRP1 was able to reduce c-Myc-induced transcription, suggesting that binding may exist, however it may be transient and hard to detect. This study, in addition to ours, may indicate that the HMG box motif is a binding site for c-Myc and that HMG box proteins have the ability to bind and inhibit c-Myc activity. The significance of the SSRP1-mediated inhibition of c-Myc activity was not followed up, but may be important to determine if this and other HMG box proteins can regulate c-Myc function in a mechanism similar to what we have observed with HBP1.

**HBP1 regulates c-Myc at multiple levels**. HBP1-mediated repression of c-Myc transactivation shares some similarities to HBP1-mediated inhibition of Wnt signaling.

Signaling by Wnt proteins regulate a number of important processes including cell proliferation and cell fate decisions and mutants within the Wnt signaling pathway have been implicated in numerous tumor types (reviewed in (Polakis 2007)). Sampson et al. demonstrated that HBP1 could inhibit transcription downstream of activation of the Wnt pathway and this inhibition was dependent on binding to the TCF4 transcription factor (Sampson, Haque et al. 2001). Specifically, binding of HBP1 to TCF4 results in the inhibition of DNA binding by TCF4/β-catenin complexes to DNA. Binding of DNA by HBP1 was dispensable in this function, as a triple point mutant unable to bind DNA was still able to repress Wnt signaling. Similarly, even though the C-terminus containing the HMG box was required for repression of c-Myc transactivation, we hypothesize that DNA binding by HBP1 is not important for c-Myc mediated repression based on the following three observations; (1) while we did not test the ability of HBP1 to bind c-Myc specific E-boxes, it has previously been shown that HBP1 can not bind the MyoD family specific E-boxes, which vary from c-Myc E-boxes by one base pair (Shih, Tevosian et al. 1998), (2) HBP1 consensus binding sites vary greatly from E-box sequences and the *e2f2* promoter region does not contain the previously described low affinity or high affinity HBP1 binding sites (Tevosian, Shih et al. 1997; Lemercier, Duncliffe et al. 2000) and (3) c-Myc binds within the DNA binding region of HBP1, perhaps preventing DNA binding by HBP1. However, additional studies will be needed to determine if this hypothesis is accurate.

One of the important aspects of HBP1-mediated inhibition of Wnt signaling is that it was shown to inhibit *c-myc* gene expression in multiple cell types (Sampson, Haque et al. 2001; Yao, Works et al. 2005; Kim, Zhang et al. 2006). This observation

coupled with our data suggests that HBP1 may work in multiple ways to inhibit c-Myc activity; (1) by inhibiting *c-myc* expression via inhibition of TCF4/β-catenin complexes and (2) by inhibiting c-Myc-induced activity as a transcription factor, as shown here. This further solidifies HBP1 as a cell cycle inhibitor and a regulator of differentiation since c-Myc expression is required for cell cycle expression and prolonged c-Myc expression results in the inhibition of differentiation.

**Inhibition of c-Myc activity as a mechanism of tumor suppression by HBP1.** As previously described, HBP1 is generally considered as a tumor suppressor protein. Recently, Paulson et al. found that HBP1 expression is reduced in a subset of invasive human breast tumors (Paulson, Rieger-Christ et al. 2007). In addition, a number of naturally occurring HBP1 mutants were found in human breast tumors. A majority of these mutants lacked the C-terminal region of HBP1 suggesting that they would be unable to inhibit c-Myc activity. Additionally, it was demonstrated that all of these mutants were defective in their ability to inhibit Wnt signaling, suggesting that loss of HBP1 would affect both c-Myc protein levels and c-Myc activity (Paulson, Rieger-Christ et al. 2007).

While we did not directly test the effect of overexpression of HBP1 on c-Myc-induced tumorigenesis, we did find that HBP1 could inhibit transactivation by a more oncogenic form of c-Myc, c-Myc$^{T58A}$. While the oncogenic properties of this mutant may not be solely linked to its transactivational activity it would be important to further pursue this course of study to determine if HBP1 could prevent tumorigenesis induced by this mutant or other oncogenic forms of c-Myc.

# Chapter Five:

# Summary and Discussion

## Use of yeast as a model system to study c-Myc

The budding yeast *Saccaromyces cerevisiae* has been widely used as a model system to study mammalian proteins. *S. cerevisiae* can stably exist with either a haploid or diploid genome and the yeast genome is easily genetically manipulated due to its high frequency of homologous recombination. Additionally, many mammalian proteins are conserved from yeast, including kinases, phosphatases and components of the ubiquitin-mediated degradation pathway. Therefore, we used yeast as a model system to both study the degradation of c-Myc, as well as to determine binding partners of the c-Myc protein in a yeast two-hybrid assay, in Chapters 2 and 3, respectively.

**Interdependent and sequential phosphorylation of c-Myc occurs in yeast.** Prior to our yeast two-hybrid assay we asked if c-Myc was phosphorylated in an interdependent and sequential manner on Serine 62 (S62) and Threonine 58 (T58) as we and others have observed in mammalian cells (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). In our yeast two-hybrid screen we hoped to isolate c-Myc interacting proteins that were important for regulating c-Myc stability and/or function. Given that phosphorylation of S62 and T58 have been implicated in both of these activities, it was important to determine if c-Myc, a protein with no known yeast homolog, could be phosphorylated in yeast. Importantly, we found that c-Myc is phosphorylated in yeast cells and that this phosphorylation appeared to be controlled in yeast as it is in mammalian cells. Specifically, we found that in yeast cells phosphorylation of c-Myc at T58 required prior phosphorylation of S62, and S62 dephosphorylation was facilitated by T58 phosphorylation. Additionally, we found that these phosphorylation events regulate

c-Myc stability in yeast cells as they do in mammalian cells, where phosphorylation at S62 stabilizes the protein and phosphorylation at T58 destabilizes the protein.

This precise regulation of c-Myc phosphorylation in yeast suggested that functional homologues to mammalian proteins that target c-Myc protein for degradation are conserved from yeast. Given that many of the key proteins that are implicated in controlling c-Myc T58 and S62 phosphorylation in mammalian cells have yeast orthologs, and strains mutated or deleted for these proteins had already been generated, we asked whether these yeast proteins were responsible for facilitating the phosphorylation and dephosphorylation events that control c-Myc protein stability in yeast. Briefly, we found that the yeast ortholog to ERK, Kss1p, and the yeast ortholog to GSK3β, Rim11p, could phosphorylate c-Myc *in vitro*. Based on examination of the phosphorylation status of c-Myc in yeast deletion mutant strains, it also appeared that Kss1p and Rim11p were at least partially responsible for phosphorylation of c-Myc at S62 and T58, respectively. Likewise, the yeast proteins Pph21p and Rts1p appeared to mediate dephosphorylation of S62 of c-Myc similar to their mammalian orthologs, the PP2A catalytic C subunit and regulatory B subunit.

**Key differences between regulation of c-Myc stability in yeast and mammalian cells.** Interestingly, we observed a few of important differences between the regulation of c-Myc stability in yeast and mammalian cells. Chiefly, we did not observe a significant dependence on peptidyl prolyl isomerase activity in the degradation of c-Myc protein in yeast cells. We have previously shown that c-Myc degradation in mammalian cells is facilitated by the activity of the peptidyl prolyl isomerase, Pin1 (Yeh, Cunningham et al.

2004). Specifically, we showed that c-Myc protein expressed in MEF cells that were homozygously deleted for *pin1* was stabilized with a half-life greater than 100 minutes, while c-Myc expressed in wildtype MEFs had an approximately nine minute half-life. In contrast, here we have found that yeast cells with a temperature sensitive mutation of Ess1, the *S. cerevisiae* Pin1 ortholog, show a similar half-life for c-Myc protein to that in a wildtype yeast strain. However, in contrast to the wildtype control, c-Myc degradation in the *ess1* mutant strain was biphasic with a sharp decrease in c-Myc protein levels in the first ten minutes. Following this initial decrease there was a subpopulation of c-Myc protein that remained stable for at least 60 minutes. This suggested to us that a majority of c-Myc protein expressed in *S. cerevisiae* was in the *trans* conformation and degraded quickly, while the remaining c-Myc protein was in a *cis* conformation and was unable to be degraded in the absence of Ess1p activity. We hypothesize that this difference may be due to the need of c-Myc to be a functional transcription factor in mammalian cells but not yeast cells. While c-Myc can act as a functional transcription factor in yeast when Max is also present there is no known yeast Myc homolog, indicating that Myc activity is not required for any yeast cell functions (Amati, Dalton et al. 1992). This hypothesis will be discussed further below.

We have recently reported a role for the scaffolding protein, Axin1, in mediating a degradation complex for c-Myc in mammalian cells (Arnold, Zhang et al. 2008. submitted for publication). Axin1 appears to coordinate interaction between c-Myc, PP2A, GSK3β and Pin1, resulting in c-Myc ubiquitination and subsequent degradation. Knockdown of Axin1 in 293 cells results in increased c-Myc protein levels, as well as an approximate three to four-fold increase in c-Myc half-life. However, it is important to

note that even though there is no known Axin1 homolog in yeast cells, c-Myc protein half-life in this system is consistently reported to be short like mammalian c-Myc (Flinn, Busch et al. 1998; Salghetti, Kim et al. 1999; Kim, Herbst et al. 2003). This difference is most likely due to the complexity of the mammalian system and the requirement for precise timing of c-Myc degradation in mammalian cells.

**A possible role for a *cis* to *trans* isomerization in regulating c-Myc activity**. MAPKs phosphorylate serines or threonines within the consensus sequence P-X-T/S-P, when the proline proceeding the serine or threonine is in the *trans* conformation (Clark-Lewis, Sanghera et al. 1991; Weiwad, Kullertz et al. 2000) (Figure 5.1). Since c-Myc is phosphorylated at S62 while the proceeding Proline 63 (P63) is in the *trans* conformation, and PP2A is a *trans*-directed phosphatase (Zhou, Kops et al. 2000), the question remains as to why c-Myc is ever converted to *cis* conformation at P63, as our data would suggest, since Pin1 facilitates PP2A-mediated dephosphorylation of S62. Given that this conformation does not seem to be required for c-Myc protein degradation in yeast it seems likely that the *cis* conformation of c-Myc is required for an important role in mammalian cells. Therefore, we hypothesize that the *cis* conformation of c-Myc may be needed for some or all of c-Myc transactivation activity. Mechanistically the exact role of this conformation in c-Myc activity is unknown, but it is possible that it is required for either DNA or co-factor binding. Based on unpublished antibody studies we believe that the c-Myc$^{T58A}$ mutant may be locked in the *cis* conformation. Interestingly, in cell fractionation studies we have observed that the c-Myc$^{T58A}$ point mutant protein is more tightly associated with chromatin than wildtype or c-Myc$^{S62A}$ (Kristi Piehl, unpublished

data), perhaps suggesting that the *cis* conformation may enhance c-Myc DNA binding. Thus far, preliminary ChIP data has demonstrated that there does not seem to be different affinities for binding the *e2f2* promoter between wildtype c-Myc and the c-Myc point mutants (Sarah Byers, unpublished data). However, this result will need to be confirmed for the *e2f2* promoter and for other c-Myc target gene promoters.

At this time it is unknown which protein is responsible for catalyzing the initial *trans* to *cis* isomerization at Proline 63. However, Pin1 is a likely candidate. Pin1 has been shown to upregulate the transcriptional activity of both c-Jun and c-Fos (Wulf, Ryo et al. 2001; Monje, Hernandez-Losa et al. 2005). Additionally, Pin1 is often described as an oncogene and is found to be upregulated in multiple tumor types (Wulf, Ryo et al. 2001; Kuramochi, Arai et al. 2006; Li, Wang et al. 2006). This data would be consistent with a role for Pin1 in increasing c-Myc activity. Determining the exact role Pin1 may play in both regulating c-Myc activity and stability is currently an important area of ongoing research in our laboratory.

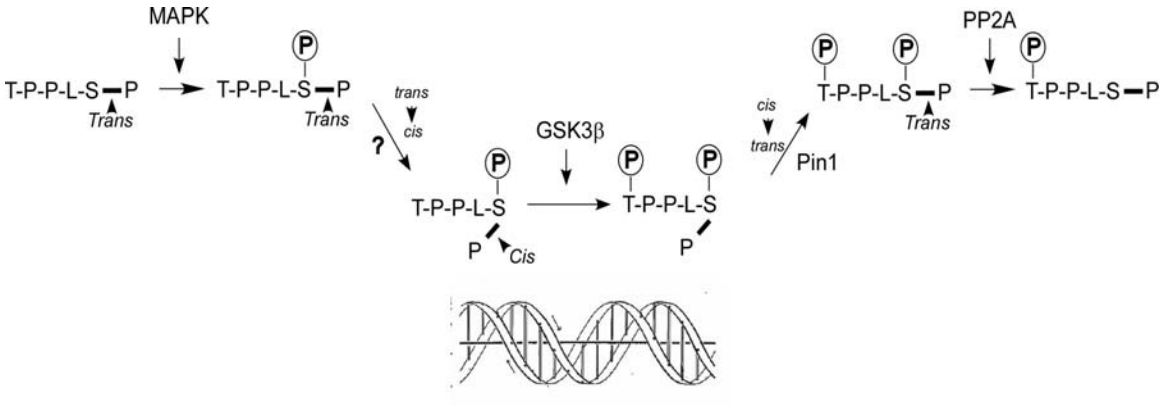



**Figure 5.1: Schematic of a role for isomerization in regulation of c-Myc phosphorylation and activity**

c-Myc is phosphorylated by MAPK at S62 when the bond preceding Proline 63 (P63) is in the *trans* configuration. A *trans* to *cis* isomerization occurs at this bond by an unknown isomerase and it is hypothesized that this isomerization increases activity by c-Myc, possibly by increasing its ability to bind DNA. c-Myc is phosphorylated at T58 by GSK3β and Pin1 catalyzes the *cis* to *trans* isomerization at the bond preceding P63, possibly resulting in the release of c-Myc from DNA. Dually phosphorylated c-Myc is then dephosphorylated at S62 by the *trans* directed phosphatase PP2A. See text for additional details.

**A signaling pathway to control protein degradation.** While the requirement for Axin1 or for peptidyl prolyl isomerase activity in the regulation of c-Myc stability does not appear to be conserved from yeast, the phosphorylation and dephosphorylation events that regulate c-Myc protein stability in mammalian cells are important in regulating c-Myc protein stability in yeast cells. This suggests that the phosphodegron that regulates c-Myc stability may be an important signal for protein degradation in both yeast and mammalian proteins. We have suggested a number of yeast and mammalian proteins that may be affected by this pathway. To date none of these proteins have been shown to follow the same signaling pathway as c-Myc, but the stability of a number of proteins have been reported to be regulated by the same players that regulate c-Myc protein

stability. For instance, c-Jun contains the same consensus phosphodegron sequence as c-Myc, T-P-P-L-S-P, within its C-terminus. c-Jun is a major component of the activator protein-1 (AP-1) transcription factor complex and is a proto-oncoprotein. Similar to T58 of c-Myc, T239 of c-Jun is phosphorylated by GSK3β and this phosphorylation is required for recognition of c-Jun by the F-box Fbw7 (Morton, Davis et al. 2003; Nateri, Riera-Sans et al. 2004; Wei, Jin et al. 2005). Additionally, it has been shown that PP2A dephosphorylates c-Jun; however, this dephosphorylation has only been reported for N-terminal negative regulatory sites (Alberts, Deng et al. 1993), suggesting that PP2A increases c-Jun activity. In contrast to this, it was found that a PP2A inhibitor increased both c-Jun levels as well as DNA binding and activity of the AP-1 transcription factor (Al-Murrani, Woodgett et al. 1999), suggesting that PP2A may play a role in regulating c-Jun stability. However, this again was suggested to be due to dephosphorylation of N-terminal sequences. Although not shown in this work, I found that the catalytic subunit of PP2A interacts with a c-Jun deletion mutant lacking all relevant N-terminal phosphorylation sites, perhaps suggesting that PP2A may also catalyze dephosphorylation of the C-terminal phosphodegron that matches c-Myc. This difference in PP2A activity and site selection may be due to different regulatory subunits within the PP2A complex. Further work will need to be performed in order to examine this possibility, as well as to identify other proteins that may be affected by this degradation pathway.

## HBP1 negatively regulates c-Myc activity

**Identification of HBP1 as a c-Myc interacting protein and a negative regulator of c-Myc activity.** In order to better understand the regulation of the proto-oncoprotein c-Myc we set out to identify new c-Myc interacting proteins by using a yeast two-hybrid assay. In this screen we identified the HMG box transcription factor HBP1 as a novel c-Myc interacting protein. HBP1 has frequently been implicated in mediating cell-cycle arrest in a number of cell types (Tevosian, Shih et al. 1997; Shih, Xiu et al. 2001; Yao, Works et al. 2005). Specifically, it is thought that HBP1 is important in inducing a required cell cycle arrest prior to terminal differentiation (Shih, Tevosian et al. 1998). Since c-Myc protein is critical for cell cycle progression and sustained expression of c-Myc inhibits terminal differentiation, we believe that HBP1 may be an important negative regulator of c-Myc activity. Indeed, we found that HBP1 could inhibit c-Myc-mediated transactivation of two different reporter plasmids and c-Myc induction of the corresponding endogenous genes. It appears that HBP1 mediates this repression of c-Myc activity by preventing c-Myc from binding the promoters of its target genes. At this time, it is unknown how HBP1 achieves this; however, we found that HBP1 interacts within the HLH-LZ region of c-Myc. Therefore, we hypothesize that HBP1 either prevents c-Myc and Max from heterodimerizing or perhaps prevents c-Myc/Max heterodimers from interacting with DNA. HBP1 has previously been shown to inhibit TCF4/β-catenin complexes from interacting with DNA, suggesting that this may be a common mode of inhibition for HBP1 (Sampson, Haque et al. 2001).

**HBP1 may inhibit c-Myc activity by multiple mechanisms.** HBP1-mediated repression of Wnt signaling has the important consequence of inhibiting *c-myc* expression (Sampson, Haque et al. 2001). Wnt signaling results in an increase in nuclear β-catenin, which forms a transcription complex with TCF/Lef-1, resulting in the expression of a number of cell cycle genes, including *c-myc* and *cyclin D* (He, Sparks et al. 1998; Shtutman, Zhurinsky et al. 1999; Tetsu and McCormick 1999). Specifically, HBP1 has been shown to bind TCF4 and prevent transcription of these genes (Sampson, Haque et al. 2001), and overexpression of HBP1 has been consistently reported to decrease *c-myc* expression in multiple cell types (Sampson, Haque et al. 2001; Yao, Works et al. 2005; Kim, Zhang et al. 2006). In addition to regulating *c-myc* expression, we have now described a role for HBP1 in negatively regulating c-Myc activity through direct binding to c-Myc, providing a second mechanism for HBP1-mediated inhibition of c-Myc activity (Figure 5.2). To add complexity to this regulation of c-Myc by HBP1, HBP1 was identified as a target gene of c-Myc (Mao, Watson et al. 2003). At this time, it is unknown whether expression of *hbp1* is activated or repressed by c-Myc but this will be important aspect of future studies to determine if a feedback loop exists. It is also important to note that the promoters of many Myc target genes also contain E2F binding sites; therefore, it is possible that HBP1 is recruited to c-Myc target genes through its interaction with pRB, which binds to the E2F family of transcription factors. It is currently unknown whether HBP1 interacts with E2F/Rb complexes; however, our observation that a HBP1 deletion mutant, which is unable to interact with c-Myc, can still confer some repression of the *e2f2* promoter, suggests secondary mechanism of repression. Therefore recruitment of HBP1 to the *e2f2* promoter through its interaction

with pRB remains a viable possibility and suggests that HBP1 may inhibit expression of c-Myc target genes by multiple mechanisms.

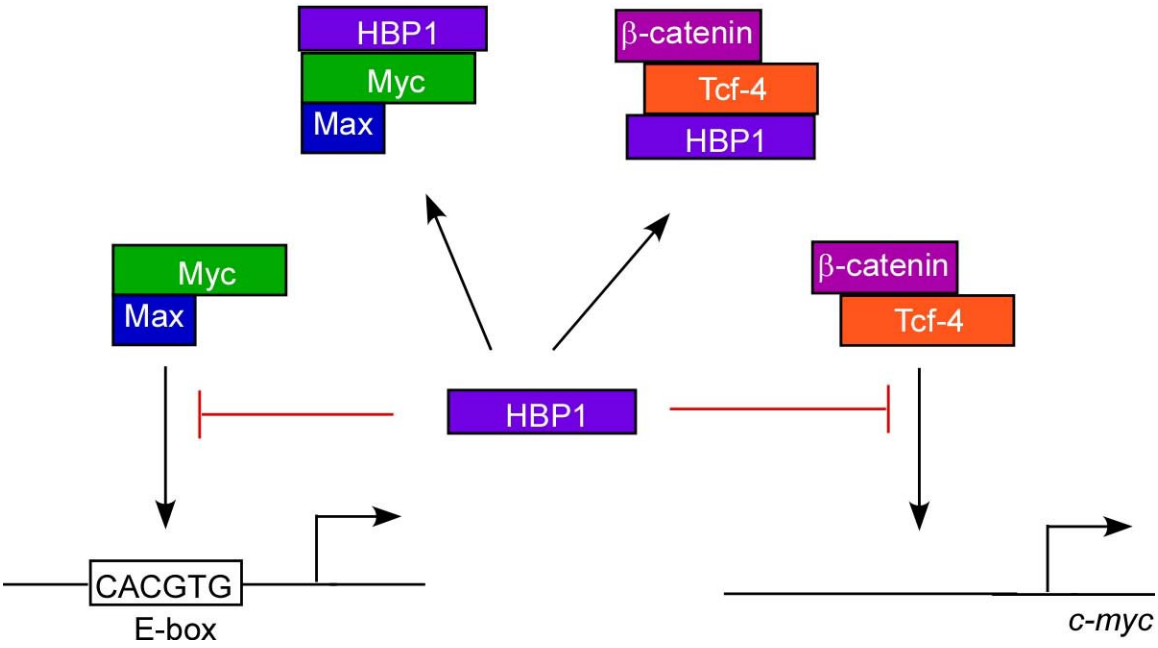



**Figure 5.2: HBP1 inhibits c-Myc activity by multiple mechanisms.**
HBP1 binds c-Myc and prevents its interaction with DNA thereby preventing activation of c-Myc target genes. Additionally HBP1 can bind TCF4 and prevent its binding to promoters of target genes including *c-myc* (Sampson, Haque et al. 2001). Therefore, HBP1 can inhibit both the activity and expression of c-Myc.

**HBP1 and c-Myc have opposing effects on the expression of cell cycle regulatory proteins.** Overexpression of HBP1 has been shown to result in the delay of onset of S phase in a number of cell types (Tevosian, Shih et al. 1997; Shih, Xiu et al. 2001; Yao, Works et al. 2005). A possible mechanism for this inhibition may be through the ability of HBP1 to alter the expression of a number of important cell cycle regulatory genes. As

mentioned above, decreased mRNA levels of *c-myc* and *cyclin D* are often observed in the presence of overexpressed HBP1 (Swanson, Knoepfler et al. 2004; Yao, Works et al. 2005). Additionally, the mRNA levels of the cyclin dependent kinase inhibitor, p21, are often reported to be elevated in the presence of overexpressed HBP1 (Shih, Xiu et al. 2001; Yao, Works et al. 2005), despite initial reports that HBP1 directly represses transcription of the *p21* gene (Gartel, Goufman et al. 1998). The exact mechanism of this increased expression is unknown, however it is interesting to note that c-Myc is reported to be a transcriptional repressor of the *p21* gene (Gartel, Ye et al. 2001; Wu, Cetinkaya et al. 2003; Brenner, Deplus et al. 2005). Therefore, we can hypothesize that this increase in *p21* mRNA, in response to HBP1 overexpression, could be due to a release of c-Myc-mediated repression of the gene. This may be due to both decreased *c-myc* expression and an inhibition of c-Myc activity. While we only tested the effect of HBP1 on c-Myc-induced gene activation, it is possible that HBP1 could also inhibit the repression function of c-Myc. One known mechanism of repression of p21 by c-Myc is by binding of the transcription factor Miz1 through the bHLH-LZ domain of c-Myc. c-Myc is then recruited to the *p21* promoter through this interaction, and here c-Myc recruits the DNA methyltransferase, Dnmt3a, through its transactivational domain (TAD). This results in the methylation of the *p21* promoter and the inhibition of transcription. Since we have shown that HBP1 can bind in both the HLH-LZ region and the TAD domain of c-Myc it is possible that binding of HBP1 to c-Myc prevents c-Myc from binding these other important regulatory proteins, thereby inhibiting c-Myc induced gene repression. Interestingly, *c/ebp*$\alpha$, another target of c-Myc repression has also been found to have elevated expression in response to overexpressed HBP1 (Yao, Works et al. 2005).

**Future avenues of study**. There still remain a number of unanswered question regarding HBP1-mediated repression of c-Myc. For example, we did not examine if pRB plays a role in this repression. pRB is a tumor suppression protein whose best characterized role is to inhibit the activity of the E2F transcription factors. HBP1 was independently identified in two yeast two-hybrid screens as an interacting protein of the RB family member p130 (Lavender, Vandel et al. 1997; Tevosian, Shih et al. 1997). It was subsequently shown that pRB could also bind HBP1 and that two pRB interaction sites reside within the HBP1 protein. A number of studies have examined the role of pRB in HBP1 function. In most cases pRB is required for, or increases HBP1 activity. For example, it was demonstrated that pRB is required for the sequence-specific repression of *n-myc* and expression of pRB was shown to enhance HBP1-induced expression of the differentiation specific histone H1(0) (Tevosian, Shih et al. 1997; Lemercier, Duncliffe et al. 2000). Interestingly, pRb binding sites were required for HBP1-mediated repression of the activity of the MyoD family of transcription factors; however, when pRB levels are increased, pRB appears to relieve this inhibition by HBP1 (Shih, Tevosian et al. 1998). Therefore, the exact role of pRB in regulating HBP1 activity may vary. The role of pRb in HBP1-mediated inhibition of Wnt signaling has not been examined and examination of the role pRB in HBP1-mediation inhibition of c-Myc activity was also beyond the scope of our study. It is likely that pRB modulates HBP1 activity by recruiting other co-factors to DNA. Since direct binding of HBP1 to c-Myc appears to inhibit c-Myc function by preventing c-Myc from binding target gene promoters, it is unlikely that pRB is required for this aspect of repression by HBP1. However, as previously mentioned, pRB may play

a role in other mechanisms of HBP1-mediated repression of c-Myc target genes. Additional studies will be needed in order to confirm these hypotheses.

Additionally, it may be important to examine the effect of overexpressed c-Myc protein on HBP1 functions. Enhanced expression of c-Myc and its family members are commonly found in many tumor types, including Burkitt's lymphoma and neuroblastoma (reviewed in (Vita and Henriksson 2006)). Therefore, it would be worth examining whether the ratio of levels of c-Myc protein to HBP1 protein affects the tumor promoting or tumor suppressing activity of either. Specifically, it may be important to determine whether overexpression of c-Myc protein can prevent some cell cycle inhibitory functions of HBP1. As c-Myc appears to interact within the DNA binding region of HBP1, it is possible that c-Myc may prevent HBP1 from interacting with its target gene promoters. This would prevent the repression of important cell cycle genes such as *n-myc*. If direct interaction with c-Myc does not inhibit DNA binding by HBP1, another possibility is that HBP1 can recruit c-Myc to its own target gene promoters, and here c-Myc could potentially changes the outcome of HBP1 binding. Both of these possibilities would be easy to examine and would be important in understanding the interplay between HBP1 and c-Myc.

**Inhibition of tumorigenesis by HBP1**. Overexpression of HBP1 has been shown to decrease the tumorigenic potential of leukemia cell lines and knockdown of HBP1 has been shown to increase tumorigenic potential in breast cancer cell lines, suggesting that it is an important tumor suppressor protein (Yao, Works et al. 2005; Kim, Zhang et al. 2006; Paulson, Rieger-Christ et al. 2007). Interestingly, Kim et al. has shown that the

green tea compound, EGCG, can stabilize *hbp1* mRNA and block the tumorigenic potential of a breast cancer cell line in an HBP1-dependent manner (Kim, Zhang et al. 2006). Inhibition of tumorigenesis by HBP1 may occur by multiple mechanisms, as overexpression of HBP1 has been shown to induce cell cycle arrest, differentiation and senescence. It has recently been shown that reduced levels of HBP1 correlates with poor relapse-free survival in breast cancer patents. Additionally, decreased *hbp1* mRNA and specific HBP1 mutant proteins have been identified in patient samples (Paulson, Rieger-Christ et al. 2007). The seven HBP1 deletion mutants isolated from breast cancer patient samples were shown to be defective in their ability to inhibit Wnt signaling, despite the fact that there was no common region deleted within all of them (Paulson, Rieger-Christ et al. 2007). This appears to be contrary to previous data from that lab demonstrating that engineered HBP1 mutants with overlapping and much larger deletions than those found in the patient samples were still able to inhibit expression by TCF4/βcatenin complexes. Therefore, more research will be needed to determine the exact effect of these deletion mutants on repression of Wnt signaling, as well as the possible mechanism of inhibition, since a number of the naturally occurring mutants retained the region described to be responsible for binding to TCF4. The smallest deletion found in patient samples encompassed the last 82 amino acids of HBP1, resulting in deletion of the HMG box, and four of the other mutants isolated also lacked the HMG box. This suggests that these proteins would be defective in their ability to inhibit c-Myc activity. Therefore, it would be important to examine whether these forms of HBP1 can repress c-Myc driven transcription.

Many studies examining the tumor suppressing effects of HBP1 have focused on its role in Wnt signaling, and therefore the effect of HBP1 on *c-myc* expression. While regulation of c-Myc expression is important, in many tumor types such as Burkitt's lymphoma, c-Myc expression is no longer under the control of its natural promoter. Additionally, we believe that regulation of c-Myc stability may be important in tumorigenesis as we have found that stabilized c-Myc protein exists in both leukemia and breast cancer cell lines and patient samples ((Malempati, Tibbitts et al. 2006) Deanne Tibbitts and Xiaoli Zhang, unpublished data). Therefore, the effect of HBP1 on c-Myc activity is also likely to be important aspect the tumor suppressing activities of HBP1. Future studies should focus on determining if HBP1 can inhibit c-Myc induced tumorigenesis and whether HBP1 mutants found in cancer are defective in binding to and repressing c-Myc activity. Further understanding of the relationship of these two proteins may be helpful in the design of targeted therapies for cancer treatment.

# Chapter Six:

# Materials and Methods

## Plasmids, shRNA and siRNA

The 2u plasmids pYESDEST52-Myc$^{WT}$, pYESDEST52-Myc$^{T58A}$, and pYESDEST52-Myc$^{S62A}$, containing V5-6xhis epitope-tagged c-Myc or c-Myc mutants, under the control of the GAL1 promoter were generated using the TOPO cloning kit purchased from Invitrogen (Carlsbad, CA). Generation of CMV-βgal, CMV-Myc, pDEST40-c-Myc$^{WT}$, pDEST40-c-Myc$^{T58A}$, pDEST40-c-Myc$^{S62A}$, pDEST40-c-Myc$^{\Delta TAD}$, pDEST40-c-Myc$^{\Delta MB1}$, as well as the reporter construct E2F2-Luc have been previously described (Sears, Ohtani et al. 1997; Yeh, Cunningham et al. 2004; Arnold and Sears 2006). pDEST40-c-Myc$^{WT\Delta C}$, pDEST40-c-Myc$^{\Delta TAD\Delta C}$, and pDEST40-c-Myc$^{\Delta MB1\Delta C}$ were generated by Topo cloning using primers indicated in Table 6.1 and pDEST40-c-Myc$^{WT}$pDEST40-c-Myc$^{\Delta TAD}$, pDEST40-c-Myc$^{\Delta MB1}$ for template DNA. pDEST40-c-Myc$^{\Delta MB1I}$ and pDEST40-c-Myc$^{\Delta MBI1\Delta C}$ were generated by TOPO cloning using pcDNA-V5-Myc$^{\Delta MB1I}$ as a template (generous gift from Dr. Mushui Dai, Indiana University Indianapolis, Indiana). pEF-BOS-HA-HBP1$^{WT}$ was kindly provided to us by Dr. Amy Yee (Tuffs Univeristy School of Medicine, Boston, Massachusetts). pEF-BOS-HBP1$^{\Delta C}$ was generated using a forward primer to the HA tag and a reverse primer that would result in the deletion of the C-terminal 84 amino acids. Both primers contained XbaI sites to allow for cloning back into pEF-BOS (Table 6.1). pEF-BOS-HBP1$^{\Delta REP}$ was generated as follows: the N-terminal fragment of HBP1$^{\Delta REP}$ was generated by PCR using a forward primer to the HA tag and an internal reverse primer. Additionally, the reverse primer contained BamHI digestion site. The C-terminal fragment was generated by digesting pEF-BOS-HA-HBP1 with XbaI and BglII. The two fragments were ligated

together and the ligated in pEF-BOS. 4xE-box-Luc and pGL2 were kindly provided by Dr. Peter Hurlin. CMV-Flag-L12 was generated by first cloning L12 into pENTR (Invitrogen). Full-length L12 was PCR amplified from a human liver cDNA library, using primers indicated in Table 6.1, and cloned into pENTR/D-Topo using the Topo cloning kit (Invitrogen). L12 was then PCR amplified from this vector using a Forward primer that generated an N-terminal Flag tag. Additionally, this primer contained a HindIII site with the reverse primer contained a BamHI site for directional cloning into pRC-CMV. pDBLeu and pEXP-AD502 were purchased from Invitrogen. pDBLeu-c-Myc/GAL4DB was constructed as follows: The GAL4DB was cloned out of the pDBLeu vector (Invitrogen) by PCR, using primers designed with the following restriction digest sites: an XbaI site (TCTAGA) on the forward primer and a BstYI site (PuGATCPy) on the reverse primer. The PCR product was digested with these restriction enzymes and then ligated into pBluescript (pBS) previously digested with XbaI (TCTAGA) and BamH1 (GGATCC), as digesting by BamHI and BstYI results in compatible cohesive ends. The resulting plasmid was designated pBS/GAL4DB. Next c-Myc was cloned into pBS/GAL4DB. Specifically, mouse c-Myc was cloned out of pRC/CMVMyc (Sears, Ohtani et al. 1997) by first digesting the plasmid with BstYI (PuGATCPy). This enzyme cuts at base pair 1138 of mouse c-Myc1. Additionally, this enzyme cuts multiple times within the pRC/CMV backbone. Following digestion of the pRC/CMVMyc plasmid with BstYI, the fragments were then filled with using the Klenow enzyme. This was done in order to be able to clone c-Myc into the blunt site, SmaI (CCCGGG), located in the pBS/GAL4DB plasmid. The digested and filled product was then further digested with HindIII (AAGCTT). This restriction enzyme digests the plasmid upstream of the Myc1

start site in pRC/CMV-Myc. Following purification of the approximately 1.2KB fragment, c-Myc was cloned into pBS/GAL4DB into the HindIII and SmaI sites. Both pBS/Myc/GAL4DB and the pDBLeu yeast expression vector were then digested with NotI (GCGGCCGC) and HindIII. This removes the approximately 2.95 KB c-Myc/GAL4DB fragment from pBS/Myc/GAL4DB and removes the GAL4DB from pDBLeu. The c-Myc/GAL4DB fragment was then ligated into the pDBLeu backbone. pDBLeu-c-Myc$^{\Delta TAD}$/GAL4DB was created by digesting pBS/c-Myc/GAL4DB with PstI and religating the linear fragment with T4 ligase (Roche, Indianapolis, IN). This results in the deletion of amino acids 40-179 of c-Myc. c-Myc$^{\Delta TAD}$/GAL4DB was removed from pBS by digesting with NotI and HindIII and then ligated into pDBLeu.

shRNA to HBP1 was generated by annealing sense and antisense oligos and ligating the resulting double stranded oligo into pENTR-H1/T0 (Invitrogen) as described by manufacturer's instructions. Control siRNA and siRNA to HBP1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Table 6.1: Primer and shRNA target sequences**

| **Yeast 2-hybrid primers** | **Sequence (5'-3')** |
|---|---|
| GAL4 BST Forward | CGCAGATCCATGAAGCAAGCCTCCTGAAAG |
| GAL4 XBA Reverse | GCTCTAGACCTCGACGATACAGTCAAC |
| pPC86 Forward (for sequencing cDNA) | TATAACGCGTTTGGATCACT |
| pPC86 Reverse (for sequencing cDNA) | GTAAATTTCTGGCAAGGTAGAC |
| **Myc primers** | **Sequence (5'-3')** |

| | |
|---|---|
| Myc Topo Reverse | TGCACCAGAGTTTCGAAGC |
| Myc2 Topo Forward | CACCATGCCCCTCAACGTG |
| MycΔ382 Reverse | GATCTGGTCACGCAGGG |
| **L12 primers** | **Sequence (5'-3')** |
| L12 Topo Forward | CACCATGCCGCCGAAGTTCG |
| L12 Topo Reverse | ACTGGCTGGGCATTCCACAGC |
| L12 Flag Forward | CGGAATTCCGATGGACTACAAGGATGACGATGACAA GGGTCCGCCGAAGTTCGACC |
| L12 BAMHI Reverse | CGGGATCCACCTTACTGGCTGGGCATTCC |
| **HBP1 primers** | **Sequence (5'-3')** |
| HBP1 HA XBA Forward | GAGGAATTCTCTAGAATGTACCC |
| HBP1 XBA Reverse | CGTCTAGAGAATTGAGGACAAATGG |
| HBP1 ΔHMG Reverse | CGTCTAGAGCTTAAGTGGCACTCACAG |
| HBP1 ΔREP Reverse | CGGGATCCGAAAATGCCAGATTC |
| **shRNA** | **Sequence (5'-3')** |
| HBPshRNA Forward | AATTCACTGTGAGTGCCACTTCTCTTCAAGAGAGAGA AGTGGCACTCACAGTC |
| HBPshRNA Reverse | TCGAGACTGTGAGTGCCACTTCTCTCTCTTGAAGAGA AGTGGCACTCACAGTG |
| **RT & ChIP primers** | **Sequence (5'-3')** |
| E2F2 RT Forward | ACAAGGCCAACAAGAGGCTG |
| E2F2 RT Reverse | TCAGTCCTGTCGGGCACTTC |
| Nucleolin RT Forward | ACTGACCGGGAAACTGGGTC |
| Nucleolin RT Reverse | TGGCCCAGTCCAAGGTAACT |

| | |
|---|---|
| E2F2 ChIP Forward | TCACCCCTCTGCCATTAAAGG |
| E2F2 ChIP Reverse | AGCAGTGTATTCCCCAGGCC |
| Nucleolin ChIP Forward | TTGCGACGCGTACGAGCTGG |
| Nucleolin ChIP Reverse | ACTCCGACTAGGGCCGATAC |
| GAPDH RT Forward | GACTTCAACAGCGACACCCAC |
| GAPDH RT Reverse | ACCACCCTGTTGCTGTAGCC |

## Cell lines and Transfections

HEK-293 cells were maintained in DMEM supplemented with 10% characterized fetal bovine serum (FBS), 2mM L-glutamine, 1x penicillin/streptomycin, Non-essential amino acids and sodium pyruvate at 37ºC and 5% CO2. Cells were plated to achieve 70-80% confluency 24 hours post-split for transfection. Transfections were performed using Metafectene (Biontex, Germany), HEK-Fectin (BioRAD, Hercules, CA.) or Lipofectamine 2000 (Invitrogen) according to manufacturer's specifications. REF52 cells were maintained in DMEM supplemented with 5% Defined Fetal Bovine Serum, 5% Bovine Calf Serum (BCS). Cells were plated to achieve 50% confluency 24 hours post split. REF52 cells were transfected by the calcium phosphate method as previously described (Sears, Ohtani et al. 1997). Total transfected DNA was held constant by the addition of empty control plasmids. All transfections included 50-200ng of CMV-βgal to determine transfection efficiency.

## Yeast strains, media and reagents

The following *S. cerevisiae* yeast strains were haploid and isogenic with BY4741 (Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*): BY4741, *Δrim11*, *Δpph21*, *Δrts*, TAP-Rim11 and TAP-Kss (Open Biosystems). The following yeast strains were haploid and isogenic with W303 (Mat**a** *ade2-1 his3-1,15 ura3-1 leu2-3,112 trp1-1 can1-100*): W303, *cdc4-1*, *Δgrr1* (generous gift of S. Lanker), $ess1^{H164R}$ and $ess1^{A144T}$ (generous gift of S. Hanes). The following yeast strains were used for the yeast two-hybrid screen: MaV103 (*MATa*, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::l*acZ*, $HIS3_{UAS\ GAL1}$::*HIS*3@*LYS*2, *can*$1^R$, *cyh*$2^R$) and MaV203 (*MATα*, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::l*acZ*, $HIS3_{UAS\ GAL1}$::*HIS*3::*LYS*2, *can*$1^R$, *cyh*$2^R$). Yeast two-hybrid yeast strains were obtained in the Proquest Two-Hybrid System with Gateway Technology kit purchased from Invitrogen. Cells were grown in selective complete (SC) dropout media or YAPD (1% yeast extract, 2% peptone, adenine, 2% glucose) at 23, 30 or 37°C as indicated. Yeast strains were transformed with the human liver cDNA library (Promega) and/or c-Myc expression constructs by lithium acetate-mediated transformation (Sherman, Fink et al. 1986). To arrest cells in G1 or M phases, cells were treated with α factor (2ug/mL) or nocodazole (15ug/mL), respectively.

## CPRG Assay

Two colonies from each transformant was grown overnight in 2.5mL SC –Leu –Trp media rotating overnight at 30ºC. 5 mL YAPD media was inoculated with 1 mL of starter

culture and grown at 30ºC until $OD_{600}$ = 1-1.5. 1.5mL from each culture was put into three 1.5mL eppendorf tubes. Cells were centrifugated at 14K rpm for 30 seconds at room temperature. Supernatant was removed and discarded. Cell pellets were resuspended in 1mL Buffer 1, pH 7.2-7.3 (100 mM HEPES, 154 mM NaCl, 4.5 mM L-aspartate, 1% BSA, 0.05% Tween) and samples were again centrifugated at 14K rpm for 30 seconds. Supernantent was removed and discarded and pellets were resuspended in 100ul Buffer 1. Autoclaved, acid-washed glass beads were added to the meniscus and samples were vortexed for two minutes. A blank sample was prepared by adding 100ul Buffer 1 and 900ul buffer 2 (2.23 mM CPRG in Buffer 1) to an empty eppendorf tube. 900ul of Buffer 2 was added to each sample, samples were vortexed and time of color development was monitored. 250ul 6 mM $ZnCl_2$ was added to each sample to stop the reaction. Samples were centrifugated at 14K rpm for one min and supernatants were transferred to cuvettes. Samples were read on a spectrophotometer at $OD_{574}$. βgal units were calculated based on the following equation: βgal units = 1000 x $OD_{574}$/(t x V x $OD_{600}$) where t = elapsed time (min) of incubation, V = volume of cultured used in assay (mL), $OD_{574}$ = absorbance by chloramphenicol red, and OD600 = cell density at the start of the assay.

## DNA extraction from Yeast

One colony was grown in 1mL SC-Leu-Trp media shaking overnight at 30ºC. The yeast culture was then centrifugated at 14K rpm for 30 seconds at room temperature and the supernatant was removed by aspiration. The yeast pellet was resuspended in 100ul of 3% SDS, 0.2N NaOH. The cell suspension was incubated at room temperature for 15

minutes, occasionally mixing by rapid inversions. 500ul TE was added and the sample was mixted by rapid inversions. 60ul of 3 M sodium acetate was added followed by addition of 300ul phenol and 300ul cholorform. The sample was then vortexed for 2 minutes and centrifugated at 14K rpm for 2 minutes at room temperature. The upper phase was transferred to a new eppendorf tube and the phenol-cholorofrom extraction was repeated. DNA was precipitated by addition of 650ul ice cold isopropanol. The sample was incubated at -20ºC for 20 minutes and then centrifugated at 14K rpm for 5 minutes at 4ºC. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. The DNA pellet was resuspended in 10ul TE. 1ul of DNA was transformed into DH10B bacteria cells by electroporation. The cells were recovered in 800ul SOC, shaking at 37ºC for one hour and then plated on Luria Broth agar plates containing Kanamycin to isolate bacterial colonies containing the cDNA plasmids. cDNA plasmids were isolated from bacterial cells and sequenced.

## Antibodies

The monoclonal V5 antibody used to detect total c-Myc protein was from Invitrogen. The c-Myc antibodies N262 and C-33 as well as the HBP1 antibodies (H-300 and C-20), the Sp1 (PEP-2) antibody, the Cdk2 (M2) antibody and the polyclonal Cdc28 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The c-Myc antibody Y69 was purchased from Abcam (Cambridge, MA) and the monoclonal V5 antibody was purchased from Invitrogen (Carlsbad, CA). The HA.11 antibody was from Covance (Berkeley, CA) and the monoclonal HA (G036) antibody was purchased from Applied Biological Materials (ABM), Inc. (Richmond, BC). The β–actin antibody, Flag (M2)

antibody and Anti-flag M2 affinity gel was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The c-Myc Serine 62 phospho-specific antisera were raised against the chemically synthesized phosphopeptide CKFELLPA/TPPLpSPSRRSG in rabbits. The antisera were purified against this phosphopeptide conjugated to Sulfolink Coupling Gel (Pierce Biotechnology Inc.) as described (Shieh, Ikeda et al. 1997). To deplete antibodies that recognized unphosphorylated c-Myc, the affinity-purified antibodies were re-purified by passing through Sulfolink Coupling Gel conjugated with the corresponding unphosphorylated peptide. The c-Myc Threonine 58 phospho-specific antibody was purchased from Cell Signaling Technology (Beverly, MA).

## Galactose Induction Assay

Cells were grown in a 2% raffinose synthetic complete medium overnight. Cells were then diluted to an optical density at 600 nm ($OD_{600}$) of 0.3 and grown for an additional 2-4 hours at the temperature indicated. A sample was removed as a negative control prior to addition of galactose. Galactose was added to the media to a final concentration of 2% to induce expression of the *c-myc* gene from the GAL1 promoter for 1-3 hours at the indicated temperature. Glucose was added to a final concentration of 5% to stop gene expression, and samples were taken at the indicated timepoints. Protein extraction and western blotting were performed as described below. The optical density at 600 nm ($OD_{600}$) of all samples was measured and the volume of each sample was adjusted to ensure that equal cell numbers were used

## *In vitro* Kinase Assay

c-Myc expression was induced in the *Δkss1* or the *Δrim11* strains as described above. Cells were lysed in Ni-NTA lysis buffer (5mM imidazole, 5mM βME and 0.5% NP-40) and c-Myc was extracted using Ni-NTA agarose (QIAGEN, Valencia, CA). c-Myc was eluted from agarose in Ni-NTA elution buffer (1:1 Ni-NTA lysis buffer:1M imidazole). The TAP-Kss1 and TAP-Rim11 strains were grown overnight in YAPD to an optical density at 600nm of 0.5. TAP-tagged proteins were extracted using Calmodulin Affinity Resin (Stragagene, La Jolla, CA.) in IPP150 Calmodulin binding buffer (Rigaut, Shevchenko et al. 1999). Beads were washed three times in Calmodulin binding buffer and one time in kinase reaction buffer (50mM Tris-HCL pH 7.5, 0.1mM EDTA, 15mM DTT). The immobilized kinases were incubated with eluted c-Myc, kinase buffer and 12uCi [$\gamma$-$^{32}$P] ATP (Perkin-Elmer, Boston, MA) in a final volume of 60 uL. Reactions were incubated at 30°C for 30 minutes. Unbound proteins were mixed with an equal volume of 2x SDS-PAGE sample buffer. Proteins were analyzed by SDS-PAGE and autoradiography.

## Western Blotting and Quantitation

Whole cell yeast extracts were prepared by using the rapid protein extraction procedure (Horvath and Riezman 1994). Protein from equal yeast cell numbers were separated by SDS-PAGE gel and transferred to Immobilon-FL membrane (Millipore, Billerica, MA). Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska) or 5% non-fat milk in PBS (P-T58 antibody). Primary antibodies were diluted in 1:1 Odyssey Blocking Buffer:PBS with 0.05% Tween20 or in 2.5% non-fat milk PBS,

0.05% Tween (P-T58 antibody). Primary antibodies were detected with secondary antibodies labeled with the near-infrared fluorescent dyes IRDye800 (Rockland, Philadelphia, Pennsylvania) and Alexa Fluor 680 (Molecular Probes, Eugene, Oregon) to allow two-color imaging and band overlay. Secondary antibodies were diluted 1:10,000 in 1:1 Odyssey Blocking buffer: PBS, 0.05% Tween or in 1.25% nonfat milk PBS, 0.05% Tween (P-T58 antibody). Blots were scanned with a LI-COR Odyssey Infrared Imager (Lincoln, Nebraska) to visualize proteins. c-Myc, HBP1, β-actin, Cdk2 and Cdc28 protein levels were quantitated using LI-COR Odyssey Infrared Imager software version 1.2. For degradation assays c-Myc total protein levels were normalized to total protein as measured by Cdc28 protein levels. Normalized c-Myc protein levels at each time point were calculated as a percentage of the first timepoint and graphed on a semi-log graph. Half-lives were calculated based on best-fit lines drawn using Microsoft Excel. Mean half-lives ± standard deviation were calculated based on three or more independent experiments.

## Co-Immunoprecipitations

Cells were resuspended in 10X cell pellet volumes of Co-IP buffer (20 mM Tris, pH 7.5, 12.5% glycerol, 0.5% NP-40, 150mM NaCl, 2mM EDTA, 2mM EGTA and 1mM DTT plus protease and phosphatase inhibitors. Cellular lysates were sonicated for 10 pulses (output = 1, 10% duty), incubated on ice for 20 minutes and cleared by centrifugation at 14K rpm for 10 minutes at 4ºC. Cleared lysates were adjusted for transfection efficiency as measure by βgal activity and incubated with either 1:1000 dilution of conjugated anti-C33, 1:1000 conjugated anti-SP1, 1:750 conjugated V5, 1:1000 aV5 or 1:500 anti-HA

(ABM) antibodies. Immunoprecipitates were washed 3 times with 10X volume of Co-IP buffer.

## Luciferase Assay

Cell pellets were resuspended in 10X volumes of 1.5X Reporter Lysis buffer (Promega) with protease and phosphatase inhibitors. Cellular lysates were sonicated for 10 pulses at output = 1 and 10% duty and incubated on ice for 20 minutes. Lysates were cleared by centrifugation at 14K rpm for 10 minutes at 4ºC and βgal and luciferase activity were analyzed. Luciferase activity was determined using the Promega Luciferase Assay Kit (Madison, WI) and Berthold luminometer (Bundoora, Australia). Luciferase activity was adjusted for βgal activity. Three of more separate experiments were performed for each luciferase assay. Fold change in luciferase activities were measure relative to empty vector or control transfections and average fold changes and standard deviations were graphed using Excel.

## Chromatin Immunoprecipitation (ChIP) Assays

Cells were crosslinked with addition of formaldehyde to a final concentration of 1% in media and incubated at room temperature for ten minutes. Glycine was added to a final concentration of 0.125 M and cells were incubated at room temperature for five minutes. Cells were collected in 1X PBS-1mM EDTA and pelleted by gentle centrifugation. Cells were resuspended in 10X cell pellet volumes of RIPA buffer (50 mM Tris, pH 8.0, 1% NP-40, 150mM NaCl, 0.5% deoxycholate, 5mM EGTA and 0.1% SDS) plus protease and phosphatase inhibitors. Cell lysates were sonicated 5X (output = 3.5, 30% duty, 10 pulses) and then cleared by centrifugation at 14K rpm for 10 minutes at 4ºC. Cell lysates

was precleared with 25ul 50% slurry of protein A beads and 25ul sheared salmon sperm DNA for 30 minutes rotation at 4ºC. Lysates were again cleared by centrifugation at 14K rpm for 5 minutes at 4ºC. V5-c-Myc was immunoprecipitation from lysates using 1:1000 dilution of anti-V5 overnight at 4ºC. 1:1000 dilution of anti-Flag was used as a negative control. Immunoprecipitates were washed two times with RIPA buffer, four times with IP wash buffer (100 mM Tris pH 8.5, 500 mM LiCl, 1% NP-40 and 1% deoxycholate) and an additional two times in RIPA buffer. Samples were rotated five minutes at room temperature in buffer in between each wash. Immunoprecipitates were eluted from beads with elution buffer (50 mM $NaHCO_3$ and 1% SDS) by rotating samples in buffer for 15 minutes at room temp. Elutions were transferred to new tubes and 5 M NaCl was added to a final concentration of 0.2M to the elutions. Additionally, 2ul of 5 mg/mL RNase A was added and samples were incubated at 65ºC overnight. DNA was precipitated overnight by adding 1ul of yeast tRNA and 650ul of 100% ethanol. DNA was isolated by centrifugation at 14K rpm for 20 minutes at 4ºC, air dried and resuspended in 100ul TE. DNA was purified by through the QIAquick PCR purification Kit (Qiagen) and used for PCR analysis with primers indicated in Table 6.1. For the ChIP assay in Figure 4.6C the EZ-ChIP kit (Millipore) was performed using manufacturer's instructions.

## RNA isolation and qRT-PCR

RNA was isolated from transfected 293 cells using TRIzol reagent (Invitrogen) according to manufacturer's protocol. cDNA was generated using the QuantiTect Reverse Transcription kit (Qiagen). qRT-PCR analysis was performed using primers indicated in Table 6.1 and Cybr Green reagent (Invitrogen) on a Step-One Real-Time PCR machine (Applied Biosystems) according to manufacturer's qRT-PCR cycle conditions.

# Appendix

## Contributions to Projects and Figures

**Figure 2.3:** The *ess1*$^{H164R}$ and *ess1*$^{A144T}$ yeast strains were a generous gift from Dr. Steven Hanes at the Wadsworth Center (Albany, New York). The W303 yeast strain was kindly provided by Dr. Stefan Lanker.

**Figure 2.5:** The *cdc4-1* and *Δgrr1* yeast strains were a generous gift from Dr. Stefan Lanker.

**Use of a Yeast Two-Hybrid Assay to identify c-Myc-interacting Proteins:** Kristi Piehl determined the optimal 3AT concentration for the yeast two-hybrid screen and assisted with the transformation of yeast with the cDNA library. William DeWitt aided in the mating screen and Moon Yoon assisted in the DNA isolation and sequencing of the cDNA plasmids.

**Figure 3.3A:** Kinrin Yamanaka cloned the CMV-Flag-L12 contstruct. Colin Daniel performed the co-immunoprecipitiation of L12 and c-Myc.

**Figure 3.3B**: The co-immunoprecipitation of c-Myc and L3 was kindly provided and performed by Dr. Mushui Dai at Indiana University (Indianapolis, Indiana).

**The Tumor Suppressor Protein HBP1 Negatively Regulates c-Myc Activity**: The pEF-Bos-HA-HBP1 construct was a generous gift from Dr. Amy Yee at Tuffs Univeristy School of Medicine (Boston, Massachusetts).

**Figures 4.2 and 4.3:** The 4xEbox-Luc and pGL2 constructs were a generous gift from Dr. Peter Hurlin.

**Figures 4.6 and 4.7B**: The co-immunoprecipitations of c-Myc and HBP1 deletion mutants were performed by Colin Daniel.

**Figure 4.7A**: The c-Myc$^{\Delta MBII}$ construct was a generous gift from Dr. Mushui Dai. Karyn Taylor generated the C-terminal c-Myc deletion mutants and cloned these, as well as c-Myc$^{\Delta MBII}$, into the pcDNA-DEST40

## Funding

This work was supported by the following grants: NIH/NCI: R02 CA100855-05 to RCS and NIH/NCI: R01 CA 100855-05S1 to RCS in support of Julie Escamilla.

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