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Regulation of Myc function by the Ubiquitin-Proteasome System.

A Dissertation Presented

by

Abhishek Amareswar Chakraborty

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Abstract of the Dissertation

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The oncogenic transcription factor c-Myc activates genes which stimulate cell cycle progression and represses genes involved in cell cycle arrest. Because alterations in Myc function often cause profound changes to cellular growth, Myc levels and activity need to be carefully regulated. One prominent way to control cellular Myc levels is via its rapid destruction by the Ubiquitin Proteasome System. The importance of this process to normal cellular homeostasis is evidenced by the fact that tumor derived mutations within Myc that allow it to escape ubiquitin-mediated proteolysis are aggressively oncogenic. Understanding how Myc is regulated, and how loss of control of this process contributes to oncogenesis, therefore, requires that we understand how Myc

stability is regulated. My thesis research is therefore centered on the regulation of Myc stability under three specific sets of conditions: During the cell cycle, via the SCF^{Fbw7} ubiquitin ligase, and in response to the adenoviral E1A oncogene.

The Fbw7 E3-ligase targets numerous important regulators of the cell cycle—including Myc. Given the importance of Myc in cell cycle progression, several groups have studied the influence of the cell cycle on Myc proteolysis, often yielding conflicting results. Whereas some studies have shown that Myc synthesis and stability are not cell cycle regulated, others concluded that phosphorylation events within Myc—at residues threonine 58 (T58) and serine 62 (S62)—that control its ubiquitylation by Fbw7 peak during late G2/M-phase, lending support to the idea that Myc destruction might be cell cycle-regulated. We reasoned that perturbations due to different synchronization techniques used in these studies may explain some of the contradictory findings on the relationship between Myc and the cell cycle, and sought to develop an independent protocol to look at the influence of the cell cycle on Myc in *unperturbed* cultures of cells. I illustrate here the utility of laser scanning cytometry (LSC), which is a technical merger between fluorescence microscopy and flow cytometry, in this analysis. By quantifying fluorophores targeted to RNA or protein relative to DNA, LSC accurately measures RNA or protein levels at specific points in the cell cycle in asynchronous cell cultures. Using the LSC approach, my work shows that Myc localization, RNA levels, and protein levels are *not* cell cycle regulated. By adapting the cycloheximide-chase protocol to LSC, I show that Myc stability is unchanged during cell cycle progression. Therefore, using the LSC-based approach I now describe the constitutive nature of Myc synthesis and destruction, suggesting the importance of Myc function at all stages of the cell cycle.

Fbw7 targets its cellular substrates by recognizing a specific phosphorylated sequence (CPD: Cdc4 Phospho-Degron). Interestingly, T58- and S62-phosphorylation events may not be the only regulatory signals targeting Myc to Fbw7. Recently, studying Myc-mutants, which should presumably be refractory to Fbw7; my work has revealed the presence of a potential second recognition

site for Fbw7 in Myc. My studies indicate that the two recognition sequences, one within residues 58-62 and the second within residues 244-248, share a large degree of functional similarity. Represented as a mutation cluster in human blood-borne-tumors, the cancer-associated mutations in region 244-248—such as P245A—escape proteolysis and induce B-cell lymphomas in animal models. This work describes the discovery of a novel regulatory element in Myc, which targets it for proteolysis via the Fbw7 pathway, and plays an important role in Myc-induced oncogenesis.

Historically it has been shown that E1A and Myc are structurally related. Recently it was reported that the adenoviral oncoprotein E1A stabilizes Myc during the course of adenoviral infection. Because Myc and E1A have been independently described to mediate several common cellular functions, we hypothesized that E1A could be an important regulator of the Myc pathway. E1A's ability to mediate specific cellular functions is often limited to small modular sequences; we thus studied a series of overlapping deletion mutants in E1A and identified that a small modular region in E1A (residues: 26-35)—important for the interaction of E1A with the transcriptional co-activator p400—is crucial for E1A's ability to stabilize Myc. We then confirmed that stabilization of Myc by E1A did not primarily occur from a global inhibition of the UPS. Instead, E1A promoted the association of p400 with Myc—both in solution and on chromatin. The association of Myc with p400 has two discernible outcomes: first, this association protects Myc from destruction by the UPS; and second, recruitment of the Myc-p400 complex on chromatin at a number of Myc target genes stimulates Myc activity and presumably plays an important role in E1A function. Indeed, E1A is unable to mediate biological functions in cells lacking Myc. Together, this work pinned Myc as the ultimate target of the E1A-p400 pathway. By simultaneously targeting two proliferation networks, Myc and E2F, E1A can profoundly influence cell growth, thereby mediating its biological roles.

Myc proteolysis has for several years been recognized as an important mechanism to control cellular Myc levels. Here, by studying several independent pathways, my work shows the striking biological importance of Myc proteolysis. I

confirm that Myc synthesis and destruction occur continuously during the cell cycle. Moreover, by studying stabilizing Myc-mutations in the Fbw7 pathway and the changes in Myc stability induced by E1A via p400, my work describes the presence of several alternate routes to regulate proteolysis and limit Myc activity. Indeed, deregulation of Myc destruction by one or more cellular pathways tightly correlates not only with the increased oncogenicity of Myc, but also potentially underlies the early events in Myc-mediated tumorigenesis.

Dedication:

**To my family and friends,
their prayers make me succeed.**

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Abbreviations

v-myc	avian myelocytomatosis virus oncoprotein
c-myc	cellular (homologue) of v-myc
bHLH	basic Helix Loop Helix
UPS	Ubiquitin Proteasome System
TAD	Transcriptional Activation Domain
Degron	Degradation motif recognized by UPS components
Skp2	S-Phase Kinase associated Protein 2
Fbw7	F-box WD-40 domain protein 7
HectH9	Homologous to E6-AP carboxy terminus
SCF	Skp-Cullin-F-box complex
G1	Gap1 phase in the cell cycle
G2	Gap2 phase in the cell cycle
S	(DNA) Synthesis phase
M	Mitosis phase
CPD	Cdc4 Phospho-degrom
WT	Wild Type
CDK	Cyclin Dependent Kinase
IgH	ImmunoGlobulin Heavy chain
LacZ	Beta-Galactosidase gene
QPCR	Quantitative-Real Time PCR
DNA	Deoxy-riboNucleic Acid
DAPI	4',6-Diamidino-2-phenylindole

PI	Propidium Iodide
shRNA	short hairpin RNA
siRNA	short interfering RNA
FITC	Fluorescein IsoThio Cyanate
RNA	RiboNucleic Acid
FISH	Fluorescent In-Situ Hybridization

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Chapter 1:

Introduction.

1.1 *The oncogenic transcription factor c-Myc*

The oncogenic transcription factor c-Myc was originally identified as the cellular homologue of the transforming gene from avian myelocytomatosis virus. The oncogenic activity of Myc stems in large part from its ability to promote cell growth and proliferation. By functioning as both a transcriptional activator and repressor, Myc directly controls the expression of numerous genes involved in cell cycle control, activating the expression of stimulatory factors such as Cyclin E and E2F, and repressing the expression of inhibitory factors such as p15 and p21. The net effect of Myc's transcriptional activities on cell cycle control is profound: Myc expression is not only necessary for cells to enter S-phase in response to growth factor signaling, but is in some cases sufficient to drive re-entry of quiescent cells into the cell cycle. Expression of Myc, therefore, is observed in most proliferating cells during normal mammalian cell growth. Conversely, deregulated expression of Myc leads to loss of growth control and is frequently observed as a hallmark of several human cancers.

Many mechanisms have been described for cancer-associated changes in Myc levels. For example, copy number variation analysis in the Myc gene locus (8q24) has revealed that the 8q24 region is frequently amplified in human cancers (Hicks et al. 2006). Expression of Myc simultaneously from many of these duplicated alleles, leads to elevated levels of Myc which in turn is believed to promote oncogenesis. Besides copy number changes, the analysis of Myc expression in blood cancers has revealed additional mechanisms to induce Myc levels. In several blood-borne cancers, especially Burkitt's lymphoma, the Myc gene translocates to the immunoglobulin locus on chromosome 14. Consequently, the expression of Myc under the control of immunoglobulin enhancers leads to an increase in the cellular levels of Myc, which in most cases is sufficient to drive oncogenesis. However, in addition to elevated levels of Myc, blood-borne cancers represent the only known cancer type where mutations have been discovered in the Myc coding sequence. Indeed, understanding the mechanism of action of these Myc mutants currently represents one of the active areas of Myc biology, and recent reports reveal that some cancer mutants attenuate Myc-induced cell death pathways and in turn are aggressively oncogenic in animal models of lymphomagenesis.

One interesting observation that has emerged from careful analysis of these Myc mutants is that, rather than being randomly distributed throughout the entire length of Myc, many of these mutations are discretely clustered. Moreover, some of these mutation clusters overlap with evolutionarily conserved regions of Myc. Because conserved regions in Myc are known to mediate many important

cellular functions of Myc the observation that cancer-associated mutation clusters occur in these regions suggests the possibility that the function of these conserved regions could be altered in cancer cells and thus play a role in the process of tumorigenesis.

1.2 Conserved regions in Myc

Myc is a basic-Helix-Loop-Helix (Amati et al.) family transcription factor and the Myc family encompasses many members encoded in the mammalian genome, such as c-Myc, N-Myc, L-Myc, etc (Adhikary et al. 2005), which probably arose from gene duplication events early during evolution. However, most of these genes play an important role in regulating cellular proliferation and feature prominently in cancers of different tissues. Consequently, it is believed that these Myc genes possibly retained important functional sequences to mediate their biological roles. One common approach to identify important functional motifs in c-Myc, therefore, is to identify evolutionarily conserved regions that it shares with other members of the Myc protein family. Indeed, besides defining the bHLH-Leucine Zipper region in its carboxyl-terminus, such analyses have revealed the presence of four conserved regions in Myc, commonly referred to as Myc-boxes (MB-I to IV). Over the years, the Myc-boxes have been demonstrated to play an important role in regulating several biological functions of Myc, such as promoting cellular proliferation, cell death, and oncogenic transformation (Sarid et al. 1987; Flinn et al. 1998; Herbst et al. 2005; Cowling et al. 2006). Moreover, conserved regions in both the amino-terminus

and the carboxyl-terminus contribute towards Myc-regulated transcription (Blackwood et al. 1991) suggesting that these conserved regions are critical for Myc function. Here, I describe the contribution of some of the important regions in the amino- and carboxyl-terminus towards Myc function (Figure 1.1).

1.2.1 *The Carboxyl-terminal region of Myc*

The carboxyl-terminus of Myc harbors two important motifs: the bHLH and the leucine-zipper (LZ). The LZ motif as part of the DNA-Binding Domain (DBD) of Myc plays an important role in Myc's DNA-binding activity whereas the bHLH mediates Myc interaction with its heterodimeric partner—Max (Blackwood et al. 1991; Amati et al. 1994). The importance of Max in Myc transcription is evidenced from the fact that the Myc-Max heterodimer serves as the core functional scaffold for Myc's transcriptional function. Myc activates transcription of its cellular targets in the context of a heterodimeric complex with Max. Conversely, one means by which Myc mediates transcriptional repression is via a ternary complex consisting of Myc, Max, and Miz-1 (Adhikary et al. 2005). Because of its importance in Myc function, several groups have studied the recruitment of Myc-Max complexes to the transcriptional target genes of Myc and identified a six-residue palindromic sequence (CACGTG), called the E-box, in the first intron of many Myc target genes (Fernandez et al. 2003; Haggerty et al. 2003). By regulating the ability of Myc to interact with Max, the motifs in the carboxyl-terminus of Myc control the recruitment of Myc to its cellular target genes and thereby its transcriptional activity.

1.2.2 *The amino-terminal region of Myc*

The amino-terminal half of Myc (residues 1-143) harbors its acidic-type Transcriptional Activation Domain (TAD). Disruption of specific regions within the TAD, especially at the two conserved regions MB-I and MB-II, affects Myc's ability to induce transcription and therefore MB-I and II are believed to play an important role in Myc function. The importance of MB-II in Myc function is especially well understood. By recruiting a slew of proteins—including transcriptional co-activators such as p300, p400, and TRRAP—MB-II contributes significantly to Myc transcription (Adhikary et al. 2005). Consistent with this notion, the absence of MB-II impairs Myc function and, unlike WT-Myc, ectopic expression of Myc lacking MB-II is unable to rescue the growth defects observed in homozygous Myc-null HO15.19 fibroblasts (Herbst et al. 2005). In contrast, the role of MB-I in Myc transcription, is not as evident. However, because MB-I constitutes an evolutionarily conserved region within the amino-terminal TAD of Myc and because numerous Myc-mutations isolated from blood-borne cancers cluster around MB-I (Bhatia et al. 1993) it is believed that residues in MB-I must play some important role in Myc function, especially in the context of Myc's ability to promote cell cycle progression.

Together, these analyses indicate that conserved regions in Myc play a critical role in the biological activities of Myc.

1.3 Biological activities of Myc

Myc, in response to different stimuli, can mediate both cellular proliferation as well as cell death. Upon DNA damage or prolonged serum starvation, Myc sensitizes cells to apoptosis. The ability of oncogenic proteins to stimulate cell death pathways, although somewhat counter-intuitive, is believed to be a quality control mechanism to limit their proliferative function. Myc induces cell death by simultaneously targeting several key pathways. For example, Myc can activate p53-induced apoptosis by regulating the p19-Arf pathway. Induction of Arf by Myc leads to the inactivation of Mdm2, a major Ub-ligase for p53, and in turn leads to p53 activation. However, Myc is known to induce apoptotic pathways independent of p53. For example, Myc transcriptionally regulates the expression of several caspases and Myc expression induces the levels of the pro-apoptotic protein, Bim. The regulation of Bim by Myc is particularly interesting. Recently, studying lymphoma-derived Myc mutants it was shown that, unlike WT-Myc, some of these mutants lose the ability to induce Bim. Lymphomas induced by these mutants retained the p53 allele yet by inactivating the pro-apoptotic Bim pathway these mutants turned aggressively oncogenic, thus confirming that apoptotic pathways induced by Myc play an important role in Myc-mediated oncogenesis.

Conversely, Myc plays an important role in regulating cell proliferation by promoting the progression of the cell cycle. The eukaryotic cell cycle is classified into four independent phases: G1 (Gap1), S (Synthesis), G2 (Gap2), and M (Mitosis). Remarkable quality control mechanisms exist to ensure the movement

of the cell cycle from one phase to another only upon completion of the previous phase. Cancer routinely emerges due to genetic changes in proteins that regulate cell cycle progression. Therefore, concerted effort has been made to understand the individual contribution of a large number of proteins to this process. One such protein is Myc, and various aspects of how Myc drives cell cycle progression are well defined.

Myc is a low-abundance protein and cellular Myc levels routinely reflect the proliferation status of cells. Consistent with this notion, Myc levels are lower in quiescent cells and induced rapidly upon mitogen-induced re-entry into the cell cycle. Importantly, ectopic expression of Myc in quiescent cells is sufficient to drive cell cycle re-entry in the absence of mitogenic stimuli, suggesting that Myc function downstream of mitogenic stimuli is one of the important biological routes to signal progression of the mammalian cell cycle.

Although much is understood about Myc's ability to promote cell cycle progression, the question about how Myc itself is regulated throughout the cell cycle is unclear. From studies in quiescent cells induced to re-enter the cell cycle, Myc was first considered a cell cycle regulated protein with its expression overlapping with the G1-phase (Kelly et al. 1983). Subsequently however, it was demonstrated in asynchronous cycling cells that Myc RNA and protein levels do not change as a function of cell cycle status (Hann et al. 1985). Conversely, several groups have demonstrated that phosphorylation of Myc at the threonine 58 residue, which mediates Myc destruction by the Ub-ligase Fbw7-dependent proteolysis pathway, could be regulated in a cell cycle dependent manner (Seth

et al. 1993; Gregory et al. 2000). This result, if true, would imply that proteolysis of Myc by the Ubiquitin-Proteasome System (UPS) could be influenced by cell cycle status. Considered as one of the major processes controlling the directionality of the cell cycle, the UPS plays an important role in limiting the expression of several key proteins, such as Cyclin E and p27, to specific stages of the cell cycle. Considering that the UPS is one of the major pathways controlling cellular Myc levels and that true cell cycle-dependent changes in Myc could have a profound impact on the mechanism through which Myc promotes cell cycle progression, it is important to carefully analyze Myc expression and proteolysis in the context of the cell cycle.

1.4 Regulation of Myc function by the Ubiquitin Proteasome System (UPS)

Originally identified as a quality control mechanism for clearing misfolded proteins, the UPS is now understood to play a far more elaborate role in various cellular processes. The basic pathway for UPS function involves the covalent conjugation of substrate proteins with a small 8.5 KDa protein Ubiquitin (Ub) and three categories of proteins are described to be relevant in this Ub-mediated proteolysis pathway: The Ub-Activating enzyme or E1 (Uba), the Ub-Conjugating enzyme or E2 (Ubc), and the Ub-ligase enzyme or E3 which controls the substrate specificity in this process (Hershko et al. 1998). The proteolysis pathway involves the systematic transfer of ubiquitin from the E1 to the E2 via high-energy thioester linkages, and ultimately leads to substrate ubiquitylation. By recognizing its substrate via specific sequences (degrons), the E3 mediates

the iterative transfer of Ub from the E2 onto the substrate, and thus flags the substrate with Ub-chains. Ubiquitylation of substrates mediates various cellular outcomes. Routinely, ubiquitylation either regulates substrate function by affecting its sub-cellular localization or targets the substrate for destruction by the multi-protein 26S proteasomal complex.

Beyond these originally identified roles, recently some novel functions of the UPS have been characterized. Of these, one interesting role of the UPS in the process of transcriptional activation is particularly well-studied in the Tansey laboratory. Several years ago, studying labile transcription factors, the Tansey lab observed that the TADs in unstable transcription factors tightly overlapped with their degrons (Salghetti et al. 2000). Thereafter, by studying an artificial transcription factor generated by tethering the TAD from a viral transcription factor (VP16) to an unrelated bacterial DBD (LexA-DBD), it was observed that tagging the TAD destabilized the transcription activator, but at the same time *induced* its ability to activate transcription. This was an apparent paradox. If indeed the transcription factor was more labile, one would predict a smaller time window for its ability to induce transcription, and thus a reduction in its transcriptional activity. Work over several years by the Tansey laboratory (and others) has helped partially resolve this paradox. Indeed, it is now believed that the process of transcriptional activation is intimately linked to the process of activator ubiquitylation and that ubiquitylation of the transcription factor serves as a licensing mechanism which induces their ability to activate transcription but simultaneously targets them for destruction. By targeting transcription factors for

destruction during the process of transcriptional activation, in this manner, the UPS limits their ability to recruit the transcriptional machinery for subsequent rounds of transcription (Tansey 2001; Lipford et al. 2003; Muratani et al. 2003).

The physical overlap between the TAD and degrons in important transcription factors such as Myc, E2F, and p53, attests to the importance and physiological relevance of the licensing model. In this context, studying the regulation of Myc has revealed numerous insights about the links between transcription activation and proteolysis. Myc is a highly unstable protein which is rapidly targeted for proteolysis after its synthesis (half-life: ~30 mins) and one prominent means for the rapid destruction of Myc is via the UPS (Salghetti et al. 1999). The amino-terminal domain of Myc harbors several key sequences which signal Myc proteolysis and a deletion of the N-terminal domain stabilizes Myc and results in reduced Myc ubiquitylation.

The amino-terminal MB's: I and II play an important role in Myc proteolysis. Additionally, MB-III, a conserved element in Myc which regulates Myc-induced cell death pathways, also affects proteolysis. Although deletion of MB-I and MB-III independently result in a modest increase in Myc half-life, compared to WT-Myc, a double deletion of both MB-I and MB-III significantly stabilizes Myc, thus suggesting that a cross-talk exists between signals mediating Myc proteolysis via these regions. Given that MB-I and MB-II also contribute towards Myc transcriptional activation and MB-III towards Myc repression, further reinforces the notion that for several unstable transcription

factors such as Myc, the seemingly disparate processes of target gene expression and proteolysis are tightly connected.

1.5 Pathways regulating Myc proteolysis

Given the importance of Myc proteolysis in its function, several groups have identified numerous proteins regulating Myc stability in the cell. I first describe the ubiquitylation pathways discovered for Myc.

Skp2 (an F-box containing E3), identified by the Tansey laboratory (and others), was the first Ub-ligase discovered for Myc (Kim et al. 2003; von der Lehr et al. 2003). Skp2 binds to Myc at two conserved sites: one within MB-II and the other within the C-terminal bHLH region. Consistent with the licensing model, Skp2 was shown to be recruited to Myc target genes and served as a co-activator for Myc transcription, thus establishing another link between Myc transcriptional activation and proteolysis.

Recently, the importance of proteolysis in Myc function has been reinforced by the identification of two other Ub-ligases for Myc: the SCF Ub-ligase Fbw7/Cdc4 (Welcker et al. 2004; Yada et al. 2004) and the Hect-domain containing E3, HectH9 (Adhikary et al. 2005). Although Skp2, Fbw7, and HectH9 ubiquitylate Myc, their downstream effects on Myc transcription and proteolysis are different. For example, although Skp2 and HectH9 ubiquitylate Myc and stimulate Myc activity; ubiquitylation by Fbw7 attenuates Myc function (Welcker et al. 2004; Yada et al. 2004). These differences reveal that there remain some gaps in our understanding of the influence of proteasomal pathways in Myc

function. By addressing several discrepancies in the Fbw7-mediated regulation of Myc I attempted to resolve some of these gaps.

1.5.1 The SCF^{Fbw7} Ubiquitin Ligase

Fbw7 was originally discovered in screens for cell-cycle regulatory proteins as its yeast homologue Cdc4, and was characterized as an important mediator of nuclear DNA synthesis and cell cycle progression (Newlon et al. 1975; Simchen et al. 1977). Nearly three decades after these observations, much is known about the yeast Cdc4, its fly homologue: Archipelago, and its human homologue: Fbw7.

Fbw7 is an F-box protein and associates with the adaptor protein Skp1, scaffold protein Cul1, and ring finger protein Rbx/Roc1 to form a functional SCF-complex, which functions as a cellular Ub-ligase (Koepp et al. 2001). By targeting key cell cycle mediators such as the yeast protein Sic1 (Feldman et al. 1997; Verma et al. 1997) and Cyclin E, Myc, and Jun in mammalian cells, Fbw7 plays an important role in timing cell cycle progression. Indeed, changes in these proteolytic events largely explain the cell cycle defects originally observed in the *cdc4* mutants.

Because many known Fbw7 substrates, including c-Myc and Cyclin E, are known to have a profound influence on cell cycle progression, how Fbw7 regulates its substrates has been systematically studied. Fbw7 targets its substrates by recognizing a degron, called the Cdc4 Phospho-Degron (CPD), in a phosphorylation dependent manner. Moreover, physiological substrates of

Fbw7 do *not* always harbor the highest affinity binding-sites for Fbw7. Instead, comparing results from *in vitro* binding studies reveals that Fbw7-substrates harbor CPDs with a wide range of binding affinities (Nash et al. 2001). Fbw7 substrates in some cases retain multiple low-affinity degrons instead of a single high-affinity CPD, presumably as a means to achieve higher fine-tuning for their proteolysis. This phenomenon is best described in the yeast substrate, Sic1, which harbors nine weak CPDs, and efficient proteolysis of Sic1 occurs only upon phosphorylation of at least six of these sequences (Nash et al. 2001; Orlicky et al. 2003).

Recently, by studying the structure of mammalian Fbw7 in complex with CyclinE, an additional explanation for the presence of multiple degrons in Fbw7-substrates was suggested (Hao et al. 2007). Fbw7, in mammalian cells, is expressed as three alternately spliced isoforms: α , β , and γ (Welcker et al. 2004; Ye et al. 2004). Although, the Fbw7 isoforms localize to different sub-cellular compartments, it has been reported that these isoforms retain the capacity to interact with each other (Zhang et al. 2006; Welcker et al. 2007). Analyzing the functional importance of Fbw7 dimerization reveals that Fbw7 monomers are sufficient for targeting strong CPDs whereas Fbw7 dimers are required for destruction of substrates via weak degrons (Welcker et al. 2007). Consistent with this notion, it is proposed that recruitment of Fbw7-dimers to substrates with weak degrons is enabled by the stabilization of this interaction via simultaneous binding of multiple CPDs. In this manner, the cumulative strength of multiple CPDs sets a biological threshold to govern their proteolysis by Fbw7.

Compared to other Fbw7 substrates, regulation of Myc by Fbw7 highlights certain differences (Figure 1.4). Fbw7 binds with high affinity to degrons phosphorylated at two amino-acids spaced four residues apart. However, this observation does not hold true for Myc. In Myc, the two phosphorylatable sites in the CPD are threonine 58 (T58) and serine 62 (S62). Fbw7 recognizes Myc primarily via T58-phosphorylation. Surprisingly, unlike other CPDs, rather than stabilizing Fbw7 interaction, the phosphorylation of the second-site S62 interferes with Fbw7 binding and in turn protects Myc from proteolysis (Sears et al. 2000; Sears 2004). Comparing biochemical data, this singly phosphorylated Myc degron at T58 which is targeted by Fbw7 would be predicted to behave as a *weak* physiological CPD. Therefore, in the absence of a second binding site, how Myc is destroyed by Fbw7 represents a paradox in the regulation of Myc function by Fbw7. Moreover, although Fbw7 can mediate Myc ubiquitylation *in vitro* and over-expression of Fbw7 leads to a reduction in cellular Myc levels; the knockdown of Fbw7 by siRNA fails to elicit any significant change either in Myc steady-state levels or half-life (Kim et al. 2003). In contrast, under the same experimental conditions, the levels of Cyclin E are readily induced by knockdown of Fbw7, raising questions about the extent of influence Fbw7 exerts on Myc proteolysis under physiological conditions.

These observations illustrate several apparent disparities in the regulation of Myc by Fbw7, as compared to other Fbw7 targets such as Cyclin E. However, given that mutations in the T58 or S62 residues, which presumably render Myc non-responsive to Fbw7, occur frequently in blood cancers and that Fbw7 itself is

inactivated in a number of cancers (Moberg et al. 2001; Strohmaier et al. 2001; Kemp et al. 2005) gives rise to the notion that deregulation of Myc due to changes in the Fbw7-mediated proteolysis pathway represents an important process in Myc-mediated control of cell proliferation, and argue for the need to investigate the regulation of Myc by Fbw7.

1.5.2 *The adenoviral E1A oncogene*

DNA tumor viruses, such as SV40 and Human Adenovirus (HAdV), have been historically exploited as tools to dissect out important cellular pathways. Several years ago, work done using DNA tumor viruses to study genome architecture, eukaryotic transcription, and DNA replication, led to the discovery of mechanisms such as mRNA splicing, transcription activator function, and RFLP mapping. Therefore, studying DNA tumor viruses, has over the years established the very foundations of modern-day molecular biology and cancer.

The rationale driving research with DNA tumor viruses has been that these viruses have evolved to target key cellular targets to mediate biological functions. Indeed, studying the oncogenic adenoviral early region 1A (E1A) gene has yielded detailed functional information about the role of important cellular pathways such as p53, Rb-E2F, p400, and CBP/p300.

The link between E1A and the Retinoblastoma (Rb) tumor suppressor is particularly well characterized (Whyte et al. 1988). Under normal conditions, Rb functions as a tumor suppressor by sequestering the cell cycle regulator E2F, thus limiting its ability to inducing cell proliferation. Upon adenoviral infection,

however, binding of E1A to Rb strips it off the E2F complex leading to ectopic activation of E2F and loss of growth control. Given our detailed understanding of this process, for many years the ability of E1A to induce cell proliferation was largely attributed to the deregulation of the Rb-E2F pathway.

The E1A protein is expressed as two alternately spliced isoforms, 243 and 289 residues in length. Aligning E1A from several different serotypes reveals four highly Conserved Regions, called CR1 to CR4. In addition to these CR's, which are believed to be functionally important and have been described to mediate distinct biological roles, several modular sequences have been identified in E1A, which mediate E1A interaction with cellular proteins. Together, such studies identifying functionally important regions in E1A have given rise to the notion of E1A being a highly modular protein which can promote key cellular functions via well defined sequence motifs.

Recently, the N-terminal region of E1A, which is important for adenovirus induced transformation and apoptosis, has been shown to interact with another class of cellular proteins, independent of Rb-E2F—the chromatin remodeling complexes p400 and CBP/p300 (Arany et al. 1994; Eckner et al. 1994). Studying the ability of the APC/C E3 ligase to interact with CBP/p300 has revealed that this interaction occurs via motifs evolutionarily conserved in E1A, suggesting that E1A could potentially regulate the interaction of CBP/p300 with the APC/C complex (particularly in mitosis or early G1-phase) and exploit APC's role in cell cycle progression (Turnell et al. 2005). Similarly, interaction of E1A with p400 is known to be indispensable for adenoviral function. Expression of p400 is essential

for the ability of E1A to induce apoptosis; and a mutant E1A protein, which fails to bind p400, is unable to co-operate with Ras and induce cellular transformation (Fuchs et al. 2001; Samuelson et al. 2005). Although, these results highlight the physiological importance of the E1A-p400 pathway there have been no mechanistic models identifying the downstream cellular target(s) of this pathway.

For several years Myc and E1A have been known to be structurally related (Ralston et al. 1983). Moreover, E1A and Myc have been described to mediate similar biological processes—including induction of cell growth, cell death, cellular transformation, and blocking differentiation. However, in the absence of any biological links, Myc and E1A were believed to function by independent pathways; E1A via regulation of Rb and Myc as a transcription factor regulating a comprehensive set of cellular target genes.

Recently, it was reported that E1A stabilizes Myc during the course of adenoviral infection (Lohr et al. 2003). This observation is not particularly surprising. The amino-terminus of E1A interacts with a slew of proteins, including Rb, 19S proteasome subunits, CBP/p300, TBP, p400, and TRRAP (Figure 1.5) (Egan et al. 1988). Considering E1A, via its N-terminus, is known to interact with numerous sub-units of the proteasome and inhibit their function (Turnell et al. 2000; Zhang et al. 2004), it is very well possible that the effect of E1A expression on Myc stability reflects the proteasomal inhibition of E1A. Given that proteolytic pathways exert a profound influence on the activity of unstable transcription factors such as Myc, and that deregulation of Myc proteolysis routinely occurs in several human cancers, this observation could be extremely important. By

studying in detail the influence of the E1A pathway on Myc stability and function, I therefore sought to expose the underlying process through which this occurs, and identify novel themes integrating the loss of proteolytic control in Myc to oncogenesis.

1.6 Thesis Objectives

For my thesis, I chose to address three important aspects about the regulation of Myc proteolysis: its regulation during the cell cycle, the role of Fbw7 in Myc function, and the extent and influence of E1A in Myc function.

My first aim was to understand the regulation of Myc expression and turnover in the context of the cell cycle. Because Myc is an important regulator of the cell cycle, I reasoned that studying the regulation of Myc expression and stability during the cell cycle could reveal important insights about the ubiquitylation pathways targeting Myc and the importance of Myc proteolysis in Myc function at specific stages of the mammalian cell cycle. My second aim was to understand the regulation of Myc by the Fbw7 Ub-ligase. In spite of the evident ties of Fbw7-mediated Myc proteolysis to tumorigenesis, many discrepancies remain in our understanding of the function of Fbw7. By studying the Fbw7 pathway, I hoped to resolve some of these discrepancies. My third aim was to understand the importance of the E1A oncogene in Myc function. Studying DNA tumor viruses, such as adenovirus, over several years has revealed many cellular pathways involved in the regulation of cell cycle progression. Given the importance of cellular pathways regulating Myc proteolysis in Myc function and

the biological similarities between Myc and E1A, I speculated that a functional interaction between E1A and Myc could prove extremely important in the process of oncogenesis. By studying the regulation of Myc stability by E1A, I sought to address this possibility.

Myc is a prominent oncogene and its deregulation is a frequent hallmark of several human cancers. Alterations in its proteolytic pathways often underlie many of the cancer-associated changes observed in Myc. Moreover, recent studies have demonstrated that for unstable transcription factors, such as Myc, proteolysis is tightly linked to their ability to regulate transcription. The goals of my thesis, therefore, were to explore in detail the influence of pathways either cellular or viral in origin, which regulate Myc stability in the cell.

Figure 1.1: The c-Myc oncogene

(A) The c-Myc oncogene is a 439 amino acid protein (MW: of 64 KDa). The Transcriptional Activation Domain spans the amino-terminal region of Myc, whereas the basic Helix-Loop-Helix leucine zipper domain, responsible for DNA-binding spans the C-terminus.

(B) Sequence alignment of Myc family proteins reveals four highly conserved regions along the entire length of Myc, commonly called the Myc-Box regions. The Myc-Boxes are functionally important for Myc and routinely mediate Myc activity by binding to a slew of cellular proteins involved in regulating either Myc transcription or turnover.

(C) Myc functions as a Transcription activator in a hetero-dimeric complex with the bHLH family protein, Max. The Myc-Max complex binds genomic DNA at several sites which are enriched for the presence of a six-bp palindromic sequence called the E-Box (CACGTG).



SCF^{Fbw7}
Usp28

SCF^{Skp2}
p400
TRRAP
Tip60

HDAC

TRRAP

SCF^{Skp2}
Max
Miz
p300

(C)

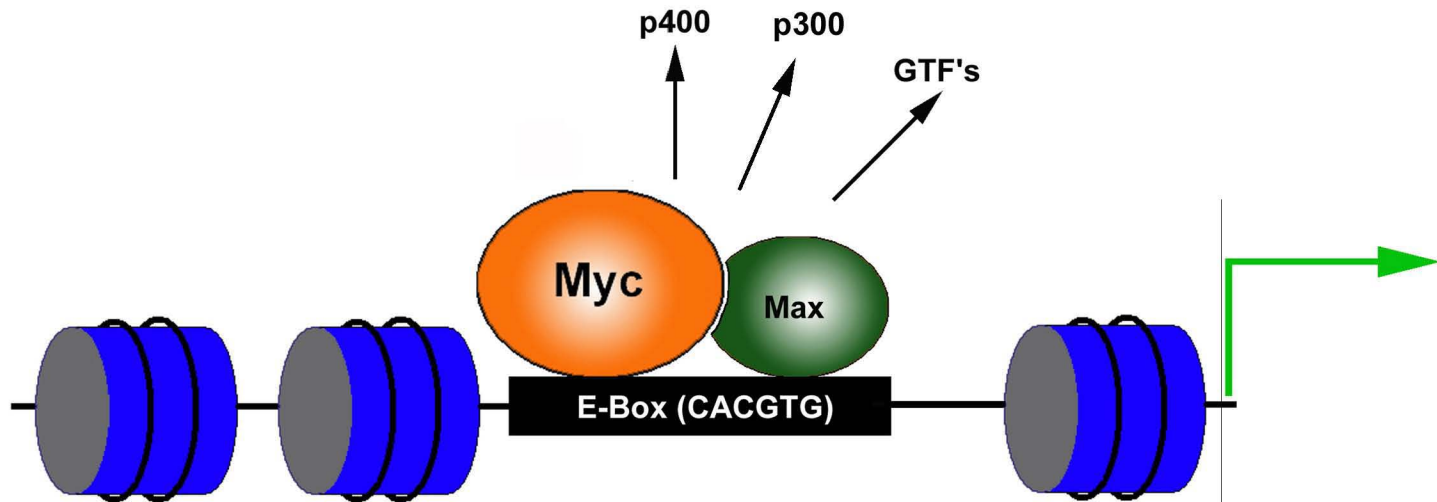


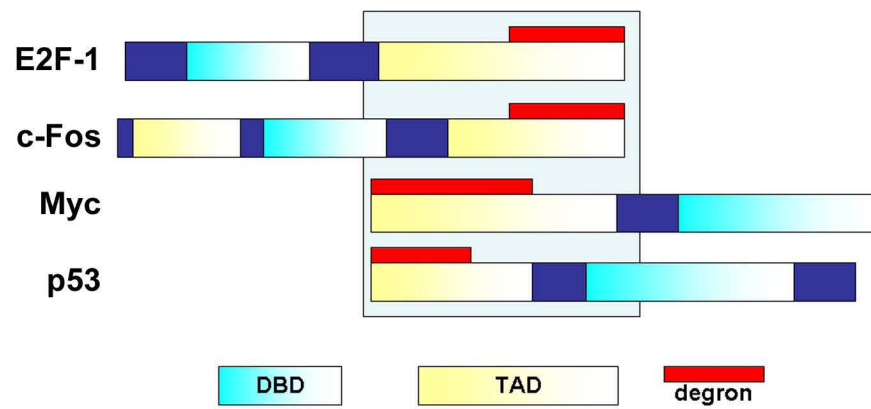
Figure 1.2: Regulation of Myc transcription by components of the Ubiquitin Proteasome System.

(A) Transcription factors possess two defined functional domains: the Transcriptional Activation Domain (TAD) and the DNA-Binding Domain (DBD). Primary sequence analysis of numerous unstable transcription factors show a tight physical overlap of regions which function as TAD's with sequences which signal proteolysis (degrons: proteasome targeting sequences).

(B) Myc is ubiquitylated by multiple components of the proteasomal machinery. Currently, three E3's are known to Ubiquitylate Myc and regulate its transcription. Skp2 and HectH9 function as co-activators of Myc transcription, whereas Fbw7 inhibits Myc transcription.

Ubiquitylation of Myc is believed to be the licensing signal to both activate transcription and concurrently target Myc to the 26S Proteasomal complex, which also localizes to genomic sequences in the vicinity of transcription regulatory elements.

(A)



(B)

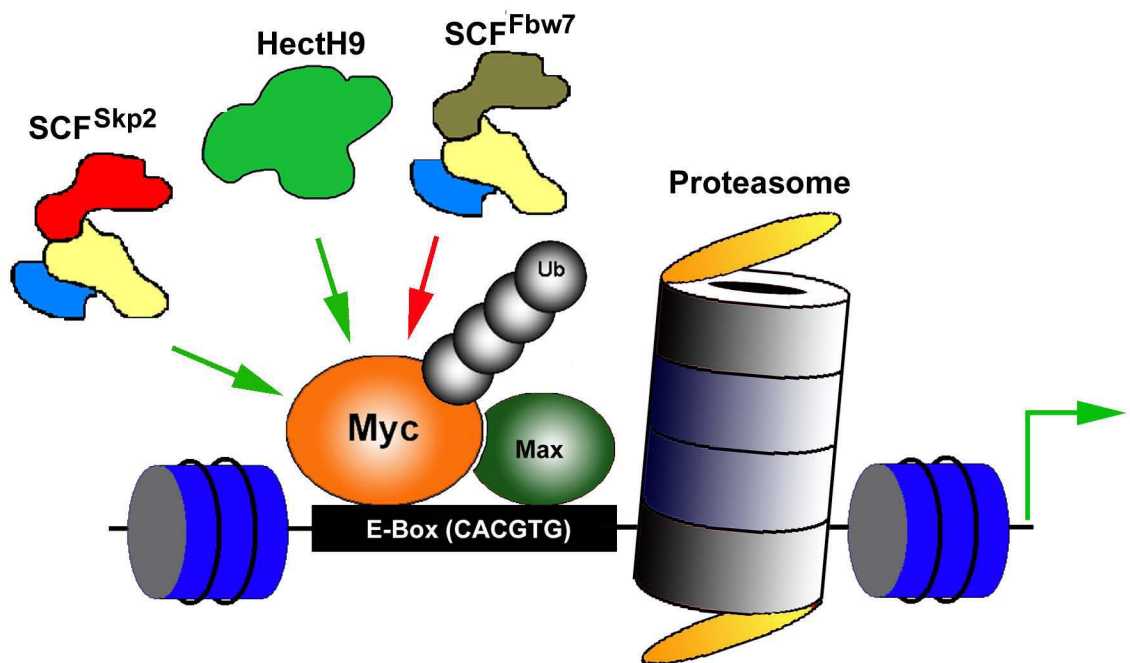


Figure 1.3: Myc-gene translocation is observed in Lymphomas

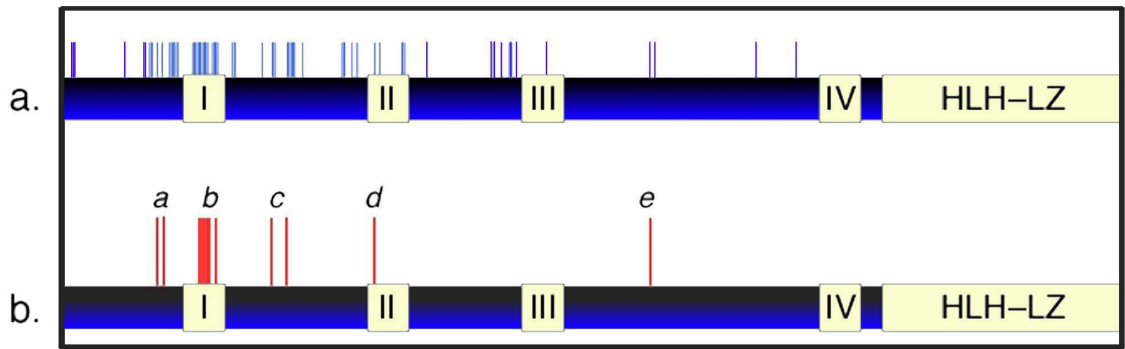
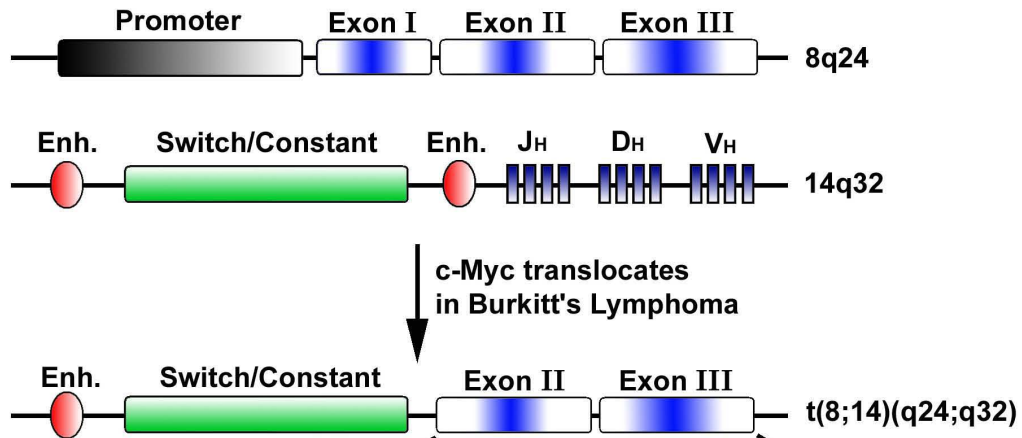
One of the best characterized cytogenetic abnormalities in neoplasms is the translocation of the c-Myc gene from chromosome 8q24 to the Immunoglobulin heavy-chain (IgH) locus 14q32 which occurs in lymphomas.

Expression of the Myc-protein under the control of the IgH enhancer elements induces Myc levels. Simultaneously the Myc gene acquires mutations, some of which confer a growth advantage.

Lower Panel (Graph): Analysis of Myc-mutation clusters.

a). Blue lines indicate the position of lymphoma-derived mutations that have been observed in Myc.

b). Red lines indicate residues where independent mutations have been observed three or more times; regions are labeled a–e.



CANCER-ASSOCIATED MYC-MUTATIONS

Figure 1.4: Regulation of Myc proteolysis by Fbw7

Myc has a complex route of destruction via Fbw7 proteolysis.

- Step I: MAP kinases downstream of the Ras-signalling pathway phosphorylate the serine 62 (S62) residue in MB-1.
- Step II: Phosphorylated S62 recruits GSK3 β and leads to phosphorylation of threonine 58 (T58)
- Step III: The double-phosphorylated Myc is recognized by the peptidyl prolyl isomerase, Pin1. Pin1 isomerizes the *cis-trans* interaction between prolines 59 and 60. This event leads to protein phosphatase PP2A mediated removal of the phospho-group on S62
- Step IV: The degron harboring the singly-phosphorylated T58 residue is recognized by Fbw7, which in turn targets Myc for destruction.

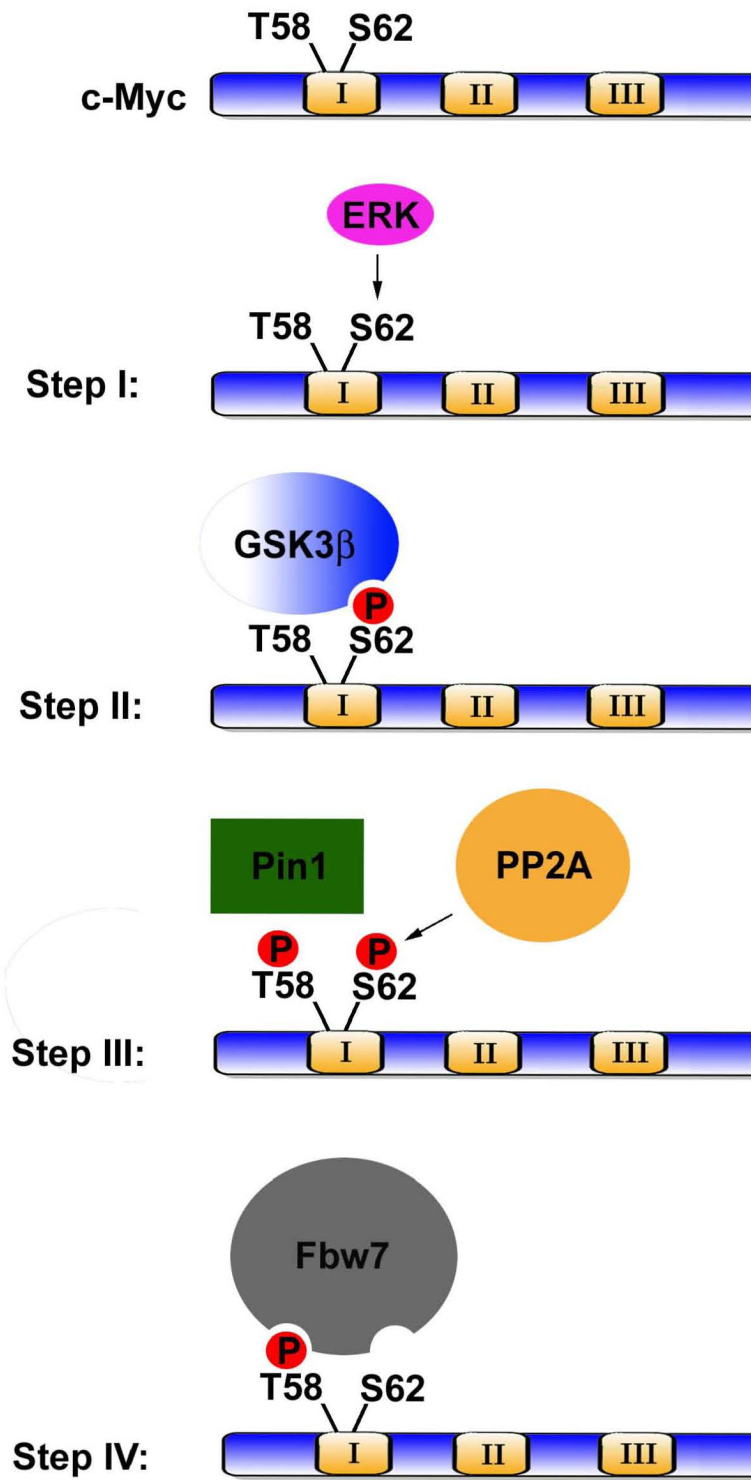
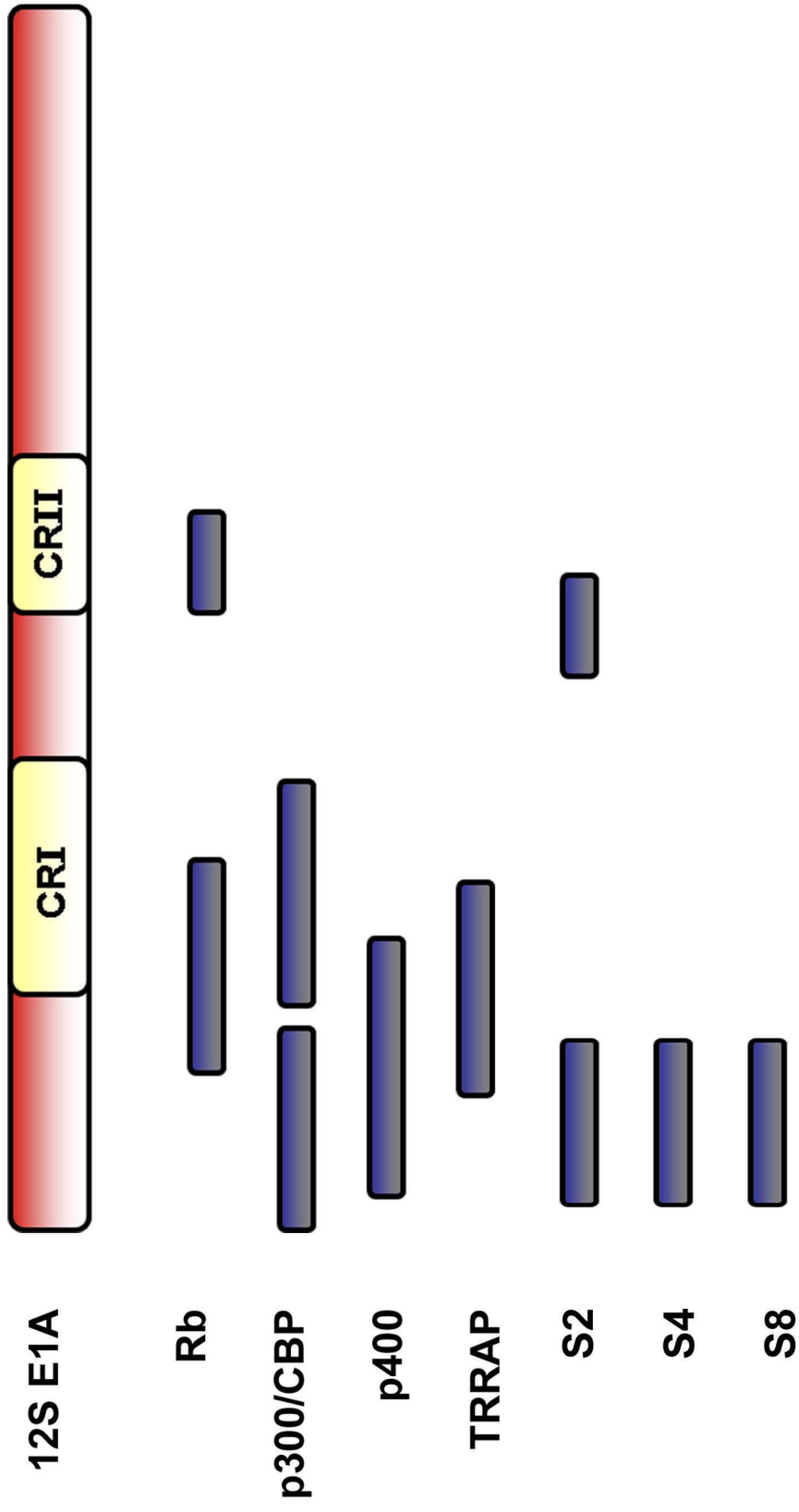


Figure 1.5: Cellular interactions of the adenoviral oncogene E1A

The adenoviral oncogene E1A mediates many of its biological roles via interactions with important cellular proteins. Figure 1.5 shows some of these proteins which interact via the amino-terminus of E1A, such as the tumor-suppressor, Rb; the chromatin remodeling complexes: p300, p400, and TRRAP; and sub-units of the proteasome: S2, S4, and S8.



Chapter 2:

Icys-Laser Scanning Cytometry (LSC): an unperturbed system to study cell cycle regulated processes.

(Chakraborty AA and Tansey WP, Exp Cell Res. 2009 Jan 26. [Epub])

2.1 INTRODUCTION

Ordered progression through the eukaryotic cell cycle depends on mechanisms that tie the abundance and activity of critical regulatory molecules to the events of DNA replication and cellular division. One of the most pervasive of these mechanisms is Ub-mediated proteolysis. By destroying proteins at specific points in the cell cycle, the Ub-proteasome system provides directionality to the events of cell duplication, and ensures that each phase of the cycle occurs after the previous one is completed (Tansey 1999).

Despite the biological importance of cell cycle-regulated proteolysis, techniques to study this phenomenon are limited. One of the most popular approaches is to arrest cells at specific cell cycle stages, either by chemical or growth-factor blockade, and to then release cells back into the cycle and monitor

them as they move synchronously through subsequent cell cycle transitions. These synchronization methods, however, involve significant disruption to normal cellular physiology and can influence the apparent behavior of molecules with respect to the cell cycle because of the perturbations involved (Davis et al. 2001). An alternative strategy, centrifugal elutriation, separates cells based on their size, which increases linearly during the cell cycle. In this way, relatively pure fractions of cells can be obtained that are enriched in either the G1, S, or G2/M cell cycle phases. Elutriation has the advantage of not requiring disruption to the cell cycle to provide synchronicity, but its practical application is restricted because it is best suited to non-adherent cells and requires relatively large volumes of cell cultures.

My thesis research focuses on the mechanisms controlling Ub-mediated proteolysis of the oncoprotein Myc. Myc is an oncogenic transcription factor with clear ties to cell cycle regulation; however, there are conflicting reports as to whether Myc levels and stability itself are influenced by the cell cycle. Some studies demonstrated that Myc synthesis and stability are not cell cycle regulated (Hann et al. 1985) whereas others concluded that Myc RNA and protein levels peak at the G1/S transition (Kelly et al. 1983), and yet others claim that Myc is stabilized during mitosis (Gregory et al. 2000). Moreover, phosphorylation events within Myc—at residues threonine 58 (T58) and serine 62 (S62)—that control its ubiquitylation by the SCF^{Fbw7} Ub-ligase have been reported to be influenced by cell cycle status (Luscher et al. 1992; Seth et al. 1993).

Fbw7 targets Myc which is phosphorylated at T58 by the GSK3 β kinase. During early G1, Ras dependent activation of the PI(3)-kinase/Akt pathway inhibits GSK3 β , preventing T58 phosphorylation and allowing a transient accumulation of Myc protein. Late in G1, however, Akt activity diminishes and GSK3 β is activated, promoting an Fbw7-dependent clearance of the Myc protein (Dominguez-Sola et al. 2004). In this way, a transient growth-regulated spike in Myc levels and stability could help to drive the entry of cells into S-phase, much in the same way as proposed for another Fbw7 target protein, Cyclin E. Moreover, T58/S62 phosphorylation also peaks in the late G2-phase of the cell cycle, suggesting that a similar periodic accumulation of Myc might also be important for the transition to mitosis (Seth et al. 1993; Niklinski et al. 2000). These results lend support to the idea that Myc destruction is cell cycle-regulated. Because cell cycle-dependent changes in Myc levels or stability could have a profound impact on the mechanism through which Myc promotes cell growth and proliferation, it is important that the issue of whether or not Myc proteolysis is cell cycle-regulated be resolved.

One way to reason some of these contradictions between the relationship of Myc and the cell cycle, is that these findings may be a result of the differences in techniques used in the various studies. In some cases, centrifugal elutriation was employed to monitor Myc levels and stability, whereas other studies used nocodazole block and release strategies. Therefore, to truly resolve this issue, it is important to develop an additional “unbiased” protocol that would allow us to take a comprehensive look at the influence of the cell cycle on Myc synthesis,

location, and stability in *unperturbed* cultures of cells. The recent development of laser scanning cytometry (LSC) created an opportunity to develop this protocol. The LSC is a technical merger between fluorescence microscopy and flow cytometry and allows for whole cell quantification of fluorophores targeted to DNA, RNA, or protein. The LSC determines the cell cycle status by quantifying total cellular DNA content using fluorescent dyes such as Hoechst 33342. By relating this cell cycle state to some other fluorescent parameter, such as the signal from a fluorescently-labeled antibody, levels of a particular RNA or protein can be measured in individual cells and expressed relative to the particular cell cycle stage. Compiling data from thousands of cells in this way, yields highly quantitative cell cycle data without disruption to normal cellular physiology. Importantly, by comparing RNA and protein levels for a particular gene product, and by monitoring protein levels after transient inhibition of protein synthesis, cell cycle dependent changes in protein stability can be inferred.

I therefore decided to utilize the LSC platform to study the influence of the cell cycle on the behavior of Myc. By studying fundamental biological processes, such as the expression of Myc RNA, Myc protein, and regulation of Myc stability at different stages of the cell cycle in an asynchronous population of cells, I hoped to resolve the apparent differences observed in Myc behavior from these earlier studies. Moreover, by comparing the cell cycle expression patterns of Myc with another Fbw7 substrate, Cyclin E, I hoped to understand the relative role of Fbw7 in regulating Myc turnover.

2.2 RESULTS

2.2.1 *Drug-induced cell cycle block perturbs Myc expression.*

Because the observed differences in the cell-cycle dependent behavior of Myc may lie in the use of different synchronization methods employed in previous studies, this study began with the investigation of the relationship between Myc and the cell cycle. I tested the notion that different methods of cell synchronization could lead to different conclusions regarding the behavior of Myc by comparing the effects of two common methods of cell synchronization on Myc—nocodazole block, which arrests cells in the G2/M stage of the cell cycle and double-thymidine (DT) block, which produces a G1/S arrest. I used Western blot to monitor steady-state Myc levels during subsequent release of cells back into the cycle; I also monitored phosphorylation of Myc at residue T58, which is required for its destruction by the Fbw7 Ub-ligase, and which has been previously reported to be cell cycle regulated. As hypothesized, these two approaches gave different results (Figure 2.1). Nocodazole arrest gave the impression that, although Myc levels are constant throughout the cell cycle, T58 phosphorylation peaks in mitosis (Figure 2.1A). DT-block, on the other hand, gave the impression that total Myc levels peak during release from G1/S, and that T58 phosphorylation does not appreciably differ from total Myc levels (Figure 2.1B). These differences were not observed when we examined another Fbw7 substrate, Cyclin E. Both nocodazole and DT block showed that Cyclin E levels were high at G1/S (Figure 2.1A: lanes 4–6; Figure 2.1B: lane 2) and low at G2/M (Figure 2.1A: lanes 2–3; Figure 2.1B: lanes 6–8). This result suggested that

apparent cell cycle-related changes in Myc levels and phosphorylation can be profoundly influenced by chemical synchronization methods. Importantly, because Cyclin E behaved consistently after both nocodazole and DT blockade, suggests that synchronization approaches can influence the status of different proteins in different ways.

2.2.2 Analysis of Myc using Laser Scanning Cytometry

The differential behavior of Myc in response to nocodazole and DT block highlighted the need to reinvestigate many of the aspects of Myc's behavior during the cell cycle. Moreover, Myc's sensitivity to existing drug-synchronization methods called for the development of an *unbiased* approach with which we could study Myc function in cellular populations, that were unperturbed by the administration of chemical agents to achieve cell cycle synchronization. I therefore studied Myc regulation using the Laser Scanning Cytometry approach, a quantitative alternative to study cell cycle related events.

I began my analysis by comparing the performance of LSC with traditional FACS analysis. I analyzed the cell cycle profiles of exponentially-growing U2OS cells using both these approaches. Results of this comparison, presented in Figure 2.2, revealed that both LSC and FACS approaches gave similar cellular distributions in the G1, S, and G2/M phase demonstrating that the cell cycle gating capabilities of the LSC were at least as good as those of traditional FACS analysis. Therefore, I used LSC-based assays to determine whether the regulation of Myc was influenced by the cell cycle.

Myc localization patterns during the cell cycle

I first asked whether the localization of Myc changes during the cell cycle. Although Myc is predominantly a nuclear protein, it can be localized to the cytoplasm in certain stages of early developmental (Gusse et al. 1989) or in certain diseased states such as endometrial cancer (Geisler et al. 2004), raising the possibility that nuclear/cytoplasmic partitioning of Myc is a regulated process. To ask if this process is regulated by the cell cycle, I combined LSC with immuno-fluorescence (counterstaining DNA with Hoechst 33342) to monitor the localization of Myc protein in cells at different cell cycle stages. This analysis (Figure 2.3) revealed that, despite its low abundance, Myc was readily detected in the nuclei of U2OS cells. The signal we obtained from Myc in these assays was reduced to near background levels by prior transfection of cells with siRNAs directed against Myc (data not shown). The results of this assay revealed that Myc was localized to the nucleus regardless of the cell cycle stage of individual cells. Together, these data support the viability of LSC for monitoring cell-cycle-dependent changes in Myc expression, and demonstrate that in asynchronous cycling cells, Myc is predominantly a nuclear protein at all cell cycle stages.

Myc RNA levels are invariant across the cell cycle

To address the issue of whether Myc synthesis is likely to be regulated in a cell cycle dependent manner, I used the LSC to monitor the steady-state levels

of Myc transcripts throughout the cell cycle in human U2OS cells. I combined LSC with RNA-fluorescence in situ hybridization (FISH) to compare the levels of Myc and (as a control) Actin RNAs, and used a detergent pre-extraction protocol to remove most of the cytosolic transcripts. I focused on nuclear RNAs to facilitate quantification, and to enrich for the population of newly-synthesized RNA molecules. Results of this analysis are shown in Figure 2.4. Using LSC/RNA-FISH, I detected robust levels of Myc and Actin RNAs using labeled anti-sense, but not the corresponding sense, probes (Figure 2.4A). Quantification of these hybridizations (Figure 2.4B) revealed that the *absolute* levels of both RNAs increase as cells passage through the cell cycle, being lowest in G1 cells, and highest in cells from the G2/M population.

This apparent increase, however, appears to be a result of the increase in cell mass that occurs during the cell cycle, because, when normalized for DNA content (Figure 2.4C), the *relative* levels of Myc and Actin RNAs do not change as a function of the cell cycle. I concluded that the levels of Myc RNA do not fluctuate during the cell cycle. Moreover, because Myc transcripts are extremely unstable with a half-life of ~25 minutes (Rabbitts et al. 1985), it is unlikely that a significant portion of Myc RNA transcribed in a given cell cycle stage would remain detectable in another. Thus, the relatively similar levels of nuclear Myc RNA in G1, S, and G2/M phase cells in all likelihood reflects constitutive transcription from the *c-Myc* gene.

Steady-state levels of Myc are unaffected by cell cycle status

To further understand the expression patterns of Myc, I employed a similar approach to examine steady-state levels of endogenous Myc protein in the different sub-populations of cells. In this analysis, as a control I decided to use Cyclin E which is known to be expressed in the G1 and S phases of the cell cycle. Moreover, Myc and Cyclin E share certain biological similarities. For example, both Myc and Cyclin E are important regulators of cell cycle progression and both of these proteins are cellular targets of the Fbw7 Ub-ligase. Therefore, I compared the expression patterns of Cyclin E with Myc during the course of the cell cycle. This analysis (Figure 2.5) revealed that Myc and Cyclin E behave differently as a function of the cell cycle. Whereas *absolute* levels of Myc increase throughout the cell cycle, the levels of Cyclin E—both relative to DNA and absolute—are considerably lower in G2/M cells than cells in other stages of the cell cycle. The behavior of Cyclin E in these experiments is consistent with previous reports showing that Cyclin E levels drop some time after cells enter S-phase, and with the notion that auto-phosphorylation of Cyclin E by the Cyclin E/CDK2 complex leads to its Ub-dependent destruction (Welcker et al. 2003). The ability to detect cell-cycle dependent changes in the levels of Cyclin E using this method further validates the use of LSC for these studies, and reinforces the notion that steady-state levels of the Myc protein do not vary as a function of the cell cycle.

Myc turnover rates are constant in all cell cycle stages

The differential behavior of Myc and Cyclin E in the preceding experiment suggests that, contrary to Cyclin E, the turnover of Myc is unlikely to be influenced by the cell cycle. However, earlier reports showing the regulation of Myc-phosphorylation by Ras and Akt signaling lead to the strong prediction that the Myc T58-phosphodegron will only be active at specific points in the cell cycle; the late G1, and possibly late G2 phases. To resolve this issue, I used the LSC as a platform for probing cell cycle-specific changes in the rate of Myc turnover. Because traditional pulse-chase methods are not adaptable to LSC-based analyses, I developed a protocol involving inhibition of protein synthesis with cyclohexamide and used the LSC to monitor the levels of Myc protein in each cell subpopulation as a function of time. This approach—which is analogous to the ‘cyclohexamide chase’ protocol that is often used to monitor Myc stability—allowed me to compare the rates with which Myc protein disappeared in each cell-cycle subpopulation. Under these conditions, Myc disappeared quickly following addition of cyclohexamide, with an apparent half-life of ~50 minutes in the total population of cells (Figure 2.6A). The estimate of Myc stability by this method compares favorably with half-life estimates derived from both pulse-chase and cyclohexamide-chase methods. Actin, in contrast, was relatively stable during the period of the experiment (Figure 2.6B). Importantly, examining the rate of Myc decay in the distinct cell subpopulations revealed that both the apparent half-life of Myc and its decay kinetics were identical in G1, S, and G2/M phase cells. This result supports early studies of Myc proteolysis which showed that the rate of Myc destruction is constant throughout the cell cycle (Hann et al.

1985). From these results I concluded that in an asynchronous cell culture system, the stability of Myc is not significantly affected by cell cycle status.

Myc is resistant to Fbw7-mediated turnover

Considering the MB-I region harbors a near-consensus phosphodegron for Fbw7, and that T58/S62 phosphorylation is presumably influenced by the cell cycle, the finding that the rate of Myc destruction does not change as a function of cell cycle progression is somewhat surprising. One possibility is that Fbw7 is responsible for the destruction of Myc at only one phase of the cell cycle, whereas other Ub-ligases participate in Myc destruction at the other phases—a situation similar to this has been reported to control destruction of the replication licensing factor Cdt1 (Nishitani et al. 2006). In this scenario, the rate of Myc destruction would appear constant, but in actuality Myc would be ‘shuffled’ between different Ub-ligases as the cell cycle progressed. If this were true, we could expect that disruption of the function of Fbw7 would result in an accumulation of Myc at a distinct phase in the cell cycle. To test this hypothesis, I used siRNA-mediated knockdown of Fbw7 to evaluate the relative contribution of SCF^{Fbw7} to cell cycle-specific levels of Myc and Cyclin E.

Results of this analysis are presented in Figure 2.7. Because there are three isoforms of Fbw7, I used siRNAs directed against a region common to all three isoforms; I also included siRNAs against either Myc or luciferase, as controls. In these experiments, Myc and Cyclin E behaved very differently. Although the levels of Myc were responsive to the anti-Myc siRNA (Figure 2.7B),

and as expected, reduced at all stages of the cell cycle; knockdown of Fbw7 had no detectable effect on Myc levels. This result was not due to a failure of the siRNA to functionally block Fbw7, because quantitative PCR analysis revealed that ~70% of Fbw7 transcripts were lost upon siRNA treatment (*data not shown*). Moreover, analysis of Cyclin E in the Fbw7 siRNA-treated cells gave the expected results: not only were total Cyclin E levels strongly induced by the Fbw7 siRNA, but there was a preferential increase in the levels of Cyclin E protein in S and G2/M phase cells (Figure 2.7A and D). Thus, despite the importance of Fbw7 for cell cycle regulation of Cyclin E, Myc appears to be resistant to the effects of Fbw7 knockdown at all stages of the cell cycle.

2.2.3 Centrifugal elutriation reveals Myc T58phosphorylation occurs throughout the cell cycle

Fbw7 targets Myc by recognizing the phosphorylated T58 residue. Although, based on earlier reports, one concludes that the phosphorylation of Myc at this residue could be regulated in a cell cycle dependent manner; the description of cell cycle dependent induction of T58-phosphorylation, in these experiments, used nocodazole arrested cells. Because my own analysis revealed that Myc is particularly vulnerable to chemical perturbations induced by these drugs and that knockdown of Fbw7 fails to yield a significant response in Myc, I reconsidered the regulation of Myc phosphorylation at T58. Given the possibility that compared to drug-synchronized cells, T58-phosphorylation may occur

differently in asynchronously cycling cells I decided to measure the levels of Myc T58-phosphorylation in unperturbed cells.

I probed for changes in Myc T58-phosphorylation in U2OS cells by LSC and western blotting. Unfortunately, my western blotting analysis revealed that the commercially available T58-phospho-specific antibody bound to several non-specific proteins; consequently I observed that the signal detected using LSC was *non-responsive* to siRNA-mediated knockdown of Myc, suggesting that a significant proportion of the signal detected by LSC was *not* Myc.

I therefore synchronized U2OS cells using centrifugal elutriation. Unlike drug-mediated synchronization techniques which can cause non-specific disruptions in the cell, centrifugal elutriation relies on the physical separation of cells at different stages of the cell cycle by their relative masses. By applying varying amounts of centrifugal force, I eluted several cellular fractions and compared their cell cycle distribution using flow cytometry. Results of this analysis (percentages shown in Figure 2.8) show that the first elutriated fractions contained predominantly the G1-phase cells and subsequent cellular fractions contained progressively increasing numbers of S- and G2-phase cells (Figure 2.8). I studied the levels of total Myc, T58-phosphorylated-Myc, Cyclin E, and Actin in these different fractions. As expected, Actin levels did not change significantly in any of the cellular fractions. In contrast, Cyclin E levels were higher in earlier fractions enriched in G1 and S-phase cells and dropped to nearly undetectable levels in the later G2/M enriched fractions (Figure 2.8: compare lanes 2-4 versus lanes 6-9). Interestingly, under these conditions both total levels

of Myc and the levels of T58-phosphorylated Myc did *not* appear to significantly change in any cell-cycle fraction. This result confirmed my own analysis using the LSC that in cycling cells, Myc was expressed constitutively throughout the cell cycle. Additionally, this analysis revealed a constant steady-state level of T58-phosphorylated Myc in the cell.

These results argue for apparent differences in the regulation of Myc and Cyclin E by Fbw7. Because my analysis of Myc T58-phosphorylation patterns reveals that the MB-I degron is constitutively phosphorylated, the limited ability of Fbw7 to target Myc cannot be explained on the basis of cell cycle regulation of T58-phosphorylation alone.

2.3 DISCUSSION

Following its discovery several decades ago, the importance of Myc as a cellular oncogene has fuelled detailed studies on its function, and the field of Myc biology continues to evolve with novel discoveries being reported to the present day. One striking example of this has been the discovery of Myc proteolysis by the Ub-proteasome system. Whereas early studies recognized Myc as a labile protein (Hann et al. 1985), the actual biochemical mechanism for this instability was unclear for many years. Work from the Tansey laboratory (and others) has in the recent past comprehensively established that Myc is destroyed by Ub-mediated proteolysis and that this process is one of the important pathways regulating Myc function in mammalian cells (Flinn et al. 1998; Gross-Mesilaty et al. 1998; Salghetti et al. 1999).

Despite the clear links between Myc and cell cycle control, it has been difficult to conclude, based on conflicting reports, whether Myc expression or stability itself is subject to cell cycle dependent changes. Given the importance of this issue, and the possibility that cell-synchronization methods can influence the outcome of cell cycle analyses, I felt that it was important to explore the metabolism of Myc during the cell cycle, using an independent method that did not require cell perturbation. Laser scanning cytometry provided this opportunity. My results demonstrate that Myc localization, synthesis, and stability are remarkably *unresponsive* to cell cycle status. Two main conclusions can be drawn from this analysis. First, the relative consistency of Myc metabolism throughout the cell cycle is consistent with the concept that Myc generally stimulates cell growth and duplication, rather than acting to drive a specific stage(s) in the cell cycle. Although early studies reported direct links between Myc and activation of the cell cycle machinery (Jansen-Durr et al. 1993; Galaktionov et al. 1996; Rudolph et al. 1996) subsequent analyses have demonstrated that Myc controls the expression of a large subset of genes, and orchestrates a broad program of gene expression that influence not only cell cycle control, but also energy production, signal transduction, DNA replication, and protein synthesis (Zeller et al. 2003). In such a model, the constitutive expression of Myc acts as a constant force to drive cell proliferation under appropriate growth conditions.

Second, my results also illustrate how two Fbw7 target proteins, Cyclin E and Myc, behave differently with respect to the cell cycle. Despite the similarity of

the Fbw7 phospho-degrons in both proteins, and the clear potential for each to be regulated in a cell-cycle-dependent manner, only Cyclin E displayed cell cycle-related changes in abundance and sensitivity to Fbw7 knockdown. Indeed, my studies found little if any evidence that Fbw7 regulates steady-state levels of Myc protein. One possible explanation for this finding is that Fbw7 regulates a small sub-population of the Myc protein. Although it is possible that the compartmentalization of Myc within the nucleus—either to discrete regions of chromatin, or perhaps the nucleolus (Welcker et al. 2004)—could direct interaction of a small population of Myc with Fbw7, it is important to note that, at steady state, elutriation data shows that a significant percentage of Myc in U2OS cells is phosphorylated at the T58 residue, and that disrupting the Fbw7 phosphodegron by point mutation [*e.g.*, T58A; (Salghetti et al. 1999)] results in a robust stabilization of the Myc protein. It seems likely, therefore, that the majority of Myc, although technically a suitable substrate for Fbw7-mediated ubiquitylation, must be shielded from the effects of this Ub-ligase.

I suggest that the differential regulation of Myc and Cyclin E by Fbw7 has significance to the maintenance of normal cellular homeostasis. Because Myc is an upstream activator of Cyclin E transcription (Jansen-Durr et al. 1993), a reduction in the expression of Fbw7—or loss of Fbw7 function, as occurs in some cancers (Minella et al. 2005)—would be expected to have a disproportionate effect on Cyclin E levels, inducing both Cyclin E transcription (via increases in Myc) and stabilization (via inactivation of Fbw7). This effect, combined with other consequences of Myc overexpression, could result in profound changes in the

regulatory status of the cell. By shielding the majority of Myc protein from Fbw7-dependent proteolysis, however, cells could precisely regulate Cyclin E levels, while at the same time modulating the function of only a subset of Myc activities, such as ribosomal RNA synthesis. Further studies will be required to determine either the precise pool of Myc proteins that are regulated by Fbw7, or the mechanistic reason for how the bulk of Myc protein remains unresponsive to this Ub-ligase.

2.4 MATERIALS AND METHODS

2.4.1 *Cell culture and immunofluorescence*

Human U2OS and HeLa cells were grown in DMEM supplemented with antibiotics and 10% fetal calf serum. For analysis, cells were plated onto glass coverslips, grown at 37°C for 24 hours, and then fixed, either using methanol or paraformaldehyde (Spector et al. 1998). Immunofluorescence was performed as described (Spector et al. 1998) using the following antibodies: (i) α -Myc (N-262, *Santa Cruz*), (ii) α -Cyclin E (HE12, *Santa Cruz*), (iii) α -Actin (AC-15, *Sigma*), and (iv) α -phospho T58 Myc (9401S, *Cell Signaling*). Immune complexes were detected using FITC-tagged secondary anti-mouse or anti-rabbit antibodies, as appropriate. DNA was stained with Hoechst 33342 (2 μ g/ml).

2.4.2 *LSC analysis*

All experiments were performed using the LSC-iCys system (Compucyte, MA) attached to an Olympus IX-71 microscope. Samples with multiple

fluorophores were compensated by measuring the spectral bleed into other channels using controls containing each individual fluorophore, and subtracting the corresponding non-specific leakage. Thresholds for measuring each fluorophore were set at signal intensities where there was negligible background signal. The fluorescence units obtained thereafter were used to compare the expression patterns within different cell cycle populations. Approximately 2000-3000 cells were scanned in each individual analysis.

Cell cycle profiles were gated based on DNA content into G1, S, and G2/M populations. For sub-cellular visualization experiments, galleries of cells in different cell cycle phases were created. The localization of the Myc was compared by merging the pictures obtained from the green (Myc), blue (DNA), and scatter (phase contrast) channels. For quantification, the mean signals of the respective proteins were measured within the given cell cycle gates. Fluorescence values were normalized either to mean signal in the G1 phase or reported as a ratio of green/blue fluorescence to quantify relative levels of protein or RNA in different cell cycle stages as a function of DNA.

2.4.3 Cell synchronization experiments

For Nocodazole arrest, cells were seeded for 24 hours, and then treated with Nocodazole (100 ng/ml) for 16 hrs. Nocodazole was removed and cells were released from G2/M block for varying timepoints. For double-thymidine (DT) arrest, cells were treated with 2.5 mM thymidine for 14 hrs, washed and released

for 12 hrs, and then re-arrested in 2.5 mM thymidine for 14 hrs before FC analysis.

Centrifugal elutriation was performed as follows. In brief, actively growing U2OS cells were elutriated using the Beckman JE-6B rotor, at a rotor speed of 1500 rpm and rotor temperature of 20°C. Cells were eluted in DMEM+1% FBS, by applying an increasing medium flow rate ranging from 40 ml/min – 150 ml/min. Approximately 10 fractions of 250 ml were collected. Cells were rapidly harvested by centrifugation and either fixed in methanol and analyzed by flow cytometry or lysate prepared for western blotting.

2.4.4 RNA FISH

RNA-FISH analysis for detection of nuclear RNAs was performed on triton-extracted, fixed, U2OS cells as described.

Four anti-sense probes were used for Myc:

Myc1:TAGTCGAGGTCATAGTTCCTG;Myc2:TCGAGGAGAGCAGAGAATCCG

Myc3:TTCAACTGTTCTCGTCGTTTC;Myc4:TGTTCGCCTCTTGACATTCTC.

Two antisense probes were used for actin:

Act1–ATAGCACAGCCTGGATAGCAA

Act2–TGGAAGCAGCCGTCGCCATCTCTTGCTCGA.

In each case, the corresponding sense probes were used as a control. Pooled probes were end-labeled with Dig-11-ddUTP using the Digoxigenin End-labeling kit (*Roche*). FISH was performed as described.

2.4.5 Protein synthesis inhibition

For experiments involving cyclohexamide, U2OS cells, growing on coverslips, were treated with 50 µg/ml cyclohexamide, fixed at the indicated time points, and Myc and Actin levels at each point quantified by immunofluorescence and LSC. The corrected fluorescence value in each of the cell cycle stages at the “0” time point was arbitrarily fixed as 100% and values of corrected fluorescence from every subsequent time point were represented as relative percentage of the “0” (100%) time point.

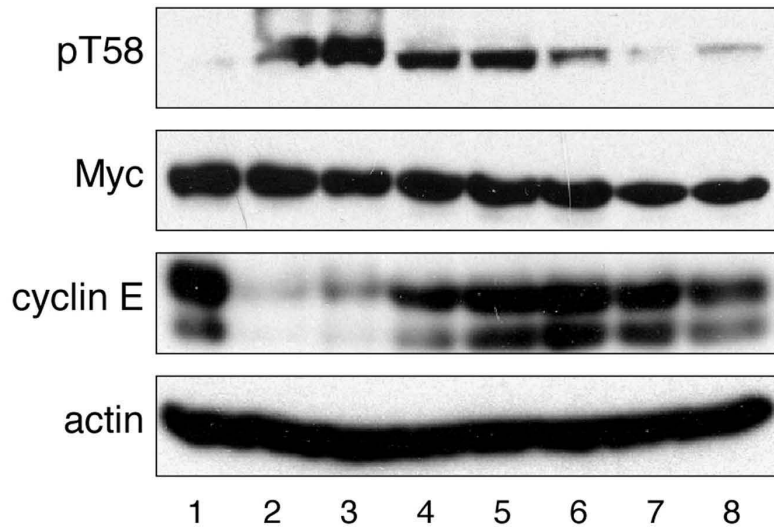
Figure 2.1: Myc expression varies in synchronized cells

(A) Nocodazole block and release experiment to follow cells released from a mitotic block showed that Myc T58-phosphorylation peaked in the G2/M arrested cells and disappeared as the cells moved into the next cell cycle whereas total Myc levels remained unchanged. In contrast, CyclinE levels were lowest in G2/M arrested cells and peaked as the cells entered the next G1-phase. Actin levels, studied as a control, do not change throughout the cell cycle.

(B) Double-Thymidine block and release to study the behavior of proteins immediately upon release from S-phase block is shown. Myc T58-phosphorylation patterns closely overlap with the expression patterns of total Myc, and show an apparent increase in S-phase and drop as cells enter G2. CyclinE patterns remain as expected, and peak in S-phase and begin to drop as cells proceed through S-phase into G2. Control Actin levels remain unchanged.

(A) NOCODAZOLE

Time (min):	asn	0	90	180	270	360	450	540
G0/G1 (%)	57	2	5	59	64	67	10	62
S (%)	19	6	4	8	10	7	8	16
G2/M (%)	21	87	88	28	22	20	15	14

**(B) DOUBLE-THYMIDINE**

Time (min):	asn	0	90	180	270	360	450	540
G0/G1 (%)	54	70	64	49	23	17	16	18
S (%)	22	15	21	31	59	67	55	23
G2/M (%)	19	10	9	11	11	14	23	53

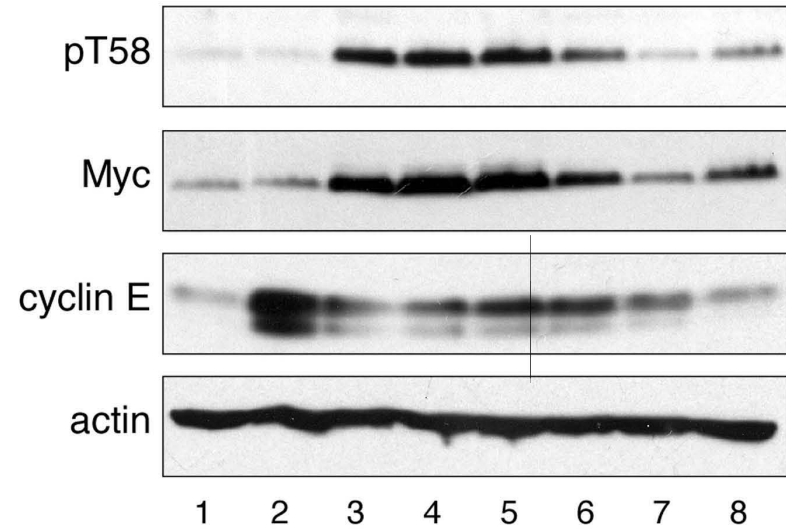


Figure 2.2: Performance of LSC compares favorably with conventional FACS analysis.

(A) Cell cycle profiles of an asynchronous population of U2OS cells obtained using by Laser Scanning Cytometry (LSC-Icys) were compared with conventional Flow Cytometry. Cell cycle histograms show similar distributions in different cell cycle stages.

(B) Quantification of cell cycle profiles from panel A showing that the gating capabilities of the LSC are largely identical to those of conventional Flow analysis. Data is Mean \pm S.D from two independent experiments.

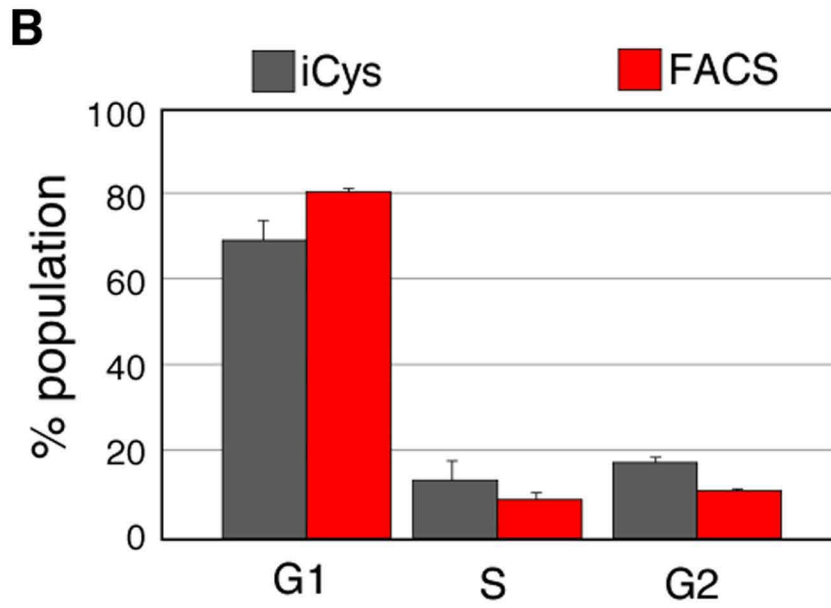
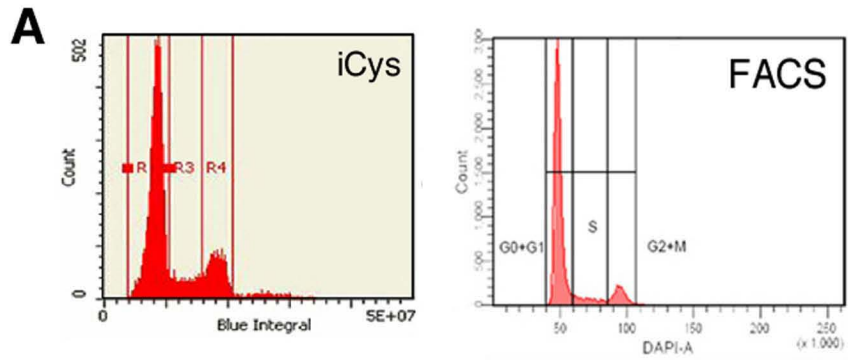


Figure 2.3: Myc remains predominantly nuclear throughout the cell cycle.

U2OS cells were immuno-stained using the anti-Myc (N262) antibody and DNA (Hoechst 33342). Slides were scanned using the LSC approach and gated into G1, S, or G2 phase of the cell cycle based on DNA-pixel intensity.

Sub-cellular localization of Myc was analyzed by creating galleries of cell binned into the specific stage of the cell cycle. Myc signal was observed predominantly in the nucleus at all stages of the cell cycle, suggesting that under active growing conditions, Myc activity is limited mostly to the nuclear compartment.

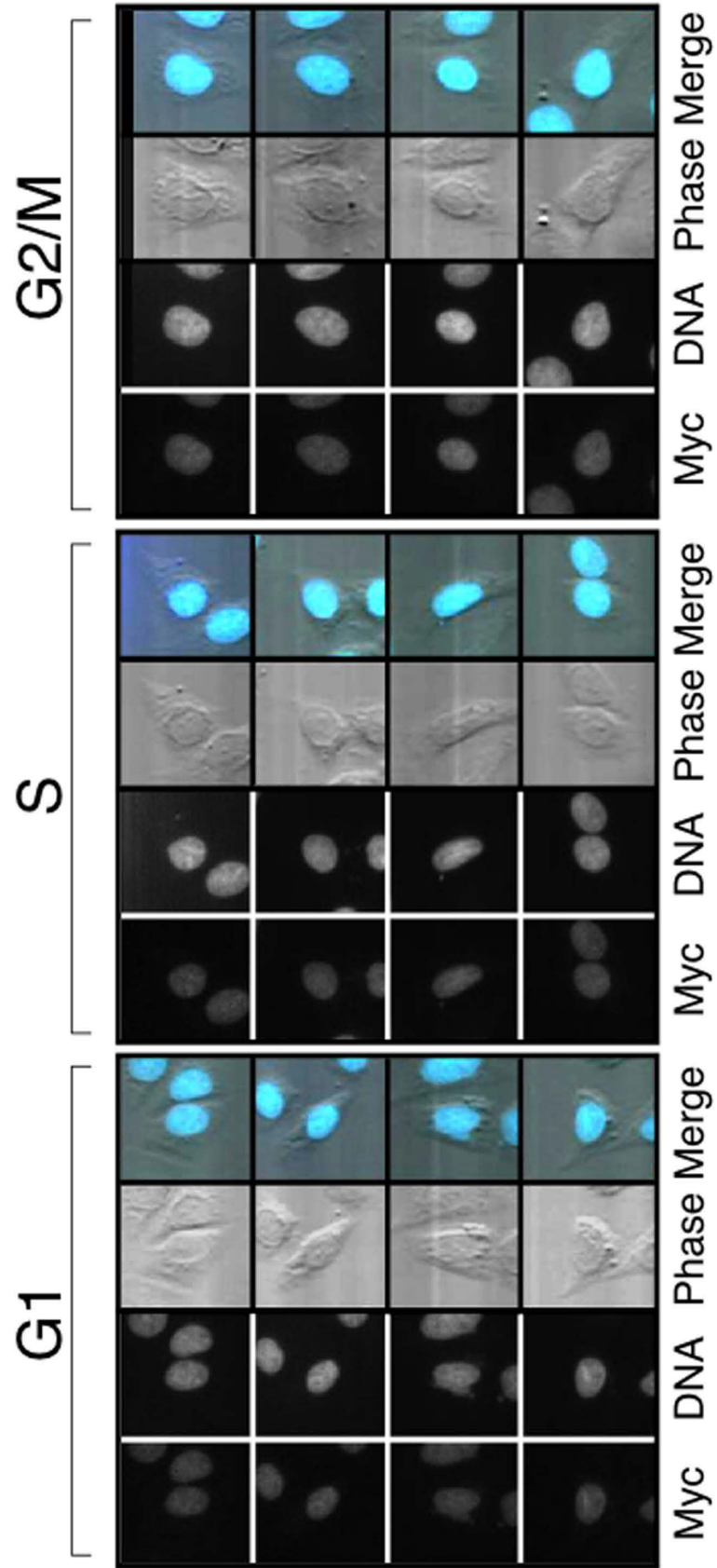


Figure 2.4: Myc and Actin RNA show cell- cycle independent expression

(A) U2OS cells were detergent pre-extracted and Myc and Actin RNA were probed by RNA-FISH with oligos labeled with Digoxigenin, followed by detection using a FITC-tagged anti-Digoxigenin antibody. Cells were counterstained using DNA-dye Hoechst 33342.

Results show a stronger fluorescence signal for both Myc and Actin RNA using the anti-sense oligo-probes as compared to the sense oligo-probes, confirming our ability to detect bonafide RNA species using the RNA-FISH approach.

(B), (C) RNA-FISH stained cells analyzed using LSC show that *absolute* levels both Myc and Actin RNA continue to increase throughout the cell cycle (Panel B). However, normalizing the RNA levels to the relative DNA content (Panel C), shows that the expression levels of Myc and Actin remain fairly constant throughout the cell cycle. Values represented are Mean \pm S.D from two independent experiments.

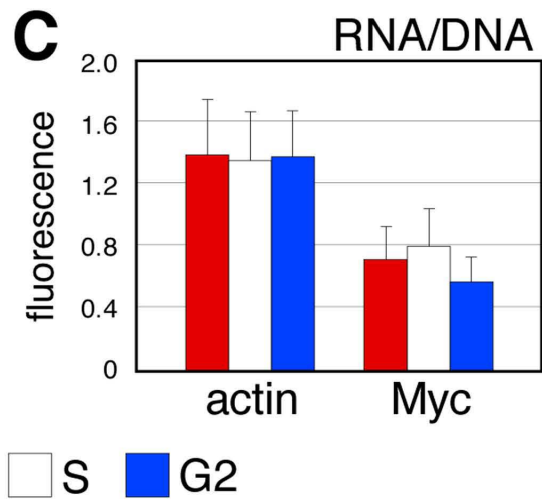
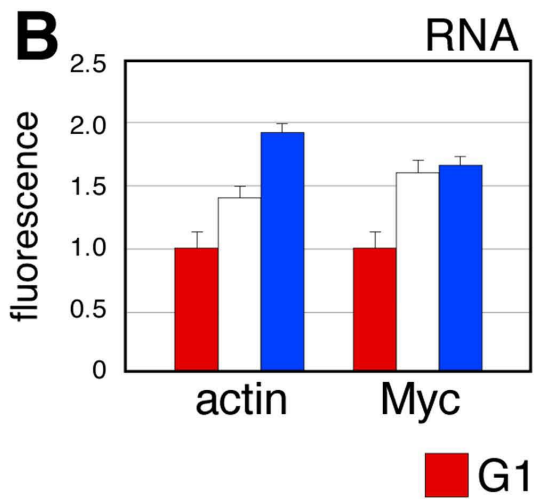
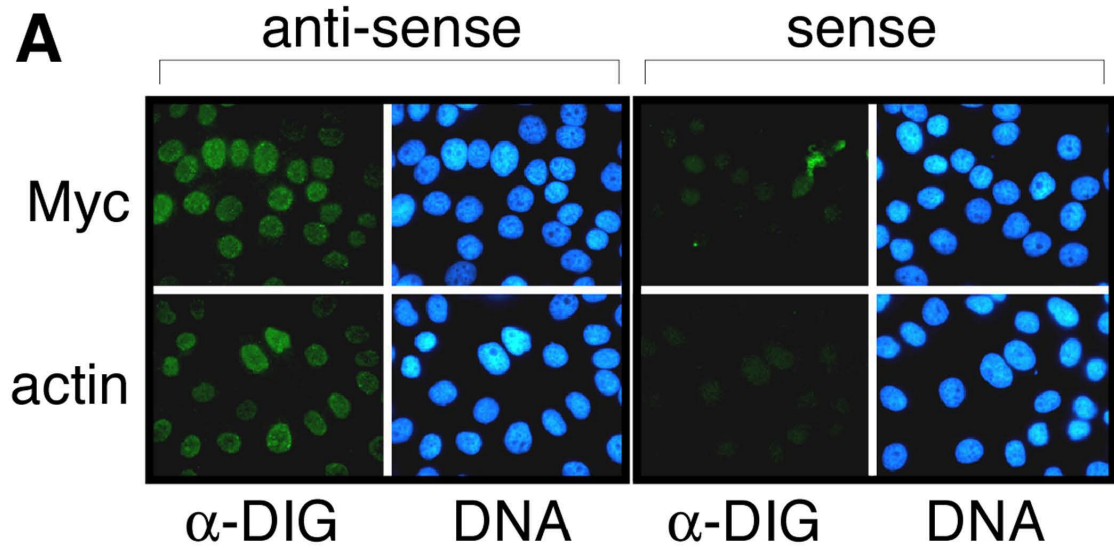
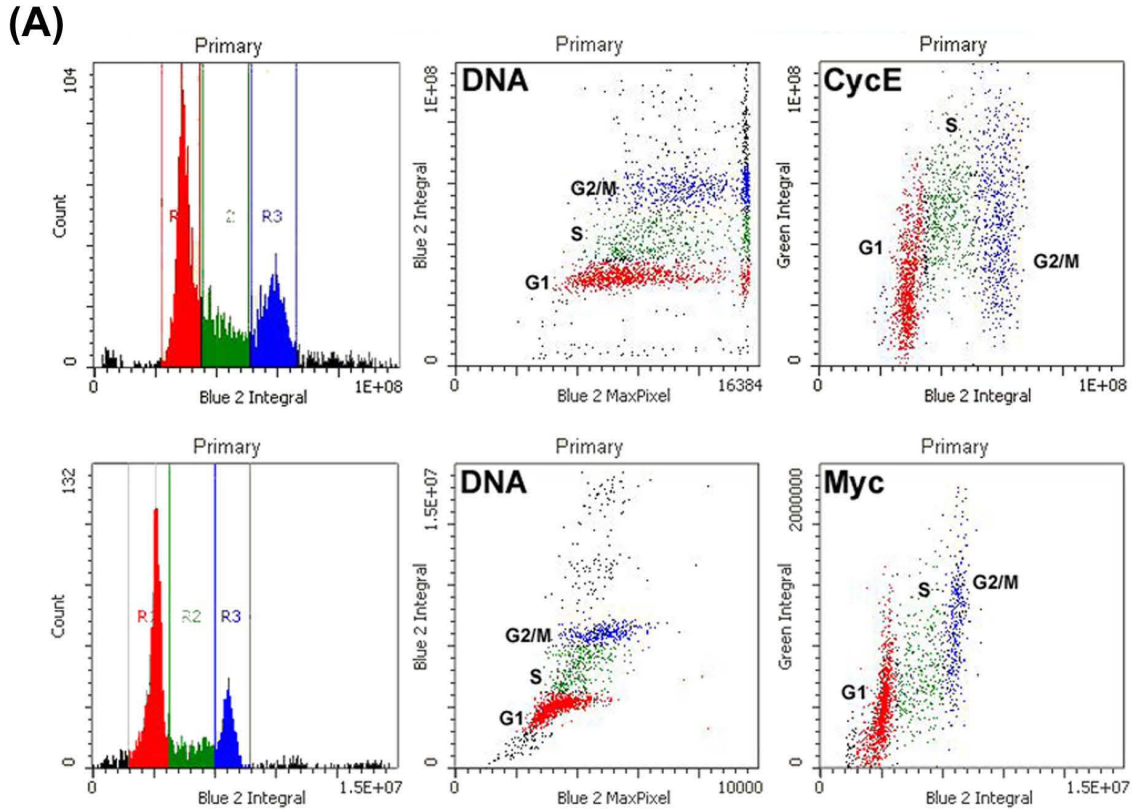


Figure 2.5: Myc protein levels increase continuously during the cell cycle

(A) Expression patterns of Myc and CyclinE were analyzed by staining Myc and CyclinE using indirect Immuno-fluorescence and measuring expression levels using LSC. Bivariate analysis, comparing the protein levels to DNA content show that the *absolute* levels of Myc continue to increase from G1 to G2/M. In contrast, CyclinE levels increase from G1 to S but are reduced in G2/M.

(B), (C) Graphical representation of mean fluorescence signals as measured by using LSC. (Panel B) Myc levels increase continuously throughout the cell cycle, whereas CyclinE levels increase from G1 to S and reduce in G2. (Panel C) Total protein levels normalized to DNA content shows Myc levels are unchanged and CyclinE levels peak in G1/S. G1 fluorescence signal was arbitrarily set to 1. Values represent Mean \pm S.D from four independent experiments.



(B)

■ G1 □ S ■ G2

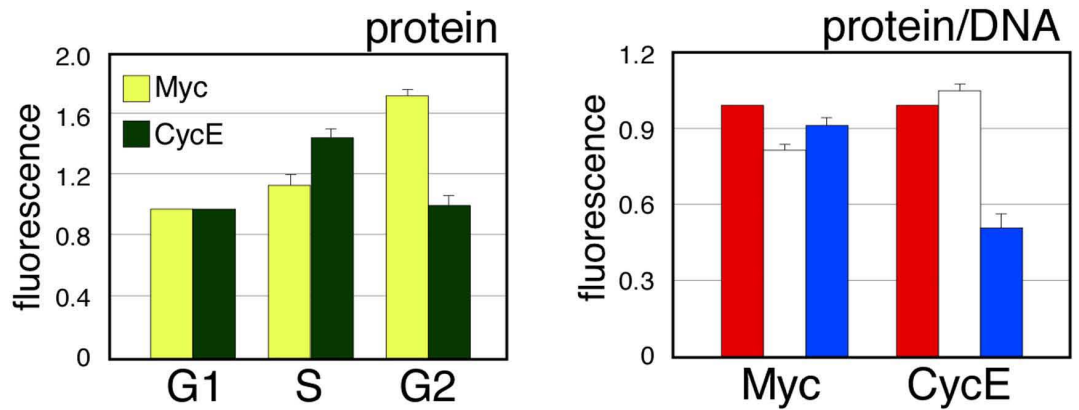


Figure 2.6: Myc decay patterns are *not* influenced by cell cycle status

U2OS cells were seeded on cover-slips and incubated overnight. Cells were treated with Cyclohexamide and cells were fixed at specific time-points. Cells were immuno-stained and analyzed using LSC for changes in Myc levels (Panel A) or levels of Actin (Panel B). The fluorescence levels at time “0” were arbitrarily fixed as 100%. Values represent Mean levels observed from two independent experiments.

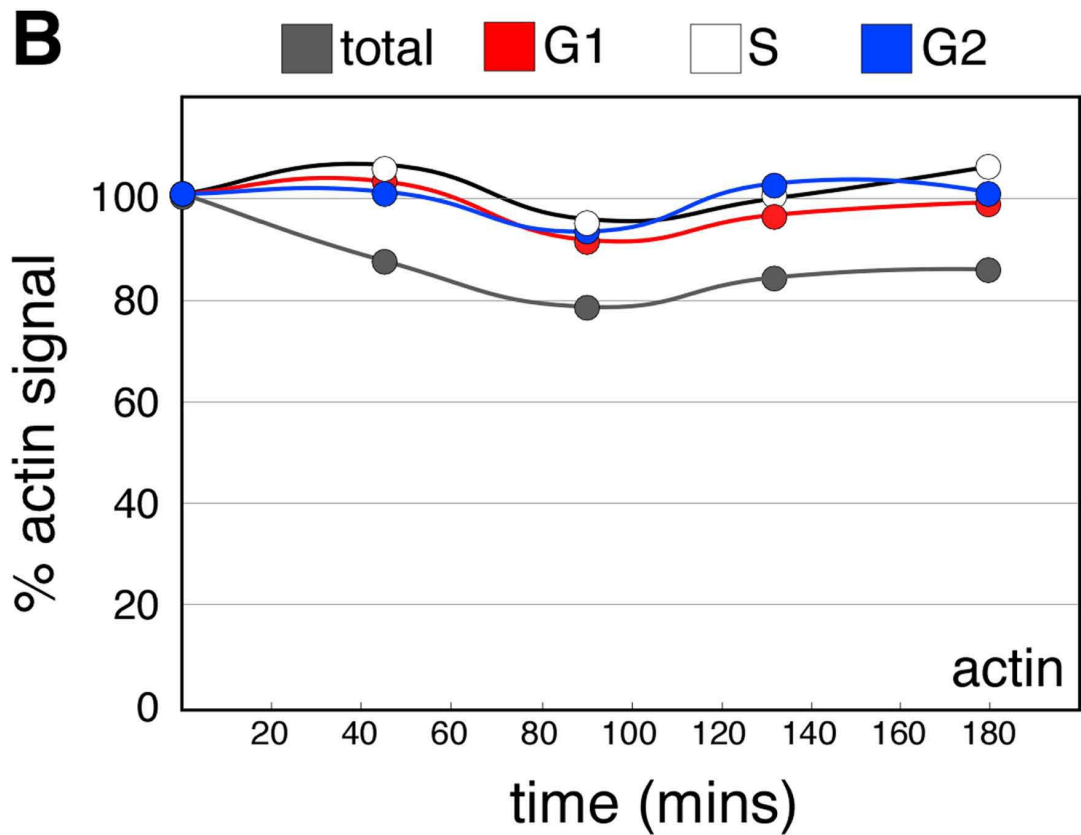
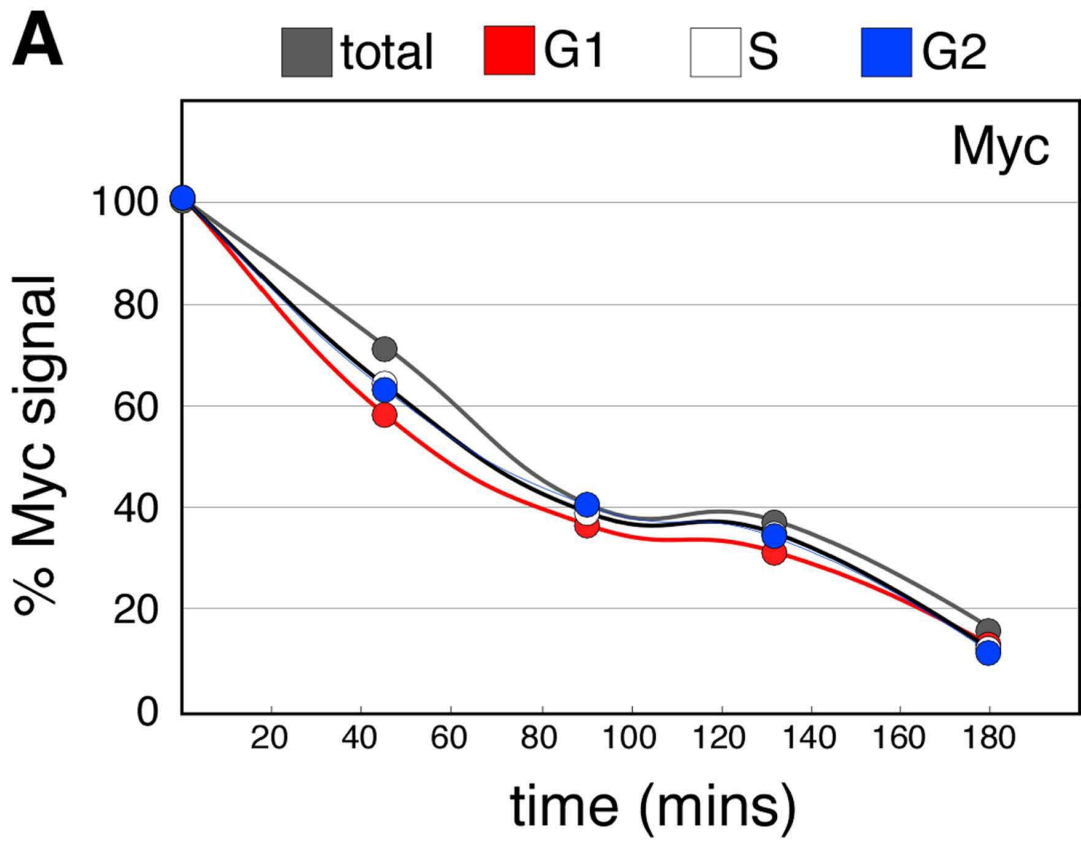


Figure 2.7: Myc is resistant to Fbw7-mediated proteolysis

(A) Analysis of Cyclin E and Myc during different stages of the cell cycle using the LSC-Icys system in U2OS cells transfected with siRNA against Luciferase (Control), Fbw7, or Myc. Knocking down Fbw7 leads to an increase in Cyclin E levels, especially in the G2/M population (note the rightward shift in the profiles). In contrast, Myc levels *do not* change significantly upon Fbw7 knockdown.

(B), (C) Quantification of LSC profiles from cells analyzed in Panel A. Cyclin E shows a near 3-fold increase in G2/M cells upon Fbw7 knockdown (compare blue bars in 2.7B), induction is lesser in G1 and S-phase. Myc levels reduce by nearly 75% upon Myc knockdown; however, Fbw7 knockdown has no discernible effect on Myc levels.

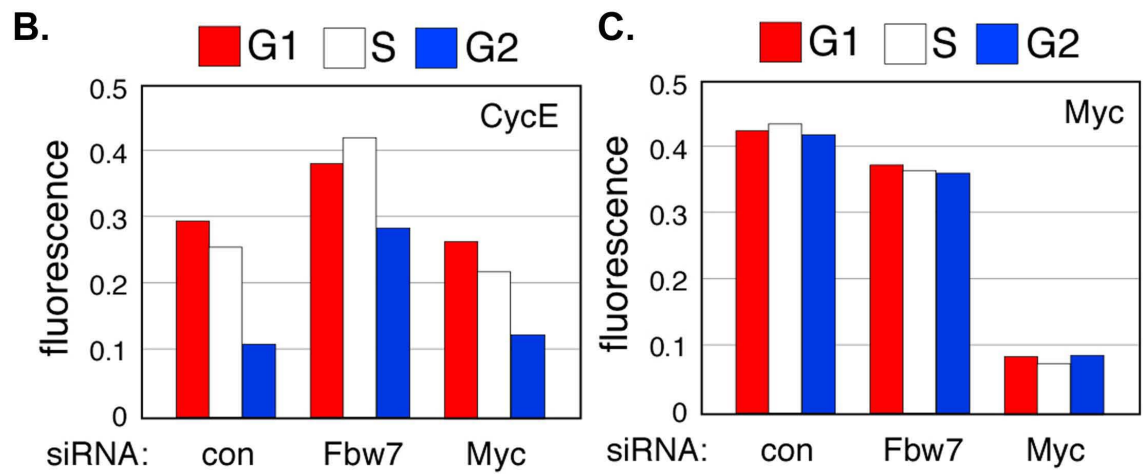
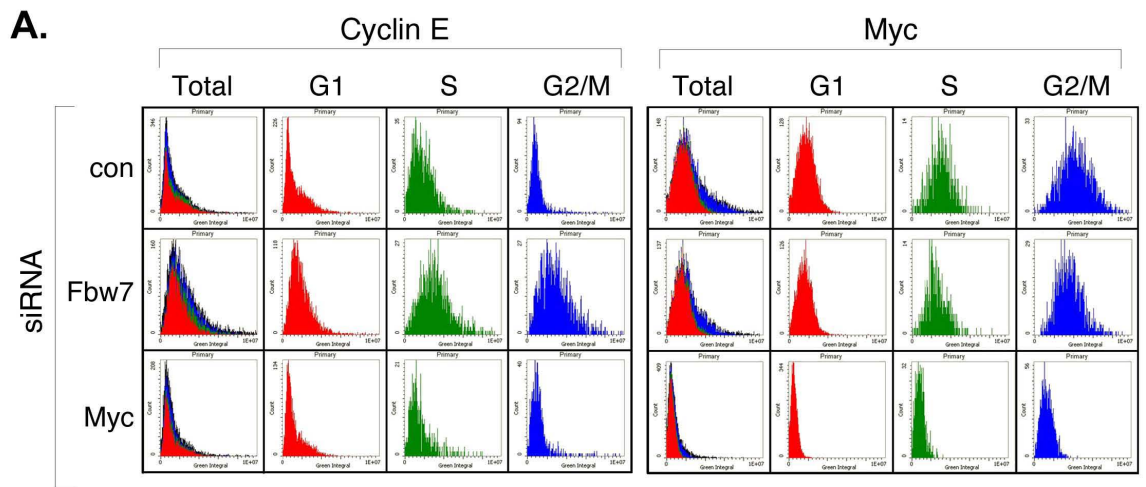
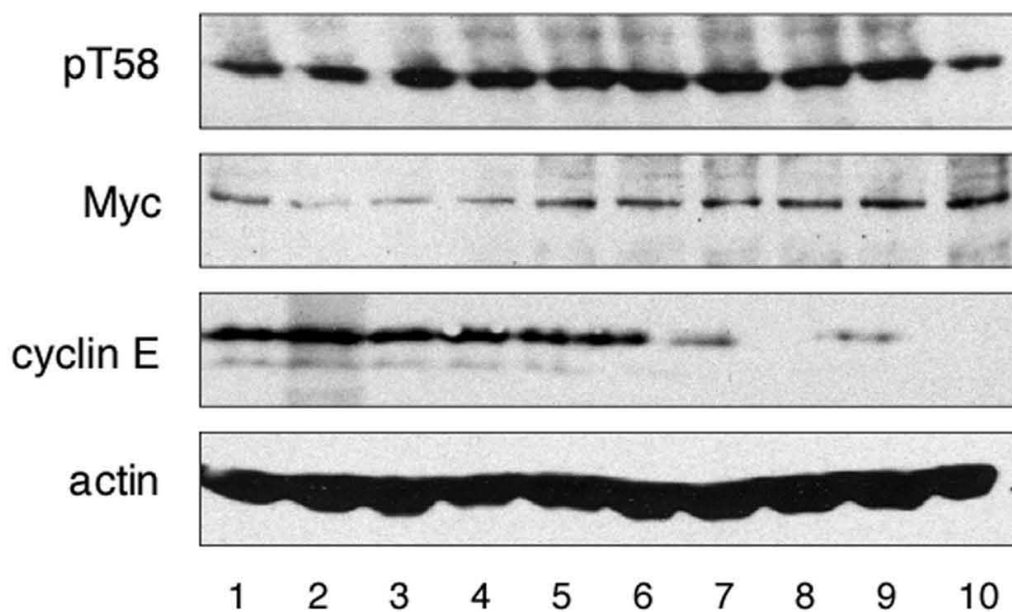


Figure 2.8: Centrifugal Elutriation reveals Myc-T58-phosphorylation occurs constitutively.

U2OS cells were separated into fractions enriched in populations of cells at specific cell cycle stages (compare cell cycle distribution percentages). Western blot analyses of the fractions show that both total-Myc levels and levels of T58-phosphorylated Myc remain unchanged throughout the cell cycle. In contrast, Cyclin E is detected in early fractions (enriched in G1/S-phase cells) and reduces to undetectable levels in fractions enriched in G2/M phase.

Fraction:	usi	1	2	3	4	5	6	7	8	9
G0/G1 (%)	58	88	63	50	57	50	47	36	32	41
S (%)	18	3	26	34	29	28	27	21	20	11
G2/M (%)	18	4	4	6	6	16	21	35	36	36



Chapter 3: Regulation of Myc function by the Fbw7-Usp28 pathway.

3.1 INTRODUCTION

The c-Myc transcription factor is an important regulator of cell growth and promotes the progression of the cell cycle (Grandori et al. 2000). Cellular Myc levels, therefore, need to be carefully regulated and elevated levels of Myc are frequently observed as a hallmark of many human cancers. Ub-mediated proteolysis defines one prominent pathway to limit Myc levels in the cell (Salghetti et al. 1999) and by studying the process of Myc destruction by the Ub-proteasome pathway several groups have reported the discovery of three Ub-ligases for Myc: Skp2 (Kim et al. 2003; von der Lehr et al. 2003), Fbw7 (Welcker et al. 2004; Yada et al. 2004), and HectH9 (Adhikary et al. 2005). Amongst these, the importance of the Fbw7 pathway in Myc turnover and its implications in oncogenesis are of particular interest to my thesis work.

Proteolysis of Myc by Fbw7 is frequently attenuated in several cancers by either the loss of Fbw7 function or by mutations in Myc which render it non-responsive to Fbw7. Despite the obvious importance of Fbw7-mediated proteolysis in Myc oncogenesis, however, our understanding of the regulation of Myc by Fbw7-mediated proteolysis is limited due to several inconsistencies in

this process (described in detail in section 3.1.3). This chapter of my thesis focuses on studying the influence of Fbw7 proteolysis on Myc function in an attempt to resolve some of these discrepancies.

3.1.1 Loss of Fbw7 function in cancers

Fbw7 is an important regulator of cell cycle progression and the Ub-ligase function of Fbw7 plays an important role in this process. By targeting key cellular proteins, including Myc, Cyclin E, and Jun, for destruction via the Ub-proteasome pathway, Fbw7 represents one of the prominent means to limit their cellular activity. Given the importance of these proteins in oncogenesis it is believed that Fbw7 functions as one of the important tumor suppressor proteins in the cell (Welcker et al. 2008). Consistent with this notion, different cellular mechanisms exist to inactivate Fbw7 function during tumorigenesis.

In the human genome, the Fbw7 gene is expressed from the short arm of chromosome 4 (4q32), and copy number analysis have revealed that 4q32 is a locus vulnerable to genomic deletions in a number of cancers (Knuutila et al. 1999). However, a more pervasive mechanism for limiting Fbw7 function involves inactivating Fbw7 via point mutations in its functional domains (Strohmaier et al. 2001). By interfering with Fbw7's proteolytic activity, these mutations interfere with the destruction of its cellular substrates, leading to an induction in their cellular levels. Given that Fbw7 regulates proteolysis of important cellular substrates such as Myc and Cyclin E, it is not surprising to find that inactivation of Fbw7 is a recurrent theme in cancers from a diverse range of tissues,

including blood-borne cancers such as B-cell and T-cell lymphomas or leukemia's (Welcker et al. 2008).

3.1.2 Cancer-associated mutations in Myc

In addition to Fbw7, the Myc protein itself is altered in certain cancers. Cytogenetic analyses from blood-borne cancers, particularly Burkitt's lymphoma, have revealed that the Myc allele frequently translocates in these cancers to the immunoglobulin (Ig) locus (Rabbitts et al. 1983). This translocation event places Myc directly under the control of the immunoglobulin (Ig) regulatory elements and results in the activation of Myc gene expression. In addition to elevated levels of Myc, blood-borne cancers frequently harbor mutations in the Myc coding sequence. Although it was earlier reasoned that Myc mutagenesis occurs because the translocation of Myc into the Ig locus subjects it to the somatic hypermutation pathway (Rabbitts et al. 1983), subsequent evidence shows that in most cases Myc mutations are homozygous (Bhatia et al. 1993). The mutation in the non-rearranged Myc locus, therefore, suggests that the mutation of Myc probably precedes the translocation event in these cancers. Although the mechanism of Myc mutagenesis is still unclear, evidence from animal models show that some tumor-derived Myc mutants are highly oncogenic (Hemann et al. 2005). This observation confirms the physiological importance of Myc mutagenesis and argues for the need of a more detailed analysis of the causes and consequences of Myc mutagenesis in lymphoid tumors.

Carefully analyzing the location of cancer-associated Myc mutations reveals that rather than being randomly interspersed throughout the coding sequence, these mutations often occur in discrete clusters. Moreover, some of these mutation clusters overlap with the evolutionarily conserved—and functionally important—regions in Myc such as MB-I and MB-II giving rise to the notion that such cancer-associated mutations probably alter specific cellular functions of Myc. Indeed, studies using the MB-I mutants P57S and T58A, reveal that unlike WT Myc, these mutants lose the ability to induce the pro-apoptotic BH3-domain containing protein, Bim, and thus attenuate the ability of Myc to induce apoptosis. Moreover, by retaining the ability to mediate proliferation, these mutants tilt the balance of Myc function in the favor of cell growth and can therefore mediate lymphomas with a significantly reduced time of onset (Hemann et al. 2005).

Several years ago, work from the Tansey lab demonstrated that many cancer mutants in Myc, including the MB-I mutants P57S and T58A, escape proteolysis and increase Myc half-life compared to WT Myc (Salghetti et al. 1999). This observation and work done by others (Flinn et al. 1998) gave rise to the idea that MB-I must be an important site for Myc proteolysis. Indeed, recently it was shown that MB-I harbors the recognition sequence for Fbw7, and that within MB-I the phosphorylation of threonine 58 (T58) and serine 62 (S62) regulate Myc binding to Fbw7. Given that non-phosphorylatable mutations at both T58 and S62 occur in many lymphoid cancers suggests that one

mechanism for stabilizing Myc in these cancers is by acquiring mutations that render Myc non-responsive to Fbw7-mediated proteolysis.

In summary, the cancer-associated alterations in both Fbw7 and Myc illustrate the potential importance of the tumor suppressive role played by Fbw7-mediated Myc proteolysis. Attenuating Fbw7 activity or acquiring mutations in Myc which escape Fbw7 proteolysis, therefore, are important routes towards cellular transformation, and represent processes which warrant careful investigation.

3.1.3 *The Myc-Fbw7 paradox*

Despite its obvious importance, the biology of Myc proteolysis via Fbw7 is plagued by numerous inconsistencies. Here I describe two such paradoxes, which I address in my thesis work:

3.1.3.1 *Myc is resistant to Fbw7 proteolysis*

Several lines of evidence suggest that Fbw7 is a bonafide Ub-ligase of Myc and that Fbw7 targets Myc via phosphorylation at T58 (Welcker et al. 2004; Yada et al. 2004). First, by studying the ubiquitylation of Myc in a reconstituted *in vitro* assay, it is observed that Fbw7 induces the accumulation of high-molecular weight Myc-Ub conjugates on WT Myc but not the T58A Myc mutant. Moreover, Fbw7 interacts strongly with WT Myc and this association is reduced by the T58A mutation. And lastly, overexpression of Fbw7 leads to a drop in cellular Myc

levels and this phenomenon is reversed by chemical inhibition of the proteasome. Whereas all these arguments are consistent with the notion that Fbw7 is an E3 for Myc, knockdown of Fbw7 using siRNA duplexes do *not* significantly alter either steady-state Myc levels (Figure 3.1) or Myc half-life (Kim et al. 2003). In contrast, levels of Cyclin E—another substrate of Fbw7, are readily induced upon reduction in Fbw7 levels. This is an apparent paradox. In the process of analyzing the possible reasons for this discrepancy, I tested the notion that Fbw7 probably targets only a small pool of Myc possibly at specific stages of the cell cycle. The results of my analysis, presented in chapter 2, revealed that although T58-phosphorylation occurred constitutively during cell cycle progression, Myc remained *non-responsive* to a reduction in Fbw7 levels, suggesting that under normal cycling conditions Myc is resistant to Fbw7 proteolysis.

This observation raises concerns about the contribution of Fbw7 to Myc proteolysis in the cell and represents one of the major paradoxes in Fbw7-mediated Myc turnover.

3.1.3.2 *Myc harbors a single “weak” Fbw7 degron*

The importance of Fbw7 proteolysis in cell cycle progression has led to the systematic identification of numerous cellular substrates in both yeast and humans. Comparing the binding properties of Fbw7 to its degron—the Cdc4 Phosphodegron (CPD)—has revealed one important theme about Fbw7 function. Fbw7 substrates can either harbor one strong CPD or function via multiple weak

degrons. For example, two CPDs have been identified on Fbw7 substrates such as Cyclin E, and as many as nine CPDs have been discovered in the yeast target Sic1 (Nash et al. 2001; Orlicky et al. 2003). Moreover, biochemical studies done using the Sic1 CPDs reveal that these sites have poor binding affinity for Fbw7. Importantly, although a Sic1 mutant with all nine degrons inactivated, escapes proteolysis by Fbw7 and results in deregulated DNA replication, this phenotype can be rescued by replenishing a single strong degron (Nash et al. 2001). This result suggests that although one strong CPD is sufficient to regulate Fbw7 proteolysis and mediate biological function, substrates have evolutionarily retained multiple weak degrons, presumably as a measure to exert higher regulatory control.

Unlike these Fbw7 substrates, Myc harbors only one degron for Fbw7. Based on the preceding discussion this suggests that the Myc degron should be a strong CPD. However, physiological evidence argues that this is probably not true. Relative to other Fbw7 substrates, Myc has a much more complex pathway for Fbw7 targeting (Figure 1.4). Consistent with the idea that CPDs are small sequences harboring either negatively charged or phosphorylatable amino-acids spaced four residues apart, phosphorylation of two residues in MB-I: T58 and S62, play an important role in Fbw7 recruitment. Whereas T58 phosphorylation is the recognition signal for Fbw7 recruitment and leads to Myc destruction, S62-phosphorylation inhibits recruitment of Fbw7 and protects Myc from destruction. The phosphorylation events at T58 and S62, therefore, counteract each other and interfere with the proteolysis of Myc by Fbw7. Given that biochemical studies

have shown that CPDs phosphorylated at both phospho-acceptor sites have higher affinities for Fbw7 than CPDs phosphorylated at a single-site, and considering Myc is presumably targeted in the context of T58 phosphorylation alone, suggests that Myc probably functions via a *weak* functional CPD. This argument is further supported by the poor response in Myc levels seen upon Fbw7 knockdown. In the absence of an additional degron, how Myc can be regulated by a single weak CPD, thus represents another gap in our understanding of Myc proteolysis via Fbw7.

Taken together, the comparison of Myc with other substrates of Fbw7 reveals that Myc-regulation *presumably* via a single degron differs markedly from the canonical route of Fbw7 proteolysis. Because Fbw7-mediated Myc proteolysis represents an important barrier for oncogenesis, I decided to address this discrepancy and clarify its underlying biological reasons.

3.2 RESULTS

3.2.1 *Usp28 shields Myc from Fbw7-mediated proteolysis*

I first addressed the limited ability of Fbw7 to target Myc for proteolysis. Fbw7 targets Myc upon T58 phosphorylation. Given that Myc is resistant to Fbw7 proteolysis despite the fact that T58-phosphorylation occurs throughout the cell cycle suggested that there must exist mechanisms in the cell to shield Myc from Fbw7-mediated destruction. Recently the Eilers lab described such a protective role for the de-ubiquitylating enzyme Usp28, in Myc proteolysis. By showing a

ternary complex between Myc, Fbw7, and Usp28, the Eilers lab argued that Fbw7 and Usp28 form a futile cycle of ubiquitin addition and removal on Myc, which in turn limits the influence of Fbw7 on Myc turnover (Popov et al. 2007). Under special cellular conditions such as DNA damage, however, Usp28 dissociates from Fbw7 and consequently allows for rapid destruction of Myc (Popov et al. 2007).

Based on my previous observation that Myc phosphorylation at T58 occurred constitutively during the cell cycle (Figure 2.8), I predicted that removal of Usp28 would relieve the shielding process and in turn would render Myc vulnerable for destruction by Fbw7 at all cell cycle stages. To address this question, I generated U2OS cells expressing either a control luciferase shRNA or shRNA against Usp28. Moreover, to test if Fbw7 was an important contributor in this process, I transiently co-expressed either control or Fbw7 siRNA in these cells. Results of this analysis are shown in Figure 3.2. I first compared the effects of changes in Fbw7 and Usp28, either alone or together, on total levels of Myc and Cyclin E by western blotting. Consistent with the shielding model knockdown of Usp28 led to a reduction in steady-state Myc levels (Figure 3.2A, compare lane 1 to 3). Moreover, Myc levels were rescued to near control levels by co-expression of Fbw7 siRNA in the cells expressing Usp28 shRNA (Figure 3.2A, compare lane 1 to 4), suggesting that in the absence of Usp28, Fbw7 was a prominent means to mediate Myc-destruction. Finally, to relate the effect of Usp28 loss in the context of the cell cycle, I studied the effect of Usp28 knockdown on Myc levels throughout the cell cycle using LSC. I stained Myc by

immunofluorescence in cells expressing either shRNA against luciferase (as a control) or Usp28. Consistent with the T58-phosphorylation patterns observed using elutriation (Figure 2.8), my LSC analysis showed that in cells with reduced levels of Usp28, Myc levels dropped consistently throughout all stages of the cell cycle (Figure 3.2B).

I concluded that the Fbw7 pathway is indeed a bonafide proteolysis pathway for Myc and can target Myc for proteolysis at all stages of the cell cycle in the absence of Usp28. In summary, therefore, these results resolved one the major paradoxes of Myc proteolysis via Fbw7 and showed that Myc resists Fbw7 proteolysis because of the shielding effects exerted by Usp28.

3.2.2 Myc-Box I mutations bind and respond to Fbw7

Because my previous analysis confirmed Fbw7 to be a context-dependent Ub-ligase for Myc, I decided to ask how Myc is targeted by Fbw7 via a single weak CPD harbored within the conserved MB-I region. Fbw7 overexpression reduces Myc levels in a proteasome-dependent manner; therefore, I analyzed the influence of Fbw7 using transient overexpression of Myc and Fbw7 in U2OS cells. I first tested the relative contribution of different residues in the Myc CPD (residues: 58-62, hereafter called CPD1) in Fbw7 proteolysis. I made systematic point and deletion mutations in Myc spanning the entire CPD1 region and characterized the effects of the following mutations: a four amino-acid deletion of L56-P59, T58A, S62A, and T58A+S62A, thus removing the phosphorylation sites at T58 and S62, either alone or together. I first tested Myc binding to Fbw7 α , the

major nucleoplasmic isoform of Fbw7, using immuno-precipitation assays with HA-tagged WT or mutant Myc protein and FLAG-tagged Fbw7 α . The results of this analysis (Figure 3.3A) show that immune-complexes containing either WT or mutant Myc were able to recover Fbw7. Conversely, Fbw7 immune-complexes were able to recover both WT and the mutant Myc proteins. I concluded that mutations in CPD1—including T58A, S62A, and the T58A+S62A double mutant—retained the ability to bind Fbw7. This result was *inconsistent* with existing literature, and I decided to test this more systematically.

I decided to carefully analyze the relative interaction of Myc with different isoforms of Fbw7. Physiologically, Fbw7 is expressed as three alternately spliced variants, Fbw7 α , β , and γ , which differ in their sub-cellular localization. Fbw7 α , the predominant isoform of Fbw7 is nucleoplasmic, Fbw7 β localizes to the cytosol, and Fbw7 γ localizes to the nucleolus. Given that Myc is predominantly nuclear, it is believed to be a substrate for both Fbw7 α and Fbw7 γ , but has been shown *not* to interact with Fbw7 β (Welcker et al. 2004). I therefore decided to co-express Myc with Fbw7 in U2OS cells and compared the interaction of Myc with Fbw7 α versus the cytosolic isoform, Fbw7 β . Figure 3.3B shows the results of this analysis. Consistent with previous reports, I observed that whereas Myc binds favorably to the α -isoform of Fbw7, its ability to interact with Fbw7 β was much lower (Figure 3.3B: compare lane 1 to 3; and lane 5 to 7). Moreover, in this assay, I compared the interaction of WT Myc and Fbw7 with the T58A mutant. This comparison revealed several important points. First, comparing the interaction of Fbw7 α with WT or T58A revealed that, as expected, WT Myc

bound Fbw7 α more favorably than T58A (Figure 3.3B: compare lane 1 to 2; and lane 5 to 6). However, comparing the association of T58A with the two Fbw7 isoforms revealed that T58A bound Fbw7 α at much higher levels than Fbw7 β (Figure 3.3B: compare lane 2 to 4; and lane 6 to 8). Given that earlier reports have shown that Fbw7 β is a cytosolic protein which does not encounter cellular Myc suggests that Fbw7 β interaction with Myc probably reflects background or non-specific levels of association. Therefore, results of this experiment show that, although diminished as compared to WT Myc, T58A retains a partial ability to bind Fbw7 α .

My interaction analysis suggests that mutations in CPD1, did not completely block Myc interaction with Fbw7, arguing that other residues outside CPD1 can possibly recruit Fbw7. I therefore tested if the CPD1 Myc mutants could respond to changes in Fbw7 α levels. I compared the effect of Fbw7 overexpression on the steady-state levels of WT and mutant Myc. Consistent with the immuno-precipitation results, the Myc mutants continued to respond to changes in Fbw7 levels, with their levels readily dropping upon Fbw7 expression. In contrast, expression of Fbw7 had no significant effect on the levels of GFP, used as a non-specific control protein in this analysis (Figure 3.3C).

Taken together, all these results are rather surprising because they show that inactivating Myc mutants in the known CPD continue to bind and respond to Fbw7. I reasoned one possible explanation for these results could be the presence of a second—as yet unknown—Fbw7 recognition sequence in Myc

outside of MB-I. Given that some other substrates of Fbw7 both in yeast and mammalian cells harbor multiple Fbw7 degrons as a means to fine-tune their proteolysis, I decided to further investigate this possibility and potentially identify additional degrons in Myc.

3.2.3 *Myc residues 244-248 harbor a consensus Fbw7-degron*

To facilitate the identification of the second recognition site, I decided to establish a consensus Fbw7-recognition sequence based on a comprehensive analysis of the best characterized Fbw7 substrates. Given the structural conservation between Fbw7 from both yeast and humans, I performed a detailed alignment using annotated phospho-degrons from several Fbw7 substrates both in yeast and humans. This analysis, illustrated in Figure 3.4, revealed a consensus five residue stretch, which is important for Fbw7 recognition: (S/T)-P-X-X-(S/T). The consensus sequence suggests that Fbw7 prefers motifs where two amino-acids spaced four residues apart (site “0” and “+4”) are either negatively-charged or can acquire a negative charge via phosphorylation. Furthermore, comparing the phospho-degrons from human Fbw7 substrates alone shows that the “+1” and “+2” sites are often prolines in numerous mammalian Fbw7 degrons. In theory therefore, a mammalian degron for Fbw7 would read as follows: (S/T)-P-P-X-(S/T).

By studying the Myc sequence for the presence of such a consensus I identified not only the previously documented degron—residues 58-62: TPPLS, but also the presence of a second potential degron harbored within residues 244-

248: TPPTT. I decided to study whether region 244-248 in Myc functions as a second Fbw7-destruction sequence. Because MB-I, the location of the first Fbw7 degron, is a well characterized mutation cluster in lymphomas, I first asked if region 244-248 also harbored cancer-associated genetic changes. Indeed, I found that three residues within this region—P245, T247, and T248—have been previously reported as sites of cancer-associated mutations in Myc and this pattern of clustered mutations (cluster *e* in Figure 1.3b) is similar to the clustering of mutations within CPD1 (cluster *a* in Figure 1.3b), suggesting that these two regions could share functional similarities, including the ability to regulate Myc proteolysis.

I therefore tested the notion that residues within region 244-248 play a role in regulating Myc proteolysis with Simone Salghetti (Tansey laboratory). I engineered 3T3 primary mouse fibroblasts stably expressing either WT or mutant Myc proteins and Simone analyzed their stability using cyclohexamide chase. In this experiment we studied the P245A cancer mutant and a deletion mutant of the entire 244-248 region for changes in stability compared to WT Myc. As a positive control, I generated 3T3 cells stably expressing the T58A mutant. Results of this experiment, presented in Figure 3.5A, show that indeed both, the P245A and the Δ 244-248 mutations increased Myc stability compared to WT-Myc and showed decay patterns similar to the T58A mutant. I concluded that region 244-248 was important for Myc proteolysis, and changes in this region either by point mutations or deletions, attenuate Myc destruction.

This analysis confirmed an important role for the 244-248 region in Myc proteolysis. Because this region harbored a consensus CPD sequence, I predicted a model where Fbw7 has two degrons in Myc: CPD1 (residues 58-62) and CPD2 (residues 244-248). This model suggests that mutations in either one of the two CPDs would still allow Myc destruction in response to Fbw7 via the other degron, as observed earlier in the case of CPD1 mutants (Figure 3.3C); however, double-mutants of Myc in both the CPDs would resist Fbw7-mediated proteolysis.

I therefore tested the role of Fbw7 in the destruction of Myc CPD2. I co-expressed Fbw7 transiently with either a CPD2 mutant alone (P245A) or with a double mutant (T58A+P245A) in U2OS cells. Additionally, as a control I expressed the Myc-interacting protein, Max. Results of this experiment are shown in Figure 3.5B. As expected, levels of the control protein, Max, were unchanged upon Fbw7 overexpression. However, although levels of the P245A mutant reduced in response to Fbw7 expression, the T58A+P245A double mutant failed to show any discernible response to changes in Fbw7 levels, thus resisting destruction via Fbw7. Results of this experiment along with the computational alignment and the results obtained from the stability experiments illustrate the presence of a second consensus-Fbw7 site in Myc within residues 244-248, which presumably functions as a degron and is represented as a mutation cluster in B-cell lymphomas.

3.2.4 *In vitro* assays show mutations of the second degron are oncogenic

Region 244-248 represents a cancer-associated mutation cluster; therefore, in collaboration with Claudio Scoppo in Scott Lowe's laboratory, I decided to address the idea that Myc mutants within region 244-248 are important in oncogenesis. We first tested the activity of these mutants *in vitro*. Because one common way to study the oncogenic potential of proteins is to study their ability to induce anchorage-independent growth in fibroblasts, we tested the Myc mutants for their ability to induce colony formation in soft agar.

For this analysis we used NIH3T3 primary mouse fibroblasts stably expressing either WT or Myc mutants. We compared the soft agar colony-forming ability of WT Myc versus Myc mutants in either CPD1 or CPD2. Results of this assay are shown in Figure 3.6A. By counting the number of colonies and visually comparing the colony morphology, we observed that Myc induced colony formation in these cells at levels much higher than background (compare empty vector control versus WT Myc). More interestingly, however, we found that mutations in Myc such as T58A and P245A, led to a nearly 1.5 – 2 fold increase in the number of colonies. The colonies formed by the T58A and P245A mutant were relatively larger and morphologically different from WT Myc. Whereas WT Myc formed more regular rounded colonies, the Myc mutants induced irregular colony growth with disorganized outgrowths breaking off the colony integrity. We concluded from these observations that mutations in CPD1 or CPD2, as measured by cellular transformation, induce the oncogenic ability of Myc.

We then tested the effects of WT and Myc mutants in proliferative assays. Because Myc mutations occur naturally in blood-borne cancers, we decided to test their influence using a competition assay in cells of the hematopoietic compartment. We cultured hematopoietic cells under previously described growth conditions which favored growth of B-cells (Whitlock et al. 1982). We transduced the cells with either WT or mutant Myc using vectors which co-expressed GFP. By following the population of GFP positive cells by FACS, we could quantitatively track the enrichment of cells expressing a certain Myc mutant. The changes in the percentage of GFP-positive cells over a time-course of several days thus correlates with the advantage conferred upon these cells by the respective Myc mutant (Figure 3.6B). Under these experimental conditions we made two important observations. First, our analysis revealed that all the Myc mutants tested in this assay scored higher than WT Myc, suggesting that cancer-associated Myc mutations confer a competitive growth advantage over WT Myc in the hematopoietic compartment. Moreover, we found that the cells expressing the $\Delta 244-248$ CPD2 mutant either alone or together with the T58A mutant scored higher than the cells expressing P245A. We concluded that under these assay conditions, although all Myc mutants confer a growth advantage compared to WT Myc, a deletion of CPD2 confers a bigger advantage compared to the point mutant, P245A, suggesting that other residues in CPD2 may also play an important role in this process.

3.2.5 P245A is an aggressive oncogene

Using *in vitro* assays, we were able to demonstrate the importance of CPD2 in Myc function. We therefore decided to extend our analysis and investigate the role of these mutations in physiologically-relevant animal models of blood-borne cancer. Because Myc has been historically identified as the oncogene driving a majority of B-cell cancers, especially Burkitt's lymphoma, there has been significant effort to recapitulate Myc-mediated lymphomagenesis in animal cancer models. Currently there exist two animal models of lymphomagenesis which have met with some degree of success: the E μ -Myc model and the adoptive-transfer model. Despite its many utilities, the E μ -Myc model system can be used to study cancer development only in the genetic context of WT Myc expression. On the other hand, the adoptive transfer model for lymphomagenesis, allows us to study tumor development either using WT or mutant Myc expression. Because our goal was to study the effects of the mutant Myc proteins, we chose the adoptive transfer method.

Using enriched hematopoietic stem cell preparations from embryonic livers, we transduced Empty Vector, WT, or mutant Myc using retroviral vectors which co-expressed GFP. We tested the relative oncogenicity of the CPD1 or CPD2 mutants compared to WT Myc, using the T58A or P245A mutant respectively. Development of tumors was followed by routinely palpating mice for the presence of enlarged lymph nodes, and oncogenicity was measured based on the survival kinetics of the animals injected with the different Myc-expressing cells. Our results, as shown in Figure 3.7, establish several important

observations. First, as previously reported we observed that T58A was more oncogenic than WT Myc and significantly increased the penetrance of lymphoma with a much smaller latency period. More interestingly, however, we observed that the P245A mutant derived from the second-degron developed lymphomas with a time-course nearly identical to T58A and in some cases with a higher disease penetrance. Although it is difficult to conclude which amongst the two CPD-mutants is more oncogenic from these results; compared to WT Myc we conclude that the P245A mutant is indeed an aggressive oncogene, suggesting that CPD2 function is important in the control of lymphomagenesis.

3.3 DISCUSSION

For many years, it has been known that Myc proteolysis is compromised in cancers. However, the full extent and mechanism of this alteration has not been appreciated. In this context, the discovery of another region in Myc which regulates its stability downstream of the Fbw7 tumor suppressor is indeed important. Although, Fbw7 has been shown to be an Ub-ligase for Myc, our understanding of Myc proteolysis by this process has been limited due to several inconsistencies in the regulation of Myc versus the regulation of other cellular substrates of Fbw7. Here, by resolving two long-standing discrepancies: the resistance of Myc to Fbw7 proteolysis under normal cycling conditions and the process of Myc targeting by Fbw7 via a single weak CPD, my analysis reinforces the importance of the Fbw7 pathway in Myc oncogenesis.

Under normal cycling conditions Fbw7 activity is attenuated by the deubiquitylating enzyme Usp28, presumably because Myc function is important to drive cell cycle progression. Conversely, [as demonstrated by others (Popov et al. 2007)] inactivation of this shielding process in response to specific cellular stimuli allows Fbw7 to rapidly destroy Myc and thus prevent cell cycle progression. The counter-acting activities of Fbw7 and Usp28 on Myc, therefore, establish a rapid cellular means to clear Myc and limit its biological activity.

Given that Usp28 exerts a protective role on Myc turnover, one would predict that Usp28 levels should be induced in cancers. Surprisingly however, copy number analyses from cancers suggest that the Usp28 containing genomic locus is a site of frequent deletions (Knuutila et al. 1999; Hicks et al. 2006). This observation is somewhat counter-intuitive, given that Usp28 loss destabilizes Myc by making Myc vulnerable to destruction by Fbw7. I speculate that this phenomenon possibly underlies the need for inactivation of Fbw7 in cancers. The loss of Fbw7 function in these cancers, therefore, could probably serve as a secondary compensatory mechanism to overcome the oncogenic barrier established by loss of Usp28 function.

Although inactivation of Fbw7 has been described in many cancers the resistance of Myc to Fbw7 proteolysis under proliferating conditions raised doubts about the importance of this process in Myc-dependent tumorigenesis. My analysis now reveals some insights about the importance of Fbw7-mediated tumor suppression in Myc biology. This analysis shows that similar to other Fbw7 substrates, Myc harbors at least two CPDs and mutations in these regions, which

occur in blood cancers, interfere with Fbw7-mediated proteolysis. Similar to the regulation of Cyclin E which also harbors two CPDs (Welcker et al. 2007), I now speculate that the two Myc degrons simultaneously mediate the recruitment of an Fbw7 dimer and that disruption of this process attenuates Myc proteolysis in several cancers.

The similarity in the oncogenic effects of the CPD1 and CPD2 mutants raises a very pertinent question: why have two degrons where one would suffice? We consider two possibilities for this observation. First, based on observations made with other cellular Fbw7 substrates, and considering both Myc CPDs occur in regions conserved throughout the Myc family (Luscher et al. 1989; Atchley et al. 1995), suggests the possibility that Myc too evolutionarily retains multiple low-affinity CPDs as a means to achieve greater regulatory control over Fbw7 proteolysis. Second, given the importance of environmental cues in Myc proteolysis at CPD1, it is not difficult to imagine that the regulation of Myc turnover via these two degrons could occur in response to different cellular needs—*independent of one another*. One possible means to achieve such a differential regulation could be via differences in the cellular kinase(s) which phosphorylate Myc at these two degrons. Indeed, by comparing the regulation of phosphorylation at CPD1 with the phosphorylation events at the T244 and T248 sites in the second degron, I continue to currently investigate this rather interesting possibility.

3.4 MATERIALS AND METHODS

3.4.1 LSC analysis

All experiments were performed using the LSC-iCys system (Compucyte, MA) attached to an Olympus IX-71 microscope. Signal from multiple fluorophores was compensated by measuring the spectral bleed into other channels using controls containing each individual fluorophore, and subtracting the corresponding non-specific leakage. Thresholds for measuring each fluorophore were set at signal intensities where there was negligible background signal. The fluorescence units obtained thereafter were used to compare the expression patterns within different cell cycle populations. Approximately 2000-3000 cells were scanned in each individual analysis and cell cycle profiles were gated based on DNA content into G1, S, and G2/M populations.

For quantification, the mean signals of the respective proteins were measured within the given cell cycle gates. Fluorescence values were normalized to a ratio of green/blue fluorescence to quantify relative levels of Myc protein in different cell cycle stages as a function of DNA.

3.4.2 RNA interference

Myc and Fbw7 knockdown was performed using RNA interference. Pools of siGenome RNA against Myc, Fbw7, and Luciferase (control) were obtained from Dharmacon, and transiently transfected into U2OS cells using the Oligofectamine reagent (Invitrogen).

The effect of USP28 was studied by transfecting shRNA pools against USP28 into U2OS cells. Knockdown of USP28 expression was confirmed using quantitative RT-QPCR, and found to be ~50% (Data not shown).

3.4.3 *Immuno-precipitation analysis*

Immuno-precipitation analysis to study the binding of Fbw7 and Myc was studied by transiently co-transfecting U2OS cells with p3xFLAG-Fbw7 (0.5 µg), pCGT-Max (250 ng), and pCGN-Myc (50 ng) using the Fugene 6.0 transfection reagent. After 48 hrs of transfection, cells were lysed under non-denaturing conditions (50 mM Tris [pH:7.5], 170 mM KCl, 1 mM EDTA, and 0.5% NP-40 with fresh protease inhibitor cocktail). Cell lysates were immuno-precipitated using either α -FLAG (M2 antibody, 1:1000) or α -HA (12CA5 antibody, 1:500). Immune complexes thus recovered were analyzed by western blotting.

3.4.4 *Anchorage-independent growth*

The ability of Myc to induce anchorage-independent growth was studied using NIH3T3 cells stably expressing either WT- or mutant-Myc oncoproteins. The assays were done by studying the growth properties of Myc in soft-agar medium. Specifically, 3.2% agarose was diluted to 0.8% using DMEM+10% Calf Serum+Pen-Step. 2.5 ml basal layer was poured in each well of a six well dish and allowed to set for 10 min at RT followed by another 10 min at 4°C. Equal volume of 0.8% agarose (pre-warmed at 55°C) was mixed with the respective 3T3 cells, to yield a final concentration of 10,000 cells/ml of 0.4% agarose. 2 ml

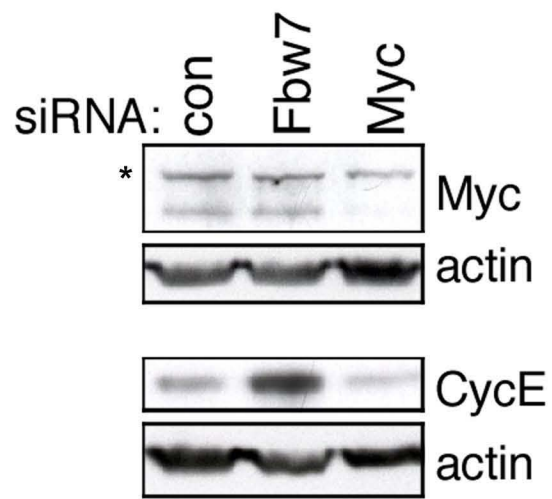
of this cell mix in soft agar was poured on top of the basal layer and allowed to set for 10 min at RT followed by 10 min. After overnight incubation, the agarose was layered with 1 ml of growth medium which was carefully replaced every four days for the entire course of the assay, usually 2 – 3 weeks. Colonies formed in the different samples were counted under a bright-field microscope and graphically represented relative to the colonies formed by WT-Myc (arbitrarily set to 100).

3.4.5 Adoptive transfer assay for lymphomagenesis

Adoptive transfer assays were done using stem cell cells isolated from embryonic fetal livers derived from pregnant E14.5 C57BL/6 WT mice. The stem cells were infected every 12 hrs four times with retroviruses expressing either WT or mutant Myc using vectors which co-expressed GFP. Retroviruses were derived using standard techniques in ecotropic phoenix packaging lines. Prior to reconstitution, the population of GFP-positive cells was measured, and cells were injected via the tail vein into cohorts of five sub-lethally irradiated C57BL/6 WT mice. Reconstituted animals were routinely monitored for signs of disease by palpating the lymph nodes and survival was defined based on the time taken from stem cell reconstitution until the animal was sacrificed. Tumors derived from the animals were processed into single cell suspension and analyzed for cell surface markers using FACS analysis.

Figure 3.1: Effect of Fbw7 knockdown on Myc and Cyclin E

U2OS cells were transfected with siRNA duplexes against luciferase-control, Fbw7, or Myc. Cell lysates were assessed using western blots for Myc, Cyclin E, and Actin. The Western blot shows that levels of Cyclin E, but not Myc, are induced upon Fbw7 knockdown. Knockdown of Myc leads to a reduction in Myc levels, as expected.



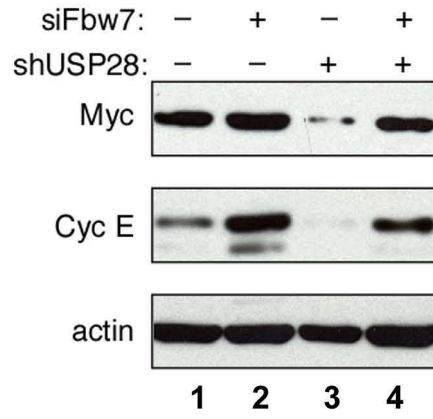
* non-specific band

Figure 3.2: Usp28 knockdown reduces Myc levels at all cell cycle stages

(A) U2OS cells were co-transfected with shRNA against control-luciferase or Usp28 with or without siRNA against Fbw7. Compared to control cells (lane 1), knocking down Fbw7 alone (lane 2) has very little effect on Myc whereas knocking down Usp28 alone (lane 3) leads to a reduction in Myc levels. Knocking down Fbw7 in cells with Usp28 knockdown, rescues Myc levels to near control levels, suggesting that in cells with lower Usp28 levels, Fbw7 represents a prominent pathway for Myc destruction.

(B) LSC-analysis of cells was done using U2OS cells expressing shRNA against either control-luciferase or Usp28. Usp28 knockdown leads a reduction in Myc uniformly at all stages of the cell cycle.

(A)



(B)

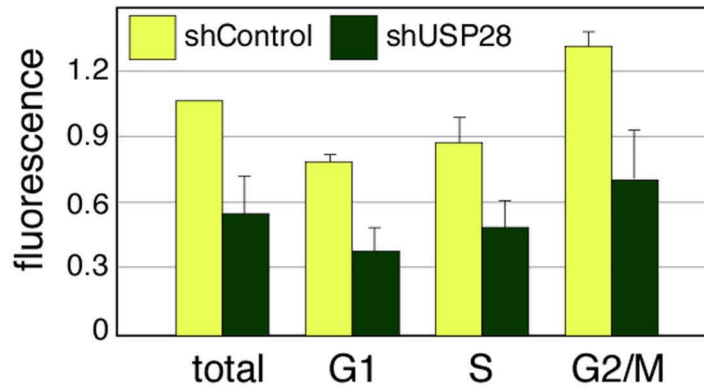


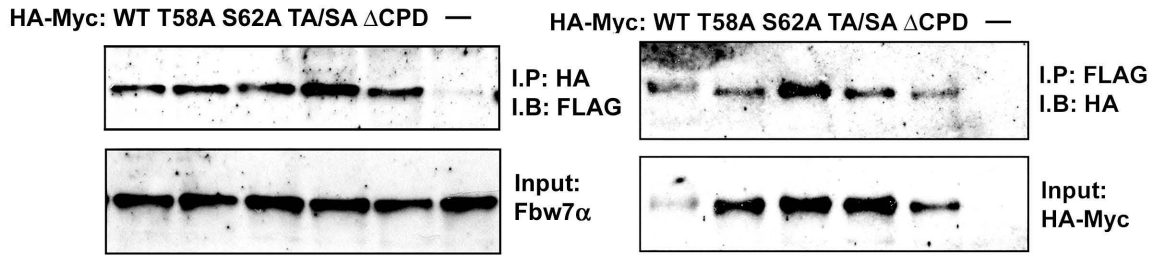
Figure 3.3: MB-I mutants retain partial ability to bind and respond to Fbw7 expression.

(A) HA-tagged Myc-Box I mutations, T58A, S62A, TA/SA (T58A+S62A), and Δ CPD1 (residues 56-59: LPTP) were co-expressed with FLAG-tagged Fbw7. Immuno-precipitations were performed by recovering either HA-Myc or FLAG-Fbw7 in parallel. Immuno-blotting shows that Myc mutants continue to recover a significant fraction of Fbw7 and *vice versa*.

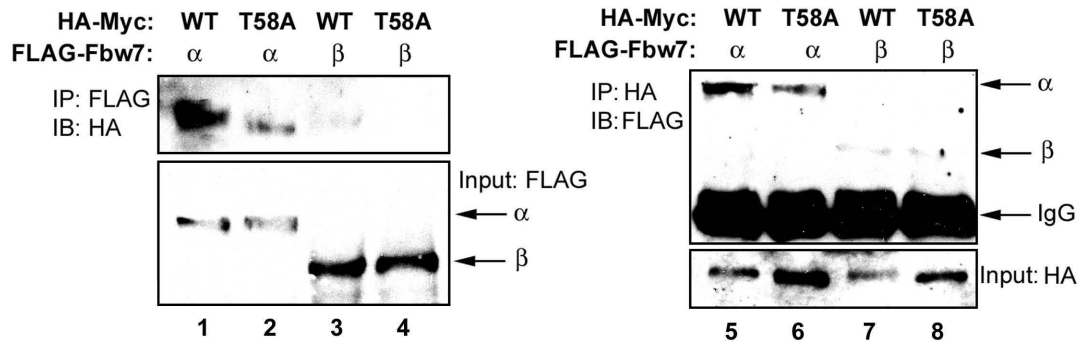
(B) Myc interaction with specific forms of Fbw7 was studied by co-expressing WT- or T58A-Myc with either Fbw7 α or Fbw7 β . Immuno-precipitations were performed in parallel for either the HA- or the FLAG-epitope and immuno-blotted. Compared to Fbw7 α , Fbw7 β interacts poorly with Myc. Although T58A binds Fbw7 α much weaker than WT-Myc; this binding is significantly more than the near-background levels of Myc recovered by Fbw7 β

(C) Consistent with the results of the immuno-precipitation experiments, steady-state levels of Myc mutants drop in response to forced expression of Fbw7. In contrast, the non-specific protein GFP does not respond to changes in Fbw7 expression.

(A)



(B)



(C)

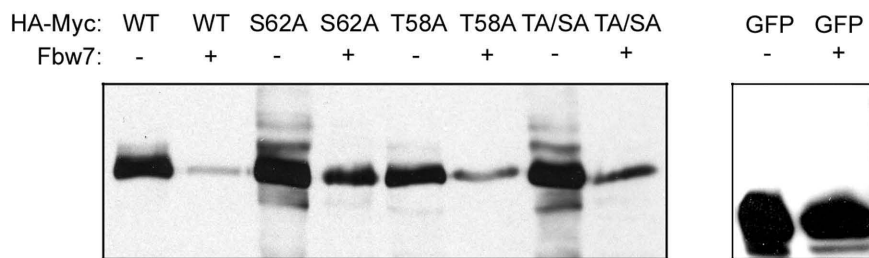


Figure 3.4: Sequence alignment of multiple Fbw7 targets reveals the presence of a second Fbw7-binding site in Myc.

(A) Multiple sequence alignment of phosphor-degrons from numerous yeast and mammalian Fbw7 substrates shows conservation of the “0”, “+1”, and “+4” sites as serine/threonine, proline, and serine/threonine respectively.

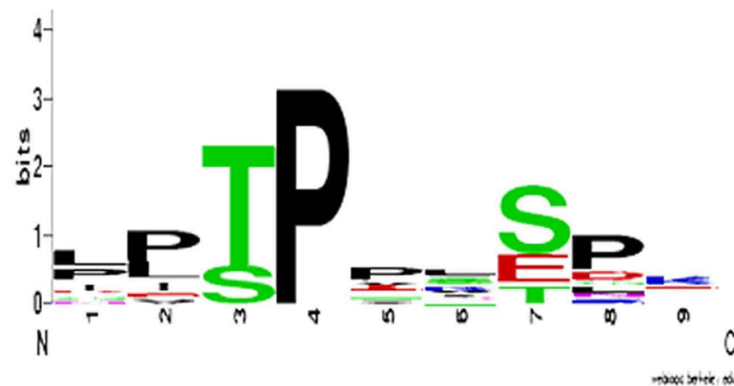
(B) Weblogo of the consensus Fbw7 recognition sequence.

(C) Primary sequence analysis of Myc shows two sequences (bold-faced) which fit the Fbw7 consensus: the originally characterized binding site at residues 58-62 and a second recognition site harbored within region 244 – 248.

(A)

<i>CyclinE_T380/1-9</i>	L	L	T	P	P	Q	S	G	K
<i>SREBP1_T456/1-9</i>	T	L	T	P	P	P	S	D	A
<i>ScFar1_S87/1-9</i>	P	I	S	P	P	P	S	L	K
<i>ScRcn1_S113/1-9</i>	L	I	S	P	P	A	S	P	P
<i>Myc_T58/1-9</i>	L	P	T	P	P	L	S	P	S
<i>Jun_T239/1-9</i>	G	E	T	P	P	L	S	P	I
<i>ScCdc6_T368/1-9</i>	P	L	T	P	T	T	S	P	V
<i>ScRum1_T58/1-9</i>	P	P	T	P	A	K	T	P	K
<i>SV40Ta_T701/1-9</i>	P	P	T	P	P	P	E	P	E
<i>ScCdc6_T39/1-9</i>	D	V	T	P	E	S	S	P	E
<i>ScClb6_T39/1-9</i>	N	L	T	P	H	S	T	N	E
<i>Notch1_T2512/1-9</i>	F	L	T	P	S	P	E	S	P
<i>CyclinE_T62/1-9</i>	I	P	T	P	D	K	E	D	D
<i>Presenilin1_T116/1-9</i>	I	Y	T	P	F	T	E	D	T
<i>ScGcn4_T165/1-9</i>	L	P	T	P	V	L	E	D	A
<i>ScClb6_S6/1-9</i>	I	P	S	P	I	S	E	R	K
<i>Myc_T244/1-9</i>	E	E	T	P	P	T	T	S	S

(B)



(C)

MPLNVSFTNRNYDLDYDSVQPYFYCDEEENFYQQQQQSEIQPPASEDIWKKFELLPTPPLSPSRRS
GLCSPSYVAVTPFSLRGDNDGGGGSFSTADQLEMVTELLGGDMVNQSFICDPDDETFIKNIIQDCMW
SGFSAAKLVSEKLASYQAARKDSGSPNPARGHSVCSTSSLYLQDLSAAASECIDPSVVFYPLNDSS
SPKSCASQDSSAFSPSSDLSLSTESSPQGSPEPLV LHEETPPTTSSDSEEEQEDEEIDVWSVEKRQ
APGKRSESGSPSAGGHSKPPHSPLV LKRVHSTHQHNYAAPPSTRKDYPAAKRVKLDVSRVLRQISN
NRKCTSPRSSDTEENVKRRTHNVLERQRRNELKR SFFALRDQIPELENNEKAPKWILKATAYILSVQ
AEEQKLISEEDLLRKRREQLKHKLEQLRNSCA

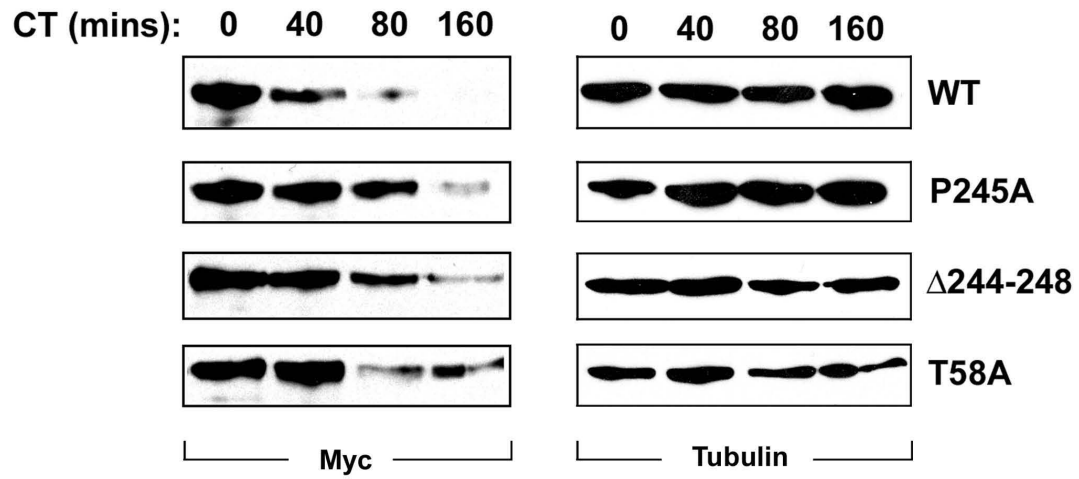
Figure 3.5: Region 244 – 248 is important for Fbw7-mediated proteolysis

*(A) Myc stability was studied using Cyclohexamide chase. The P245A and D244-248 mutants are more stable compared to WT-Myc, and follow decay patterns similar to the T58A mutant. These results confirm that the 244-248 region is important for Myc proteolysis.

(B) Overexpression of Fbw7 reduces P245A levels. However, the double-mutant T58A+P245A is resistant to Fbw7 proteolysis. As a control, T7-tagged Max is expressed and shows no change in response to Fbw7.

(* This experiment was done by Simone Salghetti, Tansey laboratory)

(A)



(B)

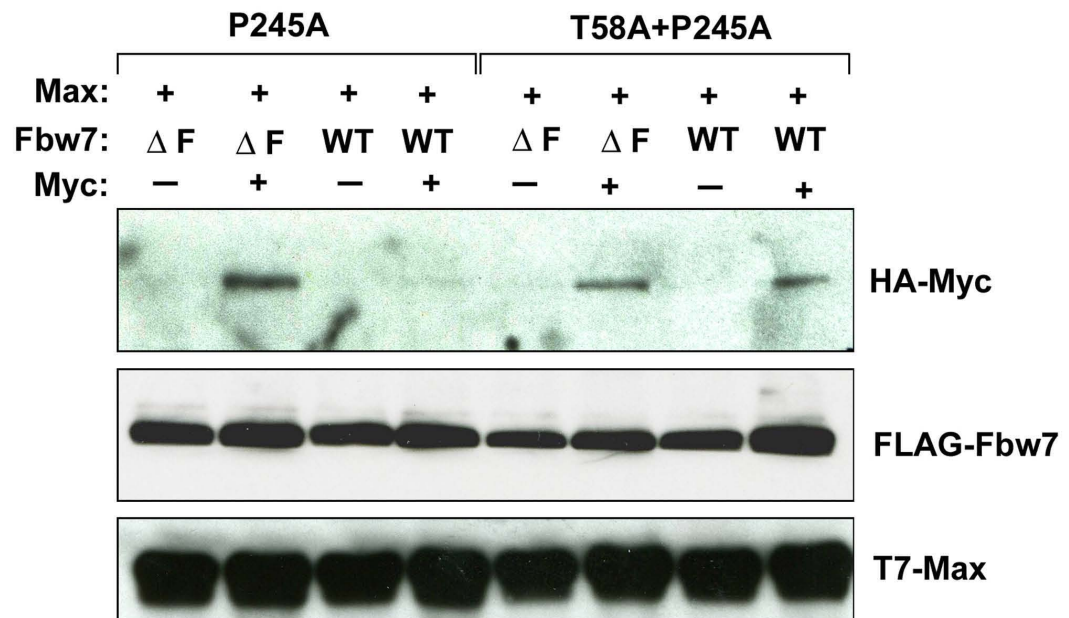


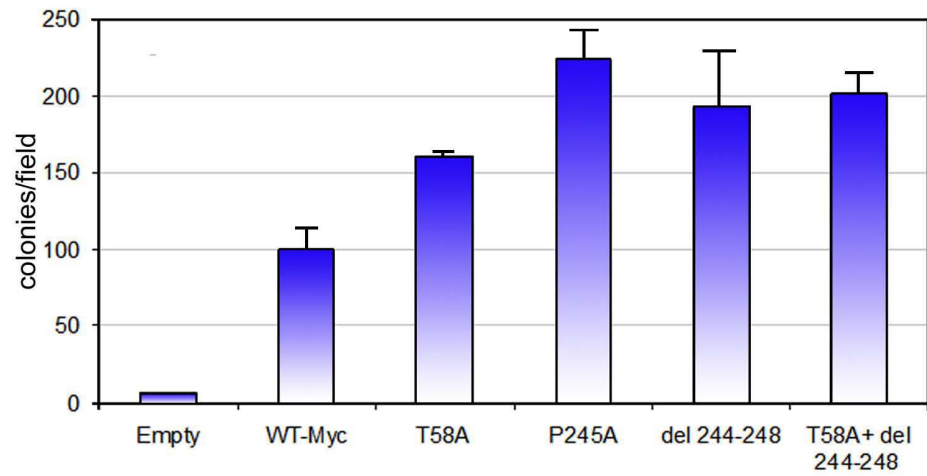
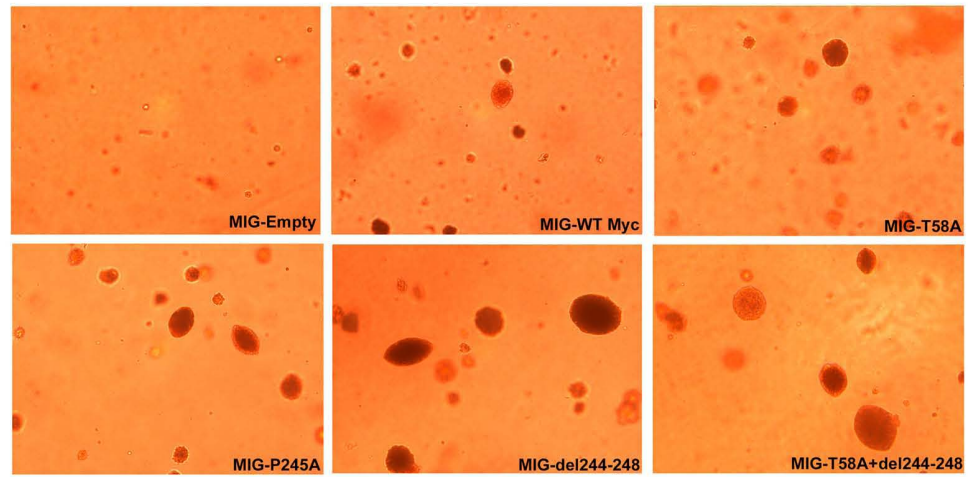
Figure 3.6: Mutants in the 244-248 region are oncogenic *in vitro*.

* (A) Soft agar colony formation assay using 3T3 cells stably expressing either WT or cancer-associated Myc mutants shows that Myc cancer mutants are more oncogenic than WT. Note in particular the much larger colony morphology in mutants of the second Myc degen. The graph depicts quantitations of colony number from three independent experiments as Mean \pm S.D.

* (B) The Whitlock-Witte assay to study the effect of Myc mutants on B-cell proliferation was done using WT and mutant Myc for a period of 8 days. The enrichment of GFP-positive cells shows that all Myc mutants score higher than WT Myc. Among the mutants, Δ 244-248 and the T58A+ Δ 244-248 double mutant yield the highest competitive advantage. Results are plotted as Mean %GFP-positive cells from six independent experiments.

(*These experiments were done in collaboration with Claudio Scoppo, Lowe laboratory)

(A)



(B)

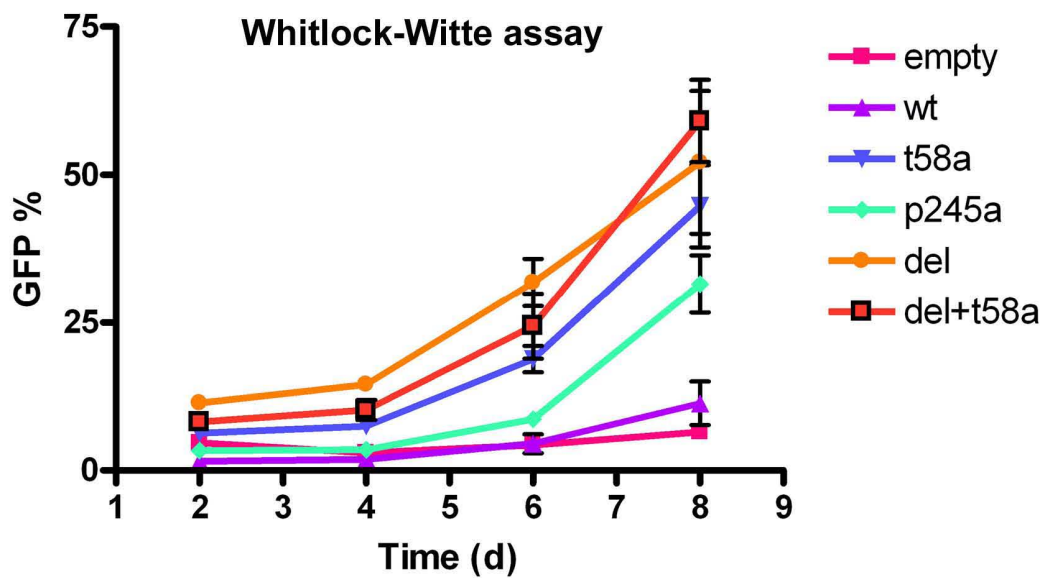


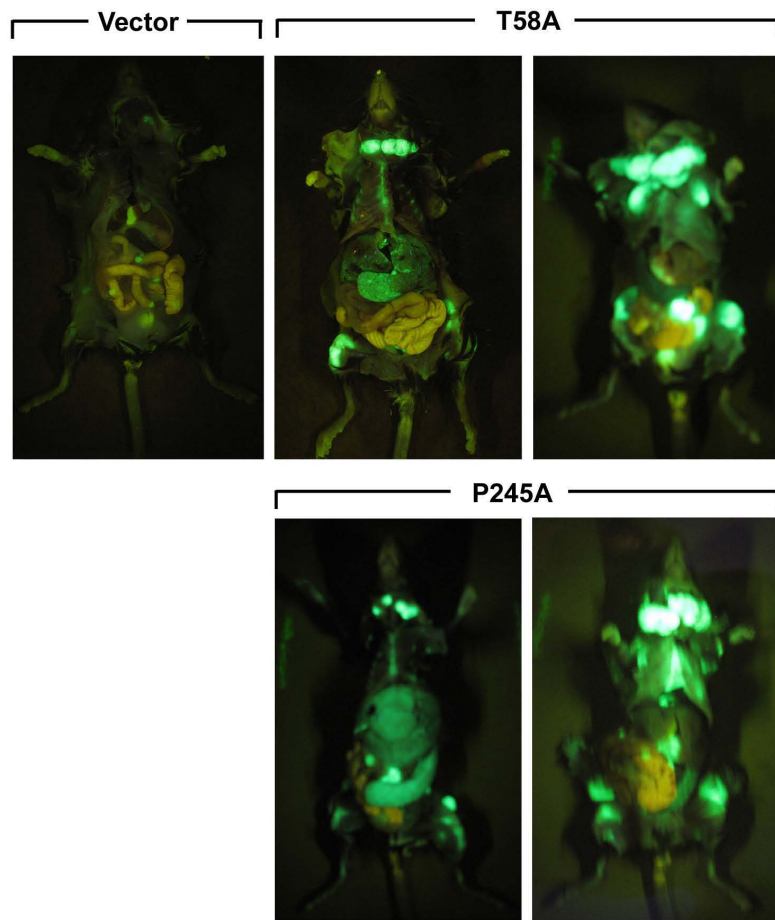
Figure 3.7: The P245A cancer mutant is highly oncogenic in animal models of lymphomagenesis.

* (A) GFP-positive lymphomas were observed in animals expressing the Myc-mutants. Animals injected with cells expressing Empty-Vector as a control, did *not* develop any tumors during the course of analysis.

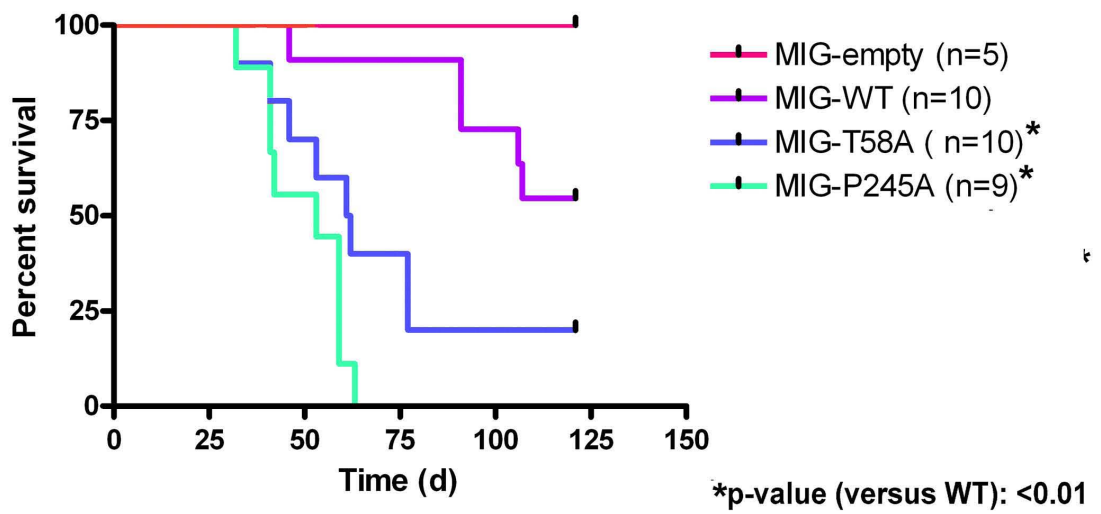
* (B) Kaplan-Meier survival curves showing the comparison between animals injected with cells expressing either Empty Vector (Control), WT, T58A, or P245A Myc. Compared to WT Myc, both the mutants tested, T58A and P245A, showed higher disease penetrance and induced tumors with reduced disease latency.

(*These experiments were done in collaboration with Claudio Scuoppo, Lowe laboratory)

(A)



(B)



Chapter 4

Regulation of Myc-proteolysis by the adenoviral E1A oncoprotein

Tworkowski KA*, Chakraborty AA*, Samuelson AV, Seger YR, Narita M, Hannon GJ, Lowe SW, Tansey WP. (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. (*Equal authors)

4.1 INTRODUCTION

DNA tumor viruses such as SV40 and adenovirus have been historically utilized to expose the cellular mechanisms underlying oncogenic transformation. It is believed that via specific regions within their small genomes, DNA tumor viruses have evolved mechanisms to hijack host machinery and drive key biological processes such as DNA replication and cell cycle progression. Because loss of growth control due to deregulation of these pathways often underlies the evolution of tumorigenesis, studying DNA tumor viruses over the years has provided a means to identify the most potent cellular pathways mediating cell proliferation, apoptosis, and oncogenic transformation.

One of the best studied oncogenes from DNA tumor viruses is the adenoviral E1A oncogene. Expressed as an early gene upon viral infection, E1A has been demonstrated to mediate several key cellular processes, including the ability to induce cell proliferation, inhibit differentiation, and regulate cell death

(Sang et al., 2002). E1A harbors discrete regions to mediate its biological functions, and consistent with their functional importance, some of these regions are evolutionarily conserved in E1A from several adenoviral serotypes, and commonly called Conserved Region (CR) 1-4.

One common means by which E1A mediates many of its functions is via the interaction with important modulators of cell growth and apoptosis (Ben-Israel and Kleinberger, 2002). Probably the most prominent of these modulators is the tumor suppressor protein Retinoblastoma (Rb). Rb regulates the cellular activity of the E2F1 protein. Under normal growth conditions, Rb-interacts with E2F at specific stages of the cell cycle and thereby limits E2F ability to induce proliferation. E1A interaction with Rb via an *LXCXE* motif located within CR2 sequesters of Rb and consequently releases the E2F transcription factor. This release from the Rb complex leads to ectopic activation of E2F and results in the activation of genes required for S-phase entry and subsequent cell cycle progression (Howe et al., 1990).

Besides CR2 other regions in the amino-terminus of E1A, including the conserved CR1 region, are also required for E1A's biological activities. However, because these regions do not participate in Rb-binding it is believed that inactivation of Rb may not be the sole mechanism through which E1A functions (Howe and Bayley, 1992). The amino-terminus of E1A binds to numerous cellular proteins; however, the interaction of E1A with a number of chromatin-remodeling factors such as p300/CBP and the SWI/SNF related protein p400 is believed to be particularly important for E1A function. This fact is best evidenced by studying

the importance of E1A-p400 interaction. p400 is essential for the ability of E1A to induce apoptosis, and an E1A mutant that can bind p300/CBP but not p400, is impaired in its ability to induce both cell death and transformation. Although, the importance of p400 in E1A is well established, the downstream cellular target(s) of this pathway was unknown for many years.

Many years ago, it was proposed that E1A and Myc are structurally related. For several years thereafter, E1A and Myc have been independently described to mediate the same biological processes. Moreover, both E1A and Myc bind to many common cellular proteins including the chromatin-remodellers p300 and p400. These results gave rise to the notion that the oncogenic pathways of E1A and Myc could partly overlap. However, no biological link was established in the E1A and Myc pathways. Recently, such a link was revealed and it was demonstrated that during the course of adenovirus infection, E1A expression inhibits the ubiquitin (Ub)-mediated destruction of Myc (Lohr et al., 2003). Considering the importance of both E1A and Myc as cellular oncogenes, and the importance of deregulation of Myc proteolysis in oncogenic pathways, I speculated that the stabilization of Myc by E1A may play a more direct role in E1A activity. I therefore investigated the mechanism through which E1A stabilizes Myc, and probed the role of this mechanism in the function of E1A.

4.2 RESULTS

4.2.1 Adenoviral E1A expression stabilizes Myc

My work to understand the importance of the Myc function in the E1A pathway was done in collaboration with Kathryn Tworkowski in the Tansey laboratory. To study the influence of E1A on Myc stability, Kathryn infected human U2OS cells with Ad5-serotype adenovirus (*d1520*) which expresses WT E1A, and followed the expression of both Myc RNA and protein at various time points following adenoviral infection. Myc protein levels were assessed by Western blot. This analysis revealed that 6 to 8 hours after infection there was a transient increase in the steady-state levels of Myc which then tapered off over the time course of 12-24 hours post-infection (Figure 4.1A). Consistent with previous reports, adenoviral infection led to a decrease in the cellular levels of Myc mRNA (Figure 4.1B). The transient increase in Myc protein levels, therefore, together with the reduction in Myc mRNA levels argue that Myc protein is stabilized upon adenoviral infection. To test this notion, U2OS cells were infected with adenovirus *d1520* and 6 hours after infection protein synthesis was inhibited using the drug Cyclohexamide (CHX). Studying the decay of Myc protein, under these conditions, revealed that adenovirus stabilized Myc considerably. Moreover, this stabilization occurred in an E1A dependent manner, as expression of a control adenovirus expressing LacZ (β -gal), instead of E1A, failed to stabilize Myc (Figure 4.1C). These results confirmed earlier observations regarding the ability of E1A to stabilize Myc and provided us with a biological setting to understand the mechanisms of this process.

4.2.2 E1A stabilizes Myc independent of global inhibition of the proteasome

The amino-terminus of E1A interacts with numerous cellular proteins including many subunits of the 19S proteasome (Turnell et al. 2000; Zhang et al. 2004). This interaction is believed to inactivate proteasomal function and has been demonstrated to result in the cellular stabilization of the tumor suppressor protein, p53. Because Myc too is a direct target for proteolysis via the Ub-proteasome pathway, we considered that the stabilization of Myc by E1A may be an indirect outcome of proteasomal inhibition.

We tested this notion in two independent ways. First, Kathryn used an artificial substrate, U-GFP, which is an unstable version of GFP and is rapidly targeted to the proteasome for destruction. Comparing the decay patterns of U-GFP in cells infected with the WT-adenovirus *d/520* versus the control cells showed that although *d/520* expression led to the stabilization of Myc, it had very little effect on the stability of U-GFP, suggesting that proteasomal activity was not globally attenuated in adenovirus infected cells (Figure 4.2).

Using an entirely different approach, I investigated the sub-cellular localization patterns of Myc in cells infected with adenovirus. Recently it was shown that inhibition of the proteasome using chemical inhibitors led to the redistribution of Myc to the nucleolus (Arabi et al. 2003). If E1A indeed caused a global inhibition of proteasomal activity, I argued that it should induce a similar sub-cellular redistribution of Myc. I addressed this question in U2OS cells following adenoviral infection and observed the localization patterns of Myc by immuno-fluorescence. Results of this experiment are shown in Figure 4.3, and reveal two important points. First, consistent with previous literature, I observed

that inhibition of the proteasome with a chemical inhibitor (MG132) led to an accumulation of Myc in the nucleolus (Figure 4.3A). Importantly however, infection with neither the control adenovirus nor the *dI520* adenovirus induced any discernible change in Myc localization patterns (Figure 4.3B). I concluded that although under some settings, E1A has been shown to inhibit proteasomal activity, this observation cannot completely account for the stabilization of Myc under our experimental conditions.

4.2.3 E1A-p400 interaction is important for Myc stabilization

E1A has been documented to interact with many cellular proteins via small modular domains, and these interactions are believed to contribute significantly towards E1A's biological functions. Because our analysis revealed that attenuation of proteasomal activity was not the predominant mechanism for E1A to stabilize Myc, Kathryn probed for regions of E1A that are required for its ability to stabilize Myc using a set of overlapping E1A deletion mutants (Figure 4.4A). Results of this experiment, presented in Figure 4.4B, revealed that out of the 11 deletion mutants tested only three mutant viruses: *dI1101*, *dI1102*, and *dI1103* failed to stabilize Myc. These three mutants span residues 4-49 in the amino-terminus of E1A, a region which mediates E1A interaction with numerous cellular proteins, including proteasomal subunits, TBP, p300/CBP, and p400. However, the smallest deletion (residues: 26-35) which fails to stabilize Myc interacts with all of these proteins with the exception of p400. These data argue that interaction of E1A with p400 is important for E1A's ability to stabilize Myc.

4.2.4 E1A requires p400 to stabilize Myc

The idea that p400 plays a role in E1A-mediated Myc stabilization was tested by comparing the ability of E1A to stabilize Myc in cells with reduced p400 levels. The expression of p400 was knocked down in U2OS cells by stably expressing shRNA against p400. Simultaneously these cells were transduced with either a control empty vector or a vector expressing WT E1A. The stability of Myc in these cells was tested using CHX chase. The results of this experiment revealed two important observations about the behavior of Myc. First, these results revealed that knockdown of p400, even in the absence of E1A expression, destabilized Myc; and second, knocking down p400 attenuated the ability of E1A to stabilize Myc. These results revealed that not only does E1A require p400 for its ability to regulate Myc, but also that p400 normally promotes Myc stability (Figure 4.5A-C).

To address the ability of p400 to protect Myc against proteolysis, I tested the influence of p400 on the destruction mechanisms of Myc. Because Ub-mediated proteolysis is one the major cellular pathways which regulates Myc destruction, I studied the effects of p400 expression on the ubiquitylation levels of Myc. Using U2OS cells, I co-transfected Myc with or without p400 alongwith a vector expressing a His-tagged version of Ubiquitin. By recovering the ubiquitylated species using Ni-NTA beads, the steady-state ubiquitylated levels

of a given protein can be detected as high-molecular weight ubiquitin conjugates by western blotting. By western blotting I probed for high-molecular weight Myc-Ub conjugates and observed that p400 over-expression led to a reduction in the formation of the Myc-Ub conjugates specifically. In contrast, p53-Ub conjugates which I analyzed simultaneously as a control were unaffected by the changes in levels of p400 (Figure 4.5D). Based on these results, I concluded that p400 expression was important for E1A to stabilize Myc and that p400 promoted Myc stability presumably by reducing the extent of Myc ubiquitylation.

4.2.5 E1A promotes the cellular association of Myc and p400

Given the importance of p400 in Myc stabilization via E1A, I sought to understand if E1A targets the ability of p400 to protect Myc from proteolysis. The chromatin-remodeling protein p400 has been shown to interact with both E1A and Myc. Therefore, I considered the possibility that E1A might regulate the association of Myc and p400. I tested this notion in U2OS cells transiently expressing HA-tagged Myc and FLAG-tagged p400. Using adenoviral infection I tested the effects of E1A expression on the association of Myc and p400 by immuno-precipitation from these cells. In parallel I infected cells with either control (β -gal) adenovirus or the p400-binding defective *d/1102* adenovirus. Recovering either Myc or p400 immune-complexes, I discovered that the association of Myc with p400 was increased in cells expressing WT E1A compared to uninfected cells or cells infected with the β -gal control adenovirus (Figure 4.6, compare lane 2 to 3; and lane 6 to 7). In contrast, the *d/1102* mutant

which is impaired in its ability to recruit p400 was attenuated in its ability to stimulate interaction between Myc and p400 (Figure 4.6, compare lane 3 to 4; and lane 7 to 8). I concluded that E1A promoted the association of Myc and p400 in solution and that E1A's interaction with p400 was important in this process.

I next asked if the co-association of Myc and p400 in response to E1A expression also occurred on chromatin at a transcriptional target of Myc. I studied the recruitment of Myc and p400 at B23 (nucleophosmin), one of the best characterized transcriptional targets of Myc. Because in my pilot experiments I found that the commercially available antibodies against p400 did not work very efficiently for ChIP, I decided to test the effects of E1A expression on Myc and p400 recruitment in cells expressing FLAG-tagged p400, which would allow me to recover p400 using anti-FLAG antibodies. Using U2OS cells expressing FLAG-tagged p400, I first performed a ChIP using an antibody against Myc and observed that expression of WT E1A, but not of the *d/1102* mutant strain, led to an increase in the association of Myc at the E-box cluster harbored within intron I of B23. To address if these Myc complexes were also enriched for p400, I recovered Myc-bound DNA complexes, released them from the beads and performed a second round of immuno-precipitation on the eluates using the anti-FLAG antibody to pull down p400 containing complexes. Results of this sequential-ChIP assay, presented in Figure 4.7, revealed that expressing WT-E1A also promoted co-association of Myc and p400 on chromatin at the B23 promoter. In contrast, the *d/1102*, which failed to induce Myc-p400 interaction in solution, did not promote the formation of these complexes at Myc target gene

promoters. I concluded that E1A promotes the formation of Myc and p400 not only in solution but also on chromatin at the Myc target gene B23. Taken together, this ability of E1A to promote recruitment of Myc to its target gene, and that too in complex with the p400 transcriptional co-activator, supports a model where E1A can functionally target Myc via its association with p400.

4.2.6 E1A functionally interacts with the Myc pathway

E1A and Myc have been described to share many functional similarities, such as the ability to induce cell proliferation and the ability to collaborate with Ras to transform primary fibroblasts. Because results from my ChIP analysis suggested that E1A might be able to stimulate Myc activity at its target gene promoters my earlier results, I considered if this overlap was partly due to the ability of E1A to function via Myc. Therefore, I tested the influence of E1A on the expression of several Myc target genes using primary human fibroblasts, IMR90. I transduced these cells with retroviruses expressing empty vector (as a control), WT E1A, or the $\Delta 26-35$ E1A mutant which fails to bind p400. Using these cells, I studied the expression of some of the best characterized Myc target genes, such as AHCY, B23, PCNA, and Rcl1. Interestingly I observed that compared to control cells, the cells expressing WT E1A showed elevated levels of the Myc target genes. Conversely, expression of the mutant E1A protein failed to evoke a comparable induction in Myc target genes. To confirm that Myc was important for the ability of E1A to induce target gene expression, I transfected these cells with siRNA-duplexes against Myc, and analyzed the target gene levels in cells with

reduced Myc levels. As expected, the ability of E1A to stimulate expression of these target genes was compromised in cells with Myc knockdown. These data support a model where E1A recruits Myc to target genes in transcriptionally active complexes with the co-activator p400 and in turn stimulates the expression of Myc target genes (Figure 4.8).

To address the functional importance of the ability of E1A to induce Myc function, we collaborated with Andrew Samuelson in the Lowe laboratory and tested the importance of Myc expression in E1A function. The requirement of Myc for the ability of E1A to induce apoptosis was tested in Rat1 fibroblasts. Expression of E1A led to a potent induction of apoptosis in Rat1 fibroblasts, upon treatment with the DNA-damaging agent, Adriamycin. However, in congenic Myc-null fibroblasts (HO15.19), where both copies of Myc are disrupted by homologous recombination, E1A failed to signal apoptosis. The inability of E1A to induce cell death was because of the absence of Myc, because reintroduction of Myc in the HO15.19 cells rescued the ability of E1A to mediate cell death in these cells (Figure 4.9). In summary, these data are consistent with the model that E1A can target the functional network of Myc and requires the expression of Myc in cells to be able to mediate its key biological functions, such as apoptosis.

4.3 DISCUSSION

Although it was known for many years that E1A and Myc share numerous biological similarities, such as the ability to induce cell proliferation, the ability to drive quiescent cells back into the cell cycle, and the ability to co-operate with

other oncogenes and promote transformation of cultured primary cells, the mechanism for this functional overlap was never understood. Our results establish that this overlapping functional repertoire emerges, at least in part, from the ability of E1A to directly hijack Myc's cellular function. By targeting a proliferative pathway, such as Myc, E1A can initiate a global transcriptional program to induce cell cycle progression in infected cells.

Additionally, these observations highlight a novel means by which E1A can target its cellular substrates. We originally approached the ability of E1A to stabilize Myc as an indirect outcome of proteasomal inhibition by E1A. However, the course of our investigations has proven that it is the regulation of the levels of Myc-p400 complexes by E1A which affect Myc-stability, rather than a global inhibition of the proteasome. Although we can never completely discount the role of E1A-mediated proteasome inhibition in our analysis, the more direct effects of p400 on Myc-ubiquitylation seem to suggest that E1A can target Myc upstream of ubiquitylation itself.

One means for E1A mediated stabilization of Myc is via the effect of E1A expression on p400 levels. Besides requiring p400 to mediate its biological functions, E1A has been previously demonstrated to induce the cellular levels of p400 (Samuelson et al. 2005). Given that our analysis suggests that p400 can act as a physiological regulator of Myc and promote its stability, it is easy to imagine that by inducing p400 levels, E1A targets the ability of p400 to regulate Myc proteolysis.

Another clue to explain this observation emerges from the fact that p400 interacts with Myc via the well conserved MB-II region. Given that MB-II also serves to recruit the Ub-ligase Skp2, I speculate that E1A could instigate a biochemical competition between p400 and Skp2 for binding to Myc. By promoting Myc-p400 association, therefore, E1A may interfere with Skp2 binding to Myc, thus protecting Myc from proteolysis.

Lastly, our body of work clarifies some previously known cellular networks regulating proliferation. For example, although it was earlier believed that knockdown of p400 leads to cellular senescence and growth arrest, primarily via induction of the p53-p21 pathway our work allows for a more comprehensive model for this process. Besides inducing p53 and p21 activity, we now propose that, loss of p400 would destabilize Myc and thereby simultaneously repress the growth-promoting function of the Myc-transcriptional program.

Historically, DNA viruses have been used in modern biology because of their ability to target the most vulnerable (and probably minimal) set of cellular pathways to mediate their function. It was therefore not surprising when E1A was demonstrated to target Ras, p53, and Rb-E2F function in the cell. Our work now demonstrates a more complete strategy by which E1A simultaneously and synergistically targets the transcriptional networks of two key transcriptional factors in the cell: E2F and Myc. This “double whammy” likely lies at the heart of the oncogenic potency of E1A and further highlights the utility of DNA tumor viruses as roadmaps to understanding cancer initiation and progression.

4.4 MATERIALS AND METHODS

4.4.1 Adenovirus culture

Adenoviruses were purified by freeze-thaw lysis followed by CsCl equilibrium centrifugation after 72 hour infections in 293 cells. For adenoviral infections, U2OS cells were incubated in a small volume of culture medium containing adenovirus particles to achieve a multiplicity of infection of approximately 15-30 plaque-forming units per cell, for 2 h at 37°C with intermittent rocking. Following this, additional media was added and infections were continued for appropriate time duration.

4.4.2 Ubiquitylation Assays.

Myc and p53 conjugates were detected by using the His-tagged Ub method after transfection of U2OS cells with pMT107 expressing His-tagged Ubiquitin, pCGN-Myc, pCGN-p53, and pCMV-p400 as indicated. Ubiquitylated species were recovered by binding Ni-NTA beads under denaturing conditions in Buffer A (6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM imidazole, pH:8). Beads were washed multiple times in Buffer A and protein samples prepared by boiling in 2x Laemmli buffer containing 300 mM Imidazole. Samples were analyzed for the levels of high molecular weight Ub-conjugates by western blotting.

4.4.3 RNAi

Duplex pools of siGenome RNA against Myc and nontargeting control sequences (Dharmacon) were transiently transfected into IMR90 cells using Oligofectamine (Invitrogen) as per the manufacturer's instructions. Knockdown of Myc RNA was at least 50% (data not shown).

4.4.4 Cell Viability

To assay the ability of E1A to induce apoptosis in *Myc*^{-/-} cells, HO.15.19, and parental Rat1 cells, TGR-1 were transduced with retroviruses to express constructs for WT E1A, E1A Δ 26–35, or Myc, in the indicated combinations. Relative apoptosis was determined by comparing cell death using the trypan blue exclusion method, 24 h after treatment with increasing doses of adriamycin. Data presented are the average of three independent experiments.

4.4.5 ChIP and Sequential-ChIP Analysis

ChIP analyses were performed in U2OS cells that had been transfected with either control (pUC119) or pCMV-FLAG-p400 construct by using Fugene 6 (Roche). After 48 h, cells were infected with control, *dl520*, and *dl1102* adenovirus for 8 h. Primary immunoprecipitation was performed by using anti-Myc (N262) antibody; for re-ChIP, a secondary immunoprecipitation using anti-FLAG (M2) antibody was performed. Co-precipitating DNAs after each round were assayed by quantitative PCR using the B23_C (specific) and B23_M (nonspecific control) amplicons. Re-ChIP DNA signals for FLAG-p400 were further normalized to those from cells transfected with the vector control.

***Figure 4.1: Adenoviral E1A stabilizes Myc**

(A) Western blot analysis to assess the effect of adenoviral infection on Myc protein levels shows that Myc levels peak transiently around the 6-8 hrs after infection around the same time as E1A expression is first detected.

(B) Myc RNA levels were measured after 6 hours of adenoviral infection by Real Time quantitative PCR. Myc RNA levels drop by nearly 50% upon adenoviral infection. Values represent Mean \pm S.D. from three experiments.

(C) E1A is important for the ability of adenovirus to stabilize Myc. Adenoviral infection with a control virus expressing β -gal does *not* significantly change Myc stability, compared to the uninfected control conditions. Chemical inhibition of the proteasome using MG132 stabilizes Myc.

(This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008).

(*These experiments were done by Kathryn Tworkowski, Tansey laboratory)

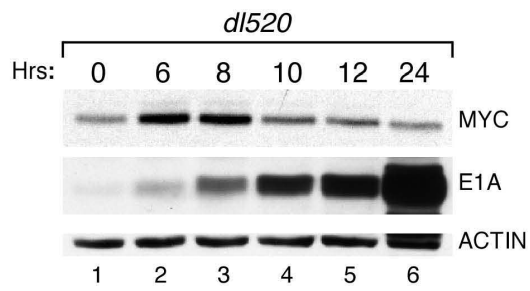
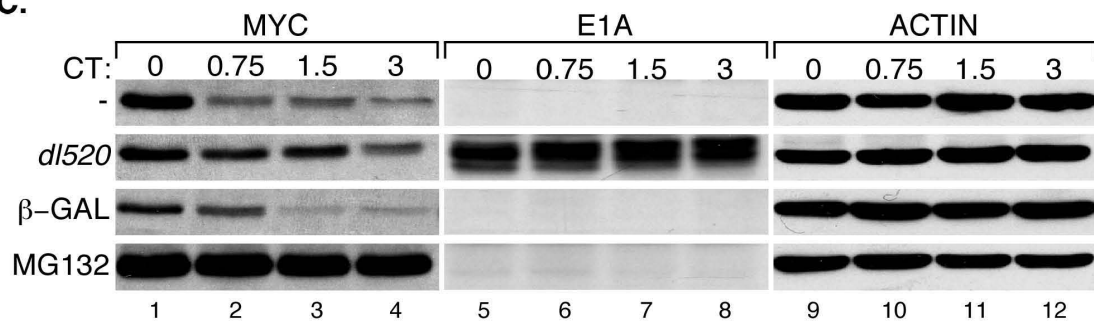
A.**B.****C.**

Figure 4.2: Adenoviral E1A does not induce global suppression of proteasome function.

The amino-terminus of E1A interacts with numerous sub-units of the proteasome and inactivates their function. The effect of E1A on global function of the proteasome was analyzed by assessing the stability of an artificial proteasomal substrate, U-GFP. Although Myc was stabilized upon adenoviral infection, the stability of U-GFP remained virtually unchanged. This observation suggests that E1A probably stabilizes Myc independent of a global shut-down of proteasome function. *(This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008).*

(*This experiment was done by Kathryn Tworkowski, Tansey laboratory)

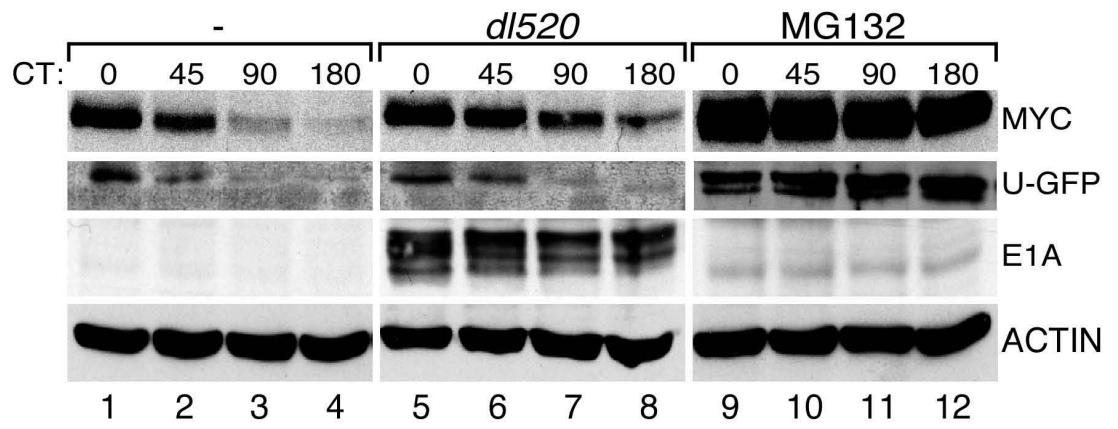


Figure 4.3: Adenoviral infection does not alter sub-cellular distribution of Myc

(A) Chemical inhibition of the proteasome by MG132 leads to an accumulation of Myc in the nucleolus.

(B) Adenoviral infection does not induce any discernible change in Myc localization patterns, showing that the effects of adenoviral infection on Myc are different from proteasomal inhibition. This data further reinforces the idea that E1A stabilizes Myc via a mechanism different from global attenuation of proteolysis.

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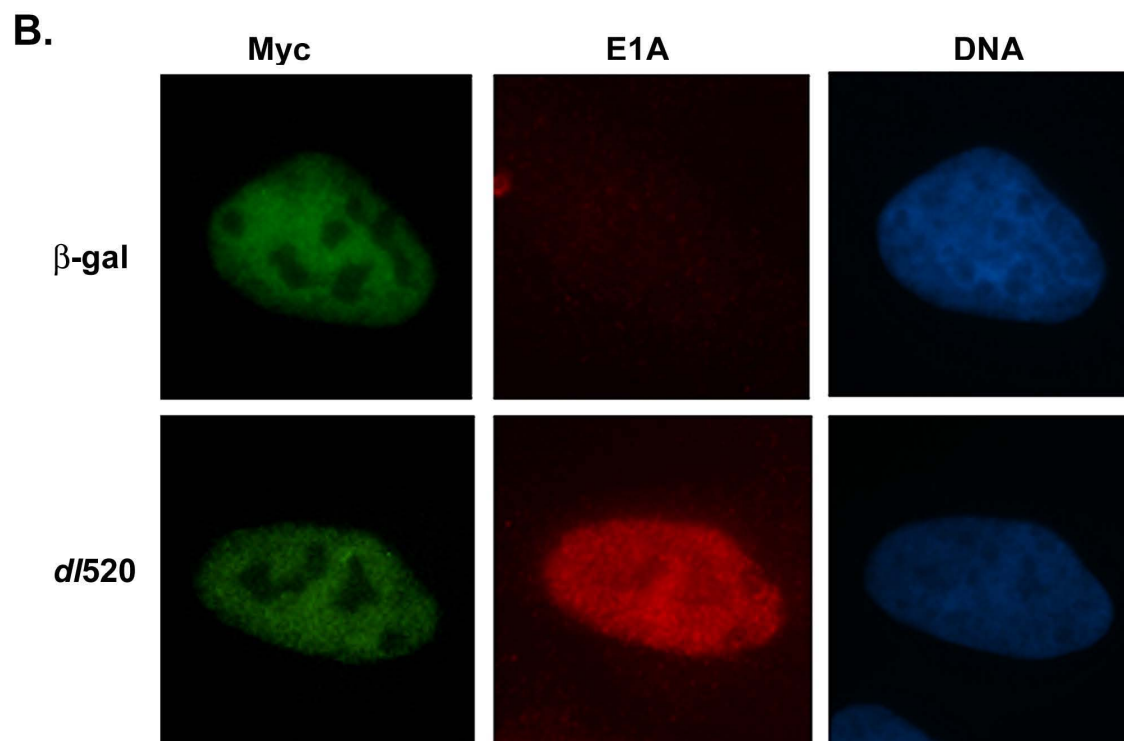
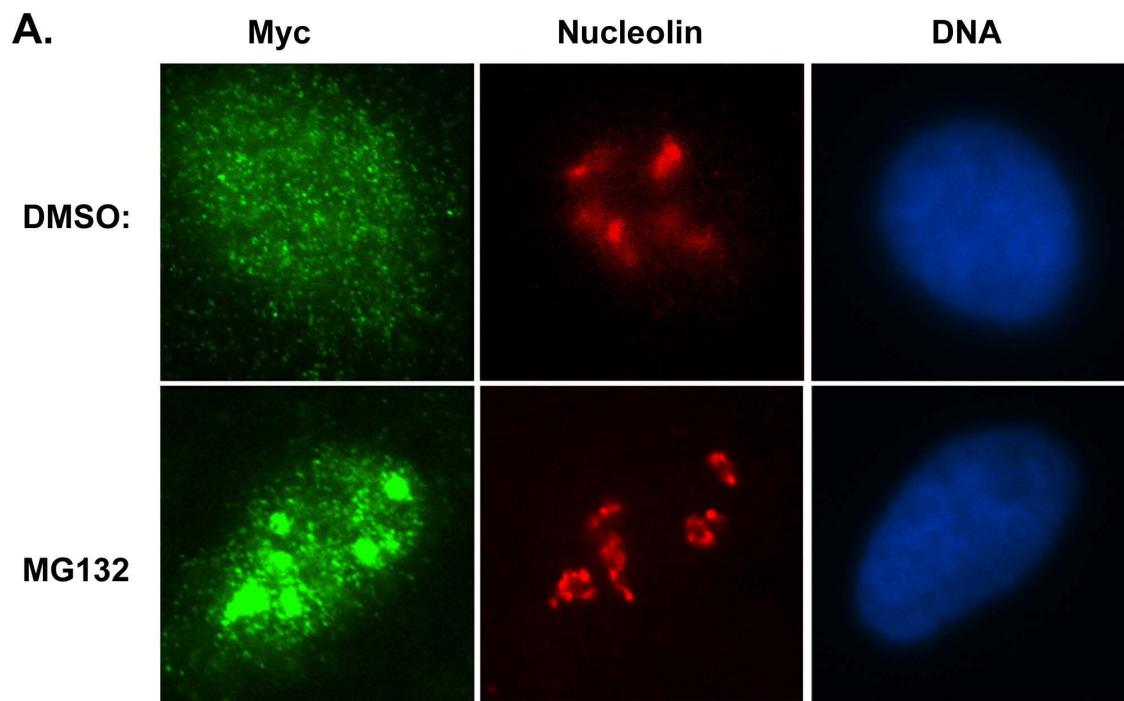


Figure 4.4: Interaction of E1A with p400 is important for Myc stabilization

(A) Schematic representation of an overlapping set of deletion mutants of E1A.

(B) Cyclohexamide chase experiment was done to assess the effect of several E1A mutants (shown in panel A) on Myc stability. Three deletion mutants: *d/1101*, *d/1102*, and *d/1103* were impaired in their ability to stabilize Myc. These mutants span residues 4-49 of E1A and overlap tightly with the region important for E1A interaction with the chromatin remodeler, p400. (*This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008.*)

(*This experiment was done by Kathryn Tworkowski, Tansey laboratory)

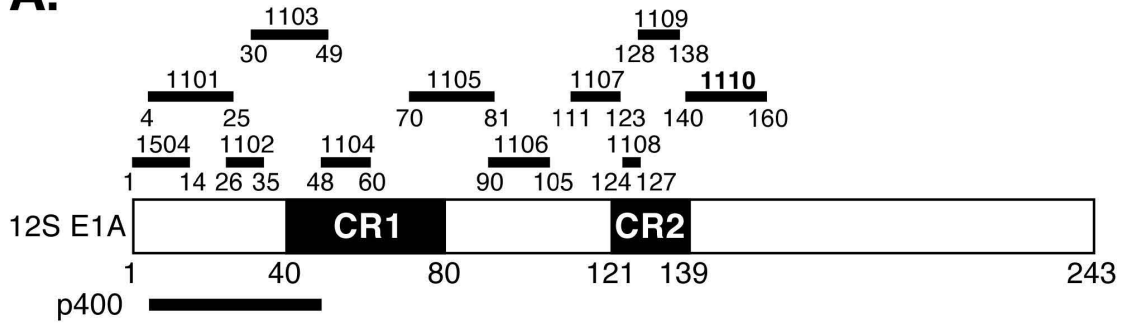
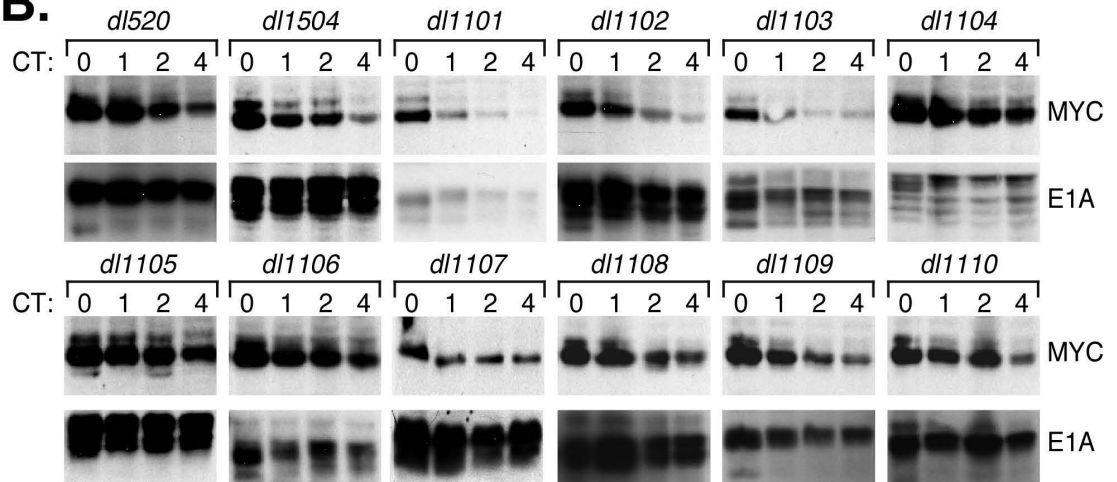
A.**B.**

Figure 4.5: p400 is a required for E1A mediated Myc stabilization.

* (A) The ability of E1A to stabilize Myc was compared in U2OS cells expressing either control shRNA or shRNA against p400. Knockdown of p400 attenuated the ability of E1A to stabilize Myc, suggesting that p400 is important in this process.

* (B), (C) p400 expression positively correlates with cellular Myc levels. Knocking down p400 by shRNA expression leads to a drop in Myc levels (panel B); in contrast, over-expression of p400 induces levels of Myc.

(D) The ability of p400 to regulate Myc destruction was analyzed by assessing the influence of p400 overexpression on Myc ubiquitylation. The results show that p400 expression leads to a reduction in the levels of high molecular weight Myc-Ub conjugates. However, p400 expression does not alter the levels of p53-Ub conjugates. *(This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008).*

(*These experiments were done by Kathryn Tworkowski, Tansey laboratory)

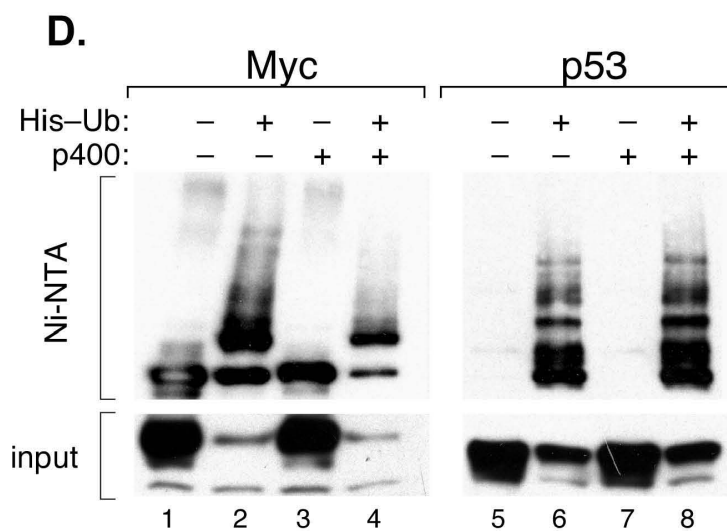
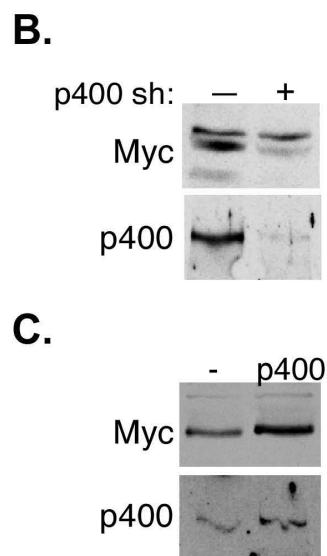
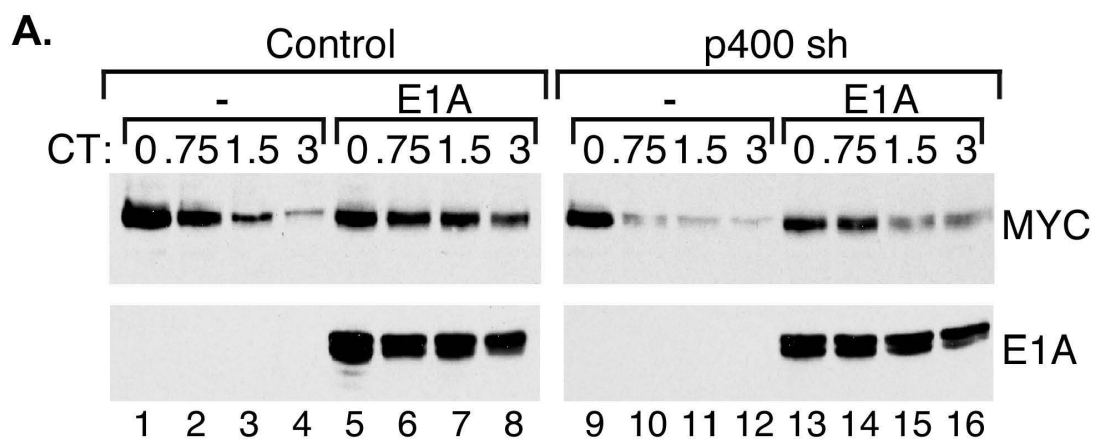


Figure 4.6: E1A promotes association of Myc and p400

The effect of E1A expression on the interaction of Myc and p400 was analyzed by immuno-precipitation in U2OS cells. Compared to mock infected control cells, or cells infected with the β -Gal expressing virus, the expression of WT E1A induced the association of Myc and p400. Conversely, the dl1102, which does not interact with p400, was impaired in its ability to stimulate the association of Myc and p400. (*This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008*).

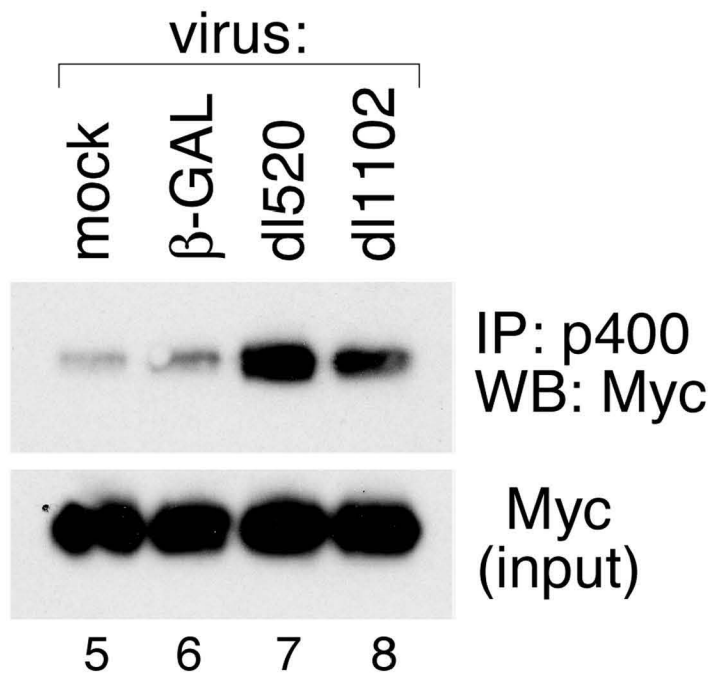
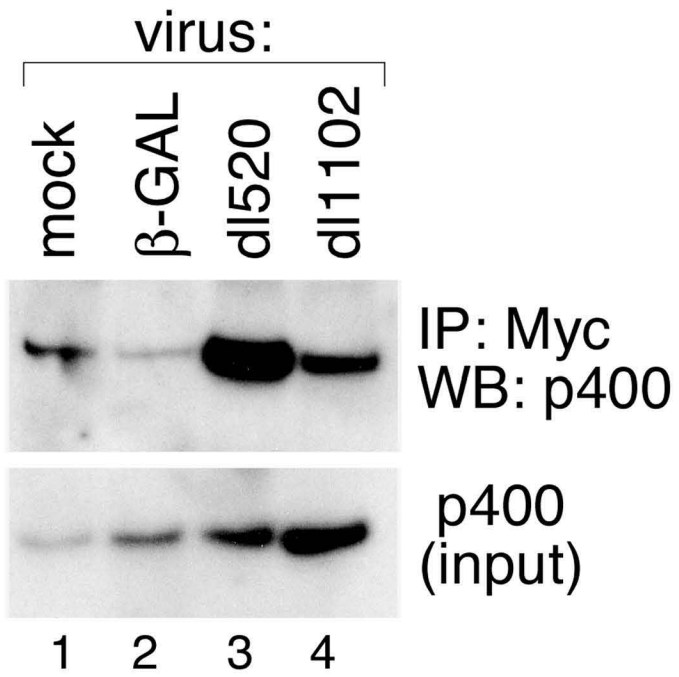
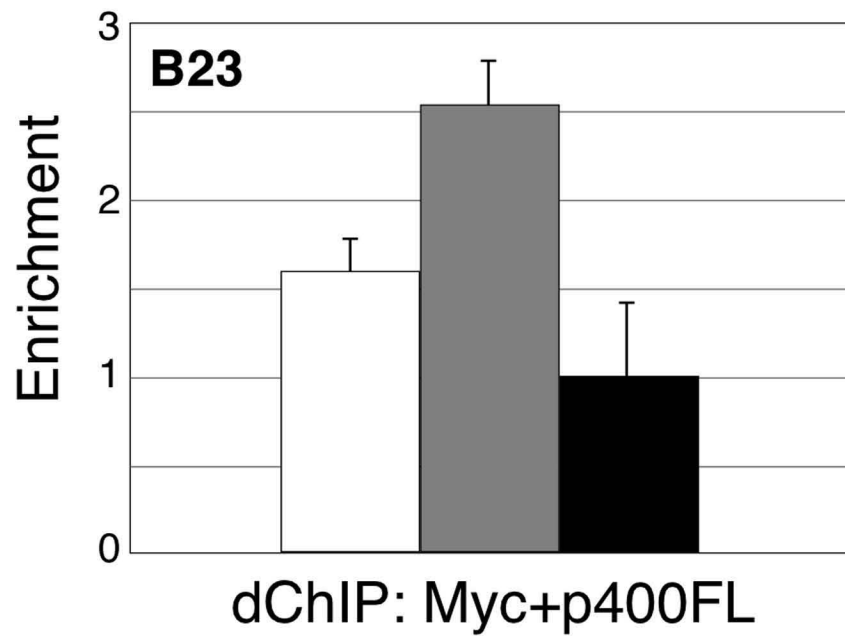
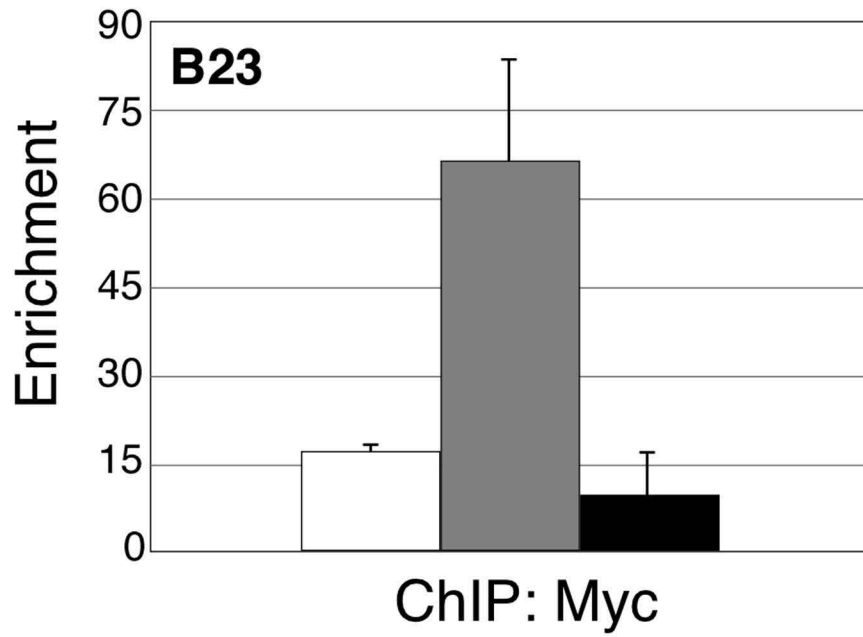


Figure 4.7: E1A promotes recruitment of Myc-p400 complexes on chromatin

The ability of E1A expression to influence Myc recruitment to its transcriptional targets was studied by ChIP analysis at the well characterized Myc target gene, B23, in U2OS cells. Infection of the *d/520* adenovirus expressing WT E1A induced recruitment of Myc. Conversely, expression of the *d/1102* mutant adenovirus failed to stimulate Myc recruitment.

The presence of p400 in the Myc complexes was analyzed by sequential-ChIP analysis. Results of this analysis show that WT E1A promotes the enrichment of Myc-p400 complexes whereas mutant E1A is unable to induce recruitment of p400-containing Myc complexes at B23.

Results are represented as Mean±S.D from three independent experiments. (*This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008.*)

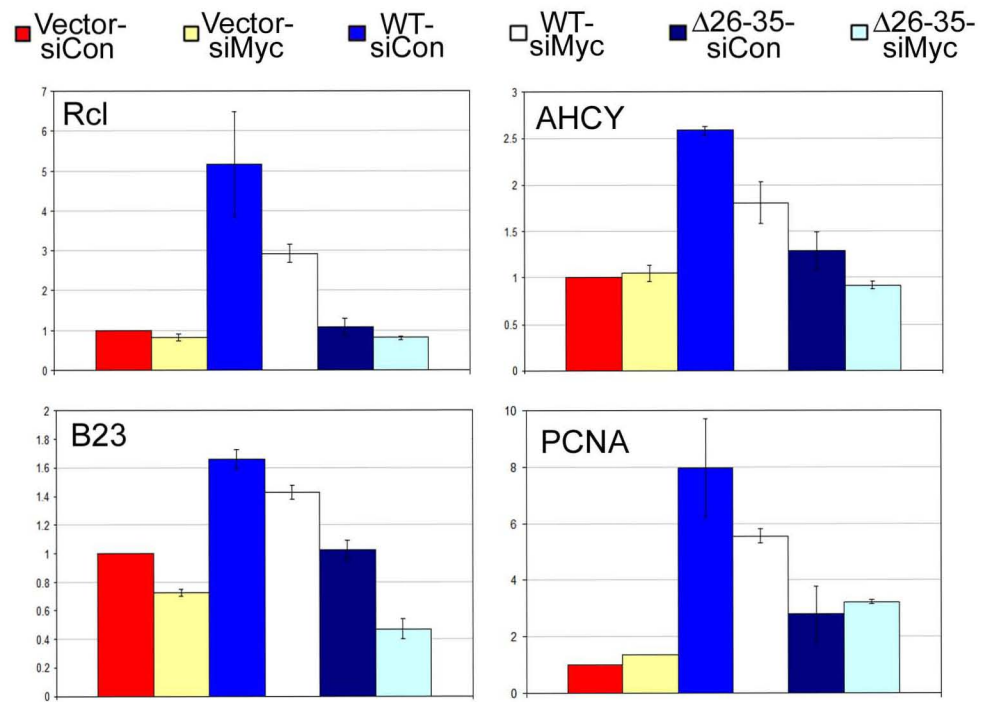


virus: \square β -GAL \square dl520 \blacksquare dl1102

Figure 4.8: E1A regulates Myc function

The effect of E1A expression on Myc function was analyzed by measuring the levels of Myc target gene mRNA in stable IMR90 cell lines, expressing either WT or the $\Delta 26-35$ E1A mutant. Compared to vector control, cells expressing WT E1A showed elevated levels of Myc target genes such as Rcl, AHCY, B23, and PCNA. This effect was not observed in cells expressing the $\Delta 26-35$ E1A mutant.

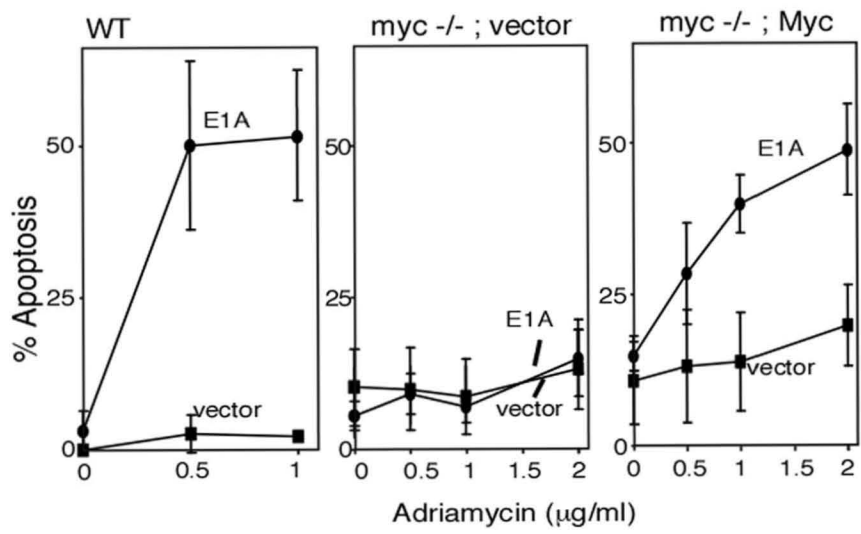
To address if this induction was due to Myc activity, the stable cells were transfected with siRNA against Myc. The results show that the ability of E1A to induce the levels of Myc target genes is attenuated upon knockdown of Myc. Results are Mean \pm S.D from three independent experiments. (*This figure was reproduced from Tworkowski KA, Chakraborty AA, et al (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008.*)



***Figure 4.9: Myc expression is important for the biological activities of E1A**

The importance of Myc expression in E1A function was assessed by studying the ability of E1A to sensitize cells for cell death in response to DNA damage. Whereas E1A readily induced cell death in WT Rat1 fibroblasts it was unable to induce apoptosis in the congenic fibroblasts HO15.19, where both copies of Myc are inactivated by homologous recombination. This loss of function was dependent on Myc and reintroducing Myc expression ectopically in the HO15.19 cells rescued the ability of E1A to mediate apoptosis. *(This figure was reproduced from Tworkowski KA, Chakraborty AA, etal (2008) Proc Natl Acad Sci U S A. 105(16):6103-8. Copyright: National Academy of Science, USA, 2008).*

(*This experiment was done by Andrew Samuelson, Lowe laboratory)



Chapter 5 Concluding Remarks

5.1 INTRODUCTION

Myc is an important oncogenic transcription factor which features prominently in many human cancers. Considering deregulation of Myc proteolysis is one of the major themes of Myc induced cancers, I decided to study the molecular mechanisms of this process. I focused on the regulation of Myc destruction under three different physiological settings: during cell cycle progression, in response to the SCF-type Ub-ligase Fbw7, and by the viral oncoprotein E1A. Here, I summarize my findings and provide concluding remarks of my thesis work.

5.2 *Constitutive regulation of Myc during mammalian cell cycle progression*

Myc is an important regulator of cell cycle progression and by virtue of its ability to control transcription Myc activates the expression of stimulatory factors, such as Cyclin E, and represses the expression of cell cycle inhibitors, such as p21. Despite the importance of Myc in the regulation of cell cycle progression, how the levels or stability of Myc itself are influenced by the cell cycle was never clearly understood due to conflicting results. Whereas some investigators

demonstrated that Myc expression and post-translational modification patterns were invariant during cell cycle progression, others showed that phosphorylation of Myc at the T58 and S62 residues is induced during G2/M transition. Because the T58 and S62 phosphorylation events are important for the recognition of Myc by the Fbw7 Ub-ligase, I decided to understand the regulation of this phosphorylation during the cell cycle.

I reasoned that many of the earlier results probably conflicted due to perturbations caused by the methods employed in those analyses. Indeed, my early experiments revealed that Myc was particularly susceptible to perturbations caused by drug-mediated cell synchronization, a common approach employed in many of the previous analysis. I therefore worked on an alternative approach which would allow me to study the cell cycle associated regulation of Myc in asynchronously growing cells. The recently developed laser scanning cytometry (LSC) technology offered me this opportunity.

My analysis using the LSC approach shows that Myc expression at both the RNA and the protein levels occurs constitutively during the progression of the cell cycle. By studying the turnover of Myc at different cell cycle stages, using an adapted cyclohexamide chase approach, I observed that Myc decay also occurred at similar rates at all cell cycle stages. Because of technical limitations I was unable to study Myc phosphorylation using LSC. I therefore used the physical separation method of centrifugal elutriation to assess Myc phosphorylation and observed that Myc phosphorylation at T58 did not occur as a function of cell cycle progression in cycling cells. Considering phosphorylation

at the T58 residue mediates the recruitment of Fbw7, I analyzed the effect of knocking down Fbw7 on Myc levels. The results of this experiment were rather striking and revealed that, unlike Cyclin E which is another cellular substrate of Fbw7, Myc was largely resistant to Fbw7 mediated proteolysis.

My analysis revealed two important insights into Myc function. First, although Myc was earlier believed to influence cell cycle progression by regulating the expression of cell cycle modulators such as cyclins, cyclin dependent kinases, etc; more recent models suggest a role for Myc in cell growth and metabolism. Consistent with this model, my data suggest that by regulating transcriptional targets involved in diverse biological processes such as DNA replication, protein translation, and metabolism throughout the cell cycle Myc possibly orchestrates a global biological program to mediate cell growth and proliferation. Second, my observation that in spite of T58 phosphorylation occurring throughout the cell cycle, the inability of Fbw7 to target Myc for proteolysis, suggests the existence of cellular mechanisms which protect Myc from destruction. These protective mechanisms are probably enforced under conditions when Myc function is important to drive growth. Conversely, relieving these shielding processes could provide a rapid means to target Myc under conditions requiring cell cycle arrest. Therefore, by studying Myc expression and proteolysis in the context of the cell cycle, my analysis supports a model where the constitutive nature of these counter-acting processes provides the cell with a rapid means to regulate the function of Myc.

5.3 Regulation of Myc proteolysis by Fbw7

Genetic changes involving inactivation of Fbw7 or mutations in Myc, which relieve it from Fbw7-mediated proteolysis, are frequently detected in neoplastic cells suggesting an important role for the Fbw7 pathway in Myc function. However, because my earlier work found that Myc was resistant to Fbw7 proteolysis in cycling cells, I decided to address the nature and importance of this resistance. The deubiquitylating enzyme Usp28 was recently shown to have a protective role for Myc proteolysis by interfering with Fbw7 function. I asked if the protective role of Usp28 could underlie the resistance of Myc to Fbw7 proteolysis. By studying Myc in cells with Usp28 knockdown, I observed that loss of Usp28 led to a reduction in Myc levels at all stages of the cell cycle and that Fbw7 was one means to target Myc under these conditions. Because Fbw7 was shown to be an Ub-ligase of Myc, the inability of Myc to respond to Fbw7 knockdown under cycling conditions represented an apparent paradox. By revealing the importance of Usp28, my analysis resolved this major discrepancy associated with Fbw7 mediated Myc proteolysis (Figure 5.1A).

Recent evidence suggests that Fbw7 substrates either harbor a single strong degron for Fbw7 or multiple low-affinity degrons. Although the presence of a single strong degron is sufficient for proteolysis, it is believed that many Fbw7 substrates have evolutionarily retained multiple low-affinity degrons as a means to exert higher regulatory control. Biochemical studies analyzing the binding characteristics of Fbw7 to several physiological degrons reveals that Fbw7 preferentially binds to degrons which harbor two negatively-charged or

phosphorylated amino acids four residues apart. Myc harbors one known Fbw7 degron and Myc interaction with Fbw7 occurs via phosphorylation of a single T58 residue. Moreover, phosphorylation of the second phospho-acceptor site S62 in the Myc CPD interferes with Fbw7 binding. This is an apparent anomaly and argues that the binding characteristics of the Myc degron would be biochemically equivalent to a weak Fbw7 degron. In the absence of a second degron, how a single weak degron of Myc mediates Fbw7 proteolysis represented another discrepancy in the regulation of Myc by Fbw7 (Figure 5.1B).

I addressed this discrepancy using mutants in the known degron of Myc. By analyzing the ability of Fbw7 to interact with these mutants and target them for proteolysis, I discovered that mutants in the Myc degron retained the ability to bind Fbw7 and were also responsive to changes in Fbw7 levels. My subsequent analysis led to the discovery of a second Fbw7 recognition sequence in Myc within residues 244-248. Similar to the first degron, the second degron also represents a mutation cluster of Myc in blood cancers. The lymphoma-derived mutants from the second degron, such as P245A, escape proteolysis and are stabilized compared to WT Myc. In animal models of lymphomagenesis, compared to WT Myc, the P245A mutant behaves like an aggressive oncogene and induces lymphomas with significantly lower latencies and at much higher penetrance. In summary, this work resolved a second paradox in the Fbw7 mediated Myc proteolysis and showed that Myc harbors two Fbw7 degrons. Changes in both these degrons occur in blood cancers and this observation

supports a model where Myc acquires mutations in its degrons to escape Fbw7 mediated proteolysis and that this process is important in oncogenesis.

5.4 Adenoviral oncogene E1A targets Myc function

Viral oncogenes from DNA tumor viruses such as adenovirus and SV40 target some of the most important cellular pathways. E1A represents one of the best studied viral oncogenes and the ability of E1A to drive cell proliferation, regulate cell death, and mediate cellular transformation is well documented. Many years ago it was shown that E1A and Myc are structurally related. Given that Myc too can mediate many of the biological roles attributed to E1A, suggested that E1A and Myc might be functionally related, however no functional link was known between these proteins for several years. Recently, such a functional link was revealed and E1A expression during the course of adenoviral infection was shown to stabilize Myc. Because Myc is a labile protein and alterations in Myc proteolysis pathways are frequently observed during oncogenesis, I speculated that regulation of Myc stability by E1A may also influence Myc function. My interest in understanding the importance of either cellular or viral processes which regulate Myc stability attracted me to investigate the regulation of Myc by this pathway.

My analysis in collaboration with Kathryn Tworkowski, a previous graduate student in the Tansey laboratory, revealed that E1A was indeed an important regulator of Myc stability. Exploring the underlying molecular mechanisms of this process by doing structure-function analysis of E1A, using a set of “classic” E1A

mutants, we identified residues 26 to 35 of E1A as being uniquely required for its ability to block Myc turnover. Given the modular nature of E1A, we reasoned that loss of interaction with a specific cellular protein might underlie the inability of the Δ 26-35 E1A mutant to stabilize Myc. The amino-terminus of E1A interacts with a slew of cellular proteins, but through comparison of different sets of E1A mutants that disrupt the various interactions, we were able to determine that p400 interaction is uniquely required for the effects of E1A on Myc. p400 is part of a nucleosome-remodeling complex that is important for the activity of both E1A and Myc. The common connection of Myc and E1A to p400, and the ability of other chromatin-remodeling proteins to regulate Myc stability, led us to hypothesize that p400 may underlie stabilization of Myc by E1A. Indeed, our subsequent studies showed that p400 is required for the ability of E1A to stabilize the Myc protein and that forced expression of p400 attenuates Myc ubiquitylation. Based on these results, we concluded that p400 is an important regulator of Myc stability and that interaction of p400 with E1A promotes its ability to stabilize Myc. Moreover, E1A promotes the stable association of Myc and p400 both in solution and, critically, on chromatin at Myc target genes. Consistent with the role of p400 as a cofactor for the transcriptional activity of Myc, this enhanced Myc-p400 interaction leads to activation of a set of Myc target genes. In a sense, therefore, E1A uses p400 to “hijack” Myc and its activities. We believe that the E1A-Myc connection is important for the activity of E1A because E1A cannot stimulate apoptosis in the absence of Myc.

In summary, we concluded that E1A drives oncogenesis, in part, by using p400 to tap into the transcriptional program of Myc and elicit a Myc-like response (Figure 5.2).

5.5 Summary

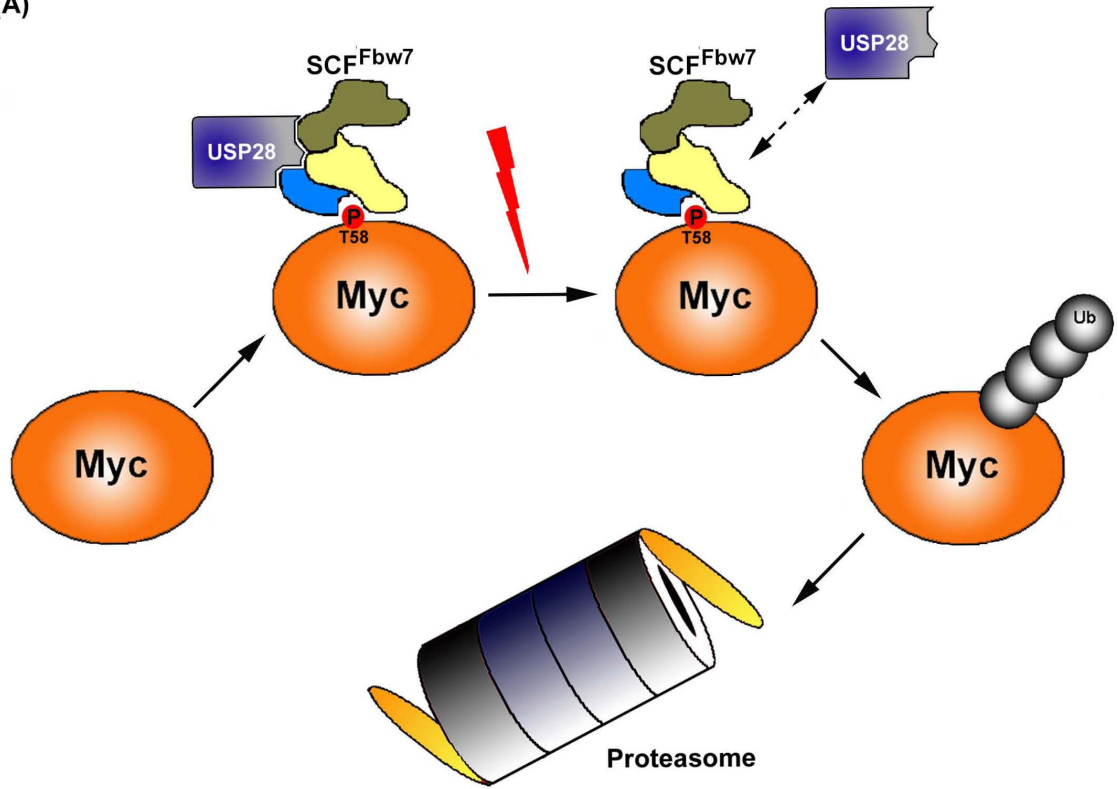
In summary, by studying independent cellular processes regulating Myc destruction, my thesis work reveals the profound importance of Myc proteolysis. My studies are consistent with a model that requires Myc expression and destruction to occur constitutively in cycling cells and demonstrate how the cells have evolved context-specific shielding mechanisms such as Usp28 to achieve this process. By studying Myc-mutants which relieve Fbw7 proteolysis and by analyzing the influence of the adenoviral E1A oncoprotein in regulating Myc stability, my work describes the existence for many cellular processes that converge on the regulation of Myc proteolysis as a means to control Myc activity. Indeed, deregulation of Myc destruction by such pathways often correlates with the increased oncogenicity of Myc and potentially defines early events during the onset of tumorigenesis.

Figure 5.1: Existing and Revised Models of Fbw7-mediated Myc turnover.

(A) Current evidence about the Fbw7-pathway implicates phosphorylation at the T58-residue in Myc as the single identification site for Fbw7 in Myc. Under normal growth conditions, the de-ubiquitylating enzyme Usp28 protects Myc from Fbw7-mediated proteolysis. However, under specific cellular conditions, such as DNA damage, Usp28 dissociates from Fbw7 and signals for rapid destruction of Myc.

(B) Our new data confirms the presence of a second Fbw7 degron in Myc. This raises interesting questions about the cross-talk or regulation of Myc by Fbw7. Considering the ability of Fbw7 to dimerize, it is easy to imagine that the Fbw7-dimer recognizes Myc in the context of both the degron simultaneously, thus enforcing a rapid response to upstream signals, which call for Myc proteolysis.

(A)



(B)

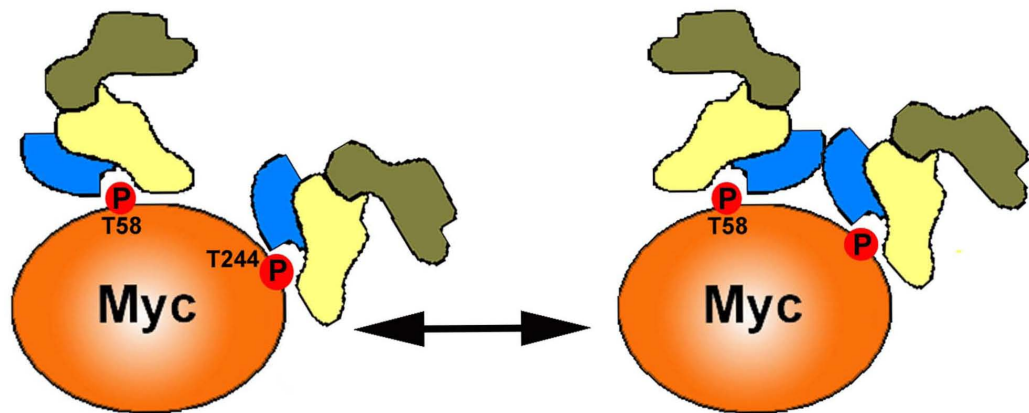
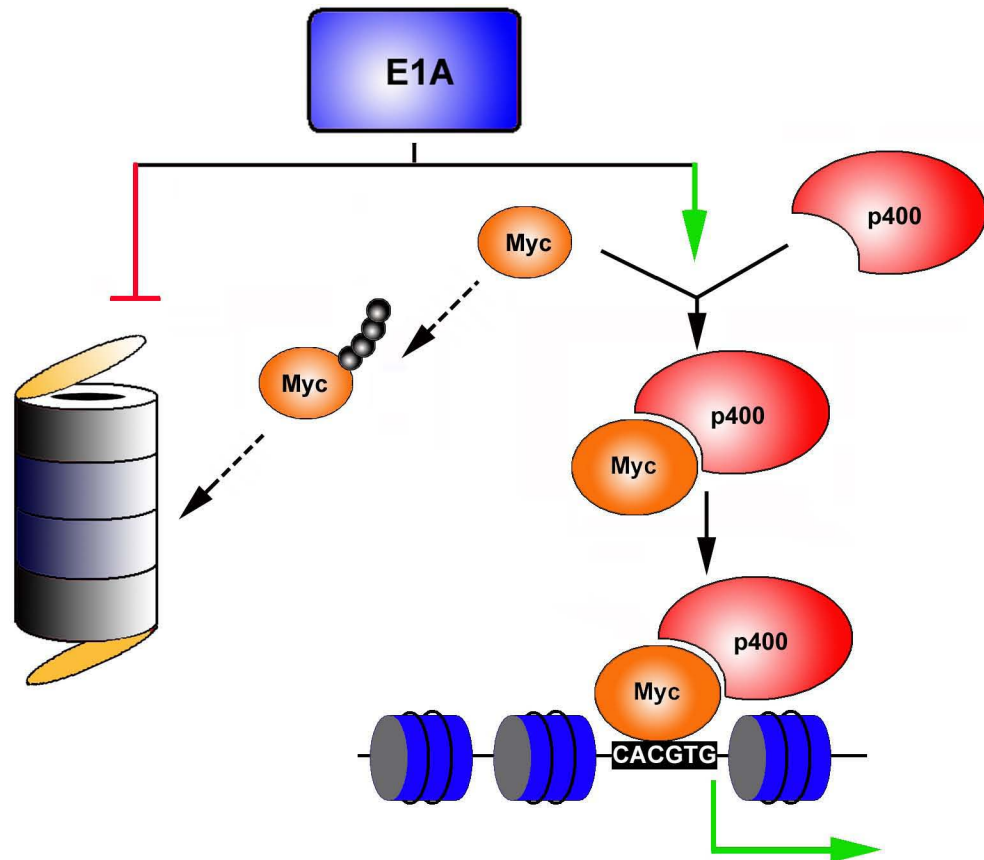


Figure 5.2: Adenoviral E1A, a regulator of proteasome function, utilizes Myc function via the p400 pathway.

The Adenoviral oncoprotein E1A interacts with numerous proteins via its N-terminus; including sub-units of the Proteasomal complex. Interaction of E1A with Proteasomal sub-units blocks their proteolytic activity and can consequently stabilize cellular substrates such as p53.

In contrast, E1A regulates Myc independent of proteasomal inhibition via the chromatin remodeling protein, p400. Association of E1A with p400 promotes its interaction with Myc. The interplay between Myc and p400 has two important consequences: it stabilizes Myc by interfering with its ubiquitylation, and it promotes the formation of Myc-p400 complexes on Myc target genes, leading to their activation. Thus, E1A is able to elicit, in part, a Myc-like cellular response that contributes to function in apoptosis and cellular transformation. *(Figure adapted from Chakraborty AA and Tansey WP (2009) Cancer Research. 69 (1):6 – 9).*



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Appendix

The work presented in Chapter 2 and Chapter 4 of this thesis resulted in the following peer-reviewed publications attached in this appendix:

- Tworkowski KA*, Chakraborty AA*, Samuelson AV, Seger YR, Narita M, Hannon GJ, Lowe SW, Tansey WP. Adenovirus (2008), E1A targets p400 to induce the cellular oncoprotein Myc, Proc Natl Acad Sci USA., 105: 6103 – 8. (* Equal Author)
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- Chakraborty AA and Tansey WP (2009), Adenoviral E1A function through Myc, Cancer Res, 69: 6 – 9.
- Chakraborty AA and Tansey WP (2009), Inference of cell cycle-dependent proteolysis by laser scanning cytometry, Exp Cell Res, 315: 1772 – 1778.

Adenovirus E1A targets p400 to induce the cellular oncoprotein Myc

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Adenovirus E1A drives oncogenesis by targeting key regulatory pathways that are critical for cellular growth control. The interaction of E1A with p400 is essential for many E1A activities, but the downstream target of this interaction is unknown. Here, we present evidence that the oncoprotein transcription factor Myc is the target of this interaction. We show that E1A stabilizes Myc protein via p400 and promotes the coassociation of Myc and p400 at Myc target genes, leading to their transcriptional induction. We also show that E1A requires Myc for its ability to activate Myc-dependent gene expression and induce apoptosis, and that forced expression of Myc is sufficient to rescue the activity of an E1A-mutant defective in p400 binding. Together, these findings establish that Myc, via p400, is an essential downstream target of E1A.

proteolysis | transcription | transformation | apoptosis

One of the most important tools for exposing the mechanisms of oncogenic transformation are DNA tumor viruses. Because these viruses, such as adenovirus, depend on the cellular DNA replication machinery to propagate, they must drive host cells into the cell cycle. It is this release from cellular growth control that promotes oncogenic transformation. The utility of DNA tumor viruses for cancer research is based on the premise that they have evolved to target the minimum number of cellular pathways necessary for virus propagation and cellular transformation. Understanding how DNA tumor viruses promote oncogenesis, therefore, can reveal the most vulnerable cellular pathways and nodes that are linked to tumorigenesis.

Adenovirus E1A is perhaps the most widely studied oncogene from a DNA tumor virus. E1A encodes proteins that have a range of activities, including the ability to induce cell proliferation and transformation, inhibit differentiation, and promote apoptosis. E1A proteins exert these effects by binding to, and modifying the function of, key cell cycle regulators (1). The most prominent of these regulators is the tumor suppressor protein Rb, but interactions of E1A with chromatin remodeling factors such as p300/CBP (2) and p400 (3) also contribute to its biological activities. The interaction of E1A with p400 is particularly important because E1A fails to induce apoptosis in cells that do not express p400 (4), and an E1A mutant that is specifically defective for p400 binding ($\Delta 26-35$), but can still interact with p300/CBP, is impaired for both transformation and apoptosis (3, 4). Although p400 is clearly required for E1A's activities, the mechanism through which it functions in this capacity is unknown.

Recently, it was reported that E1A can inhibit the ubiquitin (Ub)-mediated destruction of Myc during the course of adenovirus infection (5). It has also been reported that E1A can interact with multiple subunits of the 19S proteasome to inhibit proteasomal proteolysis (6). Although global proteasome inhibition could account for the stabilization of Myc by E1A, the exact mechanism through which E1A stabilizes the Myc protein is unknown. We have investigated how E1A attenuates Myc proteolysis and find that, contrary to expectations, stabilization of Myc does not occur via widespread proteasome inhibition. Instead, E1A stabilizes Myc by promoting its association with

p400, which in turn reduces Myc ubiquitylation and promotes formation of a Myc-p400 complex on promoter DNAs. Consistent with these findings, we also show that E1A can activate Myc target genes and that Myc is an essential downstream effector of E1A. Together, these data reveal that stabilization of Myc by E1A is a specific targeted effect of the adenoviral protein and establish that the E1A-p400-Myc connection is important for oncogenesis.

Results and Discussion

To examine the effects of E1A on Myc protein stability, human U2OS cells were infected with the Ad5 adenovirus *dl520*, which expresses WT 12S E1A, and endogenous Myc protein levels were assessed by Western blot (WB; Fig. 1A). Six to 8 h after infection, at the point at which E1A expression was first detected, we observed an increase in steady-state Myc levels, which gradually subsided over a 12- to 24-h period. Consistent with previous reports (5), the increase in Myc protein levels at 6 h was accompanied by a decrease in the levels of Myc mRNA (Fig. 1B). Although Baluchamy *et al.* (7) have reported that adenovirus activates Myc gene expression, these experiments were done in quiescent cells; our assays, and those of Lohr *et al.* (5), were performed in cycling cells, suggesting that regulation of Myc transcription by adenovirus is influenced by the growth status of the cells.

The transient increase in Myc levels we observed, together with the decline in Myc mRNA, suggested that Myc is stabilized during the course of adenovirus infection. This notion was confirmed by treating infected cells with cyclohexamide (CHX), and monitoring Myc levels by WB (Fig. 1C). Under these conditions, adenovirus stabilized Myc considerably, and in a manner that depended on E1A; a virus that expresses *LacZ* (β -gal) instead of E1A did not induce Myc stability (Fig. 1C). Importantly, expression of E1A alone was sufficient to stabilize Myc; retroviral expression of 12S E1A in U2OS cells resulted in a potent stabilization of Myc (Fig. 1D) and a commensurate decrease in Myc mRNA levels (Fig. 1E). Thus, confirming earlier work (5), E1A promotes Myc stability.

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The authors declare no conflict of interest.

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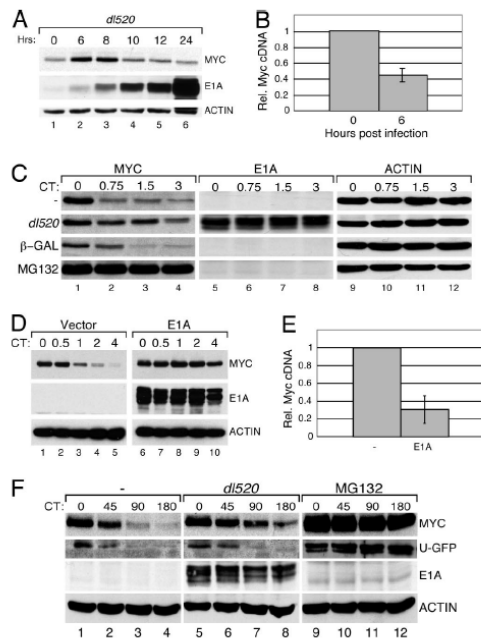


Fig. 1. Adenovirus E1A stabilizes Myc. (A) U2OS cells were infected with adenovirus *dl520* and harvested at the indicated time points. Levels of Myc, E1A, and actin were determined by WB. (B) U2OS cells were infected with adenovirus, as in A, and RNA was harvested. Levels of *c-Myc* cDNAs were analyzed by quantitative PCR and normalized to those of an actin control. (C) U2OS cells were either infected with *dl520* or a control virus expressing β -gal, mock-infected, or treated with the proteasome inhibitor, *MG132*, and incubated for 6 h. CHX was added to inhibit protein synthesis, and protein samples were taken at the indicated times (CT; hours). WB analysis shows the levels of Myc, E1A, and actin. (D) U2OS cells were transfected with a retroviral expression construct encoding WT 12S E1A (pLPC E1A). CHX chase, followed by WB, was used to analyze Myc turnover in E1A expressing cells and control (vector) cells. (E) RNA was isolated from cells in D, and levels of *c-Myc* cDNAs relative to actin, determined by quantitative PCR. (F) U2OS cells were transiently transfected with a plasmid encoding unstable GFP (U-GFP) and incubated for \sim 20 h. These cells were then either infected with *dl520*, mock-infected, or treated with *MG132* and incubated for 6 h. CHX chase was used to analyze the levels of GFP, Myc, E1A, and actin.

E1A has been shown to interact with multiple subunits of the 19S proteasome, and this interaction has been proposed to inhibit proteasomal proteolysis (6). Because Myc proteolysis requires proteasome function (8), global inhibition of proteasome activity by E1A could account for its ability to stabilize Myc. It is important to note, however, that E1A has not been shown to elicit widespread proteasome inhibition *in vivo*. We therefore asked whether adenoviral infection stabilizes the synthetic protein U-GFP (9), a substrate that is widely used as an *in vivo* reporter of proteasome activity (Fig. 1F). Under conditions where adenovirus stabilized Myc, there was little, if any, change in the rate of destruction of U-GFP, demonstrating that proteasome function is not generally attenuated in adenovirus-infected cells. This notion was supported by comparing the effects of adenovirus infection and proteasome inhibition on Myc localization [supporting information (S1) Fig. S1]. Whereas

proteasome inhibition results in the redistribution of Myc from the nucleoplasm to the nucleolus (10), infection of cells with adenovirus expressing E1A did not significantly alter the nuclear distribution of the Myc protein. Thus the consequences of stabilization of Myc by E1A are different from those of proteasome inhibition. Taken together, these data support a model in which the ability of E1A to stabilize Myc does not involve general inhibition of proteasome function.

E1A Stabilizes Myc via p400. We next probed for the regions of E1A that are required to stabilize Myc. We analyzed a panel of adenovirus E1A deletion mutants, depicted in Fig. 2A, for their ability to stabilize Myc at 6 h after adenovirus infection (Fig. 2B). This experiment revealed that, of the 11 mutant viruses tested, all but three were able to stabilize Myc efficiently. The three viruses that failed to stabilize Myc (*dl1101*, *dl1102*, and *dl1103*) encode E1A proteins with deletions that span residues 4–49. This region of E1A is important for its interaction with p400, CBP/p300, TBP, and Rb, but the smallest deletion that disrupts Myc stabilization, Δ 26–35 (*dl1102*), interacts with all of these proteins, with the exception of p400 (4). Although the Δ 26–35 mutation also disrupts interaction of E1A with the coactivator TRRAP (11), interaction with TRRAP additionally requires CR1; disruption of CR1 (as in the *dl1104* and *dl1105* viruses) did not block the ability of E1A to stabilize Myc (Fig. 2B). Importantly, we also observed that WT E1A, but not the Δ 26–35 mutant, stabilized Myc in Rat1 and IMR90 cells (Fig. S2), demonstrating that the ability of E1A to interact with p400 correlates with Myc stabilization in a variety of cell types. Together, these data indicate that adenoviral-mediated stabilization of Myc requires interaction of E1A with p400.

To determine whether p400 plays a role in E1A-mediated stabilization of Myc, we knocked down expression of p400 in U2OS cells by using short-hairpin-mediated gene silencing and examined the effects of E1A on Myc turnover. This analysis (Fig. 2C) showed that knockdown of p400 attenuates the ability of E1A to stabilize Myc. Moreover, we found that knockdown of p400, in the absence of E1A expression, increased the rate of Myc proteolysis and decreased steady-state Myc protein levels (Fig. 2D), indicating that p400 normally acts to promote Myc stability. Consistent with this idea, overexpression of p400 promoted accumulation of Myc protein (Fig. 2E) and specifically reduced the formation of high-molecular-weight Myc-Ub conjugates (Fig. 2F); p53-Ub conjugates, which we assayed as a control, were unaffected by p400 expression. Based on these results, we conclude that p400 acts to stabilize Myc by reducing the extent of Myc ubiquitylation and that E1A targets this process.

E1A Promotes Formation of a p400–Myc Complex. Given the ability of p400 to stabilize Myc, a simple model to explain our observations is that E1A promotes the association of Myc and p400. To test this model, U2OS cells expressing HA-tagged Myc and FLAG-tagged p400 were infected with various adenoviruses, and Myc–p400 complexes were detected by coimmunoprecipitation analysis (Fig. 3A). By recovering either Myc or p400 immune complexes, we found that WT E1A, but not the Δ 26–35 mutant, promoted coassociation of both proteins. Importantly, WT E1A could also stimulate the association of Myc and p400 on promoter DNA, as assayed by ChIP. Because commercially available anti-p400 antibodies did not function for ChIP (data not shown), we expressed FLAG-tagged p400 in cells infected with various adenoviral vectors for these analysis. We first performed ChIP by using an antibody against Myc and found that expression of WT E1A (encoded by *dl520*), but not the Δ 26–35 mutant (*dl1101*), promoted the association of Myc with the nucleophosmin (B23) promoter (Fig. 3B). We then recovered Myc–DNA complexes from the ChIP reaction and performed a second round of immunoprecipitation with anti-FLAG antibody to recover chromatin that was bound by both Myc

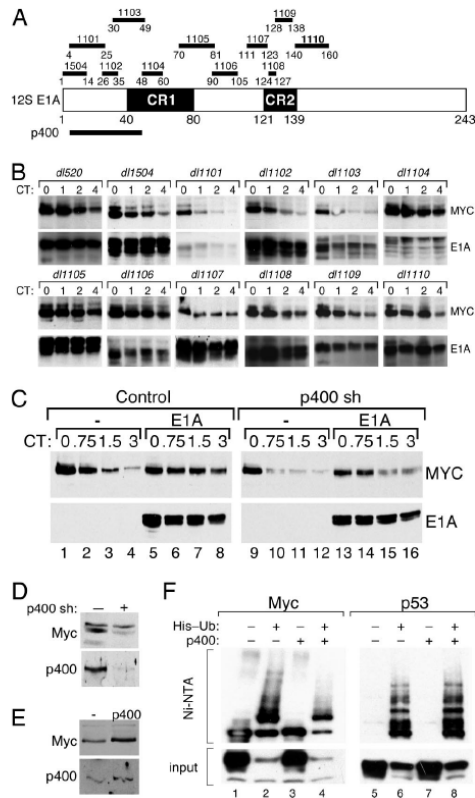


Fig. 2. p400 is required for E1A to stabilize Myc. (A) 12S E1A is depicted, showing conserved regions 1 (CR1) and 2 (CR2). The positions of adenovirus E1A deletion mutants *dI1504* and *dI1101*–*dI1110* are shown above, and residues required for interaction with p400 are shown below. (B) U2OS cells were transfected with adenovirus deletion mutants, or *dI520*, and incubated for 6 h, and CHX chase was performed. WB was used to monitor levels of E1A and endogenous Myc. (C) U2OS cells were transfected with retroviral expression constructs encoding (i) a short hairpin directed against p400 (sh p400), or vector control, and (ii) WT 12S E1A, or vector control (–). CHX chase followed by WB analysis was used to determine levels and stability of Myc and E1A. (D) WB analysis was used to determine levels of Myc and p400 in control (–) and p400 hairpin (+)-expressing cells. (E) U2OS cells were transiently transfected with HA-tagged Myc, in the absence (–) or presence of an expression construct encoding p400. Levels of Myc and p400 were determined by immunoblotting. (F) U2OS cells were transfected with expression vectors encoding either Myc or p53, together with FLAG-tagged p400 (or vector control), and a plasmid expressing His-tagged Ub (20). Ub conjugates were recovered by Ni-NTA chromatography, and ubiquitinated Myc and p53 proteins were detected by WB.

and p400. Consistent with the ability of E1A to promote association of Myc and p400 in solution, expression of E1A also promoted an enrichment of Myc–p400 co-complexes at the B23 promoter (Fig. 3C); as expected, this enrichment was not observed with the $\Delta 26$ –35 E1A mutant. The ability of E1A to promote both the association of Myc with a target gene, and, beyond this, to promote corecruitment of Myc and a coactivator, supports a model in which E1A functionally targets Myc via its interactions with p400.

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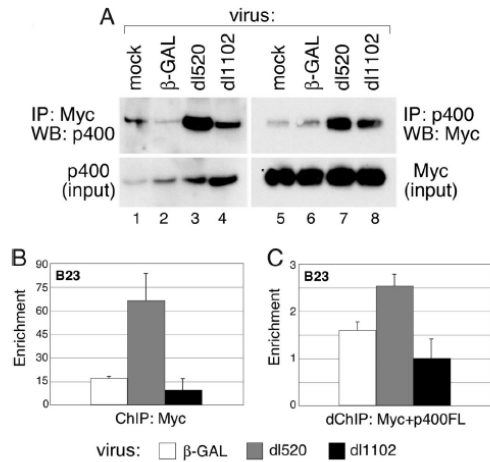


Fig. 3. E1A expression promotes the formation of Myc–p400 complexes. (A) U2OS cells were transiently transfected with HA-tagged Myc and FLAG-tagged p400 for 48 h. Transfected cells were infected for 6 h with β -GAL, *dI520*, or *dI1102* adenovirus. Coimmunoprecipitation was performed as described (4) by using anti-Myc (N262) or anti-FLAG (M2) antibodies, and Myc and p400 were detected by WB. (B) U2OS cells were transfected with FLAG-p400 or control DNA for 48 h and then infected with β -GAL, *dI520*, or *dI1102* adenovirus for 6 h. ChIP analysis was performed by using the anti-Myc antibody N262, and coprecipitating DNAs corresponding to the B23 E-box were detected by quantitative PCR. Enrichment is calculated relative to a non-Myc binding sequence. (C) Protein–DNA complexes recovered in B were eluted and subject to a second round of ChIP with anti-FLAG antibodies to recover p400-containing chromatin. Levels of coprecipitating DNA from the B23 E-box were determined by quantitative PCR. To control for nonspecific background, signal in the re-ChIP was normalized to that from an identical, parallel, experiment from cells not expressing FLAG-tagged p400.

Functional Interaction Between Myc and E1A. E1A and Myc share a number of functional similarities, including the ability to promote ectopic S-phase entry, sensitize cells to apoptosis, and collaborate with oncogenes like Ras to transform rat fibroblasts (12). Given our demonstration that E1A can induce both Myc levels and the Myc–p400 interaction, we speculated that the overlapping activities of these proteins may result, in part, from the ability of E1A to function through Myc. To probe this idea, we first asked whether E1A can stimulate Myc’s transcriptional activity. We examined expression of two Myc target genes (*Rcl1* and *Cad*) in diploid IMR90 cells transduced to express either WT E1A or the $\Delta 26$ –35 E1A mutant (Fig. 4A). Compared with vector control, we found that both genes were induced by expression of WT E1A, to a level comparable to that observed upon overexpression of Myc. The $\Delta 26$ –35 E1A mutant, in contrast, failed to activate either gene. Importantly, activation of *Rcl1* (Fig. 4B) and *AHCY* (Fig. 4C) by E1A depended on Myc, as siRNA-mediated knockdown of Myc in these cells attenuated the ability of E1A to activate these genes. Similar behavior was observed at the *B23* and *PCNA* genes (Fig. S3). Thus E1A can activate a Myc target gene in a Myc-dependent manner, supporting the notion that it functionally stimulates the Myc protein.

The E1A–p400 interaction has recently been found to be important for the ability of E1A to promote apoptosis (4). If, as our model predicts, the E1A–p400 interaction functions through Myc, we would expect that Myc will also be required for induction of apoptosis by E1A. We therefore asked whether E1A

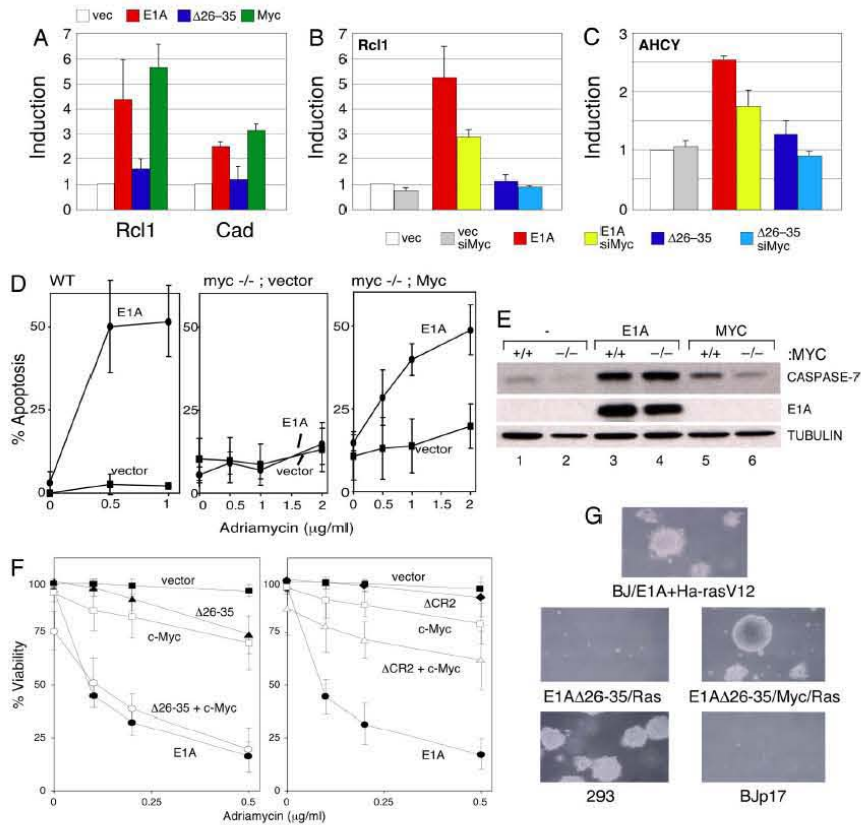


Fig. 4. Myc is a downstream effector of E1A. (A) IMR90 cells were transfected by retroviral vectors expressing 12S E1A, the E1A $\Delta 26-35$ mutant, or Myc. RNA was harvested, and levels of *Rcl1* and *Cad* cDNAs were determined by quantitative PCR. (B) IMR90 cells were transfected with the indicated retroviruses, and then transfected with either nontargeting siControl, or siMyc RNA, duplexes for 48 h. RNA was then harvested and levels of *Rcl1* cDNA were analyzed by quantitative PCR. Fold induction is normalized to an actin control for each sample. (C) As in B but assaying for levels of *AHCY* cDNA. (D) WT (TGR-1 cells) or congenic *myc*^{-/-} Rat-1 cells (HO.15.19 cells) were transfected with retroviruses expressing E1A, E1A $\Delta 26-35$, or empty vector, and the resulting cell populations were treated with adriamycin and assessed for viability after 24 h by trypan blue exclusion. The *myc*^{-/-}; Myc panel represents *myc*^{-/-} cells where full-length Myc was reintroduced by retroviral gene transfer. (E) WB analysis of samples derived from C showing expression of caspase-7, E1A, or tubulin. (F) IMR90 cells were transfected with retroviral expression constructs encoding E1A (●), E1A $\Delta 26-35$ (▲), E1A $\Delta CR2$ (◆), Myc (□), E1A $\Delta 26-35$ + Myc (○), E1A $\Delta CR2$ + Myc (△), or an empty vector (■). Cell populations were treated with the indicated doses of adriamycin for 24 h, and viability was determined by trypan blue exclusion. (G) BJ fibroblasts were retrovirally transfected with the indicated constructs. The resulting cells were assayed for colony formation in soft agar.

can induce apoptosis in Rat1 fibroblasts in which both copies of the *c-myc* gene were disrupted by homologous recombination (13). In Rat1 cells, E1A was a potent inducer of apoptosis triggered by adriamycin (Fig. 4D; see Fig. S4A for expression data on the E1A and Myc proteins). In congenic *Myc*-null cells, however, E1A was unable to induce apoptosis. This deficit was caused by a loss of Myc and not a secondary mutation, because reintroduction of Myc into *Myc*-null cells restored the ability of E1A to induce apoptosis. Importantly, this deficit was not caused by a general defect in E1A activity in *Myc*-null cells. Our previous studies have shown that binding of E1A to Rb is important for inducing the expression of several caspases, and that this induction potentiates cell death in E1A-expressing cells (14). When we examined caspase-7 levels in our system (Fig. 4E), we found that

E1A was capable of inducing caspase-7, and that this induction was not diminished in *Myc*-null cells. This result demonstrates clearly that the ability of E1A to function via the Rb pathway does not depend on Myc expression and reveals that only a subset of E1A activities require Myc.

A key prediction of our model is that overexpression of Myc should rescue defects in E1A that are associated with loss of the p400 interaction. To challenge this prediction, we asked whether overexpression of Myc can restore the ability of the E1A $\Delta 26-35$ mutant to sensitize cells to apoptosis (4). As reported (15), expression of E1A in IMR90 fibroblasts sensitizes them to apoptosis in the presence of adriamycin (Fig. 4F Left) and results in the induction of both ARF and p53 (Fig. S4B). Under these conditions, E1A is a more potent inducer of apoptosis than Myc, and its proapoptotic

activity (Fig. 4F), and ability to induce ARF and p53, is disrupted by the $\Delta 26$ –35 mutation. As predicted from the model, overexpression of Myc in the presence of the $\Delta 26$ –35 E1A mutant rescued both E1A's ability to trigger apoptosis (Fig. 4F) and induce ARF and p53 (Fig. 4B). The rescue of mutant E1A function was specific, because overexpression of Myc did not rescue the apoptotic defects of an E1A mutant ($\Delta CR2$) that still interacts with p400 but fails to interact with Rb (Fig. 4F). Thus, increased expression of Myc can specifically rescue apoptotic defects associated with loss of the E1A–p400 interaction.

Finally, we asked whether the same phenomenon applied to the ability of E1A to drive human cell transformation. Our previous studies have shown that expression of E1A and activated Ha-RasV12 in early passage normal human foreskin fibroblasts (designated BJ) allows the formation of colonies in soft agar (Fig. 4G and ref. 16). Blocking the ability of E1A to interact with p400 also attenuates its ability to collaborate with Ras to drive human cell transformation in this assay (Fig. 4G). Importantly, this activity can be restored by overexpression of Myc. Thus, overexpression of Myc can rescue both the transformation and apoptotic defects that result when the ability of E1A to interact with p400 is blocked. These data are consistent with the idea that the critical function of the E1A–p400 interaction in transformation and apoptosis is to induce Myc.

Conclusions

Together, our data support a model in which the binding of E1A to p400 promotes the formation of a Myc–p400 complex at Myc-target gene promoters. The increase in interaction of Myc and p400 is associated with stabilization of the Myc protein and an induction of Myc target genes. These functions of E1A are required for its ability to induce the ARF/p53 pathway, promote apoptosis, and drive cellular transformation, revealing that the downstream arm of the E1A–p400 interaction is mediated via Myc. Although it has long been known that E2F functions as the downstream target of the E1A–Rb interaction, the molecular processes downstream of the E1A–p400 connection have remained obscure. Our data indicate that Myc is the ultimate target of this connection. This finding not only provides an explanation for the overlapping biological functions of Myc and E1A, but also reveals an interesting viral strategy for promoting oncogenesis. By targeting and activating both the E2F and Myc transcriptional networks, E1A can provoke a synergistic response in parallel pathways to efficiently couple cell cycle progression, transformation, and apoptosis. Moreover, given that E1A targets cellular pathways relevant to transformation, our data also support an important role for the Myc–p400 connection in human cancer.

Methods

Antibodies. The antibodies used in this study were: anti (α -)Ad5 E1A antibodies M73 and M58 (17) and sc-430 (Santa Cruz); α -p400 monoclonal antibody RW144 (2); α -caspase-7 (14); α -Myc 9E10 (Oncogene) and N262 (Santa Cruz); α -Actin AC-15 (Sigma); α -GFP PC408 (Novagen); α -FLAG M2 (Sigma); α -p53 CM1 (Novocastar); α -ARF sc-8340 (Santa Cruz); α -HA 12CA5 (Roche); α -tubulin B512 (Sigma); and α -nucleolin (Santa Cruz).

Cells and Adenoviruses. U2OS, IMR90, HEK293, HO.15.19, and TGR-1 (13) cells and BJ human fibroblasts were grown under standard conditions. WT adenovirus 5

(Ad5), *d1520*, was obtained from ATCC. Ad5 mutants were obtained from Arnold Berk (University of California, Los Angeles) (18) and Philip Branton (McGill University, Quebec, Canada) (19). Adenoviruses were propagated in 293 cells and purified by CsCl equilibrium centrifugation. For adenoviral infections, U2OS cells were incubated with virus at a multiplicity of infection of 15 plaque-forming units per cell, for 1 h at 37°C with intermittent rocking.

Protein Assays. Myc protein turnover was measured by adding CHX (100 μ g/ml) to cells, collecting protein samples at the indicated time points, and assaying relevant protein levels by WB. Myc and p53 conjugates were detected by using the His-tagged Ub method after transfection of U2OS cells with pMT107 (20), pCGN-Myc (8), pCGN-p53 (unpublished work), and pCMV-p400 (3) as indicated.

cDNA measurement. Where appropriate, total cellular RNA was harvested with TRIzol (Invitrogen) and reverse-transcribed with the TaqMan kit (Applied Biosystems), and cDNA levels from the indicated genes were quantified by using the SYBR Green PCR Master Mix (Applied Biosystems) in conjunction with a MJ Research CFD-3240 Chromo 4 Detector. Transcript levels for target genes were normalized to those of actin. Primer sequences are available on request.

RNAi. Duplex pools of siGenome RNA against Myc and nontargeting control sequences (Dharmacon) were transiently transfected into IMR90 cells via Oligofectamine (Invitrogen). Knockdown of Myc RNA was at least 50% (data not shown).

Cell Viability. To assay the ability of Myc to rescue the apoptotic defect of the E1A $\Delta 26$ –35 mutant, IMR90 cells were stably transduced with pLPC, pLPC E1A (21), pLPC E1A $\Delta 26$ –35 (4), pLPC $\Delta CR2$ (4), or pBabe Hygro HAM Myc (22) by retroviral infection. The resulting cell lines were plated into 12-well dishes at a density of 1×10^5 cells per well. Twenty-four hours later, cells were treated with adriamycin for 24 h. Adherent and nonadherent cells were then pooled and analyzed for viability by trypan blue exclusion. At least 200 cells were counted for each data point. To assay the ability of E1A to induce apoptosis in *Myc*^{-/-} cells, HO.15.19, and parental Rat1 cells, TGR-1 (13) were transduced with retroviral expression constructs for E1A, E1A $\Delta 26$ –35, or Myc, in the indicated combinations. Relative apoptosis was determined by comparing cell death 24 h after treatment with increasing doses of adriamycin. Data presented are the average of three independent experiments.

Anchorage-Independent Growth. BJ normal human primary foreskin fibroblasts were stably transduced with pBabe-Puro Ha-RasV12, pWZL-Neo E1A, or Hygro-MarXII-Myc in the indicated combinations by retroviral infection and analyzed for anchorage-independent growth in semisolid media as described (16).

ChIP and Re-ChIP Analysis. ChIP analyses were performed in U2OS cells that had been transfected with either control (pUC119) or pCMV-FLAG-p400 construct by using Fugene 6 (Roche). After 48 h, cells were infected with control, *d1520*, and *d1102* adenovirus for 8 h. Primary immunoprecipitation was performed by using anti-Myc (N262) antibody; for re-ChIP, a secondary immunoprecipitation using anti-FLAG (M2) antibody was performed. Coprecipitating DNAs after each round were assayed by quantitative PCR using either the B23.C (specific) and B23.M (nonspecific control) amplicons (23). Re-ChIP DNA signals for FLAG-p400 were further normalized to those from cells transfected with the vector control.

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Adenoviral E1A Function through Myc

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Abstract

The study of DNA tumor viruses has been invaluable in uncovering the cellular nodes and pathways that contribute to oncogenesis. Perhaps one of the best-studied oncoproteins encoded by a DNA tumor virus is adenovirus E1A, which modifies the function of key regulatory proteins such as retinoblastoma (Rb) and the chromatin remodeling protein p400. Although the interaction of E1A with Rb has long been known to target regulation of the E2F transcription factors, the downstream target of the E1A-p400 interaction has remained elusive. We have recently reported that a critical downstream link of the E1A-p400 nexus is the oncoprotein transcription factor c-Myc. Through its interaction with p400, E1A stabilizes Myc and promotes formation of Myc-p400 complexes on chromatin, leading to activation of Myc target genes. These findings point to an important role for p400 in Myc function and reveal that E1A drives oncogenesis by tapping into two important transcriptional networks: those of E2F and Myc. [Cancer Res 2009;69(1):6-9]

DNA Tumor Viruses: Keys to Unlocking Molecular Mechanisms

DNA tumor viruses, such as SV40 and adenovirus, have long been used as tools with which to probe critical cellular processes, including oncogenic transformation. Because of their small genomes and striking biological effects, it is generally assumed that DNA tumor viruses have evolved to target the minimal number of cellular nodes and pathways required for transformation. Thus, by studying these viruses over the years, researchers have been able to probe mechanisms of cancer initiation as well as expose the underlying biological events. There is a rich legacy of exploiting DNA tumor viruses to study genome architecture, eukaryotic transcription, DNA replication, and oncogenesis. Work done on DNA tumor viruses in the 1970s and 1980s led to the development of techniques such as the use of ethidium bromide staining for DNA and RFLP analysis, as well as revealing pre-mRNA splicing, transcriptional regulation, and oncogene cooperation (1). Thus, much of what we take for granted in modern-day molecular biology and cancer research has come from studying DNA tumor viruses.

One of the best-studied DNA tumor viruses is adenovirus, which usually causes upper respiratory tract infections, particularly in children. Although adenovirus is not thought to cause cancer in humans, its early gene products are particularly effective at transforming mammalian cells *in vitro*. Much of the research on adenovirus has focused on the E1A protein of adenovirus-5 (Adv-5),

a small, tractable protein that efficiently collaborates with the adenovirus E1B protein to drive oncogenic transformation. Adv-5 E1A is expressed as two alternately spliced isoforms, referred to as 12S and 13S, and shares four highly conserved regions (CR1-CR4) with E1A proteins from other serotypes. These four conserved regions have been studied extensively and are thought to influence distinct cellular processes that, together, contribute to tumorigenesis (2). By focusing on these regions, researchers were able to move beyond E1A to the cellular proteins with which it interacts and exploits to exert its effects. The most notable of these cellular proteins is the retinoblastoma (Rb) protein (3), which was identified as a cellular factor that interacts with E1A via CR1 and CR2, regions essential for the ability of E1A to promote cell cycle progression. It is now clear that, under normal conditions, Rb functions as a tumor suppressor by sequestering the cell cycle regulator E2F and limiting its role in inducing proliferation. On adenoviral infection, however, binding of E1A to Rb strips it away from the E2F complex, which in turn leads to ectopic activation of E2F and its target genes and loss of growth control. These early studies of the E1A-Rb connection thus revealed an important regulatory pathway that has subsequently been found to be deregulated in most human cancers.

In addition to Rb, E1A interacts with an impressive collection of cellular proteins, including TBP, CBP/p300, p400, YY1, and CDK8, to name a few (2). Although it can be difficult to dissect the individual contributions of each of these interactions to E1A function, a growing body of evidence indicates that interaction of E1A with chromatin remodeling proteins such as CBP/p300 (4) and p400 (5) is important for the biological activities of E1A. In the first case, it has been argued that interaction of E1A with CBP/p300 regulates its association with the anaphase-promoting complex APC, which in turn influences cell cycle progression during mitosis or early G₁ phase of the cell cycle (6). For the E1A-p400 interaction, however, the downstream effector network was unknown. Our recent studies (7) have led to the realization that the cellular oncoprotein c-Myc is an important target of the E1A-p400 nexus and that E1A can function through p400 to exploit the transcriptional program of Myc.

Myc as a Downstream Effector of E1A

Myc is an oncoprotein transcription factor that promotes tumorigenesis by activating and repressing a wide set of target genes that control cell growth and proliferation (8). The amount of Myc in a normal cell is very low and is tightly controlled at both the transcriptional and posttranscriptional levels. We have previously found that one mechanism regulating Myc levels is ubiquitin-mediated proteolysis and that tumor-derived mutations within Myc that subvert this process are associated with aggressive oncogenic activity (9, 10). Moreover, we and others have also found that the activity of transcription factors such as Myc can be profoundly regulated by their ubiquitylation status (11-15) and that in some cases proteolysis of these factors is linked to their

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ability to activate transcription (16). Because of the importance of ubiquitin-mediated proteolysis in influencing Myc levels and activity, therefore, it is important to identify and understand the molecular processes through which Myc ubiquitylation and stability are regulated.

Recently, Lohr and colleagues (17) reported that E1A stabilizes Myc during the course of adenoviral infection. To explore the underlying molecular mechanism through which this occurs, we performed a structure-function analysis of E1A, using a set of "classic" E1A mutants developed by others, and probed for regions in the protein that are necessary for Myc stabilization. This analysis identified residues 26 to 35 of E1A as being uniquely required for its ability to block Myc turnover. Given the modular nature of E1A, we reasoned that loss of interaction with a specific cellular protein might underlie the inability of the $\Delta 26-35$ E1A mutant to stabilize Myc. The NH₂ terminus of E1A interacts with a slew of proteins, including Rb, 19S proteasome subunits, CBP/p300, TBP, p400, and TRRAP, but through comparison of different sets of E1A mutants that disrupt the various interactions, we were able to determine that p400 interaction is uniquely required for the effects of E1A on Myc. p400 is part of a nucleosome-remodeling complex that the Livingston group has shown is important for the activity of both E1A and Myc (5). The common connection of Myc and E1A to p400, and the ability of other chromatin-remodeling proteins to regulate Myc stability (18), led us to hypothesize that p400 may

underlie stabilization of Myc by E1A. Indeed, our subsequent studies showed that p400 is required for the ability of E1A to stabilize the Myc protein and that forced expression of p400 attenuates Myc ubiquitylation. Based on these results, we have concluded that p400 is an important regulator of Myc stability and that interaction of p400 with E1A promotes its ability to stabilize Myc.

What is the functional significance of the E1A-p400-Myc connection? Our subsequent studies showed that E1A promotes the stable association of Myc and p400 both in solution and, critically, on chromatin at Myc target genes. Consistent with the role of p400 as a cofactor for the transcriptional activity of Myc, this enhanced Myc-p400 interaction leads to activation of a set of Myc target genes. In a sense, therefore, E1A uses p400 to "hijack" Myc and its activities. We believe that the E1A-Myc connection is important for the activity of E1A because E1A cannot stimulate apoptosis in the absence of Myc and because increased expression of Myc can compensate for transformation defects that are associated with loss of the E1A-p400 interaction. Taking our observations together, we conclude that E1A drives oncogenesis, in part, by using p400 to tap into the transcriptional program of Myc and elicit a Myc-like response (Fig. 1).

Coopting a Cellular Oncoprotein

The functional similarities between Myc and E1A have been appreciated for many years (19). Both proteins have the ability to

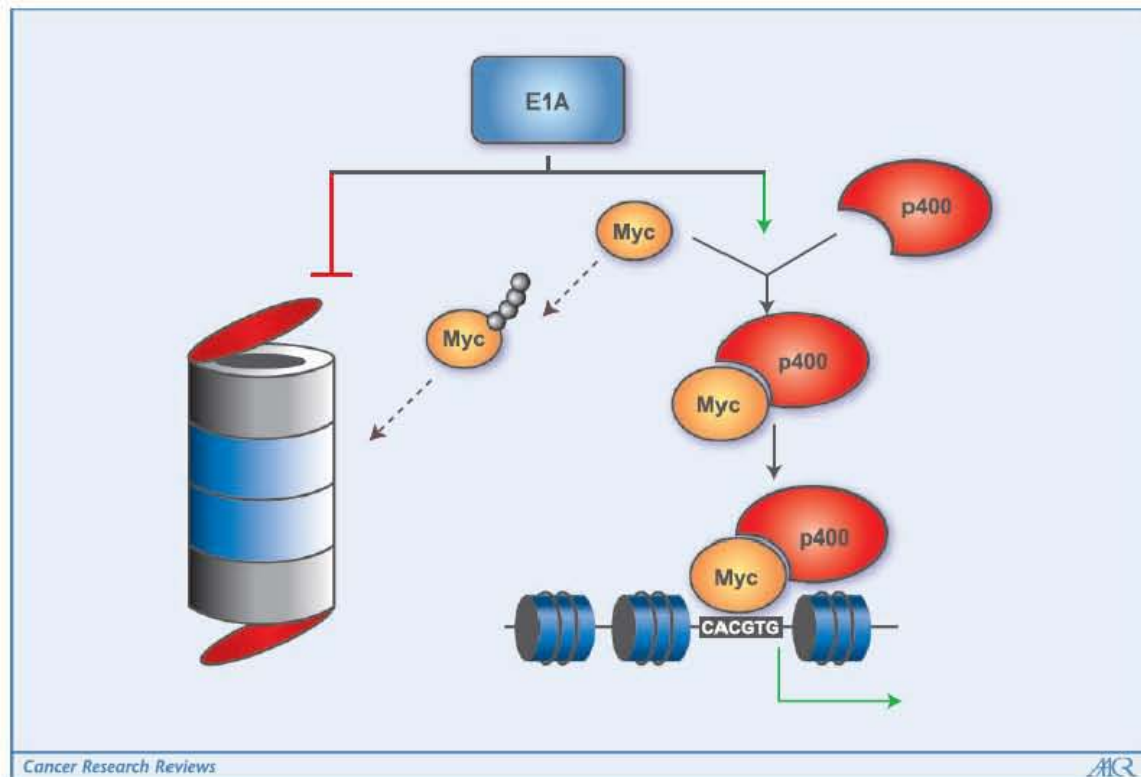


Figure 1. E1A regulates Myc via p400. Association of E1A with the chromatin regulator p400 promotes its interaction with Myc. The interplay between Myc and p400 has two important consequences: it stabilizes Myc by interfering with its ubiquitylation, and it promotes the formation of Myc-p400 complexes on Myc target genes, leading to their activation. Thus, E1A is able to elicit, in part, a Myc-like cellular response that contributes to function in apoptosis and cellular transformation.

promote ectopic S-phase entry and cell proliferation. Both can block differentiation, and both cooperate with oncogenes such as Ras to drive cellular transformation. Despite the overlapping repertoires of Myc and E1A, the underlying biological basis of this phenomenon has been unclear. Our demonstration that E1A can function through Myc, thus regulating Myc target genes, provides one explanation for this overlap and shows how a viral oncoprotein can recruit a cellular oncoprotein to exert its effects.

The regulation of Myc by adenovirus E1A raises several interesting issues about the underlying processes at work and how these processes affect cell growth control pathways. One intriguing issue is the mechanism through which E1A acts on p400 to stimulate its interaction with Myc. We favor the idea that E1A acts by forming a ternary complex between itself, p400, and Myc and that it is this complex that assembles on Myc-regulated genes to activate their expression. Although the Livingston group (5) has shown that p400 associates with distinct populations of E1A and Myc in solution, it is possible that such a complex forms only transiently or is stabilized within the context of chromatin. The concept that E1A acts within the context of chromatin is not without precedent. Work from the Harter group (20) has shown that E1A associates with E2F-dependent target genes and that this association leads to changes in chromatin structure that permit gene activation. In this instance, E1A is not simply acting to sequester Rb family proteins but is playing an active role in setting the appropriate epigenetic state for E2F-dependent transcription. We hypothesize that E1A similarly acts at Myc target genes to influence their chromatin structure via p400. Indeed, we have detected E1A at several Myc target genes by using chromatin immunoprecipitation,³ consistent with the idea that E1A acts on Myc that is promoter bound. If this notion is correct, it raises the interesting possibility that E1A could influence Myc function not simply through p400 but also through the unique set of other transcription factors with which it interacts (2). In this way, E1A does not just stimulate Myc activity but, through recruitment of new cofactors, could qualitatively change the manner in which Myc functions and how its target genes are regulated.

An additional unanswered question is the mechanism through which p400 stabilizes the Myc protein. Curiously, Myc stability is known to be regulated by the histone acetyltransferases hGCN5 and TIP60 (18), establishing a precedent for how a chromatin modifier can influence Myc turnover. In the latter instance, however, hGCN5/TIP60 function by acetylating Myc, which could possibly block availability of lysine residues within the protein for ubiquitin conjugation. Given that p400 resides within the TIP60 complex (5), it is possible that Myc acetylation is a relevant mechanism in the E1A-p400-Myc nexus. Alternatively, it is possible that p400 binds directly to the transcriptional activation domain of Myc, the same region that signals Myc ubiquitylation (21). In this case, stabilization could occur by steric hindrance, with p400 blocking binding of either the Fbw7 (22) or Skp2 (12, 15) ubiquitin ligases to their cognate degrons within Myc.

Regardless of the mechanism through which p400 stabilizes Myc, however, the observation itself raises a curious paradox. Numerous studies have implied that the ubiquitylation/destruction of proteins such as Myc is coupled to their activity and in some cases may be required for activator function (13, 14).

At least two ubiquitin ligases that target Myc also serve as coactivators for Myc function (8, 11, 12, 15). If indeed ubiquitylation promotes Myc activity, how can E1A, which attenuates Myc ubiquitylation via p400, stimulate Myc activity? We suggest that stimulation of the p400-Myc interaction by E1A serves to obviate the requirement of Myc ubiquitylation in Myc activity. This could occur either if p400 bypasses the need for ubiquitylation by initiating a different mode of Myc activity or if ubiquitylation itself is a signal that normally acts to promote or stabilize the p400-Myc interaction. In either case, E1A both stimulates Myc activity and disconnects it from a potent mode of cellular regulation that integrates multiple signaling pathways (23, 24) that typically keep Myc in check. Interestingly, E1A may have multiple means to disconnect Myc activity from destruction. Myc is stabilized by phosphorylation of Ser⁶² (24) within its degron. This phosphorylation is carried out by extracellular signal-regulated kinase (ERK), which in turn is induced by E1A (25). Thus, in addition to promoting the p400-Myc interaction, E1A may also stabilize Myc by activation of ERK. As it is likely that some cancer-associated Myc mutants are stabilized by accumulation of Ser⁶² phosphorylation (24), further study of E1A and p400 may thus provide important insight into the mechanism through which highly aggressive mutants of Myc function. An important future goal will be to study the parallels between the effects of E1A on Myc versus the tumor-derived Myc cancer mutants. It will also be important to determine how p400 and Myc ubiquitylation figure in this context.

As mentioned earlier, the utility of studying DNA tumor viruses stems in large part from their ability to shed light on the most influential and vulnerable cellular processes that modulate growth control. Just as the interaction of E1A with Rb revealed the importance of the Rb-E2F pathway in control of the cell cycle and cancer progression, we believe that interaction of E1A with p400 highlights the importance of the p400-Myc connection to the same processes. Previous work has shown that p400 is a novel regulator of the p53-p21 cellular senescence pathway (26), and led to the notion that up-regulation of p400 could subvert normal senescence mechanisms. Our data imply that, conversely, increases in p400 levels would also lead to a profound deregulation of Myc target gene expression, which could collaborate with a block in senescence to drive transformation. Although there are no data currently pointing to altered p400 expression in human cancers, the duality with which perturbations in p400 could influence tumorigenesis makes an examination of p400 expression and regulation in tumors an attractive idea.

A Left Jab, Right Hook Strategy for Transformation

E1A is a remarkable protein. Despite its small size (~250 amino acids), it has evolved to target a surprising number of host cell factors. As a viral strategy, the ability of E1A to tap into the Myc pathway is both highly effective and economical; as little as nine amino acids in E1A (26-35) could allow it to modulate expression of many of the hundreds to thousands of genes that are under the control of Myc. Together with a plethora of previous studies, our work reveals that E1A simultaneously and synergistically activates at least two critical growth-controlling transcriptional networks: Myc and E2F. This "double whammy" likely lies at the heart of the oncogenic potency of E1A and further highlights the utility of DNA tumor viruses as roadmaps to understanding cancer initiation and progression.

³ Unpublished data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Research Article

Inference of cell cycle-dependent proteolysis by laser scanning cytometry

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ABSTRACT

Mechanisms that couple protein turnover to cell cycle progression are critical for coordinating the events of cell duplication and division. Despite the importance of cell cycle-regulated proteolysis, however, technologies to measure this phenomenon are limited, and typically involve monitoring cells that are released back into the cell cycle after synchronization. We describe here the use of laser scanning cytometry (LSC), a technical merger between fluorescence microscopy and flow cytometry, to determine cell cycle-dependent changes in protein stability in unperturbed, asynchronous, cultures of mammalian cells. In this method, the ability of the LSC to accurately measure whole cell fluorescence is employed, together with RNA fluorescence *in situ* hybridization and immunofluorescence, to relate abundance of a particular RNA and protein in a cell to its point at the cell cycle. Parallel monitoring of RNA and protein levels is used, together with protein synthesis inhibitors, to reveal cell cycle-specific changes in protein turnover. We demonstrate the viability of this method by analyzing the proteolysis of two prominent human oncoproteins, Myc and Cyclin E, and argue that this LSC-based approach offers several practical advantages over traditional cell synchronization methods.

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Introduction

Ordered progression through the eukaryotic cell cycle depends on mechanisms that tie the abundance and activity of critical regulatory molecules to the events of DNA replication and cellular division. One of the most pervasive of these mechanisms is ubiquitin (Ub)-mediated proteolysis, a process in which covalent attachment of Ub to target proteins signals their destruction by the 26S proteasome. By destroying proteins at specific points in the cell cycle, the Ub–proteasome system (UPS) provides directionality to the events of cell duplication, and insures that each phase of the cycle occurs after the previous one is completed [1].

Despite the biological importance of cell cycle-regulated proteolysis, techniques to study this phenomenon are limited. One

of the most popular approaches is to arrest cells at specific cell cycle stages, either by chemical or growth-factor blockade, and to then release cells back into the cycle and monitor them as they move in synchrony through subsequent cell cycle transitions. These synchronization methods, however, involve significant disruption to normal cellular physiology, and—because of the perturbations involved—can influence the apparent behavior of molecules with respect to the cell cycle [e.g., [2–4]]. An alternative strategy, centrifugal elutriation [5], separates cells based on their size, which increases linearly during the cell cycle. In this way, relatively pure fractions of cells that are in either G1, S, or G2/M cell cycle phases can be obtained. Elutriation has the advantage of not requiring disruption to the cell cycle to provide synchronicity, but its practical application is restricted because it is best

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Abbreviations: DT, double-thymidine; FC, flow cytometry; IF, immunofluorescence; LSC, laser scanning cytometer; RNA-FISH, RNA-fluorescence *in situ* hybridization; Ub, ubiquitin; UPS, ubiquitin–proteasome system

suiting to non-adherent cells and requires relatively large volumes of cell cultures.

We are interested in the mechanisms controlling Ub-mediated proteolysis of the oncoprotein Myc [6]. There are conflicting reports as to whether Myc levels and stability are influenced by the cell cycle. Some studies demonstrated that Myc synthesis and stability are not cell cycle regulated [e.g., [7]], whereas others concluded that Myc RNA and protein levels peak at the G1/S transition [e.g., [8,9]], or that Myc is stabilized during mitosis [10]. Moreover, phosphorylation events within Myc—at residues threonine 58 (T58) and serine 62 (S62)—that control its ubiquitulation by the SCF^{Fbw7} Ub-ligase [11] have been reported to peak during late G2/M-phase [e.g., [12]], lending support to the idea that Myc destruction is cell cycle-regulated [13]. Because true cell cycle-dependent changes in Myc levels or stability could have a profound impact on the mechanism through which Myc promotes cell growth and proliferation, it is important that the issue of whether or not Myc proteolysis is cell cycle-regulated be resolved.

We reasoned that some of the contradictory findings on the relationship between Myc and the cell cycle may have resulted from the different techniques used in the various studies. In some cases, centrifugal elutriation was employed to monitor Myc levels and stability [e.g., [7]], whereas other studies used either Nocodazole or double-thymidine block and release strategies. We sought to develop an additional protocol that would allow us to take a comprehensive look at the influence of the cell cycle on Myc synthesis, location, and stability in *unperturbed* cultures of cells. The recent development of laser scanning cytometry (LSC) created an opportunity to develop this protocol. The LSC, which is a technical merger between fluorescence microscopy and flow cytometry [14], allows for whole cell quantification of fluorophores targeted to DNA, RNA, or protein. By accurately quantifying total cellular DNA content (using fluorescent dyes such as Hoechst 33342), the LSC can determine the cell cycle state of an individual cell, and then relate this state to some other fluorescent parameter, such as the signal from a fluorescently-labeled antibody. In this way, levels of a particular RNA or protein can be measured in individual cells and expressed relative to the particular cell cycle stage. By compiling data from thousands of cells in this way, highly quantitative cell cycle analysis can be performed without disruption to normal cellular physiology. Importantly, by comparing RNA and protein levels for a particular gene product, and by monitoring protein levels after transient inhibition of protein synthesis, cell cycle dependent changes in protein stability can be inferred.

Here we describe how LSC-based assays can be used to monitor cell cycle-dependent changes in protein stability in small numbers of unperturbed cells growing on a coverslip. We demonstrate the utility of this approach by analyzing the cell cycle expression profile of Myc and comparing it with that of another prominent human oncoprotein, Cyclin E.

Materials and methods

Cell culture and immunofluorescence

Human U2OS and HeLa cells were grown in DMEM supplemented with antibiotics and 10% fetal calf serum. For analysis, cells were

plated onto glass coverslips, grown at 37 °C for 24 h, and then fixed, either using methanol or paraformaldehyde [15]. Immunofluorescence was performed as described in [15] using the following antibodies: (i) α -Myc (N-262, *Santa Cruz*), (ii) α -Cyclin E (HE12, *Santa Cruz*), (iii) α -Actin (AC-15, *Sigma*), and (iv) α -Phospho T58 Myc (9401S, *Cell Signaling*). Immune complexes were detected using FITC-tagged secondary anti-mouse or anti-rabbit antibodies, as appropriate. DNA was stained with Hoechst 33342 (2 μ g/ml). For Nocodazole arrest, cells were seeded for 24 h, and then treated with Nocodazole (100 ng/ml) for 16 h. Nocodazole was removed and cells were released from G2/M block for varying time points. For double-thymidine (DT) arrest, cells were treated with 2.5 mM thymidine for 14 h, washed and released for 12 h, and then re-arrested in 2.5 mM thymidine for 14 h before FC analysis.

Centrifugal elutriation

Centrifugal elutriation was performed as described in [16]. Briefly, actively growing U2OS cells were elutriated using the Beckman JE-6B rotor, at a rotor speed of 1500 rpm and rotor temperature of 20 °C. Cells were eluted in DMEM + 1% FBS, by applying an increasing medium flow rate ranging from 40 ml/min–150 ml/min. Approximately 10 fractions of 250 ml were collected. Cells were rapidly harvested by centrifugation and either fixed in methanol and analyzed by flow cytometry or lysate prepared for Western blotting.

RNA FISH

RNA-FISH analysis for detection of nuclear RNAs was performed on triton-extracted, fixed, U2OS cells as described in [15]. Four anti-sense probes were used for Myc: Myc1-TAGTCGAGGTCATAG-TTCCTG; Myc2-TCGAGGAGAGCAGAGAATCCG; Myc3-TTCAACTGTTCTCGTCTTTC; Myc4-TGTTCCCTCTTGACATTCT. Two antisense probes were used for actin: Act1-ATAGCAGCCTG-GATAGCAA; Act2-TGGAAGCAGCCGTCGCATCTCTTGCTCGA. In each case, the corresponding sense probes were used as a control. Pooled probes were end-labeled with Dig-11-ddUTP using the Digoxigenin End-labeling kit (*Roche*). FISH was performed as described in [15].

LSC analysis

All experiments were performed using the LSC-iCys system (Compucyte, MA) attached to an Olympus IX-71 microscope. Samples with multiple fluorophores were compensated by measuring the spectral bleed into other channels using controls containing each individual fluorophore, and subtracting the corresponding non-specific leakage. Thresholds for measuring each fluorophore were set at signal intensities where there was negligible background signal. The fluorescence units obtained thereafter were used to compare the expression patterns within different cell cycle populations. Approximately 2000–3000 cells were scanned in each individual analysis.

Cell cycle profiles were gated based on DNA content into G1, S, and G2/M populations. For sub-cellular visualization experiments, galleries of cells in different cell cycle phases were created; localization of the signal was compared by merging the pictures obtained from the Green, Blue, and Scatter channels. For quantification, the mean signals of the respective proteins were

measured within the given cell cycle gates. Fluorescence values were normalized either to mean signal in the G1 phase, or reported as a ratio of green/blue fluorescence to quantify relative levels of protein or RNA in different cell cycle stages as a function of DNA.

RNA interference and protein synthesis inhibition

Myc and Fbw7 knockdown was performed using RNA interference. Pools of siGenome RNA against Myc, Fbw7, and Luciferase (control) were obtained from Dharmacon, and transiently transfected into U2OS cells using the Oligofectamine reagent (Invitrogen). Transfected cells were analyzed by LSC and Western blotting. For experiments involving cyclohexamide, HeLa cells, growing on coverslips, were treated with 50 $\mu\text{g}/\text{ml}$ cyclohexamide, fixed at the indicated time points, and Myc and actin levels at each point quantified by immunofluorescence and LSC. The corrected fluorescence value in each of the cell cycle stages at the “0” time point was arbitrarily fixed as 100% and values of corrected fluorescence from every subsequent time point were represented as relative percentage of the “0” (100%) time point. The effect of USP28 was studied by transfecting shRNA pools against USP28 into U2OS cells. Knockdown of USP28 expression was confirmed using quantitative RT-QPCR, and found to be ~50% (not shown).

Results and discussion

We began our study of the relationship between Myc and the cell cycle by testing the notion that different methods of cell synchronization can lead to different conclusions regarding the behavior of Myc. We compared two common methods of cell synchronization—Nocodazole block, which arrests cells in the G2/M stage of the cell cycle—and double-thymidine (DT) block, which produces a G1/S arrest. We used Western blot to monitor steady-

state Myc levels during subsequent release of cells back into the cycle; we also monitored phosphorylation of Myc at residue T58, which is required for its destruction via the SCF^{Fbw7} Ub-ligase, and which has been reported to be cell cycle regulated [12]. As hypothesized, these two approaches gave different results (Fig. 1 and Supplemental Fig. 1). Nocodazole arrest gave the impression that, although Myc levels are constant throughout the cell cycle, T58 phosphorylation peaks in mitosis (Fig. 1A). DT-block, on the other hand, gave the impression that total Myc levels peak during release from G1/S, and that T58 phosphorylation does not appreciably differ from total Myc levels (Fig. 1B). These differences were not observed when we examined another SCF^{Fbw7} substrate, Cyclin E—both Nocodazole and DT block showed that Cyclin E levels were high at G1/S (Fig. 1A, lanes 4–6; Fig. 1B, lane 2), and low at G2/M (Fig. 1A, lanes 2–3; Fig. 1B, lanes 6–8). We conclude that apparent cell cycle-related changes in Myc levels and phosphorylation can be profoundly influenced by chemical synchronization methods. Importantly, because Cyclin E behaved consistently after both Nocodazole and DT blockade, we further conclude that synchronization approaches can influence the status of different proteins in different ways.

Given the discrepancy between the two synchronization approaches, we sought to develop an LSC-based protocol that would allow us to measure the influence of the cell cycle on Myc levels and stability in unperturbed, asynchronous, cultures of cells growing on a glass coverslip. As an initial validation, we found that cell cycle profiles determined by LSC were comparable to those determined by traditional flow cytometry (FC; Supplemental Figs. 2A, B); we also found that we could detect a robust signal for Myc using immunofluorescence (IF), and that Myc was predominantly nuclear through all stages of the cell cycle (Supplemental Fig. 2C). We therefore chose to use the LSC determine whether steady-state Myc RNA and protein levels, or stability, are influenced by the cell cycle. By triangulation of all three parameters, we hoped to make an informed conclusion regarding cell cycle control of Myc.

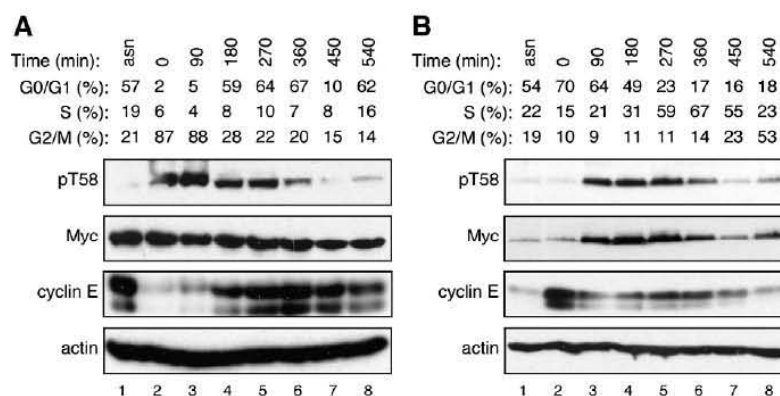


Fig. 1 – Myc levels and phosphorylation are influenced by cell-synchronization. (A) U2OS cells were synchronized by treatment with Nocodazole to arrest at the G2/M transition. Cells were released from arrest, and samples were taken at the indicated time-points for analysis of cell cycle by FC, and protein levels by Western blot. The numbers for G0/G1, S, and G2/M percentages were derived from FC. ‘pT58’ refers to an antibody that recognizes the phosphorylated form of residue threonine 58 within Myc. (B) As in (A), except that cells were subjected to double-thymidine (G1/S) block and release. FC profiles are presented in Supplemental Fig. 1. Detailed methods are presented in Supplemental Information.

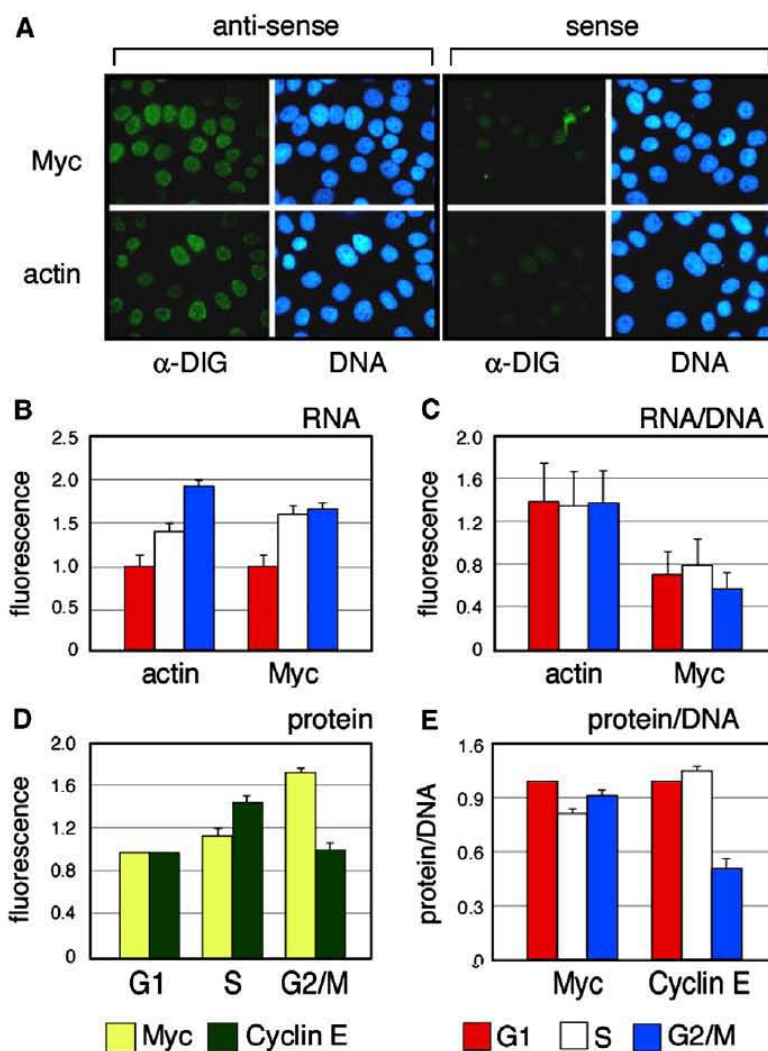


Fig. 2 – Parallel analysis of Myc RNA and protein levels throughout the cell cycle. (A) Visualization of nuclear Myc and actin transcripts in U2OS cells by RNA FISH. Anti-sense probe cocktails detect bona-fide transcripts; sense probe cocktails reveal background. (B) Quantification of absolute FISH signals. Fluorescence intensities were quantified by LSC in cells binned into G1, S, or G2/M populations, and expressed relative to the signal from G1 phase cells. (C) Quantification of relative FISH signals. Fluorescence intensities were quantified by LSC in cells binned into G1, S, or G2/M populations, normalized to the signal for DNA content in those cells, and expressed relative to the signal from G1 phase cells ($n = 2$, mean \pm SD). Arbitrary units of fluorescence are used. (D) Quantification of absolute levels of Myc and Cyclin E protein. Fluorescence intensities were quantified by LSC in cells binned into G1, S, or G2/M populations, and expressed relative to the signal from G1 phase cells. (E) Quantification of relative protein levels. Fluorescence intensities were quantified by LSC in cells binned into G1, S, or G2/M populations, normalized to the signal for DNA content in those cells, and expressed relative to the signal from G1 phase cells ($n = 4$, mean \pm SD). Detailed methods are presented in [Supplemental Information](#).

We combined LSC with RNA-fluorescence *in situ* hybridization (RNA-FISH) to compare the levels of Myc and (as a control) actin RNAs in human U2OS cells (Fig. 2). For these studies, we used a detergent pre-extraction protocol to remove most of the cytosolic transcripts [15], allowing us to enrich for the popula-

tion of newly-synthesized RNA molecules. Results of this analysis, performed in human U2OS cells, are shown in Fig. 2. Using LSC/RNA-FISH, we were able to detect robust levels of Myc and actin RNAs using labeled anti-sense, but not the corresponding sense, probes (Fig. 2A). Quantification of these

hybridizations (Fig. 2B) revealed that the *absolute* levels of both RNAs increase as cells passage through the cell cycle, being lowest in G1 cells, and highest in cells from the G2/M population. This apparent increase, however, appears to be a result of the increase in cell mass that occurs during the cell cycle, because, when normalized for either cell volume (*data not shown*) or DNA content (Fig. 2C), the *relative* levels of newly-synthesized Myc and actin RNAs are equivalent in each of the cell cycle populations. We conclude, therefore, that the levels of Myc RNA do not fluctuate during the cell cycle. Moreover, because Myc transcripts are extremely unstable, with a half-life of ~25 min [17], we infer that the relatively similar levels of nuclear Myc RNA in G1, S, and G2/M phase cells reflect constitutive transcription from the *c-Myc* gene.

We next examined endogenous Myc protein in the different sub-populations of cells. We compared Myc with Cyclin E, because both proteins are targets for the SCF^{Fbw7} Ub-ligase [11], and because Cyclin E is tagged for destruction soon after cells enter S-phase [*e.g.*, [18]]. In these assays (Figs. 2D, E), Myc protein levels mirrored those of Myc RNA and—after normalization to DNA content—were unaffected by cell cycle status (Fig.

2E). Cyclin E, in contrast, displayed pronounced cell cycle dependency, with both its relative (Fig. 2D) and absolute (Fig. 2E) levels being lowest in G2/M cells, as expected. We validated this pattern of expression independently, by comparing the bivariate distribution of DNA content versus Myc or Cyclin E immunofluorescence as described by Gong *et al.*, [2] and presented in Supplemental Fig. 2D. Comparison of these results with those from analysis of parallel U2OS cell cultures by centrifugal elutriation (Supplemental Fig. 3) showed remarkable consistency between the LSC and elutriation approaches, with Myc levels relatively fixed throughout the cell cycle, and Cyclin E being lowest in G2/M cells. From these data, we conclude that Myc stability is likely to be unchanged throughout the cell cycle, and that LSC-based methods combining RNA-FISH and IF provide a viable way to reveal cell cycle-dependent changes in protein levels and stability.

To more directly probe for cell cycle-dependent changes in Myc turnover, we transiently inhibited protein synthesis with cyclohexamide and used the LSC to monitor the levels of Myc protein in each cell subpopulation as a function of time. This approach—which is analogous to the ‘cyclohexamide chase’ protocol that is often used to monitor Myc stability [19–21]—allowed us to compare the rates with which Myc protein disappeared in each cell-cycle subpopulation of cells. Under these conditions, Myc disappeared quickly following addition of cyclohexamide, with an apparent half-life of ~50 min (Fig. 3A). Actin, in contrast, was relatively stable during the period of the experiment (Fig. 3B). Importantly, when we examined the rate of decay of Myc in the distinct cell subpopulations, both the apparent half-life of Myc and its decay kinetics were identical in G1, S, and G2/M phase cells. These results support early studies of Myc proteolysis [7,17,22] which showed that the rate of Myc destruction is constant throughout the cell cycle, and are consistent with the conclusions made from our parallel analysis of Myc RNA and protein levels.

One of the most practical advantages of the LSC-based approach is that it can be performed on a small number of cells (typically less than 5000), allowing investigators to quickly and economically examine the effects of agents such as drugs or siRNAs on cell cycle-dependent proteolysis; these type of experiments are difficult or prohibitively expensive with the scale needed for centrifugal elutriation. To illustrate this point, we compared the effects of siRNA-mediated knockdown of Fbw7 on two of its substrate proteins, Myc and Cyclin E (Fig. 4). For Myc, we also examined the effects of knock-down of USP28, a Ub-specific protease that antagonizes Fbw7-dependent Myc destruction [23]. Knockdown of Fbw7 had little if any effect on levels of Myc protein detected by either LSC (Fig. 4A) or Western blot (Fig. 4B), and no influence on the cell cycle distribution of Myc (Fig. 4C). This lack of an effect is likely due to the reported antagonism between Fbw7 and USP28, as knockdown of USP28 reduced steady-state Myc levels (Fig. 4D) equally in all stages of the cell cycle (Fig. 4E), and this effect was reversed by simultaneous knockdown of Fbw7 (Fig. 4D). We thus conclude that the Fbw7–USP28 pathway is unlikely to act in a cell cycle-dependent way on Myc. In contrast, however, Fbw7 did appear to act in a cell cycle-dependent manner upon Cyclin E. Knockdown of Fbw7 increased Cyclin E levels in total cell populations (Figs. 4B, E), with the most pronounced effects observed in the S and G2/M populations of cell, as expected. This result

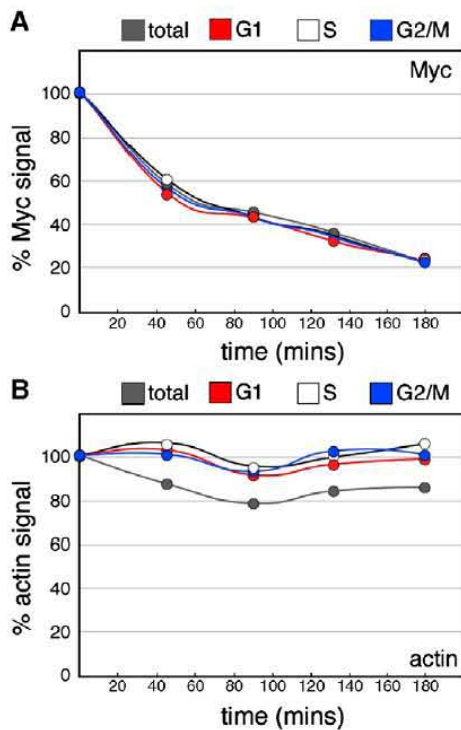


Fig. 3 – The metabolic stability of Myc is constant throughout the cell cycle. HeLa cells were treated with cyclohexamide for the indicated time points, fixed, and Myc (A) and actin (B) levels for each cell cycle subpopulation measured by immunofluorescence and LSC. In each case, the relative signals are normalized to DNA content and presented as a percentage of the zero-time point samples. Detailed methods are presented in Supplemental Information.

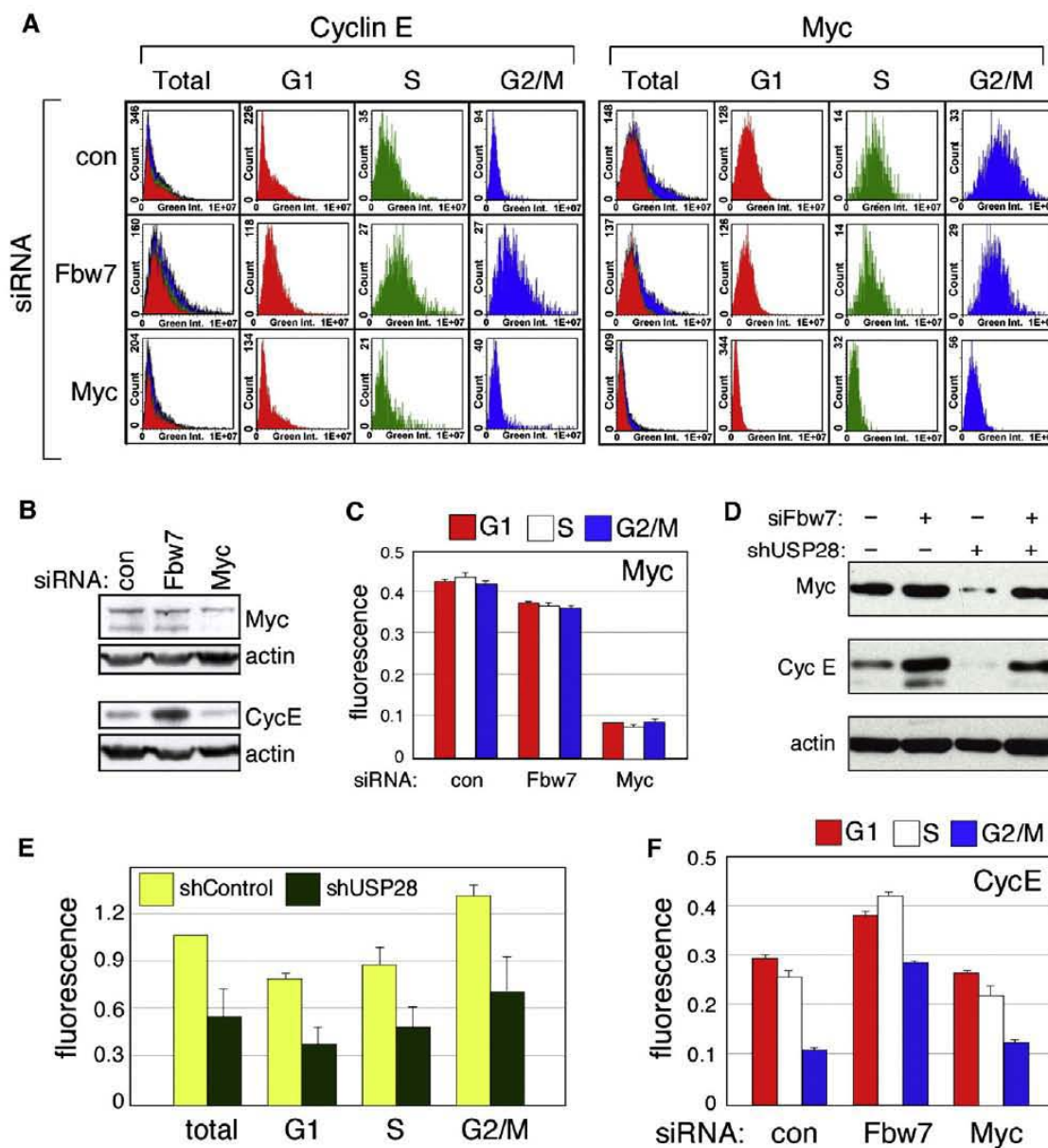


Fig. 4 – Differential regulation of Myc and Cyclin E by Fbw7 during the cell cycle. (A) Effects of siRNA-mediated knockdown of Fbw7 on Myc and Cyclin E. U2OS cells were transfected with the indicated siRNAs [control (= luciferase); Fbw7, and Myc], and Myc and Cyclin E levels determined in each cell cycle subpopulation of cells. Protein expression histograms show that Fbw7 knockdown substantially increases Cyclin E levels in S and G2 phase (rightward shift), whereas it has no significant effect on Myc profiles. (B) Analysis of total steady-state levels of Myc and Cyclin E following Fbw7 knockdown. Western blot analysis of cells analyzed in (A). (C) Quantification of relative Myc protein levels. Fluorescence intensities for Myc were quantified by LSC in cells binned into G1, S, or G2/M populations, normalized to the signal for DNA content in those cells, and expressed relative to the signal from G1 phase. (D) Combined effect of USP28 and Fbw7 knockdown. U2OS cells stably expressing an shRNA against USP28 (or control luciferase shRNA) were transfected with siRNA against Fbw7 (or control luciferase siRNA). Cells were harvested and Myc, Cyclin E, and actin levels detected by WB. (E) Effect of USP28 on Myc levels during the cell cycle. Immunofluorescence of endogenous Myc was performed in U2OS cells expressing either cells stably expressing an shRNA against USP28, or control luciferase shRNA. Cells were counterstained with Hoechst 33342 and analyzed by LSC. (F) Cell cycle-dependent effects of Fbw7 on Cyclin E. Experiment was performed as in (C), except that Cyclin E was detected by IF. Detailed methods are presented in [Supplemental Information](#).

demonstrates that the LSC-based approach can be used to address physiologically important questions relating to the role of specific Ub-ligases in cell cycle-dependent proteolysis.

Conclusions

The LSC-based approach that we describe here is a simple and reliable way to determine how turnover of a specific protein is influenced by the cell cycle. Our comparison with chemical synchronization methods, and the different results that we obtained with the different methods, illustrates the importance of being able to probe cell cycle changes in protein levels in asynchronous cultures of cells. The LSC-based method can obtain highly quantitative data from a much smaller number of cells fixed on a coverslip. The reduced scale of this protocol makes it particularly suited to studying the effects of agents such as siRNAs on cell cycle-related protein turnover (as we did with Cyclin E); an approach that is not practical with larger cultures. Finally, the LSC-based method also offers the advantage of being able to simultaneously monitor intracellular protein distribution, offering the potential to expose relationships between the cell cycle, protein localization, and stability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2009.01.011.

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