OREGON HEALTH & SCIENCE UNIVERSITY
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DIFFERENTIAL REGULATION OF N-MYC AND C-MYC SYNTHESIS, DEGRADATION, AND TRANSCRIPTIONAL ACTIVITY BY THE RAS/MAPK PATHWAY

By

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LIST OF ABBREVIATIONS

4E-BP	eIF4E-binding protein
AREs	A-U rich elements
BDNF	brain-derived neurotrophic factor
bHLH-LZ	basic region helix-loop-helix leucine zipper
BR	basic region
CHX	cycloheximide
CRD	coding region instability determinant
degron	degradation domain
E	embryonic day
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
eRF	eukaryotic release factor
ERK	extracellular signal-regulated kinase
FS	Feingold Syndrome
FGF	fibroblast growth factor
GAP	GTPase-activating protein
GEF	guanine-nucleotide exchange factor
GSK3β	glycogen synthase kinase 3β
HLH-LZ	helix-loop-helix leucine-zipper
HSC	hematopoietic stem cell
IGF2BP1	IGF-II mRNA binding protein 1

LiCl	lithium chloride
MAPK	mitogen-activated protein kinase
MB	Myc homology boxes
miRNA	microRNA
Miz1	Myc-interacting zinc finger protein-1
Mnk	MAPK-interacting serine/threonine kinase
myc	myelocytomatosis
PI3K	phosphoinositide 3-kinase
Pin1	prolyl isomerase 1
PP2A	protein phosphatase 2A
RBP	RNA binding protein
RTK	Receptor tyrosine kinase
Ser54	Serine 54
Ser62	Serine 62
TAD	transactivation domain
Thr50	Threonine 50
Thr58	Threonine 58
Trk	tropomyosin receptor kinase
UPS	ubiquitin-proteasome system
UTR	untranslated region
WT	wild-type

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ABSTRACT

Members of the Myc family – N-Myc, c-Myc, and L-Myc – have prominent roles in embryonic development and tumorigenesis. Myc proteins are transcription factors that control the expression of genes involved in diverse cellular processes such as proliferation, growth, inhibition of differentiation, metabolism, and apoptosis. Overexpression of N-Myc, in particular, contributes to the malignant progression of the pediatric cancer neuroblastoma and is a strong predictor of poor prognosis. *MYCN*-gene amplification is one mechanism that contributes to N-Myc overexpression. However, mechanisms that deregulate N-Myc at the post-transcriptional level are less well understood.

The purpose of this thesis is to understand how the small GTPase Ras controls N-Myc at the post-transcriptional level. Ras communicates extracellular signals to the intracellular portion of the cell by activating a number of signal transduction pathways. In turn, these signaling pathways regulate the expression and activity of multiple factors, including N-Myc. Mutations within Ras or, more commonly, alterations to upstream components that activate Ras, are observed in N-Myc-associated cancers. Therefore, understanding how Ras activation regulates N-Myc may provide points of intervention to suppress aberrant N-Myc levels and its oncogenic activity in cancer. Here I show that Ras activation promotes both the synthesis and degradation of N-Myc protein. The translational upregulation exceeds the destabilizing effect Ras has on N-Myc, such that higher levels of N-Myc protein are the net result. Interestingly, Ras-mediated proteolysis of N-Myc is associated with an increase in N-Myc transcriptional activity. These findings suggest that upregulation of N-Myc translation coupled with increased degradation is an

underlying mechanism by which Ras stimulates N-Myc transcriptional and oncogenic activity.

The current understanding of the process by which Ras controls Myc proteolysis has largely been determined using c-Myc as a model. However, this model cannot account for the observation that Ras destabilizes rather than stabilizes N-Myc. Two critical phosphorylation sites within c-Myc are involved in its proteolysis, Thr58 and Ser62. Ras activity modulates the phosphorylation of these sites to prevent proteolysis of c-Myc. In N-Myc, the equivalent sites, Thr50 and Ser54, are perfectly conserved. For this reason, it is generally thought that N-Myc is stabilized by Ras in a manner similar to c-Myc. Since my studies demonstrate that Ras does not stabilize, but rather destabilizes N-Myc, I directly tested whether Thr50 and Ser54 of N-Myc were involved in N-Myc degradation and found that they indeed serve the same proteolytic role as Thr58 and Ser62. Furthermore, the mechanism by which Ras destabilizes N-Myc does not require phosphorylation of N-Myc at Thr50. These studies show that N-Myc degradation is controlled by at least two separate mechanisms: one involving Ras and another involving phosphorylation of N-Myc at Thr50 and Ser54.

Taken together, these studies provide mechanistic insight into how oncogenic Ras augments N-Myc expression and activity. Further, they suggest that upregulation of N-Myc translation and transcriptional activity may be an important mechanism underlying the oncogenic activities of hyperactivated Ras.

CHAPTER 1 | Introduction

1.1 OVERVIEW

Myc oncoproteins are among the most potent regulators of cell cycle progression and cellular transformation. They also play important roles in cell differentiation, apoptosis, angiogenesis, and metabolism. The Myc family consists of c-Myc, N-Myc, and L-Myc, and are collectively referred to as Myc. Myc proteins exert their effects on such diverse processes by regulating the expression of thousands of target genes, which represent ~10-15% of the genome (1–3). Efforts to understand the biological attributes of Myc proteins have focused primarily on c-Myc and secondarily on N-Myc for they play critical roles in development and tumorigenesis. Both are essential for normal development, as germline deletion of either c-Myc or N-Myc in mice leads to lethality at embryonic day (E) 10.5 or E11.5, respectively, due to multi-organ failure (4–8). Furthermore, many cancers are caused by deregulation of c-Myc, N-Myc, and less frequently, L-Myc (9).

The expression and activity of Myc is tightly regulated by the presence of growth and survival signals. Deregulation of Myc, due to mutation of Myc or aberrant activity of upstream regulatory constituents, allows a cell to proliferate aberrantly and evade apoptosis, ultimately leading to malignant transformation. The Ras/mitogen-activated protein kinase (MAPK) pathway regulates c-Myc through multiple mechanisms, including transcription, translation, post-translational modifications, and protein stability. With regard to protein stability, the Ras/MAPK pathway controls the phosphorylation of c-Myc at two highly conserved residues to prevent its ubiquitin-mediated proteolysis. These phosphorylation sites in c-Myc are completely conserved in N-Myc and across

multiple organisms. Thus it has been proposed that Ras/MAPK signaling controls N-Myc stability in similar manner; however, this has not been directly tested.

The purpose of this thesis is to understand how Ras/MAPK signaling regulates N-Myc expression and activity. The central hypothesis is that the Ras/MAPK pathway regulates N-Myc and c-Myc by similar post-translational modifications. In Chapter 2, I show that this hypothesis is not correct: Ras does not control N-Myc and c-Myc by a similar mechanism. Instead, I demonstrate that Ras regulates N-Myc expression by two competing mechanisms involving increased translation and increased degradation. Studies in Chapter 3 aim to further elucidate this translational mechanism by investigating a potential role for glycogen synthase kinase 3β (GSK3β) — a target of Ras activity— in this process.

Chapter 1 provides an introduction to the function of N-Myc and c-Myc, the mechanisms that control their expression, and the biological consequences that ensue when these mechanisms are compromised. Many of the studies in this thesis examine N-Myc and c-Myc in parallel to identify regulatory mechanisms that are common or distinct between the two proteins. Therefore, I emphasize the similarities and differences in N-Myc and c-Myc regulation.

Finally, in Chapter 4 I propose a model that integrates the findings presented in Chapters 2 and 3 into the context of other published results. The implications of these findings with regard to embryonic development and Myc-driven tumorigenesis are discussed.

1.2 MYC STRUCTURE, FUNCTION, AND REGULATION

The transforming gene of the avian myelocytomatosis retrovirus (MC29), *v-myc*, was identified as causing various cancers such as carcinomas, leukemias, and sarcomas in chickens (10). Following the theory that viral oncogenes could be captured from normal cellular DNA, *myc* (myelocytomatosis) genes were subsequently identified due to their homology to *v-myc* and are among the earliest examples of proto-oncogenes (11). The cellular homolog of *v-myc*, *c-myc* (cellular), was first to be discovered (12), followed by N-*myc* (neuronal) and L-*myc* (lung) that were later isolated in the amplified sequences of neuroblastoma cells and small cell lung tumors, respectively (13, 14). Vertebrate organisms express all three myc family members to varying degrees of homology, whereas invertebrate organisms, such as flies and worms, express one myc gene (15). Gene duplication of a single myc gene likely occurred soon after the origin of vertebrates (16). This event gave rise to a lineage we know as c-myc and another lineage that undergone a second gene duplication giving rise to N-myc and L-myc. The proteins encoded by *myc* genes have crucial roles in cell fate decisions, such as proliferation, apoptosis, and differentiation. Deregulation of Myc family members is associated with many types of cancers, and as demonstrated with various mouse models, is implicated in development of tumors.

1.2.1 Myc Proteins are Transcription Factors

Myc proteins exhibit all the hallmarks of transcription factors. As depicted in Figure 1.1, Myc proteins have one or two nuclear localization signals that sequester the protein to the nucleus. The C-terminus of the protein contains a basic region helix-loop-helix

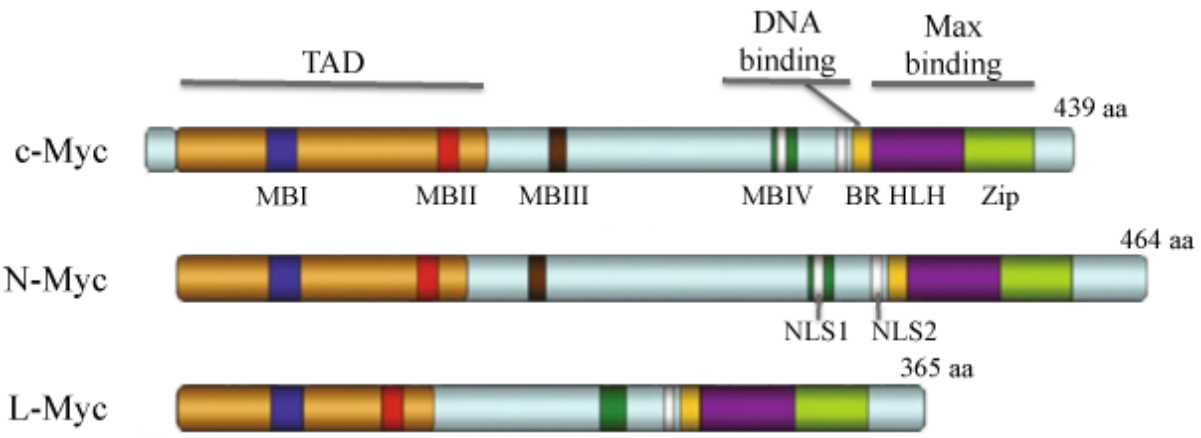

Figure 1.1 Schematic presentation of the Myc family protein structure. Myc oncoproteins, c-Myc, N-Myc, and L-Myc, have several conserved domains that are typical of transcription factors. The N-terminus of Myc protein contains the transactivation domain (TAD). The C-terminus contains the elements involved in DNA binding; the basic region (BR) and helix-loop-helix leucine-zipper (HLH-LZ) domain mediate the protein's interactions with DNA and Max, respectively. Myc homology boxes (MB) I-V interact with various proteins and harbor sites of posttranslational modification, which influence the expression and activity of the protein. The nuclear localization signal (NLS) directs the protein to the nucleus.

leucine zipper (bHLH-LZ) domain. The basic region creates an affinity for DNA and the HLH-LZ domain allows Myc to dimerize with another bHLH-Zip protein called Max (17). Myc-Max complexes bind to canonical (CACGTG) and non-canonical E-box sequences within the promoter region of target genes, and recruit transcriptional coactivators, histone modifying enzymes, and RNA polymerases to stimulate transcription (18–21). Max is required for Myc-induced transactivation since disruption of the bHLH-Zip domain, which abolishes Max interactions, prohibits Myc from binding E-box sites and inducing transcription (22, 23). At the N-terminus of Myc proteins is the transactivation domain (TAD), which contains conserved domains called the Myc homology boxes (MBs). MBs are critical for many aspects of Myc function, including transcriptional activity and proteolysis of the protein, which will be discussed later in this chapter.

The transcriptional activity of Myc can be influenced by a set of bHLH-LZ proteins that include Mnt and members of the Mxd family (Mxd1-4) (24). Like Myc proteins, Mnt and Mxd1-4 require association with Max to bind E-box promoter sequences. Mnt-Max and Mxd-Max complexes, however, function to transcriptionally repress their gene targets by associating with the corepressor Sin3. Sin3 recruits histone deacetylases, which causes the chromatin structure to adopt a “closed” or inactive state. Mnt and Mxd proteins are proposed to antagonize the transactivating functions of Myc (25) by competing for Max dimerization and common E-box binding sites.

Myc-Max complexes also repress transcription of several key genes that regulate the cell cycle. By interacting with the transcriptional activator Miz1 (Myc-interacting zinc

finger protein-1), Myc-Max complexes disrupt the ability of Miz1 to recruit the coactivator complex CBP/p300, thereby blocking transactivation of Miz1 target genes (26, 27).

1.2.2 Myc proteins control diverse biological processes

It is estimated that Myc proteins influence the expression of thousands of genes. For c-Myc, this represents 10-15% of the human genome (1–3). Myc target genes are involved in a broad range of biological processes including cell proliferation, growth, differentiation, apoptosis, metabolism, and angiogenesis (28–31). A key biological function of Myc is its ability to promote proliferation. During embryogenesis, high Myc expression is intimately link to actively proliferating tissues whereas reduced Myc expression generally corresponds to cells undergoing terminal differentiation or quiescence (32). Accordingly, loss of c-Myc or N-Myc was shown to greatly reduced rate of cell proliferation in rat fibroblasts or granule neuron precursors, respectively (33, 34). Re-introduction of either c-Myc or N-Myc into c-Myc-deficient rat fibroblasts results in an increase in cell growth and proliferation (35).

Myc carries out its proliferative function by, in part, controlling the expression of key cell cycle regulators to promote the G_1-S phase transition. For example, Myc induces the expression of Cyclin E and Cyclin D and their kinase partners Cdk2 and Cdk4/6, respectively (36–38). The activities of the CyclinE-Cdk2 and CyclinD2-Cdk4 complexes permit activation of genes required for S phase (39–41). Myc also promotes cell cycle progression by controlling the expression of two critical cell cycle inhibitors, $p15^{Ink4b}$ (*cdkn2b*) and $p21^{Cip1}$ (*cdkn1a*). Both $p15^{Ink4b}$ and $p21^{Cip1}$ maintain cells in an arrested

state in the absence of growth factors (26, 42–44). Myc-Max complexes associate with Miz1 to block Miz1-mediated activation of *cdkn2b* and *cdkn1a* to promote cell cycle entry (45).

Another important function of Myc is to sensitize cells to apoptosis through a process called oncogene-induced apoptosis (46, 47). The seemingly disparate functions of Myc – cell proliferation and apoptosis – are intimately linked such that the apoptotic function serves as a safety mechanism to defend against inappropriate proliferation. The apoptotic functions of Myc are suppressed as long as pro-survival signals are present (48, 49). Myc-induced apoptosis occurs in response to a number of other signals, such as growth factor deprivation, hypoxia, and cytotoxic drugs, indicating that Myc is a general regulator of apoptosis (50). One mechanism by which c-Myc induces apoptosis involves c-Myc-dependent transactivation of the tumor suppressor p19Arf, which activates p53 by binding and sequestering Mdm2 (51). Mdm2 is an E3 ligase that targets p53 for degradation (52). In turn, activated p53 upregulates the expression of pro-apoptotic factors such as *BAX*, *BIM*, and *PUMA*. Mutations in p53 or its regulators that inactivate p53 are often observed in cancers also having deregulated c-Myc or N-Myc (53, 54). In a separate mechanism, c-Myc represses the expression of anti-apoptotic BCL-2 family members, which control cytochrome c release from the mitochondria and formation of the apotosome complex (55).

Our understanding of the cellular functions of Myc proteins has been largely characterized using c-Myc, but it appears that N-Myc performs similar functions as c-Myc. As observed with c-Myc, forced expression of N-Myc can induce the re-entry of quiescent cells into the cell cycle in the absence of mitogenic stimulation, and when

misexpressed at later stages, N-Myc can prevent cell cycle exit (49). Similar to the apoptotic function of c-Myc, N-Myc can sensitize neuroblastoma cells to apoptosis (56). Since c-Myc and N-Myc have a similar protein structure and perform similar functions, they are generally thought to be functionally redundant and subjected to similar regulatory mechanisms. As discussed in following sections, c-Myc and N-Myc do not appear to be completely redundant and are differentially regulated at multiple several levels. Studies presented in Chapter 2 describe how c-Myc and N-Myc are differentially regulated at the level of translation and protein stability.

1.2.3 Role of Myc proteins in embryonic development

Myc proteins have prominent roles in embryogenesis since they regulate many biological processes that are essential for development. During mouse embryogenesis, the spatial and temporal expression patterns of individual Myc members are generally distinct with some overlap by c-Myc or N-Myc with L-Myc (8, 57, 58). For N-Myc and c-Myc, their expression patterns are nearly reciprocal (59). c-Myc expression is ubiquitous and corresponds to tissues undergoing rapid proliferation, while N-Myc and L-Myc expression is more specialized (57). N-Myc expression is initially observed at the onset of organogenesis in the primitive streak, and as development progresses, is highest in the developing kidney, retina, lung, limb buds, heart, and various parts of the central nervous system (5, 60, 61). L-Myc expression is detected in the developing kidney, lung, brain, and neural tube (62). Germline deletion of c-Myc or N-Myc, but not L-Myc, is embryonic lethal at E10.5 or E11.5, respectively, caused by failure of organs that strongly express the particular Myc homolog (4–8). In normal adult mice, c-Myc expression is

restricted to cells undergoing rapid turnover (e.g., skin and gut epithelial cells and hematopoietic progenitor cells) and is undetectable in differentiated cells. N-Myc and L-Myc are generally not detected post-development (63, 64).

N-Myc is the only Myc family member implicated in a birth defect. Haploinsufficiency of N-Myc causes Feingold Syndrome (FS). Mutations within N-Myc that result in premature stop codons are detected in a large subset of FS patients (65). Deletions within *MYCN* have also been observed (66). FS patients display variable combinations of microcephaly, limb malformations, esophageal and duodenal atresia, and learning disabilities (67). By employing conditional mouse models, several labs have demonstrated an important role for N-Myc in the development the organs affected in this syndrome, such as the central nervous system (68) and limbs (69).

With regard to development, there are two important questions in the Myc field. First, are the Myc family members functionally redundant? And second, do they (as opposed to can they) functionally compensate for one another? These questions specifically pertain to N-Myc and c-Myc since their biochemical attributes appear to be most similar and both are required for development. Support for N-Myc and c-Myc being functionally redundant came from a study in which the coding region of the *c-myc* gene was exchanged for the *N-myc* coding sequence in mice (*c-Myc*$^{N/N}$) (70). A fraction of mice homozygous for the *c-Myc*$^{N/N}$ allele survived into adulthood, demonstrating that *N-myc* was able to rescue the ~E10.5 lethality caused by loss of *c-myc*. *c-Myc*$^{N/N}$ mice were also fertile. It was therefore concluded that N-Myc could functionally replace c-Myc and that divergence of *N-Myc* and *c-Myc* must have evolved to facilitate their different (reciprocal) expression patterns. However, it is important to note that the *c-myc*$^{N/N}$ mice

displayed subtle phenotypic abnormalities, including an overall reduction in body size, periodic dystrophy of skeletal muscles, and lethality of a fraction of *c-myc*$^{N/N}$ mice soon after birth. This suggests that N-Myc can substitute for a majority of activities performed by c-Myc, but that N-Myc and c-Myc have some unique functions.

Support for unique functions of c-Myc and N-Myc came from studies using conditional knockout mouse models where *N-Myc*, *c-myc*, or both genes were deleted in a tissue-specific manner. Laurenti et al. (64) compared the function of N-Myc and c-Myc in hematopoiesis and found that N-Myc and c-Myc have overlapping roles in hematopoietic stem cell proliferation and survival. However, c-Myc had a specific function in promoting differentiation of hematopoietic stem cells that could not be compensated for by N-Myc. A similar type of study conducted by Hurlin and colleagues found that c-Myc and N-Myc operate independently and do not compensate for one another in limb development (71).

Taken together, these studies indicate that N-Myc and c-Myc have both overlapping and unique functions during development and, to a large extent, can compensate for one another in the development of certain organs/systems. Presumably, regulatory mechanisms that determine the predominance of one Myc over the other are critical in establishing the proper expression patterns of N-Myc and c-Myc. In Chapter 2, I present findings that describe how the Ras/MAPK pathway differentially regulates N-Myc and c-Myc expression at the post-transcriptional level.

1.2.4 Myc expression is regulated at multiple levels

The precise amount of Myc available in the cell greatly influences its biological activity such that small changes in Myc expression have significant biological consequences. Regulatory mechanisms must be in place to ensure that the level of Myc production, timing, location, and activity is appropriate for the cellular context. Such strict control is accomplished by controlling Myc expression at multiple levels, i.e. transcription, translation, and mRNA and protein stability. Post-translational modification of Myc (e.g., with phosphate or ubiquitin) and interaction with other proteins are additional mechanisms that modulate the expression and activity of Myc.

Transcription of *N-myc* and *c-myc* genes is induced by diverse physiological signals – both mitogenic and anti-mitogenic – that operate through an array of transcription factors, chromatin remodelers, and regulatory RNAs (72). A partial list of stimuli, the signaling pathway they function through, and the transcription factor(s) they activate to regulate *N-myc* and *c-myc* expression is provided in Table 1.1. Although *N-myc* and *c-myc* are differentially expressed, their promoters share common binding elements and can be regulated by similar transcription factors. This implies that signals that uniquely affect the transcription of N-Myc or c-Myc but not both are primarily responsible for establishing distinct N-Myc and c-Myc expression patterns. In neuronal cells, for example, which require N-Myc for cell proliferation, sonic hedgehog signaling activates the expression of *N-myc*, but not *c-myc* (73, 74). In mesenchymal cells of the development chick limb bud, *Wnt3a* and *Fgf8* induce *N-myc* in a synergistic fashion; *c-myc* does not appear to be upregulated by either molecule alone or in combination (75). Negative autoregulation by *myc* genes has been observed during high levels of Myc

Table 1.1 A partial list of transcription factors (TFs) that regulate *N-myc* and *c-myc* gene expression. Signal transduction pathways are activated in response to various stimuli and influence association of these transcription factors to *myc* promoters. For some entries, the stimulus was not mentioned in the respective reference (Ref).

N-Myc

TF	Signaling	Stimulus	Ref.
β-catenin	Wnt	Wnt3a	(75)
Gli3	Shh	Shh	(73,74)
Pax5	Jak/Stat	IL-7	(187)
Smad1/5	Bmp	Bmp2	(188)
Smad2/3	TGF-β	TGF-β	(189)

c-Myc

TF	Signaling	Stimulus	Ref.
Estrogen receptor	Ras/MAPK	Estrogen	(190)
β-catenin	Wnt/PI3K TGF-β	Wnt3	(191)
E2F	Ras/MAPK		(72)
LEF	Wnt/PI3K	Wnt	(72)
NF-κB	NF-κB, Ras/MAPK JAK/Stat		(72)
Estrogen receptor	Ras/MAPK	Estrogen	(190)
Smad2/3	TGF-β	TGF-β	(192)

expression (76, 77). c-Myc and N-Myc also appear to negatively regulate each other's gene expression of (78). The extent to which negative regulation of one Myc by another family member contributes to their distinct expression pattern is unclear.

The stability and translation rate of *myc* mRNA is also tightly regulated by multiple mechanisms acting through cis-acting elements (untranslated region (UTR) and internal ribosome entry sequence) and trans-acting factors like RNA binding proteins (RBPs) and microRNAs (miRNAs). *N-myc* and *c-myc* transcripts are relatively unstable (approximate half-lives of 33-38 and 30-48 minutes, respectively) and their stability fluctuates in response to developmental or environmental stimuli (79, 80). The half-life of *c-myc* mRNA is primarily controlled through its 3' UTR (81, 82). A-U rich elements (AREs) within the *c-myc* 3' UTR provide binding sites for several ARE-binding RBPs that can either suppresses (83, 84) or promote (85) *c-myc* translation. ARE-binding RBPs often compete for the same binding sites, effectively antagonizing the activity of each other (85). The *c-myc* 3' UTR is also targeted by several miRNAs, including miR-145, miR132, and let-7, that function alone or in combination to repress c-myc expression (86–88). Furthermore, the interplay between RBPs and miRNAs, like HuR and let-7, can positively or negatively influence the activity of the RBP and miRNA (89). A decay element termed the coding region instability determinant (CRD) is located within the coding region of c-myc at the 5' terminal end. CRD is targeted by IGF-II mRNA binding protein 1 (IGF2BP1), resulting in stabilization the *c-myc* mRNA (90). IGF2BP1 forms a complex with other RBPs (HNRNPU, SYNCRIP, YBX1, and DHX9) and blocks c-myc mRNA from associating with polysomes (91). It is postulated that stabilization of *c-myc*

by IGF2BP1 limits the transfer of the transcript to polysomes thereby inhibiting translation-coupled decay.

Regulation of *N-myc* mRNA is poorly understood. The RBPs HuD and MDM2 have been shown to regulate *N-myc* stability (79, 92). The CRD within *c-myc* is similar to a region within *N-myc* (68% homology). Not surprisingly, IGF2BP1 can bind to *N-myc in vitro*, albeit with less affinity and specificity than compared to *c-myc* (93). Whether this interaction is present *in vivo* has yet to be tested. N-Myc is regulated by several miRNAs, including miR-34a, let-7, and miR-101, all of which target the 3' UTR of *N-myc* (94, 95).

Proteolysis is also an important mechanism that controls Myc expression. Prolonged half-lives of c-Myc and N-Myc protein have been observed in several types of cancers (96–99) and may suggest that a failure to regulate Myc protein turnover contributes to pathogenesis. Myc proteins are unstable with half-lives of 20-30 minutes (100, 101). c-Myc contains several determinants that influence its stability (Fig. 1.2). Degrons, elements that are necessary and sufficient to trigger ubiquitin-mediated proteolysis, have been mapped to MBI, MBII, and MBIII of c-Myc (102–105). MBI and MBII of c-Myc contain binding sites for the Fbw7 and Skp2 ubiquitin ligases, respectively, which mark the protein for ubiquitin-mediated degradation by the proteasome (102, 106–109). Several other ubiquitin ligases have been shown to target c-Myc for ubiquitin-mediated degradation (reviewed in (110) and Fig. 1.2). Since MBI, MBII, and MBIII are conserved within c-Myc and N-Myc, it is generally thought that these degrons regulate N-Myc turnover in a manner similar to c-Myc. However, studies by several groups indicate that this is not completely true (described in more detail below). The PEST element – enriched in proline, glutamic acid, serine, and threonine residues – resides within c-Myc

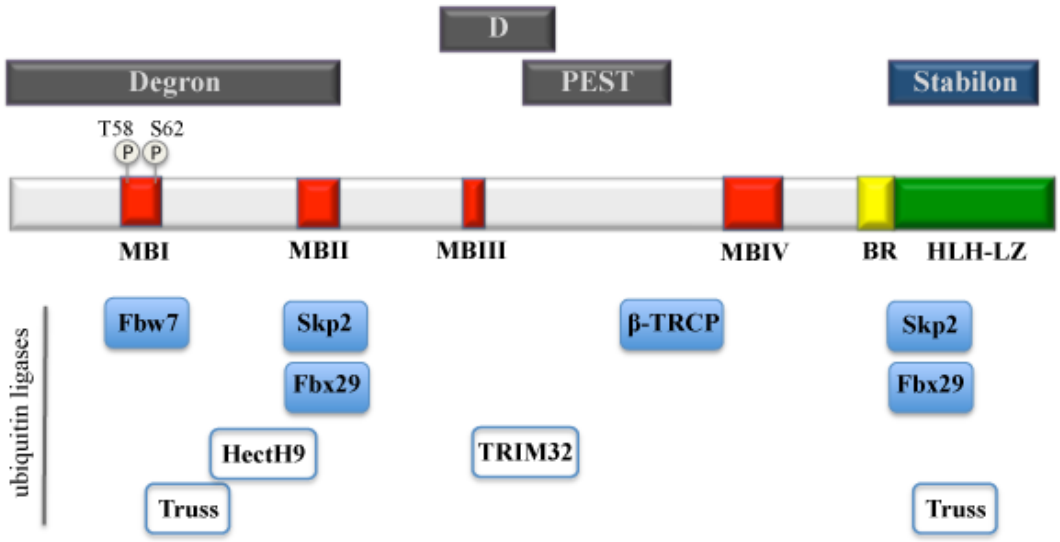

Figure 1.2 Map of instability elements and E3 ligase binding sites within c-Myc. Schematic is adapted from Thomas and Tansey (110). The major instability determinants within c-Myc are the degron, D-element, and PEST element. Two phosphorylation sites, Threonine 58 (T58) and Serine 62 (S62), reside within the MBI (overlaps with the degron) and have critical roles in mediated Myc turnover. A determinant conferring stability called the stabilon resides in the C-terminus of c-Myc. c-myc is a substrate for multiple E3 ligases that regulate its turnover. E3 ligases with filled-in boxes have well-defined binding sites and there position along the protein indicates these binding sites. E3 ligases with unfilled boxes also mediate c-Myc turnover, however their binding sites are less well characterized.

and is often found in unstable proteins (111). The PEST and D elements appear to influence c-Myc stability in a manner that does not require ubiquitin-mediated turnover (112, 110). Finally, the C-terminus of c-Myc contains an element called the stabilon, which stabilizes the protein in two ways. First, the stabilon allows for binding to Miz1, an interaction that enables c-Myc to transcriptionally repress select target genes and stabilizes c-Myc (103). Second, the stabilon is involved in partitioning c-Myc to a metabolically stable pool where the protein is tightly associated with the chromatin (113). It is possible that c-Myc is associated with a stable pool when it is engaged in the process of transcriptional repression. Interestingly, proteolysis of c-Myc has been linked to its transactivating activity (103) and suggests that c-Myc is associated with an unstable pool when it is engaged in the process of transcriptional activation. The relationship between the activity and proteolysis of Myc is discussed in more detail in Section 1.2.7.

Degradation of Myc proteins is regulated through post-translational modifications – namely phosphorylation, acetylation, and ubiquitination – that signal to prevent or promote proteolysis (114). Figure 1.2 illustrates the ubiquitin ligases that have been shown to target Myc. Some of the Myc modifications are mechanistically interconnected, resulting in cross talk between phosphorylation and ubiquitination or acetylation and ubiquitination (115). An important mechanism that requires cross talk between phosphorylation and ubiquitination to control c-Myc proteolysis involves interdependent phosphorylation of two residues that reside within MBI, Threonine 58 (Thr58) and Serine 62 (Ser62). These phosphorylation events determine the interaction of c-Myc (and presumably N-Myc) with the Fbw7 E3 ligase resulting in ubiquitination and proteasomal degradation of c-Myc (116). This degradation mechanism is described in further detail in

the next section (Section 1.2.5). In addition to the Thr58/Ser62 degradation pathway, c-Myc is also degraded by additional mechanisms since the phosphorylation mutant c-MycT58A, which prevents its ubiquitination, is also degraded by the proteasome albeit at a slower rate than WT c-Myc (103, 117). One mechanism involves the E3 ligase Skp2, which directs ubiquitin-mediated proteolysis of c-Myc in a manner that does not require phosphorylation at Thr58 (108). The kinase Pim-2 has been shown to stabilize c-Myc by a mechanism that involves phosphorylation at Ser329, but not Thr58 or Ser62 (118). Another mechanism by which c-Myc stability is regulated involves cross talk between acetylation and ubiquitin modifications. Because lysines can be modified by both acetylation and ubiquitination, these modifications can impede the effects of one another. Indeed promoting c-Myc acetylation was shown to decrease c-Myc ubiquitination and enhance its stability (119–121). Acetylation and ubiquitination of c-Myc can also function in a synergistic fashion to regulate c-Myc stability. For example, acetylation of c-Myc by the histone acetyltransferases p300 appeared to accelerate protein turnover in a manner that required ubiquitin-mediated proteasomal degradation (121). The extent to which these and other mechanisms contribute to c-Myc proteolysis is not well understood. It is likely that different Myc degradation mechanisms are employed in response to different cellular signals and in a cell-specific manner (112).

1.2.5 The Myc degradation pathway involving Thr58 and Ser62 phosphorylation

Myc expression, and thus activity, is tightly regulated with respect to the proliferative status of the cell. In non-proliferative, quiescent cells, Myc expression is very low. Upon mitogen stimulation and entry into G_1 phase, Myc rapidly accumulates at both the

transcript and protein levels (116, 122). This sharp induction is transient and is followed by an equivalent sharp reduction within hours. By late G_1, Myc levels are low and maintained at this level through the cell cycle (123, 124). Modulation of Myc expression in this setting is achieved, in part, by regulating its protein stability through the mechanism involving phosphorylation at Thr58 and Ser26. This mechanism is illustrated in Fig. 1.3.

In response to mitogen stimulation, the Ras/MAPK and PI3K/Akt pathways are activated downstream of receptor tyrosine kinases (RTKs). Activation of Ras triggers the Raf/MEK/Erk kinase cascade, resulting in phosphorylation of c-Myc at Ser62 and stabilization of the protein (125). At the same time activation of the PI3K/Akt pathway, either by Ras or RTKs, results in phosphorylation and inactivation of GSK3β. GSK3β phosphorylates c-Myc at Thr58, a signal for ubiquitin-mediated proteolysis (125, 126). Overall, activation of the Ras/MAPK and PI3K/Akt pathways maintain c-Myc in a singly phosphorylated, stabilized form upon mitogen stimulation.

As growth factor signals diminish, the activities of the Ras/MAPK and PI3K/Akt pathway decline relieving the inhibitory effect on, and thus activating, GSK3β (127). As a 'priming' kinase, GSK3β recognizes phosphorylated c-Myc at Ser62 as a priming site for phosphorylation at Thr58 (125, 126). The coordinated activities of the peptidylprolyl isomerase Pin1 and protein phosphatase 2A (PP2A) act on doubly phosphorylated c-Myc to direct dephosphorylation of Ser62 (128). Finally, singly phosphorylated c-Myc at Thr58 is targeted for ubiquitination by the E3 ubiquitin ligase Fbw7 directing the proteasomal-mediated degradation of c-Myc (106, 107, 125).

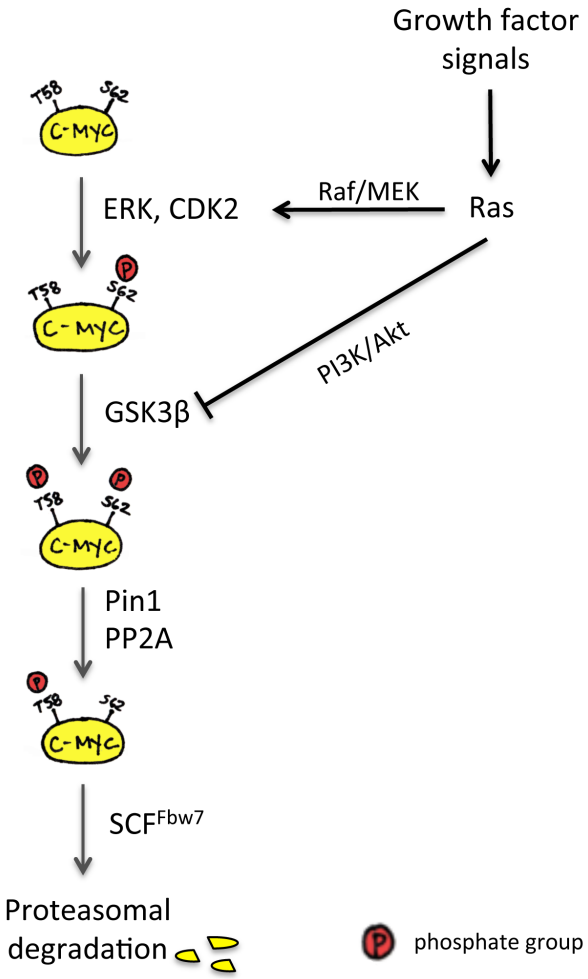

Figure 1.3 Model of the Myc degradation pathway involving Ras signaling. Schematic is adapted from Sears et al. (125). Ras is activated in response to growth factors, and signals through MAPK pathway (Raf/MEK) and the PI3K/Akt pathway to modulate the phosphorylation of c-Myc at Ser62 and Thr58. Phosphorylation of c-Myc at Ser62 stabilizes the protein. As growth factor signals decline, repression of GSK3β is relieved. GSK3β-mediated phosphorylation of c-Myc at Thr58 triggers subsequent events (involving Pin1, PP2A, and Fbw7) that target c-Myc for ubiquitin-mediated degradation by the proteasome.

Enhanced stabilization of Myc due to impairment of this degradation pathway has been observed in several cancer cell lines and tumor samples, and underscores the relevance of this regulatory mechanism to cancer. Mutations that prevent phosphorylation of c-Myc at Thr58 or Ser62 have been identified in Burkitt's lymphoma and AIDS-associated lymphoma tumor samples (117, 129). Enhance c-Myc stability was also observed in some leukemia and breast cancer cell lines and patient samples that also displayed altered phosphorylation Thr58 and Ser62 (96, 130). In addition, aberrant activation of the Ras/MAPK or PI3K/Akt pathways are frequently observed in cancers and may contribute to tumor development by enhancing c-Myc stability (97).

1.2.6 Are N-Myc and c-Myc degraded by the same mechanism?

It is currently thought that the mechanism by which Ras/MAPK signaling regulates c-Myc degradation also applies to N-Myc (131). This notion is supported by the fact that the phosphorylation sites in c-Myc that mediate its proteolysis, Thr58 and Ser62, are conserved in N-Myc. The equivalent sites in the mouse N-Myc homolog are Threonine 50 (Thr50) and Serine 54 (Ser54). In humans, these sites in N-Myc are Thr58 and Ser62. Most studies presented in this thesis use the mouse N-Myc homolog; therefore I will refer to the phosphorylation sites in N-Myc as Thr50 and Ser54 for the remainder of this thesis. Further support that N-Myc is degraded in a manner similar to c-Myc comes from observations that, similar to c-Myc, N-Myc protein levels are induced by Ras signaling (131) and that N-Myc is phosphorylated at Thr50 by GSK3β (132).

Several lines of evidence suggest that N-Myc and c-Myc are not degraded by the same mechanism that is illustrated in Fig. 1.3. Kenney et al. (132) found that

phosphorylation of Ser54 destabilizes N-Myc, rather than enhancing stability as observed with c-Myc. In another study, the same group showed that, in contrast to c-Myc, N-Myc is stabilized by PP2A (133). Pin1 has been shown to destabilize c-Myc but does not appear to affect N-Myc stability (133). To date, the role of Ras/MAPK signaling on N-Myc protein stability has not been directly tested. In Chapter 2, I present studies that examine the role of the Ras/MAPK pathway in N-Myc stability and provide evidence that Ras/MAPK signaling does not regulate N-Myc and c-Myc degradation by the same mechanism.

1.2.7 Control of Myc activity by ubiquitin-mediated proteolysis

The rapid and tightly regulated degradation of Myc by the ubiquitin-proteasome system (UPS) ensures that cellular levels of Myc are kept low and responsive to stimuli. Interestingly, the UPS has also been implicated in regulating the transcriptional activity of Myc such that accelerated proteasomal degradation of Myc correlates with enhanced transcriptional activity (103). An intimate connection between UPS-mediated degradation and transcriptional activity has been documented for several proteins (134). Many unstable transcription factors, especially those with TADs such as Myc, appear to engage the UPS machinery when they activate transcription (134, 135). Indeed c-Myc was shown to recruit UPS machinery – the E3 ligase Skp2 and components of the 20S core, 19S lid, and 19S base – to the *CYCLIN D2* promoter (108, 109). Furthermore, recruitment of the UPS machinery to the *CYCLIN D2* promoter was dependent on c-Myc and was necessary for activation of *CYCLIN D2* gene expression (109). c-Myc has also been shown to

interact with Sug1, a subunit of the 20S proteasome, and this interaction is speculated to influence c-Myc's transcriptional activity (136).

The mechanistic basis for why Myc recruits UPS components to the *CYCLIN D2* promoter, and presumably other Myc target promoters, is not fully understood. Zhu et al. (143) showed that ubiquitination and proteasomal degradation of p53 were required for p53 transactivation of the $p21^{waf1}$ promoter. Similar to p53, ubiquitination and proteasomal degradation of Myc are likely involved in simulating the transcriptional activity of Myc, and recruitment of UPS components to Myc-bound promotes would facilitate this process. There are several potential mechanisms by which ubiquitination and proteolysis of Myc could induce transcription. First, recruitment of UPS components to Myc-bound promoters may recruit RNA polymerase II. This mechanism was described for progesterone receptor-dependent transcription, such that inhibition of the 26S proteasome resulted in a failure to recruit RNA polymerase II to select promoters (144). c-Myc interacts with several basal transcription factors that function to assemble the preinitiation complex (145). A second potential mechanism would involve proteolytic removal of promoter-bound c-Myc, and possibly other coactivators, promotes disassembly of the preinitiation complex and promoter release of the RNA polymerase II for transcription to proceed to the elongation step. Alternatively, promoter-bound c-Myc may recruit UPS machinery to promote ubiquitination and degradation of corepressors. Proteolytic removal of corepressors could relieve their repressive transcriptional effects and permit transactivation. Finally, promoter-bound c-Myc may recruit E3 ligases that ubiquitinate histones to remodel the chromatin structure in a manner that facilitates transcription (146).

The functional importance for coupling the proteolysis of Myc with its transcriptional activity is not fully understood. It is speculated that coupling an activator's activity with its proteolysis may be necessary for transcription to proceed properly by influencing the composition of protein complexes on the chromatin (147, 148). Coupling an activator's activity with its proteolysis may also establish limits of transcription for potent activators such that periods of high activator activity are kept relatively short (149). This feature may be particularly important for Myc, since prolong durations of highly active Myc can cause excessive proliferation and contribute to cellular transformation. This suggests that disrupting the link between Myc proteolysis and activity may trigger the oncogenic functions of Myc. Indeed mutations in Myc that are associated with cancer appear to have enhanced transcriptional activity, but also increased protein stability (108). These Myc mutants were capable of interacting with the E3 ligase Skp2 and were ubiquitinated to a greater extent than WT c-Myc. This finding suggested that the cancer-associated mutants are still capable of engaging the UPS to activate transcription but evade proteolysis.

1.2.8 Role of N-Myc in neuroblastoma development

Neuroblastoma is a pediatric cancer of the peripheral sympathetic nervous system and accounts for 15% of all childhood cancer deaths (161, 140). The clinical hallmark of neuroblastoma is its vast heterogeneity, which results in a broad spectrum of disease stages ranging from spontaneous regression to aggressive metastasis (150). Overexpression of N-Myc is a prominent genetic feature of neuroblastoma, indicating an aggressive phenotype and poor prognosis, and has been shown to directly contribute to the development of neuroblastoma (105, 151). High levels of N-Myc are also associated

with other types of cancers, such as medulloblastoma, retinoblastoma, small cell lung carcinoma, glioblastoma, and certain embryonic tumors (163).

Amplification of the *MYCN* gene is the most common genetic defect that causes N-Myc overexpression in neuroblastoma (152). However, *MYCN* amplification does not account for all cases. In some neuroblastoma cell lines, elevated levels of N-Myc protein were not accompanied by gene amplification (153). This suggests that impairment of post-transcriptional regulatory mechanisms can also contribute to N-Myc overexpression (101), perhaps by enhancing the synthesis or stability of N-Myc protein. Hyperactivation of Ras signaling is observed in neuroblastoma (discussed in Section 1.3.4) and may be required for the oncogenic activities of N-Myc in neuroblastoma development (131). Ras signaling can induce N-Myc protein expression through post-transcriptional mechanisms (131, 132). Our studies described in Chapter 2 suggest that translational upregulation of N-Myc is one mechanism by which Ras signaling post-transcriptionally controls N-Myc expression in neuroblastoma cells.

1.3 RAS SIGNALING

Ras proteins are highly regulated GTPases that have key roles in regulating cell growth, survival, senescence, adhesion, and motility (154). In response to extracellular stimuli, the primary role of Ras proteins is to assemble transient signaling complexes at the cell membrane to activate signal transduction pathways, which coordinate the aforementioned cellular processes.

1.3.1 Structure and function of Ras proteins

In humans, three *RAS* genes encode for four distinct but highly homologous Ras proteins: H-Ras, N-Ras, and K-Ras (A and B). The first 85 amino acids of Ras proteins are identical and contain the G domain, which binds guanosine nucleotides and regulates binding to Ras regulators and effectors. The major differences in the Ras proteins are confined to the C-terminal called the hypervariable region and are sites of post-translational modifications.

Ras proteins (hereafter Ras) are synthesized as biologically inactive precursors. Post-translational modification of newly synthesized Ras directs the protein through the trans-Golgi network to the plasma membrane anchorage (reviewed in (155). Briefly, Ras is farnesylated in the endoplasmic reticulum at the CAAX motif (C denotes cysteine, A refers to any aliphatic amino acid, and X may be any amino acid). N-Ras, H-Ras, and K-RasA undergo a second modification, palmitolyation, in the Golgi apparatus. These modifications ensure that Ras is securely anchored to the membrane. Localization to the plasma membrane likely positions Ras in close proximity to activated RTKs, which in turn triggers an activation cascade involving Ras and its effector proteins.

Ras functions as a binary switch, alternating between an active and inactive state. In the inactive form, Ras is bound by GDP and is localized to the plasma membrane. In response to mitogens, cytokines, and growth factors, guanine exchange factors (GEFs) are activated, associate with Ras, and trigger the release of GDP from Ras. Ras-GEFs include SOS and RasGRP (156). Ras is then free to bind GTP. Binding of GTP to Ras causes disassociation of the GEF and conformational changes to Ras that increase its affinity for multiple effector proteins. Activation of these effector proteins trigger distinct

signal transduction pathways that are described in more detail below. Inactivation of Ras requires hydrolysis of bound-GTP to GDP. Although Ras has intrinsic GTPase activity (157), this process is too slow and biologically inefficient. Therefore, hydrolysis of bound-GTP is carried out by guanine activating proteins (GAPs). Ras-GAPs include NF1 and Rheb (156).

1.3.2 Ras signals through multiple pathways

Activation of Ras occurs downstream of RTKs, such as fibroblast growth factor receptors (FGFRs). Binding of ligands to RTKs, for example fibroblast growth factor binding to FGFRs, causes receptor dimerization. The protein kinase of each receptor monomer then phosphorylates specific tyrosine residues in the cytoplasmic domain of its dimer partner resulting in receptor activation. Phosphorylation of tyrosine residues creates recognition sites for many intracellular intermediates, which assist in transmitting the ligand signal to many signal transduction pathways. The adaptor proteins GRB2 and SOS are recruited to activated RTKs and function to activate Ras (158).

Ras stimulates a myriad of downstream signaling pathways through activation of multiple effector proteins. Raf serine/threonine kinases (Raf-1, A-Raf, and B-Raf) and phosphoinositide-3-kinases (PI3K) are two well-characterized Ras effector proteins that trigger the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathway, respectively (Fig. 1.4). Additional effector proteins include RasGDS, p120GAP, Rin1, Tiam, Af6, Nore1, PLCε, and PKCζ (159). Ras activates effector proteins, in part, by promoting their translocation to the plasma membrane (160), where additional steps are

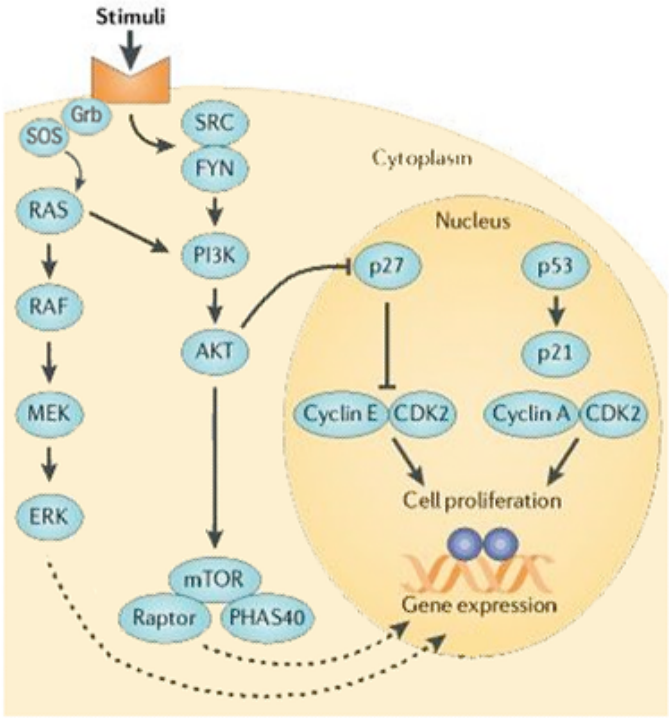

Figure 1.4 Activation of the MAPK and PI3K/Akt pathways by Ras signaling. Schematic is adapted from Lee et al. (165). Binding of receptor tyrosine kinases by various stimuli causes their activation. Recruitment of adaptor and effector proteins to the activated receptor leads to activation of Ras, and subsequent activation of the MAPK pathway via Raf and the PI3K/Akt pathway. The MAPK and PI3K/Akt signaling pathways influence the expression of many proteins, some of which are illustrated in the figure, which function in cell proliferation, growth, survival, and migration, and differentiation.

required for full effector activation. Activation of Raf initiates a cascade of protein phosphorylation by first phosphorylating MEK1/2. Phosphorylated MEK in turn phosphorylates ERK1/2 (161). Phosphorylated ERK1/2 migrates to the nucleus where it subsequently phosphorylates and activates number of transcription factors (162). The Ras/Raf/MEK/ERK cascade is also referred to as the MAPK pathway. As discussed above, activation of the MAPK pathway leads to phosphorylation of c-Myc causing enhanced stability (125) and increased transcriptional activity (163). Ras-mediated activation of another well-known effector, PI3K, generates the second messenger lipid phosphatidylinositol (3,4,5) triphosphate (PIP_3). PIP3 recruits PDK1 and AKT to the plasma membrane, where PDK1 phosphorylates and activates Akt (164). In turn, Akt phosphorylates a number of substrates, including GSK3β and the tuberous sclerosis complex 2 (TCS2). Akt substrates are involved in cell proliferation, growth, and survival (166). As discussed previously, Erk1/2 and GSK3β play important roles in the proteolysis of c-Myc (131, 132). The studies presented in Chapter 3 suggest that GSK3β also plays a role in the proteolysis of N-Myc.

1.3.3 Ras signaling controls translation through multiple mechanisms

Ras activation can stimulate translation by signaling through the MAPK and PI3K/Akt pathways (167). The primary method of controlling protein synthesis is modulating the phosphorylation state of translational components to alter their activity (168). This allows a cell to rapidly respond (within minutes) to physiological and environmental changes. Notably, the global effects of acute Ras/MAPK and PI3K/Akt signaling have been linked to changes in translation rather than transcription (169),

indicating that translational control is a major mechanism by which these pathways control gene expression.

To understand how Ras signaling controls translation, we must understand the factors involved in translation. Translation in mammalian cells proceeds in three stages (initiation, elongation, and termination) and involves eukaryotic initiation factors (eIFs), elongation factors (eEFs), and release factors (eRFs). Translation initiation involves recruitment of the 40S ribosomal subunit to mRNA. This process is coordinated by the eIF4F complex, which consists of eIF4A, eIF4E, and eIF4G, and interacts with both the 40S subunit and the mRNA (168). eIF4E availability is regulated by eIF4E-binding proteins (4E-BPs) whereby 4E-BPs bind to and prevent eIF4E from interacting with eIF4F complex (168). eIF4B assists in recruiting the 40S ribosomal subunit to mRNA by stimulating eIF4F activity (170). Recruitment of the initiator $tRNA^{Met}$ to the 40S subunit requires eIF2, a GTP-binding protein (171). Following association of the $tRNA^{Met}$, the 60S ribosomal subunit is recruited, forming of the final 80S ribosome complex, which is required for advancement to translation elongation. Translation elongation requires eEF1 and eEF2, which recruit aminoacyl-tRNAs to the 80S ribosome and mediate translocation of the 80S ribosome to the next codon, respectively (168). Finally, termination of translation is mediated by eRFs that recognize the stop codon within mRNA and catalyze release of the ribosome complex from the protein and mRNA.

Ras modulates that activity of numerous proteins involved in translation by activating the MAPK and PI3K/Akt pathways (Fig. 1.5). Through the MAPK pathway, Ras

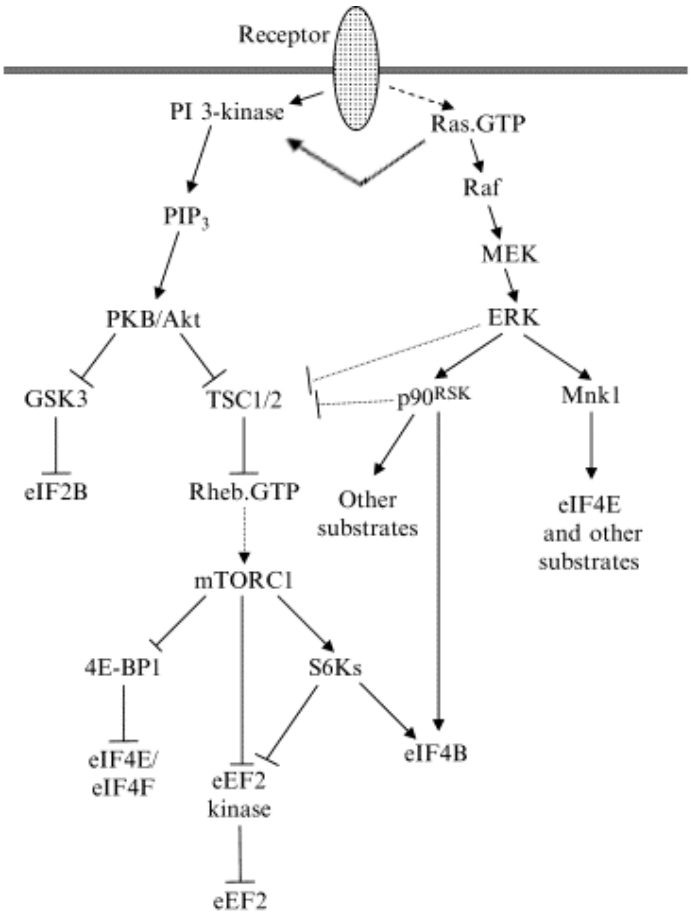

Figure 1.5 Regulation of protein synthesis by Ras signaling. Schematic is adapted from CG Proud (168). Ras activates the MAPK and PI3K/Akt pathways via Raf and PI3K effector proteins, respectively. Downstream targets of these signaling pathways control several factors that function in translation initiation and elongation (see text for more detail).

activates MAPK-interacting serine/threonine kinase (Mnk) that in turn phosphorylates eIF4E (172). Although the exact mechanism is unclear, it is thought that phosphorylation of eIF4E by Mnk increases the affinity of eIF4E for capped mRNA and for eIF4G (173) thereby promoting cap-dependent translation. Also via the MAPK pathway, Ras activates the p90 ribosomal S6 kinase (RSK) family of serine/threonine kinases (174). As the name implies, $p90^{RSK}$ proteins phosphorylate ribosomal protein S6, a component of the 40S ribosomal subunit (175). Phosphorylation of ribosomal protein S6 correlates with, but is not required for, increased translation of transcripts containing an oligopyrimidine tract in their 5' UTR (176). Additional $p90^{RSK}$ substrates are eIF4B (177) and eEF2 kinase (178), whose phosphorylation results activation of eIF4B and eEF2, promoting translation initiation and elongation, respectively.

Ras signaling also regulates the translational machinery through activation of the PI3K/Akt pathway. Phosphorylation of GSK3β by Akt attenuates GSK3β activity thereby relieving the inhibitory activity of GSK3β toward eIF2Bε (179). Since eIF2Bε is required for establishing additional rounds transcriptional initiation events, this is one mechanism by which PI3K/Akt pathway stimulates translation. The PI3K/Akt pathway also activates mammalian target of rapamycin (mTOR), a large multidomain protein that interacts with several proteins, including raptor and rictor, to form mTORC1 and mTORC2 complexes, respectively. mTORC1 modulates the phosphorylation of several proteins involved in translation, including 4E-BP1, the S6 kinases (S6K1 and S6K2), and eEF2 kinase. Phosphorylation of 4E-BP1 by mTORC1 alleviates the inhibitory effect of 4E-BP1 on eIF4E, thereby promoting translation initiation (180). Phosphorylation of S6K1 and S6K2 by mTORC1 results in their activation. In turn, S6K1 and S6K2 phosphorylate several

proteins involved in translation, such as ribosomal protein S6, eEF2 kinase, and eIF4B, to positively influence translation (181).

Ras signaling can also control translation of specific transcripts by influencing their association with ribosomes. Polysomes are complexes of multiple ribosomes bound to a single mRNA (182). Transcripts associated with polysomes, in effect, have increased rates of translation. In contrast, monosomes are complexes mRNA bound by a single ribosome and have slower rates of translation. Ras signaling appears to differentially control the polysome association of transcripts. In glial cells, for example, Ras activation promoted polysome association of mRNAs that encode proteins involved in process that promote malignant transformation, including cell proliferation, growth, and migration (169). Similarly, Ras activity preferentially associated mRNAs that encode proteins involved in oncogenesis in transformed prostate epithelial cells (183). Furthermore, Ras signaling redistributed transcripts encoding ribosomal proteins and RBPs involved in apoptosis and p53 signaling away from polysome complexes (183). It is unclear exactly how mRNAs are actively recruited or excluded from polysomes in these settings, but RBPs and miRNAs have been implicated in the process (91, 184). Alternatively, $p90^{RSK}$, a downstream target of Ras signaling, has been shown to translocate to polysome and stimulate phosphorylation of several ribosome-associated proteins (185).

1.3.4 Oncogenic activities of Ras signaling in neuroblastoma and other cancers

Hyperactivation of Ras signaling is frequently observed in many types of cancers. *RAS* oncogenes were originally discovered as cellular homologs to the v-ras oncogene (186). Multiple activating mutations within *RAS* genes are found in various types of

human tumors (187). It is estimated that activating mutations in *RAS* genes occur in 15% of all human tumors (188).

Activation mutations in H-Ras and N-Ras have been observed in some neuroblastoma cell lines or patient samples (189, 190); however mutations in *RAS* genes appear to be a rare event (191, 192). Instead, overexpression upstream and/or downstream signaling components that activate Ras signaling are frequently observed in neuroblastoma. The tropomyosin receptor kinases (Trk) A, B, and C are RTKs that signal through the Ras/MAPK and PI3K/Akt pathways and are often overexpressed in neuroblastoma (150). The ligands that activate Trk proteins are also overexpressed in neuroblastoma, both separately and concomitantly with Trk proteins. Specifically, nerve growth factor, brain-derived neurotrophic factor (BDNF), and neruotrophin-3, respectively stimulate TrkA, TrkB, and TrkC, respectively (99). Overexpression of TrkB and BDNF, in particular, is associated with poor prognosis (193). More over, some patients with neuroblastoma having both *MYCN* gene amplification and high TrkB expression have even worse prognoses (193, 194). Given that N-Myc and Ras can cooperate in cellular transformation (195), this provides a potential mechanism by which N-Myc and hyperactivated Ras signaling (through TrkB activation) contribute to the development of neuroblastoma. Our studies presented in Chapter 2 examine a mechanism by which Ras signaling can augment N-Myc expression and activity.

1.4 AIMS OF THESIS

The overall goal of this thesis is to further our understanding of how N-Myc is regulated and how this regulation might be relevant to the oncogenic activity of N-Myc. Specifically, I have focused on the role of the Ras/MAPK pathway in controlling the synthesis, proteolysis, and activity of N-Myc. The aims of this thesis are:

1. To elucidate the mechanism by which the Ras/MAPK pathway controls N-Myc expression,
2. To understand how regulation of N-Myc by the Ras/MAPK pathway affects N-Myc activity, and
3. To determine whether N-Myc and c-Myc are subject to similar post-transcriptional regulation by the Ras/MAPK pathway.

CHAPTER 2 | Differential Regulation of N-Myc and c-Myc Synthesis, Degradation, and Transcriptional Activity by the Ras/MAPK Pathway

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Author contributions: KK and PJH devised experiments, performed data analysis, and wrote the manuscript. KK performed all experiments.

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2.1 ABSTRACT

Myc transcription factors are important regulators of proliferation and can promote oncogenesis when deregulated. Deregulated Myc expression in cancers can result from *MYC* gene amplification and translocation, but also from alterations in mitogenic signaling pathways that affect Myc levels through both transcriptional and post-transcription mechanisms. For example, mutations in Ras family GTPase proteins that cause their constitutive activation can increase cellular levels of c-Myc by interfering with its rapid proteasomal degradation. Although enhanced protein stability is generally thought to be applicable to other Myc family members, here we show that c-Myc and its paralog N-Myc respond to oncogenic Ras (Ras^{G12V}) in very different ways. Ras^{G12V} promotes accumulation of both c-Myc and N-Myc, but whereas c-Myc accumulation is achieved by enhanced protein stability, N-Myc accumulation is associated with an accelerated rate of translation, which overcomes a surprising Ras^{G12V}-mediated destabilization of N-Myc. We show that Ras^{G12V}-mediated degradation of N-Myc functions independently of key phosphorylation sites in the highly conserved Myc homology box I region that controls c-Myc protein stability by oncogenic Ras. Finally, we found that N-Myc and c-Myc transcriptional activity is associated with their proteasomal degradation, but that N-Myc may be uniquely dependent on Ras-stimulated proteolysis for target gene expression. Taken together, these studies provide mechanistic insight into how oncogenic Ras augments N-Myc levels in cells and suggest that enhanced N-Myc translation and degradation-coupled transactivation may contribute to oncogenesis.

2.2 INTRODUCTION

N-Myc is a member of the *Myc* family of proto-oncogenes that also include *c-Myc* and *L-Myc*. *Myc* genes encode basic helix-loop-helix/leucine zipper proteins that associate with their binding partner Max (1). Myc-Max heterodimers bind to E-box motifs in DNA and, together with a variety of co-activator proteins that interact with Myc, influence chromatin structure and the activities of RNA polymerase I, II and III to promote transcription (196, 21). Myc can also repress transcription by binding to Miz1 and blocking Miz1-dependent activation (4). The potential transcriptional effects of Myc expression are far reaching as it has been shown that, depending on its levels, Myc can directly or indirectly influence the expression of thousands of genes controlling diverse cellular processes (197).

Deregulated expression of Myc family proteins is found in diverse types of tumors. Deregulated N-Myc plays a particularly important role in the malignant progression of tumors derived from the nervous system including neuroblastoma, medulloblastoma, and glioblastoma (105, 198). Medulloblastoma and neuroblastoma are the first and third most common pediatric cancers, respectively, and together, they account for 35% of all childhood cancer deaths (105, 198). In addition to N-Myc gene amplification, deregulation of growth factors or downstream signaling constituents are frequently observed in neurological cancers (105, 199) and may contribute to upregulation of N-Myc. N-Myc is also essential for embryonic development (9) and mutations that inactivate N-Myc cause Feingold syndrome, a pleomorphic birth defect syndrome (10). Consistent with the critical roles of both N-Myc and c-Myc in controlling proliferation and fate determination during development and their well-established oncogenic potential

when deregulated, multiple regulatory mechanisms have evolved to tightly control their levels and activity.

Rapid degradation of Myc proteins by the ubiquitin-proteasomal pathway is one mechanism thought to contribute to maintaining physiologically appropriate levels of Myc. Our current understanding of the process by which Myc proteins are degraded has been largely determined using c-Myc. One mechanism of c-Myc degradation requires sequential phosphorylation of two key residues in the N-terminal Myc homology box I (MBI) region of c-Myc: Threonine 58 (Thr58) and Serine 62 (Ser62) (11). In response to growth factor signals the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway activates extracellular signal-regulated kinase (ERK), which in turn phosphorylates c-Myc at Ser62 (131). This phosphorylation event transiently stabilizes c-Myc (12). As growth factor signals diminish, the activities of the PI3K/Akt pathway decline and result in activation of glycogen synthase kinase-3β (GSK3β) (133), which recognizes phosphorylated Ser62 as a priming site for phosphorylation of c-Myc at Thr58 (12, 14). The coordinated activities of the peptidylprolyl isomerase Pin1 and protein phosphatase 2A (PP2A) act on doubly phosphorylated c-Myc to direct dephosphorylation of Ser62 (134). Finally, singly phosphorylated c-Myc at Thr58 is targeted for ubiquitination by the E3 ubiquitin ligase Fbw7, which directs the proteasomal-mediated degradation of c-Myc (112, 113, 131).

This mechanism of c-Myc degradation can be blocked by oncogenic mutations in Ras family GTPases that constitutively maintain Ras in an active state (200). Oncogenic Ras signaling promotes c-Myc stability in two ways: first, it induces Ser62 phosphorylation by activating ERK through the MAPK pathway and second, it blocks Thr58

phosphorylation by activating the PI3K/Akt pathway, which inhibits GSK3β (12). The well-established cooperation between c-Myc and oncogenic Ras in promoting cellular transformation (201) may in part be due to Ras-mediated stabilization of c-Myc (200).

Like c-Myc, N-Myc can cooperate with oncogenic H-Ras and K-Ras to transform normal embryonic fibroblasts into cells with tumorigenic potential (195, 202). The mechanism underlying cooperation between N-Myc and oncogenic Ras was suggested to be due to Ras-mediated stabilization of N-Myc (21). This is a reasonable assumption, since the Ser and Thr phosphorylation sites in MBI of c-Myc that control its degradation are conserved in N-Myc (the equivalent sites in the mouse N-Myc homolog being Thr50 and Ser54). However, several studies call into question whether N-Myc degradation is controlled in the same manner as c-Myc. First, Kenney et al. (22) found that mutating N-Myc Ser54 to alanine stabilized N-Myc, while mutation of the equivalent site in c-Myc, Ser62, to alanine destabilizes c-Myc (12). Second, PP2A activity promotes c-Myc degradation (14), but appears to stabilize N-Myc (23). Finally, while the prolyl isomerase Pin1 appears to control PP2A-mediated dephosphorylation of Ser62 of c-Myc (15), Pin1 does not alter the phosphorylation status of the equivalent Ser54 of N-Myc in mouse neuronal precursors (23). While these differences in regulation might be attributable to different cell types or the specific conditions in which the studies were conducted, they might also reflect fundamental differences in how c-Myc and N-Myc respond to the activation of growth factor receptors and the activities of oncogenic Ras. To address this issue, we specifically examined how growth factor signaling and oncogenic Ras controls N-Myc protein levels and activity and compared this to c-Myc. Fibroblast growth factor (FGF) signaling and oncogenic Ras similarly triggered robust N-Myc and c-Myc

accumulation, but in contrast to c-Myc, we found no evidence that N-Myc accumulation was due to enhanced protein stability. To the contrary, oncogenic Ras promoted N-Myc turnover, and its increased accumulation was instead associated with a marked increase in N-Myc translation. Further, we show that proteasomal degradation of N-Myc and c-Myc is associated with enhanced transcriptional activity. Our results suggest that hyperactivated growth factor and Ras signaling may promote oncogenesis in N-Myc expressing cells by coupling augmented N-Myc translation with N-Myc proteolysis and transcriptional activity.

2.3 RESULTS

FGF signaling upregulates N-Myc expression post-transcriptionally through the MAPK pathway

Our finding that loss of N-Myc disrupted proximal-distal patterning of the developing mouse limb buds (27), a process controlled by FGF signaling, prompted our initial interest in investigating if and how FGF signaling controls N-Myc expression. N-Myc is not expressed in C3H 10T½ cells, and we first tested whether FGF stimulation of the mouse mesenchymal cell line C3H 10T½ could induce N-Myc transcription. FGF2 treatment activated FGF signaling based on phosphorylation of FGF receptor (FGFR) (Fig. 2.1), but N-Myc was not induced. However, when an N-Myc cDNA containing only the coding region was expressed ectopically, FGF2 addition induced high levels of N-Myc protein (Fig. 2.2A). Induction of N-Myc protein occurred post-transcriptionally since *N-myc* mRNA levels were unaffected by FGF2 (Fig. 2.2B). Both the MAPK and PI3K pathways were stimulated in response FGF2 addition (Fig. 2.1) and we used

pharmacological inhibitors to delineate which pathway(s) were involved in the induction of N-Myc. Inhibition of the MAPK pathway with PD98059 or UO126 prevented N-Myc induction, while inhibition of PI3K signaling with LY294002 had no effect on N-Myc protein levels (Fig. 2.2C). Therefore, in this system FGF signaling functions through the MAPK pathway to increase N-Myc protein levels.

To further establish a link between FGFR activation and N-Myc upregulation, we examined the effect of two FGFR2 mutants associated with cancer and developmental defects: $FGFR2^{S252W}$ and $FGFR2^{K659E}$. $FGFR2^{S252W}$, which has broadened ligand-binding specificity and increased ligand affinity, causes the developmental disorder Apert Syndrome and is found in some cancers (203, 204). $FGFR2^{K659E}$ is a ligand-independent, constitutively active mutant that has been found in uterine cancer (205). Introduction of $FGFR2^{S252W}$ or $FGFR2^{K659E}$ into C3H 10T½ cells elevated ectopic N-Myc protein levels over that of WT FGFR2 (Fig. 2.2D), and N-Myc induction by the mutant receptors required activation of the MAPK pathway (Fig. 2.2E). Taken together, these results indicate that the MAPK pathway is responsible for the post-transcriptional-mediated increase in N-Myc that is stimulated by FGFR signaling.

Oncogenic Ras stabilizes c-Myc but destabilizes N-Myc

Previous studies conducted with c-Myc showed that mutant activated H-Ras promoted its stability as mentioned above (200). We therefore used a constitutively active form of H-Ras, Ras^{G12V} (or oncogenic Ras), to further investigate how the MAPK signaling upregulates N-Myc and whether this was due to increased N-Myc protein stability. Similar to what was observed with FGF2 treatment, Ras^{G12V} increased N-Myc

protein levels, but had little effect on *N-myc* transcript levels (Fig. 2.3A, B). Using cycloheximide (CHX) to block *de novo* protein synthesis, we monitored the rate of N-Myc degradation and were surprised to find that Ras^{G12V} consistently reduced the half-life of N-Myc (Fig. 2.3C). To control for potential off-target effects by CHX, N-Myc turnover was also examined by ^{35}S-methionine pulse-chase analysis. Like the CHX experiments, pulse-chase analyses consistently showed that Ras^{G12V} accelerated N-Myc turnover (Fig. 2.3D). Because these findings are opposite to what has been described for c-Myc (12), we performed parallel studies with c-Myc. Ras^{G12V} increased the amount of ectopic c-Myc protein, but did not affect *c-myc* transcript levels (Fig. 2.3A, B), and consistent with published data, ^{35}S-methionine pulse-chase analysis showed that Ras^{G12V} slowed c-Myc degradation (Fig. 2.3E). We conclude that oncogenic Ras has the opposite effect on N-Myc and c-Myc protein stability: it destabilizes N-Myc while stabilizing c-Myc.

Oncogenic Ras increases N-Myc protein synthesis

The observation that Ras^{G12V} elevated steady-state N-Myc protein levels without affecting *N-myc* mRNA levels but also accelerated N-Myc turnover, suggested that Ras^{G12V} might act at the level of translation. To test this, we examined the rate of N-Myc protein synthesis by ^{35}S-methionine pulse analysis. Newly synthesized, ^{35}S-methionine-labeled N-Myc protein accumulated faster in the presence of Ras^{G12V} and peaked at 20 minutes (Fig. 2.4A, left). The slight decrease in ^{35}S-methionine-labeled N-Myc at 30 minutes was consistently observed in the Ras^{G12V}-expressing cells and is likely due to its already short, but further shortened half-life (17-23 minutes) in the presence of oncogenic

Ras. In this 30 minute assay, Ras^{G12V} increased the rate of N-Myc protein accumulation ~2-fold (Fig. 2.4A, right), but the rate of synthesis is likely greater since the accelerated rate of protein degradation is not factored into this calculation. In contrast to N-Myc, Ras^{G12V} did not affect the rate of ectopic (Fig. 2.4A) or endogenous c-Myc protein synthesis (Fig. 2.5).

We considered that the increased rate of N-Myc protein synthesis might be due to Ras^{G12V} activity targeting elements within the vector used to expresses N-Myc (i.e. 3' LTR promoter, 3' untranslated region [UTR]). To control for this possibility, the gene encoding green fluorescent protein (GFP) was expressed in the same expression vector, and the rate of GFP protein synthesis was determined. In contrast to N-Myc, GFP translation was not altered by Ras^{G12V} (Fig. 2.4A). In addition, because it was possible that stable expression of Ras^{G12V} and/or N-Myc could lead to secondary effects that might influence how Ras^{G12V} regulates N-Myc during cell passaging, we examined N-Myc translation in cells two days after transfecting Ras^{G12V} and N-Myc expression vectors. In this context, Ras^{G12V} also consistently increased N-Myc protein synthesis (Fig. 2.4B).

Inhibition of the Ras/MAPK pathway suppresses N-Myc translation

The Ras/MAPK cascade signals through multiple pathways several of which are involved in translational control (167). We used a panel of inhibitors that target Ras effectors to determine what steps are involved in controlling N-Myc translation. C3H 10T½ cells transiently expressing N-Myc alone or with Ras^{G12V} were treated with UO126, LY294002, or SB230508 to inhibit MEK, PI3K, or p38/MAPK. Treated cells were pulsed with ^{35}S-methionine to examine the effects of these inhibitors on N-Myc

protein synthesis. Inhibition of MEK with UO126 reduced the amount of newly synthesized N-Myc protein (Fig. 2.4C). LY249002 also reduced the amount of newly synthesized N-Myc protein, but this was likely a non-specific effect since it concomitantly reduced total protein synthesis (Fig. 2.4C). Inhibition of p38/MAPK with SB230508 did not significantly affect N-Myc translation (Fig. 2.4C). Treatment with the proteasome inhibitor MG132 did not increase N-Myc levels indicating that protein degradation did not contribute to the changes in N-Myc levels (Fig. 2.4C). Collectively, these data show that Ras^{G12V} upregulates N-Myc translation via MEK activation and are consistent with MEK activation being required for increased steady-state levels of N-Myc in Ras^{G12V}-expressing cells (Fig. 2.2C).

N-myc mRNA is redistributed to polysomes by oncogenic Ras

To further characterize Ras^{G12V}-driven N-Myc translation, we next examined the translational status of *N-myc* mRNA by monitoring its association with ribosome complexes isolated following sedimentation through sucrose gradients. Ras^{G12V} caused *N-myc* mRNA to shift to the heaviest sucrose fractions (n=2), indicating enhanced association of *N-myc* mRNA with polysomes (Fig. 2.6A, B). Consistent with Ras^{G12V} preferentially affecting N-Myc protein synthesis, ectopic *CMYC* and *Actin* transcripts were not redistributed toward polysomes (fractions 5-11 – Fig. 2.6C, D) to the same extent as *N-myc* mRNA.

K-Ras, and possibly H-Ras and N-Ras, alters the polysome distribution of many targets (169, 183). This may account for the larger polysome population observed in C3H 10T½ cells expressing Ras^{G12V} (Fig. 2.6A) and calls into question the extent to which

Ras^{G12V} upregulates N-Myc translation in a general or selective manner. Ras^{G12V} did not increase global protein synthesis (Fig. 2.7), a result consistent with translational upregulation of N-Myc being at least partly selective. Moreover, redistribution of *N-myc* mRNA to polysomes by Ras^{G12V} is consistent with some level of specificity for *N-myc* mRNA since Ras^{G12V} did not alter the polysome association of *CMYC* (Fig. 2.6C) or *Cyclin D1* (Fig. 2.8).

Translational control by microRNAs and RNA binding proteins (RBPs) often requires the UTRs of transcripts (206, 207). However, our studies characterized ectopically expressed *N-Myc* transcripts lacking endogenous UTRs. This suggested that N-Myc UTRs were not required for Ras^{G12V}-mediated translational upregulation of N-Myc. To determine whether the 5' and 3' UTRs of N-Myc influenced N-Myc protein expression in the presence or absence of Ras^{G12V}, we compared levels of N-Myc expressed from a "full-length" N-Myc cDNA that included the complete UTR sequences to the "coding-region only" cDNA expressed from the same vector. Ras^{G12V} increased N-Myc protein level to a similar extent with both versions of N-Myc cDNA (Fig. 2.9). Taken together, these results suggest that Ras^{G12V} promotes N-Myc translation by targeting the N-Myc mRNA coding region for redistribution toward actively translating polysomes.

Oncogenic Ras promotes translation of endogenous N-Myc in neuroblastoma cells

While Ras^{G12V} increased N-Myc translation and accelerated N-Myc protein degradation in C3H 10T½ cells using ectopically expressed N-Myc, we wanted to know whether this was also true for endogenous N-Myc. To our knowledge, there are no non-tumor derived cell lines that express N-Myc. Therefore, we utilized two different human

neuroblastoma cell lines, SMS-KCNR and SK-N-BE(2)C, which express N-MYC from *MYCN*-amplified loci. In both of these cell lines, ectopic expression of Ras^{G12V} increased N-MYC protein, but not mRNA levels (Fig. 2.10A, B). As in C3H 10T½ cells, Ras^{G12V} increased the rate of N-MYC degradation in both neuroblastoma cell lines (Fig. 2.10C). Moreover, Ras^{G12V} caused a redistribution of *MYCN* mRNA, but not *CMYC* or *ACTIN* mRNA, to polysome fractions in SK-N-BE(2) cells (Fig. 2.10D). These results suggest that the effects of oncogenic Ras on N-Myc translation and degradation may be generally applicable.

Role of Conserved Thr and Ser Phosphorylation sites in c-Myc and N-Myc degradation

Our results indicated that Ras^{G12V} regulates the stability of N-Myc and c-Myc in an opposing manner. This is surprising because the key phosphorylation sites in MBI that are involved in Ras-mediated stabilization of c-Myc (Thr58 and Ser62) are conserved in N-Myc (Thr50 and Ser54). We therefore directly compared how mutations at the equivalent phosphorylation sites in N-Myc and c-Myc affected their stability using a panel of phosphorylation defective and mimetic mutants. Phosphorylation-specific antibodies were used to assess and compare how the various mutations at one phosphorylation site affected phosphorylation at the adjacent non-mutated site for both N-Myc and c-Myc. We found that the phosphorylation pattern of N-Myc and c-Myc was similar among the various mutant proteins (Fig. 2.11A, B). Phosphorylation of N-Myc at Thr50 and c-Myc at Thr58 was observed for both WT N-Myc and c-Myc proteins, but not for any of the mutant proteins (Fig. 2.11A, B). Phosphorylation of Ser54 and Ser62

was elevated in the N-Myc^{T50A} and c-Myc^{T58A} mutants, respectively, but was not detected in the N-Myc and c-Myc Serine mutants (Fig. 2.11A, B). These observations suggest that N-Myc undergoes the same hierarchical phosphorylation that has been delineated for c-Myc, such that phosphorylation of N-Myc at Thr50 requires prior phosphorylation of Ser54 (131, 208).

Next, we compared how mutation of the conserved phosphorylation sites affected the half-life of N-Myc and c-Myc. Both N-Myc^{T50A} and c-Myc^{T58A} displayed prolonged half-lives compared to their WT counterparts (Fig. 2.11C, D). Similarly, N-Myc^{T50D} and c-Myc^{T58D} had longer half-lives compared to their WT counterparts, but for c-Myc^{T58D}, this was not statistically significantly different (Fig. 2.11C, D). While the half-lives of N-Myc^{S54A} and c-Myc^{S62A} were longer than their WT counterparts, they were not statistically significantly different. Finally, the half-lives of N-Myc^{S54D} and c-Myc^{S62D} were longer than their WT counterparts, but for c-Myc^{S62D}, this was not statistically significantly different.

Since both N-Myc^{T50A} and c-Myc^{T58A} exhibited prolonged half-lives compared to their WT counterparts, this suggested that oncogenic Ras functions independently of Thr50 to promote N-Myc degradation. Indeed, N-Myc^{T50A} retained sensitivity to Ras^{G12V}-dependent destabilization (compare Fig. 2.11C for T50A to Fig. 2.11E, top). Finally, Ras^{G12V} did not alter the half-life of c-Myc^{T58A} (compare Fig. 2.11D for T58A to Fig. 2.11E, bottom), which is consistent with the observation that the activities of oncogenic Ras function through this site to block c-Myc proteolysis (12). Together, these data show that while phosphorylation at Thr50 or Ser54 are involved in regulating N-Myc stability, oncogenic Ras functions independently of these sites to promote N-Myc degradation.

N-Myc degradation affects its transcriptional activity

The unexpected finding that Ras^{G12V} accelerated N-Myc turnover seemed in discordance with the observation that N-Myc and mutant activated Ras cooperate in oncogenic transformation of cells in culture (195). However, a functional link between transcriptional activity and protein degradation has been documented for numerous transcription factors, including c-Myc (109, 209, 143), raising the possibility that the process of proteasomal degradation might be important for N-Myc transcriptional activity. To test this, we measured the activity of the Myc-responsive pGL-M4 luciferase reporter (26) in response to transfected N-Myc or c-Myc in the presence or absence of proteasomal inhibitor MG132. Consistent with being substrates of the proteasome, N-Myc and c-Myc protein levels accumulated in the presence of the MG132 (Fig. 2.12A). Despite this accumulation in Myc protein, MG132 inhibited N-Myc- and c-Myc-induced luciferase activity (Fig. 2.12B). Reporter activity was reduced in the presence or absence of Ras^{G12V} for both N-Myc and c-Myc, suggesting that proteasomal degradation in general contributes to N-Myc and c-Myc transcriptional activity (Fig. 2.12B). Further, MG132 inhibited transcriptional activity of both N-Myc^{T50A} and c-Myc^{T58A}, suggesting that degradation-coupled transcription functions independent of Myc proteolysis governed by this conserved site in MBI (Fig. 2.12B). We next examined how N-Myc and c-Myc proteolysis might affect the expression of a subset of Myc target genes and whether oncogenic Ras was involved in this process. The transcriptional induction of a subset of Myc target genes (*eIF4E* (210), *Mnt* (211), and *Cad* (212)) by c-Myc alone or when combined with Ras^{G12V} was reduced by MG132 (Fig. 2.12C, right), supportive of a link between c-Myc transcription and protein degradation (38). This effect was not

observed for *Max*, a gene not regulated by Myc, and for the Myc target gene *Nucleolin* (*Ncl*) (213), indicating that degradation-coupled transcription is not relevant to all Myc targets. In contrast to c-Myc, N-Myc induced the expression of *eIF4E*, *Mnt*, and *Cad* with MG132 treatment, but like c-Myc, their induction was suppressed by MG132 treatment only in the presence of Ras^{G12V} (Fig. 2.12C, left). Induction of these genes was dependent on N-Myc and not MG132 treatment since, in the absence of N-Myc, their expression was reduced by MG132 treatment (Fig. 2.12D). Together, these data suggest that the transcriptional activity of both N-Myc and c-Myc are coupled to their proteasome-mediated degradation, but that degradation-coupled activation of N-Myc target genes may occur only in the presence of hyperactivated Ras signaling.

2.4 DISCUSSION

Mutations in Ras family members that lead to their constitutive activity are frequently found in a wide range of cancers and various mouse models have confirmed their oncogenic activity (214). This study suggests a novel mechanism by which constitutively active Ras proteins promote oncogenesis. We made the unexpected observation that oncogenic Ras signaling strongly promotes the accumulation of N-Myc while also stimulating N-Myc proteolysis. The increased accumulation of N-Myc was associated with an accelerated rate of N-Myc translation. Although clearly accelerated, a precise calculation of the rate of N-Myc synthesis in the presence of oncogenic Ras is confounded by a concomitant increase in the rate of N-Myc degradation. Nonetheless, because *N-myc* mRNA levels were not significantly altered by oncogenic Ras, it is likely that an elevated rate of translation is the underlying mechanism that overcomes the

increased rate of N-Myc degradation and accounts for the strong net increase in N-Myc accumulation observed. These data, together with results linking N-Myc and c-Myc degradation to their transcriptional activity, suggest that the simultaneous stimulation of N-Myc translation and stimulation of N-Myc degradation may be a mechanism underlying the oncogenic activity of mutant activated Ras, as well as a variety of other oncoproteins that hyperactivate the MAPK pathway.

Control of N-Myc proteolysis by Ras signaling

Our observation that Ras/MAPK signaling has the opposite effect on N-Myc and c-Myc turnover has likely gone unnoticed because c-Myc has generally served as the Myc prototype for describing the biochemical attributes of Myc family members. This is particularly true when considering the function of phosphorylation sites in MBI that regulate c-Myc stability since these sites are so highly conserved in Myc family proteins. The critical Thr and Ser residues in c-Myc that are phosphorylated in response to oncogenic Ras signaling and lead to enhanced protein stabilization, as well as their immediately surrounding amino acids, are identical in N-Myc. By comparing the half-lives of phosphorylation-defective and phosphomimetic Myc mutants, we show that the equivalent Thr and Ser residues in c-Myc and N-Myc, to some extent, modulate protein stability in a similar fashion. However, our data indicate that oncogenic Ras can act independent of these sites to regulate N-Myc protein stability. Indeed, our finding that oncogenic Ras destabilizes both WT and N-Myc^{T50A}, but does not affect c-Myc^{T58A} stability supports this hypothesis. Interestingly, the ubiquitin ligase Huwe1 has been shown to destabilize N-Myc, but not c-Myc (215), and may be a candidate for mediating

the differential effect oncogenic Ras has on N-Myc and c-Myc protein stability. One possibility is that oncogenic Ras/MAPK signaling leads to modifications of Huwe1, or other proteins involved in proteasomal degradation of N-Myc, to increase their binding affinity toward N-Myc.

Myc proteins are highly dynamic molecules, interacting with numerous proteins in both the nucleus and cytoplasm and in a context-dependent manner. These actions, in effect, create different populations of Myc that may be targeted for proteolysis at different efficiencies. This has been observed for c-Myc such that, in mitotic cells, there is an unstable and stable pool of c-Myc (45). Our protein synthesis studies using cells stably expressing N-Myc and Ras also suggest that there is more than one population of N-Myc protein subjected to different rates of degradation. A single cellular population of N-Myc protein would have a specific rate of synthesis and degradation such that, in our labeling assay, newly synthesized N-Myc protein would exponentially increase and then plateau as the rates of synthesis and degradation reach equilibrium. However, in the presence of oncogenic Ras, the accumulation of newly synthesized N-Myc protein did not plateau but rather declined at 30 minutes of synthesis (Fig. 2.4A, left), indicating that there are different populations of labeled N-Myc being degraded at different rates. There are at least two cellular populations of N-Myc: recently synthesized and pre-existing N-Myc protein. One possibility is that Ras targets them for degradation at different rates and our pulse-labeling results (Fig. 2.4A, left) would predict that the pre-existing pool of N-Myc protein is degraded faster. The two different methods used to determine the half-life of N-Myc, ^{35}S-methionine pulse-chase and cycloheximide treatment, measured the half-life of recently synthesized and pre-existing N-Myc protein, respectively. Contrary to our

prediction, the pre-existing population of N-Myc had a slightly longer half-life (17.6 ± 0.9 minutes) than the recently synthesized population of N-Myc protein (13.85 ± 2.7 minutes) in the presence of Ras (Fig. 2.3 C, D). However it is important to note that these half-lives were not statistically different. A simple explanation of our contradicting findings is that the decline in labeled N-Myc (at 30 minutes) in the presence of Ras is an artifact. Our pulse-labeling experiments using transient expression of N-Myc and Ras (Fig. 2.4B) supports this explanation, since we did not observe a decline in protein expression at 30 minutes of labeling. Since we did not examine the half-life of transiently expressed N-Myc with or without Ras, we cannot rule out the possibility that the method of gene expression (transient verses stable) affects the degradation kinetics of N-Myc. Additional labeling and cycloheximide studies measuring N-Myc synthesis and decay are required to address the possibility of artifactual results and to increase our sample size to derive accurate half-lives for N-Myc in the different populations.

Control of N-Myc synthesis by Ras signaling

Ras-mediated signaling modulates the activity of factors associated with the translational machinery to alter the translation of numerous transcript targets (175, 216), and our data indicate that N-Myc transcripts are one of those targets. Translational upregulation of N-Myc by oncogenic Ras was observed following stable and transient Ras^{G12V} expression (Figure 2.4A, B), indicating that this mechanism is not a result of secondary events acquired during chronic activation of Ras. Several studies have also demonstrated that oncogenic Ras influences the association of transcripts with polysomes (169, 183). Oncogenic Ras increased the relative amount of total RNA in the polysome

fractions (Fig. 2.6A), but this was not associated with global increases in translation (Fig. 2.7). Therefore it appears that oncogenic Ras may selectively increase the translation of certain mRNAs, with N-Myc being one of them. We observed increased N-Myc translation when expressing N-Myc cDNA lacking UTRs and an internal ribosome entry segment, suggesting that oncogenic Ras targets elements within the coding region of N-Myc. While mechanisms that control the expression or activity of a transcript most commonly target non-coding regions, there is a growing number of examples of RBPs and microRNAs that target the coding regions of transcripts (217–220). It will now be important to obtain a more precise understanding of how Ras/MAPK signaling targets the N-Myc coding region to stimulate N-Myc translation.

Ras-mediated proteolysis is associated with enhanced N-Myc transcriptional activity

The idea that the ubiquitin proteasome pathway is involved in gene transcription is supported by the fact that a number of components of the proteasome are recruited to promoter regions of target genes and that proteasome-dependent degradation of nuclear hormone receptors is coupled to efficient transcriptional activity (221). Indeed, it is now well documented that transcriptional activation domains (TADs) and degradation domains (degrons) overlap in many short-lived transcription factors and this arrangement may function to couple transcriptional activation and proteasomal degradation (53). Moreover, a link between protein degradation and transcriptional activity has been suggested for c-Myc (109, 222). In an effort to identify regions that target c-Myc for proteolysis, Salghetti et al. (38) identified a region comprising the first 143 amino acids c-Myc that increased both its transcriptional activity and degradation. The MBI and Myc

homology box II (MBII) regions of c-Myc, which reside within the first 143 amino acids, are important for its transcriptional activity (222), transforming activity (55), and stability (56), and it was postulated that these nearby regions might act to link c-Myc transcription with its degradation. MBI and MBII are highly conserved between c-Myc and N-Myc, and MBI and MBII in N-Myc might also function to link N-Myc transcription with its degradation. Our data support a link between both c-Myc and N-Myc degradation and their transcription activity, but suggest that degradation of N-Myc may not require residues residing within the MBI (Fig. 2.12B). Further, our results suggest that degradation-coupled transcriptional regulation of some Myc target genes in the presence of oncogenic Ras may operate differently for N-Myc and c-Myc (Fig. 2.12C).

A mechanism for oncogenic activities of Ras and N-Myc in neuroblastoma

Elevated levels of N-Myc are associated with a number of cancers, including neuroblastoma, where deregulated N-Myc expression is frequently caused by *MYCN* gene amplification and is associated with a poor prognosis (194, 223). Some patients with neuroblastoma having both *MYCN* gene amplification and high expression of tropomyosin-receptor kinase (Trk) A or TrkB have even worse prognoses (193, 194). Trk receptor signaling, like other receptor tyrosine kinase receptors, activates a number of different downstream pathways, including the Ras/MAPK pathway (223). Our results predict that the combination of hyperactive Ras/MAPK activity driven by elevated Trk and *MYCN* amplification would result in strongly augmented N-Myc protein levels and transcriptional activity, and thus provide a potential molecular basis for why neuroblastomas with combined *MYCN* amplification and high Trk/Ras expression are so

aggressive. Future studies designed to delineate the mechanisms by which hyperactivated Ras/MAPK and Trk receptor signaling controls N-Myc translation and protein turnover, and how N-Myc degradation is coupled to enhanced N-Myc transcriptional activity, may provide insights into specific points of intervention for inhibiting N-Myc oncogenic activity in neuroblastoma and other cancers.

2.5 FIGURES AND FIGURE LEGENDS

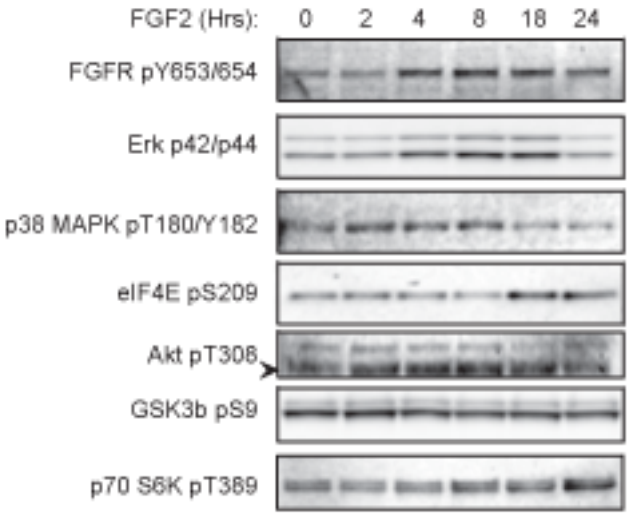


Figure 2.1 Activation of the MAPK and PI3K/Akt pathways by FGF2 stimulation. C3H 10T½ cells stably expressing N-Myc were treated with FGF2 for the indicated time. Cell lysates were analyzed by immunoblotting for the expression of the indicated phosphorylated proteins. Arrowhead indicates bands referring to Akt pT308.

Figure 2.2. **FGF signaling induces N-Myc expression through the MAPK pathway**. A, C3H 10T½ cells stably expressing N-Myc or containing control vector were treated with FGF2 for the indicated times. Cell lysates were analyzed by immunoblot analysis for the expression of N-Myc and Actin (loading control). B, C3H 10T½ cells stably expressing N-Myc were treated as in (A) and analyzed for *N-myc* mRNA levels by qRT-PCR. Error bars show S.D. for biological duplicates. There is no significant difference between time points as determined using the one-way ANOVA test. C, N-Myc-expressing C3H 10T½ cells were treated with FGF2 for 2 hours, followed by treatment with H_2O, DMSO, PD98059, UO126, or LY294002 for an additional 6 hours. Lysates were collected for immunoblot analysis. D, cellular extracts from C3H 10T½ cells stably expressing N-Myc and WT FGFR2 (WT), $FGFR2^{S252W}$ (S252W), or $FGFR2^{K659E}$ (K659E) were analyzed by immunoblot analysis for the expression of the indicated proteins. E, C3H 10T½ cells described in (D) were treated with UO126 for 6 hours, then harvested for immunoblot analysis. All experiments were performed in triplicate unless otherwise stated.

Figure 2.2 Continue

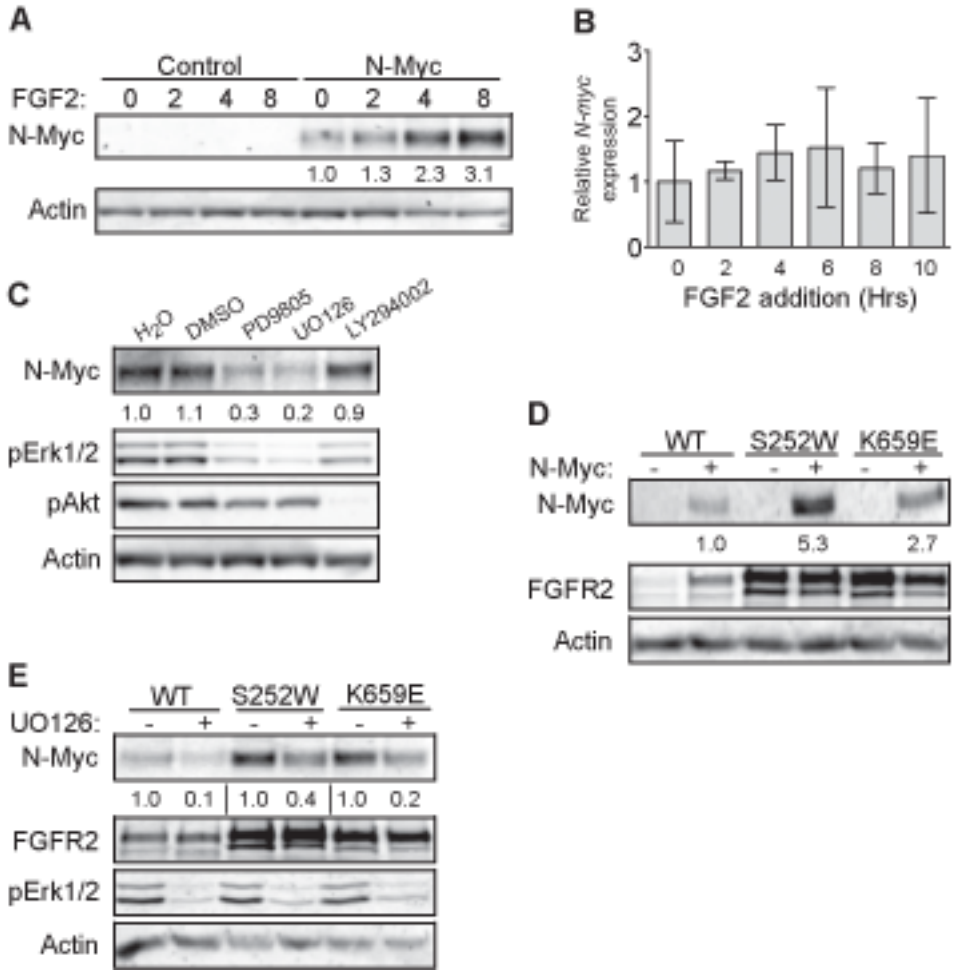


Figure 2.3. **Oncogenic Ras promotes N-Myc degradation**. A, C3H 10T½ cells containing control vector, stably expressing ectopic N-Myc or c-Myc alone, or stably expressing N-Myc or c-Myc combined with Ras^{G12V}, were analyzed by immunoblot analysis for expression of the indicated proteins. B, relative levels of *N-myc* and *c-myc* mRNA were assessed by qRT-PCR for the cell lines described in (A). Error bars show S.D. for biological triplicates. C, N-Myc-expressing C3H 10T½ cells containing control vector or stably expressing Ras^{G12V} were pulsed with CHX and lysates were collected at the indicated times for immunoblot analysis. Note that the Control panel is a longer exposure to equalize the signal at the 0 min time points. Quantifications for the blots shown are to the right. For four independent experiments, the average half-life of N-Myc was significantly greater ($p < 0.04$) in Control cells ($T½ = 24.4 \pm 1.1$ minutes) than in Ras^{G12V}-expressing cells (17.6 ± 0.9 minutes). D, pulse-chase analysis with C3H 10T½ cells described in (C). Radiolabeled N-Myc was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. Quantifications for the gels are shown to the right. The average half-life of N-Myc, calculated from two independent experiments, was 27.6 ± 5.9 minutes in Control cells and 13.85 ± 2.7 minutes in Ras^{G12V}-expressing cells. The half-lives were not statistically different. E, pulse-chase analysis with c-Myc-expressing C3H 10T½ cells containing control vector or stably expressing Ras^{G12V}. Radiolabeled c-Myc was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. Quantifications for the blots are shown to the right and are representative of two independent experiments.

Figure 2.3 Continue

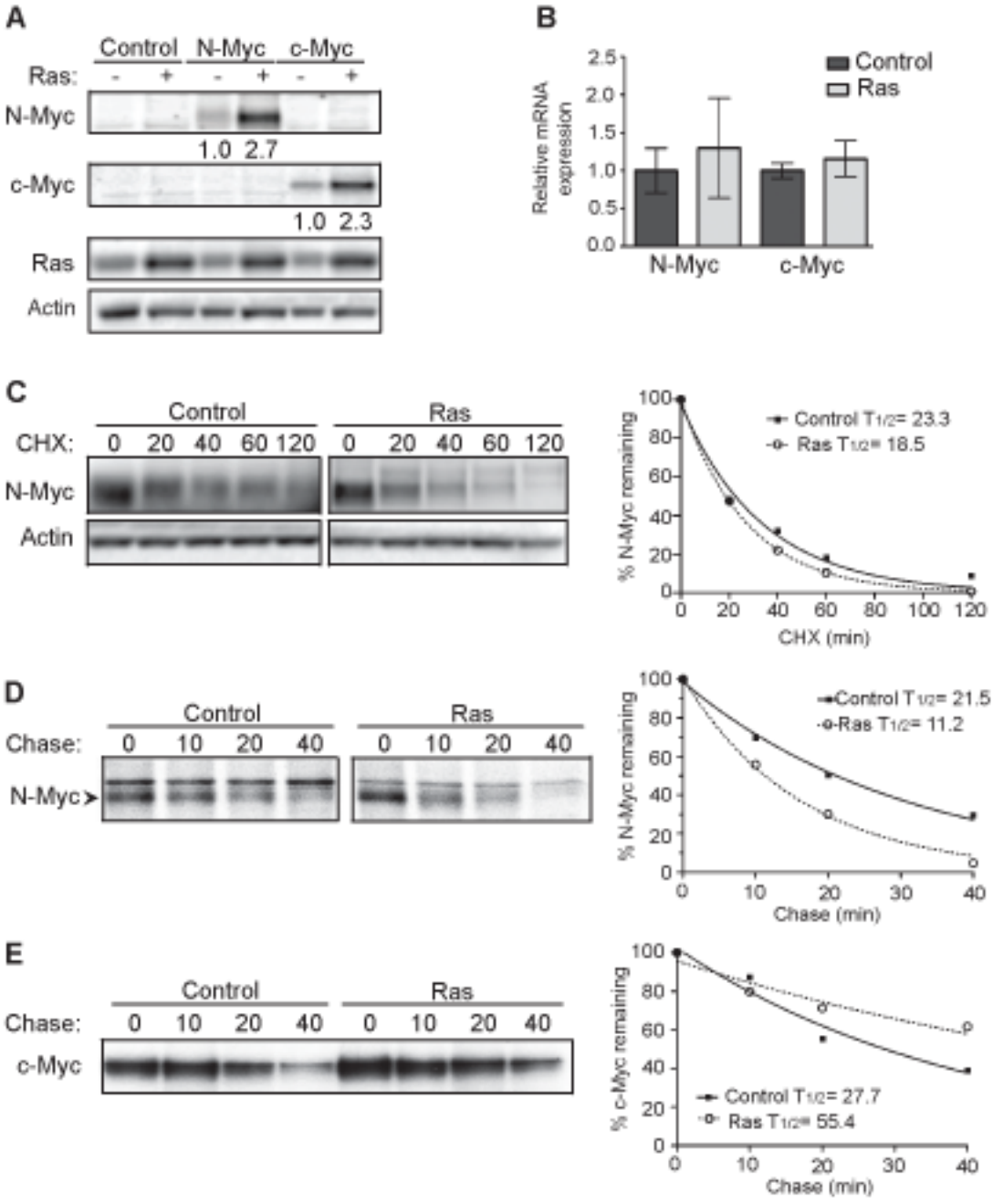
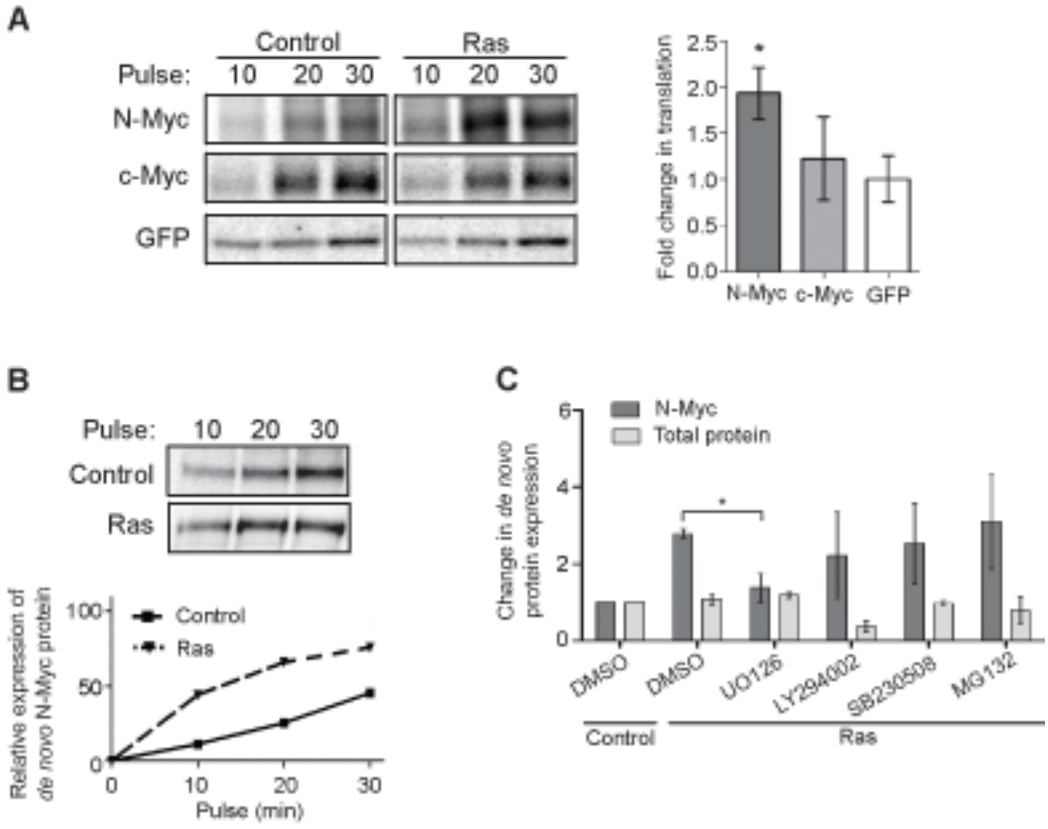


Figure 2.4. **Activation of the MAPK pathway by oncogenic Ras promotes N-Myc protein synthesis**. A, pulse analysis was performed with C3H 10T½ cells expressing the indicated proteins. Radiolabeled N-Myc, c-Myc, or GFP was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. The fold change in translation rate (right) is significantly different between N-Myc and GFP (asterisk, $p< 0.017$), but not between c-Myc and GFP. Error bars show S.D. for biological triplicates. B, pulse analysis was performed with C3H 10T½ cells transfected with N-Myc and control vector (pcDNA6) or Ras^{G12V}. Quantifications are graphed below. A representative image of four independent experiments is shown. C, C3H 10T½ cells transfected with N-Myc and control vector or Ras^{G12V} were treated with DMSO (vehicle control), UO126, LY294002, SB230508, or MG132 for 90 minutes. Cells were pulsed with ^{35}S-methionine for 20 minutes and radiolabeled N-Myc protein was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. Total protein synthesis was measured as described in the Experimental Procedures section. The expression of *de novo* N-Myc protein (dark bars) and total protein (light bars) is relative to the Ras^{G12V}-DMSO treated sample. Labeling experiments were performed in triplicate. Asterisk indicates $p=0.03$.

Figure 2.4 Continue



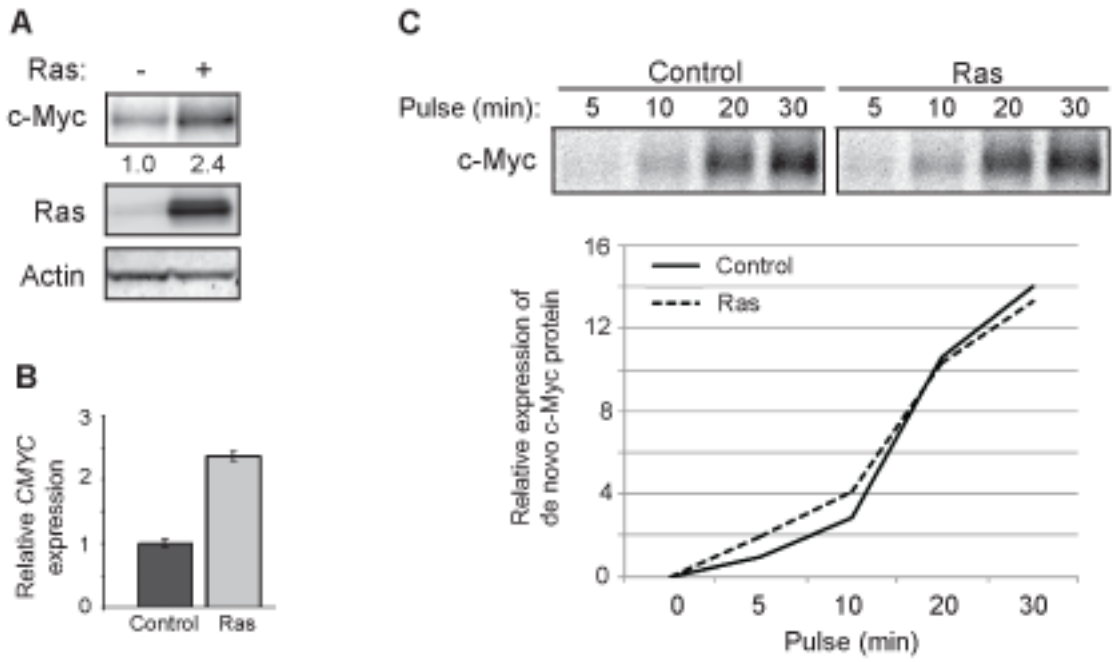


Figure 2.5 Oncogenic Ras does not affect the protein synthesis of endogenous c-Myc. A, C3H 10T½ cells stably expressing control vector or Ras^{G12V} analyzed for the expression of endogenous c-Myc, Ras, and Actin by immunoblot analysis. B, qRT-PCR analysis documenting *c-myc* mRNA expression in the cells described in (A). Error bars show S.D. from two independent experiments. C, Pulse analysis of C3H 10T½ stable cell lines described in (A). Labeled c-Myc was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. Quantifications are graphed below. A representative image of two independent experiments is shown.

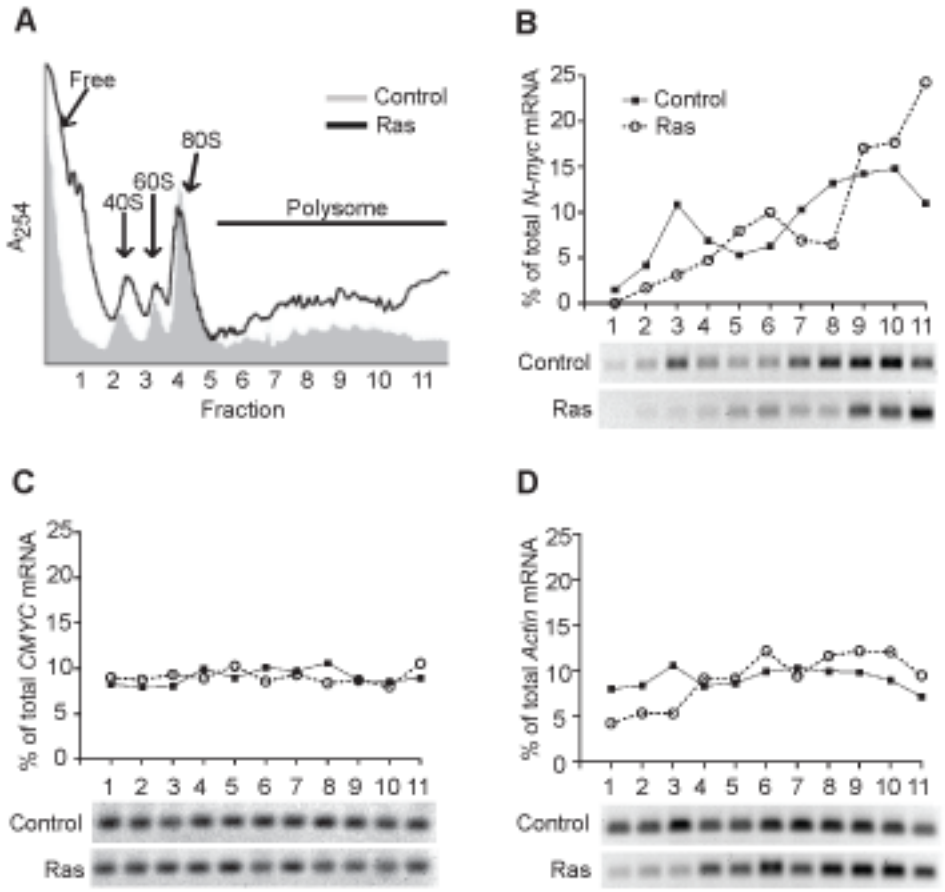


Figure 2.6. **Oncogenic Ras promotes redistribution of *N-myc* mRNA to polysomes**. A, representative polysome absorbance (254-nm) traces from C3H 10T½ cells expressing N-Myc plus containing control vector or Ras^{G12V} is shown, with positions of free ribonucleotide particles (free), 40S and 60S ribosomal subunits, 80S ribosomes, and polysomes indicated. B-D, relative distribution of *N-myc* (B), *CMYC* (C) and *Actin* (D) mRNAs was assessed by RT-PCR (bottom). Quantifications are shown above. A representative experiment of two independent experiments is shown.

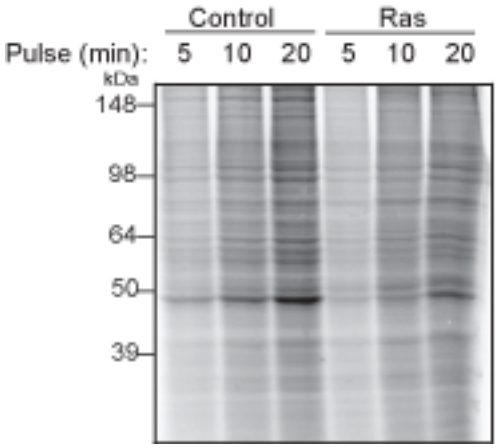


Figure 2.7. Global protein synthesis is not affected by Ras^{G12V}. C3H 10T½ cells expressing control vector or Ras^{G12V} were pulsed with ^{35}S-methionine for the indicated times and radiolabeled extracts were separated by SDS-PAGE. A representative image of two experiments is shown.

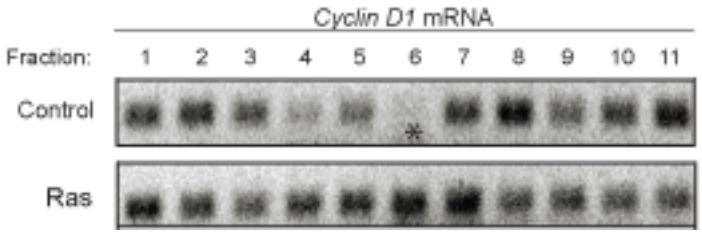


Figure 2.8. Oncogenic Ras does not redistribute *Cyclin D1* mRNA to polysomes. RT-PCR analysis of *Cyclin D1* mRNA from cytoplasmic lysates of C3H 10T½ cells expressing N-Myc plus containing control vector or Ras^{G12V} that were separated through a sucrose gradient. Fraction 6 Control sample (asterisk) was improperly processed.

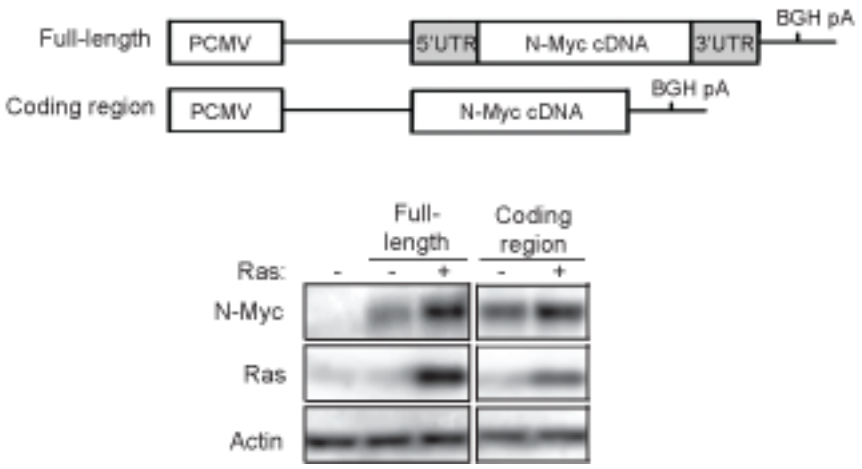


Figure 2.9 N-Myc UTRs are not required for N-Myc upregulation by Ras^{G12V}. Design of constructs containing the full-length or coding region of N-Myc (top). The promoter (PCMV) and polyadenylation signal (BGH pA) are depicted. Constructs were transfected into C3H 10T½ cells and N-Myc expression was documented by immunoblot analysis.

Figure 2.10. **Oncogenic Ras promotes N-Myc translation, but not protein stability, in *MYCN*-amplified neuroblastoma cells**. A, SMS-KCNR (KCNR) and SK-N-BE(2)C (BE(2)C) cells transfected with control vector or Ras^{G12V} were analyzed for expression of N-Myc and Ras by immunoblot analysis. B, relative expression of *MYCN* mRNA levels was assessed by qRT-PCR in the cells described in (A). Error bars show S.D. for biological triplicates. C, SMS-KCNR and SK-N-BE(2)C cells transfected with control vector or Ras^{G12V} were pulsed with CHX for the indicated times or with DMSO (D) for 90 minutes. N-Myc expression was documented by immunoblot analysis. Quantifications of the blot are shown below. Data are representative of two independent experiments. D, polysome analysis documenting the relative distribution of *MYCN* (top), *CMYC* (middle) and *ACTIN* (bottom) mRNA by RT-PCR in SK-N-BE(2) cells containing control vector or stably expressing Ras^{G12V}.

Figure 2.10 Continue

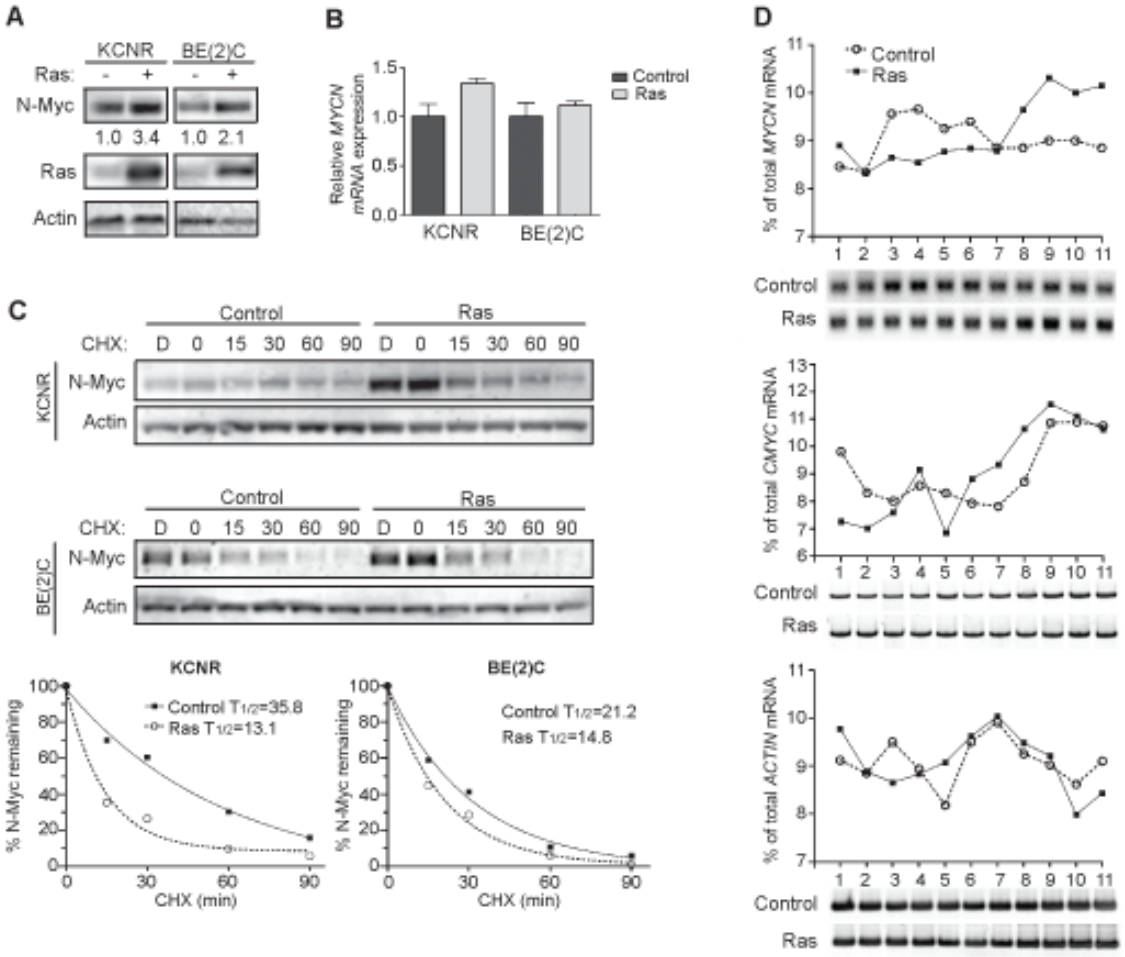


Figure 2.11. **Thr50 and Ser54 of N-Myc serve the same proteolytic function as Thr58 and Ser62 of c-Myc**. A, C3H 10T½ cells stably expressing WT or mutant N-Myc proteins were examined for the expression of the indicated proteins by immunoblot analysis. Arrowhead indicates the band representing N-Myc pS54. Asterisk indicates a non-specific band. B, C3H 10T½ cells stably expressing WT or mutant c-Myc proteins were examined for the expression of the indicated proteins by immunoblot analysis. C, C3H 10T½ cells expressing WT or mutant N-Myc proteins were pulsed with CHX for the indicated times. Lysates were analyzed by immunoblotting for N-Myc. D, C3H 10T½ cells expressing WT or mutant c-Myc proteins were pulsed with CHX for the indicated times. Lysates were analyzed by immunoblotting for c-Myc. Half-lives (mean ± S.D.) reported in (C) and (D) are calculated from three independent experiments. *$p < 0.01$ and **$p = 0.03$. E, C3H 10T½ cells stably expressing N-Myc^{T50A} or c-Myc^{T58A} and control vector (Cntl) or Ras^{G12V} were pulsed with CHX for the indicated times and lysates were analyzed by immunoblotting for N-Myc (top) or c-Myc (bottom). Half-lives (mean ± S.D.) are calculated from two independent experiments. *$p = 0.04$.

Figure 2.11

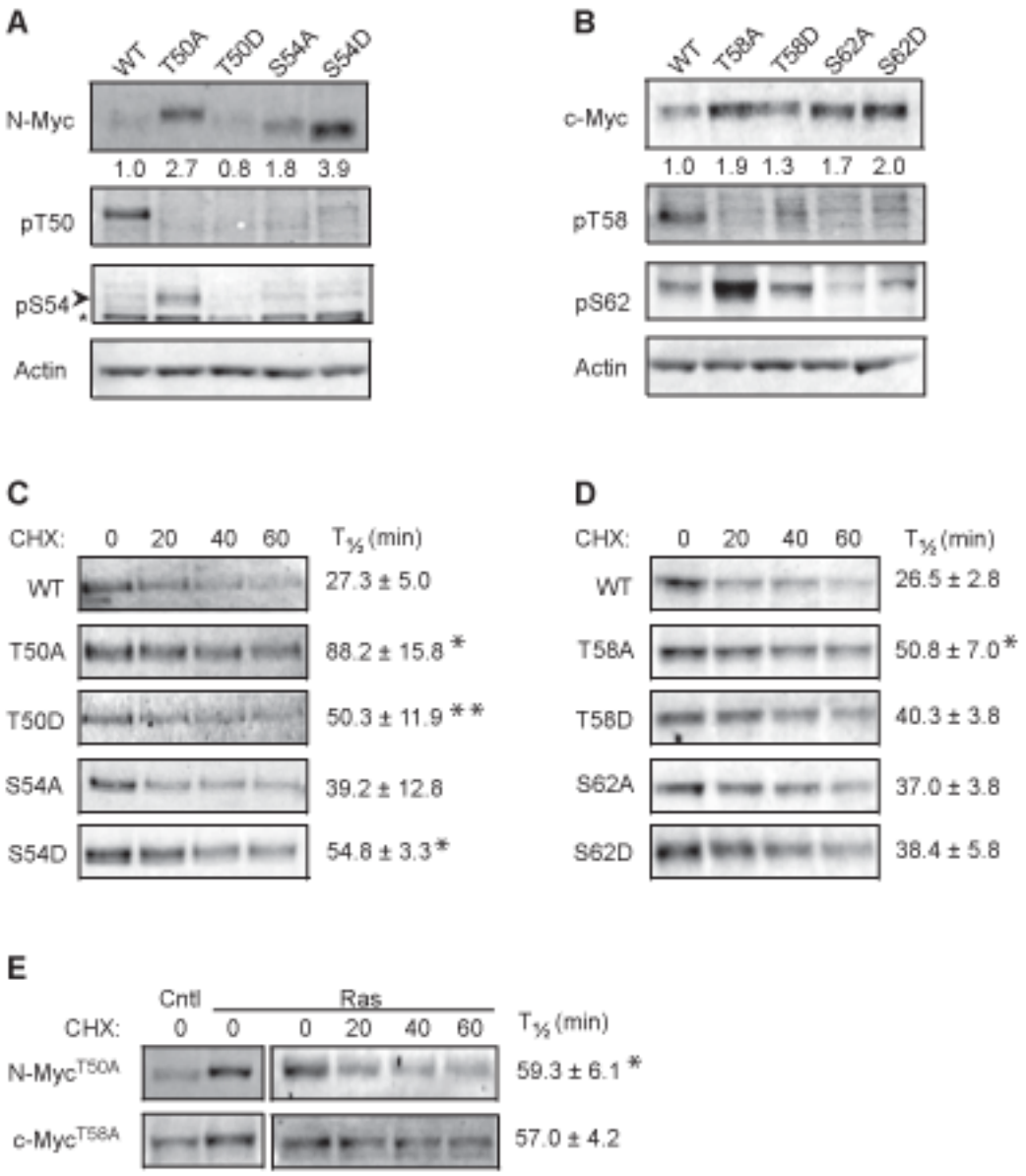
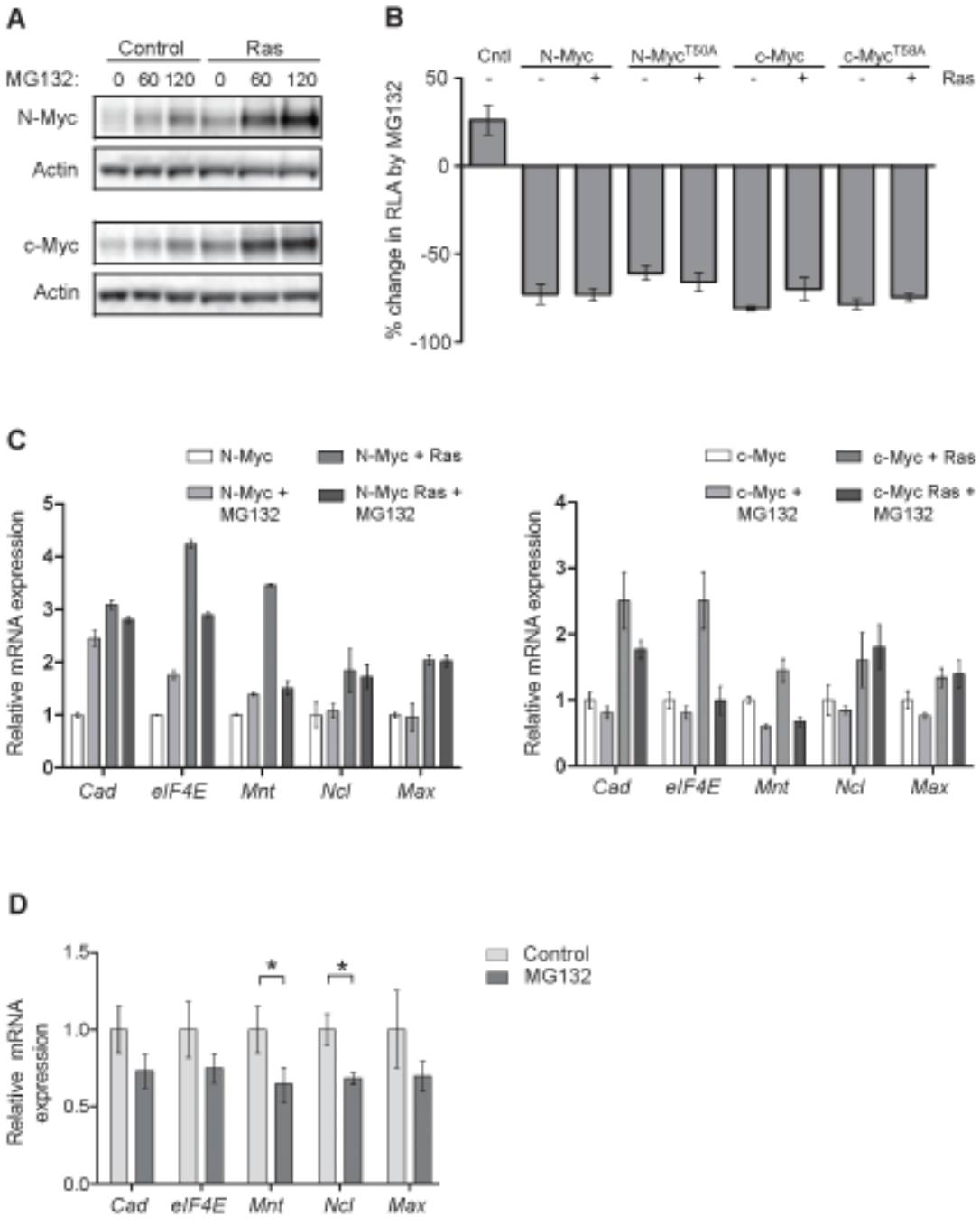

A
WT
T50A
T50D
S54A
S54D
N-Myc
1.0 2.7 0.8 1.8 3.9
pT50
pS54
Actin
B
WT
T58A
T58D
S62A
S62D
c-Myc
1.0 1.9 1.3 1.7 2.0
pT58
pS62
Actin
C
CHX: 0 20 40 60
T½ (min)
WT
27.3 ± 5.0
T50A
88.2 ± 15.8 *
T50D
50.3 ± 11.9 **
S54A
39.2 ± 12.8
S54D
54.8 ± 3.3*
D
CHX: 0 20 40 60
T½ (min)
WT
26.5 ± 2.8
T58A
50.8 ± 7.0*
T58D
40.3 ± 3.8
S62A
37.0 ± 3.8
S62D
38.4 ± 5.8
E
Cntl
Ras
CHX: 0 0 0 20 40 60
T½ (min)
N-Myc T50A
59.3 ± 6.1 *
c-Myc T58A
57.0 ± 4.2

Figure 2.12. Proteolysis of N-Myc and c-Myc correlates with enhanced transcriptional activity. A, C3H 10T½ cells stably expressing N-Myc or c-Myc and Ras^{G12V} or containing control vector were treated with MG132 (10 μM) for the indicated times. Lysates were analyzed by immunoblotting for expression of the indicated proteins. B, HEK 293 cells were transfected with the indicated Myc protein, control vector (Cntl) or Ras^{G12V}, and a Myc-responsive luciferase reporter. Following treatment with MG132 (5 μM) for 8 hours, cells were analyzed for luciferase activity. The graph shows the % change in relative luciferase activity (RLA) by MG132 calculated from three independent experiments. C, C3H 10T½ cells stably expressing N-Myc or c-Myc alone or in combination with Ras^{G12V} were treated with MG132 (10 μM) for one hour. Expression of the indicated genes was determined by qRT-PCR analysis. Error bars show S.D. for biological duplicates. D, C3H 10T½ cells containing control vector or stably expressing Ras^{G12V} were treated with MG132 for one hour and mRNA was harvested for analysis of *Cad*, *eIF4E*, *Mnt*, *Ncl* (*Nucleolin*) and *Max* expression by qRT-PCR. Error bars show S.D.

Figure 2.12 Continue



2.6 EXPERIMENTAL PROCEDURES

Cell culture and inhibitor assays—SK-N-BE(2) cells were maintained in DMEM/F12 (Invitrogen), SMS-KCNR cells were maintained in RPMI 1640 (Invitrogen), and C3H 10T½ cells (ATCC) and EcoPack 2-293 cells (Clontech) were maintained in DMEM (Invitrogen). All cell lines were supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin. For inhibitor assays, cells were treated with the following compounds: cycloheximide (CHX; 100 μg/ml), MG132 (10 μM, unless otherwise indicated), PD98059 (50 μM), UO126 (10 μM), SB230508 (10 μM) or LY249002 (50 μM) (all from Sigma).

Expression plasmids—The following plasmids have been previously described (224): pBABEpuro-c-Myc (c-Myc NCBI Accession no. ABW69847), pBABEpuro-GFP, pFBneo-WT-FGFR2c (Bek), pFBneo-FGFR2c^{K659E}, pFBneo-GFP, pLXSN-FGFR2c^{S252W} and pBABEpuro-Ha-Ras^{G12V}. For pBABEpuro-N-Myc, the mouse N-Myc cDNA (coding sequence only - NCBI Accession no. AAH49783) was cloned into BamHI and EcoRV sites of pBABEpuro. Mutant N-Myc (N-Myc^{T50A}, N-Myc^{T50D}, N-Myc^{S54A}, and N-Myc^{S54D}) and c-Myc (c-Myc^{T58A}, c-Myc^{T58D}, c-Myc^{S62A}, and c-Myc^{S62D}) genes were generated from the pBABEpuro-N-Myc or pBABEpuro-c-Myc plasmids using the Change-IT Multiple Mutation Site Direct Mutagenesis Kit (USB). To construct pcDNA6 N-Myc, N-Myc was amplified from pBABEpuro-N-Myc with primers that added NotI sites, and cloned into the NotI site of pcDNA6. The pcDNA6-H-Ras^{G12V} plasmid was constructed by removing H-Ras^{G12V} from pBABEpuro-H-Ras^{G12V} using BamHI and EcoRI and inserting it into pcDNA6. The full-length N-Myc mouse cDNA (obtained from OpenBiosystems) was cloned into pcDNA6 using EcoRI and NotI sites.

Retroviral infections and transfections—Viral infection and the establishment of stable cell lines were performed as previously described (224). For stable cell lines expressing more than one ectopic gene, cells were infected with a second virus 24 hours after the initial infection. Cells were selected with puromycin (1 mg/ml), geneticin (1 mg/ml), or both after all rounds of infection. Transient transfections in the C3H 10T½ and neuroblastoma cell lines were performed with Lipofectamine 2000 (Invitrogen) according the manufacture's protocol.

Immunoblot Analysis—Cells were lysed with RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% SDS, 1.0% NP-40, 0.5% sodium deoxycholate) containing 1x COMPLETE Protease Inhibitor cocktail (Roche). Equal amounts of protein were separated on 4-12% Novex Bis-Tris acrylamide gels (Invitrogen) and Western Blot analysis was performed as previously described (25) using the following antibodies: N-Myc (C-19), c-Myc (9E10), c-Myc (N-262), BEK (C1-7) and Phospho-Erk1/2 (E-4) from Santa Cruz Biotechnology; Ras (Ras10) from Upstate Biotechnology; Actin (Ac-40) from Sigma; GFP (ab290) and Phospho-c-Myc (S62) from Abcam; Phospho-Akt (S473), Phospho-p38 MAP Kinase (T180/Y182), Phospho-eIF4E (S209), Phospho-GSK-3β (S9), Phospho-c-Myc (T58/S62), and Phospho-p70 S6 Kinase (T389) from Cell Signaling Technology. Quantitation of protein expression was performed by densitometry.

RNA Isolation, semi-quantitative RT-PCR, and quantitative RT-PCR—Total RNA was isolated using TriZol (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined using a NanoDrop 2000c instrument (Thermo Scientific). cDNA was generated from 1 μg of RNA sample using SuperScript III first-strand cDNA synthesis kit (Invitrogen) and semi-quantitative RT-PCR was carried out using 1 μL of

cDNA per sample and 0.2-0.3 μM of each primer. Each target was amplified using an empirically determined cycle number allowing for gel-based visualization of samples within the exponential amplification phase of each reaction. Equivalent volumes of each PCR reaction were separated through 1.2% TBE agarose gels containing ethidium bromide and photographed under UV illumination.

Quantitative RT-PCR (qRT-PCR) expression analysis was performed with 10-30x diluted cDNA (prepared as described above) using IQ SYBR Green Supermix (Bio-Rad) and analyzed with an iQ5 Real-Time PCR Detection System (Bio-Rad). All reactions were carried out in triplicate and measurements were analyzed using the relative quantization $2^{-\Delta\Delta Ct}$ method with *Actin* as the calibrator.

Metabolic labeling—Stable C3H 10T½ cell lines were seeded at $6x10^5$ per 10-cm dish 48 hours prior to labeling. Transfected cells were labeled 24 hours after transfection. Where indicated, transfected cells were treated with DMSO or the indicated inhibitor 2 hours prior to and during labeling. Cells were starved of L-methionine and L-cysteine by incubation for 15 minutes in L-methionine/L-cysteine-free D-MEM (Invitrogen) supplemented with 10% dialyzed fetal bovine serum and L-glutamine (20 mM). Following starvation, cells were labeled with 250 μCi/mL (for stable cells) or 100 μCi/mL (for transfected cells) of ^{35}S-methionine/cysteine (MP Bio) at 37° C for 30 minutes for protein stability assays or as indicated in protein synthesis assays. For stability assays, metabolically labeled cells were washed twice with cold "chase" media (D-MEM supplemented with 10%FBS, 15 mg/L L-methionine, and 20 mg/L L-cysteine) and then incubated in chase media for the indicated times. Cells were washed twice with cold PBS and lysed with RIPA buffer containing protease inhibitors. Lysates were

incubated at 4° C overnight with 1 μg of antibody against N-Myc (NCM100-II, Santa Cruz Biotechnology), c-Myc (9E10, Santa Cruz Biotechnology for ectopically expressed c-Myc or N-262, Santa Cruz Biotechnology for endogenous c-Myc), or GFP (ab290, Abcam), and then incubated at 4° C for an additional 2 hours with G plus-agarose beads. Immune complexes were separated by 10% SDS-PAGE and visualized using a low-intensity phosphor screen. Quantification of immunoprecipitated proteins was performed by densitometry using ImageQuant software (GE Healthcare). Background values were calculated from an equivalent area in each lane and subtracted from the signal value for the labeled protein. Data points were plotted on a linear scale with Time 0 being set at 100%. The protein half-life was calculated using a one-phase decay equation (GraphPad Prism). The fold change in translation rate was defined as the rate of protein accumulation for oncogenic Ras expressing cells divided by the rate of protein accumulation for Control cells.

Polysome Analysis—Cells (5×10^6) were plated in 15-cm culture dishes and grown for 48 hours. Prior to harvest, cells were washed twice with cold PBS and collected into Wash Buffer A (110 mM potassium acetate, 2.0 mM magnesium acetate, 10 mM Hepes pH 7.3, 2 mM DTT, 100 μg/mL CHX). Cells were pelleted, resuspended in a hypotonic lysis buffer (10 mM potassium acetate, 2 mM magnesium acetate, 5 mM Hepes pH 7.3, 2 mM DTT, 100 μg/mL CHX, 1x protease inhibitor cocktail) and lysed by passing cells through a 22-gauge needle. Lysates were centrifuged and the resulting cytoplasmic fraction (supernatant) was separated through a sucrose gradient (17-51%) by centrifugation at 40,000 rpm with a SW-41-Ti Rotor (Beckman Coulter, Fullerton, CA) for 120 minutes at 4° C. Gradients were then collected into 12 equal fractions (Bio-Rad

system) while the absorbance at 254 nm was continuously monitored (Kipp & Zonen chart recorder). Total RNA from each fraction was isolated by phenol-chloroform extraction and ethanol precipitation, followed by DNase treatment. One-third volume of total RNA was separated on a 1.2% formaldehyde-agarose gel and stained with ethidium bromide to assess RNA quality and the distribution of tRNA, 18S rRNA, and 28S rRNA. The remaining two-thirds of total RNA was used for RT-PCR analysis as described above. Semi-quantitative analysis of the polysome gradients was performed by measuring band intensities and subtracting the background readings from equivalent areas using ImageQuant Ver7.

Luciferase Assay—HEK 293 cells were transfected using the calcium phosphate precipitation method (225) with expression vectors or control empty vector together with pGL2M4 reporter plasmid (26) and pCMV β-galactosidase reporter. Forty-eight hours after transfection, cells were treated with MG132 (5 μM) for one hour and the luciferase activity was assessed using the Dual-Light system (Applied Biosystems) on a Centro XS^3 LB Microplate Luminometer (Berthold Technologies). Luciferase activity was adjusted for β-galactosidase activity.

Acknowledgments—We thank Sara Ota for her initial observations that led to this work, Dr. Bruce Magun and Kristin Diez-Sauter for their assistance with polysome assays, and Sara Ota, William C. Hooker, and William L. Walker for insightful discussions.

CHAPTER 3 | Mechanistic insight into Ras-mediated translation and proteolysis of N-Myc involving GSK3β and the N-Myc Thr50 codon

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Author contributions: KK and PJH devised experiments and performed data analysis. KK performed all experiments and wrote the manuscript.

3.1 INTRODUCTION

The Myc family of transcription factors (c-Myc, N-Myc, and L-Myc) controls the expression of genes involved in diverse cellular processes including proliferation, growth, apoptosis, and differentiation (197). Temporal regulation of Myc expression is critical for normal cell function, while deregulation of Myc is frequently observed in cancer (226). Overexpression of N-Myc is a common genetic feature of neuroblastoma, a pediatric cancer of the peripheral sympathetic nervous system (125). High N-Myc expression is associated with aggressive tumor behavior and poor prognosis (227, 228). Several studies have implicated N-Myc in the malignant progression of neuroblastoma: ectopic expression of *MYCN* in neuroblastoma cells accelerated their progression through the cell cycle (229); additionally, targeted overexpression of *MYCN* in the neuroectoderm of mice results in tumors that biologically and genetically resemble aggressive neuroblastoma (230).

One mechanism that leads to abnormally high expression of N-Myc involves aberrant activation of the Ras/MAPK pathway (141). The Ras family of GTPases, comprised of H-Ras, K-Ras, and N-Ras, functions to communicate extracellular signals to the intracellular portion of the cell. Ras proteins activate a number of signaling pathways to regulate cell proliferation, differentiation, survival, and apoptosis (154). Activating mutations within Ras family members are rarely observed in neuroblastoma, however disruption of upstream signaling components that cause Ras activation is frequently observed (105, 191, 192). N-Myc and H-Ras can cooperate to transform normal embryonic fibroblasts into cells with tumorigenic properties (195). This cooperation may be relevant to the pathogenesis of neuroblastoma. Yaari et al. (141) demonstrated that blocking Ras activity with a small molecule inhibitor arrested the growth of *MYCN*-amplified neuroblastoma cells. The Ras inhibitor also reduced N-Myc expression,

suggesting that upregulation of N-Myc by Ras signaling is an underlying mechanism of their cooperation in transformation.

Our understanding of how Ras controls N-Myc protein expression has largely been based on studies that use c-Myc as a model. Ras has been shown to increase c-Myc protein expression by slowing c-Myc proteolysis through mechanisms that involve two key residues, Thr58 and Ser62 (136). Ras stabilizes c-Myc protein in two ways. First, Ras activates the Raf/MEK/ERK signaling cascade, to phosphorylate c-Myc at Ser62 and stabilize the protein (136). Second, Ras activates the PI3K/Akt pathway, which in turn inhibits GSK3β. GSK3β phosphorylates c-Myc at Thr58 and targets c-Myc for ubiquitin-mediated proteasomal degradation (137). By inactivating GSK3β, Ras prevents phosphorylation of c-Myc at Thr58 and its subsequent proteolysis (136).

Ras is thought to stabilize N-Myc by the same mechanism that has been delineated for c-Myc, especially since the phosphorylation sites involved in this process are conserved between c-Myc and N-Myc (Thr58 and Ser62 in c-Myc and Thr50 and Ser54 in N-Myc). However, we have previously shown that Ras does not stabilize, but rather destabilizes N-Myc (Chapter 2). These findings call into question the role of other factors implicated in the control of N-Myc proteolysis. Here, we examined the role of GSK3β in Ras-mediated proteolysis of N-Myc and show that GSK3β is not required. GSK3β was also not required for Ras-induced translation of N-Myc. Unexpectedly, we found that the nucleotide region encoding Thr50, the GSK3β phosphorylation site, may be involved in N-Myc translation.

3.2 RESULTS

Oncogenic Ras does not require GSK3β to direct N-Myc proteolysis

According to the degradation model that has been delineated for c-Myc (Fig. 1.4), GSK3β is thought to destabilize N-Myc by phosphorylating N-Myc at Thr50, thereby signaling for its destruction. To date, however, the effect of GSK3β on N-Myc half-life has not been directly tested. We sought to determine if inhibition of GSK3β indeed stabilizes N-Myc and whether Ras functions through GSK3β to control N-Myc degradation. C3H 10T½ mouse mesenchymal cells expressing ectopic N-Myc and control vector or a constitutively active form of H-Ras (Ras^{G12V}) were treated with lithium chloride (LiCl) to inhibit GSK3β (231, 232). Inactivation of GSK3β by LiCl had little effect on N-Myc protein levels in the presence or absence of Ras^{G12V} (Fig. 3.1A). The effect of GSK3β on c-Myc turnover has been examined (137), therefore we performed parallel experiments with c-Myc for a comparison. Similar to N-Myc, c-Myc protein expression did not significantly change in response to LiCl in the presence and absence of Ras^{G12V} (Fig. 3.1B). Phosphorylation of N-Myc at Thr50 and c-Myc at Thr58 was reduced by LiCl treatment, which is consistent with these sites being substrates for GSK3β (Fig. 3.1A,B).

To determine whether GSK3β activity is required for N-Myc proteolysis, we monitored the rate of protein turnover following addition of LiCl-treated cells with CHX. LiCl treatment increased the half-life of N-Myc from 27 to 45 min (Fig. 3.2A). In agreement with published reports (137), LiCl treatment increased the half-life of c-Myc from 25 to 40 min (Fig. 3.2B). In the presence of Ras^{G12V}, however, LiCl treatment did not affect the half-life of N-Myc (Fig. 3.2C). These data indicate that GSK3β targets both

N-Myc and c-Myc for proteolysis. Since LiCl treatment did not overcome the effects of Ras^{G12V} on N-Myc turnover, this suggests that Ras^{G12V} functions independently of GSK3β to enhance N-Myc turnover.

To further characterize the role of GSK3β in N-Myc proteolysis, we examined the effect of LiCl on the $N\text{-}Myc^{T50A}$ mutant. The equivalent c-Myc mutant, $c\text{-}Myc^{T58A}$, is stabilized because it cannot be targeted for GSK3β-mediated phosphorylation and is unaffected by LiCl treatment (137). Steady-state levels of $N\text{-}Myc^{T50A}$ were higher than WT N-Myc and were not affected by LiCl treatment (Fig. 3.3A). Similar to what has been observed for $c\text{-}Myc^{T58A}$, the half-life of $N\text{-}Myc^{T50A}$ was longer than WT N-Myc and was not affected by LiCl treatment (Fig. 3.3B,C).

Collectively, these data suggest that GSK3β-mediated phosphorylation of N-Myc at Thr50 promotes N-Myc turnover. Furthermore, oncogenic Ras functions independently of GSK3β to promote N-Myc turnover.

Oncogenic Ras does not require GSK3β to enhance N-Myc translation

In Chapter 2, we demonstrated that oncogenic Ras increases N-Myc protein expression by promoting its translation. GSK3β can control protein synthesis through several mechanisms, including regulation of the translation factor eIF2Bε (233). Therefore, we considered the possibility that Ras functions through GSK3β to promote N-Myc translation. Using MG132 to block proteasomal degradation, we examined how LiCl treatment affects the rate of N-Myc protein accumulation. LiCl reduced N-Myc protein accumulation in the presence of Ras^{G12V}, but did not significantly affect N-Myc protein accumulation in the absence of Ras^{G12V} (Fig. 3.4). Since, in the presence of

Ras^{G12V}, LiCl did not affect the degradation of N-Myc, these findings suggest that LiCl may alter the synthesis of N-Myc. To directly test if LiCl treatment affects Ras-mediated N-Myc translation, we pulse-labeled LiCl-treated and untreated C3H 10T½ cells with ^{35}S-methonine to monitor *de novo* protein synthesis. In the presence of Ras^{G12V}, LiCl reduced both N-Myc protein synthesis and total protein synthesis to a similar extent, suggesting that LiCl inhibits N-Myc translation in a non-specific manner (Fig. 3.5A).

Although LiCl is a potent inhibitor of GSK3β (231, 232), it has also been shown to inhibit the activity Akt and the 26S proteasome (234, 235). Both Akt and the 26S proteasome have been implicated in controlling global translation (236–238) and may account for the LiCl-mediated reduction in global protein synthesis (Fig. 3.5A). However, N-Myc protein synthesis was minimally affected by $GSK3\beta^{K85R}$, a dominant negative GSK3β mutant (239), either in the presence or absence of Ras^{G12V} (Fig. 3.5B).

We conclude that oncogenic Ras does not act through GSK3β to increase the translation of N-Myc.

Translation of WT N-Myc is different from N-Myc^{T50A} and may be Ras-dependent

We previously showed that oncogenic Ras increases N-Myc protein levels through a translational mechanism that involves regulation of the *N-myc* mRNA. It was therefore hypothesized that Ras^{G12V} would have the same translation effect on *N-myc*^{T50A} mRNA, i.e. Ras^{G12V} would increase N-Myc^{T50A} protein expression to a similar extent as WT N-Myc. Ras^{G12V} increased the expression of WT N-Myc 3.2-fold while increasing the expression of N-Myc^{T50A} 1.5-fold (Fig. 3.6A). Since Ras^{G12V} did not affect the levels of WT *N-myc* mRNA (Fig. 2.3) or *N-myc*^{T50A} mRNA (Fig. 3.6B), we tested the possibility

that oncogenic Ras has differential effects on the translation of WT N-Myc and N-Myc^{T50A}. Ras^{G12V} caused a 3.1-fold induction of newly synthesized WT N-Myc protein, but did not affect the amount of newly synthesized N-Myc^{T50A} protein (Fig. 3.6C). This result suggests that mutation of the Thr50 codon disrupts the ability of Ras^{G12V} to increase N-Myc translation. More so, *de novo* N-Myc^{T50A} protein levels were 3-fold higher than that of WT N-Myc (Fig. 3.6C). It was possible that the shorter half-life of WT N-Myc compared to N-Myc^{T50A} accounts for this difference (Fig. 3.6C); however, protein degradation did not significantly affect the expression of *de novo* WT N-Myc (Fig. 2.4C, compare DMSO to MG132 treated samples). Together, these data suggest that ribonucleotides encoding Thr50 of N-Myc potentially mediates N-Myc translation and may be required for Ras-induced translation of N-Myc.

Silent mutations at Thr50 codon alter N-Myc protein expression

To determine whether ribonucleotides that encode Thr50 of N-Myc affect N-Myc protein synthesis, we generated three silent N-Myc mutants (Fig. 3.7A) that produce identical protein products and examined their expression in several cell lines. Expression of N-Myc$^{T50T\ 'ACA'}$ and N-Myc$^{T50T\ 'ACT'}$ were generally lower than WT N-Myc (Fig. 3.7B, compare lane 2 to lanes 5 and 7). N-Myc$^{T50T\ 'ACC'}$ expression was lower than WT N-Myc in the mouse cell lines (C3H 10T½ and MEF), but not in the human cell line (HEK 293) (Fig. 3.7 A, compare lane 2 to 6). We also generated c-Myc mutants with silent mutations at Thr58 (Fig. 3.7A). Interestingly, the protein expression of the c-Myc^{T58T} silent mutants was not significantly different to WT c-Myc (Fig. 3.7C). The exception was c-Myc^{T58T}

'ACG', which was expressed slightly higher than WT c-Myc in C3H 10T½ cells (Fig. 3.7B, compare lane 2 to 6).

Next, we examined whether silent mutations at Thr50 affected N-Myc protein synthesis *in vitro*. Equal amounts of *in vitro* transcribed *N-myc* mRNA (WT, T50A, and T50T mutants) were translated in rabbit reticulocyte lysate (Fig. 3.8). All N-Myc^{T50T} silent mutants were translated at the same rate as WT N-Myc.

Mutations at the Thr50 codon of N-Myc may effect the translation of the protein by altering the RNA structure (240, 241) or codon usage (242). Using mFold (243), the mutations (T50A and T50T) were not predicted to alter RNA structure (Fig. 3.9). The differences in codon usage among the four Thr codons (Table 3.1) did not correlate with the difference in protein expression among the N-Myc^{T50T} mutants, suggesting that codon usage bias may not account for the differences in expression patterns of the N-Myc^{T50T} mutants.

In summary, these data show that mutation of the Thr50 codon of N-Myc, which preserves the protein sequence, affects expression the protein. This effect may be attributed to changes in translation of the mutant N-Myc protein that involve trans-acting factors within the cell.

3.3 DISCUSSION

We have previously shown that oncogenic Ras promotes both the synthesis and degradation of N-Myc protein. To further characterize the mechanisms by which Ras carries out these activities, we examined whether GSK3β is required for Ras-mediated N-Myc translation and degradation. In this study, we show that oncogenic Ras does not

require GSK3β to control N-Myc translation or degradation. Unexpectedly, we found that mutations in N-Myc at the Thr50 codon, which preserve the protein sequence, change the expression of N-Myc protein. These data suggest that the ribonucleotides encoding Thr50 of N-Myc may be involved in N-Myc translation.

Ras and GSK3β control N-Myc proteolysis through separate mechanisms

N-Myc and c-Myc contain several conserved domains, one of which harbors a consensus GSK3β site (244). GSK3β directs c-Myc for destruction by phosphorylating c-Myc at Thr58 (208, 245) to signal the protein for ubiquitin-mediated proteasomal degradation (137). The target residue within the consensus GSK3β site in N-Myc is Thr50. Several studies have shown an association between GSK3β activity and phosphorylation of N-Myc at Thr50 (137, 138, 246); however, phosphorylation of N-Myc at Thr50 by GSK3β has never been formally tested (e.g., by chymotryptic phosphopeptide mapping). As demonstrated with c-Myc, GSK3β is thought phosphorylate N-Myc Thr50 thereby signaling for its proteolysis; yet, to date, this has not been formally tested. We demonstrated that inactivation of GSK3β with LiCl increased N-Myc half-life. This indicates that, similar to c-Myc, GSK3β facilitates N-Myc turnover. GSK3β operates downstream of Ras signaling. Therefore we hypothesized that inactivation of GSK3β by LiCl should rescue the accelerated turnover of N-Myc caused by activated Ras; however, this was not the case. A possible explanation that accounts for these observations is that Ras controls N-Myc protein through two mechanisms: first, Ras destabilizes N-Myc in a GSK3β-independent manner and second, Ras stabilizes N-Myc through inhibition of GSK3β (Fig. 3.10). The destabilizing effect of Ras activity on N-

Myc must be dominant over its stabilizing effect, since net effect of Ras activity is to destabilize N-Myc. In summary, we propose that, in our system, N-Myc degradation proceeds by at least two Ras-dependent mechanisms: one that involves Ras/MAPK signaling and another that involves phosphorylation of Thr50 by GSK3β.

Ras regulates N-Myc translation independently of GSK3β

GSK3β phosphorylates several proteins that are involved in RNA processing and translation (233, 247–249). Most notably, GSK3β phosphorylates eIF2Bε and inhibits its activity (233). eIF2Bε is responsible for recycling eIF2 to an active state. eIF2 recruits initiator $tRNA^{Met}$ to the 40S ribosome and is therefore required for every translation initiation event (250). While inhibition of eIF2Bε by GSK3β should have broad effects on protein synthesis, polysome-bound mRNAs would theoretically be most affected since they undergo more initiating events. Accordingly, GSK3β activity would negatively effect translation of polysome-associated transcripts. We previously demonstrated that oncogenic Ras promotes N-Myc translation by associating *N-myc* mRNA with more ribosomes (Chapter 2). Results from this study indicate that Ras does not function through GSK3β to promote N-Myc translation. While the GSK3β inhibitor LiCl reduced N-Myc protein synthesis, it concomitantly reduced global protein synthesis. Inhibition of GSK3β with the dominant negative $GSK3\beta^{K85R}$ mutant did not affect N-Myc protein synthesis. It is important to note that these studies were conducted with ectopic N-Myc expressing only the coding region. It is possible that GSK3β regulates N-Myc translation in a manner that requires the N-Myc UTRs.

A mechanism for controlling N-Myc translation involving the Thr50 codon

Point mutations, including a case involving a silent mutation, have been shown to alter translation of the respective protein (251, 252). We demonstrated that mutation of the Thr50 codon in N-Myc, which preserves the protein sequence, affects expression the protein. This effect may be attributed to changes in the translation of the mutant N-Myc protein that involve trans-acting factors within the cell. Furthermore, our data suggest that oncogenic Ras controls N-Myc translation by targeting the Thr50 codon through a yet unidentified mechanism.

Our data supporting a translational mechanism that involves the Thr50 codon of N-Myc are preliminary and several important experiments are required to confirm this mechanism. First, translation rates of WT N-Myc, N-Myc^{T50A}, and the N-Myc^{T50T} silent mutants must be determine to evaluate how mutations at Thr50 affect N-Myc protein synthesis. In addition, the effect of Ras^{G12V} on the protein synthesis of N-Myc^{T50A} and the N-Myc^{T50T} silent mutants needs to be addressed. To control for the effects of proteasomal degradation, the metabolic labeling experiments should be performed in the presence and absence of the proteasomal inhibitor MG132. By using MG132 in these studies, we can examine how protein synthesis of N-Myc is affected under the specific condition. Second, we must determine if Ras^{G12V} or the T50T silent mutations themselves alters mRNA expression of the respective mutant transcript. If changes to mRNA expression are observed, then it will be important to determine if this is due to altered mRNA stability. *N-myc*^{T50A} mRNA levels are comparable to that of WT *N-myc* mRNA and are not altered by Ras^{G12V}. Thus, we hypothesize that *N-myc*^{T50T} mRNA levels would also be comparable to that WT *N-myc* mRNA and would not be altered by Ras^{G12V}. Lastly, if

altered mRNA stability does not account for the changes in protein expression observed with the N-Myc silent mutants, then it will be important to compare the polysome association of WT *N-myc* mRNA to those harboring T50A and T50T mutations. Since Ras^{G12V} redistributes WT *N-myc* mRNA to polysomes (Fig. 2.6), it will be important determine if mutations at Thr50 of N-Myc impair *N-myc* mRNA polysome association.

If the experiments outlined above support a translational mechanism involving Ras and the Thr50 codon, then this work identifies a critical region of N-Myc that functions at both the nucleic and amino acid level to control N-Myc translation and degradation, respectively. Regarding translation, the ribonucleotides that encoding Thr50 may serve as a binding site for a RBP or miRNA, and mutations at Thr50 may disrupt these interactions (253). Our data may also suggest that the putative RBP or miRNA is controlled by Ras/MAPK signaling. According to SiteSifter, a program that predicts putative miRNA binding sites based on sequence homology, there are no miRNAs that are predicted to target the ribonucleotide region encoding Thr50 (219). However, it must be experimental tested whether a RBP, miRNA, or both target *N-Myc mRNA*. Taken together, these data provide insight into a novel mechanism that controls N-Myc translation and may implicate Ras/MAPK signaling in this process.

3.4 FIGURES AND FIGURE LEGENDS

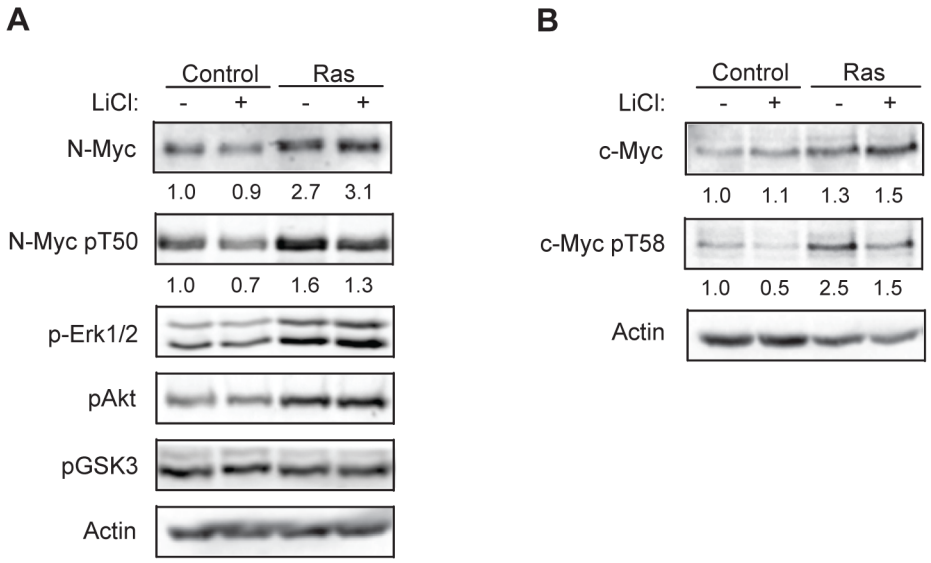

Figure 3.1 The effect of LiCl on N-Myc and c-Myc protein expression. A, C3H 10T½ cells stably expressing N-Myc alone or with Ras^{G12V} were treated with LiCl for one hour. Cell lysates were analyzed by immunoblotting for the expression of the indicated proteins B, C3H 10T½ cells stably expressing c-Myc alone or with Ras^{G12V} were treated as in (A) and lysates were analyzed by immunoblot analysis for the expression the indicated proteins.

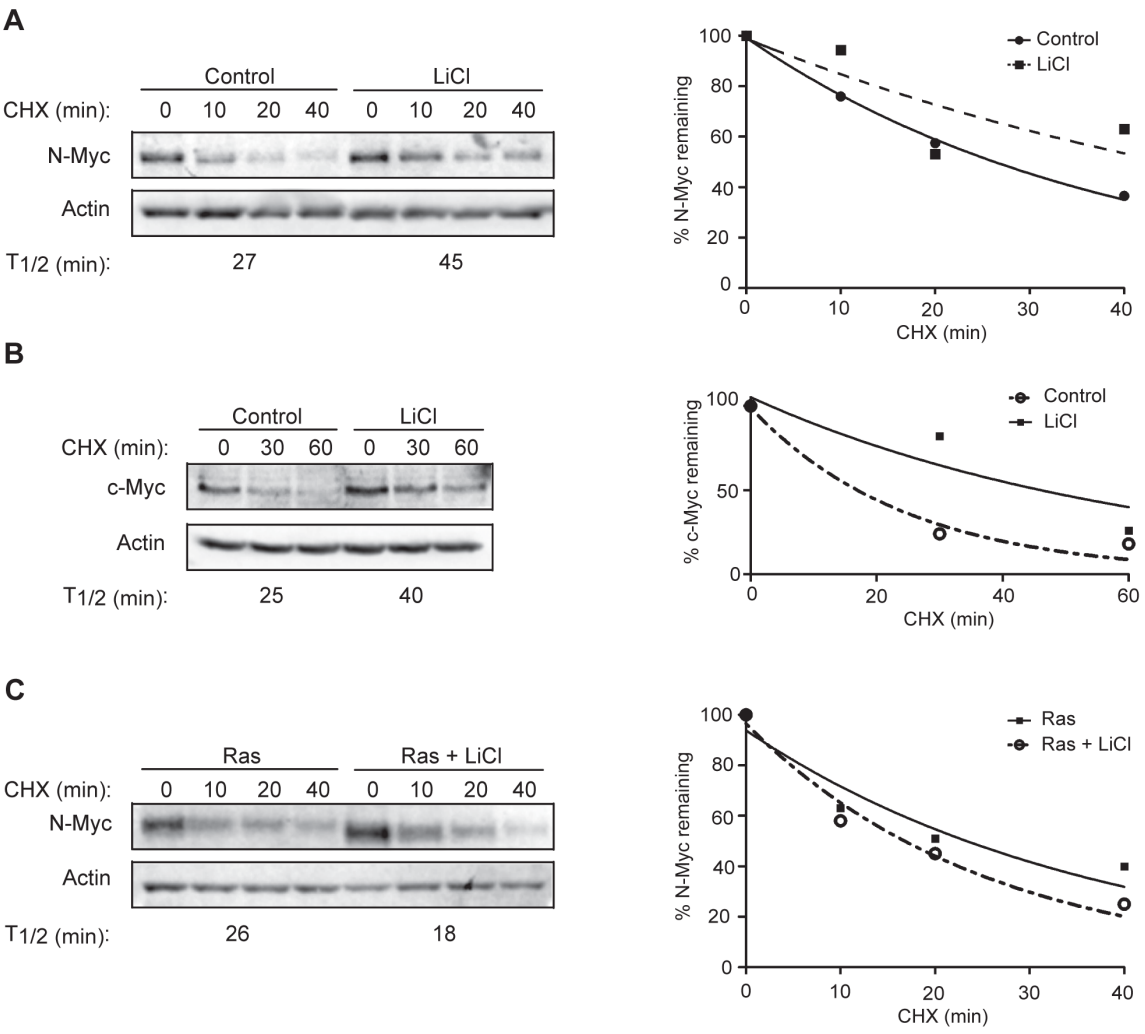

Figure 3.2. LiCl fails to stabilize N-Myc in the presence of oncogenic Ras. A, N-Myc-expressing C3H 10T½ cells were untreated (Control) or treated with LiCl for one hour then pulsed with CHX. Cell lysates were analyzed by immunoblotting for N-Myc and Actin expression. B, c-Myc-expressing C3H 10T½ cells were treated as in (A). Cell lysates were analyzed by immunoblotting for c-Myc and Actin expression. C, C3H 10T½ cells stably expressing N-Myc and Ras^{G12V} were treated as in (A). Half-lives are reported below the respective blots in (B) and (C). All data are representative of two independent experiments.

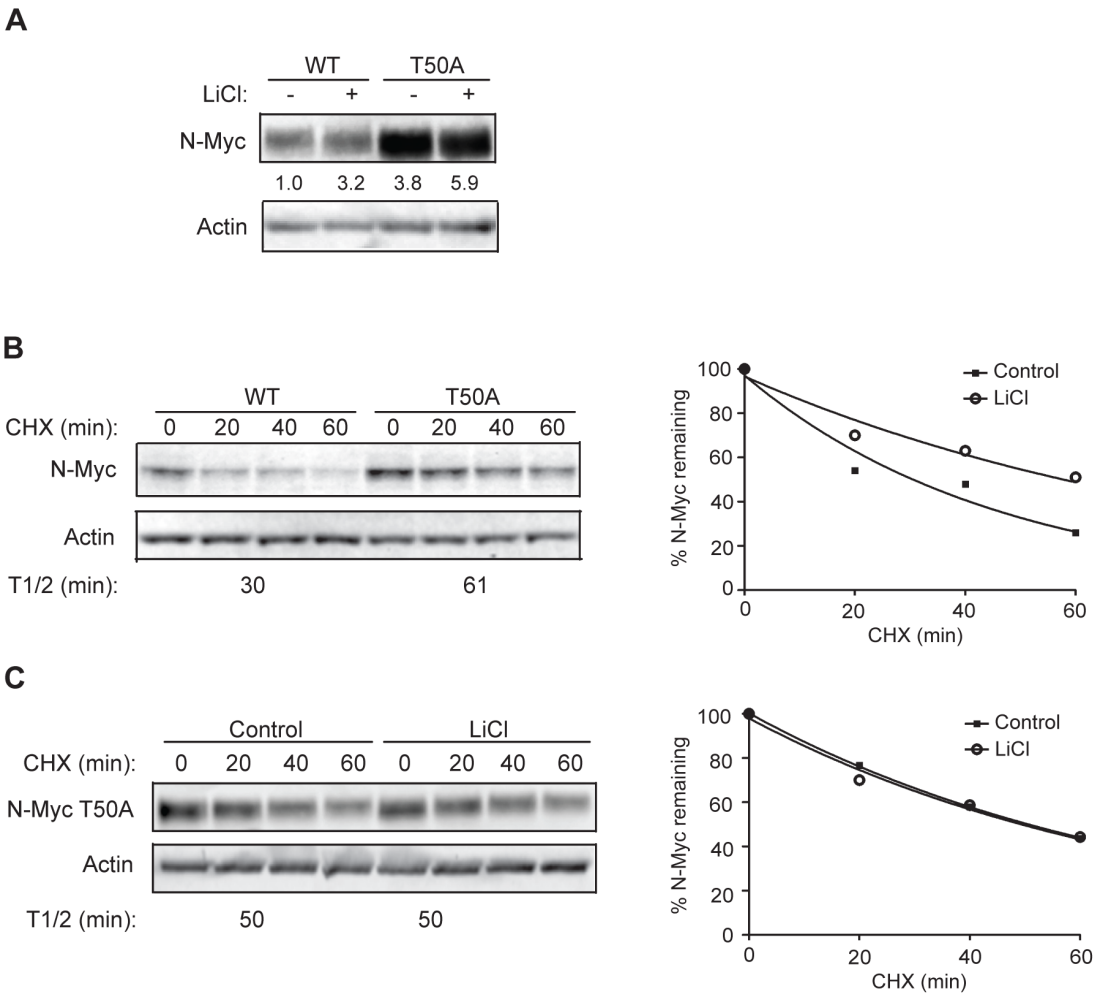

Figure 3.3 The effect of oncogenic Ras and LiCl on N-Myc^{T50A} stability. A, C3H 10T½ cells stably expressing WT N-Myc (WT) or N-Myc^{T50A} (T50A) were treated with LiCl for one hour. Cell lysates were analyzed by immunoblotting for N-Myc and Actin expression. B, C3H 10T½ cells described in (A) were pulsed with CHX for the indicated times. Cell lysates were analyzed by immunoblot analysis for N-Myc and Actin expression. C, N-Myc^{T50A}-expressing C3H 10T½ cells were treated with LiCl or H_2O (vehicle control) for one hour then pulsed with CHX. Cell lysates were analyzed by immunoblotting for N-Myc and Actin expression. Half-lives are reported below the respective blot. All data are representative of two independent experiments.

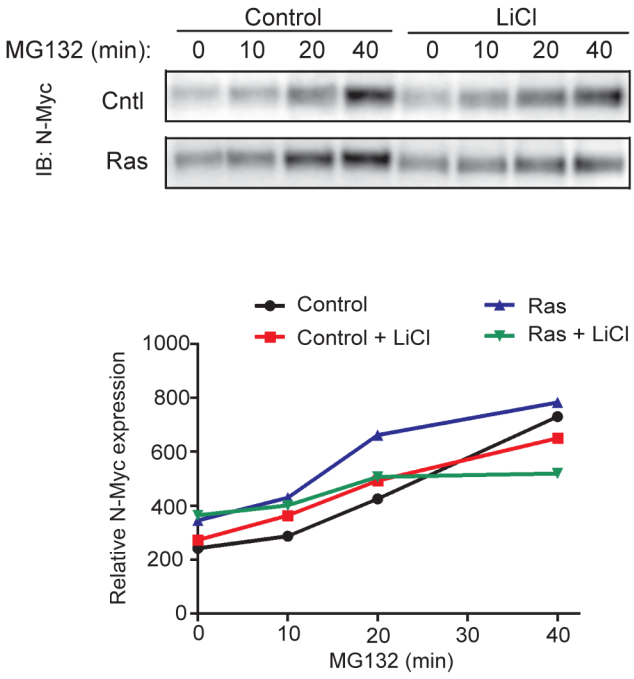

Figure 3.4 LiCl inhibits Ras-induced accumulation of N-Myc. C3H 10T½ cells stably expressing WT N-Myc (Cntl) or Ras^{G12V} were untreated (Control) or treated with LiCl for one hour then treated with MG132 for the indicated times. Cell lysates were analyzed by immunoblotting (IB) for N-Myc. Quantification of protein expression is shown below the blots. Data are representative of two independent experiments.

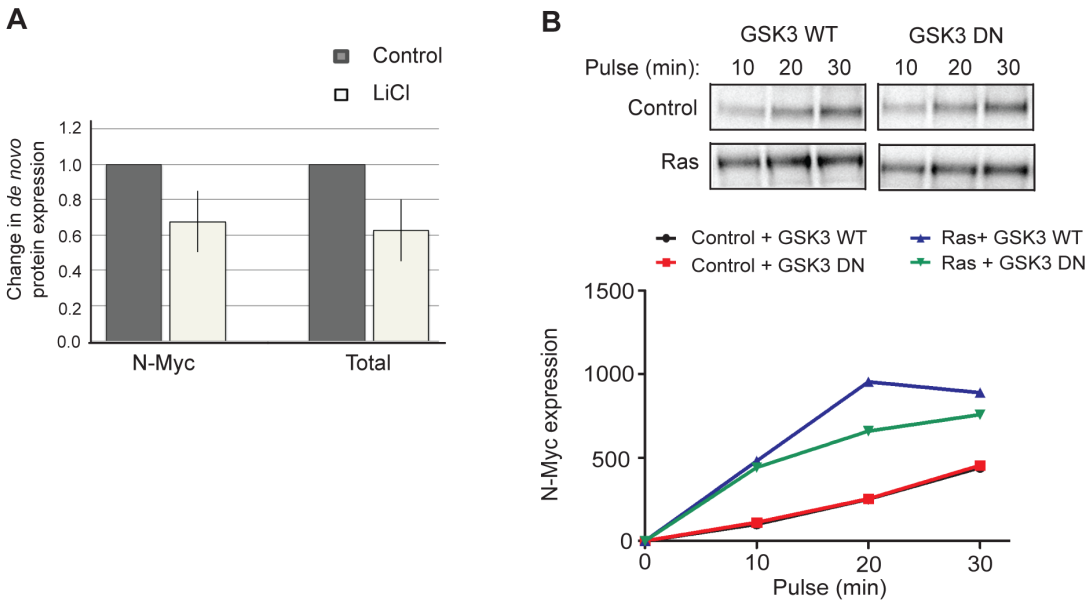

Figure 3.5 GSK3β does not affect N-Myc protein synthesis. A, C3H 10T½ cells transfected with N-Myc and Ras^{G12V} were untreated (Control) or treated with LiCl for one hour then pulsed with ^{35}S-methionine for 20 minutes. Radiolabeled N-Myc was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. Total protein synthesis was measured as described in the Materials and Methods section. The graph shows mean ± S.D. (n = 3) change in expression of *de novo* N-Myc protein (left) and total protein (right) relative to Controls, which are set to 1. B, C3H 10T½ cells transfected with N-Myc, control vector or Ras^{G12V}, and WT GSK3β or dominant negative (DN) GSK3β were pulsed with ^{35}S-methionine for 10, 20, or 30 minutes. Radiolabeled N-Myc analyzed as in (A). Quantification of blots is shown below. A representative image of two independent experiments is shown.

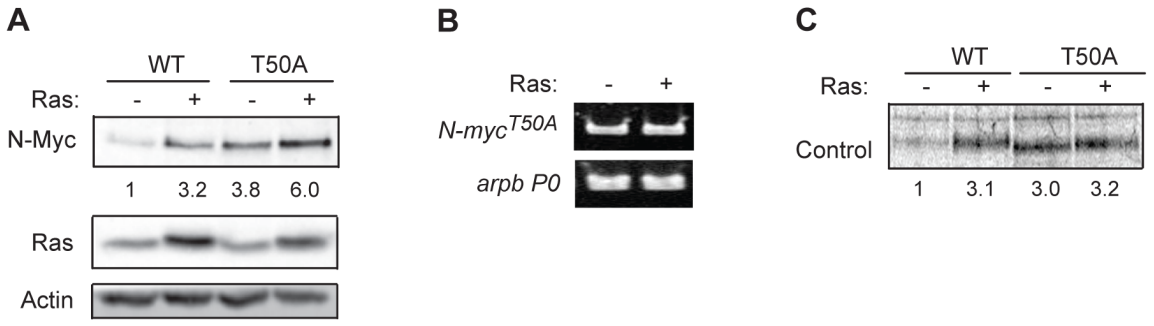

Figure 3.6 Oncogenic Ras increases the protein synthesis of WT N-Myc, but not N-Myc^{T50A}. A, C3H 10T½ cells stably expressing WT N-Myc (WT) or N-Myc^{T50A} (T50A) alone or with Ras^{G12V} were analyzed by immunoblot analysis for expression of the indicated proteins. B, relative transcripts levels of *N-myc^{T50A}* and *arpb P0* (loading control) were assessed by RT-PCR in the cell lines described in (A). C, C3H 10T½ cells transfected with WT N-Myc or N-Myc^{T50A} and control vector or Ras^{G12V} were pulsed with ^{35}S-methionine for 20 minutes. Radiolabeled N-Myc was immunoprecipitated from equal concentrations of total protein and analyzed by SDS-PAGE. Quantifications are shown below the image.

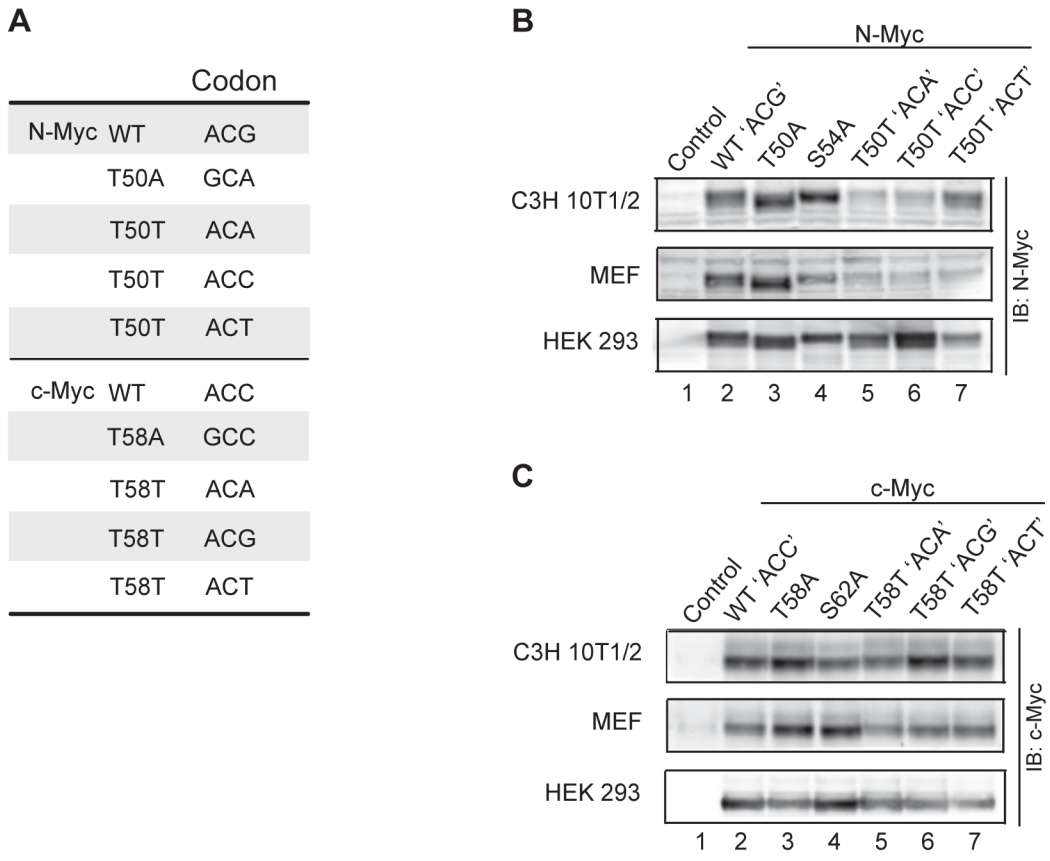

Figure 3.7 Characterization of N-Myc^{T50T} and c-Myc^{T58T} silent mutants. A, a summary of Thr codon usage for WT and mutant N-Myc and c-Myc constructs. B, C3H 10T½ cells (top panel), MEF cells (middle panel), and HEK 293 cells (bottom panel) were transfected with control vector, WT N-Myc, or various N-Myc mutants. N-Myc expression levels were assessed by immunoblot analysis. B, the cell types described in (A) were transfected with control vector, WT c-Myc, or various c-Myc mutants. c-Myc expression levels were assessed by immunoblot analysis. Data shown are representative of two independent experiments.

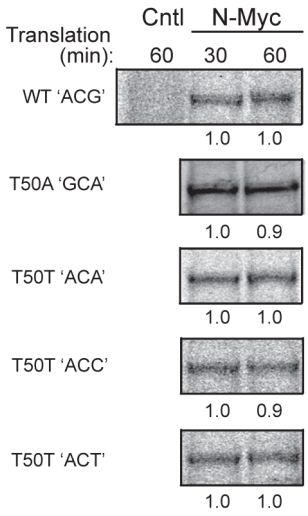

Figure 3.8 Silent mutation of Thr50 does not affect *in vitro* protein synthesis of N-Myc. *In vitro* transcribed mRNAs were translated in the presence of ^{35}S-methionine in nuclease-treated rabbit reticulocyte lysate at 30° C for 30 or 60 minutes. A reaction with no mRNA served as the negative control (Cntl). Translation reactions subjected to SDS-PAGE and visualized on a phosphor screen. Quantifications are shown below each blot.

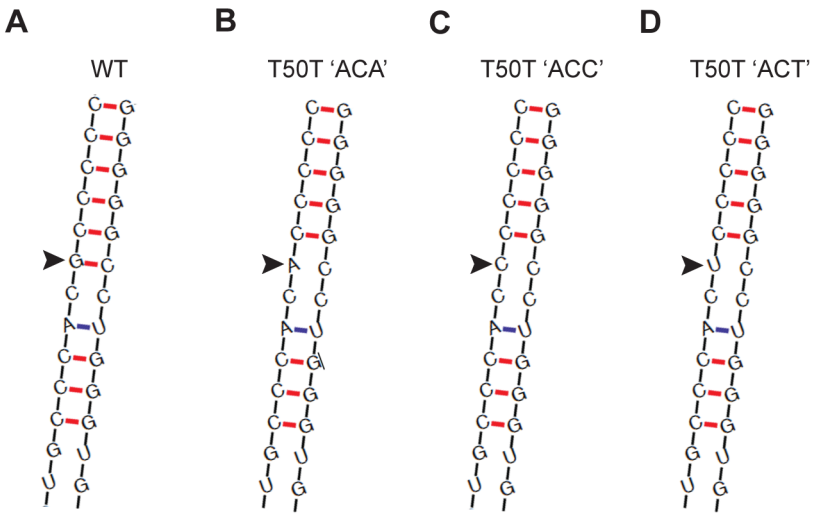

Figure 3.9. Predicted mRNA structure of the local region encompassing the Thr50 codon for WT and mutant N-Myc. The local secondary structure encompassing the Thr50 codon was predicted with the Mfold software using the default settings. Arrowhead indicates the third position of the Thr50 codon.

Table 3.1. Codon usage for all Thr codons and one Ala codon in mice and humans. Codon usage is defined as the frequency (per thousand codons) of codon occurrence from protein sequences maintained in GenBank and is reported as the codon adaptation index (254).

			Codon Adaptation Index*	
		Codon 50	Mus	Homo
N-Myc	WT	ACG	5.6	6.1
	T50A	GCA	15.8	15.8
	T50T	ACA	16.0	15.1
	T50T	ACC	19.0	18.9
	T50T	ACT	13.7	13.1

* codon frequency/thousand codons

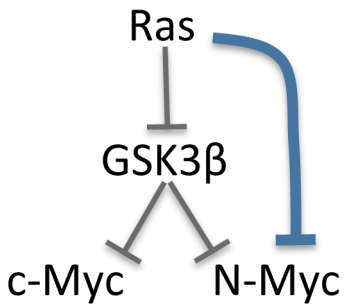

Figure 3.10. A model for the regulation of N-Myc proteolysis by Ras. Ras activity controls N-Myc through two mechanisms – one that stabilizes N-Myc and another that destabilizes N-Myc. Ras stabilizes N-Myc (and c-Myc) through inhibition of GSK3β, thereby preventing phosphorylation of N-Myc at Thr50 (or c-Myc at Thr58) and subsequent proteolysis (gray lines) (136,141). Ras activity also destabilizes N-Myc through a GSK3β- and Thr50-independent mechanism (blue, bold line). Since the stabilizing effect of Ras on N-Myc is not experimentally observed, suggesting that the destabilizing effect of Ras is dominant.

3.5 EXPERIMENTAL PROCEDURES

Cell culture and inhibitor assays—C3H 10T½ cells (ATCC) and Ecopack 2 293 cells (Clontech) were maintained in DMEM (Invitrogen). All cell lines were supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin. For inhibitor assays, cells were treated with the following compounds (Sigma): LiCl (20 mM), cycloheximide (100 μg/ml) and MG132 (10 μM).

Expression plasmids—The following plasmids have been previously described (224): pBABEpuro-c-Myc and pBABEpuro-H-Ras^{G12V}. For pBABEpuro-N-Myc, the mouse N-Myc cDNA (coding sequence only) was cloned into BamHI and EcoRV sites of pBABEpuro. pBABEpuro-N-Myc^{T50A} was generated from the pBABEpuro-N-Myc plasmid using the Change-IT Multiple Mutation Site Direct Mutagenesis Kit (USB). To construct pcDNA6-N-Myc, N-Myc was amplified from pBABEpuro-N-Myc with primers that added NotI sites, and cloned into the NotI site of pBABEpuro. Mutant N-Myc constructs (T50A, S54A, T50T 'ACA', T50T 'ACC', and T50T 'ACT') were generated from the pcDNA-N-Myc construct using the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (USB). pcDNA6-c-Myc was constructed by digesting pBABEpuro-c-Myc with BamHI and SalI to isolate human c-Myc cDNA, and the fragment was ligated into pcDNA6, which was linearized with BamHI and XhoI. Mutant c-Myc constructs (T58A, S62A, T58T 'ACA', T58T 'ACG', and T58T 'ACT') were generated from the pcDNA-c-Myc construct using the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (USB). The pcDNA6-H-Ras^{G12V} plasmid was constructed by digesting H-Ras^{G12V} with BamHI and EcoRI sites from pBABEpuro-H-Ras^{G12V} and inserting the fragment into pcDNA6. pcDNA6-GSK3β-DN-V5/His$_{6x}$ was generated by

amplifying a catalytically inactive mutant GSK3β protein, K85R, from pGSK3myc-pXT1 (a gift from Dr. Sokol, Harvard Medical School, Boston, MA (239)) with primers that removed the myc tag and stop codon and added KpnI and NotI restriction sites for ligation into pcDNA6. pcDNA6-GSK3β-WT-V5/His_{6x} was generated by mutating pcDNA6-GSK3β-DN-V5/His_{6x} with the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (USB).

Retroviral infections and transfections—The establishment of stable cell lines was performed as previously described (224). For stable cell lines expressing more than one ectopic gene, cells were infected with a second virus 24 hours after the initial infection. Cells were selected with puromycin (1 mg/ml), geneticin (1 mg/ml), or both after all rounds of infection. Transient transfections were performed with Lipofectamine 2000 (Invitrogen), according the manufacture's protocol.

Immunoblot Analysis—Cells were lysed with RIPA buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 0.1% SDS, 1.0% NP-40, 0.5% sodium deoxycholate) containing 1x COMPLETE Protease Inhibitor cocktail (Roche). Equal amounts of protein were separated on 4-12% Novex Bis-Tris acrylamide gels (Invitrogen) and Western Blot analysis was done as previously described (224) using the following antibodies: N-Myc (C-19), c-Myc (9E10), and Phospho-Erk1/2 (E-4) from Santa Cruz; Ras (Ras10) from Upstate Biotechnology; Actin (Ac-40) from Sigma; Phospho-Akt (S473), Phospho-GSK-3β (S9), and Phospho-c-Myc (T58/S62) from Cell Signaling Technology.

RNA Isolation and semi-quantitative RT-PCR—Total RNA was isolated using TriZol (Invitrogen) and reverse transcribed from 1 μg of RNA using SuperScript III first-strand cDNA synthesis kit (Invitrogen). The resulting material was amplified by PCR using

primers that recognize mouse-specific N-Myc or Arbp P0 (loading control) cDNA. The number of PCR cycles used was empirically determined to be within the linear range of N-Myc amplification. Equivalent volumes of each PCR reaction was run through 1.2% TBE agarose gels containing ethidium bromide and photographed under UV illumination.

In vivo Metabolic labeling—Cells labeling was performed 24 hours after transfection. Cells were starved of L-methionine and L-cysteine by incubation for 15 minutes in L-methionine/L-cysteine-free D-MEM (Invitrogen) supplemented with 10% dialyzed fetal bovine serum and L-glutamine (20 mM). Following starvation, cells were labeled with 100 μCi/mL of ^{35}S-methionine/cysteine (MP Bio) at 37° C for the indicated times. Cells were washed twice with cold PBS and lysed with RIPA buffer containing protease inhibitors. Lysates were incubated at 4° C overnight with 1 μg of N-Myc antibody (NCM100-II, Santa Cruz), and then an additional 2 hours with G plus-agarose beads. Immune complexes were separated through 4-12% acrylamide gels (Invitrogen) and visualized using autoradiography. Quantification of immunoprecipitated proteins was performed by densitometry using ImageQuant software (GE Healthcare). Background values were calculated from an equivalent area in each lane and subtracted from the signal value for the labeled protein.

In vitro Transcription and Translation—*N-myc* mRNA was transcribed from 1 μg of linearized pcDNA6 N-Myc (WT or indicated mutation) with T7 RNA polymerase (Invitrogen) according to manufacture's protocol. N-Myc protein was synthesized using the Rabbit Reticulocyte Lysate (RRL) System (Promega) according to manufacture's instructions. Briefly, 1 μg of non-denatured *N-myc* mRNA was combined with RRL, amino acid mixture (-methionine), 10 μCi ^{35}S-methionine, and RNase Inhibitor

(Invitrogen) and incubated at 37° C for 30 or 60. Equal aliquots of reactions were combined with SDS sample buffer, subjected to SDS-PAGE, and ^{35}S-methionine-labeled N-Myc protein was visualized using a low-intensity phosphor screen. Quantification of protein expression was performed by densitometry using ImageQuant software (GE Healthcare).

RNA Structure Prediction—The Mfold software (243) was used to predict the RNA structure of N-Myc using the first 800 nucleotides (starting at ATG start site) obtained from NCBI (NCBI Accession no. AAH49783). The default parameters were used without constraints.

CHAPTER 4 | Discussion and Conclusions

4.1 SUMMARY

N-Myc serves critical functions during embryogenesis, particularly in the development of the murine nervous system, hematopoietic system, limbs, and lungs (68, 64, 69, 255). *MYCN* haploinsufficiency in humans causes Feingold Syndrome, a disorder in which patients exhibit cognitive disabilities and have heart, limb, duodenum, and esophageal defects (5). In contrast, overexpression of N-Myc is implicated in the formation of several pediatric cancers, including neuroblastoma and medulloblastoma (105, 199). Thus, understanding the mechanisms that control N-Myc expression and activity is critical to address N-Myc-related disorders.

N-Myc is regulated at the transcriptional and post-transcriptional levels by several signaling pathways (8–13). The Ras/MAPK has been implicated in the post-transcriptional regulation of N-Myc; however the mechanism by which this occurs is not well understood. The purpose of this thesis was to determine how Ras signaling controls N-Myc expression at the post-transcriptional level. Using several cell lines to model normal and tumorigenic cellular states, I have demonstrated that oncogenic Ras concomitantly accelerates the synthesis and degradation of N-Myc protein. The translational upregulation exceeds the destabilizing effect that oncogenic Ras has on N-Myc, such that higher levels of N-Myc protein are the net result. Ras-dependent upregulation of N-Myc translation is achieved by associating *N-myc* mRNA with actively translating polysomes (Fig. 4.1). GSK3β, a kinase whose activity is negatively regulated by Ras signaling, has been shown to alter N-Myc expression (137, 138, 246). Our studies

indicate that oncogenic Ras does not require GSK3β to control the translation or degradation of N-Myc. Instead, we uncovered a potential role for the Thr50 codon of N-Myc in controlling the translational of N-Myc.

The finding that oncogenic Ras promoted N-Myc turnover was unexpected since oncogenic Ras is thought stabilize N-Myc by a similar mechanism that has been delineated for c-Myc. Ras modulates the phosphorylation status of two key residues within c-Myc – Thr58 and Ser62 – to stabilize the protein. These residues are perfectly conserved in N-Myc, the equivalent sites being Thr50 and Ser54. Because I found that oncogenic Ras does not stabilize, but rather destabilizes N-Myc, I performed parallel studies for N-Myc and c-Myc to determine if Ras signaling controls N-Myc and c-Myc by similar or different mechanisms. In contrast to my observations with N-Myc, oncogenic Ras stabilized c-Myc protein and did not effect the translation of c-Myc. I also examined whether Thr50 and Ser54 of N-Myc were involved in N-Myc degradation and found that Thr50 and Ser54 of N-Myc serve the same proteolytic role as Thr58 and Ser62 of c-Myc. These studies did not rule out the possibility that Ras controls phosphorylation of N-Myc at Thr58 and Ser54 resulting in stabilization of N-Myc. Interestingly we found that oncogenic Ras destabilizes N-Myc in a manner that does not require phosphorylation of N-Myc at Thr50. These studies suggest that oncogenic Ras has both stabilizing and destabilizing effects on N-Myc (Fig. 4.1). Furthermore, I showed that Ras-mediated proteolysis of N-Myc is associated with an increase in N-Myc transcriptional activity. This provides an explanation as to how Ras-mediated proteolysis of N-Myc promotes the oncogenic activity of both Ras and N-Myc.

In summary, the overall effect of Ras signaling on N-Myc and c-Myc is similar (*i.e.* increased protein expression and transcriptional activity); however, these effects are achieved by very different mechanisms (Fig. 4.1). Taken together, these studies provide mechanistic insight into how oncogenic Ras augments the expression and activity of N-Myc through enhanced translation and degradation-coupled transactivation.

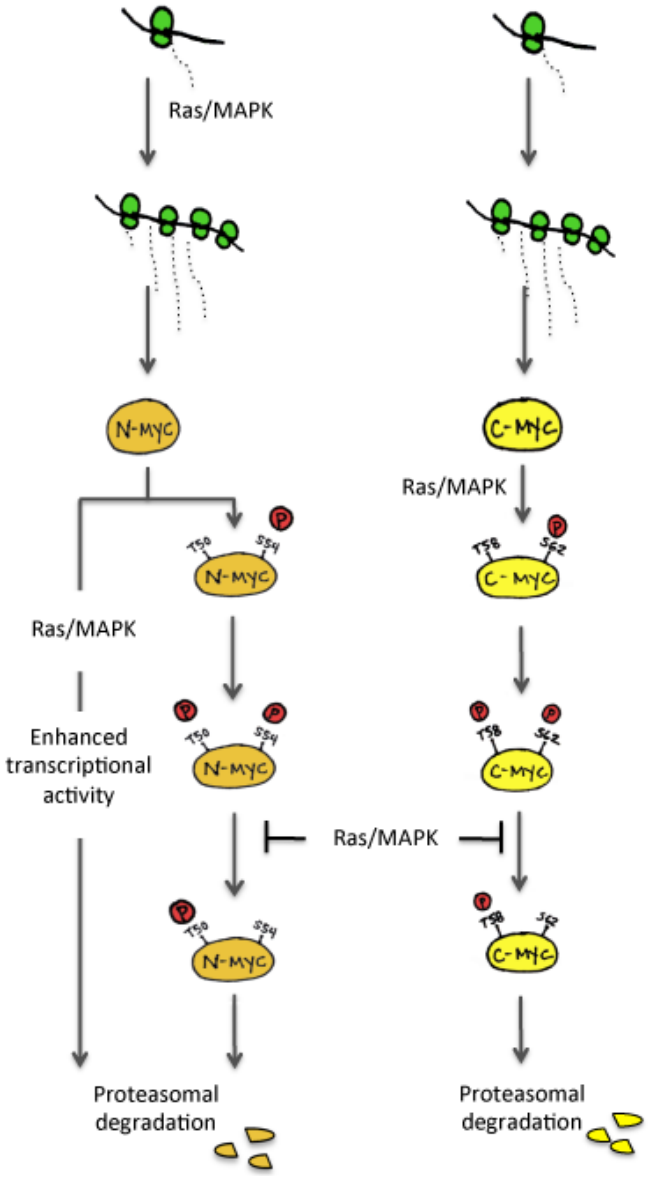

Figure 4.1 A model summarizing the findings. We propose that Ras signaling controls N-Myc expression through multiple mechanisms involving increased translation (promotes polysome association), enhanced stability (right arm), and accelerated proteolysis (left arm). Ras-mediated proteolysis of N-Myc may enhance the transcriptional activity of N-Myc. In contrast, Ras signaling does not promote c-Myc translation, but promotes stabilization of c-Myc protein by promoting phosphorylation at Ser62 and inhibiting GSK3β-mediate phosphorylation at Thr58, subsequently slowing c-Myc proteolysis.

4.2 DISCUSSION

Experimental approach to understanding the effect of Ras signaling on N-Myc

The system in which we used to characterize the effects of Ras signaling on N-Myc expression involved stable expression of a constitutively active form of Ras, Ras^{G12V}, in C3H 10T½ cells. Stable cell lines were established by retroviral infection, theoretically resulting in the expression of one gene copy per cell, and pooled cells were subjected to drug selection. Selected cells were stored in aliquots so that we could perform our studies with the original batch of established cell lines. This system is advantageous for maintaining consistent expression levels of Ras^{G12V} across experiments. Since we wanted to study post-transcriptional regulation of N-Myc, we used C3H 10T½ cells that do not express endogenous N-Myc. Continual passaging of cells overexpressing a potent oncogene like Ras^{G12V} may result in selection of a population of cells that exhibit a growth advantage in tissue culture conditions. This is scenario is especially likely given that we are using a heterologous population of stable cells. Therefore a potential caveat of this system is that we are characterizing artifactual effects of Ras^{G12V} on N-Myc due to the tissue culture conditions. To control for this possibility, we limited the number of passages for cells used in our experiments and went back to our original stock of stable cell lines when this limit was reached. In addition, our results regarding N-Myc translation using the stable cell lines corroborated with results using transient expression of Ras^{G12V} in C3H 10T½ cells (Fig. 2.4A, B), indicating that our findings are reflective of the effect of Ras^{G12V} itself, and not the nature in which it is expressed.

Our studies on the effect of FGF signaling on N-Myc expression (Chapter 2) indicated that the Ras/MAPK pathway was involved. Subsequent experiments therefore

used cells stably expressing Ras^{G12V} to focus on the Ras/MAPK pathway in regulating N-Myc expression. In a physiological setting, however, FGF signaling has built in negative feedback mechanisms to limit the signal duration and keep the pathway responsive to stimuli (256). Therefore, our studies using constitutive activation of Ras may not be completely relevant to physiological settings. Some activating mutations in Ras are associated or cause certain developmental disorders (257), indicating that constitutive Ras/MAPK signaling is not permissive during development. Our study using transient expression of Ras^{G12V} (Fig. 2.4B) is more relevant to a physiological setting with regards of FGF signaling. Since we found that activated Ras promoted N-Myc protein synthesis when being expressed stably and transiently, we reasoned that our studies using stable expression of Ras might have some relevance to FGF signaling in a physiological setting. Future studies that perform similar studies conducted in this thesis in response to transiently expressed Ras^{G12V} or FGF2 stimulation would clarify the effect of FGF and Ras/MAPK signaling on N-Myc expression in a physiological setting.

Control of N-Myc translation

Translation is an energetically demanding process. Not surprisingly, cells have evolved elaborate mechanisms to control the precise amount of protein synthesized. Ras signaling controls the translation of numerous transcripts (169, 175, 183, 216), and my studies show that *N-myc*, but not *c-myc* or *Cyclin D1*, mRNA is one of those targets. While oncogenic Ras increased the amount of total RNA in the polysome fractions, this was not associated with an increase in global translation. This suggests that oncogenic Ras modulates the polysome association, and therefore the translation rate, of specific

mRNAs. The translational studies conducted with C3H 10T½ cells expressed a form of N-Myc that contained the coding region of N-Myc and lacked both UTRs. This indicates that Ras signaling targets elements within the coding region of *N-myc* mRNA to promote N-Myc translation. It is possible that, by characterizing an N-Myc construct lacking its endogenous UTRs, I have inadvertently disabled a translational mechanism that requires one or both of them. However, oncogenic Ras upregulated the translation of endogenous *MYCN* mRNA in human neuroblastoma cells, further validating that Ras upregulates N-Myc translation by targeting elements within the N-Myc coding region. Translational regulation of transcripts often involve their UTRs (258), but there is a growing number of examples of RBPs and miRNAs that target the coding regions of transcripts (217–219).

Toward identifying an element within *N-myc* mRNA that is targeted by Ras signaling, I have provided preliminary evidence that suggests the Thr50 codon of N-Myc is involved in Ras-mediated translation of N-Myc; however, several factors make this mechanism highly speculative. First, the mechanism by which Ras mediates N-Myc translation is not applicable to c-Myc. A putative translational mechanism would, presumably, require elements within N-Myc that are distinct from c-Myc. The nucleotide region immediately surrounding the N-Myc Thr50 codon (i.e. five nucleotides upstream and downstream) differs by two nucleotides (~85% homology) to the equivalent region in c-Myc. The lack of nucleotide variation reduces the possibility that this region in N-Myc, but not c-Myc, is subject to translational control. However, single nucleotide polymorphisms have been shown to disrupt binding of a RBP (251, 252), indicating that single nucleotide changes are sufficient to alter binding affinity of RBPs. Second, my proposed translational mechanism requires recognition of a miRNA (or RBP) in the

coding region. Binding sites for miRNAs commonly reside within UTRs, and less frequently, within coding regions (220). Third, silent mutation of N-Myc at Thr50 alters protein expression, but does not alter the predicted mRNA secondary structure of *N-myc* mRNA. RNA secondary structure may play an important role in defining RBP binding sites. This may indicate that the nucleotide region encompassing the Thr50 codon is not RBP binding site. Some RBPs can, however, recognize unstructured mRNAs (259). Lastly, despite a thorough search of the literature, I was unable to identify any experimentally validated/predicted consensus RBP or miRNA binding sites within N-Myc that surround the Thr50 codon. Nevertheless, future studies are required to determine whether the Thr50 codon is involved in Ras-mediated N-Myc translation. These future studies are briefly outlined below and were described in Chapter 3.

Control of N-Myc degradation

The metabolic instability of N-Myc has been known for many years (260), but the molecular mechanism(s) that control N-Myc proteolysis are largely unknown. Data provided within this dissertation suggest that Ras signaling can both slow and accelerate the proteasomal degradation of N-Myc by mechanisms that either involve phosphorylation at Thr50 and Ser54 or operate independently of those phosphorylation sites, respectively (Fig. 4.1). Our results show that the destabilizing effect of Ras signaling on N-Myc overrides the stabilizing effect of Ras.

My observations regarding the effect of oncogenic Ras on N-Myc turnover may have been overlooked because c-Myc has typically served as the prototype for understanding the biochemical attributes of the Myc family members. This is particularly true with

regard to the Myc degradation model (Fig. 1.4) since it involves two critical residues that are conserved between N-Myc and c-Myc. In agreement with my findings, several other studies demonstrated that the Myc degradation model is not completely generalizable to N-Myc (9, 32). Furthermore, several proteins have different effects on N-Myc and c-Myc proteolysis. For example, the E3 ubiquitin ligase HuweI affects N-Myc degradation, but does not affect c-Myc degradation (215, 261). One possibility is that Ras/MAPK signaling regulates Huwe1 or another protein involved in proteasomal degradation of N-Myc. This study reinforces the notion that the biochemical attributes of c-Myc, at least regarding proteolysis and translation, are not generalizable to all Myc relatives.

Myc proteins are highly dynamic molecules that interact with more than 40 proteins (34) and localize to different subcellular domains (262, 263). These activities create different populations of Myc within the cell and it has been shown that different populations of Myc are targeted for proteasomal degradation at varying efficiencies (37). As discussed in greater detail in the Discussion of Chapter 2, our protein synthesis studies using the stable cell lines suggested that there is more than one population of N-Myc protein subjected to different rates of degradation (Fig. 2.4A). Specifically, these studies suggested that there are two populations of N-Myc in the presence of oncogenic Ras, a newly synthesized population and a pre-existing population of N-Myc, and predicted that the pre-existing population was degraded at a faster rate. Our half-life studies, however, did not support the notion that the pre-existing population of N-Myc was degraded at a faster rate. An explanation for this conflicting data is that the notion that there are two populations of N-Myc is based on an artifactual result. Additional studies that examined the protein synthesis rate of transiently expressed N-Myc suggested that there was one

population, not two populations, of N-Myc in the presence of oncogenic Ras. The metabolic labeling and cycloheximide assays used to determine the protein synthesis and degradation rates of N-Myc are highly variable in nature. To definitively address whether there is one or multiple populations of N-Myc with varying rates of proteolysis, these assays need to be repeated multiple times to derive accurate and statistically significant results.

Implications of correlating the activity and Ras-mediated proteolysis of N-Myc

Our finding that one oncoprotein (Ras^{G12V}) reduces the stability of another (N-Myc) is difficult to reconcile given that they cooperate in cellular transformation (195). However, our finding that inhibition of Ras-mediated N-Myc degradation corresponds to a reduction in N-Myc transcriptional activity is consistent with their oncogenic properties. The idea that activity of an unstable transcription factor is coupled to its proteasomal degradation has gained considerable support (143, 264, 209, 265) and may be a general phenomenon for unstable transcription factors (264). Associating proteolysis with activity may serve as a 'fail-safe' mechanism to limit the activity of potent transcription factors or may prevent accumulation of excess activators that would sequester the basal transcriptional machinery.

Many transcription factors contain overlapping TADs and degrons (47). This overlap is thought to facilitate the coupling of transcription and proteasome-mediated degradation. Consistent with my observations, the transcriptional activity of c-Myc appears to be linked to its proteolysis (43–45). The overlapping TAD and degrons in c-Myc are confined to residues 1-146 and 1-143, respectively, which include the conserved

MBI-IV domains (264). Defined regions within first 146 residues of c-Myc were found to be necessary for its transcriptional activity (46) and proteolysis [(43, 47, 48) and depicted in Fig. 1.2]. The TADs and degrons within N-Myc are not well characterized. A TAD may be located within the first 73 amino acids of N-Myc, since this region is required for transcriptional activation of N-Myc (49). To date, only Thr50 and Ser54 (Thr58 and Ser62 in the human homolog) of N-Myc are implicated in N-Myc turnover (112, 139, 261). Since Ras-mediated proteolysis of N-Myc operates independently of Thr50, this suggests that N-Myc contains additional degrons. Future studies that identify elements within N-Myc that are required for its transcriptional activity and Ras-mediated proteolysis will provide insight into how this process is mediated by Ras and why it is specific to N-Myc, and not c-Myc.

A limitation of our studies that correlate Ras-mediated proteolysis and enhanced transcriptional activity of N-Myc is that they do not provide insight to a mechanism by which this phenomenon occurs. Prior to this study, N-Myc transcriptional activity has not been correlated to its destruction. Ras signaling, as well, has not been implicated in enhancing the activity of a transcription factor through modulation of the factor's proteolysis. As discussing in Chapter 1, I described several mechanisms by which ubiquitination and proteolysis of Myc could promote its transcriptional activity. Briefly, these mechanisms involve promoter recruitment of the RNA polymerase II, disassembly of the pre-initiation complex to release RNA polymerase II, and/or remodeling of chromatin structure. Activation of Erk, which occurs downstream of Ras signaling, has been shown to induce transcription of the mouse mammary tumor virus transgene by promoting recruitment of basal transcription factors and RNA polymerase II (266). The

involvement of Myc in this study was not examined, but one possibility is that Ras signaling would require Myc-mediated recruitment of UPS components to recruit RNA polymerase II. Another possibility is that Ras signaling controls UPS components that would be recruited to active promoters by Myc. The E3 ligase Huwe1, which directs N-Myc for proteolysis, is predicted to be a MAPK substrate by several prediction algorithms. Upon activation by Ras signaling, HuweI could target promoter-bound N-Myc to direct N-Myc ubiquitination and proteolysis. Recruitment of HuweI to the promoter could promote ubiquitination and degradations of additional promoter-associated proteins to facilitate transcription. As noted in the previous section, N-Myc is a stronger target than c-Myc for Huwe1-mediated proteolysis (215). If Ras signaling regulates the proteolytic functions of Huwe1, this would provide an explanation as to why Ras-mediated proteolysis and enhanced transcriptional activity is specific to N-Myc and not c-Myc. Future studies that define the mechanism by which Ras signaling promotes N-Myc degradation (as outlined in Section 4.3 of this chapter) is required to determine the how Ras signaling is controlling N-Myc's transcriptional activity.

Finally it is important to note that several therapeutic strategies aim to exploit Myc degradation pathways to target Myc in cancer (267). Findings from this study, as well as others, suggest that targeting N-Myc or c-Myc for degradation may not substantiate a therapeutic advantage, for these strategies may inadvertently elevate Myc's transcriptional activity, and thus, its oncogenic activity.

Implications for unique functions of N-Myc and c-Myc

N-Myc and c-Myc are thought to be partially redundant (21). But, as described in the Introduction, they also have unique functions (2, 53, 54). Indeed, gene-replacement mouse studies in which the coding and intervening intron sequences of *c-myc* were replaced by those of *N-myc* suggest that N-Myc does not fully compensate for the activates of c-Myc (54). These unique functions may, in part, contribute to their reciprocal patterns of expression (55) since one can negatively regulate the other (56). Regulatory mechanisms that establish predominance of one Myc over the other may be important for proper development and homeostasis. These studies raise the possibility that Ras/MAPK signaling assists in establishing the predominance of one Myc over the other through differential effects on N-Myc and c-Myc protein synthesis, proteolysis, and activity.

Implications for embryonic development

N-Myc is involved in the development of diverse tissues and organs during embryogenesis, where it has critical roles in cellular expansion and fate determination of various stem and progenitor cell populations (268). In the developing limb bud, for example, N-Myc is required for expansion of limb bud mesenchyme containing progenitors that form the limb skeleton (3, 12, 53). The expansion and fate of limb bud mesenchyme is strongly influenced by FGF signaling (269). Previous work from our lab suggested that FGF signaling acts through N-Myc to control proper limb patterning (3). Data from this study suggests that an important function of FGF signaling is to control the level and activity of N-Myc through post-transcriptional mechanisms described here.

Transcriptional upregulation of N-Myc by FGF and Ras/MAPK signaling was not observed in our systems. This suggests that additional signaling pathways, which induce *N-myc* gene expression, coordinate with FGF signaling to control N-Myc expression. Indeed, Wnt and FGF signaling operate synergistically to induce N-Myc expression the developing chick limb (12). Signaling by SHH, which is critical for limb bud development, regulates N-Myc expression at both the transcriptional and post-translational stages in mouse neuron precursor cells (9, 11). In summary, the combinatorial actions of Wnt, FGF, and SHH signaling provide a mechanism to fine-tune the level and activity of N-Myc during limb development, and potentially other settings (e.g. neurogenesis (68, 270) and lung development (271, 272)).

Theoretical implications for N-Myc-related diseases

Overexpression of N-Myc is implicated in the malignant progression of neuroblastoma and predicts an unfavorable prognosis (150, 230). Other tumors associated with elevated levels of N-Myc include medulloblastoma, retinoblastoma, small cell lung carcinoma, glioblastoma, and certain embryonal tumors (273). *MYCN g*ene amplification most often contributes to N-Myc overexpression in neuroblastoma, but does not account for all cases (150, 153). High expression of TrkB and/or its ligand, BDNF, is strongly associated with *MYCN*-amplified tumors and is associated with poor prognosis (193). Trk proteins, like other RTKs, activate a number of different downstream pathways, including the Ras/MAPK pathway (150, 193). Based on my studies in neuroblastoma cells, BDNF/TrkB signaling is predicted to stimulate N-Myc translation and transcriptional activity. This would provide a molecular basis for why neuroblastomas with combined

MYCN-amplification and high Trk/Ras activity are so aggressive. Furthermore, the molecular mechanisms delineated in this study may be relevant to other N-Myc-associated cancers, since activation of the Ras/MAPK pathway was also observed in these some tumors (274, 275). Future studies designed to delineate the mechanisms by which activated Ras/MAPK and Trk receptor signaling control N-Myc translation and protein turnover, and how N-Myc degradation is coupled to its activity, may provide insights into specific points of intervention for suppressing N-Myc levels and oncogenic activity in neuroblastoma and in other cancers.

4.3 FUTURE DIRECTIONS

The studies presented in this dissertation indicate that Ras/MAPK signaling promotes the synthesis, degradation, and activity of N-Myc. These findings raise a number of questions regarding the molecular mechanisms and biological consequences of these events. The following section describes future directions of this work that addresses these questions.

Investigation of the Molecular Basis for Ras-Mediated Translation

The mechanism by which Ras promotes N-Myc translation requires further investigation. Several observations made in this study provide a starting point for such an investigation. First, Ras promotes the association of N-Myc transcripts with polysomes. Second, the coding region of N-Myc is sufficient to allow for its translational regulation by Ras. Lastly, the ribonucleic region that encodes Thr50 may be required for Ras-mediated translation of N-Myc. In the Discussion section of Chapter 3, I outlined several

important experiments designed to test the validity of the latter observation. In short, these experiments involve a comparison of protein synthesis rate and polysome association of the N-Myc mutants (T50A and T50T silent mutants) to WT N-Myc. These experiments will also examine the effect of Ras signaling on the rate of protein synthesis and polysome association of the N-Myc mutants (T50A and T50T silent mutants). These experiments should be performed in multiple cell types to determine the generality or specificity of the observations. Together, these studies will determine if the Thr50 codon affects N-Myc translation and whether Ras acts through this nucleic region to control N-Myc translation.

It will be important to identify the sequences, perhaps in addition to those encoding Thr50, that are required for Ras-mediated translation of N-Myc. This will involve examining a series of truncation/intragenic deletion N-Myc mutants for altered translation rates and polysome distribution profiles. Recall that, in our system, Ras signaling did not affect the translation rate or polysome association of c-Myc. Thus, regions within N-Myc that are shown to effect its translation can be genetically swapped into the equivalent c-Myc region. This will determine whether these N-Myc regions are sufficient for mediating its translation. Identification of the regions required for Ras-mediated translation of N-Myc can subsequently be exploited to identify potential miRNAs or RBPs that bind this region (276).

Initially, these experiments should be performed using C3H 10T½ cells since they have been used throughout this study. Once the molecular mechanism has been established, it should be confirmed in settings where N-Myc is endogenously expressed (e.g., neuroblastoma cells or cerebellar granule neuron precursors). These studies will

provide significant insight to the molecular mechanism underlying Ras-mediated translation of N-Myc.

Investigation of the Molecular Basis for Ras-Mediated Proteolysis of N-Myc

Another outstanding question prompted by this study pertains to the molecular mechanism underlying Ras-mediated proteolysis of N-Myc. I have shown that GSK3β and Thr50 of N-Myc are not required in this process. Additional regulators involved in the Myc degradation pathway (Fig. 1.3), such as Pin1, PP2A, and Fbw7, should be investigated for their effects on N-Myc half-life. By using a series of truncation/intragenic deletion N-Myc mutants, one can identify the region(s) that are required for its proteolysis. Fusion of candidate regions to relatively stable proteins (e.g., GFP or GAL4(43)) will determine whether these regions are sufficient for Ras-directed proteolysis of the fusion protein. Gene-swapping of candidate regions into c-Myc may be misleading or may obscure results since Ras has a stabilizing effect on c-Myc. Identification of the region(s) in N-Myc that are necessary and/or sufficient for its Ras-directed proteolysis will be valuable identifying proteins that interact with that region or critical residues that are modified in response to Ras activation.

Ubiquitination of N-Myc directs it proteolysis by the 26S proteasome (277). Another question raised by this study is whether Ras signaling affects the ubiquitination status of N-Myc, specifically with regard to different types of ubiquitin chain linkages. K48-linked polyubiquitin chains mainly target the protein for degradation while K63-linked polyubiquitin chains affect functional aspects of the protein, including transcriptional activity (278). It is possible that Ras signaling does not affect total ubiquitination of N-

Myc, but affects ubiquitination of specific chain linkages. Future studies must take this into consideration. Understanding if and how Ras signaling affects N-Myc ubiquitination will provide valuable information regarding the mechanism of Ras-mediated proteolysis of N-Myc.

Investigation of Ras-Mediated Coupling of N-Myc Turnover and Activity

Studies within this dissertation provide a correlation between Ras-mediated proteolysis of N-Myc and an increase in its transcriptional activity. However, I do not directly demonstrate that Ras signaling couples N-Myc turnover and transcriptional activity. To directly demonstrate this, chromatin-immunoprecipitation studies are required to show that Ras influences N-Myc promoter occupancy. It is predicted that Ras would increase N-Myc promoter occupancy and that inhibition of the proteasome by MG132 would block this effect.

The previous section described studies that are designed to identify N-Myc degrons through which Ras operates. It should therefore be examined whether deletion of these regions affects the transcriptional activity of N-Myc. It is predicted that, in the presence of Ras, these N-Myc deletion mutant are stabilized but also have reduced transcriptional activity.

A common feature of unstable transcription factors whose activity is coupled to their proteolysis is overlapping TADs and degrons (264). It will be interesting to examine whether N-Myc degrons (identified in studies described above) and TADs overlap.

Evaluating the Biological Significance of Ras-Induced N-Myc Translation, Degradation, and Transcriptional Activity

A broad goal of this and future work is to understand how N-Myc and Ras function together at the molecular level in tumorigenesis. In the context of cancer, it will be important to evaluate the biological significance of the effect of Ras on N-Myc synthesis, degradation, and transcriptional activity. Once a molecular basis for each of these interactions has been established, one can systematically disable the mechanism and examine the effect on cellular transformation, proliferation, differentiation, and survival.

4.4 CONCLUSIONS

The main conclusions of this study are:

1. Ras/MAPK signaling promotes the accumulation of N-Myc and c-Myc by different mechanisms,
2. Ras signaling induces both the synthesis and proteolysis of N-Myc. The overall effect is elevated levels of N-Myc protein,
3. Ras signaling elevates c-Myc protein levels by preventing its degradation. Ras does not affect c-Myc protein synthesis, and
4. The transcriptional activity of N-Myc and c-Myc is coupled to their proteasomal degradation. Specifically for N-Myc, this process depends on Ras activity.

This work clarifies the current understanding of how Ras regulates N-Myc stability and expands the functions of Ras to include a role in N-Myc translation and transcriptional activity. By examining N-Myc and c-Myc under similar conditions, I was

able to demonstrate that Ras signaling has differential effects on N-Myc and c-Myc. This work supports the notion that Myc family members, despite their strong homology, are not regulated in the same manner. Specifically, the biochemical attributes of c-Myc should not be generalized to other family members. Future studies that further define the mechanisms by which Ras signaling regulates N-Myc may provide insight into points of therapeutic intervention in neuroblastoma and other cancers.

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