Differential Regulation of N-Myc and c-Myc Synthesis, Degradation, and Transcriptional Activity by the Ras/Mitogen-activated Protein Kinase Pathway

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Background: Oncogenic Ras signaling promotes c-Myc accumulation by slowing its degradation.
Results: Unlike c-Myc, oncogenic Ras promotes N-Myc protein degradation but stimulates its accumulation by increasing N-Myc translation.
Conclusion: Ras signaling stimulates N-Myc translation and proteolysis, and Ras-stimulated N-Myc proteolysis correlates with increased N-Myc transcriptional activity.
Significance: Increased N-Myc translation and proteolysis may underlie the oncogenic activity of activated growth factor receptors and Ras.

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-transcriptional 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 H-Ras (H-RasG12V) in very different ways. H-RasG12V promotes accumulation of both c-Myc and N-Myc, but although c-Myc accumulation is achieved by enhanced protein stability, N-Myc accumulation is associated with an accelerated rate of translation that overcomes a surprising H-RasG12V-mediated destabilization of N-Myc. We show that H-RasG12V-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.

N-Myc is a member of the Myc family of proto-oncogenes that also includes 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 (2, 3). 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 (5).

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 (6, 7). Medulloblastoma and neuroblastoma are the first and third most common pediatric cancers, respectively, and together they account for 35% of all childhood cancer deaths (6, 7). In addition to N-Myc gene amplification, deregulation of growth factors or downstream signaling constituents are frequently observed in neurological cancers (6, 8) and may contribute to up-regulation 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 mech-
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anism of c-Myc degradation requires sequential phosphorylation of two key residues in the N-terminal Myc homology box I (MBI)2 region of c-Myc: Thr-58 and Ser-62 (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 Ser-62 (12). 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 (13), which recognizes phosphorylated Ser-62 as a priming site for phosphorylation of c-Myc at Thr-58 (12, 14). The coordinated activities of the peptidylprolyl isomerase Pin1 and protein phosphatase 2A act on double-phosphorylated c-Myc to direct dephosphorylation of Ser-62 (14). Finally, single-phosphorylated c-Myc at Thr-58 is targeted for ubiquitination by the E3 ubiquitin ligase Fbw7, which directs the proteasomal-mediated degradation of c-Myc (12, 15, 16).

This mechanism of c-Myc degradation can be blocked by oncogenic mutations in Ras family GTPases that constitutively maintain Ras in an active state (17). Oncogenic H-Ras signaling promotes c-Myc stability in two ways; first, it induces Ser-62 phosphorylation by activating ERK through the MAPK pathway, and second, it blocks Thr-58 phosphorylation by activating the PI3K/Akt pathway, which inhibits glycogen synthase kinase-3β (12). The well established cooperation between c-Myc and oncogenic Ras in promoting cellular transformation (18) may in part be due to Ras-mediated stabilization of c-Myc (17).

Like c-Myc, N-Myc can cooperate with oncogenic H-Ras and K-Ras to transform normal embryonic fibroblasts into cells with tumorigenic potential (19, 20). 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, because 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 Thr-50 and Ser-54). 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 Ser-54 to alanine stabilized N-Myc, whereas mutation of the equivalent site in c-Myc, Ser-62, to alanine destabilizes c-Myc (12). Second, protein phosphatase 2A activity promotes c-Myc degradation (14) but appears to stabilize N-Myc (23). Finally, although the prolyl isomerase Pin1 appears to control protein phosphatase 2A-mediated dephosphorylation of Ser-62 of c-Myc (15), Pin1 does not alter the phosphorylation status of the equivalent Ser-54 of N-Myc in mouse neuronal precursors (23). Although 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. Furthermore, 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.

EXPERIMENTAL PROCEDURES

Cell Culture and Inhibitor Assays—SK-N-BE(2) cells were maintained in DMEM/F-12 (Invitrogen), SMS-KCN 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; 10 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 (24): pBABEpuro-c-Myc (c-Myc NCBI accession number ABW69847), pBABEpuro-GFP, pHBeo-WT-FGFR2c (Bek), pFBneo-FGFR2cK659E, pFBneo-GFP, pLXSN-FGFR2cS252W, and pBABEpuro-H-RasG12V. For pBABEpuro-N-Myc, the mouse N-Myc cDNA (coding sequence only, NCBI accession number AAH49783) was cloned into BamHI and EcoRV sites of pBABEpuro. Mutant N-Myc (N-MycT50A, N-MycT50D, N-MycS54A, and N-MycS54D) and c-Myc (c-MycT58A, c-MycT58D, c-MycS62A, and c-MycS62D) genes were generated from the pBABEpuro-N-Myc or pBABEpuro-c-Myc plasmids using the Change-IT Multiple Mutation site-directed mutagenesis kit (U. S. Biochemical Corp.). 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-RasG12V plasmid was constructed by removing H-RasG12V from pBABEpuro-H-RasG12V 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 (24). For stable cell lines expressing more than one ectopic gene, cells were infected with a second virus 24 h 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.

2 The abbreviations used are: MBI and MBII, Myc homology box I and II, respectively; CHX, cycloheximide; FGFR, FGF receptor; qRT, quantitative RT; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
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Immunoblot Analysis—Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing 1× Complete Protease Inhibitor mixture (Roche Applied Science). 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; green fluorescent protein (GFP; ab290) and phospho-Myc (Thr-58/Ser-62), and phospho-p70 S6 kinase (Thr-389) from Cell Signaling Technology. Quantitation of protein expression was performed by densitometry.

RNA Isolation, Semiquantitative 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 the SuperScript III first-strand cDNA synthesis kit (Invitrogen), and semiquantitative RT-PCR was carried out using 1 μl of cDNA per sample and 0.2–0.3 μM concentrations 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% Tris borate-EDTA-agarose gels containing ethidium bromide and photographed under UV illumination.

Quantitative RT-PCR (qRT-PCR) expression analysis was performed with 10–30×-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−ΔΔCt method with actin as the calibrator.

Metabolic Labeling—Stable C3H 10T½ cell lines were seeded at 6 × 10⁵ per 10-cm dish 48 h before labeling. Transfected cells were labeled 24 h after transfection. Where indicated, transfected cells were treated with DMSO or the indicated inhibitor 2 h before and during labeling. Cells were starved of l-methionine and l-cysteine by incubation for 15 min in l-methionine/l-cysteine-free DMEM (Invitrogen) supplemented with 10% dialyzed fetal bovine serum and l-glutamine (20 mM). After starvation, cells were labeled with 250 μCi/ml (for stable cells) or 100 μCi/ml (for transfected cells) of [35S]methionine/cysteine (MP Bio) at 37 °C for 30 min for protein stability assays or as indicated in protein synthesis assays. For stability assays, metabolically labeled cells were washed twice with cold “chase” media (DMEM supplemented with 10% FBS, 15 mg/liter l-methionine, and 20 mg/liter l-cysteine) and then incubated in chase media for the indicated times. Cells were washed twice with cold PBS and lysed with radioimmune precipitation assay 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 h 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 × 10⁶) were plated in 15-cm culture dishes and grown for 48 h. Before 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), 1× protease inhibitor mixture), 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 min at 4 °C. Gradients were then collected into 12 equal fractions (Bio-Rad system), whereas 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. A 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, 18 S rRNA, and 28 S rRNA. The remaining two-thirds of total RNA was used for RT-PCR analysis as described above. Semiquantitative analysis of the polysome gradients was performed by measuring band intensities and subtracting the background readings from equivalent areas using ImageQuant Version 7.

Luciferase Assay—HEK 293 cells were transfected using the calcium phosphate precipitation method (25) 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 1 h, and the luciferase activity was assessed using the Dual-Light system (Applied Biosystems) on a Centro XS LB Microplate Luminometer (Berthold Technologies). Luciferase activity was adjusted for β-galactosidase activity.

RESULTS

FGF Signaling Up-regulates 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
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A, C3H 10T\(^{1/2}\) 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 10T\(^{1/2}\) 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 analysis of variance. C, N-Myc-expressing C3H 10T\(^{1/2}\) cells were treated with FGF2 for 2 h followed by treatment with H\(_2\)O, DMSO, PD98059, UO126, or LY294002 for an additional 6 h. Lysates were collected for immunoblot analysis. D, cellular extracts from C3H 10T\(^{1/2}\) cells stably expressing N-Myc and WT FGFR2 (WT), FGFR2\(^{2S252W}\) (S252W), or FGFR2\(^{K659E}\) (K659E) were analyzed by immunoblot analysis for the expression of the indicated proteins. E, C3H 10T\(^{1/2}\) cells described in D were treated with UO126 for 6 h, then harvested for immunoblot analysis. All experiments were performed in triplicate unless otherwise stated.

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 (12). We, therefore, used a constitutively active form of H-Ras, H-Ras\(^{G12V}\) (or oncogenic Ras), to further investigate how MAPK signaling up-regulates N-Myc and whether this was due to increased N-Myc protein stability. Similar to what was observed with FGF2 treatment, H-Ras\(^{G12V}\) increased N-Myc protein levels but had little effect on N-myc transcript levels (Fig. 2, A and B). Using CHX to block de novo protein synthesis, we monitored the rate of N-Myc degradation and were surprised to find that H-Ras\(^{G12V}\) consistently reduced the half-life of N-Myc (Fig. 2C). To control for potential off-target effects by CHX, N-Myc turnover was also examined by \(^{35}\)Smethionine pulse-chase analysis. Like the CHX experiments, pulse-chase analyses consistently showed that H-Ras\(^{G12V}\) accelerated N-Myc turnover (Fig. 2D). Because these findings are opposite to what has been described for c-Myc (12), we performed parallel studies with c-Myc. H-Ras\(^{G12V}\) increased the amount of ectopic c-Myc protein but did not affect c-myc transcript levels (Fig. 2, A and B), and consistent with published data, \(^{35}\)Smethionine pulse-chase analysis showed that H-Ras\(^{G12V}\) slowed c-myc degradation (Fig. 2E). 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 H-Ras\(^{G12V}\) elevated steady-state N-Myc protein levels without affecting N-myc mRNA levels but also accelerated N-Myc turnover suggested that H-Ras\(^{G12V}\) might act at the level of translation. To test this, we examined the rate of N-Myc protein synthesis by \(^{35}\)Smethionine pulse analysis. Newly synthesized \(^{35}\)Smethionine-labeled N-Myc protein accumulated faster in the presence of H-Ras\(^{G12V}\) and peaked at 20 min (Fig. 3A, left). The slight decrease in \(^{35}\)Smethionine-labeled N-Myc at 30 min was consistently observed in the H-Ras\(^{G12V}\)-expressing cells and is likely due to its already short, but further shortened half-life (17–23 min) in the presence of oncogenic Ras. In this 30-min assay, H-Ras\(^{G12V}\) increased the rate of N-Myc protein synthesis ~2-fold (Fig. 3A, right), but this rate is likely greater as the accelerated rate of protein degradation is not factored into this calculation. In contrast to N-Myc, H-Ras\(^{G12V}\) did not affect the rate of ectopic or endogenous c-Myc protein synthesis (Fig. 3A and supplemental Fig. S2).

We considered that the increased rate of N-Myc protein synthesis might be due to H-Ras\(^{G12V}\) activity targeting ele-
ments within the vector used to expresses N-Myc (i.e. 3’-untranslated region promoter). To control for this possibility, the gene encoding GFP was expressed in the same expression vector, and the rate of GFP protein synthesis was determined. In contrast to N-Myc, the translation rate of GFP was not altered by H-RasG12V (Fig. 3A). In addition, because it was possible that stable expression of H-Ras G12V and/or N-Myc could lead to secondary effects that might influence how H-RasG12V regulates N-Myc during cell passage, we examined N-Myc translation in cells 2 days after transfecting H-RasG12V and N-Myc expression vectors. In this context, H-RasG12V also consistently increased N-Myc protein synthesis (Fig. 3B).

**Inhibition of the Ras/MAPK Pathway Suppresses N-Myc Translation**—The Ras/MAPK pathway signals through multiple pathways, several of which are involved in translational control (31). We used a panel of inhibitors that target Ras effectors to determine what pathway(s) is involved in controlling N-Myc translation. C3H 10T½ cells transiently expressing N-Myc alone or stably expressing H-RasG12V were treated with UO126, LY294002, or SB230508 to inhibit MEK, PI3K, or p38/MAPK, respectively. Treated cells were pulsed with [35S]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. 3C). LY294002 also reduced the amount of newly synthesized N-Myc protein, but this was likely
a nonspecific effect as it concomitantly reduced total protein synthesis (Fig. 3C). Inhibition of p38/MAPK with SB230508 did not significantly affect N-Myc translation (Fig. 3C). 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. 3C). Collectively, these data show that H-RasG12V up-regulates N-Myc translation via MEK activation and are consistent with MEK activation being required for increased steady-state levels of N-Myc in H-RasG12V-expressing cells (Fig. 1C).

**N-myc mRNA Is Redistributed to Polysomes by Oncogenic Ras—**To further characterize H-RasG12V-driven N-Myc translation, we next examined the translational status of N-myc mRNA by monitoring its association with ribosome complexes isolated after sedimentation through sucrose gradients. H-RasG12V caused N-myc mRNA to shift to the heaviest sucrose fractions (n = 2), indicating enhanced association of N-myc mRNA with polysomes (Fig. 4, A and B). Consistent with H-RasG12V preferentially affecting N-Myc protein synthesis, ectopic CMYC and actin transcripts were not redistributed toward polysomes (fractions 5–11; Fig. 4, C and 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 (32, 33). This may account for the larger polysome population observed in C3H 10T½ cells expressing H-RasG12V (Fig. 4A) and calls into question the extent to which H-RasG12V up-regulates N-Myc translation in a general or selective manner. H-RasG12V did not increase global protein synthesis (supplemental Fig. S3), a result consistent with translational up-regulation of N-Myc being at least partly selective. Moreover, redistribution of N-myc mRNA to polysomes by H-RasG12V is consistent with some level of specificity for N-myc mRNA as H-RasG12V did not alter the polysome association of CMYC and cyclin D1 despite elevating their protein levels (Figs. 2A and 4C and data not shown).

Translational control by microRNAs and RNA-binding proteins often requires the UTRs of transcripts (34, 35). However, our studies characterized ectopically expressed N-Myc transcripts lacking endogenous UTRs. This suggested that N-Myc UTRs were not required for H-RasG12V-mediated translational up-regulation 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 H-RasG12V, 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. H-RasG12V increased N-Myc protein level to a similar extent with both versions of N-Myc cDNA (supplemental Fig. S4). Taken together, these results suggest that H-RasG12V 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—**Although H-RasG12V 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 neuroblastoma cell lines, SMS-KCNR and SK-N-BE(2)C, that express N-MYC from MYCN-amplified loci. In both of these cell lines, ectopic expression of H-RasG12V increased N-MYC protein but not mRNA levels (Fig. 5, A and B). As in C3H 10T1/2 cells, H-RasG12V increased the rate of N-MYC degradation in both neuroblastoma cell lines (Fig. 5C). Moreover, H-RasG12V caused a redistribution of MYCN mRNA, but not CMYC or actin mRNA, to polysome fractions in SK-N-BE(2) cells (Fig. 5D). 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 H-RasG12V 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 (Thr-58 and Ser-62) are conserved in N-Myc (Thr-50 and Ser-54). 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. 6, A and B). Phosphorylation of N-Myc at Thr-50 and c-Myc at Thr-58 was observed for both WT N-Myc and c-Myc proteins but not for any of the mutant proteins (Fig. 6, A and B). Phosphorylation of Ser-54 and Ser-62 was elevated in the N-MycT50A and c-MycT58A mutants, respectively, but was not detected in the N-Myc and c-Myc Serine mutants (Fig. 6, A and 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 Thr-50 requires prior phosphorylation of Ser-54 (12, 36). Mutation of Thr-58 to Ala and Ser-62 to Asp in c-Myc increased the protein half-life as previously shown (12, 37, 38), and the equivalent mutations in N-Myc (Thr-50 to Ala and Ser-54 to Asp) increased protein half-life (Fig. 6C). The increased half-life of the Ala-54 mutant is consistent with previous results (22). Contrary to previous observations (12, 36, 37), mutation of c-Myc from Thr-58 to Asp or Ser-62 to Ala resulted in protein stabilization (Fig. 6D). In our system, however, the equivalent c-Myc and N-Myc mutants displayed the same phosphorylation patterns and half-lives, suggesting that phosphorylation of the conserved Thr and Ser residues in MBI of N-Myc and c-Myc have essentially equivalent roles in controlling protein stability. These results suggested that onco-

FIGURE 5. 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 H-RasG12V 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 H-RasG12V were pulsed with CHX for the indicated times or with DMSO (D) for 90 min. 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 H-RasG12V.
Regulation of N-Myc Stability, Translation, and Activity by Ras

Genic H-Ras functions independently of Thr-50 or Ser-54 to promote N-Myc degradation. Indeed, N-Myc<sup>T50A</sup> retained sensitivity to H-Ras<sup>G12V</sup>-dependent destabilization (compare Fig. 6C for T50A to Fig. 6E, top). Finally, H-Ras<sup>G12V</sup> did not alter the half-life of c-Myc<sup>T58A</sup> (compare Fig. 6D for T58A to Fig. 6E, bottom), which is consistent with the observation that the activities of oncogenic Ras function through this site to control c-Myc stability (12). Together, these data show that although phosphorylation at Thr-50 or Ser-54 are involved in regulating N-Myc stability, oncogenic H-Ras functions independently of these sites to promote N-Myc degradation.

N-Myc Degradation Regulates Its Transcriptional Activity—The unexpected finding that H-Ras<sup>G12V</sup> 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 (19). However, a functional link between transcriptional activity and protein degradation has been documented for numerous transcription factors, including c-Myc (38–40), 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. 7A). However, while MG132 initially increased luciferase activity, it later decreased luciferase activity despite its increased accumulation (Fig. 7B). Reporter activity was similarly affected in the presence or absence of H-Ras<sup>G12V</sup> for both N-Myc and c-Myc, suggesting that proteasomal degradation in general contributes to N-Myc and c-Myc transcriptional activity (Fig. 7B). Furthermore, MG132 inhibited transcriptional activity of both N-Myc<sup>T50A</sup> and c-Myc<sup>T58A</sup>, suggesting that degradation-coupled transcription functions independent of Myc proteolysis governed by this conserved site in MBI (data not shown). 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, Mnt, and Cad (43)) by c-Myc alone or when combined with H-Ras<sup>G12V</sup> was reduced by MG132 (Fig. 7C, 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) (44), 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 H-Ras<sup>G12V</sup> (Fig. 7C, left). Induction of these genes was dependent on N-Myc and not MG132 treatment, as in the absence of N-Myc, their expression was reduced by MG132 treatment (supplemental Fig. S5). 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.

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 (45). This study suggests a novel mechanism by which constitutively active Ras proteins promote oncogenesis. We made the surprising observation that oncogenic H-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.

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 (46). 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 (47). Moreover, a link between protein degradation and transcriptional activation has been suggested for c-Myc (38, 48). 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 of c-Myc that increased both its transcriptional activity and degradation. The MBI and MBII regions of c-Myc, which reside within the first 143 amino acids, are important for its transcriptional activity (48), transforming activity (49), and stability (50), and it was postulated that these nearby regions might act to link c-Myc transcription with its degradation. MBI and MBII regions of N-Myc, which reside within the first 143 amino acids, are important for its transcriptional activity (48), transforming activity (49), and stability (50), and it was postulated that these nearby regions might act to link N-Myc transcription with its degradation. However, our data indicate that oncogenic Ras can act independently of these sites to regulate N-Myc protein stability. Indeed, our finding that oncogenic Ras destabilizes both WT and N-MycT50A but does not affect c-MycT58A stability supports this hypothesis. Interestingly, the ubiquitin ligase Huw1 has been shown to destabilize N-Myc, but not c-Myc (51), and is a potential 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 Huw1 or other proteins involved in proteasomal degradation of N-Myc to increase their binding affinity toward N-Myc. By increasing their association with N-Myc, oncogenic Ras might promote N-Myc degradation but also potentially stimulate N-Myc transcriptional and oncogenic activity as well.

FIGURE 7. Proteolysis of N-Myc and c-Myc is associated with enhanced transcriptional activity. A, C3H 10T1/2 cells stably expressing N-Myc or c-Myc and H-RasG12V 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 or H-RasG12V, and a Myc-responsive luciferase reporter. After treatment with MG132 (5 μM) for the indicated times, cells were analyzed for luciferase activity. The graph shows the % change in relative luciferase activity (RLU) by MG132 calculated from three independent experiments. C, C3H 10T1/2 cells stably expressing N-Myc or c-Myc alone or in combination with H-RasG12V were treated with MG132 (10 μM) for 1 h. Expression of the indicated genes was determined by qRT-PCR analysis. Error bars show S.D. for biological duplicates.
Although results from this study are clearly relevant to the oncogenic activity of constitutively activated Ras, they may also be relevant to the normal function of growth factor signaling that leads to Ras activation. Stimulation of C3H 10T½ cells with FGF2 or ectopic expression of mutant-activated FGF2 resulted in strong up-regulation of exogenously expressed N-Myc that was mediated by MEK activation (Fig. 1, A–C). Therefore, signaling that modulates Ras/MAPK activity during development or tissue homeostasis is predicted to heavily influence the dynamics of N-Myc synthesis and degradation. N-Myc is involved in the development of diverse tissues and organs during embryonic development, where it plays essential roles in regulating the cellular expansion and fate of various stem and progenitor cell populations (52). For example, in the developing limb bud, N-Myc is required for expansion of limb bud mesenchyme-containing progenitors that form the limb skeleton (27, 52, 53). The expansion and fate of limb bud mesenchyme is strongly influenced by FGF signaling (54), and data from this study suggest that this FGF signaling in the limb bud, depending on its source, strength, and persistence, may promote the appropriate level of limb bud mesenchyme proliferation by modulating the levels and activity of N-Myc through post-transcriptional mechanisms. Because neither FGF signaling nor oncogenic Ras activity leads to N-Myc transcription, these signals would need to be coordinated with signals that induce N-Myc transcription. In the developing limb, Wnt signaling can induce N-Myc transcription (53), and the combinatorial actions of Wnt and FGF signaling in the limb bud and perhaps at other sites as well may provide a mechanism to fine-tune the level and activity of N-Myc and its potent ability to regulate cell proliferation. Moreover, Wnt signaling can strongly influence the activity of glycogen synthase kinase-3β (55), the kinase that phosphorylates N-Myc at Thr-50 (22) and c-Myc at Thr-58 (36) to, as shown for c-Myc, promote proteasomal degradation (56). In light of our findings, the effect of glycogen synthase kinase-3β on N-Myc, which is thought to be inhibitory, may need to be further substantiated because, by targeting N-Myc for degradation, it may also stimulate its transcriptional activity. And this may be true for c-Myc as well (Fig. 7C, right). Furthermore, because promoting Myc degradation may actually potentiate its activity, the notion that strategies that enhance Myc degradation would be therapeutically effective against cancer may need to be reconsidered.

Ras-mediated signaling modulates the activity of factors associated with the translational machinery to alter the translation of numerous transcript targets (57, 58), and our data indicate that N-Myc transcripts are one of those targets. Translational up-regulation of N-Myc by oncogenic Ras was observed after transient and stable H-RasG12V expression (supplemental Fig. S2 and Fig. 3A), 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 (32, 33). In our polysome assays, oncogenic Ras increased the relative amount of total RNA in the polysome fractions (Fig. 4A), but this was not associated with global increases in translation (supplemental Fig. S3). Therefore, it appears that oncogenic H-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 H-Ras targets elements within the coding region of N-Myc. Although mechanisms that control the expression or activity of a transcript most commonly target non-coding regions, there is a growing number of examples of RNA-binding proteins and microRNAs that target the coding regions of transcripts (59–62). 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.

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 (63, 64). Some patients with neuroblastoma having both MYCN gene amplification and high expression of tropomyosin receptor kinase A (TrkA) or TrkB have even worse prognoses (63, 65). Tropomyosin receptor kinase signaling, like other receptor tyrosine kinase receptors, activates a number of different downstream pathways, including the Ras/MAPK pathway (64). Our results predict that the combination of hyperactive Ras/MAPK activity driven by elevated tropomyosin receptor kinase 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 tropomyosin receptor kinase/Ras expression are so aggressive. Future studies designed to delineate the mechanisms by which hyperactivated Ras/MAPK and tropomyosin receptor kinase 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.

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